DOCTOR OF PHILOSOPHY

Studies on the ant-fungus mutualism in leafcutting ants, Formicidae: attini.

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STUDIES ON THE ANT-FUNGUS MUTUALISM IN LEAFCUTTING ANTS, FORMICIDAE: ATTINI.

A thesis submitted to the University of Wales

by

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in candidature for the degree of

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Dedicated to

my parents,

Harold and Rita Bass.

SUMMARY

The mutualism between leafcutting ants (*Atta* spp.) and their fungus was studied in the laboratory. The dynamics and internal structure of fungus gardens were examined. *Atta* practised a continual temporal monoculture, with a turnover rate of 20-70 days. Worker access to internal areas of the garden was restricted and internal cavities had larger standing crops of staphylae than did outer surfaces of the garden.

The behaviour of workers and alates was examined. Combining observations made with behaviours reported in the literature showed that *Atta sexdens* workers perform 76 acts. Alate females (*Atta cephalotes*) performed 13 acts and males, 9 acts, when on the garden surface.

Workers produced three types of infrabuccal pellet; one containing detritus, one fungus and one was intermediate. Fungal pellets probably resulted from the ingestion of hyphae during fungus garden licking, an activity which engaged up to 30% of workers on the garden surface.

The role of fungus garden licking was studied by temporarily preventing worker access to the garden. This led to subsequent increases in the numbers licking these previously restricted areas, but this effect declined if garden was isolated for more than 6 days. However, fungus garden was successfully isolated from workers for up to 12 days before contaminant growth occurred, although alien spores were present.

Fungus garden licking was studied as a possible pruning mechanism and as a possible source of nutrients. Pruning hyphae on the garden surface artificially, or by licking workers, led to increases in the crops of staphylae subsequently produced. Experiments using worker longevity and weight gain on different diets, as well as fluorescence microscopy to trace material into worker crops showed that workers, particularly minima , could utilise hyphae as food and that these supplied 0.7-2.8% of worker respiratory energy requirements.

Finally, dying nests were used to provide insights into the ant-fungus mutualism.

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CONTENTS

Chapter 1: General Introduction

1. The Attine-fungus mutualism

A mutualism is an obligate mutually beneficial interdependence between unrelated symbionts and such relationships are common between arthropods and fungi. Ambrosia beetles of the Tribe Xyleborini, of which there are 1,500 described species, use a mutualistic fungus, often a *Fusarium*, as a food source and successfully attack hundreds of species of woody plants throughout the world, especially in the tropics and sub-tropics (Norris 1979).

Ants of the Tribe Attini, which are confined to tropical and neotropical regions of the New World (Weber 1972), have an obligate mutualism with a fungus which is found only in their nests. Moeller (1893) first isolated the symbiotic fungi of Attines and found them to be Basidiomycetes, although Wheeler (1910) disputed this, claiming that they were Ascomycetes. Many further attempts have since been made to isolate the ant fungi for the propagation of fruit bodies, but with little success since the mycelium is usually vegetative only. Kriesel (1972, 1975) named the symbiont of *Atta insularis*, '*Attamyces bromatificus*' and placed it in the Mycelia Sterilia. However, Powell (1984) showed the presence of dolipore septation, a Basidiomycete characteristic.

Leafcutting ants of the genera *Acromyrmex* and *Atta*, culture their fungus on fresh vegetable substrate to produce a 'fungus garden' (Plate 1a). Initially it was believed that the ants ate the plant material they cut (Buckley 1860, Lincecum 1867), but Belt (1874) suggested that they cultured a fungus on it which then served as their food. This was later confirmed by Tanner (1892) and Moeller (1893). Most authors continued to believe that the fungus provided the sole food source for the colony (Wheeler 1907, Walton *et al.* 1938, Weber 1966, 1972, Mariconi 1970) until Quinlan and Cherrett (1979) showed that the nutritive bodies produced by the fungus, the

'staphylae', provided only 4.8% of worker respiratory needs, the majority of which were obtained by drinking plant sap. In contrast, the larvae were found to be monophagous, consuming only staphylae. Staphylae are clusters of swollen hyphae called 'gongylidia' (Plate 1b and c) and provide a high quality food with high levels of assimilable carbohydrates and proteins rich in essential amino acids (Martin *et al.* 1969).

The Attini also include primitive genera, such as *Apterostigma* and *Cyphomyrmex*, which use insect frass and dead vegetable matter as fungal substrate (Weber 1972). It remains unclear how much of their energy needs are supplied by their fungus.

Attines are not the only ants which utilise fungi. Malyshev (1968) believed that queens of *Lasius niger* feed on the fungi which grow on the walls of their claustral chambers during colony foundation and fungus-eating is also a minor feature of the diet of *Formica rufa* (Wellenstein 1952). *Lasius fuliginosus* uses a fungus (*Cladosporium myrmecophilum*) to construct carton nests, although the fungus is not actually consumed (Maschwitz and Holldobler 1970).

When workers are removed from the Attine fungus garden, it is rapidly over-run by competing fungi. Leaf cutting ants maintain their pure fungus culture through strict hygiene and assiduously lick leaf surfaces before using them as fungal substrate. These licked surfaces are less contaminated with alien spores than unlicked ones (Quinlan and Cherrett 1977). Licking also removes leaf waxes, some of which have antibiotic properties (Martin and Juniper 1970). The fungus itself may also produce antibiotics (Hervey and Nair 1979, Angeli-Papa 1984) as do the ant metathoracic glands, which secrete myrmicacin and phenylacetic acid (Schildknecht and Koob 1970) and these may also assist in maintaining the monoculture.

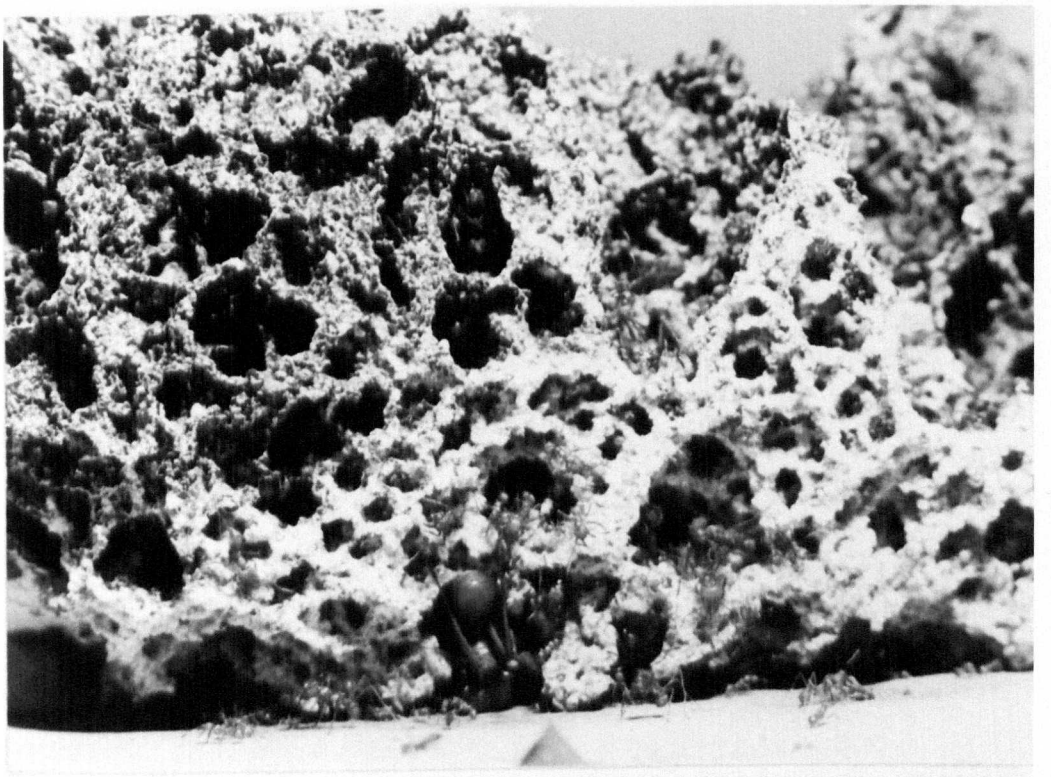
Attine workers continually defaecate on the fungus garden and their rectal fluid contains enzymes which degrade protein, chitin and starch (Martin and Martin

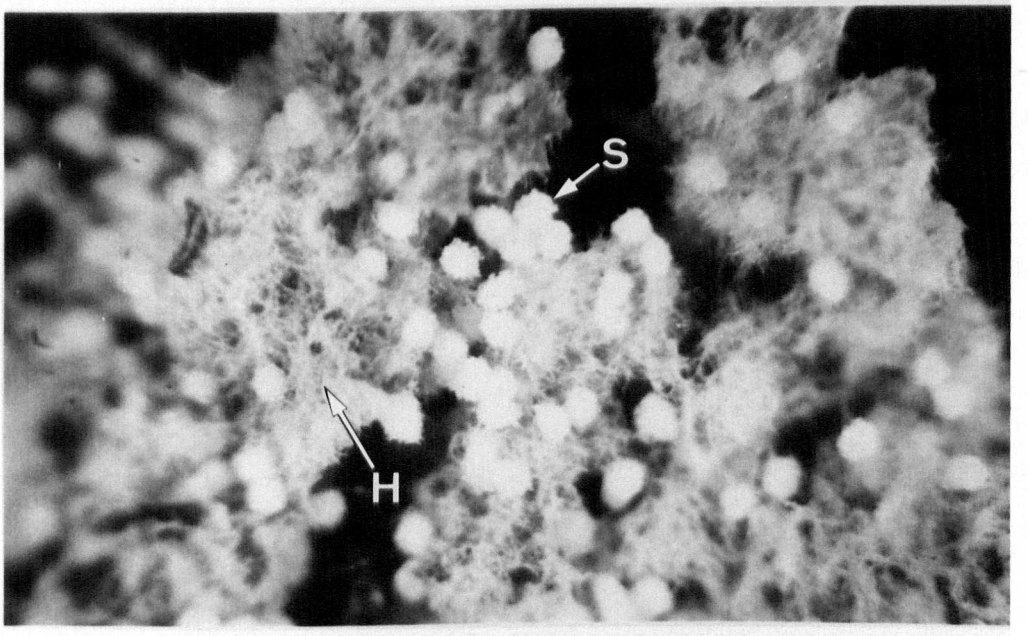
Plate 1:

a) The fungus garden of *Atta cephalotes*, showing darker, larger-celled young areas at the top (x2).

b) Close-up of the fungus garden, showing staphylae (S) and the mat of hyphae (H), which covers the garden surface (x10).

c) Single staphyla, stained and viewed microscopically, showing the individual swollen hyphae or 'gongylidia' (x100).




S
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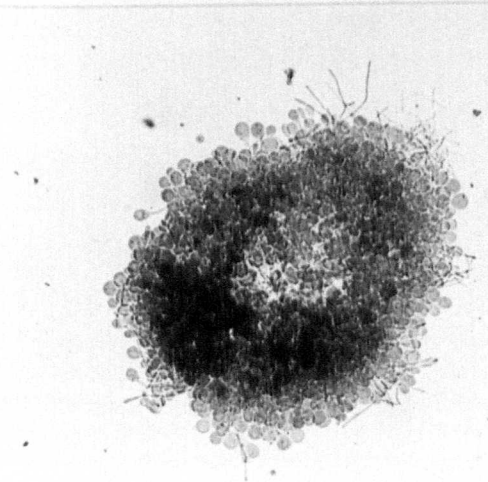


1970a, b, 1971, Martin *et al.* 1973) as well as promoting fungus growth. The proteinases found in the ant faeces actually originate from the ant fungus (Martin *et al.* 1975). The faeces also contain growth-promoting nitrogeneous compounds (Martin and Martin 1970b). Attines have become physiologically adapted to mycophagy and chitinase is produced by the labial glands and lipase, maltase, trehalases and proteases by the gut, for fungus digestion (Febvay and Kermarrec 1981a, Febvay *et al.* 1984).

Attines have also developed behavioural strategies for culturing fungus. They excavate nest chambers in the soil at depths providing suitable temperatures and they control nest humidity, adjusting air flows by opening and closing entrance holes, regurgitating water on to fungus gardens or digging deeper in the soil (Weber 1972). They also select suitable substrate for fungus growth, although the extent to which they adjust their diet to the needs of their fungus is controversial (Cherrett *et al.* 1989).

Quinlan and Cherrett (1978a) found no evidence that the ants chose material to suit their fungus and there was no apparent learning behaviour. The ants do tend to avoid materials more acidic than the fungus garden and so obtain substrate which is probably low in tannins and high in available protein (Powell 1984). However, past selection pressures may have determined preferences for material which is not deleterious to fungus growth, since if the fungus garden was destroyed by toxic forage, the ant colony would not reproduce. The broad range of leaves harvested by leafcutting ants and their changing preferences with time also provides a safety margin against poisoning of the whole garden. Introduced species of crop plant are attractive to leafcutting ants because these plants have not evolved defences against them. Similarly, the ants find jackbean, *Canavalia ensiformis*, attractive, but Mullenax (1979) showed that when fed solely on a diet of this plant, laboratory colonies died, due to the presence of toxins. Similarly, extracts of sesame, *Sesamum indicum*, inhibit

growth of the ant fungus in artificial culture (Pagnocca *et al.* 1990).

2. The transmission of fungus between generations

The ant fungus is found nowhere else but in living Attine nests and is transmitted to newly-founded colonies via the single queen. It is unable to produce fruiting bodies in the nest and is propagated vegetatively by the workers. Gynes accumulate fragments of fungal mycelia in the infrabuccal pocket, a small cavity beneath the opening of the oesophagus. After mating and completing her mating flight, the queen digs a chamber in the soil, expels the pellet of mycelium and tends it assiduously. This was first discovered by von Ihering (1898) and Huber (1905).

Different species of Attines appear to grow different strains of fungus, with different productivities (Stradling and Powell 1986). Thus the fungus of *Atta cephalotes*, which has up to 0.7×10^6 workers per colony, is 7.62 times as productive as that of *Trachymyrmex urichi*, which has only 800 workers per colony. The fungus of *Acromyrmex octospinosus* (14×10^3 workers per colony) is 3.77 times as productive and that of *Atta sexdens rubropilosa* (3×10^6 workers per colony) is 30 times as productive as that of *Trachymyrmex urichi*. Stradling and Powell also found that when fungal isolates from different species of *Atta*, *Acromyrmex octospinosus* and *Trachymyrmex urichi* were grown together in pairs on culture plates, there were no interactions. These authors therefore suggest that the different fungal strains constitute clones which may be shared by more than one ant species and are therefore of great antiquity. However, this may not necessarily be true for the lower Attines, since Hervey *et al.* (1977) found clamp connections in the fungal mycelium of *Apterostigma mayrii*.

However, for these strains to remain pure, no recombination or transfer of genes between the fungi of different ant species can have taken place for millions of years, since the different ant species arose. Vast numbers

of new colonies are founded within the territories of established nests, which subsequently destroy them (Jutsum *et al.* 1979). It is likely that fragments of fungi from these new colonies are brought back to the mother nest, providing the opportunity for fungal recombination. Stradling and Powell (1986) found no evidence that recombination could occur, but an experiment conducted over a period of days may not necessarily be comparable to events in the field. Extremely rare events become increasingly probable over long periods of time. Recombination and the development of new strains may have contributed to the diversity of species among the Attines.

The recombination of fungus clones to produce new strains could take place if queens are readopted or if multiple colony foundation (pleometrosis) occurred. Primary pleometrosis does occur in *Atta texana* (Mintzer and Vinson 1985), but there is no evidence that this occurs in any other species. The readoption of queens either from the mother colony or from strange nests occurs in some ant species (Sudd and Franks 1987) but there is no evidence that this takes place in Attines.

3. Benefits and costs of the mutualism

The mutualism provides benefits for both parties. The ants use the fungus to circumvent insect defences in leaves and to break down plant material to provide them with a source of protein. The fungus in turn is protected from competitors and is able to exploit a rich nutrient source, i.e. fresh leaves. Living leaves usually defend themselves from fungal attack by physical defences, such as waxy cuticles (Agrios 1988). However, substrate preparation by the ants removes such barriers, allowing the mycelium to penetrate (Quinlan and Cherrett 1977). Most biotrophic fungal parasites of plants are specific to single species or genera (Agrios 1988) and similarly, most phytophagous insects have narrow diet tolerances (Strong *et al.* 1984). Leafcutting ants however, are highly polyphagous and take more than 50% of local plant species (Cherrett 1968). They

do exhibit preferences for different types of substrate and deterrents such as toughness (Cherrett 1972a, Waller 1982a), latex (Stradling 1978) and secondary defensive chemicals (Littledyke and Cherrett 1978, Waller 1982b, Hubbell *et al.* 1983, Febvay *et al.* 1985) all inhibit cutting. However, it is likely that workers drink sap from a much narrower range of species than the ones they cut (Littledyke and Cherrett 1976).

The evolution of the mutualism has been costly for both parties. The ants must invest large amounts of resources in maintaining the fungus culture and at any one time, 30% of *Acromyrmex octospinosus* workers may be licking the garden surface (Quinlan and Cherrett 1979). The fungus has lost the ability to survive without the ants and produces large numbers of nutritive staphylae, which serve only as a food source for the ants.

4. The success of the mutualism

The success of this 'unholy alliance' (Cherrett *et al.* 1989) has led to leafcutting ants becoming dominant exploiters of living vegetation and they are important crop pests (Cramer 1967). In tropical rainforest, they may harvest 17% of total leaf production (Cherrett 1989) and in Panamanian rainforest, up to 80% of leaf damage was caused by *Atta* spp. (Wint 1983). Introduced crop species are particularly susceptible to attack, since they have not coevolved with Attines and have not developed deterrents.

Atta colonies may grow to considerable sizes, with several million workers and more than 2,000 nest chambers (Weber 1966, Jonkman 1978, 1980). *Acromyrmex colonies* in contrast, are smaller with few nest chambers and worker populations of several thousand (Weber 1972). However, *Acromyrmex* colonies often occur at higher densities than *Atta* nests, which may cover large areas. Stahel and Geijskes (1939) excavated a nest of *Atta cephalotes* with a surface area of 256 m^2.

5. The origins of the mutualism

The origins of the mutualism remain unclear. Weber (1958) speculated that the Attines may have arisen 50 million years ago and Baroni-Urbani (1980) reported the first confirmed Attine fossils, which were 35 million years old. von Ihering (1894) believed that the ancestral Attines were harvester ants, which then switched to feeding upon fungi growing on harvested grain. However, there are major morphological differences between harvesters and Attines. Forel (1902) believed that Attines evolved from predacious arboreal Dacetini, which often nest in rotting wood where moulds grow in abundance. The Dacetini and Attini are closely related, but this theory requires an extreme change in diet. Weber (1958, 1972) believed that early Attines switched to eating fungi growing on nest refuse and faeces and began to collect insect frass and eventually plant material to culture this fungus. Garling (1979) suggested that fungus-culturing behaviour began when a generalist forager began to feed upon and subsequently propagate ectotrophic mycorrhizae associated with plant roots. There are striking similarities between the taxonomies of mycorrhizae and Attine fungi.

6. Study aims

Few animals have discovered agriculture and the majority remain hunter-gatherers. Human agriculture arose only 10,000 years ago (Diamond 1991). Many ant groups practise animal husbandry, using aphids or mealy bugs for example, as sources of carbohydrate-rich honeydew. However, only one group of ants, the Attini, cultivate fungi as a source of food.

Animals seeking to grow their own food have a fundamental set of problems to face. Firstly, they must find a suitable organism which can be domesticated and which can provide them with a sustaining food source. Growing crops can also lead to problems with pests and diseases. In the natural situation, plants are usually widely distributed and mixed with other species, but in

agriculture, they may be concentrated together as monocultures. Such monocultures can be either spatial or temporal. Spatial monocultures, such as a large field of potatoes for example, are susceptible to pests and diseases because these have a greater opportunity to encounter food plants and to spread between them. Epidemics of pests and diseases can therefore spread rapidly, devastating the crop. In the 1840's, such an epidemic of potato blight, *Phytophthora infestans*, led to the Irish potato famine, in which about a million Irish farmers and their families starved to death (Diamond 1991).

Temporal monocultures, where a crop is grown for successive years in the same place, can also lead to problems as pests build up in the soil, like the beet cyst nematode, *Heterodera schachtii*, which damages sugar beet crops (MAFF 1978). Some crops may be both temporal and spatial, when a crop is grown over large areas for long periods.

Attines appear to practise a temporal monoculture, but in the natural situation, gardens of *Atta* are constructed in separate chambers. They therefore seem to avoid a spatial monoculture, but it is unclear whether fungus garden culture is continuous or discontinuous in the absence of environmental stimuli. Discontinuous culture can be used to reduce pest problems and improve hygiene, which might be important for nests which continually import forage contaminated with alien spores. There is some evidence that *Atta* species move fungus gardens between chambers at different seasons (Weber 1972). The macroscopic culture of fungus gardens in the laboratory over time is examined in Chapter 3.

Having domesticated and successfully grown the crop, an agriculturalist must also be able to harvest it, dispose of crop residues and cope with surpluses and shortages.

Another important aspect of culture is the rate of turnover, or how long substrate can continue to support fungal growth. Similarly, although much work has been done on the ant-fungus interactions on the outer surface of the garden, the way in which workers exploit the internal areas

of the garden remains unclear. These internal areas make up the largest part of the fungus garden and thus provide large potential areas for staphyla production and for workers to lick. Substrate turnover and the internal structure of fungus garden are both examined in Chapter 4.

Cherrett *et al.* (1989) drew an analogy between the intensive production of cereals in agriculture and the intensive fungus gardening of *Atta*. Caring for the fungus garden demands enormous input by the ants and Quinlan and Cherrett (1979) reported that up to 30% of the workers present on the garden surface were licking it at any one time. Chapter 5 attempts to catalogue the entire behavioural repertoire of *Atta sexdens* and to ascertain the relative frequencies of licking on different areas of the garden by different worker castes. Weber (1972) assumed that fungus garden licking was for hygienic purposes and Cherrett *et al.* (1989) suggested that they might be ingesting the breakdown products of extra-cellular digestion. 'Licking' might provide workers with food in the form of hyphae, but Wilson (1980a) stated that licking workers did not ingest hyphae. Ants in fact, can only utilize liquid food and solid particles are trapped in the infrabuccal pocket. There is therefore no clear explanation for this major activity and its importance and purpose in the ant-fungus mutualism are examined in Chapters 6-10.

Fungus-culturing by *Atta* is then considered as an agricultural activity in Chapter 12.

Chapter 2: General materials and methods

INTRODUCTION

All studies described in this thesis were made under laboratory conditions. With this kind of research, efforts must therefore be made to ensure that results obtained in the laboratory reflect what actually happens in the field.

This chapter describes how colonies were maintained in the laboratory to approximate natural conditions and how workers and garden were classified and prepared for use in experiments. Statistical methods used are also described.

GENERAL MATERIALS AND METHODS

1. Culture of ant colonies

Laboratory nests were maintained in culture rooms at 27 (±2) ^{0}C and 80% (± 10%) relative humidity. The ants built their fungus gardens in clear plastic 'domes' of 2.5 litre capacity, inverted on porous ceramic tiles and with a coarse wire mesh tube inside to encourage building. These domes were placed on tables, the legs of which were standing in dishes of engine oil to prevent the ants from escaping.

The culture rooms were illuminated with light from fluorescent tubes for 12 hrs daily. However, in the field, fungus gardens are built underground and only foraging workers are exposed to light.

Each nest was fed once daily on a variety of in-season leaves. Under natural conditions, forage preferences vary with time (Cherrett 1972b) and nests are highly polyphagous (Cherrett 1968, Rockwood 1976) therefore nests were provided with a varied diet (Table 2.1).

Nest sizes were not restricted and fresh domes were supplied as necessary. Large amounts of forage were supplied (0.6-0.9 kg per day for the large *Atta sexdens* nest), plus extra sources such as fruit, which take longer

for workers to cut up and acted as a buffer forage source. This allowed nests to develop more like those in the field, encouraging natural behaviour and caste ratios and ensured that large amounts of fungus garden were available for experimentation. However, excavations of field nests of *Atta vollenweideri* have shown that 3,000 chambers may be present (Jonkman 1980), making even the largest laboratory nests appear small by comparison, although not all of these chambers contain fungus garden.

Table 2.1: Typical substrates used to feed the laboratory leafcutting ant colonies over one year.

MONTH	TYPE OF FORAGE SUPPLIED		
	MAJOR SOURCE	OCCASIONAL SOURCE	MINOR CONSTANT SOURCE
January February	Privet	*Escalonia*, Holly, Portugal laurel, *Rhododendron*, Veronica	Cabbage, various fruit and vegetables, flowers, dried products (oat-flakes, bread, lentils, soya meal)
March April May	Ash, Elder, Sycamore, Knotweed	Alder, Beech, Lime, Oak, Willow	
June July August September October	Ash, Beech, Chestnut, Elder, Knotweed	Alder, Birch, Lime, Oak, Willow	
November December	Privet	*Escalonia*, Holly, Portugal laurel, *Rhododendron*, Veronica	

- continued overleaf

Key to Latin names:

Alder, *Alnus* spp.
Ash, *Fraxinum excelsior*
Beech, *Fagus sylvatica*
Birch, *Betula* spp.
Chestnut, *Aesculus hippocastanum* and *Castanea sativa*
Elder, *Sambucus nigra*
Escalonia sp.
Holly, *Ilex aquifolium*
Knotweed, *Reynoutria japonicum* and *Reynoutria sachalinensis*
Lime, *Tilia* spp.
Oak, *Quercus* spp.
Portugal laurel, *Prunus lusitanica*
Privet, *Ligustrum ovalifolium*
Rhododendron sp.
Sycamore, *Acer pseudoplatanus*
Veronica, *Hebe* sp.
Willow, *Salix* spp.

2. Culture material available for study

Most studies were made using a large *Atta sexdens* (L.) nest. This was approximately 15 years old in 1989 and had 75 gardens. By September 1992, it had 92 gardens. Other nests were also used, including a 10 year old nest of *Atta cephalotes* (L.) (initially with 40 gardens, but with 57 at the conclusion of studies), a young nest of *Atta laevigata* (Smith) (brought from Brazil in 1990, with 1 garden) and a colony of *Acromyrmex octospinosus* (Reich). Three more *Atta sexdens* nests were also used, two of which were brought from Venezuela in 1989, grew to sizes of 7 and 8 gardens respectively, then declined and died in 1990. The third nest, aged 15 years and with 22 gardens, also died in 1990.

A queenless colony of *Myrmica ruginodis* Nyl., an omnivorous Myrmicine ant found abundantly in Britain (Donisthorpe 1915), was also used. A colony of approximately 300 workers was maintained in a 1 litre box, half-full of damp soil which was re-moistened daily. It was fed on sucrose

crystals and freshly-killed insects (cockroaches and mealworms). The colony died after 5 months, when the soil was accidentally allowed to dry out.

3. Classifying fungus garden into age types

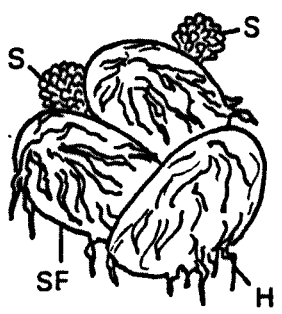
Fungus garden is heterogeneous and Weber (1972) described how structure and colour vary from the top to the base of a garden. For these investigations, three ages of garden were defined (Fig. 2.1). Young garden, where the majority of new substrate is added, is usually found at the top, with aging garden at the bottom, but if fungus gardens are disturbed this pattern can be upset (pers.obs).

MICROSCOPIC APPEARANCE

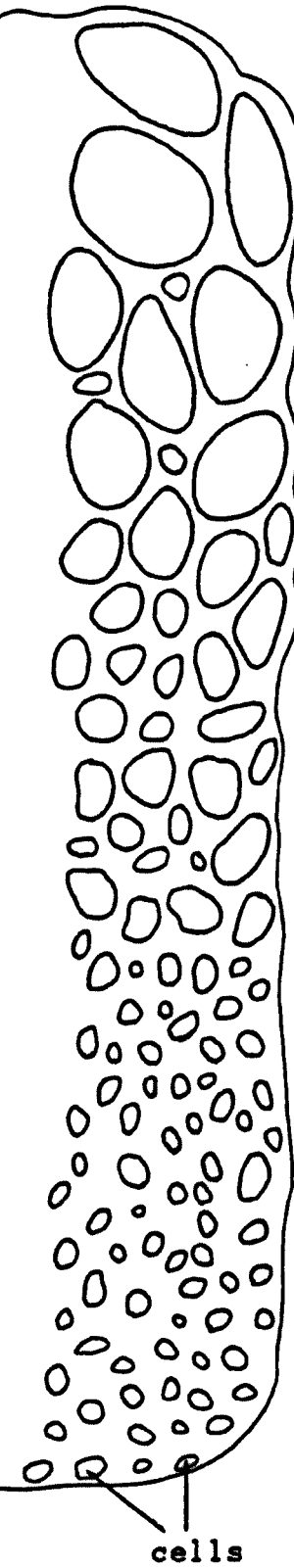
PHYSICAL APPEARANCE

YOUNG GARDEN
Large thin-walled cells
>1 cm diameter
Colour: green/grey
Few or no staphylae
Luxuriant hyphal growth
Loosely packed substrate particles

MATURE GARDEN
Medium-sized cells
Colour: khaki
Staphylae common
Hyphae form a mat;
no longer luxuriant



AGING GARDEN
Small thick-walled cells,
<0.5 cm diameter
Colour: yellow/brown
Staphylae common
Hyphae form thick ropy masses
Densely packed substrate particles



H = Hyphae
S = staphyla
SF = Substrate fragment
Scale: ⊢——⊣ 1 mm

Figure 2.1: The structure of a typical fungus garden and its division into three ages.

4. Defining the castes of *Atta*

In *Atta*, there is a continuous size range from 2-15 mm in body length. Head size increases disproportionately with body length, due to a diphasic allometry (Oster and Wilson 1978). Workers can be divided up into four groups based on behavioural role (Wilson 1980a); 'minima ', based around headwidth 1 mm are gardener-nurses, those with headwidths 1.4-1.6 mm are within-nest generalists, workers centred around headwidth 2.2 mm are forager-excavators and those with the largest headwidths are defenders. Weber (1972) divided *Atta* workers into four arbitrary groups based upon body length; minima (2-3 mm), media (4-6 mm), maxima (7-9 mm) and soldiers (10-15 mm)*.

In this thesis, both measures were used, depending upon the type of observations being made. Headwidth is a more accurate measure of size in terms of behaviour, but if many observations are being made in a short space of time, estimating body length may be more useful. Headwidths were measured using a binocular microscope and eyepiece graticule, while body lengths were either estimated by sight or measured with a ruler to classify workers into four groups. The latter are however, only a crude measure.

However, Weber and Wilsons' classifications (1972 and 1980a, respectively) do not directly correspond. Regressing the headwidths of 50 *Atta sexdens* workers against their body lengths yielded the regression equation:

headwidth (mm) = 0.489 body length (mm) - 0.538

$p<0.001$, $Rsq_{(adj)}$ 97.1 %, df 49 (plotted in Fig. 2.2)

Using this equation to calculate headwidths from Weber's arbitrary classes shows that minima are smaller than for Wilson's classification and the other three castes are larger (Table 2.2). However, the regression may not be valid for the largest workers, since head size increases disproportionately to the rest of the body (Oster and Wilson 1978).

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* These are referred to as 'castes' throughout this thesis. Strictly speaking however, they are arbitrary sub-divisions of a single variable worker caste - the true castes of *Atta* are gyne, male, worker and soldier.

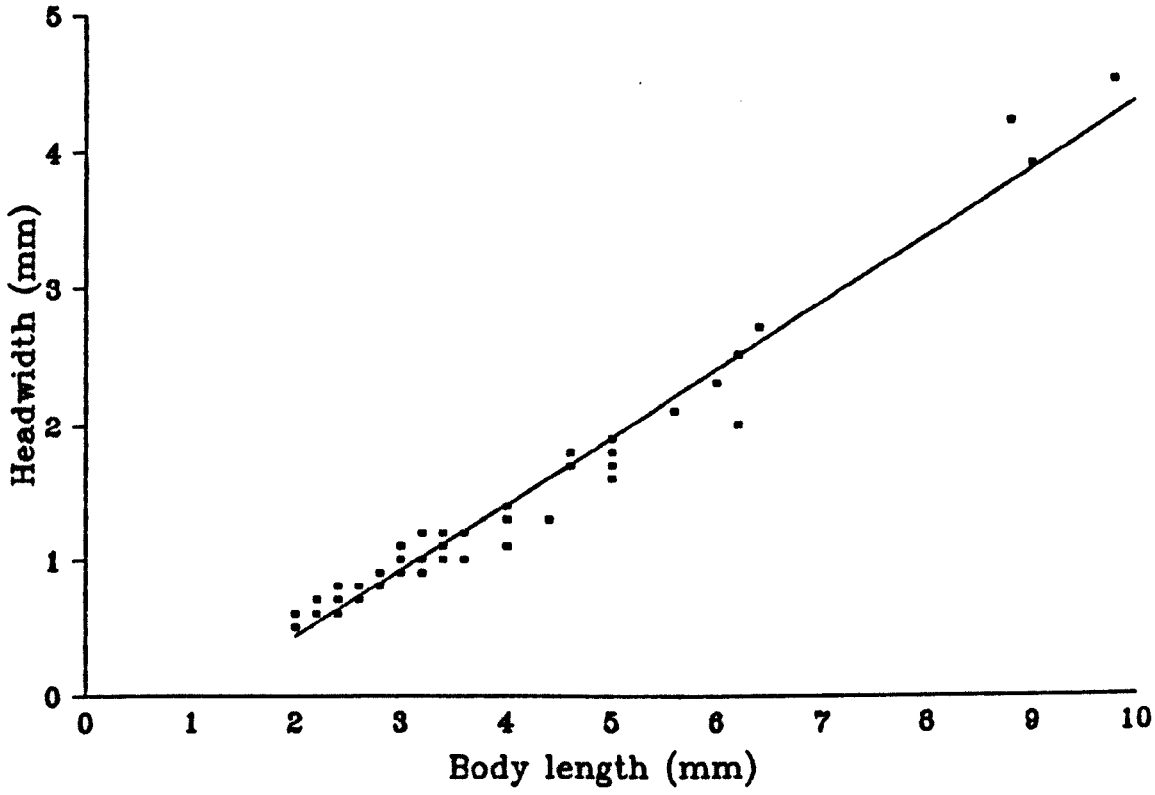


Figure 2.2: Headwidths (mm) of *Atta sexdens* workers plotted against body lengths (mm), shown with the regression line ($p<0.001$, $Rsq_{(adj)}$ 97.1%, df 49).

Table 2.2: Headwidths of workers of Weber's (1972) arbitrary caste sizes calculated from a regression equation for body length and headwidth, compared with Wilson's (1980a) definitions.

CASTE	BODY LENGTH (mm)	HEADWIDTH (mm)	WILSON'S CASTE DEFINITIONS - HEADWIDTHS (mm)
Minima	2 - 3	0.4 - 0.9	around 1.0
Media	4 - 6	1.4 - 2.4	around 1.4
Maxima	7 - 9	2.9 - 3.9	around 2.2
Soldier	10 - 15	4.4 - 6.8	> 3.0

5. Separating workers from fungus garden

Fungus garden and workers were removed from the nest by lifting domes away carefully and gently pulling out pieces of garden with a spatula. These were dropped into containers with Fluon-coated sides. 'Fluon' (P.T.F.E, ICI) provides a surface too slippery for insects to walk on.

Two methods were used to separate workers from garden:

a) Cooling

To obtain workers, fungus garden freshly removed from the nest was placed in a container with Fluon-coated sides in the freezer compartment of a refrigerator at 0^{0}C, for 1-2 minutes. Immobilised workers could then be removed from the garden using fine forceps.

b) Carbon dioxide anaesthesia

Cooling damaged the fungus garden, so where ant-free fungus garden was required for subsequent studies, garden and attached workers were placed into a container with a lid and carbon dioxide gas was introduced.

The second method could also be used to obtain workers, but was more stressful and caused rectal evacuation. Workers rapidly recovered from both methods of anaesthesia with no ill effects. The resilience of these ants was demonstrated by a group of maxima workers left overnight at 0^0C to kill them, more than half of which recovered when they were returned to room temperature.

When ant-free fungus garden was required, sterile containers and implements were used to reduce the risks of contamination by alien fungi. Workers were most easily separated from young and early-mature garden, as the brown colour and denseness of aging garden made separation more difficult. Consequently, garden from the upper areas was used for experiments.

6. General statistical analysis of results

All data were analysed using the MINITAB statistical package. All data were checked for normal distributions and non-normally distributed data were transformed to achieve normality where possible. Data were then analysed using either parametric or non-parametric tests, as appropriate. Before statistical analysis, percentages were transformed by dividing them by 100 and calculating arcsine values, to normalize them.

Non-parametric tests are applicable wherever parametric tests are and also where they are not (i.e. on non-normal data). Parametric tests can be quite sensitive to a few outlying observations, whereas non-parametric tests are more resistant to distortion from a few gross errors. However, where a parametric test is applicable, it will be more efficient than the corresponding non-parametric test (larger samples may be required to detect differences when using non-parametric tests). Non-parametric tests are also based on summary statistics rather than the actual numerical values of the data (Whitaker 1990). However, if significance is found using a non-parametric test, then transforming the data to obtain a normal distribution and analyzing with a parametric test

will provide no clarification. In many cases, non-parametric tests are therefore quicker and simpler than transforming data to produce a normal distribution. Non-parametric tests also mean that the data remain in their original form rather than being transformed to the logarithmic equivalent or similar. This makes it more immediately understandable to the reader.

The parametric tests used were Chisquare analysis, analyses of variance, multiple comparisons and regression. Both one-way and two-way analyses of variance were employed, depending upon whether one or two types of variable were present. Where one-way analyses of variance (referred to throughout as 'ANOVA') were carried out between more than two columns, multiple comparisons were used to determine the statistically significant (95% confidence) relationships between them. Tukey's multiple comparison was used where replicate numbers were the same and Scheffe's multiple comparison was used where replicate numbers varied between treatments.

Non-parametric tests used were Mann-Whitney U and Mood tests. The first, equivalent to a parametric T-test and almost as efficient, was carried out between pairs of columns, while the latter, equivalent to ANOVA, was used when comparing several columns (Whitaker 1990).

Parametric data were described using means and standard errors, although 95% confidence limits were used in figures, enabling significant differences to be more easily seen. Non-parametric data were expressed using medians and 95% sign confidence limits (Whitaker 1990).

Chapter 3: The dynamics of fungus gardens

INTRODUCTION

In agriculture, many crops require discontinuous culture. Sugar beet for example, can only be grown on the same land for one in every four years, because of the soil-borne beet cyst nematode, *Heterodera schachtii* and this rotation is enforced by law (MAFF 1978). Without rotation, the pest builds up as crops are grown in successive years, until total crop failure results. In contrast, leafcutting ants seem to practise just such a temporal monoculture. Some monocultures such as winter wheat, which can be directly drilled into the stubble of the previous crop, are successful in agriculture but these are intensive systems and require high inputs of resources. The Attine fungus monoculture also requires a high input and if the workers are removed, the fungus garden is quickly overrun by alien organisms (Weber 1972). The workers continually bring contaminant spores into the nest on imported forage material. However, as Powell (1984) pointed out, there is a spatial separation in a fungus garden between metabolite-rich aging garden and nutrient-rich young garden. The ants can easily remove potentially dangerous exhausted substrate.

When *Atta* nests are excavated, large numbers of chambers may be found and many of these are empty. Jonkman (1980) excavated a nest of *Atta vollenweideri* and 339 out of 614 mapped chambers were empty, although these were in an inactive part of the nest. In a second nest with no inactive zone, only 65 out of 481 mapped chambers contained no fungus garden. Some species under desert conditions build gardens in different chambers during different seasons (Weber 1972), which suggests that some leafcutting ants practise discontinuous culture. A nest of *Trachymyrmex urichi* in Panama had seven small chambers in a vertical series, which allowed them to move upwards or downwards

according to season, with no additional excavation (Weber 1982).

This chapter examines whether continuous or discontinuous culture is the primary method used by leaf cutting ants in the laboratory, in the absence of environmental stimuli. Discontinuous culture reduces pest carryover problems but might require more organisation than continuous culture, since different gardens would be at different stages. Worker populations on the fungus garden surface are large (Weber 1972) and workers produce antibiotic chemicals like myrmicacin and phenylacetic acid (Maschwitz *et al.* 1970, Schildknecht and Koob 1970, 1971), which protect the fungus garden against alien fungi (Powell 1984). This suggests that the ants could cope with continuous culture. Another reason why continuous culture may occur is that fungus gardens can be divided into young, mature and aging zones, indicating that they persist for some time and that material is added gradually rather than all at once.

In this chapter, the development of new fungus gardens is examined, while established gardens are followed over time to see if they are built up at different rates.

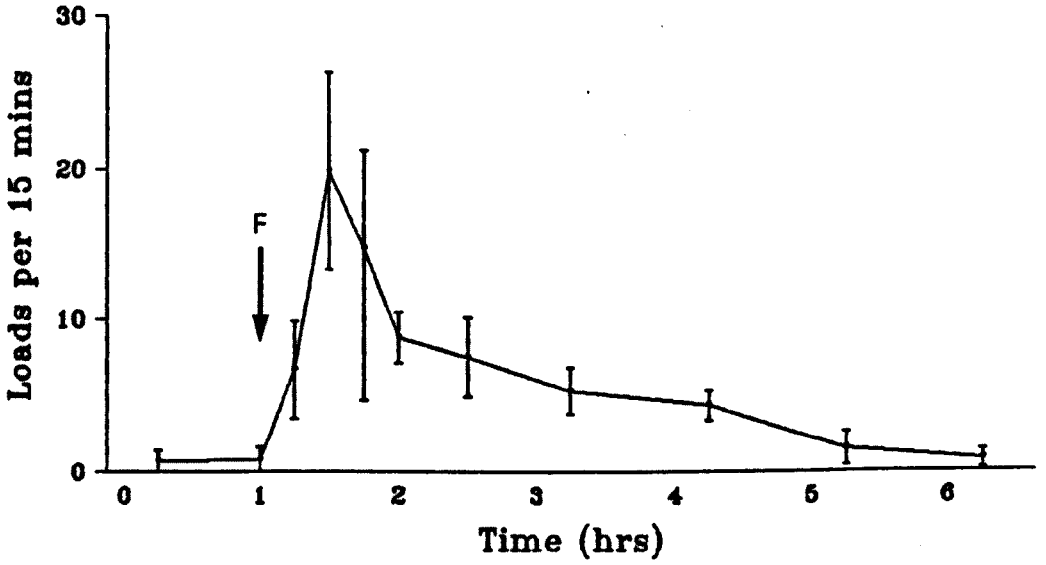
MATERIALS AND METHODS

The intakes and outputs of developing and established fungus gardens of an *Atta sexdens* nest (initially of 75 gardens) and an *Atta cephalotes* nest (initially 40 gardens) were recorded. At least one clean unoccupied dome was available to each nest at any time.

1. Determining optimum observation times

Because forage intake into gardens is not constant over 24 hrs, an optimum observation time for recording it was defined. Comparing peak intakes was preferable to

a) Privet



b) Sycamore

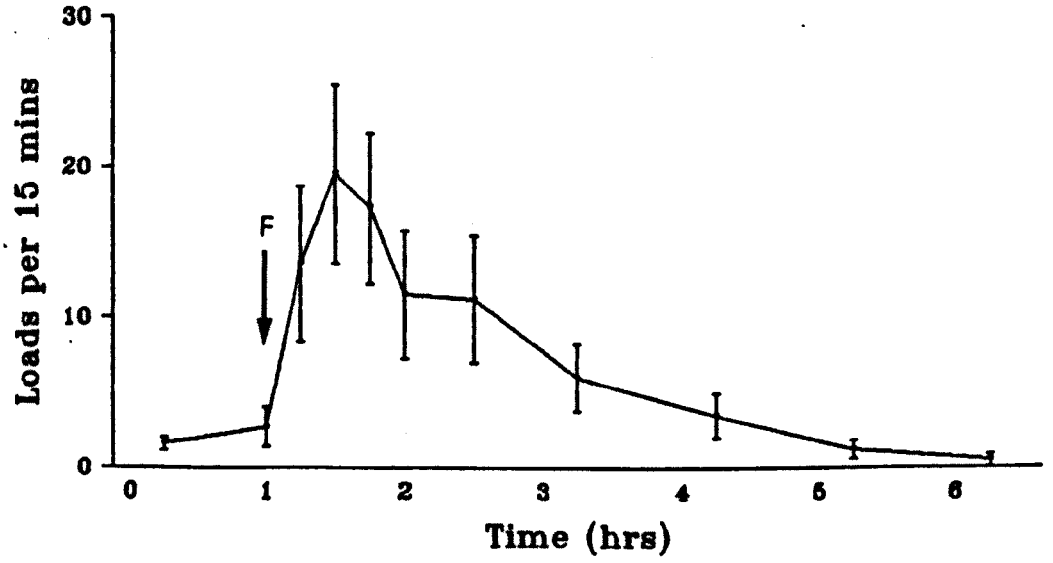


Figure 3.1: Mean intakes (± 95% confidence limits) of two types of forage, a) privet (*Ligustrum ovalifolium*) and, b) sycamore (*Acer pseudoplatanus*) per 15 minute observation period, for six fungus gardens of the same nest, over 6 hrs. Forage was supplied after 1 hr, at 'F'.

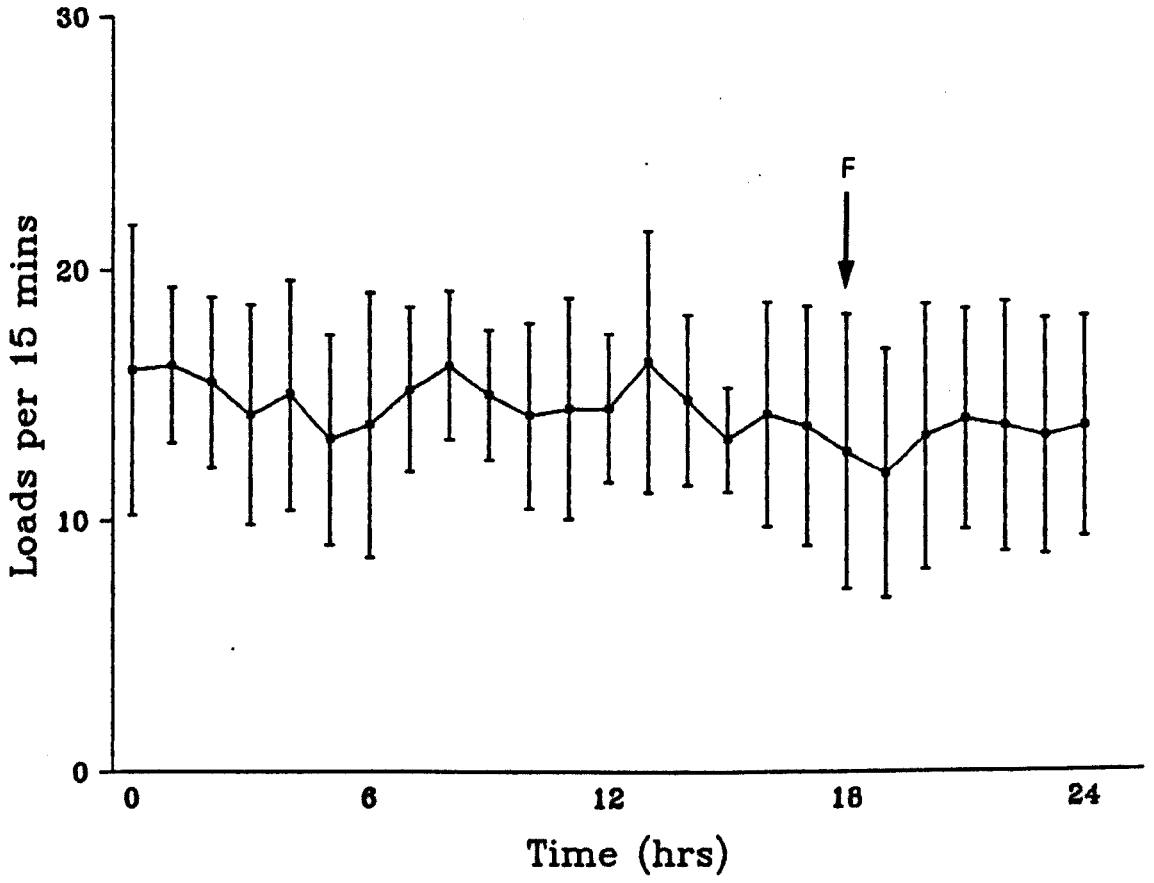


Figure 3.2: Mean outputs (± 95% confidence limits) of refuse loads per 15 minute observation period, for six fungus gardens of the same nest over 24 hrs, beginning at 1700 hrs. Forage was supplied at 'F'.

comparing random ones, which would range from peak to zero intake, when no forage was available.

Numbers of loads of forage carried into six gardens during 15 minute periods were recorded every hour from 1000 to 1600 hrs on one day, forage being supplied at 1100 hrs. This was carried out for two types of forage; 'tough' forage, using mature privet leaves (*Ligustrum ovalifolium*) and 'soft' forage, using young sycamore leaves (*Acer pseudoplatanus*). Similarly, numbers of loads of refuse carried out of six gardens during 15 minute periods were recorded every hour for 24 hrs, starting at 1700 hrs. These refuse output observations were carried out only once.

Plotting numbers of loads carried into or out of gardens at different times showed the times of peak forage intake and refuse output. Peak intakes for both privet and sycamore were between 15 and 45 minutes after forage was supplied (Fig. 3.1). This period was therefore used as the optimum observation time for forage intake. Refuse output over 24 hrs was constant, therefore any observation time was equally suitable (Fig. 3.2). However, during the peak forage intake period, forage loads obscured other types of load, making observations difficult. To ensure that all loads were recorded, refuse output and other types of load (e.g. brood, fungus garden, staphylae) were recorded after 1600 hrs, when forage intake was low (forage was provided daily at around 1100 hrs).

2. Examining optimal observation times for foraging on different substrates

To illustrate how peak forage removal times vary with forage type, the rates of removal of ten different substrates by the *Atta sexdens* nest were examined. Samples of forage weighing 20 g (wet weight) were placed on a forage table connected to the nest via a single bridge with a mark half way along its length. Numbers of loads of forage being carried across this mark per minute were

recorded at intervals over the next 2 hours. No other forage was available. The substrates supplied were:

(1) Cabbage leaves (*Brassica oleracea*)
(2) Camellia petals (*Camellia* sp.)
(3) Citrus peel (*Citrus sp.*)
(4) Elder leaves (*Sambucus nigra.*)
(5) Holly leaves (*Ilex aquifolium*)
(6) Knotweed leaves (*Reynoutria sachalinensis*)
(7) Magnolia petals (*Magnolia x soulangeana*)
(8) Oat flakes (Quaker Porridge Oats)
(9) Privet leaves (*Ligustrum ovalifolium*)
(10) Sycamore leaves (*Acer pseudoplatanus*)

Mean wet weights of loads of each substrate were recorded by removing 100 loads at random as they were carried across the bridge and weighing them on a Unimatic SN1 balance. Percentage dry weights were measured by drying 5 g samples of each substrate in an incubator at 50°C, then reweighing. The ratio of wet to dry weight can be used as a measure of toughness, therefore these figures could be compared with the removal rates.

3. Recording the presence of brood on the garden surface

Numbers of brood present on the garden surface were recorded by counting groups of brood, since brood items are usually clumped together. Three faces of each dome were visible, therefore total numbers of brood groups were counted for all these for each garden. An estimate of brood group size was obtained by counting individuals in a random sample of groups, using a binocular microscope and a cold fibreoptic light source (Schott KL 1500-T).

4. Recording fungus garden sizes

Fungus garden sizes were recorded by tracing silhouettes of the frontal faces (with entrance holes) of the observation domes on to cellulose acetate sheets. An

acetate sheet was placed against the dome and the outline of the garden quickly drawn on to it with a marker pen. Gloves were worn to protect against ant bites. 'Silhouette' areas were then measured by tracing these images with a digitiser cursor.

5. Analyses of results

Time series analysis was used, which investigates the dependence between observations made at different times (Diggle 1990). Series of data with no missing values and spaced at regular intervals are required. Calculations were carried out using the MINITAB statistical package.

a) Autocorrelation

This measures the linear dependence between observations. The autocorrelation coefficient, r_k is then plotted against time (expressed as 'lags') to produce a correlogram, the appearance of which can be used to interpret the data (Fig. 3.3). For a random time series, r_k will be approximately zero for all non-zero values of the lag, although occasional significant values may occur by chance. Significance levels (approximately 95% confidence) are determined by $\sqrt{N}$, where N is the number of observations in the series.

Where data have a short-term correlation and the most recent observation is related to the one before it rather than to observations a long time ago, r_k has a fairly high value at lag 1, followed by two or three values which, while significantly larger than zero, tend to get successively smaller, or 'decay'. Where data alternate around the mean, r_k values also alternate around zero, although the alternating peaks decay to zero after a few lags. If a time series contains a trend, then r_k will stay on one side of zero and will not approach it except at very large lag values. These are non-stationary series, where an observation on one side of the mean tends to be followed by many more on the same side of the mean. In stationary

LAG	r_k	-0.8 -0.6 -0.4 -0.2 0.0 0.2 0.4 0.6 0.8 r_k
1	0.580*	XXXXXXXXXXXXXXXXX
2	0.157	XXXXX
3	-0.045	XX
4	-0.113	XXXX
5	-0.191	XXXXXX
6	-0.100	XXX
7	0.010	X
8	0.044	XX
9	0.035	XX
10	0.005	X
11	-0.058	XX
12	-0.034	XX
13	-0.000	X
14	0.005	X
15	0.038	XX

Figure 3.3: An autocorrelation correlogram produced by MINITAB for a series with 26 observations (improvised data) showing autocorrelation coefficients (r_k) plotted against time (lags). Values of r_k larger than ± 0.39 are significant ($p<0.05$) and are marked with a *. Unless otherwise specified, MINITAB prints out r_k values up to $\sqrt{(n)}+10$ lags, where n = number of observations. This series shows a short-term correlation, since the only significant r_k value is at lag 1 and is followed by rapid decay.

series, the observations fluctuate around the mean (Chatfield 1984).

b) Differencing

'Differencing' removes trends in time series data and renders non-stationary data stationary. The difference Dy_t of a time series y_t is defined by:

$$Dy_t = y_t - y_{t-1}$$

This is a measure of the change between one observation and the next (Diggle 1990). Second order differencing D^2y_t, was used for the data obtained in this study and is defined by:

$$D^2y_t = Dy_t - Dy_{t-1}$$

c) Cross correlation

Cross correlation can be used to examine the relationship between two time series and calculates the cross-correlation function, $r_{xy}(k)$, between observations in the two series which were made at the same time. These are then plotted against time (expressed as 'lags') (see Fig. 3.4). Independent series will have $r_{xy}(k)$ values of zero for all lags. Positive or negative coefficients occur depending upon whether two related series are in or out of phase. The significance (approximately 95% confidence) of these values is determined by $\sqrt{N}$, N being the number of observations in the series (Diggle 1990).

If a significant coefficient occurs at lag 0, the two series are directly correlated. If significant values occur after several lags, then one of the series may be related to the other when delayed by this number of lags. If N is quite large (approximately 100) two uncorrelated series may produce significant coefficients by chance. However, the presence of large numbers of significant values generally indicates that the two series are related.

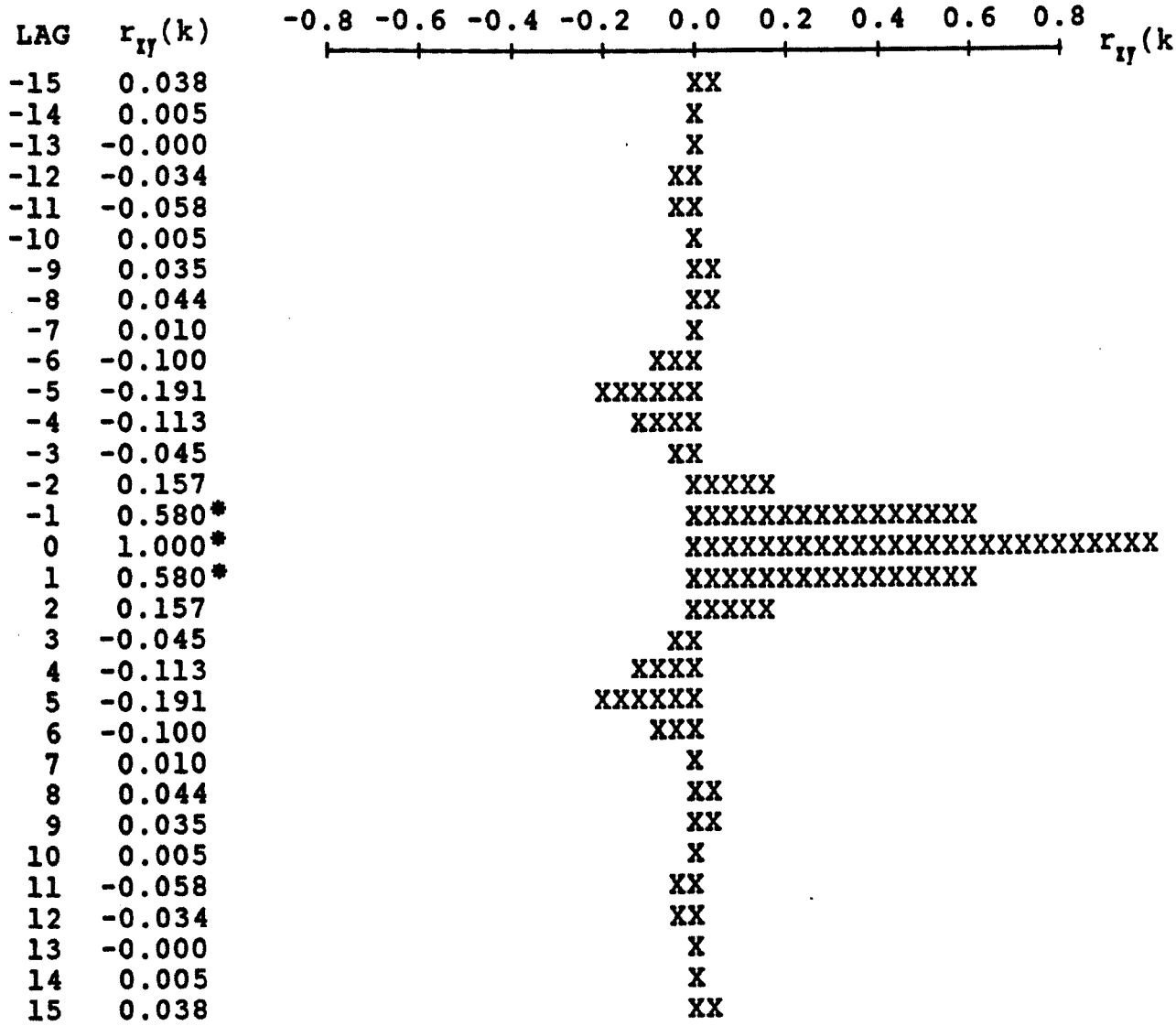


Figure 3.4: A cross-correlogram produced by MINITAB for two closely related series, each with 26 observations, showing cross-correlation coefficients ($r_{xy}(k)$) plotted against time (lags). Values greater than ± 0.39 are significant ($p<0.05$) and are marked with a *. Unless otherwise specified, MINITAB prints out values up to $\sqrt{(n)}+10$ lags, where n = number of observations. These series have a direct positive correlation, with significant coefficients around 0 lags.

RESULTS

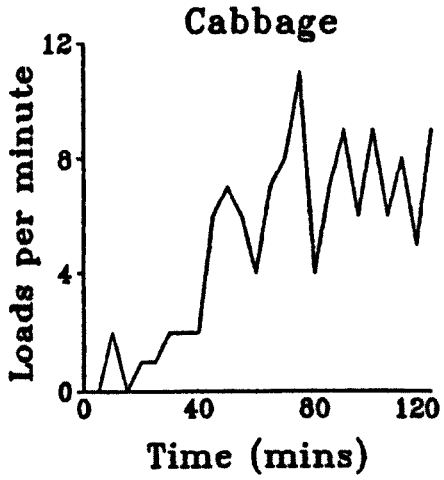
1. Comparing the removal rates of different types of forage

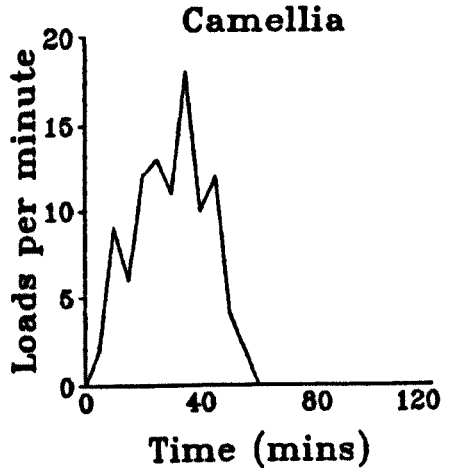
This experiment illustrated how removal rates vary with forage type, thereby affecting the observational windows defined earlier. Because this was just an illustration, each substrate was tested only once.

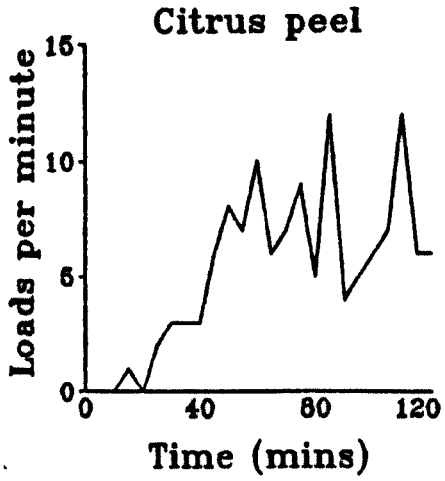
Different substrates were removed at widely differing rates (Fig. 3.5). Some were removed rapidly (knotweed, sycamore and elder leaves) while others were removed slowly (cabbage, citrus peel and holly leaves). Oat flakes were picked up in large numbers immediately, but overall were removed slowly because they were small and light. Camellia and magnolia petals and privet leaves were removed at intermediate rates.

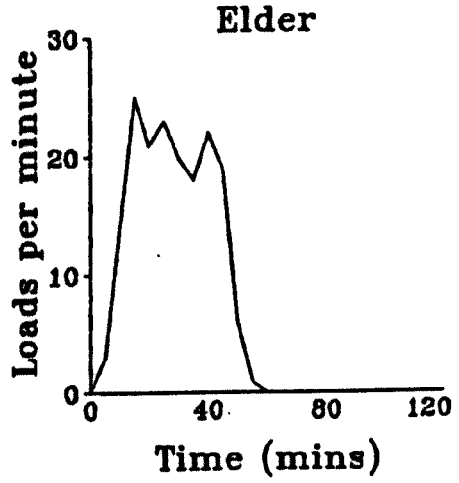
Comparing median load size (data were not normally distributed and could not be transformed) and percentage dry weight (Fig. 3.6) of each substrate with their removal rates showed that there was little correspondence between removal times, load sizes and dry weights. Regressing these data showed no significant relationship ($p>0.5$, $Rsq_{(adj)}$ 0%, df 9) However, holly leaves did have large load size and a high percentage dry weight. In theory, high percentage dry weight might mean large load size and possibly slow removal time. However, if workers seek to cut loads of a given weight, then load size will be independent of density.

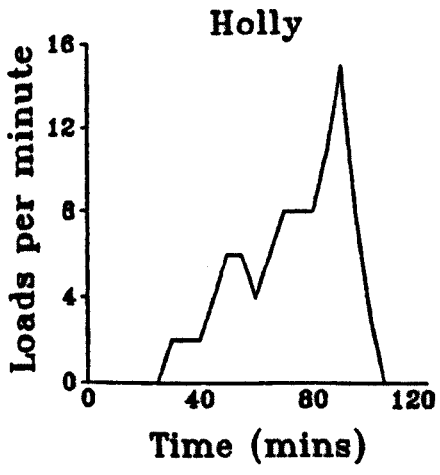
Figure 3.5: Removal rates (loads per minute over 120 minutes) of ten types of forage offered to an *Atta sexdens* nest.

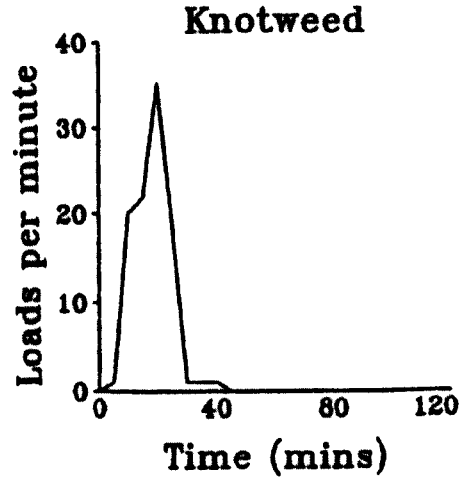












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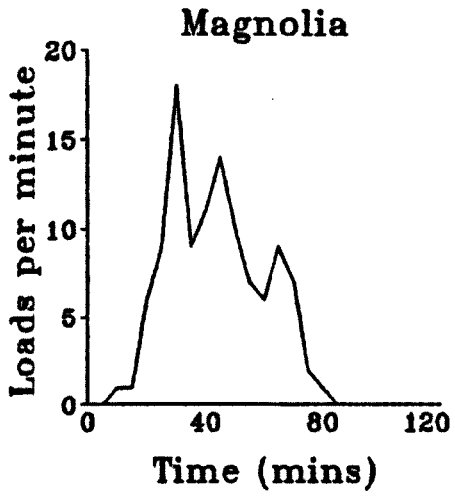
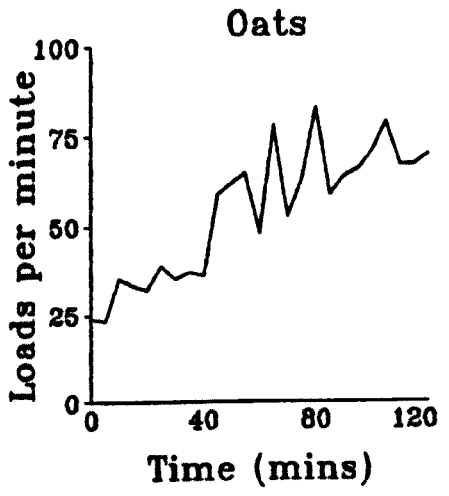
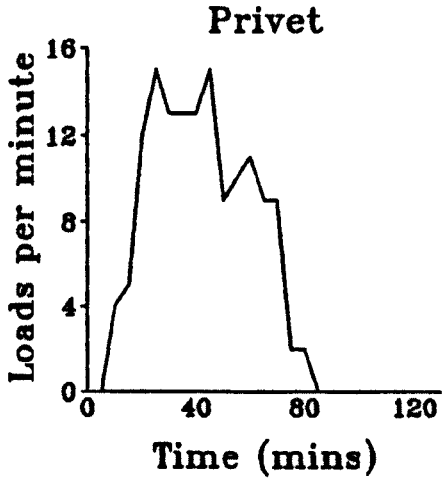
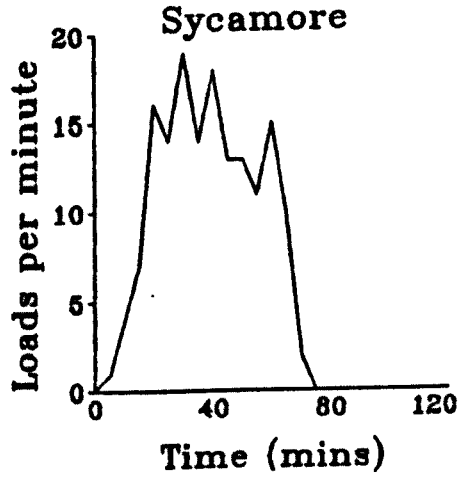

Magnolia
Loads per minute
20
15
10
5
0
0
40
80
120
Time (mins)


Oats
Loads per minute
100
75
50
25
0
0
40
80
120
Time (mins)


Privet
Loads per minute
16
12
8
4
0
0
40
80
120
Time (mins)


Sycamore
Loads per minute
20
15
10
5
0
0
40
80
120
Time (mins)

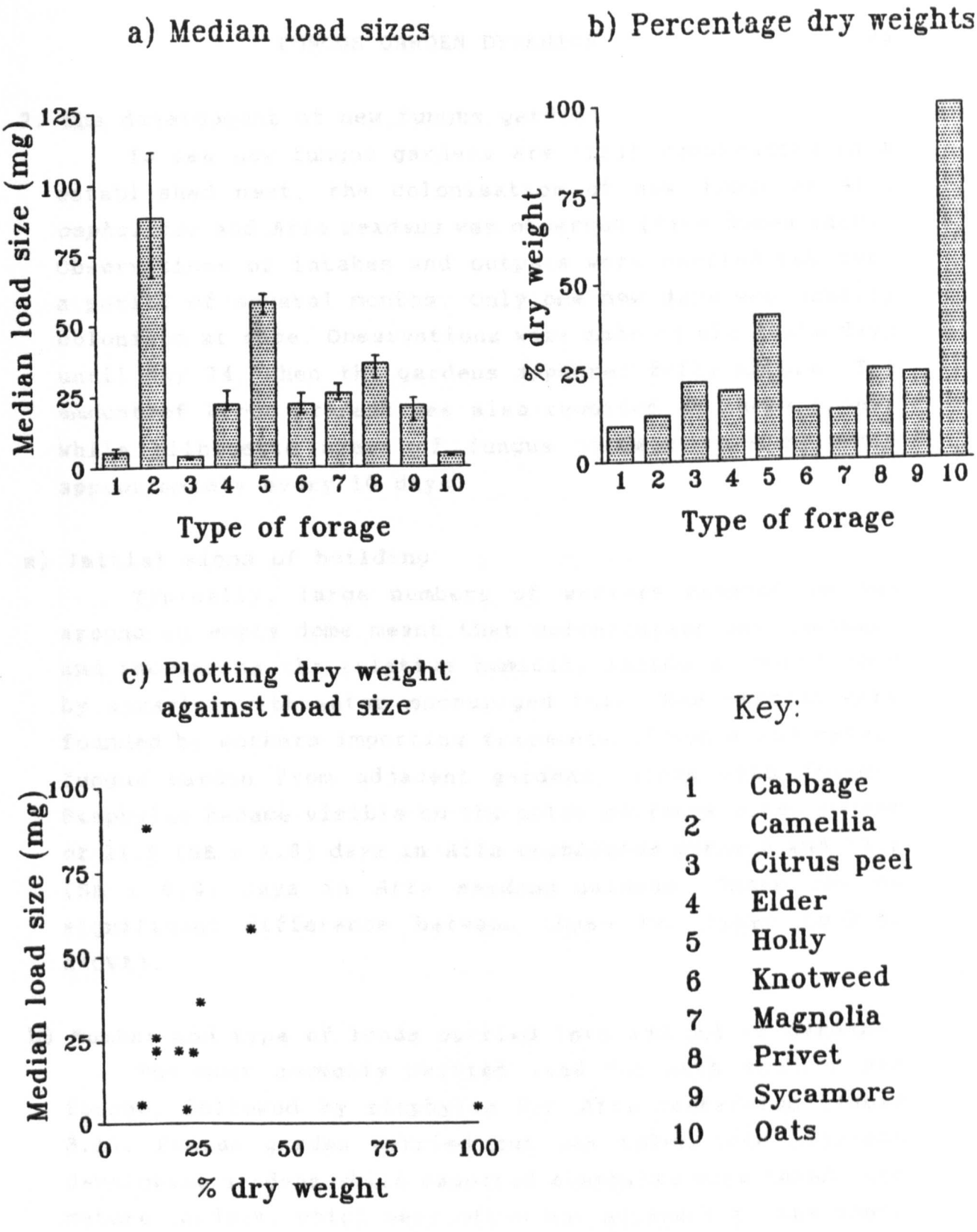


Figure 3.6: Median load sizes (mg, ± 95% confidence limits, 100 replicates) of ten types of forage offered to an *Atta sexdens* nest, compared with their percentage dry weights (one 5 g sample for each forage type).

2. The development of new fungus gardens

To see how fungus gardens are first constructed in a established nest, the colonisation of new domes by *Atta cephalotes* and *Atta sexdens* was observed (five domes each). Observations of intakes and outputs were carried out over a period of several months. Only one new dome was usually colonised at once. Observations were made on alternate days until day 74, when the gardens appeared fully mature. The amount of brood present was also recorded at these times, while silhouette areas of fungus gardens were recorded approximately every 10 days.

a) Initial signs of building

Typically, large numbers of workers present in and around an empty dome meant that colonisation was imminent and increasing the relative humidity inside an empty dome by spraying with water encouraged this. New gardens were founded by workers importing fragments of young and mature fungus garden from adjacent gardens, along with forage. Staphylae became visible on the outer surfaces after a mean of 11.8 (SE ± 1.8) days in *Atta cephalotes* gardens and 13.2 (SE ± 0.8) days in *Atta sexdens* gardens. There was no significant difference between these two times ($p>0.4$, ANOVA).

b) Number and type of loads carried into and out of gardens

The most commonly carried load for both species was forage, followed by staphylae for *Atta cephalotes* (Table 3.1). Fungus garden carried out was taken into adjacent developing gardens while exported staphylae were taken into mature gardens, which were often not adjacent to the donor young garden.

Table 3.1: Mean numbers (± SE) of each type of load recorded being carried into and out of five developing fungus gardens of two species of *Atta*, over 74 days of observation (recorded on alternate days).

TYPE OF LOAD	MEAN NO'S OF LOADS (± SE) CARRIED INTO OR OUT OF FUNGUS GARDENS OF:			
	ATTA CEPHALOTES		*ATTA SEXDENS*	
	IN	OUT	IN	OUT
Forage	451.8 ± 52.5*	ǂ	417.6 ± 11.2*	ǂ
Refuse	0	98.2 ± 20.8*	0.2 ± 0.2	89.6 ± 10.8*
Fungus	37.4 ± 19.2*	2.2 ± 2.0	39.3 ± 11.1*	0.4 ± 0.2
Staphylae	15.4 ± 5.8*	218.8 ± 77.6*	15.8 ± 1.5	3.4 ± 0.9
Larvae	2.0 ± 0.3	3.2 ± 1.6	4.2 ± 2.0	9.4 ± 5.2
Pupae	0.8 ± 0.8	1.2 ± 0.6	2.2 ± 1.3	25.2 ± 22.5
Mature workers	0	0	0	0
Callow workers	2.0 ± 1.4	0	0	3.4 ± 2.9

ǂ These were not recorded, although some forage was carried out of domes.

* Significant differences ($p<0.005$, Chisquare) occurred between individual gardens.

Total numbers of loads of each type entering or exiting were compared between gardens using a 5 x 2 Heterogeneity Chisquare test on the numbers of loads versus the total of all other loads carried. For *Atta cephalotes*, forage, fungus and staphyla intakes and refuse and particularly staphyla outputs all varied significantly between gardens ($p<0.005$). For *Atta sexdens*, forage and fungus intakes and refuse outputs all varied significantly between gardens ($p<0.005$), but staphyla intakes did not ($p>0.05$). Staphyla outputs could not be compared for this species, because the figures were too small.

c) Amount of brood

There were three types of brood groupings; larvae only, pupae only and mixed (prepupae also occur, but were too difficult to distinguish). Numbers of individuals in about 50 of each type were examined on the surface of established fungus gardens using a binocular microscope.

Pupal groups tended to be found in upper areas of the garden, whereas larvae were more common in lower areas. Dissecting a fungus garden showed that brood were found both on the exterior and within internal cavities.

The three types of group had different sizes (Table 3.2) and these were significantly different ($p<0.001$, Mood). Larval and mixed groups were significantly larger than pupal ones ($p<0.01$, Mann-Whitney) but larval groups were not significantly different in size to mixed ones ($p>0.1$, Mann-Whitney). Mixed groups contained significantly more larvae than pupae ($p<0.01$, Mann-Whitney).

Table 3.2: Median numbers (± 95% confidence limits) of larvae and pupae in the three types of brood group observed. (N = number of replicates).

GROUP TYPE	MEDIAN NUMBERS OF BROOD ITEMS	N	95% CONFIDENCE INTERVALS
LARVAL	16	57	11.0,23.7
PUPAL	9	50	5.0,13.0
MIXED - Total	22	47	16.0,24.6
- Larvae	12	47	9.0,16.0
- Pupae	7	47	6.0,8.6

d) Changes over 74 days

Figs. 3.7a and b show mean forage intakes, refuse outputs, silhouette areas, brood numbers and fungus intakes for the two species (see Appendix 1.1 for raw data). During

the first few days of construction, forage intake rose sharply in both *Atta cephalotes* (Ac) and *Atta sexdens* (As) gardens, but little refuse was expelled until after 40-50 days. Brood numbers present also increased gradually with time, although more were present in As than in Ac gardens during the first 10 days. Silhouette areas increased rapidly and reached an approximate maximum size by 25 days. As gardens appeared to increase more smoothly in size than Ac gardens. Fungus intake was high during the first 15 days, with both species having a large peak during the first 5 days, just before forage intake reached its maximum levels. A second peak also occurred after 10 days, which was small in Ac gardens but large for As gardens. Regressing peak forage intakes for all ten gardens during the first 20 days against peak fungus intakes, showed no significant relationship ($p>0.8$, $Rsq_{(adj)}$ 0%, df 9).

Small numbers of staphylae were carried into As gardens around day 10, but few were taken in at other times (Fig. 3.8b). Ac gardens showed no such pattern and staphylae were carried into gardens at variable times (Fig. 3.8a). Few staphylae were carried out of As gardens but they were taken out of Ac gardens in large numbers at certain times (Fig. 3.9). Staphylae were taken out of Ac Garden 1 (Ac1) after day 70, out of Ac2 after day 50, Ac3 after day 40 and out of Ac4, after day 10, while almost none was taken from Ac5. However, individual gardens were observed at different times since the ants built only one new garden at a time. Outputs of staphylae from Ac1-4 all began around April 24th, while Ac5 was constructed from May 5th onwards.

The data were then subjected to time series analysis, except for silhouette area data, which were collected at approximately 10 day intervals rather than on alternate days. Raw data were used, since there were no missing values and data were taken at regular intervals.

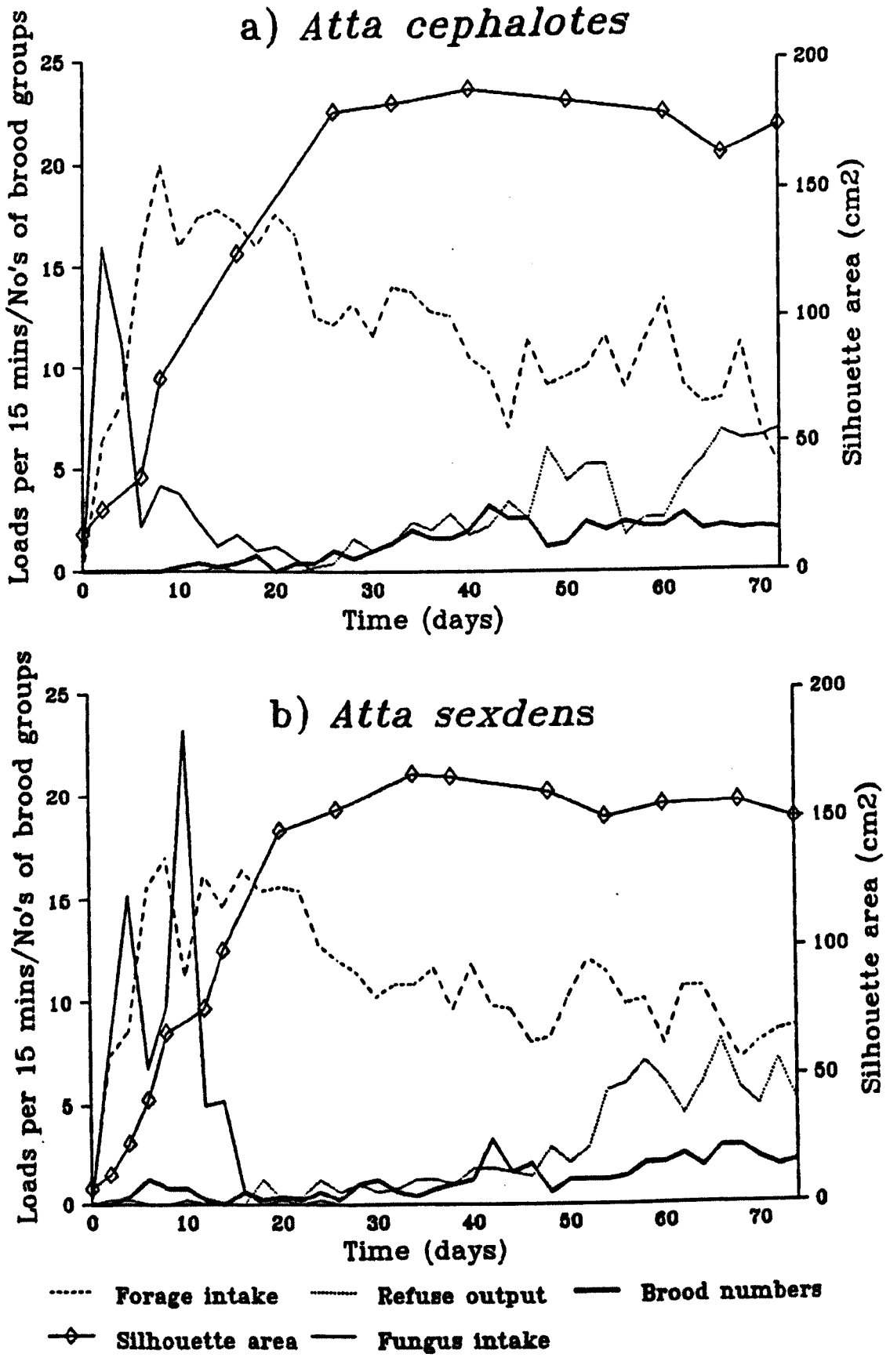


Figure 3.7: Mean numbers of loads of forage and refuse carried into and out of five young gardens of two species of *Atta*, during 15 minute observation periods on alternate days over the first 74 days of development. Mean garden sizes (silhouette areas, cm^2) and numbers of brood groups visible on the three observable garden surfaces are shown. Raw data are shown in Appendix 1.1.

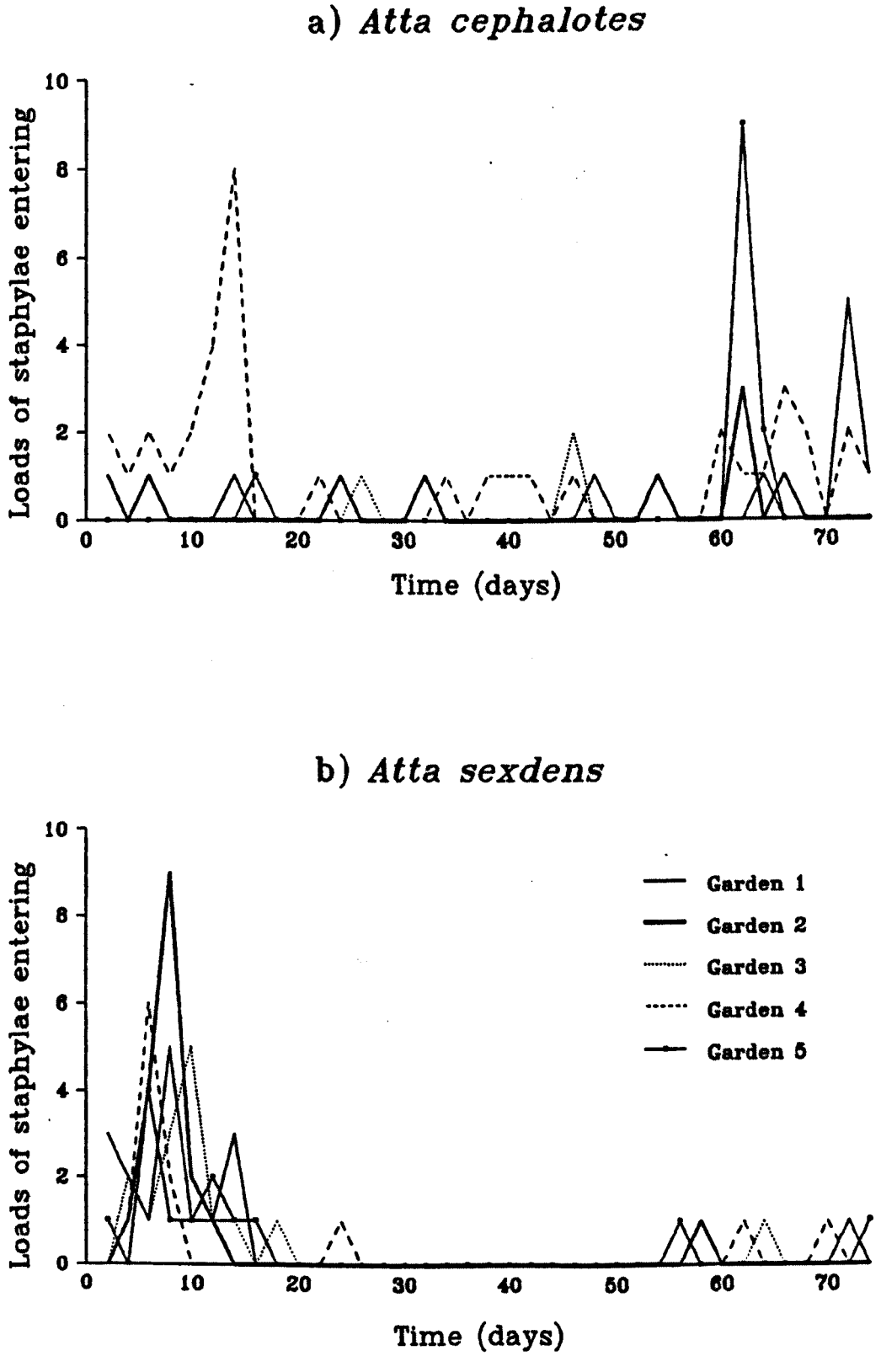


Figure 3.8: Numbers of loads of staphylae carried into five young gardens of two species of *Atta*, during 15 minute observation periods on alternate days over the first 74 days of development.

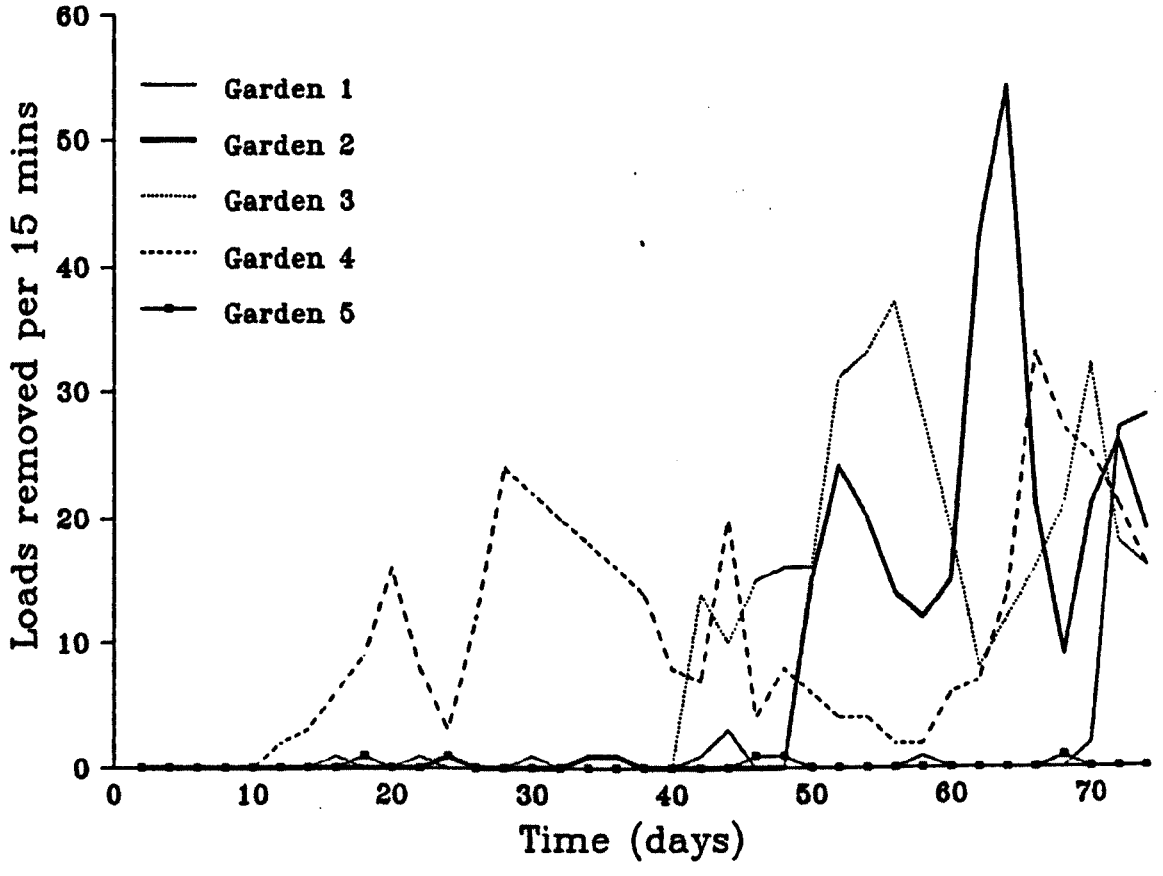


Figure 3.9: Numbers of loads of staphylae carried out of five young gardens of *Atta cephalotes*, during 15 minute observation periods on alternate days over the first 74 days of development.

(i) Autocorrelations

Refuse output data revealed strong trends and Fig. 3.7 showed increasing refuse output with time for both species. Most gardens appeared to have two opposite trends in forage intake and Fig. 3.7 showed that forage intake increased over the first few days and then declined slightly. Fungus intake data had significant values at lag 1, which decayed rapidly, indicating short term correlations. Brood numbers in As1, 2 and 3 and Ac2 showed decaying, slightly alternating series with significant r_k values around lags 1 and 2, while As4 and Ac1 and 5 showed strong trends. No brood were present in Ac4, while the data for Ac3 and As5 were random. Staphyla intake data formed rapidly decaying series with significant values around lag 1 for As1-5. However, the data for Ac1-5 were random. Staphyla outputs were random for As1-5 and Ac1 had a short term correlation, while Ac2, 3 and 4 contained trends, also shown by Fig. 3.9.

Table 3.3 shows, as an example, a set of auto-correlation values obtained for forage intake, refuse output and brood numbers for As3 (a garden chosen at random). Similar tables were constructed for each of the ten gardens, for all six factors. These were too extensive to present in this thesis. However, the raw data are available in Appendix 1.1).

Table 3.3: Sample autocorrelations obtained for *Atta sexdens* Garden 3, for forage intake, refuse output, fungus intake and brood numbers.

LAG	AUTOCORRELATION COEFFICIENTS FOR:			
	FORAGE INTAKE	REFUSE OUTPUT	FUNGUS INTAKE	BROOD NUMBERS
1	0.64*	0.82*	0.42*	0.49*
2	0.53*	0.68*	0.27	0.40*
3	0.27	0.62*	0.01	0.31
4	0.15	0.50*	-0.01	0.18
5	0.10	0.45*	-0.01	0.14
6	0.09	0.44*	-0.01	0.21
7	0.09	0.34*	-0.01	-0.04
8	-0.01	0.28	-0.02	-0.04
9	-0.01	0.23	-0.02	-0.03
10	-0.06	0.12	-0.02	-0.02
11	-0.03	0.04	-0.02	-0.02
12	0.01	-0.03	-0.02	-0.07
13	0.02	-0.09	-0.02	-0.11
14	0.01	-0.11	-0.03	-0.12
15	-0.15	-0.12	-0.03	0.00
16	-0.18	-0.17	-0.03	0.13

* Significant autocorrelation coefficients ($p<0.05$).

(ii) Differencing

Trend removal by differencing the data showed that forage intakes changed most dramatically during the first 10 lags for As1-3, but in As4, this occurred around 4, 12, 20 and 34 lags, while As5 changed at around 5 and 34 lags. Major changes occurred in Ac1 between 13 and 19 lags and similarly for Ac4, which also had major changes around 36 lags. Ac3 was variable around 32 lags and Ac5; at 5, 14 and 25 lags. Changes in forage intake therefore occurred at different times for different gardens.

Refuse outputs were all constant for the first 20 or 30 lags, then showed major variability between 20 and 38 lags for all except As5, which had a low refuse output.

a) Autocorrelation of data

Time (lags)	r_t
1	-0.69
2	0.24
3	-0.10
4	0.14
5	-0.15
6	0.02
7	0.14
8	-0.18
9	0.12
10	-0.06
11	0.08
12	-0.12
13	0.11
14	-0.01
15	-0.13
16	0.19

b) Time plot of data

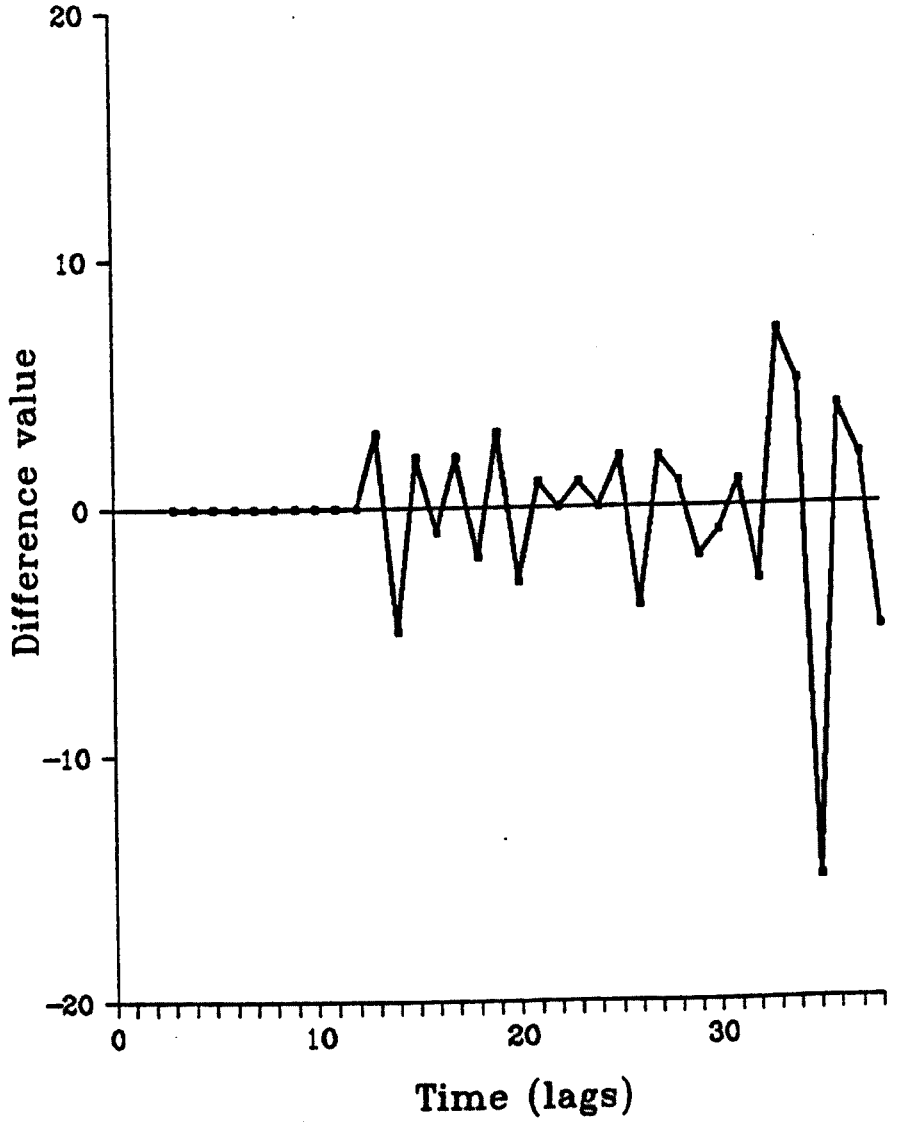


Figure 3.10: a) The autocorrelation coefficients (r_t) obtained for twice-differenced refuse output data for *Atta sexdens* Garden 3 (a rapidly decaying, short-term correlation), compared with, b) the time plot of the twice-differenced data.

Similarly, fungus intakes were all variable around lags 3-7. However, brood numbers showed variations at different times for every garden, as did staphyla outputs. Changes in staphyla intakes occurred consistently between 4 and 8 lags for As1-5, but at different times for Ac1-5. An example of differenced data is shown in Fig. 3.10.

(iii) Cross correlations

An example of the results obtained from cross correlations is shown in Table 3.4, which shows the cross correlations between forage intake and other factors for *Atta sexdens* Garden 3.

Table 3.4: Sample cross-correlations obtained for *Atta sexdens* Garden 3, between forage intake and other factors.

LAG	CROSS CORRELATION COEFFICIENTS FOR:				
	A	B	C	D	E
-16	0.18	0.12	0.13	-0.14	0.02
-15	0.18	0.06	0.15	-0.03	-0.02
-14	0.19	0.02	0.18	-0.00	0.01
-13	0.16	0.05	0.18	0.13	-0.09
-12	0.13	0.03	0.15	0.07	-0.02
-11	0.07	0.06	0.08	0.03	-0.12
-10	0.03	0.18	0.02	-0.00	-0.03
-9	-0.03	0.24	-0.05	0.06	-0.03
-8	-0.06	0.21	-0.10	0.15	0.10
-7	-0.12	0.32*	-0.14	0.16	0.10
-6	-0.15	0.32*	-0.23	0.29	-0.03
-5	-0.19	0.48*	-0.26	0.27	-0.04
-4	-0.31	0.44*	-0.31	0.31	-0.09
-3	-0.39*	0.79*	-0.27	0.40*	-0.03
-2	-0.42*	0.39*	-0.31	0.61*	-0.05
-1	-0.44*	0.17	-0.30	0.59*	0.02
0	-0.42*	-0.07	-0.36*	0.60*	0.07
1	-0.39*	-0.21	-0.29	0.62*	-0.01
2	-0.39*	0.10	-0.29	0.09	0.18
3	-0.39*	-0.07	-0.21	-0.00	0.12
4	-0.36*	-0.02	-0.21	-0.15	0.28
5	-0.34*	-0.03	-0.20	-0.12	0.21
6	-0.30	-0.03	-0.21	-0.09	0.57*
7	-0.31	-0.03	-0.20	-0.11	0.02
8	-0.26	-0.03	-0.10	-0.08	-0.10
9	-0.33*	-0.04	-0.07	-0.12	-0.15
10	-0.35*	-0.04	-0.01	-0.08	-0.21
11	-0.38*	-0.04	-0.06	-0.11	-0.11
12	-0.38*	-0.04	-0.23	-0.09	-0.07
13	-0.35*	-0.04	-0.30	-0.11	-0.12
14	-0.24	-0.05	-0.30	-0.08	-0.08
15	-0.13	-0.05	-0.27	-0.11	-0.09
16	-0.03	-0.06	-0.20	-0.12	-0.02

* Significant cross-correlation coefficients ($p<0.05$).

- continued overleaf

A - Forage intake versus refuse output = a strong negative correlation.

B - Forage versus fungus intake = a strong positive correlation, with fungus intake preceding forage intake.

C - Forage intake versus brood numbers = a strong positive correlation.

D - Forage versus staphyla intake = a strong positive correlation.

E - Forage intake versus staphyla output = probably no correlation.

Ac1 and 5 and As2, 3 and 5 all had strong negative correlations between forage intakes and refuse outputs, while Ac2 and 4 and As1 had significant values between lags 20 and 33, indicating that refuse output peaked 20-33 lags (40-66 days) after forage intake. However, when coefficients were significant only at large lag values, this suggested that they were significant only because the two compared series had only one large peak each. Refuse output in Ac3 was sporadic and in small quantities, which led to a significant positive correlation between lags 3 and 7 when no refuse was being produced and forage intake levels were low. This garden was unusual because the ants started to build it, then abandoned it for a few days before resuming building after day 8. As4 showed no correlation at all. Fig. 3.7 shows that forage intakes tended to decrease after day 10 while refuse outputs gradually rose. However, cross correlating the differenced data, with trends removed, showed very few significant correlations.

Cross correlating forage and fungus intakes showed significant positive correlations for As1 and 2, which had peaks around -8 lags; fungus peaks preceded forage peaks by around 16 days. Similarly, As3 had significant values between -5 and -2 lags; the fungus intake peak preceded that of forage by 4-10 days. Except for Ac5, which had almost no fungus intake, the other gardens also showed

significant correlations, the lags of the significant values reflecting the distances between the forage and fungus peaks. Fig. 3.7 shows that these peaks closely coincided and cross correlations of the differenced data supported these results. Similarly, cross correlating forage and staphyla intakes showed significant relationships for As1-5 and Ac4, but cross correlations of forage intakes and staphyla outputs showed very variable results, with significant values often at very large lags. Again, cross correlations of the differenced data supported these results.

There were no significant correlations between refuse outputs and fungus intakes, except at very large values of the lag, while cross correlations with brood numbers were difficult because Ac2, 3 and 4 and As1, 3 and 5 all had very small brood numbers. However, Ac4 had a strong negative correlation between forage intake and brood numbers, with many significant values. As2 and 4 also had significant values around lag -2 and at lags 13 and 16 respectively. On day 5, in As2, the culture room overheated to 30^0C and large numbers of brood were temporarily moved into this garden. They were then removed again over the next few days, after room temperature had decreased. Small numbers of brood were also present in As4 around days 4-6, in contrast to other gardens. Ac1 and 5 and As2 and 4 had strong significant correlations between brood numbers and refuse output, with 5-16 significant peaks per correlation. Ac1 also had a strong negative correlation between fungus intake and brood numbers with more than 10 significant peaks. However, differenced data showed no significant relationship for Ac5.

Cross correlations with staphyla intakes or outputs were difficult because Ac gardens had low intakes while As gardens had low outputs. As1-5 did show positive correlations between refuse output and staphyla intake, but the significant values were at large values of the lag. Similarly, Ac1-4 were all significantly correlated with staphyla outputs, but the significant peaks occurred at different lags for each garden and this variation suggested

that there was no real relationship. These conclusions were supported by cross correlating the differenced data.

As1-5 had significant positive correlations between fungus garden and staphyla intakes, with significant values from -1-6 lags, depending upon how closely the intake peaks coincided. Fungus and staphyla intakes both peaked during the first 15 days (Figs. 3.7 and 3.8). Ac4 had significant peaks around lag 6, indicating that the staphylae peak followed that of fungus by 12 days. Ac5 had significant peaks around 30 lags and had a staphylae peak after 70 days (Fig. 3.8). As gardens therefore showed a constant relationship between fungus garden and staphyla intakes, but Ac gardens did not and significant coefficients obtained were probably random. Similarly, although fungus intakes and staphyla outputs in Ac1-5 were all significantly correlated with many significant values, the lag values of these peaks varied depending on the relative distances between fungus intake and staphyla output peaks, suggesting that there was no real relationship. These results were confirmed by cross correlating differenced data.

Cross correlating forage intakes between gardens showed significant correlations, with many significant values. This was also true for refuse output and fungus intakes; all gardens therefore showed similar patterns of these. Cross correlating the differenced data between gardens showed that forage intakes and refuse outputs were more closely correlated between Ac gardens than between As gardens. Ac garden cross correlations had a mean of 3.2 (SE ± 0.6) significant values (10 comparisons) for forage intake, while As gardens had only 1.6 (SE ± 0.4). Similarly, for refuse output, Ac gardens had a mean of 4.9 (SE ± 0.3) significant values per cross correlation, while As gardens had only 2.6 (SE ± 0.8).

Cross correlating brood numbers between gardens produced many significant peaks but most gardens had small numbers of brood and those gardens which did have large

numbers of brood showed no consistent pattern. Staphyla intakes were significantly correlated between the As gardens but in Ac gardens the lag positions of the significant values were very variable; the only relationship between them was that most had small intake peaks at some time. Staphyla outputs in Ac1-4 were all correlated, with significant values at widely varying lags. Again, no consistent relationship was present. Cross correlating differenced data tended to confirm these results.

To summarise, refuse outputs were poorly correlated with forage intake. Fungus garden and forage intakes were closely correlated but refuse output was probably not related to fungus intake. Fungus, forage and staphyla intakes were all correlated in As gardens but not in Ac gardens. All gardens had similar patterns of forage intake, fungus intake and refuse output, but brood numbers and staphyla outputs varied between gardens. Staphyla intakes were consistent between As but not between Ac gardens. Ac gardens were more closely correlated for forage intakes and refuse outputs than As gardens.

3. The dynamics of established fungus gardens over 700 days

To examine long-term changes in fungus gardens, the forage intake and refuse output of six adjacent established gardens were observed on alternate days for 160 days; then every fifth day until day 400 and then every fifth day during days 490-510, 570-590 and 680-700. These last three periods were used to check previous observations made before day 400. The amount of brood present was also recorded at these times, while silhouettes of the fungus garden areas were recorded every 10 days.

a) Numbers and types of loads carried in and out of gardens

Forage and refuse were the most commonly carried loads, but staphylae, brood, fungus garden and workers were also transported (Table 3.5). Fragments of fungus garden

were usually carried out of domes into ones where new gardens were being founded. Staphylae were carried in bundles of two or three and were recorded as loads.

The total numbers of loads of each type recorded entering or exiting, were compared between the six gardens using a 6 x 2 Heterogeneity Chisquare analysis on the numbers of loads versus the total of all other loads carried. Forage intakes, refuse outputs and staphyla intakes all differed significantly between gardens ($p<0.005$) but there were no significant differences between numbers of staphylae exiting, larvae entering or pupae exiting different gardens ($p>0.25$). Similarly, numbers of larvae exiting were not significantly different ($p>0.05$) and neither were numbers of pupae entering ($p>0.1$).

Table 3.5: Mean numbers per garden (± SE) of each type of load recorded being carried into and out of six mature fungus gardens, over 700 days of observation.

TYPE OF LOAD	MEAN NO'S OF LOADS (± SE) CARRIED:	
	INTO GARDENS	OUT OF GARDENS
Forage	1726.7 ± 115.0*	!
Refuse	1.5 ± 0.8	1057.8 ± 43.7*
Fungus garden	0.7 ± 0.3	2.3 ± 1.6
Staphylae	10.8 ± 2.4*	9.5 ± 1.5
Larvae	9.5 ± 1.0	13.7 ± 2.1
Pupae	10.0 ± 1.2	18.0 ± 2.6
Mature workers	0.5 ± 0.2	0.2 ± 0.2
Callow workers	0	0.3 ± 0.5

! These were not recorded, although some forage was carried out of domes.

* Significant differences ($p<0.005$, Chisquare) occurred between gardens.

b) Changes over 700 days

Fig. 3.11a-f shows the variations in forage intake, refuse output, brood numbers and silhouette area for the six gardens over 700 days. Means for 20 day periods were plotted, to show the major changes occurring, without the short-term fluctuations (see Appendix 1.2 for errors).

All six gardens had low forage intakes between days 50 and 100 (March to April). This also occurred 1 year later (day 400). Then, all gardens showed one or two small forage intake peaks between days 100 and 200 (May to August). Levels 1 year later (day 500) were similar.

Silhouette areas at the start were quite large in all gardens (except G2, which was being built up), but decreased sharply around day 150 (June 12th). This coincided with peaks in refuse output and drops in the numbers of brood present. In fact, the culture room overheated on day 158 and dead pupae and fungus garden were discarded with refuse.

Forage intake decreased around day 200 (August 2nd) and in G1, 3, 4 and 6 this coincided with a peak in refuse output. However, the highest forage intakes were recorded between days 200-300 (August to November) for all six gardens. G2 also started with a very high forage intake, but was being rapidly built up at that time. A year later (day 600), this late summer foraging peak seemed to occur again.

Between days 300-400, (November 1990 to February 1991) forage intake decreased, but silhouette areas continued to increase and stayed high until day 400. Areas then decreased again by day 500 (May 1991). Numbers of brood present followed a similar pattern to that of silhouette area.

To summarise, forage intake and garden size decreased during the early spring and were then variable until August. Forage intake then increased during the late summer (days 200-300) and garden sizes increased to a maximum between days 300-400. Brood numbers also increased at this

Figure 3.11: Mean numbers of loads of forage and refuse carried into and out of six fungus gardens during 15 minute observation periods, averaged over 20 day periods. Mean garden sizes (silhouette areas, cm^2) and numbers of brood groups on the three visible garden surfaces are also shown. Errors are shown in Appendix 1.2.

Key:

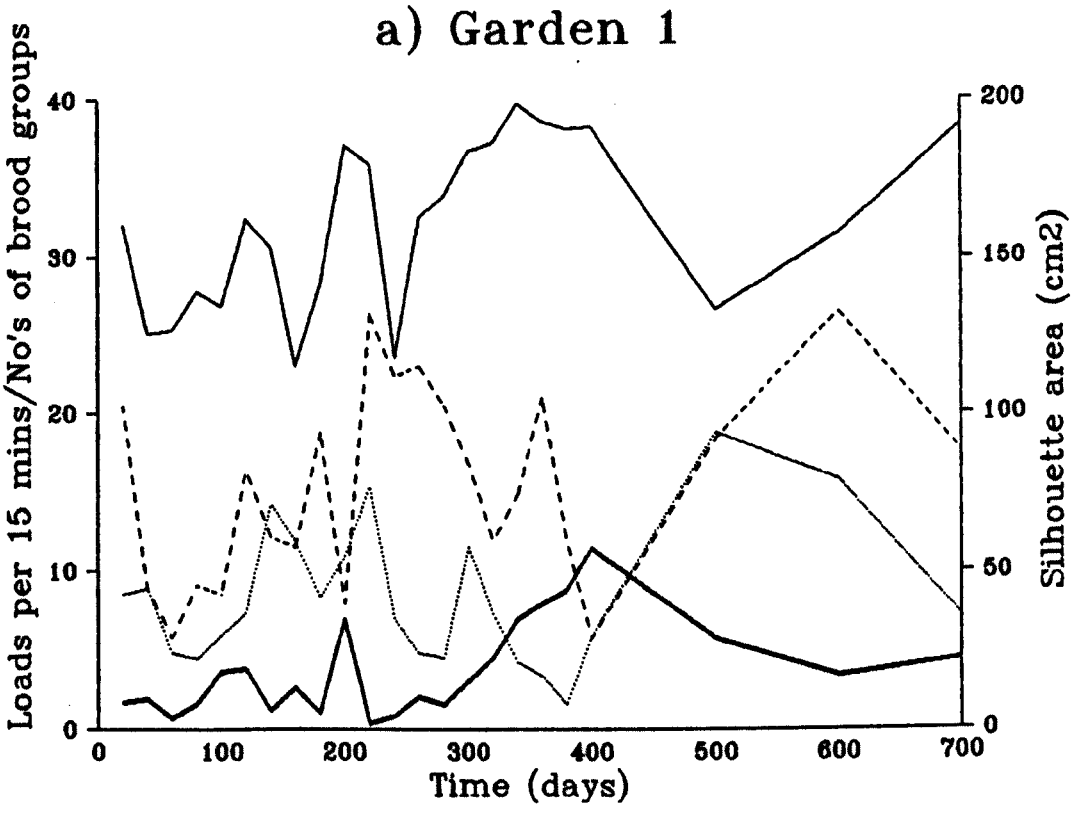
---- Forage intake

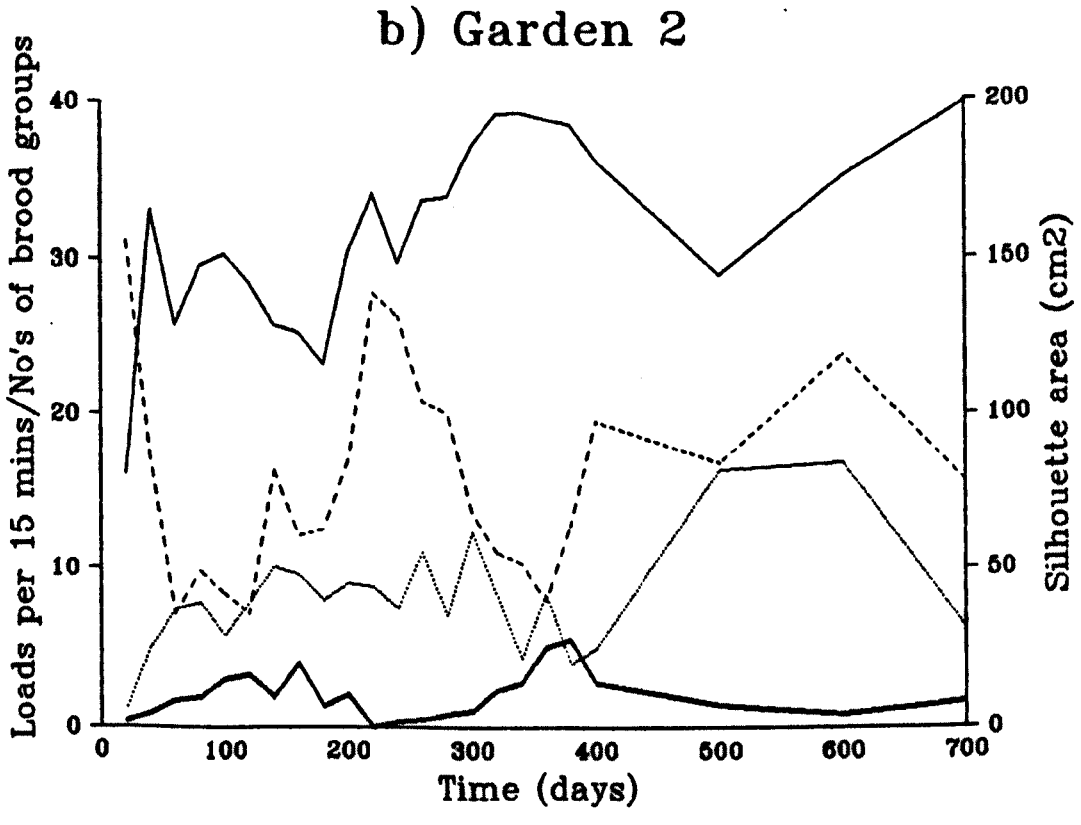
..... Refuse output

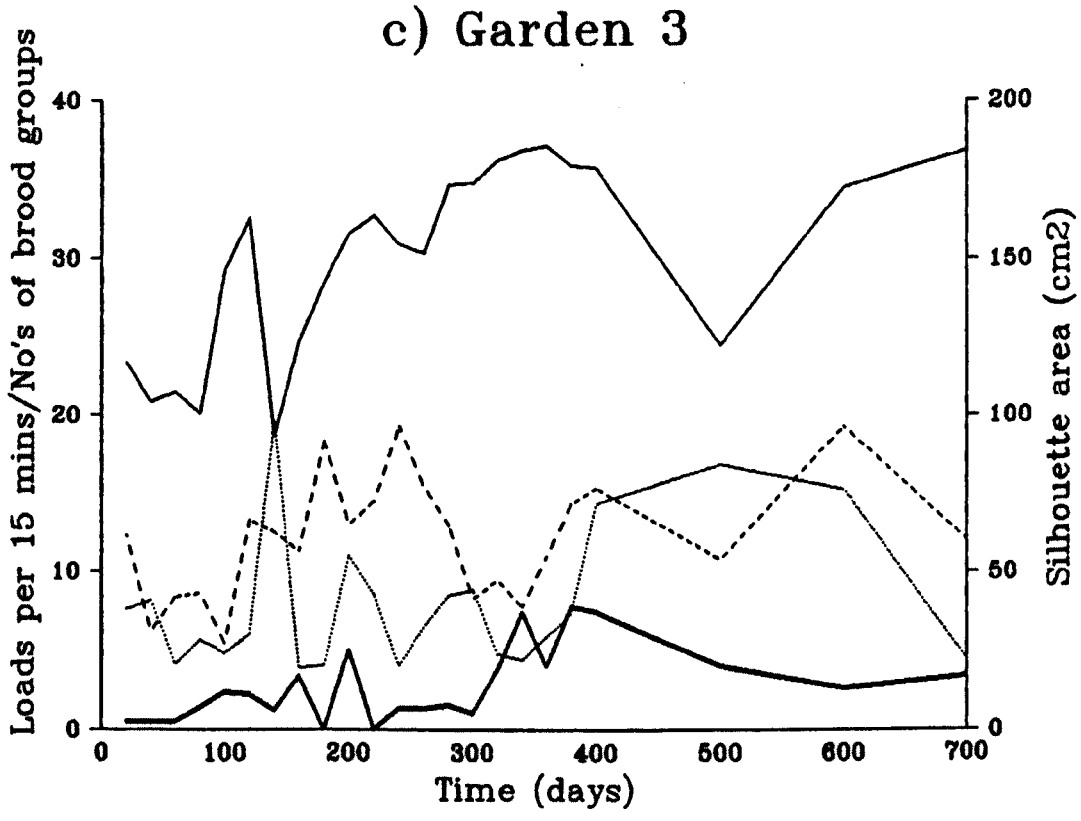
—— Brood numbers

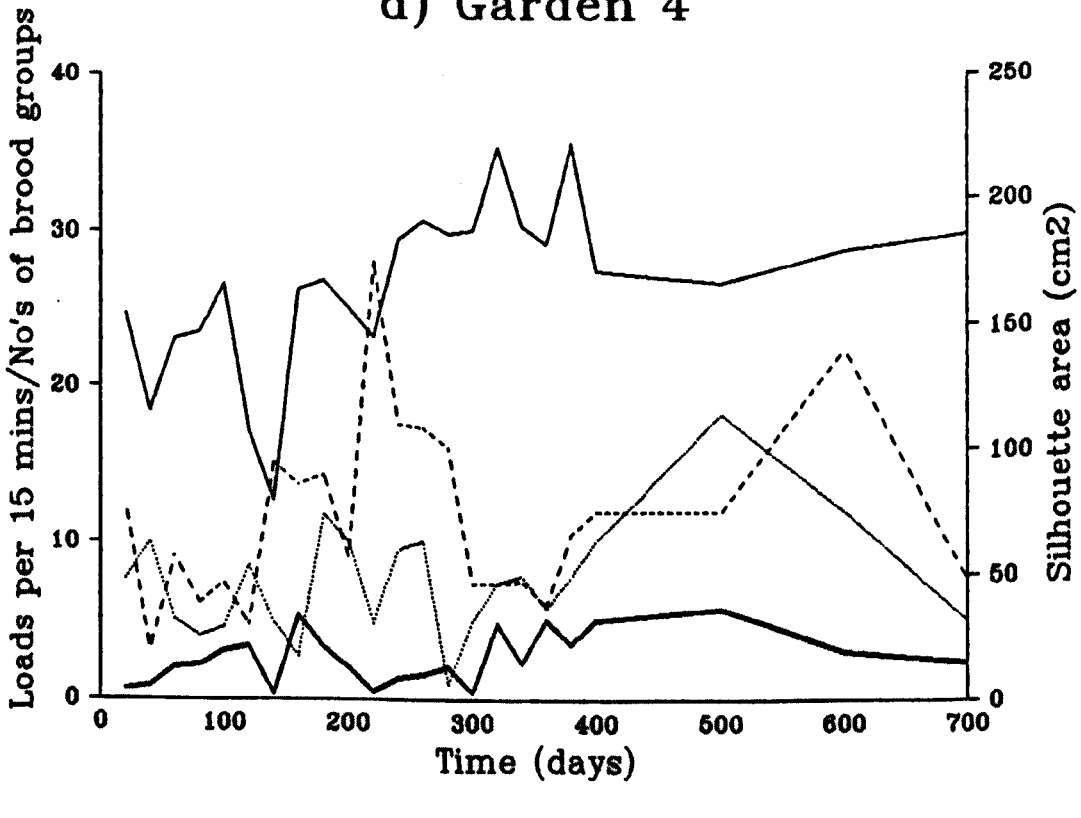
—— Silhouette area

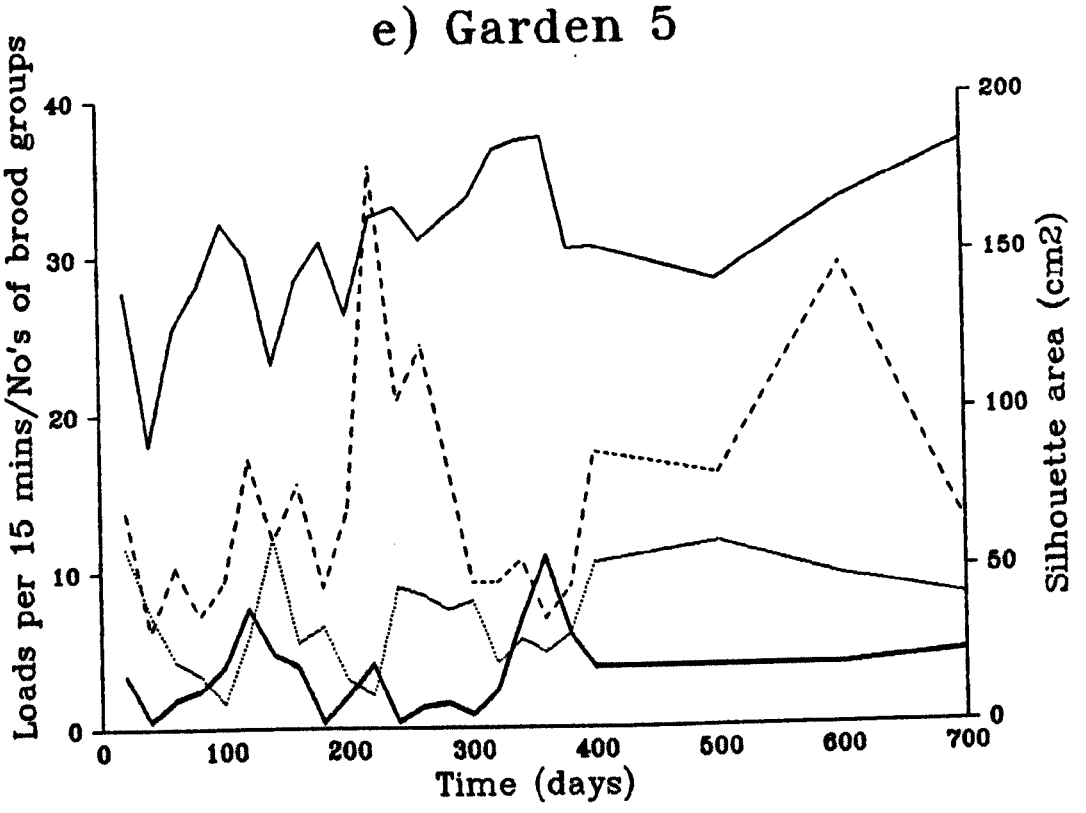
DAY NUMBER	CALENDAR DATE
0	13th January 1990
100	23rd April 1990
200	1st August 1990
300	9th November 1990
400	17th February 1991
500	28th May 1991
600	5th September 1991
700	14th December 1991

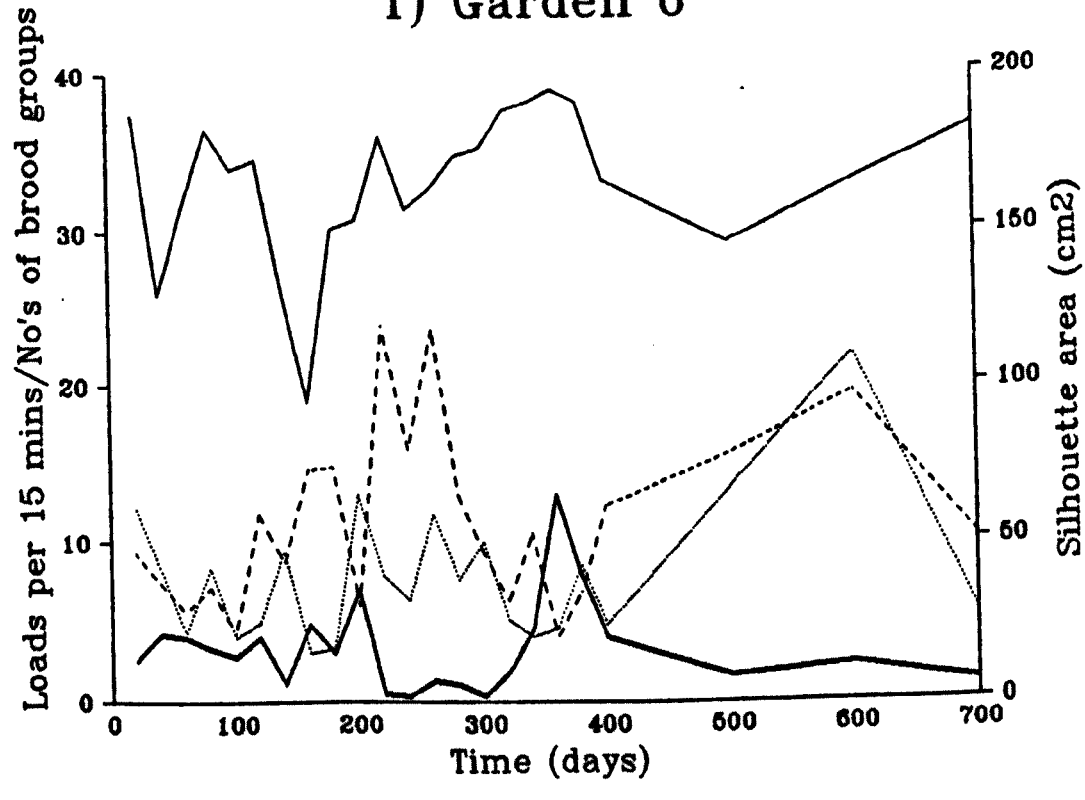

a) Garden 1
Loads per 15 mins/No's of brood groups
Silhouette area (cm2)
Time (days)
0
10
20
30
40
50
100
150
200
300
400
500
600
700


b) Garden 2
Loads per 15 mins/No's of brood groups
Silhouette area (cm2)
Time (days)
0
10
20
30
40
50
100
150
200
300
400
500
600
700


c) Garden 3
Loads per 15 mins/No's of brood groups
Silhouette area (cm2)
Time (days)
0
10
20
30
40
50
100
150
200
300
400
500
600
700


d) Garden 4
Loads per 15 mins/No's of brood groups
Silhouette area (cm2)
Time (days)
0
10
20
30
40
50
100
150
200
250
300
400
500
600
700


e) Garden 5
Loads per 15 mins/No's of brood groups
Silhouette area (cm2)
Time (days)
0
10
20
30
40
50
100
150
200
300
400
500
600
700


f) Garden 6
Loads per 15 mins/No's of brood groups
Silhouette area (cm2)
Time (days)
0
10
20
30
40
50
100
150
200
300
400
500
600
700

time. Refuse outputs followed no obvious pattern, but peaks sometimes coincided with decreases in silhouette area or forage intake.

The data for the first 400 days were subjected to time series analysis (using the 20 day means). Because time series analysis requires regular intervals, with no missing values, days 500-700 were not analyzed.

(i) Autocorrelations

Autocorrelations of refuse output showed that it alternated either side of the mean at irregular intervals. Some significant values ($p<0.05$) were present for G4-6, at lags 2 and 5, 4 and 6 and lag 7 respectively, but these were probably due to random variations.

Forage intake data alternated either side of the mean, but less randomly than refuse. G2 and 3 had significant values at lag 1, indicating short-term correlations. Brood numbers data were also alternating series, except for G4 data which appeared random. Garden silhouette area data were again all alternating series, although G6 had no significant values. No trends were visible.

(ii) Differencing

Differencing the data showed that the variations in all four factors for each garden in Fig. 3.11 were still evident. This was generally true for all six gardens, since the autocorrelations showed that no trends were present, except possibly for silhouette areas.

(iii) Cross correlations

Forage intake showed little correlation with refuse output, with only occasional significant peaks ($p<0.05$) which were probably due to chance. Fig. 3.11 showed little relationship between forage intake and refuse output.

Cross correlating forage intake with brood numbers showed that G1 and 6 had only one significant peak each at lags 6 and 0 respectively (around day 120 and day 0), while G2-5 had two or more significant peaks each, indicating

that forage intake and brood numbers were correlated. These peaks were present around lag 0 (at the start of the series) and at lags 6-9 (days 120-180), suggesting that the two series were related, with brood numbers peaking more than 100 days after forage intake peaked, as in Fig. 3.11.

There were no significant relationships between forage intake and silhouette areas for G2 and 3, but the other gardens all had significant peaks at lag 5, indicating that the two series were related, but that silhouette area peaked 5 lags (100 days) after forage intake.

Cross correlating refuse output with brood numbers showed no significant correlations except for G1. This had significant coefficients at 1 and 9 lags, probably due to chance. Similarly, when refuse output was correlated with silhouette area, only G1 showed any significant correlation with peaks at lags -3 and 1. Fig. 3.11a showed that refuse output and silhouette area followed very similar patterns for the first 350 days and then diverged. However, since only one of the six gardens showed this relationship, it was probably due to chance.

Cross correlating brood numbers with silhouette areas showed no significant values for G2, 4 or 5 but G1, 3 and 6 all had two or more peaks. G1 had peaks at lags -3, -1, 0 and 1, G3; at lags -2, 1, 0 and G6; at lags -11 and -10. G1 and 3 therefore showed a strong short-term correlation, while G6 showed a correlation with a delay of 10 lags (200 days). This latter correlation was probably due to chance. Fig. 3.11 showed that G1, 3, 5 and 6 all had strong brood peaks around day 400, which coincided with the end of the large silhouette peak, while G2 and 4 showed little increase in brood numbers at this time. However, G5 and 6 had the least well defined silhouette area peaks.

Cross correlating forage intakes between the six gardens showed 1-6 significant values per correlation, indicating that all gardens followed the same pattern. However, cross correlating refuse outputs between the six gardens showed very little correlation, with only occasional significant coefficients. This suggested that

refuse is discarded randomly from different gardens at different times. Cross correlating brood numbers between gardens showed 1-3 significant values per correlation, which indicated that brood numbers fluctuate in different gardens at the same times. Silhouette areas were also very strongly correlated between gardens, with 1-5 significant peaks per correlation.

DISCUSSION

1. Observation times used

The best time to observe forage intake was after initial forage retrieval and before most of the forage had been removed. Peak forage intakes rather than total forage intakes were used because estimating the latter would have meant 24 hr surveillance of each garden.

Looking at the removal rates of different substrates showed how the observations may have been affected by the forage types provided (see Table 2.1, Chapter 2, page 12). The observation 'window' used was from 15-45 minutes after forage was provided, but peak removal times for cabbage and holly both occurred after this time (Fig. 3.5), while knotweed was removed very rapidly. Some substrates were removed as large numbers of loads per minute (knotweed, oats), while others were removed at low rates per minute (cabbage, citrus peel), due to differences in sizes of loads cut and rates of cutting. Physical leaf toughness can affect cutting by workers (Cherrett 1972a, Waller 1982a), as can the presence of repellent chemicals (Littledyke and Cherrett 1978, Waller 1982b, Hubbell *et al.* 1983, Febvay *et al.* 1985). Load sizes were not generally determined by forage dry weights but sometimes this may have been a factor (Fig. 3.6). For example, *Camellia* petals had a low percentage dry weight but large load size, because workers simply picked up individual petals. Cabbage and citrus peel had moderate percentage dry weights but small load sizes,

because workers had to cut chunks of thick material rather than cutting through a thin leaf.

Fortunately, there were few days when only one type of forage was used and the mixture of substrates normally taken in probably cancelled out many of the effects of slowly-removed or fast-removed substrates. Any effects were further minimised by using 20 day means for the 700 days study on established gardens. Using means meant that short-term fluctuations were lost, but the 700 days study was a long term study of major changes and short-term fluctuations were not important. Forage type was less important for the developing garden data, since new gardens were observed over a much shorter period (74 days) and there was less chance of season affecting leaf quality.

Unlike forage intake, refuse output was relatively constant over 24 hours. Refuse output in the laboratory is frequently cyclic (Weber 1972) and taking 20 day means (for established gardens) masked short-term changes in output from individual gardens. Unfortunately, developing gardens were probably not observed for long enough to obtain a true picture of fluctuations in refuse output over time.

2. The development of new gardens

All gardens were founded in the same way. Workers cleaned the inner surfaces of the new chamber, then imported fungus garden and substrate. *Atta sexdens* gardens also imported staphylae, which may have been a response to larvae present. However, larvae were also seen in *Atta cephalotes* gardens, but few staphylae were imported. Imported staphylae may have been used as fungal inoculum and planted on to fresh substrate.

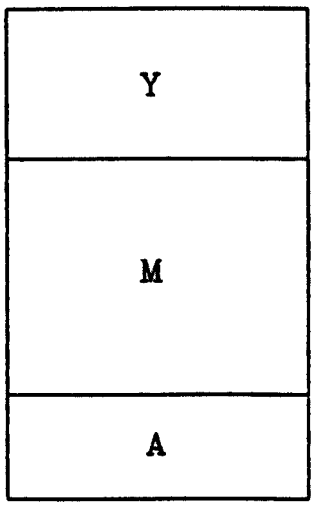
Small numbers of immature callow workers were sometimes moved into or out of new gardens during the first few days. These are often involved in caring for fungus garden or brood (Wilson 1980a) and this may have been their role here.

Staphylae were visible on outer surfaces of young gardens after 11.8 days (*Atta cephalotes*) or 13.2 days (*Atta sexdens*). Angeli-Papa and Eyme (1979) found that staphylae developed in agar culture after 20 days, but in the fungus garden a large fungal inoculum is available to ensure rapid growth and development. Staphylae probably developed much sooner on inner garden surfaces, because new gardens are small and the outer surface is where the majority of new substrate is added, while mature gardens fill their domes and fresh material is added to the top (Fig. 3.12). A young garden only appears visibly mature when it begins to fully occupy its dome. Staphylae are seldom found on young garden but occur in large numbers on older areas (See Fig. 2.1, Chapter 2). Hence young gardens, with outer surfaces comprised of young garden, will not have visible staphylae.

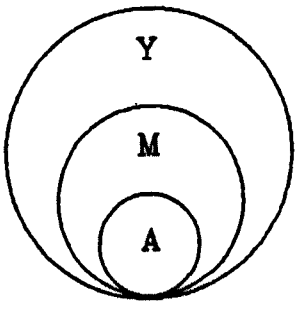
All developing gardens went through a building-up stage, initially of high fungus garden intake, then of high forage intake, both of which then declined. Workers therefore build the new garden very quickly and it may have to pass a critical size to be viable. Small pieces of fungus garden dry out quickly, even under the humid conditions of the culture room. Once the garden has filled its dome, it cannot continue to grow at the same rate unless faster turnover occurs, due to lack of space. Forage intake must therefore decrease to a level which keeps pace with refuse output. Similarly, once the garden has been founded, there is no point in bringing more fungus from other gardens, so fungus intake decreases.

The garden may however, continue to grow by infilling. Even though a young garden may fill the dome in silhouette, it may not be very dense. Young garden consists of large, thin-walled cells, while older garden has smaller thick-walled cells (see Fig. 2.1, Chapter 2). A developing garden may therefore continue to import relatively large amounts of forage to 'fill in gaps' and some substrate fragments are inserted into mature garden, although seldom into aging garden (pers.obs). The weight of fresh material added to

Mature garden



Developing garden



Y = Young garden
M = Mature garden
A = Aging garden

Figure 3.12: Comparative structures of mature and developing gardens. Mature gardens fill the chamber (dome) and fresh substrate is mostly added to the top. Developing gardens are small and fresh substrate is added to all surfaces.

the top of the garden is also likely to compress garden below, reducing its cavity size. The fungus garden does continually slump downwards and young garden moves down more rapidly than older areas, at a rate of 0.176 (SE ± 0.021) mm hr^{-1}, compared to 0.114 (SE ± 0.005) mm hr^{-1} for mature garden and 0.126 (SE ± 0.014) mm hr^{-1} for aging garden (Bass 1989). The compaction of older garden probably occurs through a mixture of infilling and compression.

Refuse output gradually increased over time, but most exhausted substrate was dumped after approximately 50 days, suggesting that gardens have turnovers of just under 2 months.

Numbers of brood present also increased over time, probably because as the garden grows, it can produce more staphylae and support more larvae. Larvae may also have been imported as eggs, which are difficult to see. However, some pupae were present, indicating that some older brood was imported too.

In *Atta cephalotes* gardens, although staphyla intakes showed no pattern, all gardens developing on April 24th exported large numbers of staphylae. The fate of these staphylae was unknown, but they were probably not used as fungal inoculum, since they were taken into mature gardens, where fungus was already abundant. This export may have been a response to hungry larvae present in the mature gardens, although no large numbers of brood were visible. Alternatively, they may have provided food for workers. A garden constructed just a few days after April 24th (Ac5) never exported staphylae in large numbers. Sexual broods were produced by this nest in the following two years, between April and June and it is possible that this massive transfer of staphylae into some gardens represented an attempt by the ants to raise a sexual brood, which subsequently failed. Producing sexual broods is a great strain on colony resources and it is likely that if this drain of resources is too severe, the sexual brood will be abandoned. No visible signs of sexual larvae were present,

but later sexual broods remained hidden inside fungus gardens and were consequently difficult to see.

Atta sexdens gardens showed no such export of staphylae and there were other differences between the two species. *Atta cephalotes* gardens followed the same patterns of forage intake and refuse output more closely than *Atta sexdens* gardens. Observations were made on *Atta sexdens* gardens developing between January and April 1990, while *Atta cephalotes* gardens were observed between February and July. This was because of the rates of colonisation of new domes in the two nests. In theory, gardens observed over a shorter period should have resembled each other more closely than those observed over longer periods, but the opposite occurred.

3. The culture of established fungus gardens

All the mature gardens observed in this study persisted for the 700 day observation period. However, although there was always at least one unoccupied dome available, due to lack of space no attempt was made to provide enough domes for the ants to move gardens at will. The results obtained did indicate that continuous culture occurs in the absence of external environmental factors (in *Atta sexdens*). The fungus requires narrow ranges of temperature to flourish (Powell and Stradling 1986) and like most fungi, requires high humidity. Changes in these conditions might stimulate the ants to relocate gardens.

In the field, the removal and addition of substrate may be cyclic, triggered primarily by season, although this does not occur in all Attine habitats (Weber 1972). In the laboratory, environmental conditions are constant so fluctuations in substrate removal or addition should not occur, if continuous culture is practised. Weber (1972) however, reports that laboratory colonies are often cyclic in carrying out their largest quantities of exhausted substrate.

The results of this study indicated that refuse output was random, with occasional peaks coinciding with decreases in intake or garden size. Individual established gardens had different schedules of casting out exhausted substrate. This was in contrast to developing gardens, which were synchronised. Outputs were sometimes coincidentally high in different gardens and this might be observed as an increase in output at nest level. Peaks in refuse output may have coincided with forage intake decreases when workers in a garden concentrated on discarding refuse and neglected substrate preparation. This is almost a form of discontinuous culture, whereby workers clear out a dome before building new garden there. However, this circumstance was rare and may have been coincidental. Similarly, peaks in refuse output would be likely to coincide with decreases in garden size, since removing substrate would reduce garden volume. Again, coinciding peaks of refuse output and silhouette area decrease were uncommon.

In an established nest, most of the gardens will have been founded at different times and will therefore not be synchronised for refuse output. Developing gardens however, were all observed at the same relative ages. The actual control and timing of refuse output was unclear, particularly since the study looked only at macroscopic changes over 700 days. It is likely that individual gardens pass through cycles of greater building than normal, later followed by a subsequently higher refuse output than normal. Little work has been done on what stimulates the output of refuse, although Jaffe (1986) considered that volatile chemical cues may be important for refuse recognition. Although forage and other loads were frequently carried in and out of gardens, once refuse loads had been carried out of gardens they were almost never carried back in and were taken straight to refuse dumping sites. This suggests that refuse is instantly recognisable to workers when it is being carried out of gardens and that they seek to dispose of it as quickly as possible. Refuse

potentially provides a reservoir of alien fungi and bacteria which could pose a threat to the garden.

Forage intake varied much more than did refuse outputs but was fairly synchronised in all gardens, showing that changes occurred at nest level. This provided evidence for continuous rather than discontinuous culture.

Forage intake was low during the early spring (February to April) but then increased. The nests received a large amount of privet between November and March and this may not be an ideal substrate for the fungus garden (Mudd and Bateman 1979). Also, Attines show shifting preferences for different substrates, which may not necessarily occur for external reasons (Littledyke and Cherrett 1975). After receiving one type of forage for a long period, they may become disinterested and intake may drop. However, the nests received approximately the same volumes of forage all year, the only differences being in the types of substrate offered, which may have had differing dry weights. Apparent decreases in intake therefore reflected slower removal rates rather than actual reductions in the amounts of imported forage. Seasonal variations in leaf compositions do occur, which may affect attractiveness of the forage. Birch in Sweden, for example has high leaf nitrogen and phosphorus levels early in the year, lower but constant levels from July to September, followed by much lower levels (Tamm 1951).

Silhouette areas also decreased during the spring, while brood numbers were quite low. However, there was no evidence for loss of garden volume through refuse dumping. The gardens did appear to follow a yearly cycle, since observations made during the second year of observations tended to reflect those made in the first year. Both silhouette areas and brood numbers were highest between November and February. Larvae consume large numbers of staphylae (Quinlan and Cherrett 1979) which may have caused a decline in garden volume during the early spring.

From late March onwards, nests began to receive young leaves and by April, these formed the majority of the

forage received. Intakes during the observation period subsequently increased after a period of delay, indicating increasing worker interest in the new forage. This delay may have been due to repellent chemicals in young leaves, although young leaves are usually more attractive to workers than older ones (Fennah 1950, Cherrett and Seaforth 1970, Barrer and Cherrett 1972). The nest may have needed a period of recovery after raising a large brood, before beginning to build gardens up again in preparation for the next large brood event, although this seems unlikely. However, silhouette areas did not subsequently increase steadily and all gardens went through a variable period between April and July, with all four factors rising and falling apparently at random. Variations in silhouette area may have occurred because the young leaves supplied at this time were very soft and low in dry weight. Fungal mycelium would easily be able to penetrate this, but such substrate would become exhausted quickly, leading to faster turnover. The low dry weight would mean that more material would be required to build up garden size. Exhausted substrate would be expelled more frequently, but in low volumes because of the original low dry weight, therefore size increases and decreases might be sporadic.

By August, mature high-dry weight leaves were being supplied, which appeared to be more attractive to workers than younger leaves, since the forage intake peak occurred during September. The young leaves provided may have contained repellent chemicals and Quinlan (1977) showed that young holly leaves contained repellent chemicals, while mature leaves were attractive but protected by spines. A more likely reason however, is that demands for forage were higher at this time as the nest prepared for a large brood.

The high dry weight of forage during the autumn meant that effectively the nest received more material, hence silhouette areas built up to a maximum and peaked after forage intake. Simultaneously, brood numbers increased with garden size. The high dry weight of forage supplied at this time also meant that it supported fungal growth for longer,

hence the peak was not punctuated by periods when large amounts of exhausted substrate were removed. Large garden size might simply mean that there was more space available for brood but larvae are a resource sink and their presence in the garden must be tied to the amount of food, in terms of staphylae available.

The mechanism by which the colony could regulate brood production with regard to available garden resources is unclear. Do the workers build up the garden to prepare for a large brood event, or does the queen lay more eggs in response to greater available garden volume? The eagerness of workers to remove eggs, or their treatment of the queen may influence her rates of egg production. Feeding of trophic eggs to the queen may also be a stimulating factor. If garden size increases in the latter part of the year due to forage quality, then numbers of brood present are also affected by forage quality, rather than being controlled by the production rate of the queen. However, it is more likely that a feedback system operates, whereby the workers take advantage of extra dry weight of forage by building up the garden and the queen takes advantage of increasing garden size by producing more eggs. Both explanations of the relationship between brood numbers and garden size will therefore be correct to a degree; workers build up the garden and inadvertently 'prepare' for a large brood, while more larvae can subsequently be raised in a larger volume of garden.

It is also possible that the forage intake peak between days 200-300 was a direct response to increased egg production by the queen. In *Atta sexdens rubropilosa*, the egg stage persists for 22 days, the larval stage for 22 days and the pupal stage for 10 days (Weber 1972). The total time taken for a worker to develop is therefore 54 days, which is a barely long enough period for increased egg production between days 200-300 to lead to large numbers of brood present between days 300-400. The queen may produce large amounts of pheromones before she begins to increase egg production, so stimulating the workers to prepare for a large brood. Brood numbers increased at the

time of year when high dry weight forage was available and it is possible that over the years, the queen's egg production has become synchronised with the annual changes in leaf quality.

Brood production in laboratory nests often appears cyclic. During the course of this study, the large *Atta sexdens* nest had greatly fluctuating numbers of brood and a flush of larvae was usually followed by a flush of pupae (pers. obs.). However, even when the majority of brood were larvae, a few pupae were visible and there were few periods when no brood at all were present. This suggests that while massive brood production may take place, perhaps in response to large food resources, it also occurs continuously, at low levels.

Both brood and staphylae were carried between gardens and this transfer might be related to larval food requirements. However, pupae were also moved and these do not require food. Different brood stages require slightly different environmental conditions; eggs and young larvae of some species need less warmth and moderate humidity, compared to large larvae which require a more humid warmth, while pupae require a drier warmth (Wheeler and Wheeler 1979). Eggs, larvae and pupae are therefore usually placed in separate groups. Brood transfer between gardens might therefore be a response to environmental stimuli. Evidence for this was provided when the culture room overheated and large numbers of pupae were moved into a dome containing a developing garden (*Atta sexdens* Garden 2). This was situated by the culture room door, where conditions were slightly cooler. However, the patterns of brood arrangement in the nest may be more important for influencing the priority with which different brood items are tended (Franks and Sendova-Franks 1992).

Staphylae may have been moved between gardens when one garden produced a surplus while another had many larvae present and was suffering a staphylae shortage. Although it might be more sensible to move larvae to the food source, there may be constraints against this. Brood items carried

outside gardens may be more vulnerable to environmental differences or accident. However, very few brood or staphylae were moved (Table 3.5) and these may have been moved by chance rather than design. Brood, particularly pupae, are continually being moved around within the garden and brood groups are fluid, continually losing or gaining members (Bass 1989). A worker carrying a brood item between gardens may just be extending this process. Similarly, workers pluck staphylae from the garden surface to take to larvae and some might wander out of gardens by chance. This is likely, because although leafcutting ants are highly organised at the colony level, at the individual level they are not intelligent and may make 'mistakes'. Evidence for this is provided by the fact that some refuse loads were carried back into gardens.

When the ants 'decide' to transport brood or staphylae between gardens, this transfer becomes very obvious. This occurred in developing gardens of *Atta cephalotes* when around April 24th, all developing gardens being observed began to export large numbers of staphylae to mature gardens.

4. The value of time series analysis

Time series analysis was useful for examining the relationships between different series. Relationships can be seen in graphs, but too much is left to personal opinion. Cross correlating to look for significant values puts an objective standard of judgement upon how closely related series are to each other. Time series analysis can also show or confirm the presence of trends or relationships which are not otherwise obvious, for example, Ac gardens were more closely correlated for forage intakes and refuse outputs than As gardens, which was not initially suspected. Equally, time series analysis is also useful for showing the absence of such trends or relationships.

Time series analysis was useful for confirming that different gardens followed the same patterns for the same factor, although the necessity of comparing pairs probably

led to error. However, time series analysis is flexible: many significant values indicate a strong relationship, while occasional peaks are more likely to be due to chance.

Modelling might have been useful (Diggle 1990) but was not carried out, since the most likely factor affecting the established garden data, leaf quality, was not assessed.

Time series analysis was less useful for immature garden data, which was partially non-stationary. Also, two series with one large peak each will show significant correlations, purely because of these single large peaks. Significant coefficients will show a delay equal to the distance between the two peaks. This makes conclusions about relationships between different factors suspect. For example, forage and fungus intake peaks coincided, but were they really related? Regressing peak intakes of forage and fungus showed no relationship. If developing gardens had been followed for a longer period, there would probably have been fewer problems with this.

SUMMARY

New gardens were founded by workers importing fungus garden and substrate. Rates of intake then declined again. *Atta sexdens* gardens also imported staphylae during the first 10 days. Staphylae were visible on the garden surface after 11 days and gardens reached their maximum size by 25 days. *Atta cephalotes* gardens exported staphylae from April 24th onwards, for unknown reasons. All gardens followed similar patterns of intake, output, size and usually brood numbers, but *Atta cephalotes* gardens were more closely related to each other in terms of forage intake and refuse output than *Atta sexdens* gardens.

The ants practised continuous rather than discontinuous culture and individual established gardens showed fluctuations in intake and output, brood numbers and size over 700 days. Refuse output was random and individual gardens followed different patterns. Changes in forage intake were caused by different rates of removal of forage,

either through changing worker preferences, or the nature of forage, since a constant amount of forage was supplied daily. Different types of forage were removed at different rates. High dry weight mature leaves led to an increase in garden size between November and February and brood numbers increased with garden size. All gardens shared the same patterns of forage intake, brood numbers and size over 700 days.

Time series analysis elucidated relationships between data series which were not always obvious from looking at graphs.

Chapter 4: The internal structure and life cycle of a fungus garden

INTRODUCTION

Studying the outer surfaces of the fungus garden may show atypical results, since all worker castes have access to it. However, the internal areas which make up most of the garden are hidden from view. Knowledge of the internal structure of a garden provides insights into how the ants obtain staphylae from its inside. If, for example, all internal cavities were closed off or too small to admit workers, then large areas which might produce a crop of staphylae would be lost. The upper areas of a garden possess large cells, which admit all worker sizes (see Fig. 2.1, Chapter 2). However, towards the base, cavity sizes become smaller, raising the question of accessibility for larger workers. Large numbers of small spaces would provide a greater surface area and hence a greater potential staphyla production than a few large cavities, but would restrict worker access. It is therefore likely that the ants have evolved a compromise, producing gardens which combine accessibility with large internal surface area.

Three-dimensional information about the internal composition of structures can be obtained by making two-dimensional sections of them and applying previously derived equations. This is 'stereology' and is a technique used widely in the study of rock, soil and tissue structures. Delesse (1848) showed that component areas in section are equivalent to component volumes in the original structure and surface areas per unit volume of structures, such as membranes in cells, can also be estimated (Williams 1977). These techniques are important because when a section is made of a cell for example, component organelles are not bisected. Instead, the section may cut diagonally through or along the side of a component. A

curving tube may therefore appear as an ellipse and a sphere will appear in sections as circles of different sizes. Stereological formulae must therefore be applied to sections to gain information from them.

Another important feature of fungus gardens is how long substrate material continues to support fungal growth. Weber (1972) found that one garden of *Atta cephalotes* had a life cycle of 7 weeks, while a second had a life cycle of 4 months and developing gardens of laboratory colonies of *Atta cephalotes, Atta sexdens* and *Atta colombica tonsipes* all had life cycles of 3-4 months. However, Chapter 3 showed that developing gardens began to discard refuse after about 50 days of growth (a 2 month life cycle). Weber (1972) 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. In the tropics, the rainy season and subsequent flush of leaves coincide with intensive leaf-cutting and garden build-up, followed by a lull and subsequent casting out of refuse. However, Weber also noted that laboratory colonies tend to be cyclic in carrying out exhausted substrate and that different colonies have different schedules.

This chapter examines the internal structure of a single garden of *Atta sexdens* and also looks at the lengths of fungus garden life cycles in the laboratory at different times of the year, to compare with Weber's (1972) figures and those obtained in Chapter 3. Different times of the year are considered because of the effect of changing forage quality. The presence of staphylae on internal surfaces of the garden is also examined.

Methods for marking areas of fungus garden are looked at, since observations made on different areas of the garden (of different ages) are not strictly comparable, due to changes in forage quality over time. Marking an area means that it can be followed through the life cycle of the garden. This method is used to follow the numbers of

staphylae present on the garden surface at different stages in the life cycle.

MATERIALS AND METHODS

All experiments were carried out using fungus gardens from a large nest of *Atta sexdens* (80 gardens).

1. Determining the internal structure of a fungus garden

This can be examined by sectioning. Fungus garden alone is too fragile to section, therefore a carrier was required to support it. Campbell and Tomkeieff (1952) stated that lung tissue could be embedded in gelatine without shrinkage or distortion for sectioning, therefore this method was tried.

Initially, several concentrations (12.5%, 15%, 20%, 25% and 30%) of gelatine solution were tested. Dry gelatine powder was mixed with distilled water, allowed to stand for 30 minutes and then warmed to 40^{0}C in an incubator. Pieces of fungus garden (approximately 50 cm^3) were then introduced into these solutions and left to soak for 1 hour before cooling in a refrigerator. Although all solutions set, none penetrated the fungus garden well, so the procedure was repeated again, this time heating solutions to 75^{0}C before introducing the fungus garden samples. The samples were then left to soak overnight at 50^{0}C in an incubator. The most suitable concentration was found to be 25% gelatine as lower concentrations did not set well and 30% gelatine was too tough to section.

To ensure worker immobilisation, the test fungus garden was first placed in a muslin bag (as rapidly as possible to avoid disruption) and dropped into liquid nitrogen (the size of the nitrogen flask neck limited the size of the garden which could be used). Once frozen, the garden was placed into the hot gelatine solution and to

facilitate penetration, the garden was weighted down by a wire gauze with three large nails attached (Fig. 4.1).

The apparatus was then cooled for several hours to allow the gelatine to solidify and the resulting block containing the garden was sectioned using the apparatus shown in Fig. 4.2.

The sections obtained were placed in 9 cm petri-dishes and photographed with a light source behind them. Slides were made and projected on to a screen. The projected images were much larger than the original sections and therefore easier to examine (the scale of size increase was noted). Outlines of the visible cavities in each section were traced on to paper from these images, along with any workers or brood visible. Stereological techniques were then applied to find the following:

a) Air to volume ratios

These can be estimated by comparing the area of the cavities with the total area of the section, according to the Delesse principle (1848), which states that the area fraction of a component lying in a random transverse section is equivalent to its volume fraction, expressed by:

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

where:

Vc = total volume of the cavities within the section
Vt = total volume of the section
Ac = total area of the cavities within the section
At = total area of the whole section
E = theoretical mean

The outlines of cavities on the paper tracings of the projected images of the sections were traced using a digitiser cursor. This measured both areas (which were fitted into the above equation) and circumferences (profile lengths) of cavities. The latter were used to calculate S_v (overleaf).

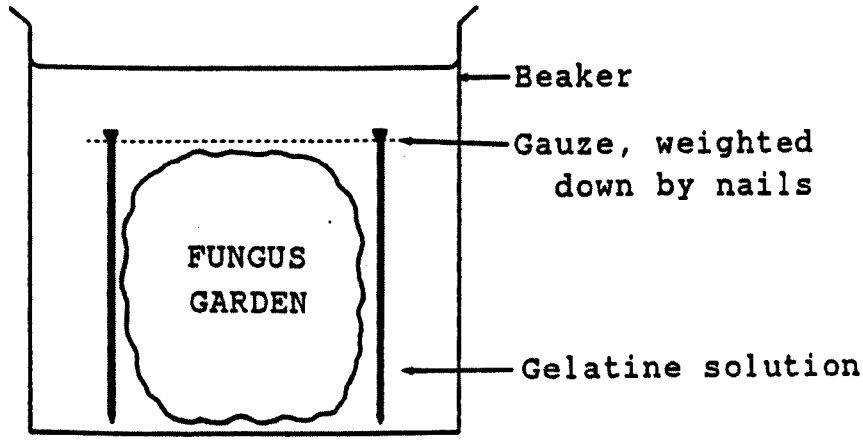


Figure 4.1: Apparatus used to impregnate a fungus garden with gelatine.

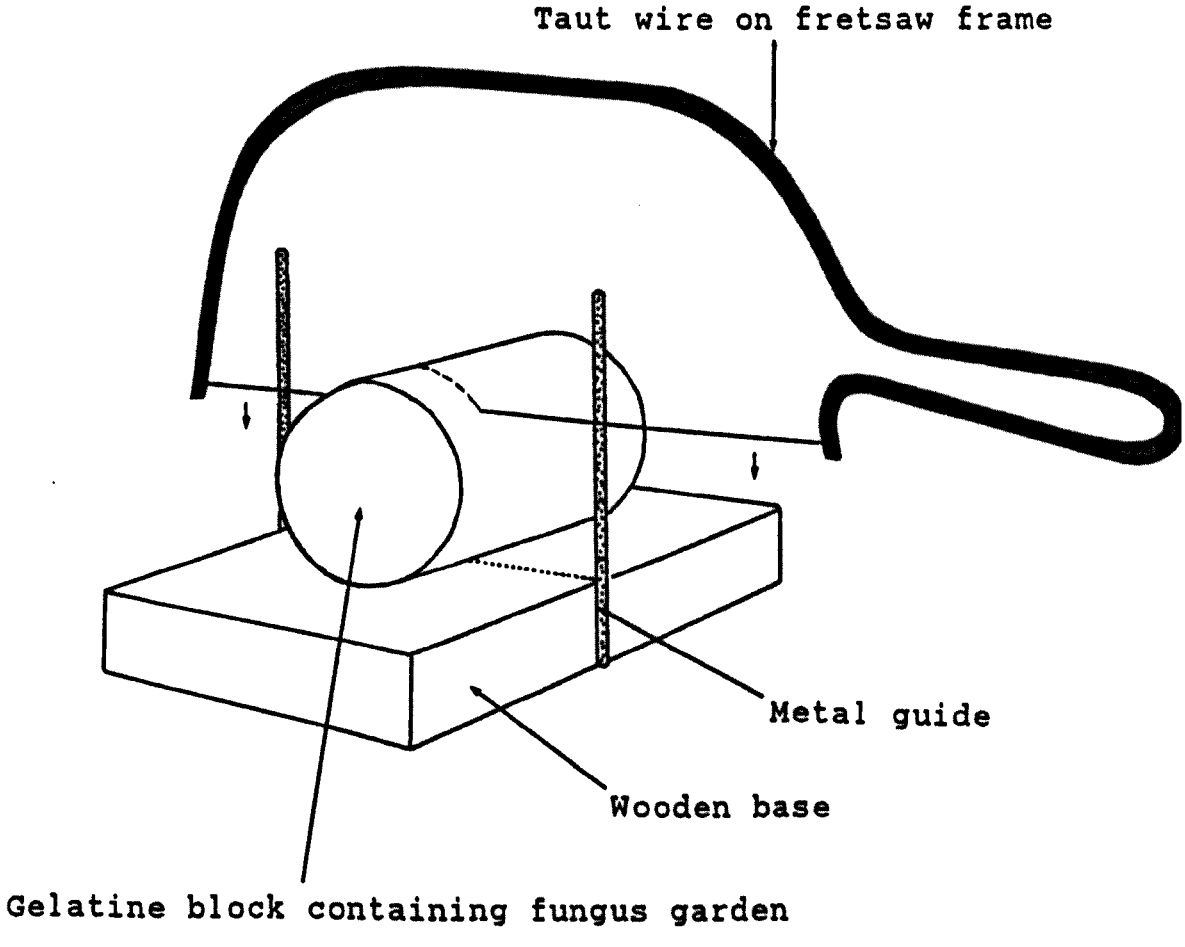


Figure 4.2: Apparatus used to section a fungus garden in a solid gelatine carrier.

b) 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 derived for S_v and the one used in this study was:

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

where:

S_v = surface area per unit volume (mm^2 per mm^3, or mm^{-1})

m = 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)

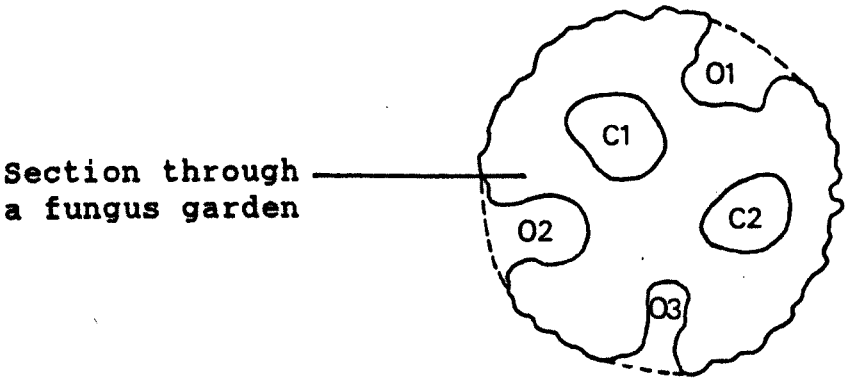
Two sets of figures were calculated for both air to volume ratios (volume spaces) and S_v. For volume space, 'percentage internal space' which excluded 'open' cells (those connected to the outer surface of the garden) and 'percentage net space' which included them were calculated (Fig. 4.3).

For S_v, both overall S_v (including external surface area) and internal S_v were calculated for each section. For the latter, a large rectangular area inside each section was examined, the sum of the enclosed profile lengths being fitted into the equation.

Visible external circumference and actual profile length of each section were examined (Fig. 4.4). The ratio between internal and external surface areas could also be examined by comparing external profile lengths with the sum of the internal cavity profiles.

2. Techniques for marking fungus gardens

In order to follow rates of turnover of substrate in fungus gardens, techniques for marking areas of garden were devised, so that a layer of garden of known age could be observed over time. Two basic approaches were employed;



$$\%internal space = \frac{Vc}{Vt} \times 100 = \left(\frac{C1}{T} + \frac{C2}{T}\right) \times 100$$

$$\%net space = \frac{Vc}{Vt} \times 100 = \left(\frac{C1}{Tx} + \frac{C2}{Tx}\right) \times 100$$

where:

Vc = total volume of cavities C1, C2
Vt = total volume of section
T = total area of section (solid outline)
Tx = total area of section, plus areas of O1, O2, O3 (broken outline)
C1, C2 = internal closed cavities
O1, O2, O3 = cavities opening on to the external surface

Figure 4.3: Definitions of percentage internal space and percentage net space, both of which are measures of percentage volume space.

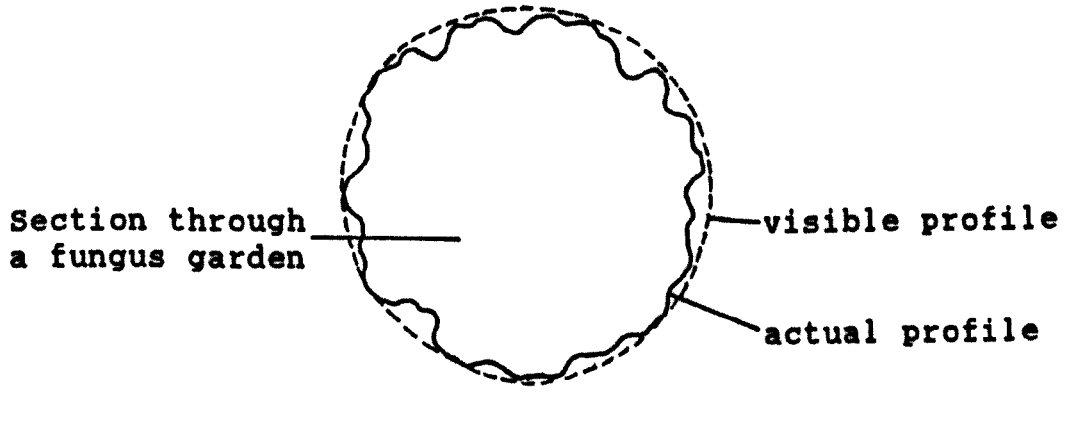


Figure 4.4: Visible and actual circumferences of the external surfaces of sections of a fungus garden.

a) Placing artificial markers

Ten plastic-headed steel pins were sterilised with alcohol, allowed to dry and inserted into the side of a fungus garden, from which the plastic dome had been temporarily removed. Ten more similarly treated pins were dipped into orange juice before being inserted and a third set was exposed to the ants on a forage table before insertion. The fates of all these pins were then observed.

The majority of pins were removed from the garden within 24 hrs. In some cases, pins were only half pulled out, but this was apparently because the dome wall was in the way. All pins had been discarded within 3 days. This method of marking was therefore abandoned.

b) Presenting markers for the ants to incorporate

Several methods were tried. Firstly, a dome was removed from a garden and 50 small squares of blue plastic sheet (4 mm^2) dipped in orange juice, were sprinkled over the top of the garden. It was hoped that the ants would treat these fragments as substrate and incorporate them into the garden. However, all plastic squares were removed from the garden surface within 3 hrs and by 24 hrs had all been dumped with refuse.

Secondly, a 0.001 g ml^{-1} solution of Neutral Red dye (Tensen's recipe, as described by Gurr 1960), a non-toxic vital stain, was used to dye knotweed leaves (*Reynoutria* sp.). The dye solution was first warmed to around 90^{0}C to ensure rapid penetration into leaves within a few seconds and the resulting red-stained leaves were acceptable to the ants after rinsing with water. Three isolated gardens were fed on these dyed leaves for 2 days and the persistence of the stain was observed. This red-dyed forage clearly marked young garden, but the colour did not persist beyond 3 days.

A third method involved an organic marker. Holly leaves (*Ilex aquifolium*) have toughened edges and sharp spines which provide protection against herbivores. Such

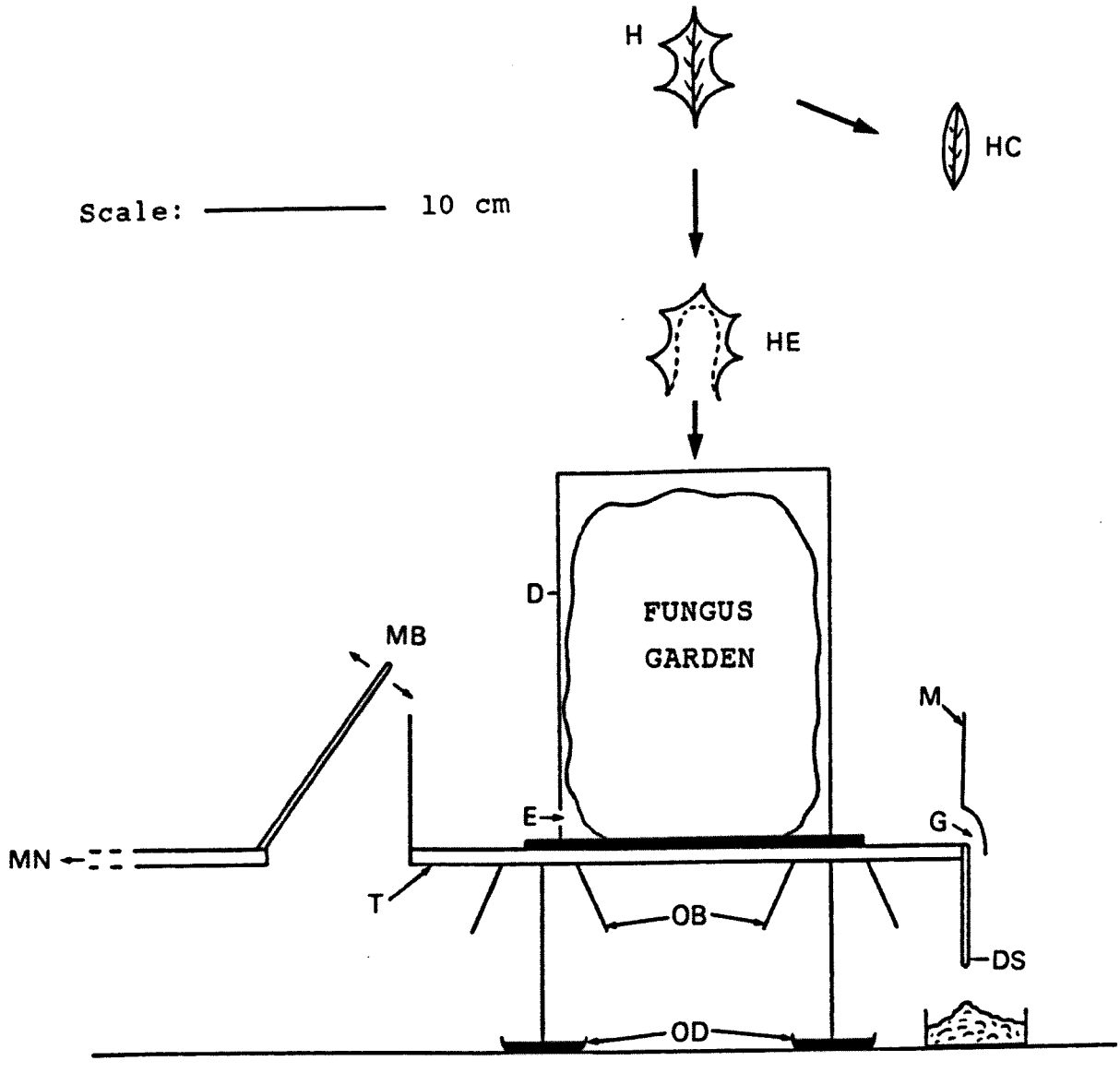
lignified leaf edges are likely to be degraded much more slowly by fungi than softer tissues and may therefore persist and remain visible in fungus gardens. A single garden was placed on a small table standing in oil-pots, disconnected from the main nest. Refuse was dumped from a wooden strip suspended from the table edge, which also had a 6.5 cm vertical metal rim to discourage dumping from the table edges. Over 4 days, 32 g of holly leaf edges containing approximately 2,500 spines were offered to this isolated garden and the workers took this substrate readily. The table was then connected to the main nest via a bridge (Fig. 4.5). Workers are reluctant to carry refuse upwards, therefore the vertical bridge to the main nest ensured that refuse produced by the test garden was dumped into the collecting dish. This refuse was then examined at intervals over the next 70 days. The presence of holly spines visible on the garden surface was also observed over this period.

Spines were clearly visible, embedded in the garden surface, with fungus actually growing on them. They were also easily visible in refuse as arrow-head shaped structures, particularly when refuse was dried and gently ground with a pestle and mortar. Holly-spine marking was therefore a simple and effective method of labelling areas of fungus garden and was consequently used in all experiments requiring marked areas of garden. Fungus gardens could therefore be marked using this method to examine their life cycles.

3. Techniques for estimating the numbers of staphylae

a) In refuse

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



D Dome containing garden, resting on ceramic tile
DS Dumping string
E Entrance hole
G Gap in metal strip surrounding table to allow access to the dumping string
H Holly leaves
HE Holly leaf edges offered to ants
HC Holly leaf centres discarded
M Metal strip surrounding table to prevent refuse from being dumped over the edges
MB Moveable bridge
MN To main nest
OB Oil-painted baffles to prevent refuse from falling into oil-filled dishes
OD Oil-filled dishes to prevent ant escape
R Collected refuse
T Table

Figure 4.5: Methods used to supply a single fungus garden with holly spine markers and to collect the spine-containing refuse discarded from this garden.

b) In a fungus garden

Vertical transects can be used to look at how numbers of staphylae present vary with garden age. Numbers of staphylae were counted by dissecting samples from different areas of the transect under a binocular microscope. Samples were removed from inner surfaces, since staphyla numbers may vary between outer and inner surfaces. They may also vary on different areas of the outer surface as some areas are used as walkways and have very few staphylae present (pers.obs.).

c) On a marked area of garden over time

A clear perspex observation chamber was set up, containing a metal grid for the ants to build on (Fig. 4.6). Initially, this chamber was sprayed inside and out with water and left on a table connected to the nest for several hours, to allow the ants to become accustomed to it. Then, 200 ml of young and mature garden with attendant workers were placed inside the chamber. Forage was supplied and the bridge connecting the table with the main nest was removed, forcing the ants to build in the new chamber. After 24 hrs, building had begun and the table was reconnected to the main nest. The chamber was then left for several weeks until the ants had completely filled it with fungus garden. The bridge was then removed again and approximately 1,000 holly spines, weighing 21 g, were supplied to the chamber. After 72 hrs, this had been incorporated into the garden in a 5 cm layer and the bridge was returned.

d) In cavities of different sizes

External garden surfaces are atypical of the garden as a whole. To look at the differences between staphylae numbers on external and internal surfaces of differently sized cavities, pieces of fungus garden were examined using a binocular microscope with a 1 cm^2 eye-piece graticule. This graticule consisted of a 1 cm^2 area divided into quarters, with millimetre divisions marked along the edges.

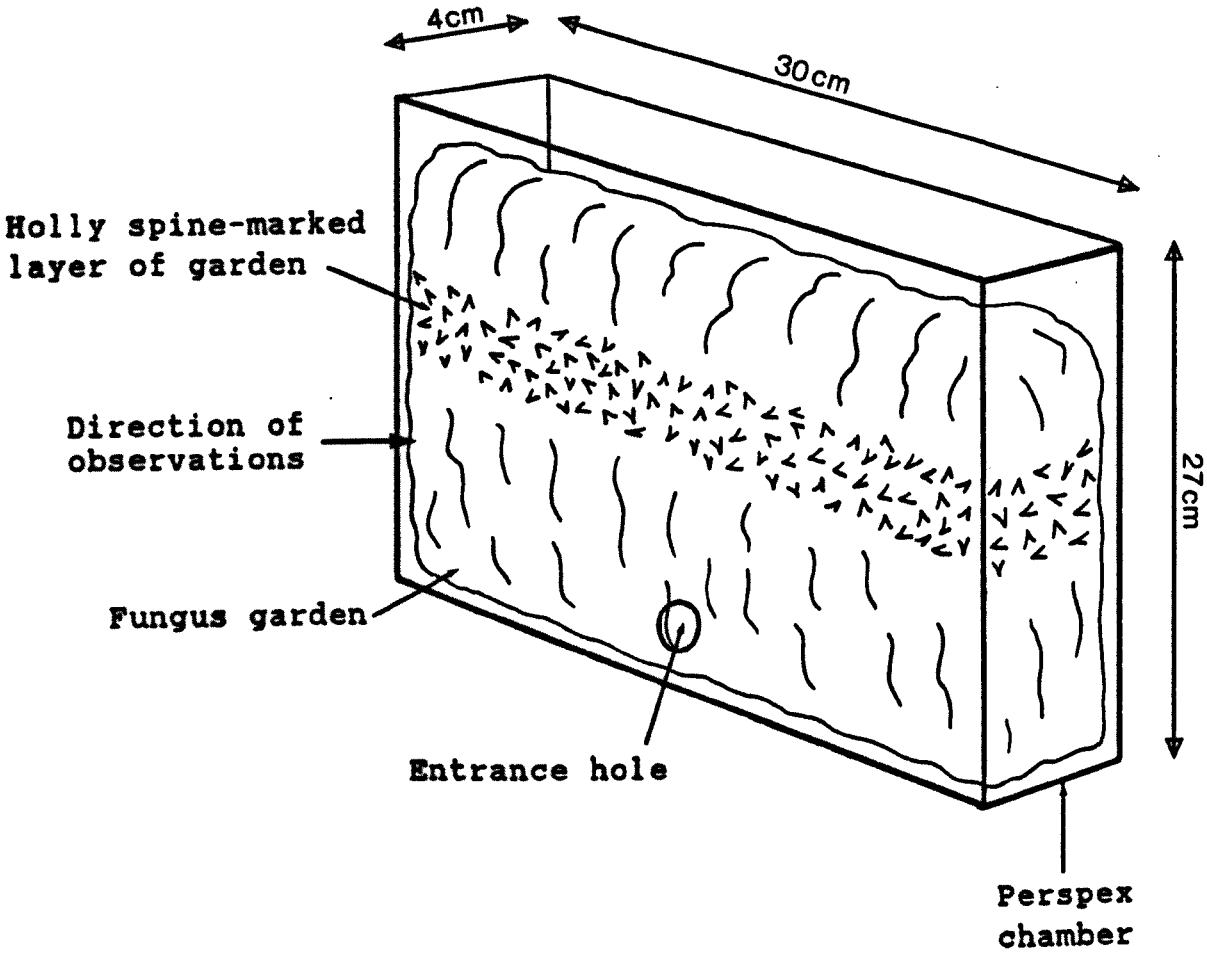


Figure 4.6: Observation chamber used to follow staphyla production with time on a holly spine marked area of fungus garden. The chamber contained a wire grid (1 cm mesh) which the ants constructed their fungus garden on.

External standing crops (staphylae per cm^2) were noted and then, taking cavities observed at random, the internal surfaces of these were exposed using dissection needles. The numbers of staphylae per 0.25 cm^2 were then counted (this being the largest area that could reasonably be exposed in a small cavity).

RESULTS

1. The internal structure of a fungus garden

A single small garden (250 cm^3) built by workers in a small container (300 cm^3) was sectioned. Sixteen slices, each approximately 3-4 mm deep were produced, but some inaccuracy was produced by large workers catching the wire and being dragged across sections, so damaging them. The rubbery texture of the gelatine block also resulted in some uneven sections. The garden was cut horizontally, so that section 1 was of young garden from the top and section 16 was of the base (aging garden). However, sections 1 and 16 were damaged by crushing and were not examined.

Sections 2, 13, 14 and 15 were the smallest, with respective areas of 7.1, 8.9, 4.8 and 3.0 cm^2 and 13, 45, 33 and 18 internal cavities each. Sections 3, 4 and 12 had areas of 13-18 cm^2 and 44-84 cavities each, while sections 5-11 all had areas of 22-32 cm^2 and more than 110 cavities each.

There were no significant differences between cavity areas or circumferences in different sections ($p>0.5$, Mood). Overall median cavity area was 2.0 mm^2 (95% confidence limits 1.8, 2.1) while median cavity circumference was 5.3 mm (95% confidence limits 5.1, 5.5).

a) Number of cavities per unit area

The total number of cavities per cm^2 of section area was highest at the base of the garden, in sections 14 and

15 (Fig. 4.7). Section from upper (young) areas had very small numbers of cavities per cm^2.

b) Air to volume ratios

Percentage net space (which included cavities opening on to the outside) was much higher than percentage internal space for sections 2 and 3 (Fig. 4.8). These were both from young garden, which has large open cells (see Fig. 2.1, Chapter 2). This means that in a young section, there will be very few apparently closed cells.

Percentage net space was fairly constant for each section (except section 15) with a mean value of 32.2% (SE ± 2.0) whereas percentage internal space with a mean value of 18.4% (SE ± 1.8) was higher for sections from the central areas of the test garden and decreased at the top and base.

c) Surface areas per unit volume (S_v)

Section 2 had no internal space, but overall S_v was greater than internal S_v for the other sections (Fig. 4.9). Both values of S_v were relatively constant for each section, although some variability was present. Mean overall S_v was 0.73 (SE ± 0.03) mm^{-1} whereas mean internal S_v was 0.51 (SE ± 0.04) mm^{-1}.

d) Visible and actual profiles

Both actual and visible profile lengths of the external surfaces of sections (see Fig. 4.4) were higher for middle sections of the garden than for 'end' sections from young or aging garden sections (Fig. 4.10). However, the ratios of actual to visible profiles were fairly constant and had a mean value of 2.07 (SE ± 0.10).

e) Worker presence in sections

In total, 139 workers were found in 'closed' internal cavities along with 36 items of brood (larvae and pupae could not be distinguished). Headwidths were measured for each worker and were compared with the areas of the

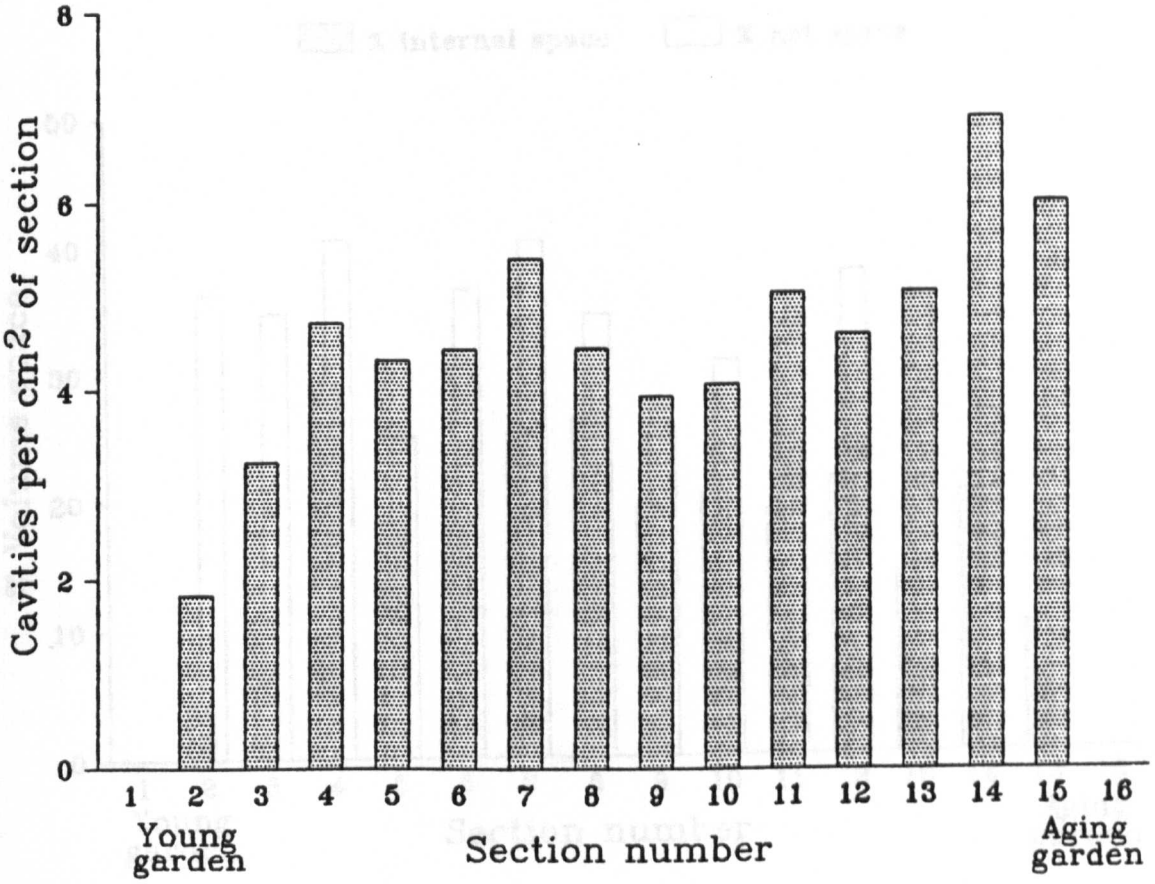


Figure 4.7: The number of cavities recorded per cm^2 of section area for different sections of a fungus garden. Sections 1 and 16 were not assessed due to damage.

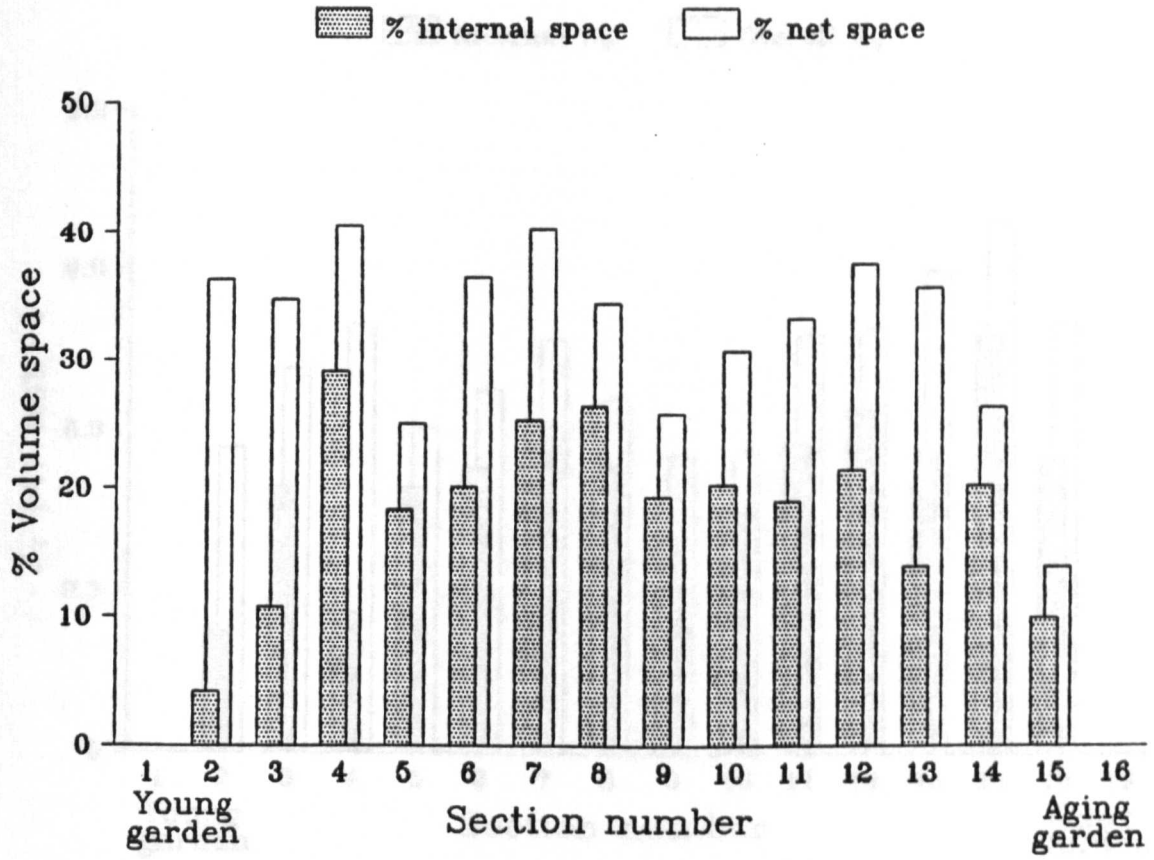


Figure 4.8: Percentage of space in different sections of a fungus garden, expressed as both % internal and % net space. Sections 1 and 16 were not assessed due to damage.

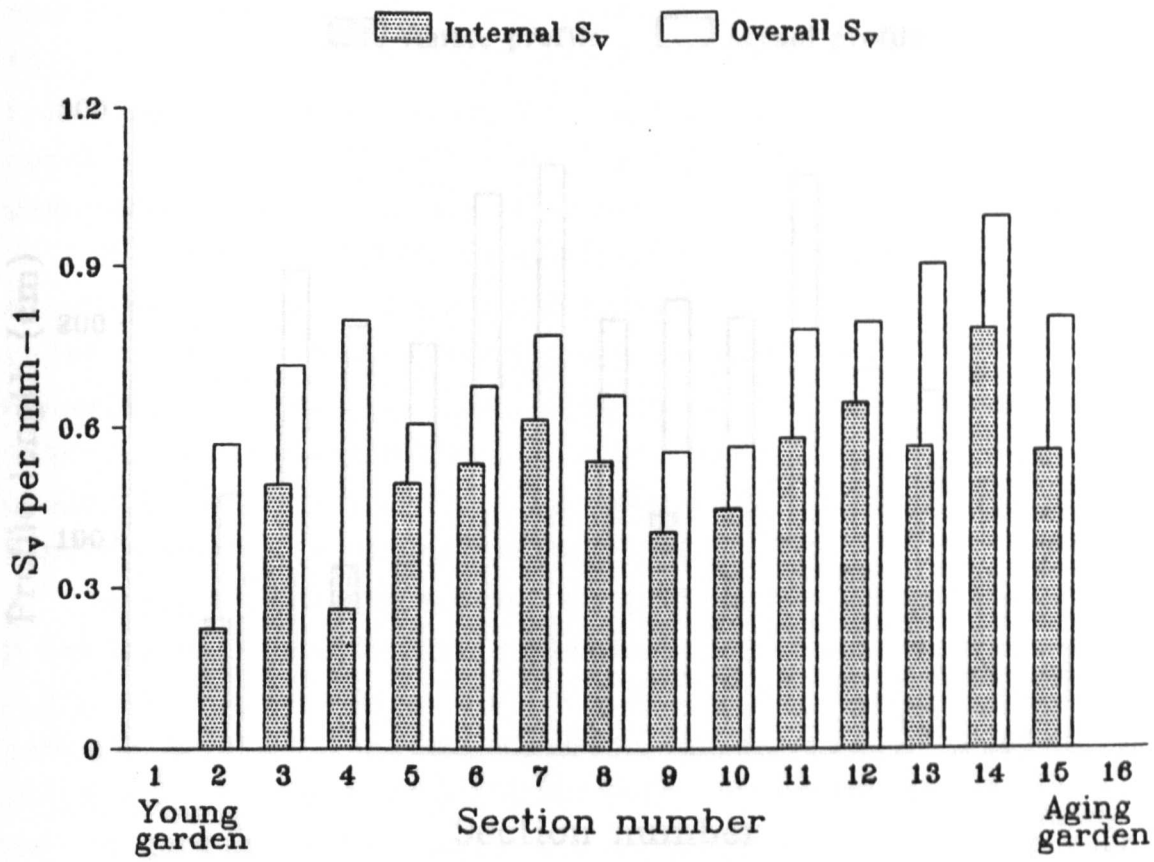


Figure 4.9: Surface areas per unit volume (S_v) for different sections of a fungus garden, expressed as both overall and internal S_v (mm^2 per mm^3). Sections 1 and 16 were not assessed due to damage.

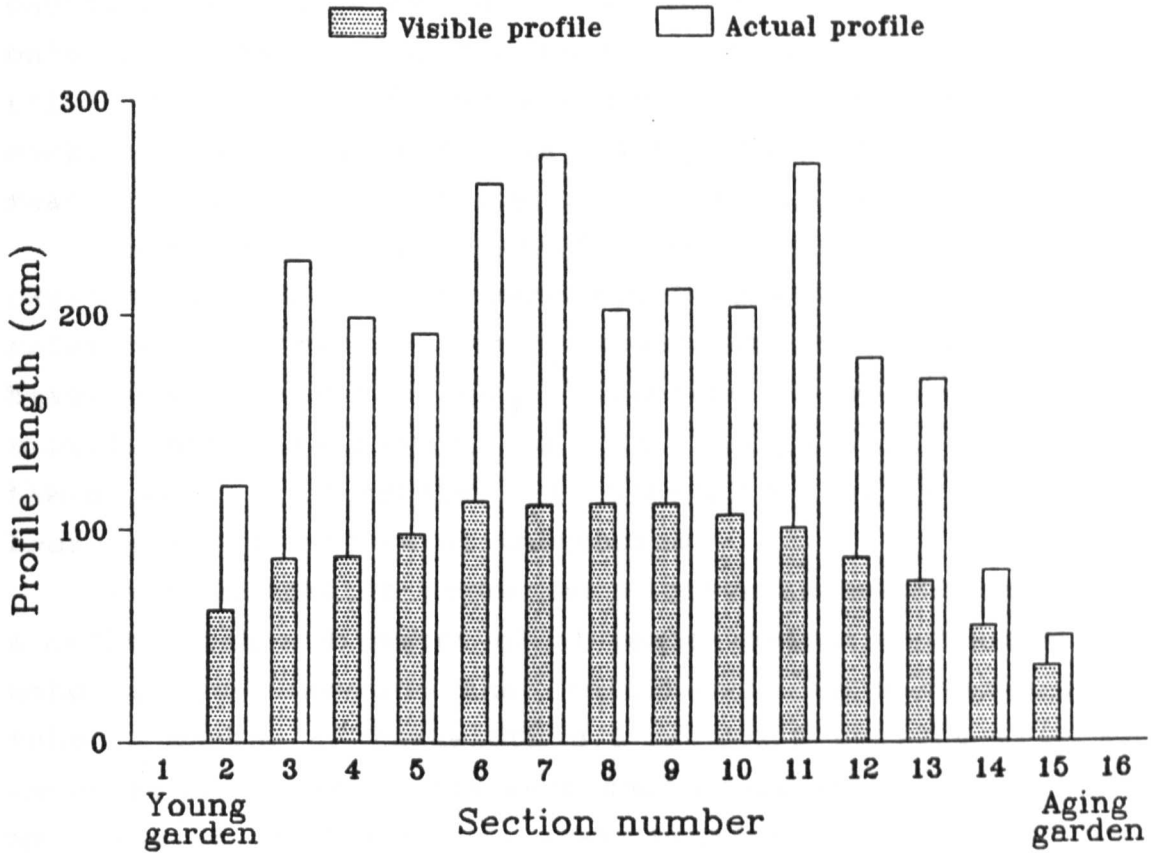


Figure 4.10: Visible and actual profiles of the external surfaces of sections of a fungus garden. Sections 1 and 16 were not assessed due to damage.

cavities in which they were found. Of all workers examined, only 10.1% had headwidths outside the minima size range (>1.2 mm). Sizes of the 139 garden cavities containing workers examined ranged from 2.4-197.0 mm^2 in area, with a mean area of 64.2 (SE ± 4.6) mm^2 and a median of 47.0 mm^2.

Regressing worker headwidths against the diameters of cavities in which they were found showed no significant relationship ($p>0.1$, $rsq_{(adj)}$ 1.1%) and regressing log_{10} headwidths against log_{10} diameters also showed no significant relationship ($p>0.08$, $rsq_{(adj)}$ 1.5%) However, there were difficulties in measuring cavity diameter because of the nature of sectioning.

Assuming that a worker of a given headwidth requires a cavity with a diameter of at least that size in order to enter it and assuming that this cavity is a cylindrical tube, a worker of headwidth 0.6 mm would only be able to enter cavities with cross-sectional areas greater than 0.28 mm^2. Figures for the cavity areas obtained for each section could therefore be used to show the maximum numbers of cavities in each section, which workers of different sizes could enter. Workers of headwidth 0.6 mm could enter a mean of 98.6% (SE ± 0.6) of available cavities, those of headwidth 1.0 mm, 83.6% (SE ± 1.5) of cavities, those of headwidth 2.2 mm, 21.5% (SE ± 1.7) of cavities and those of headwidth 4.0 mm, only 4.3% (SE ± 0.8) of cavities. Although cavity sizes seen in sections were not the same as actual cavity sizes, these figures do give some indication of how accessible the garden is to workers of different castes.

Further evidence that workers can enter the cavities within the garden was provided using a dried 800 cm^3 garden from an *Atta cephalotes* nest. This dried out naturally in the culture room when its plastic dome was removed, the workers present preventing contaminant growths from developing. Talcum powder was gently blown into a cavity on the side of this garden (in a mature area) using a straw. When the garden was subsequently dissected with forceps, the powder had penetrated deep into it and was found in

cavities from the base to the top. The cell structure of the dried garden was still intact.

2. The life cycles of fungus gardens

Four gardens were marked with holly spines and numbers of spines discarded per day were recorded at regular intervals. The first garden was marked during November 1990 using 2,500 spines, the second during June 1991 using 2,500 spines and the third and fourth during August 1991 with 1,000 spines each. Refuse was collected on alternate days until no more spines were discarded.

Two further sets of gardens were marked with 500 spines each. Four were marked simultaneously in October 1991 and five more were marked during November 1991. The rates of output of holly spines could then be assessed simultaneously for several gardens.

Three gardens marked with holly spines during June and August 1991, had similar peaks of spine dumping, between 20 and 30 days after marking (Fig. 4.11). However, the garden marked during November 1990 had a much later peak of holly spine dumping, between 60 and 80 days after marking. Regressing the times from spine incorporation to peak spine output, against the number of days between the summer equinox and spine incorporation, showed no significant relationship ($p>0.1$, $Rsq_{(adj)}$ 62.7%, df 3).

In gardens marked during October and November 1991, peak dumping times coincided closely, although peak spine outputs varied between different gardens (Figs.4.12 and 4.13). Gardens marked during October had a mean peak spine output 36.0 (SE ± 2.8) days post-marking, whereas those marked during November had peak output times after 42.8 (SE ± 1.8) days, although these were not significantly different ($p>0.07$, ANOVA). These results do, however, suggest that gardens marked at the same time will have similar rates of turnover, but that rate of turnover varies with time of year, being longer during the winter and shorter during the summer (see Fig. 4.11).

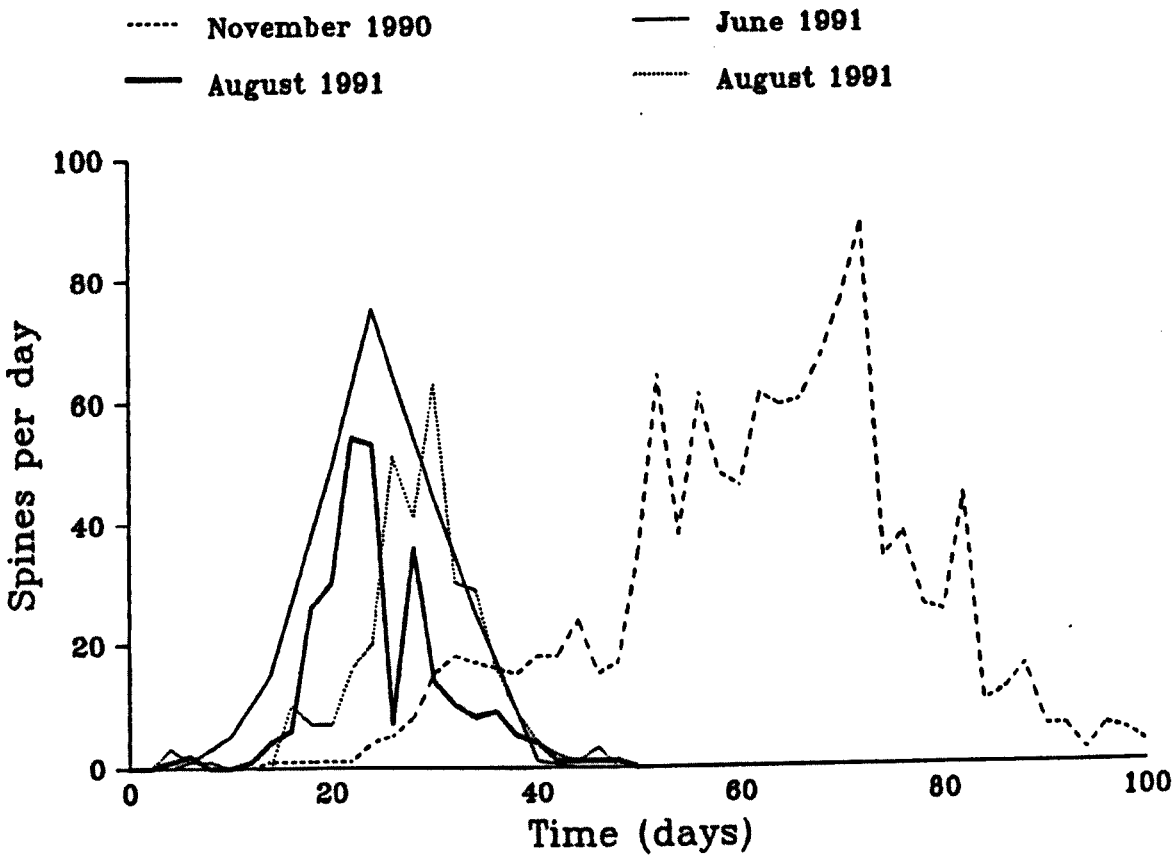


Figure 4.11: The numbers of holly spines discarded daily in refuse from four fungus gardens marked at three different times of the year.

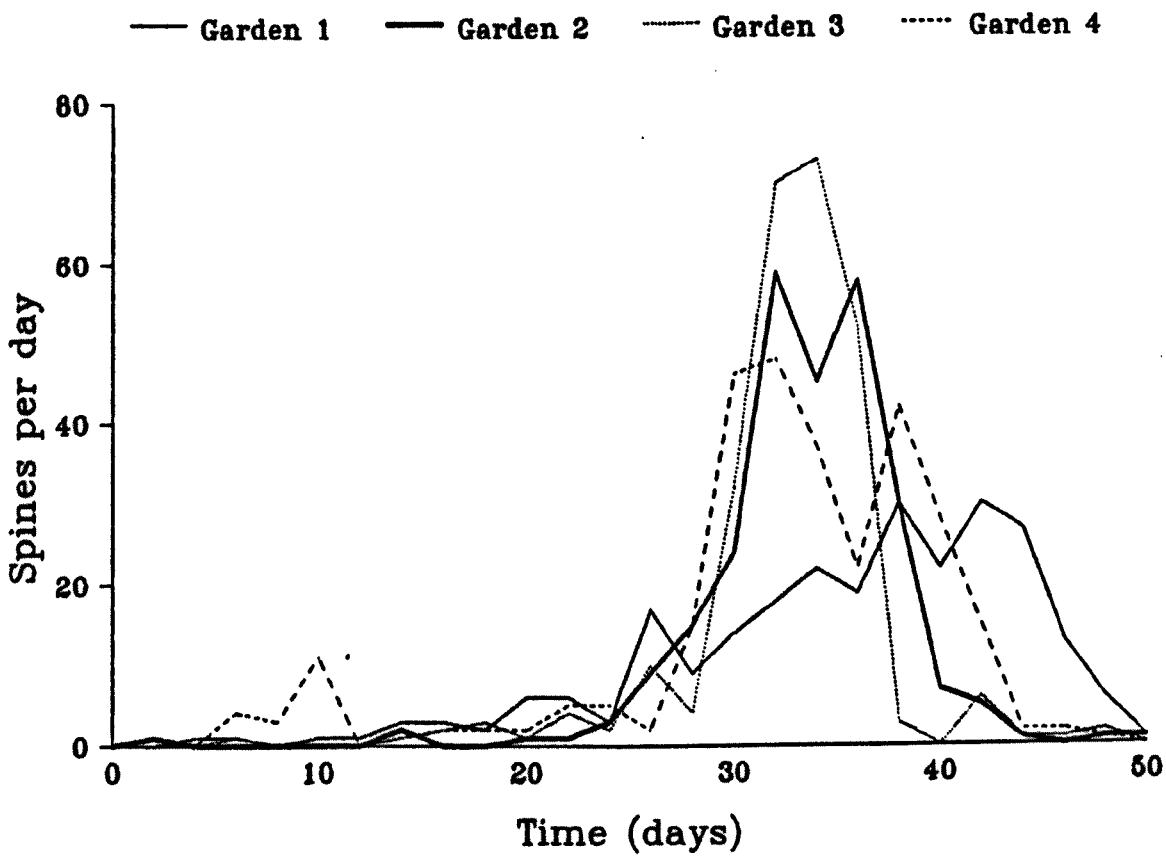


Figure 4.12: The numbers of holly spines discarded daily in refuse from four fungus gardens marked simultaneously during October 1991.

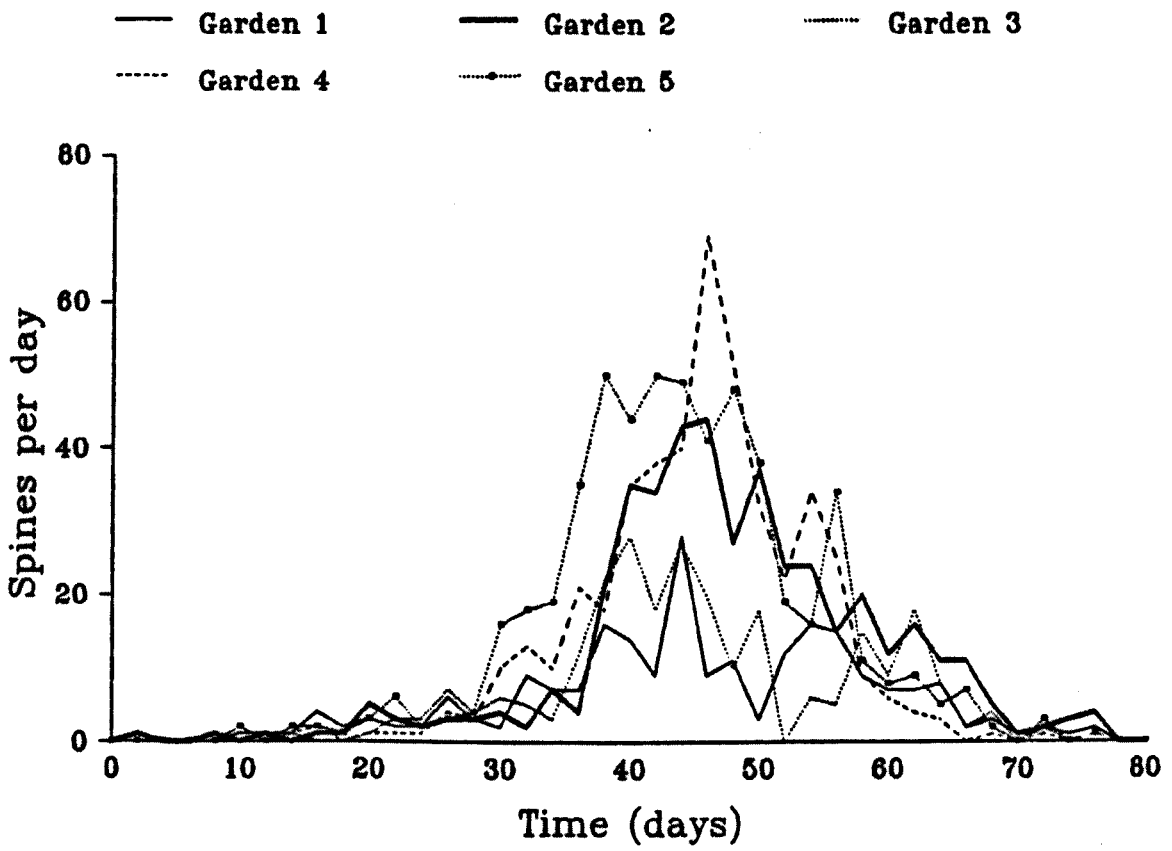


Figure 4.13: The numbers of holly spines discarded daily in refuse from five fungus gardens marked simultaneously during November 1991.

3. The relationship between standing crop of staphylae and the age of the garden

A vertical transect was obtained by cutting off the corner of a large mature garden. This transect was 12 cm deep and could be divided into young, mature and aging layers. It was divided into 1 cm deep zones and 0.05 g samples were removed from the inner surfaces of each zone.

Staphyla numbers varied from the top to the base of this garden (Fig. 4.14). Young garden had almost no staphylae, in contrast to mature garden, which had the largest numbers. To confirm this, the numbers of staphylae present on garden marked with holly spines were counted at intervals as the garden aged.

From the first day of spine-incorporation until the spiny layer disappeared, up to 50 x 1 cm^2 areas on the marked layer were selected at random and the numbers of staphylae in each counted using a binocular microscope (by observing through the side of the dome). The progress of the spine-marked layer down the garden profile was also recorded. Three gardens were marked to observe changes in staphylae numbers; in April, October and November 1991. The second and third replicates were also backed up by sets of four and five simultaneously marked gardens. These were used to look at how the rate of spine removal corresponded with numbers of staphylae present on the garden surface and to assess differences in turnover rates between individual gardens.

Few staphylae were present during the first 5 days in the April-marked garden, then numbers rose sharply over the next 20 days (Fig. 4.15). By day 10 the whole spine-marked layer had reached the mature stage and by day 20 was in the centre of the mature garden zone. By day 30, staphyla numbers had peaked and were just beginning to fall, while the spiny layer had reached the base of the mature garden zone and was on the verge of becoming aging garden. By day 40, staphyla numbers per cm^2 had decreased to day 10 levels and the spiny layer had become aging garden. After this,

the spiny layer was rapidly dumped and by day 46 had almost disappeared, precluding further staphylae counts. By day 50 all visible holly spines had disappeared.

Figs.4.16 and 4.17 show similar data to Fig. 4.15 and also show that peak output of holly spines in refuse took place after the decline of staphyla numbers per cm^2. Spines were collected from refuse for much longer than spiny layers were visible in the staphyla observation chamber, particularly in Fig. 4.17.

Examining 50 refuse loads showed that a mean of 0.5 (SE ± 0.1) staphylae were dumped per load. Refuse loads had a mean weight of 3.6 (SE ± 0.1) mg. In contrast, five 0.1 g samples of mature fungus garden contained a mean of 81 (SE ± 4.0) staphylae each, therefore a 3.6 mg sample of mature garden would theoretically contain 2.9 staphylae; a much higher figure than that obtained for refuse loads.

4. The relationship between the size of a garden cavity and the standing crop of staphylae supported

Eighty cavities were examined and regressing $\log_{10}$ staphyla numbers per 0.25 cm^2 against $\log_{10}$ cavity diameter showed a significant relationship ($p<0.001$, $Rsq_{(adj)}$ 51.4%, df 79). Smaller cavities possessed larger numbers of staphylae per 0.25 cm^2 than larger ones (Fig. 4.18). In cavities of less than 4 mm diameter, there were 48-72 staphylae per cm^2 while on external surfaces, there were 28.1 (SE ± 0.9) staphylae per cm^2. Larger cavities, above 10 mm diameter, contained only 24-32 staphylae per cm^2, a similar figure to that obtained for external surfaces.

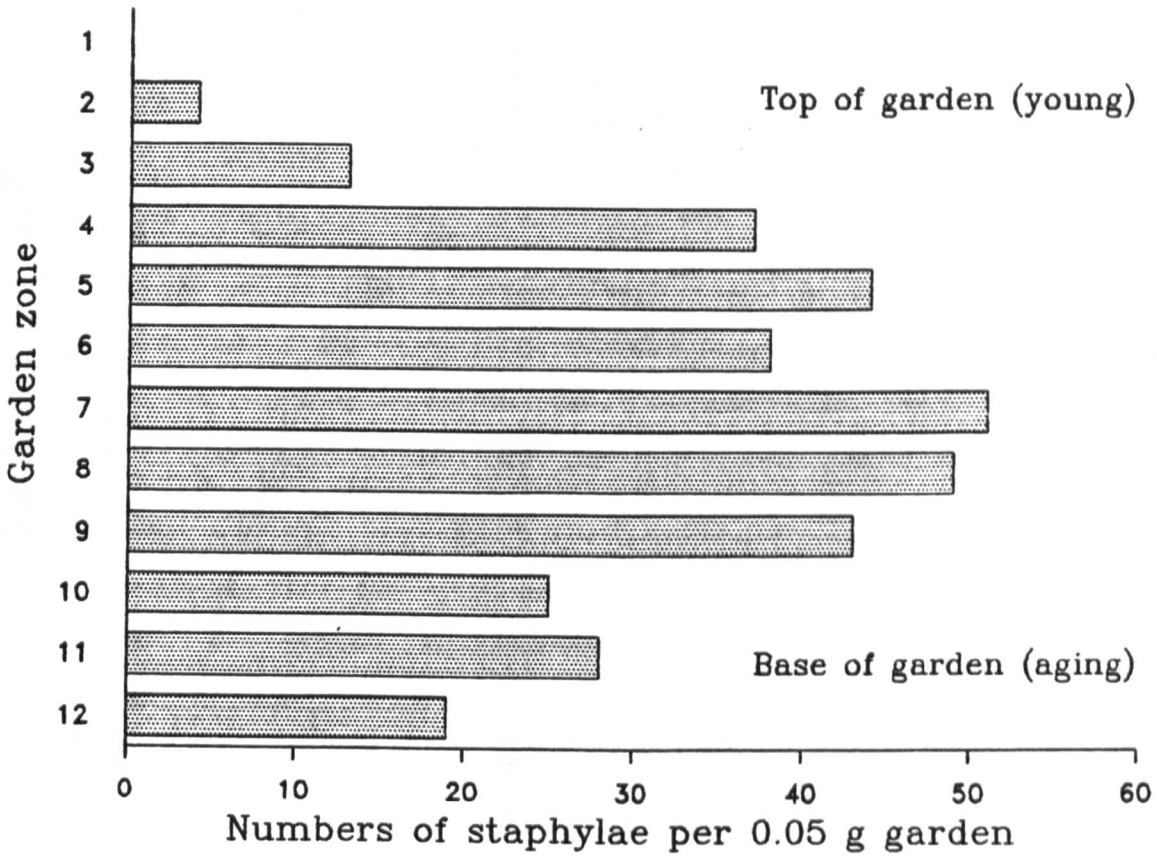


Figure 4.14: Changes in staphylae numbers per 0.05 g sample from the top of a fungus garden (young garden) to its base (aging garden). Each zone of garden examined was 1 cm deep.

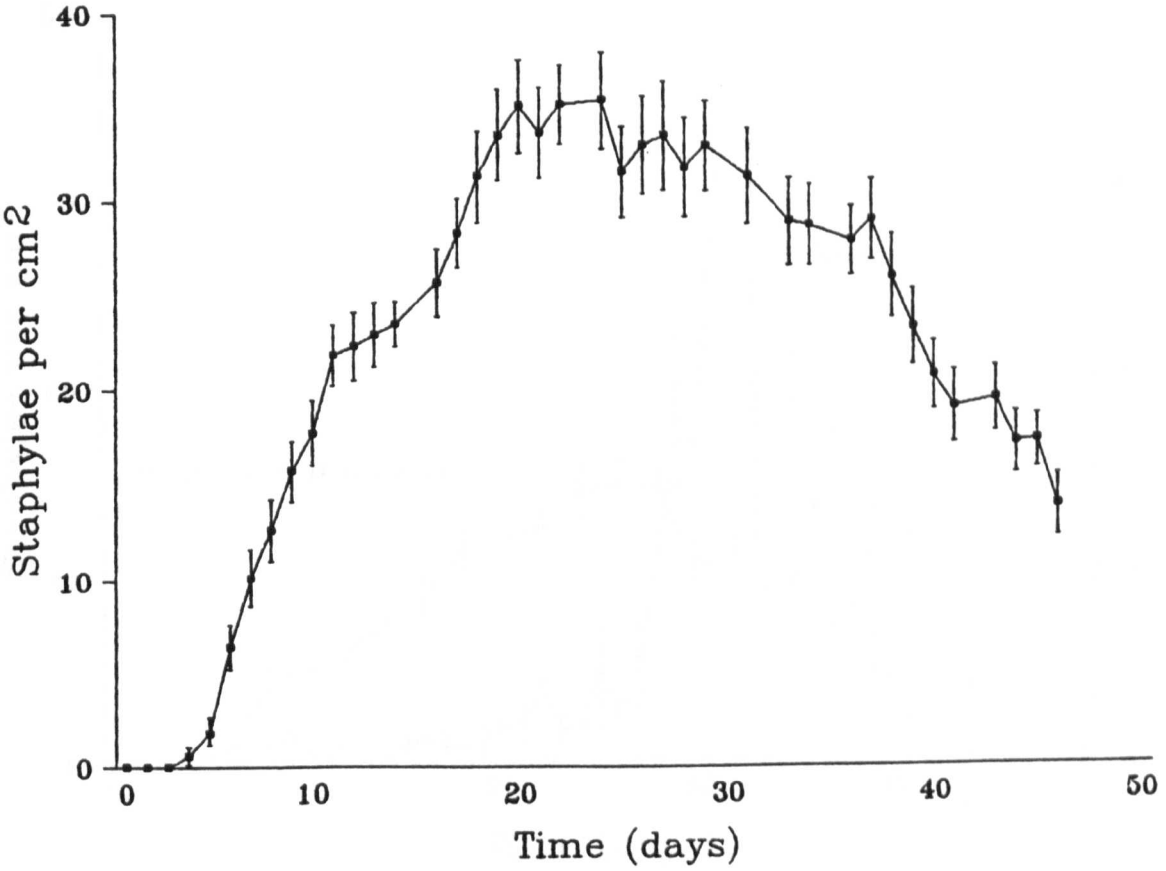


Figure 4.15: Mean numbers of staphylae per cm^2 (± 95% confidence limits, 50 replicates) on fungus garden marked with holly-spines during April 1991, from spine incorporation (days 0-4) until it was discarded (after day 40).

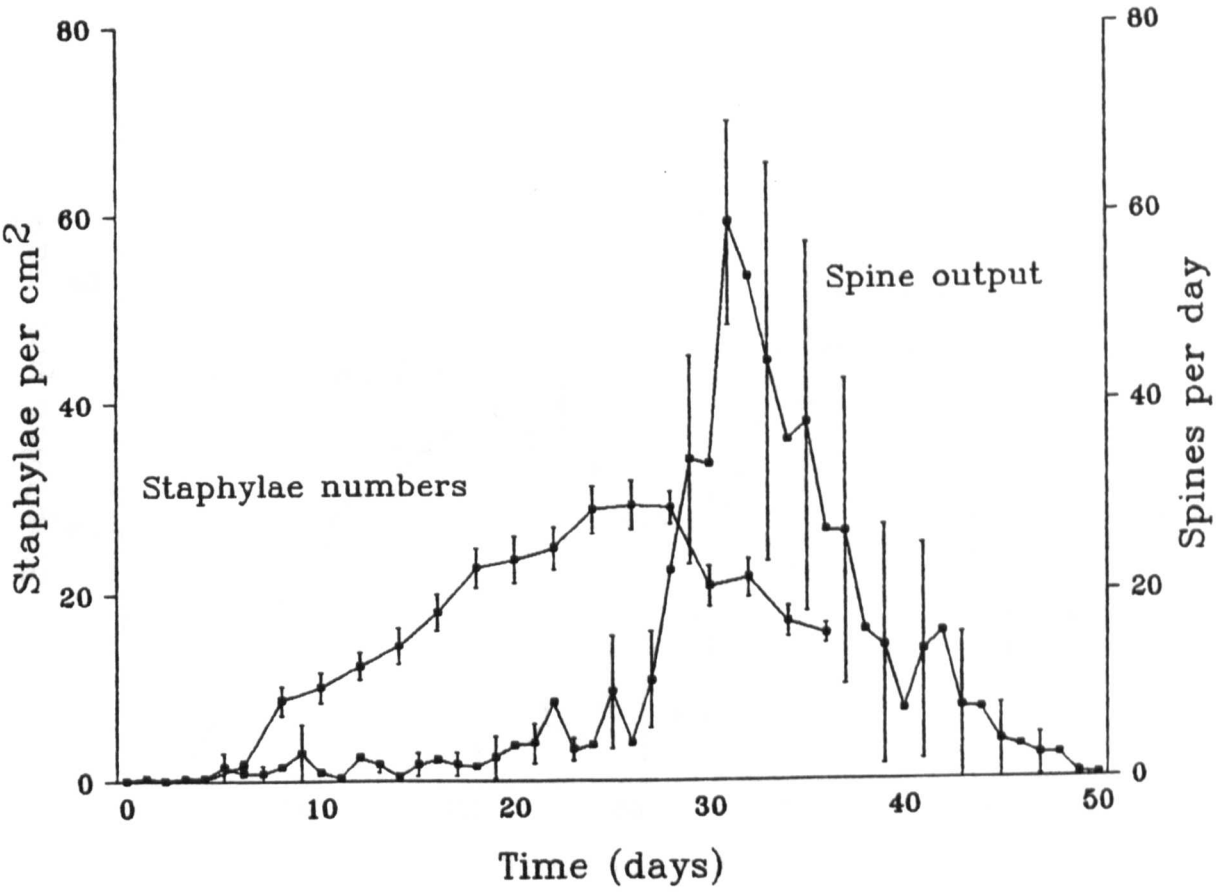


Figure 4.16: Mean numbers of staphylae per cm^2 (± 95% confidence limits, 50 replicates) on fungus garden marked during October 1991, compared with mean numbers (± 95% confidence limits) of spines discarded daily from four simultaneously marked gardens.

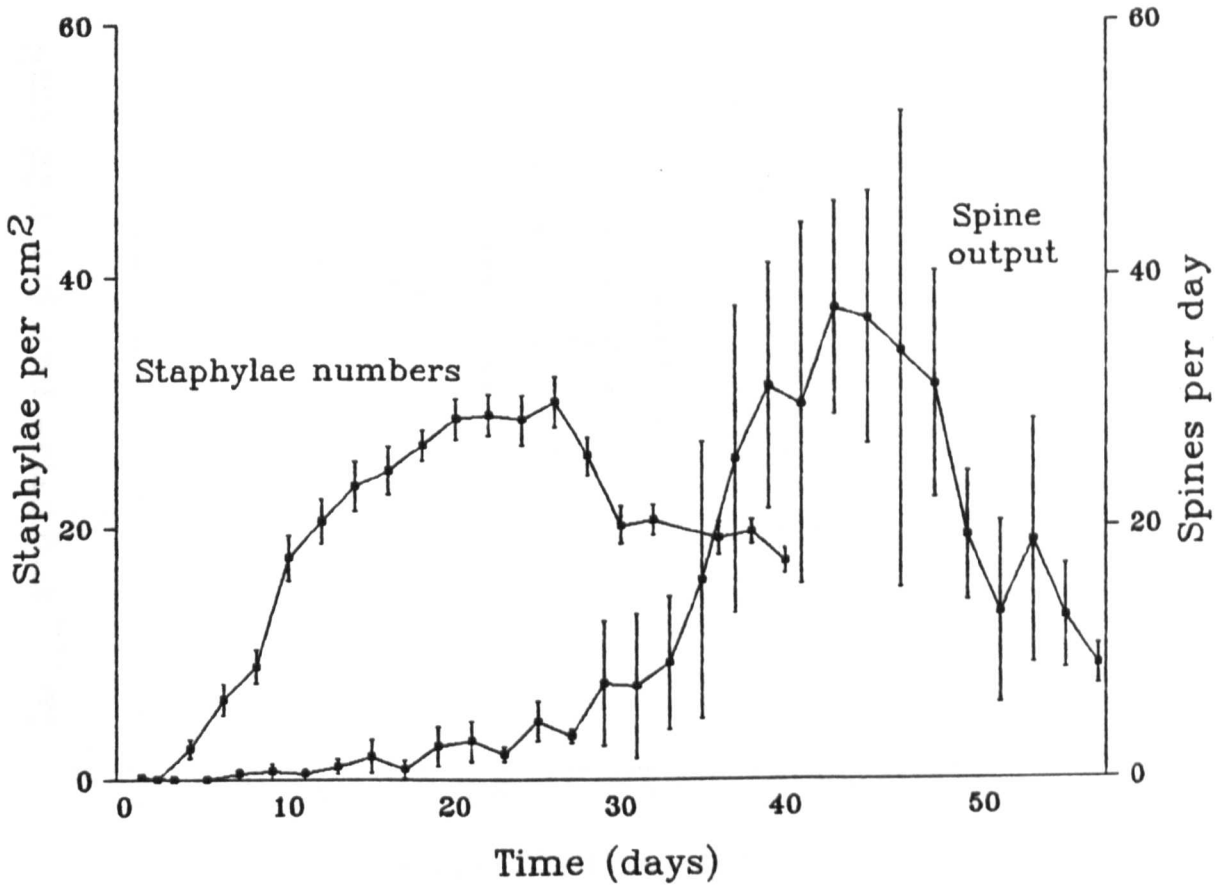


Figure 4.17: Mean numbers of staphylae per cm^2 (± 95% confidence limits, 50 replicates) on fungus garden marked during November 1991, compared with mean numbers (± 95% confidence limits) of spines discarded daily from five simultaneously marked gardens.

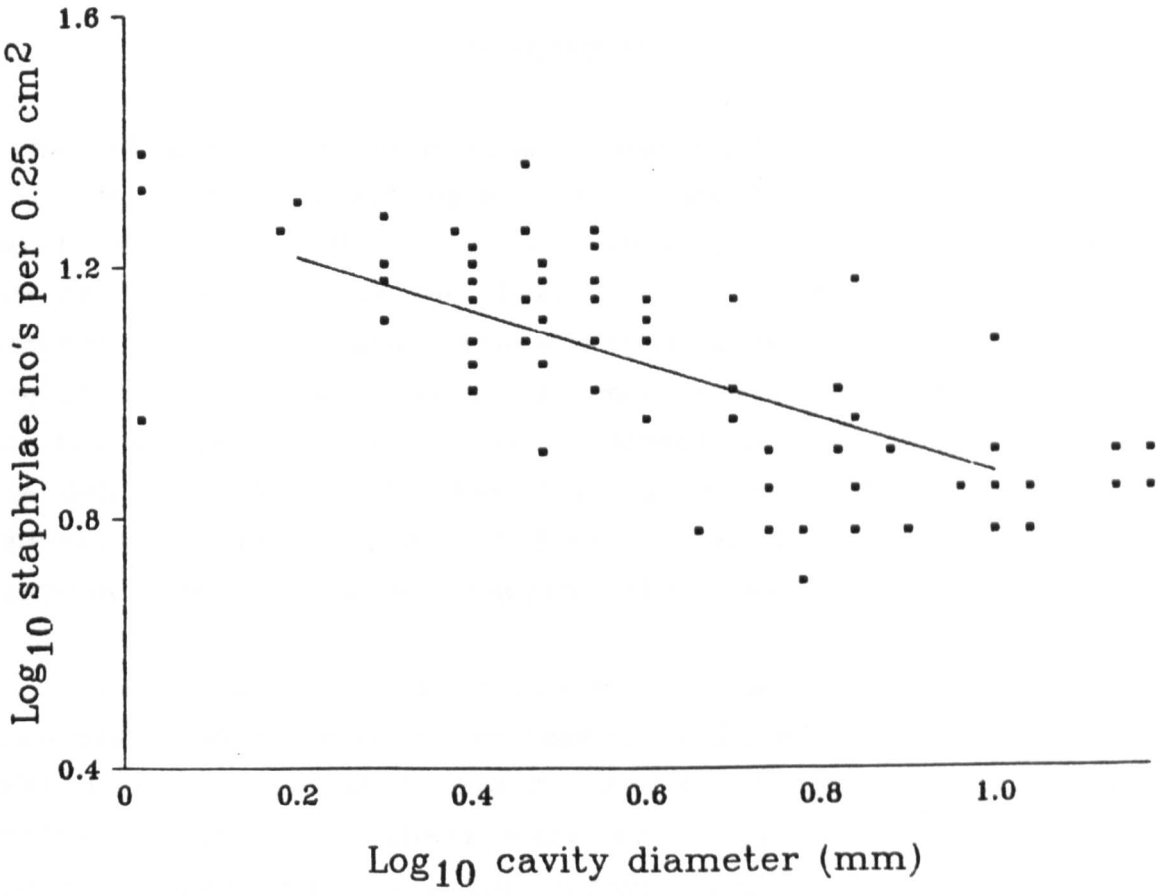


Figure 4.18: Log_{10} numbers of staphylae per 0.25 cm^2 area of fungus garden cavity wall, plotted against the log_{10} diameters (mm) of cavities in which they were found (regression equation, $y = 1.3 - 0.431x$, $p<0.001$, $Rsq_{(adj)}$ 51.4%, df 79).

DISCUSSION

1. The internal structure of a fungus garden

Numbers of cavities per unit area of section increased with garden age (Fig. 4.7) and this has also been recorded in the literature (Weber 1972). It is obvious when fungus gardens are examined, since young areas have few large cells, while aging garden has many small ones. This leads to the question of why cavity numbers should increase with garden age. The ants may fill in some areas of cells, decreasing their size, or divide them up. The weight of garden above may also compress them (see Chapter 3, page 43).

Percentage net space was much higher than percentage internal space because the former included 'open' cavities which are especially common in young garden with its diffuse structure. There were two types of 'open' cell; some plunged deep into the garden section and had narrow connections with the outside, whereas others were wide and shallow. In these cases, the problems of two-dimensional observations on three-dimensional structures became clear, since it was difficult to decide whether a cavity was an open cell or merely a depression in the garden surface. Cross-sectioning also caused problems by cutting through cavities at oblique angles, making them appear larger or smaller than they actually were. Percentage net space was a more realistic figure because all cavities are likely to be connected to the outer surface and workers were found deep within the garden. Similarly, talcum powder blown into an external cavity penetrated deep into a garden. Also, the larger the cavity, the more likely it is to be an 'open' one in section.

Internal S_v (surface area per unit volume) was considerably smaller than overall S_v which included external surfaces. However, the garden used in these observations was very small, with a large external area to volume ratio. For a larger garden, overall S_v would more closely resemble internal S_v, since it would have a much lower external area

to volume ratio. Internal S_v was therefore a general measure which could be applied to the inside of any fungus garden, whereas overall S_v was a specific measure for the garden used and gardens of different total volume have different ratios of volume to external surface area.

Actual profiles of the external surfaces of sections were 2.1 times longer than visible profiles. This means that 1 cm^2 of visible area on a garden surface is really equivalent to 2.1 x 2.1 cm^2 (4.4 cm^2). This was probably an over-estimate since it included open cells which plunge deeply into the garden and would not be relevant in external observations. However, it did illustrate that an observed area of garden surface may be much larger than it actually appears due to corrugations in the garden surface.

Only simple stereological analysis was carried out since few sections were used and each was quite thick. Also, only one garden was examined, which may have been atypical due to its small size. However, the results obtained do provide some insights into the internal structure of fungus gardens. Workers were found deep inside the fungus garden, indicating that most of the cavities were connected with the outside. Most of these workers were of small headwidth, suggesting that most internal cavities were too small to allow the entry of large workers. A large proportion of the staphylae-bearing area inside the garden was therefore available only to small workers and calculations showed that workers of headwidth 4.0 mm could enter only 4.3% of cavities, while foragers of headwidth 2.2 mm (Wilson 1980b) could enter only 21.5% of cavities. However, fungus-gardening minima could enter 83.6-98.6% of available cavities. These calculations assumed that the cavities inside fungus garden are cylindrical tubes and that all sections across cavities were exact cross-sections. In fact, cavities are unlikely to be perfectly tubular and sections probably cut diagonally through cavities, or along their sides, making them appear smaller or larger than they really were. However, assuming that

cavity sizes in the sections were representative of actual garden cavity sizes, this does demonstrate how small workers can enter almost all cavities, while large ones can enter few.

The mean values for S_v (surface area per unit volume) calculated from the 14 sections examined showed that the fungus garden had a large available surface area per unit volume. The ants therefore maximise surface area and hence potential cropping area, whilst maintaining accessibility for smaller workers. This illustrates the division of labour practised by the Attines; small workers are gardener-nurses and larger ones forage, excavate or defend (Wilson 1980a).

A fungus garden of approximately 2 litres in volume will therefore contain 368 cm^3 of internal air space. If internal S_v is 0.51 mm^2 per mm^3, then total S_v will be 1,020 cm^2 (for 2 litres). Similarly, visible external surface area will be 953 cm^2, but actual external surface area will be 2.1^2 times larger, i.e. 4,200 cm^2. Total surface area will therefore be 5,220 cm^2.

2. Fungus garden life cycles

The best method found for marking fungus garden was using holly leaf spines. Workers treated them like any other substrate and unlike the citrus albedo used by Powell (1984), they were easily visible when incorporated into fungus garden or ejected in refuse.

However, the times taken for holly-marked areas to be dumped varied between gardens marked at different times of the year. Gardens marked simultaneously had similar life cycles, but gardens marked during the summer months (June or August) had shorter life cycles than those marked in October, which in turn had shorter life cycles than those marked in November (Figs.4.11-4.13). The longest life cycle recorded for a November-marked garden was 70 days, which was still shorter than Weber's (1972) figure of 3-4 months.

Nests in the laboratory are maintained under constant environmental conditions and the only possible factor causing these changes in garden life cycle length was forage quality. Chapter 3 showed how forage intake of fungus gardens during 15 minute observation periods varies with time of year, peak intakes occurring during the late summer (August and September) when fungus garden size is also increasing. So, the short June and August life cycles coincided with increasing forage intake and garden size and the long October and November life cycles coincided with decreasing forage intake and large garden size.

The early summer is therefore a time of rapid turnover and build-up whereas by November turnover has slowed, probably due to changing forage quality. During the early summer, the nests receive young, soft leaves which, when incorporated into fungus garden, are easily colonised by the fungus and quickly exhausted. This will lead to rapid turnover and a short life cycle. However, leaves supplied during the summer become progressively more mature and consequently tougher, containing more lignified and sclerotised tissues. The ants therefore take longer to cut them up, leading to apparently lower rates of foraging and when such mature leaves are incorporated into the garden, the fungus takes longer to colonise them, turnover slows and the life cycle lengthens. Because of the slow turnover, garden size tends to increase.

Another important aspect of changing forage quality is that different substrate types may affect the growth of the fungus in culture (Powell 1984) and may therefore affect fungus growth in the garden itself. This might be expressed by differences in mycelial colonization of substrate, or by the numbers of staphylae produced per unit area. Mullenax (1979) supplied *Atta* colonies with jackbean, *Canavalia ensiformis*, and after several days these colonies became inactive. This plant contains demethylhomopterocarpin, which has fungicidal properties (Lampard 1974). Howard *et al.* (1988) also suggested that secondary metabolites present in some plants may be harmful to the ants and their fungus.

Unfortunately, most studies of the effect of different substrates on fungal growth have been carried out using plant extracts in agar culture, which does not truly reflect the situation in the fungus garden. Ideally, a study should be undertaken to examine the growth of hyphae and staphylae on fungus garden derived from known types of substrate. This could be carried out by supplying individual gardens with test substrates, then holly spines to mark layers of garden above the test layers. Staphyla numbers could be recorded as in the experiments described in this chapter and estimates of hyphal production could be obtained using the agar film technique, as described by Thomas *et al.* (1965).

Few staphylae were dumped with refuse, indicating that efficient removal of these must take place from aging garden before it is discarded. Those that are dumped may be old and unpalatable anyway.

Staphyla numbers also varied with garden age. Weber (1972) reported that they are produced in large quantities in the lower regions of a fungus garden and this was confirmed (see Figs. 4.14-4.17). Nests were maintained on a variety of substrates, with different quantities of each being received at different times. When a transect of garden was examined (Fig. 4.14), changes in staphyla numbers with garden age may simply have reflected these dietary fluctuations and replication would have reinforced this. However, this difficulty was overcome when staphyla productivity was examined on one type of forage with time (holly spines).

Marking garden with holly spines showed that staphylae were produced around 5 days after forage incorporation, indicating the period taken by the fungus to colonise and establish itself on the new material. However, just before major dumping of the observed areas began to take place, staphyla numbers began to decline. Either the fungus garden was producing fewer staphylae or the workers were harvesting them more intensely on aging areas that were about to be dumped. In either case, the same result is

obtained; staphylae are scarce on garden which is about to be discarded. Dumping garden after it has passed its peak productivity would indicate an optimal strategy on the part of the ants; it would be better to wait until staphyla production dropped to non-worthwhile levels before dumping substrate rather than wasting resources collecting fresh forage. However, there may be other factors, like decreases in the quality of staphylae with garden age or increased risk of contamination. Another problem is that staphyla standing crops were measured rather than actual production rates. The latter are difficult to measure since the ants continually remove staphylae. It is possible that areas of garden with low standing crops of staphylae may simply have higher harvesting rates.

Another important aspect of staphyla production is where they are produced in the fungus garden. As discussed earlier, gardens have a large available internal surface area which is accessible to the minima workers. Smaller cavities also contain more staphylae per unit area than larger ones or external surfaces (Fig. 4.18). This suggests that there is a large demand for staphylae from larger workers, which can only reach large cavities or external surfaces and staphyla numbers are consequently lower in these places. Internal cavities are visited only by small workers and harvesting pressure is therefore lower. However, staphyla numbers in small cavities never became very high, suggesting that the majority are removed sooner or later.

SUMMARY

A sample fungus garden contained many small cavities and there were more cavities per unit area in sections from aging garden than in those from young garden. Most of these cavities (83.6%) were accessible to workers with headwidths less than 1 mm, but workers of headwidths larger than 2.2 mm could enter less than 21.5% of cavities. These cavities provided a large internal surface area and had larger

numbers of staphylae per unit area than external surfaces, indicating a heavy 'harvesting pressure' from large workers on the external surfaces.

The best method for marking areas of fungus gardens was by allowing the ants to incorporate holly leaf spines into their gardens as substrate. This method was used to show that lengths of garden life cycles varied with time of year and this appeared to coincide with quality of forage supplied.

Staphylae were most common in the central regions of the fungus garden and numbers decreased before aging garden was discarded as refuse, indicating that either efficient removal of these occurs just before dumping, or that aging garden produces fewer staphylae than younger areas.

Chapter 5 : The behaviour of *Atta* on the fungus garden.

INTRODUCTION

In solitary bees and wasps, females must carry out a series of sequential acts to successfully raise their young. The development of eusociality in some insect groups has led to the evolution of a more efficient system whereby different tasks are carried out by specialised groups of individuals within the colony; a 'division of labour'. A typical ant colony has a wide range of tasks which must be carried out, including brood-care, queen-care, nest-care and foraging. *Leptothorax curvispinosus* workers perform 29 types of act (Wilson and Fagen 1974), *Leptothorax longispinosus* workers perform 37 acts (Herbers and Cunningham 1983) and those of *Formica polyctena, Camponotus sericeiventris* and *Pheidole dentata* all have 28 acts (Brandao 1979, Calabi *et al.* 1983 and Wilson 1976a, respectively). One of the largest repertoires is possessed by the turtle ant, *Zacryptocerus varians*, which has 40-42 acts (Wilson 1976b, Cole 1980). However, Oster and Wilson (1978) suggested that *Atta laevigata* may perform more than 50 acts. Wilson (1980a) recorded 29 acts for *Atta sexdens* workers but was not attempting to show the entire repertoire and excluded acts of personal behaviour like resting and self-grooming.

Repertoire sizes can be calculated by fitting behavioural abundance data to probability distributions (Fagen and Goldman 1977). However, one major problem with comparing repertoires is the definition of an act, since one observer may see several acts where another sees only one. Similarly, some authors do not include personal acts like resting while others do. Exclusion of personal acts may lead to false impressions of activities since workers may spend 70% of their time at rest (Otto 1958, Cole 1986).

Attine ants are likely to have larger repertoires than other species because of their fungus-culturing habit. Forage material brought back to the nest must be processed, incorporated into the fungus garden, planted with tufts of hyphae and continually cared for to prevent contamination by alien fungi and bacteria (Weber 1972). Quinlan and Cherrett (1979) reported that 30% of the workers on a fungus garden are licking it, which emphasizes the level of commitment required to maintain the fungus culture. If the workers are removed, the garden is rapidly overcome by alien organisms (Weber 1972).

With a division of labour, ultimately the most ergonomically efficient system would be to have one caste per task. However, there seem to be constraints against this since only 44 out of 263 extant ant genera contain species with more than one physical caste and only three genera have more than two castes (Oster and Wilson 1978). Only one species, *Eciton burchelli*, has four physically distinct worker castes (Sudd and Franks 1987). In such polymorphic species, major workers tend to be specialists for tasks like foraging, seed-milling or defence (Oster and Wilson 1978). They often perform limited numbers of acts; majors of *Solenopsis geminata* have 2 behavioural acts whereas minor workers have 17 (Wilson 1978) and majors of *Pheidole hortensis* have 6 acts while minors perform 26 (Calabi *et al.* 1983). Some genera such as *Atta, Eciton and Pheidologeton* have also developed minima castes which are specialised for brood-care, or in the case of *Atta*, for garden-care (Oster and Wilson 1978).

Temporal division of labour in ants occurs where young workers tend the brood, nest and queen while old workers, close to the end of their physiological lives, undertake the dangerous business of foraging. Foragers of *Pogonomyrmex owyheei* survive only 15 days on average (Porter and Jorgensen 1981) and in *Oecophylla smaragdina*, the oldest workers are the first to engage in territorial battles with neighbouring colonies (Holldobler 1983). Temporal division of labour is important in *Pheidole*

dentata, the minor workers being divided into three temporal castes (Wilson 1976a).

Workers may also exhibit individual traits. Otto (1958) found that up to 40% of 'Innendienst' workers of *Formica polyctena* showed specialisation or preferences for certain tasks. This may be for genetic reasons and some authors (Calderone and Page 1988) have suggested that bee division of labour is affected by genotype. Alternatively, individuals may specialise in different tasks because of rapid localised learning or 'imprinting' during the callow stage (Jaisson 1975, in *Formica polyctena*).

Atta exhibits both physical and temporal divisions of labour. The maximum size range of the worker caste is the greatest found in ants and is continuous (Oster and Wilson 1978). Four recognisable types can be defined, which are minima , media , maxima and soldiers. In species like *Atta cephalotes*, soldiers are physically distinct from other workers and possess ocelli, which are usually restricted to sexual forms. Wilson (1980a) defined four behavioural castes centred around different headwidths; gardener-nurses (headwidth 1 mm), within-nest generalists (1.4 mm), forager-excavators (2.2 mm) and defenders (>3 mm).

Behaviour patterns therefore change with increasing size. Three of these physical castes can also be divided into two temporal castes, making a total of seven castes overall (Wilson 1980a). Young (callow) workers engage in brood-care, whereas older ones are involved in other tasks like foraging. However, callows of the larger castes tend to be inactive.

Primitive Attines like *Cyphomyrmex* are monomorphic, so the complex caste system in *Atta* has probably developed as an adaptation to collecting fresh vegetation as a substrate (Wilson 1980a). The most efficient leaf cutting size is centred on headwidth 2.2 mm (Wilson 1980b) and successively smaller workers process the cut fragments until they can be inserted into the garden. Minima are more efficient at tending fungus garden than larger castes, since they can

enter tiny cavities in the spongy garden mass and manipulate small tufts of mycelium.

This chapter attempts to catalogue the behaviour of *Atta* workers associated with the fungus garden. The activities of sexuals are also examined. Attine queens are capable of carrying out the acts required to culture a garden and raise the first brood, when founding a new colony (*Atta* colonies are monogynous and usually founded by haplometrosis). Once the first worker brood has been raised, the queen becomes primarily an egg-layer but can revert to worker roles if required (Weber 1960). In contrast, males generally do not contribute to the daily maintenance of ant colonies and Wilson (1971) described them as little more than 'flying sperm dispensers'.

Although *Acromyrmex* frequently produces sexual broods in the laboratory, *Atta* colonies seldom achieve sufficient size to do so. Most studies on sexual broods have therefore been based on *Acromyrmex* or have examined mating flights or insects caught post-mating flight. However, Jutsum and Cherrett (1977) reported the production of sexual forms, including microgynes in laboratory colonies of *Atta cephalotes*. During the course of this work, a sexual brood of *Atta cephalotes* was produced and the activities of these alates are also examined.

MATERIALS AND METHODS

Observations of worker behaviour were all made using a large *Atta sexdens* nest (80 gardens).

1. Examining the effects of light on worker behaviour

When studying behaviour, observations must be made without causing disturbance. The fungus garden can be damaged by excessive heat (Powell and Stradling 1986) and even slight temperature increases cause workers to move brood (Quinlan and Cherrett 1978a). Under natural

conditions no light reaches the garden, but in the laboratory gardens are exposed to light for 12 hours per day. This level of light is unlikely to seriously affect behaviour, otherwise nests could not be successfully maintained. However, to observe workers on the garden surface, a higher level of light is required. The effects of this 'extra' light were therefore examined, using the movement of brood by workers as a measure of disturbance.

Test lights were shone from 15 cm away, on to one side of a dome containing a garden with known numbers of brood present on the surface. After 1 hour brood numbers were recounted. Warm and cold light sources with different coloured filters were used since ants may be insensitive to red light and Wehner and Toggweiler (1972) demonstrated that *Cataglyphis* workers cannot detect red light. These treatment lights were:

(1) control - no extra light
(2) warm white light - 40 W bulb
(3) warm red light - 40 W bulb plus a red filter
(4) cold white light - cold fibreoptic light-source (Schott KL 1500-T)
(5) cold red light - fibreoptic light with a filter
(6) cold blue light - " " "
(7) cold green light - " " "

There were significant differences between the amounts of disturbance caused by different lights ($p<0.001$, ANOVA) and Tukey's multiple comparison showed that warm light caused significantly more disturbance than other lights, large numbers of brood being removed from treated areas of garden ($p<0.05$, see Table 5.1). In contrast, cold lights caused no significant changes in brood numbers ($p>0.05$). Cold blue light caused the most disturbance of the cold lights, indicated by an influx of brood during the experiment. Cold red light caused the least disturbance and was therefore used to observe ant behaviour on the garden surface.

Table 5.1: The effects of different observational lights on worker behaviour; mean arcsine-transformed percentage changes (arc%'s, ± SE) in numbers of brood items present in areas of garden treated with different test lights. Non-significantly different means are joined by vertical lines ($p>0.05$, Tukey's multiple comparison, 5 replicates).

TYPE OF LIGHT	MEAN ARC% BROOD MOVEMENT (± SE)
Warm white	39.5 ± 9.0
Warm red	34.3 ± 4.3
Cold blue	18.6 ± 6.6
Cold white	8.6 ± 5.4
Cold green	7.1 ± 4.2
Control	5.8 ± 5.9
Cold red	3.6 ± 1.5

2. Studying worker behaviour on the fungus garden

a) On the outer surface

Areas of garden, each of 16 cm^2, were scanned with a binocular microscope to record instantaneously all acts in progress by workers.

b) On inner surfaces

The fungus garden is sponge-like, with many small inter-connected internal cavities (see Chapter 4) and an attempt was made to look at what happens inside these cavities. To obtain data comparable with that gained from the outer surface study, the following method was employed:

Some gardens fill domes completely and press against the wall, preventing general ant traffic and these areas are effectively 'inner surfaces'. Gently moving a dome so that one wall presses against the garden also creates such surfaces. After allowing the ants to settle for several hours, worker castes and activities could then be examined on these 'inner surfaces', by scanning 16 cm^2 areas.

3. Studying the existence of temporal castes

The ideal way to see if temporal castes occur or if workers specialise in different tasks, is to mark them in some way that does not affect their behaviour and record their activities over a period of time.

Individuals can be labelled by spots of paint on the legs, corresponding to numbers. Alternatively, a piece of paper with a number can be glued to the thorax or a wire loop can be placed around the petiole. The latter labels groups however, rather than individuals. The thoracic spines can also be cut to mark workers, but this is difficult to carry out and treated ants are difficult to see.

The first three methods of marking were attempted, with a view to looking at individual behaviour over time. Groups of 20-30 workers (media and maxima) were treated and confined in petri-dishes with damp filter paper to check that marks remained intact and that the treatment did not kill them.

4. Examining the behaviour of *Atta* sexuals

These were observed over periods of up to 30 minutes using a binocular microscope and cold red light source. One fertile queen in a small nest of *Atta sexdens* (7 gardens) was visible occasionally and could be used for observations (other nests were too large to see the queen). During February and March 1991, unusually large larvae which appeared flaccid and starved were observed in an *Atta cephalotes* nest (40 gardens). Some matured into an alate brood of mixed males and females and their behaviour was also examined.

5. Analysis of results

Good's (1953) estimate of sample coverage was calculated for behavioural acts recorded (as described by Fagen and Goldman 1977). This is defined by:

$$\theta g\text{-}1\text{-}\frac{N1}{I}$$

where θ_g is an estimate of sample coverage (what proportion of total behavioural acts present have been observed). N1 is the number of acts observed only once and I is the total number of ants observed. If θ_g is 0.99, then missing (non-observed) behaviour types will have an aggregate probability of only 0.01; sample coverage is essentially complete. However, a sample coverage of only 0.1 would indicate that the majority of behaviours had not been observed.

Observations were also checked for normality and analyzed statistically.

RESULTS

1. The behaviour of workers on the fungus garden surface

a) Worker behaviour on the outer surface

The behaviours of the four size groups defined by Weber (1972) were examined on areas of young, mature and aging garden (see Fig. 2.1, Chapter 2) at 12 observation times over 24 hrs. This was repeated five times over a period of several days for each garden age at each observation time, ensuring that both active (foraging) and inactive periods were included. Separate areas of garden were used where possible for different observations (80 gardens were available). Activities of workers in each observation were recorded in tables compiled from the literature (Weber 1972, Wilson 1980a) and from personal observation.

Numbers and types of brood present were also noted for each replicate, along with the numbers and occupations of immature callow workers.

(i) Numbers of each worker caste present on different garden ages

Minima were the most common caste, making up 70.6% of the 8,648 workers observed. Media were the second-most common caste, with 16.0%, followed by maxima , with 11.7%. Soldiers were the least common caste (1.7%).

The data for caste numbers on different garden ages were not normally distributed. However, rather than using transformation and subsequent parametric tests, the data were compared non-parametrically. This ensured that the results were comparable with later, parametric analyses. Caste numbers were compared between different garden ages (pooling replicates for the different observation times) and differed significantly ($p<0.001$, Mood). Numbers on aging garden were significantly higher than those on young ($p<0.001$, Mann-Whitney) for minima and maxima (soldier numbers were combined with maxima numbers for convenience, since the former were so low). Media numbers were also significantly higher on aging than on young garden ($p<0.02$, Mann-Whitney). Minima, media and maxima numbers were all significantly higher on aging than on mature garden ($p<0.01$, Mann-Whitney). There were however, no significant differences between young and mature garden for minima ($p>0.09$, Mann-Whitney), media ($p>0.6$) or maxima ($p>0.5$) The largest numbers of workers were therefore observed on aging garden and the fewest on young (Fig. 5.1a).

Arcsine-transformed percentages (arc%'s) were also examined and did not differ significantly between the three garden ages for any caste ($p>0.2$, Mood) (Fig. 5.1b).

(ii) Comparing caste numbers present at different times

Caste numbers were also compared between foraging and non-foraging periods, using Mann-Whitney tests. Observations made between 1000 and 2000 hrs were used for the former and those made between 0000 and 0800 hrs were used for the non-foraging period (forage was supplied at 1000 hrs). Observations made at 1000 and 2000 hrs were excluded, since these were intermediate between the two

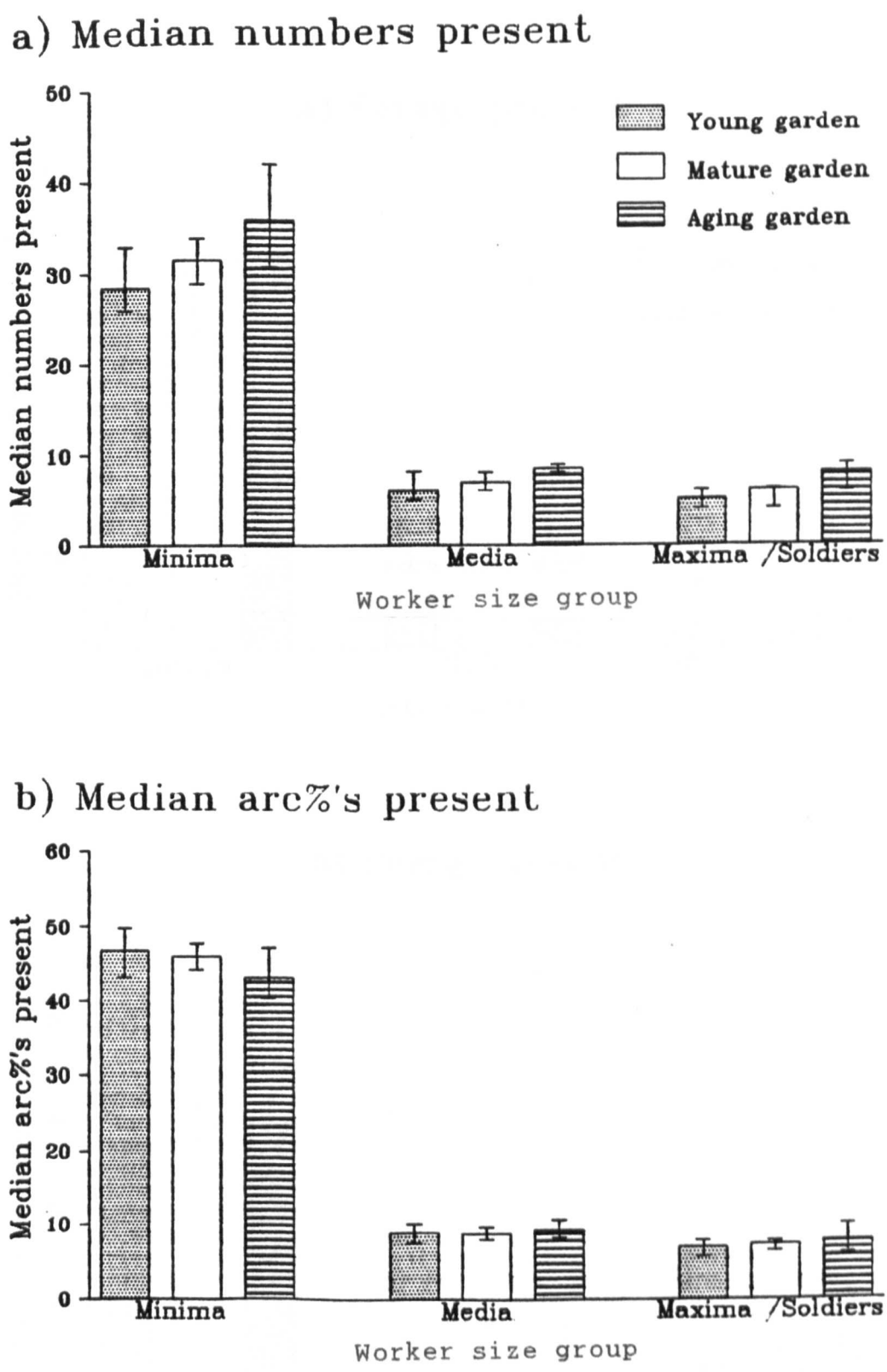
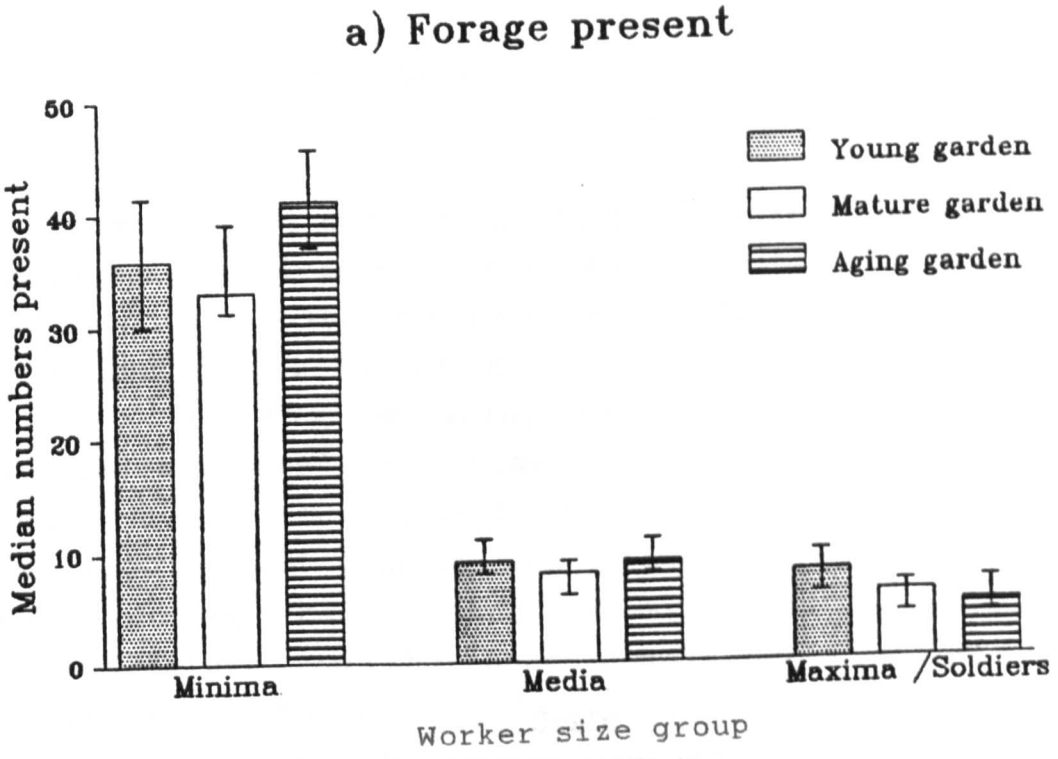


Figure 5.1: Numbers of each worker size observed on three ages of fungus garden per 16 cm^2 area, expressed as, a) Median numbers present and, b) Median arcsine transformed percentages present (arc%'s) (± 95% confidence limits, 60 replicates).



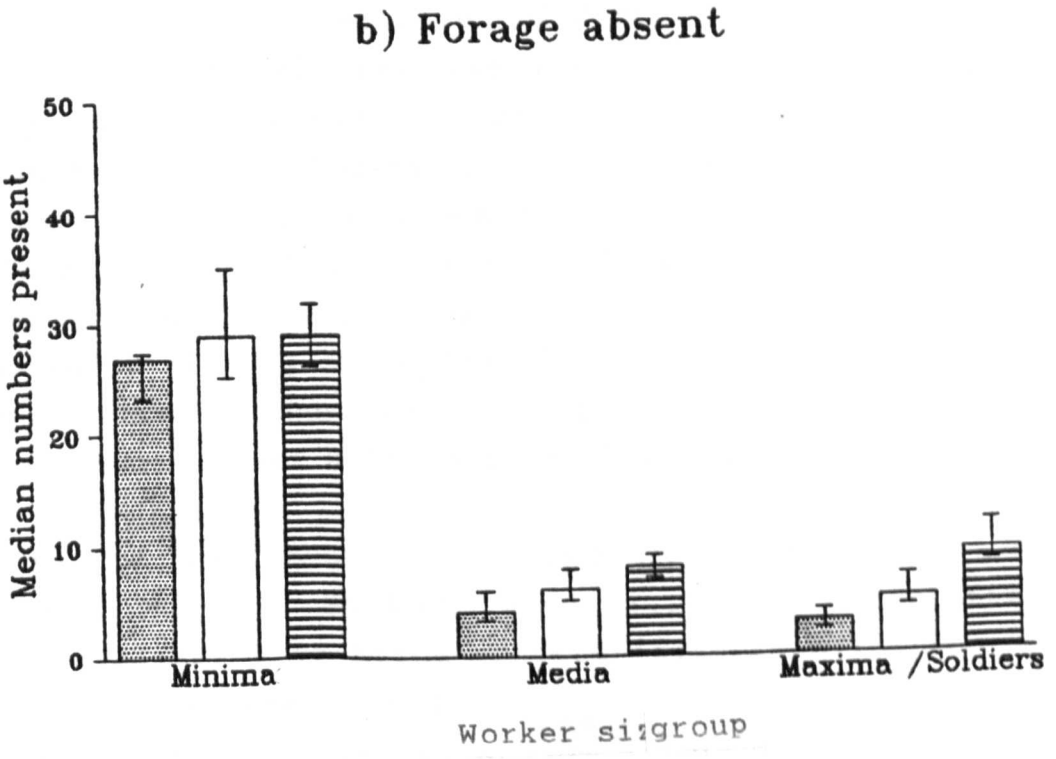


Figure 5.2: Median numbers (± 95% confidence limits) of each worker size present on three ages of fungus garden, when forage was either present or absent (25 replicates).

periods. Twenty-five replicates were therefore used for comparisons.

Minima numbers were significantly higher on young and aging garden when forage was available ($p<0.01$), but not on mature ($p>0.05$). Media numbers were significantly higher on young garden during the foraging period ($p<0.001$) but not on mature ($p>0.08$) or aging ($p>0.2$). Maxima numbers (including soldiers) were significantly higher on young garden ($p<0.001$), not significantly different on mature garden ($p>0.6$) and significantly lower on aging garden ($p<0.01$) during the foraging period. Total numbers of workers present were therefore significantly higher on young garden during the foraging period ($p<0.001$) but not on mature ($p>0.08$) or aging garden ($p>0.1$). These differences are illustrated in Fig. 5.2.

(iii) Behavioural repertoires of different castes

Percentage relative frequencies of acts for each worker caste are shown in Table 5.2. Most acts are self-explanatory, but 'guarding brood' refers to workers standing motionless on or beside brood and workers counted as 'carrying staphylae' also included workers plucking staphylae (an act rarely seen, due to the short time taken to actually pluck a staphyla). 'Licking' refers to workers rasping the garden surface with their glossae and manipulating hyphae with their maxillae and mandibles.

Minima performed 28 acts, media ; 26, maxima ; 24 and soldiers; only 13. Soldiers were usually either resting, moving restlessly or being groomed (passive allogrooming) but were the least common caste. Different types of behavioural act occurred at different relative frequencies. Overall, there were four very common acts; restless movement, resting, licking garden and antennating garden. Conversely, acts like defaecation and trophallaxis were rare and some, like interactions with the queen, were not observed in this study, although they were seen at other times.

For minima , Good's (1953) estimate of sample coverage was 1.0 (100 % coverage) while for media it was 0.99855, for maxima , 0.99704 and for soldiers, 0.97203. Coverage was therefore best for minima , good for media and maxima and moderate for soldiers.

Numbers of workers of each caste involved in each act were compared using a 4 x 2 Heterogeneity Chisquare analysis on the numbers of workers engaged in each act versus the numbers engaged in all other acts. For some acts, there were no significant differences between relative numbers of each caste involved ($p>0.05$), such as trophallaxis, eating staphylae mutually, being allogroomed (passive allogrooming), self-grooming and licking leaf. However, many acts did show differences between castes, such as numbers eating staphylae alone ($p<0.005$), due to lower than expected numbers of media . Numbers antennating workers also showed significant differences ($p<0.005$), because of more medias than expected. Numbers actively allogrooming, antennating garden, carrying staphylae, licking garden or caring for brood were significantly different ($p<0.005$), due to higher numbers of minima and lower numbers of larger workers than expected. However, numbers crimping, carrying or inserting leaf, moving restlessly or at rest showed significant differences ($p<0.005$) due to fewer minima and more media and maxima than expected from the null hypothesis.

Table 5.2: Percentage relative frequencies of observed behaviours of different worker castes on the outer surface of the fungus garden (n = number of acts recorded).

BEHAVIOURAL ACT	RELATIVE FREQUENCY FOR:-				
	MINIMA n=6,109	MEDIA n=1,382	MAXIMA n=1,014	SOLDIERS n=143	TOTAL n=8,648
At rest	6.040	15.123	30.473	51.049	11.101
Move restlessly	20.740	40.593	31.657	24.476	25.254
Self-groom	5.778	4.631	4.931	2.797	5.446
Allogroom -active	4.436	1.664	0.493	0	3.457
Allogroom -passive	2.832	3.401	3.649	6.993	3.087
Trophallaxis	0.376	0.434	0.690	0	0.416
Eat staphyla alone	2.603	1.158	1.677	0.699	2.232
Eat staphyla mutually	0.638	0.507	0.789	0.699	0.636
Defaecate	0.033	0	0.099	0	0.035
Antennate worker	1.457	3.184	1.972	1.399	1.792
Feed larva	0.566	0.289	0.099	0	0.451
Lick larva	2.095	0.289	0.394	0	1.573
Lick pupa	0.262	0.217	0	0	0.220
Carry larva	0.737	0.579	0.394	0	0.659
Carry pupa	0.393	0.796	0.592	0	0.474
Guard brood	4.829	4.993	3.748	3.497	4.706
Lick garden	27.304	2.098	0.099	0	19.635
Lick staphyla	0.098	0	0	0	0.069
Antennate garden	11.360	7.959	4.832	3.497	9.921
Carry staphyla	4.420	1.520	0.592	0	3.434
Carry refuse	0.049	0.072	0	0	0.046
Carry hyphae	0.164	0.145	0	0	0.139
Drink droplets on garden surface	0.098	0	0	0.699	0.081
Cut leaf section	0	0.217	1.381	0	0.197
Crimp leaf	0.426	4.052	5.030	0	1.538
Lick leaf	1.031	1.375	0.690	1.399	1.052
Rest on leaf	0.066	0.072	0.296	0.699	0.104
Carry leaf	0.802	3.763	4.536	2.098	1.735
Insert leaf fragment	0.376	0.868	0.888	0	0.509
TOTALS	100.0	100.0	100.0	100.0	100.0

Table 5.3a-d shows the relative frequencies of acts for different castes on three ages of garden. Numbers of workers engaged in each act were compared between ages using 3 x 2 Heterogeneity Chisquare analyses on the numbers of workers engaged in each act versus the numbers engaged in all other acts.

For minima (Table 5.3a) some acts had relatively constant frequencies over the whole garden like moving restlessly, self-grooming, trophallaxis and antennating with workers. Many increased in relative frequency with increasing garden age, such as eating and carrying staphylae, allogrooming, aspects of brood-care and resting. Some acts decreased in relative frequency on older garden, for example, most aspects of substrate preparation, antennating garden and licking garden. Comparing numbers involved showed that these differences were all significant ($p<0.01$).

For media workers (Table 5.3b), numbers at rest, moving restlessly, self-grooming, actively allogrooming and antennating workers were not significantly different between garden ages ($p>0.05$). Numbers of workers being allogroomed, caring for brood (all aspects), licking garden, antennating garden or preparing substrate (all aspects) showed significant differences between garden ages ($p<0.025$). In the first two cases, this was due to lower than expected numbers on young garden and in the latter three, due to higher than expected numbers on young garden. Numbers of media engaged in other acts were too low for analysis.

For maxima (Table 5.3c) there were no significant differences between garden ages for workers allogrooming, self-grooming, moving restlessly, at rest or carrying staphylae. However, numbers preparing substrate did show significant differences between garden ages due to higher than expected numbers on young garden ($p<0.005$).

For soldiers (Table 5.3d) there were no significant differences between garden ages for numbers at rest or moving restlessly ($p>0.05$). Numbers of soldiers engaged in other acts were too low for analysis.

Table 5.3: Percentage relative frequencies of observed behaviours of different worker castes on the outer surfaces of three ages of fungus garden (n = total numbers of acts recorded).

a) Minima

BEHAVIOURAL ACT	RELATIVE FREQUENCY FOR:-		
	YOUNG GARDEN n=1,838	MATURE GARDEN n=2,004	AGING GARDEN n=2,267
At rest	4.570	6.886	6.484
Move restlessly	19.260	22.206	20.644
Self-groom	5.441	5.938	5.911
Allogroom -active	1.306	5.888	5.690
Allogroom -passive	0.707	3.743	3.749
Trophallaxis	0.326	0.449	0.353
Eat staphyla alone	0.871	3.693	3.044
Eat staphyla mutually	0.054	0.749	1.015
Defaecate	0.109	0	0
Antennate worker	1.469	1.148	1.720
Feed larva	0	0.299	1.235
Lick larva	0	1.597	4.235
Lick pupa	0	0.150	0.573
Carry larva	0	0.499	1.544
Carry pupa	0	0.200	0.882
Guard brood	0.218	3.244	9.969
Lick garden	45.811	23.054	16.056
Lick staphyla	0	0.150	0.132
Antennate garden	13.493	11.477	9.528
Carry staphyla	1.415	5.489	5.911
Carry refuse	0	0	0.132
Carry hyphae	0.490	0.050	0
Drink droplets on garden surface	0	0.200	0.088
Cut leaf section	0	0	0
Crimp leaf	1.088	0.299	0
Lick leaf	1.143	1.048	0.926
Rest on leaf	0.054	0.100	0.044
Carry leaf	1.632	0.798	0.132
Insert leaf fragment	0.544	0.649	0
TOTALS	100.0	100.0	100.0

b) Media

BEHAVIOURAL ACT	RELATIVE FREQUENCY FOR:-		
	YOUNG GARDEN n=445	MATURE GARDEN n=415	AGING GARDEN n=522
At rest	13.933	15.663	15.709
Move restlessly	38.427	43.373	40.230
Self-groom	5.393	4.096	4.406
Allogroom -active	0.899	1.928	2.107
Allogroom -passive	1.348	4.337	4.406
Trophallaxis	0.225	0.964	0.192
Eat staphyla alone	0.674	1.205	1.533
Eat staphyla mutually	0	0.482	0.958
Defaecate	0	0	0
Antennate worker	3.596	1.446	4.215
Feed larva	0	0.241	0.575
Lick larva	0	0	0.766
Lick pupa	0	0.241	0.383
Carry larva	0	0.482	1.149
Carry pupa	0	1.687	0.766
Guard brood	0.899	4.337	9.004
Lick garden	4.045	1.446	0.958
Lick staphyla	0	0	0
Antennate garden	10.337	7.229	6.513
Carry staphyla	0.449	2.169	1.916
Carry refuse	0.225	0	0
Carry hyphae	0.225	0.241	0
Drink droplets on garden surface	0	0	0
Cut leaf section	0	0.482	0.196
Crimp leaf	10.337	1.687	0.575
Lick leaf	1.348	1.446	1.341
Rest on leaf	0	0.241	0
Carry leaf	6.067	3.855	1.724
Insert leaf fragment	1.573	0.723	0.383
TOTALS	100.0	100.0	100.0

c) Maxima

BEHAVIOURAL ACT	RELATIVE FREQUENCY FOR:-		
	YOUNG GARDEN n=322	MATURE GARDEN n=293	AGING GARDEN n=399
At rest	27.640	32.765	31.078
Move restlessly	28.882	33.447	32.581
Self-groom	5.280	6.485	3.509
Allogroom -active	0.621	0.683	0.251
Allogroom -passive	2.484	5.119	3.509
Trophallaxis	0.311	0.341	1.253
Eat staphyla alone	0.621	0.683	3.258
Eat staphyla mutually	0.311	0.683	1.253
Defaecate	0	0.341	0
Antennate worker	2.484	2.048	1.504
Feed larva	0	0	0.251
Lick larva	0	1.024	0.251
Lick pupa	0	0	0
Carry larva	0	0.341	0.752
Carry pupa	0	1.365	0.501
Guard brood	0	2.730	7.519
Lick garden	0.311	0	0
Lick staphyla	0	0	0
Antennate garden	4.037	5.461	5.013
Carry staphyla	0.311	0.683	0.752
Carry refuse	0	0	0
Carry hyphae	0	0	0
Drink droplets on garden surface	0	0	0
Cut leaf section	0	0.341	3.258
Crimp leaf	13.975	1.024	0.752
Lick leaf	0.932	1.024	0.251
Rest on leaf	0.311	0.341	0.251
Carry leaf	9.938	2.048	2.005
Insert leaf fragment	1.555	1.024	0.251
TOTALS	100.0	100.0	100.0

d) Soldiers

BEHAVIOURAL ACT	RELATIVE FREQUENCY FOR:-		
	YOUNG GARDEN n=25	MATURE GARDEN n=58	AGING GARDEN n=60
At rest	64.000	44.828	51.667
Move restlessly	16.000	25.862	26.667
Self-groom	0	3.448	3.333
Allogroom -active	0	0	0
Allogroom -passive	0	8.621	8.333
Trophallaxis	0	0	0
Eat staphyla alone	0	0	1.667
Eat staphyla mutually	0	1.724	0
Defaecate	0	0	0
Antennate worker	0	3.448	0
Feed larva	0	0	0
Lick larva	0	0	0
Lick pupa	0	0	0
Carry larva	0	0	0
Carry pupa	0	0	0
Guard brood	0	3.448	5.000
Lick garden	0	0	0
Lick staphyla	0	0	0
Antennate garden	12.000	1.724	1.667
Carry staphyla	0	0	0
Carry refuse	0	0	0
Carry hyphae	0	0	0
Drink droplets on garden surface	4.000	0	0
Cut leaf section	0	0	0
Crimp leaf	0	0	0
Lick leaf	0	3.448	0
Rest on leaf	0	0	1.667
Carry leaf	4.000	3.448	0
Insert leaf fragment	0	0	0
TOTALS	100.0	100.0	100.0

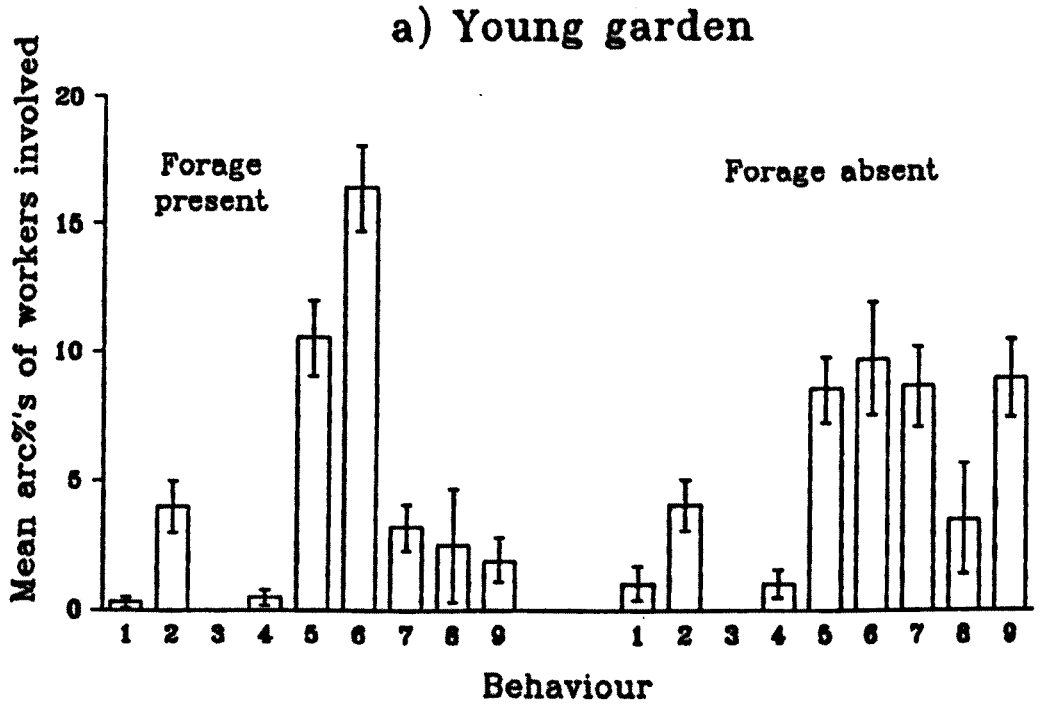
(iv) Comparing activities at different times

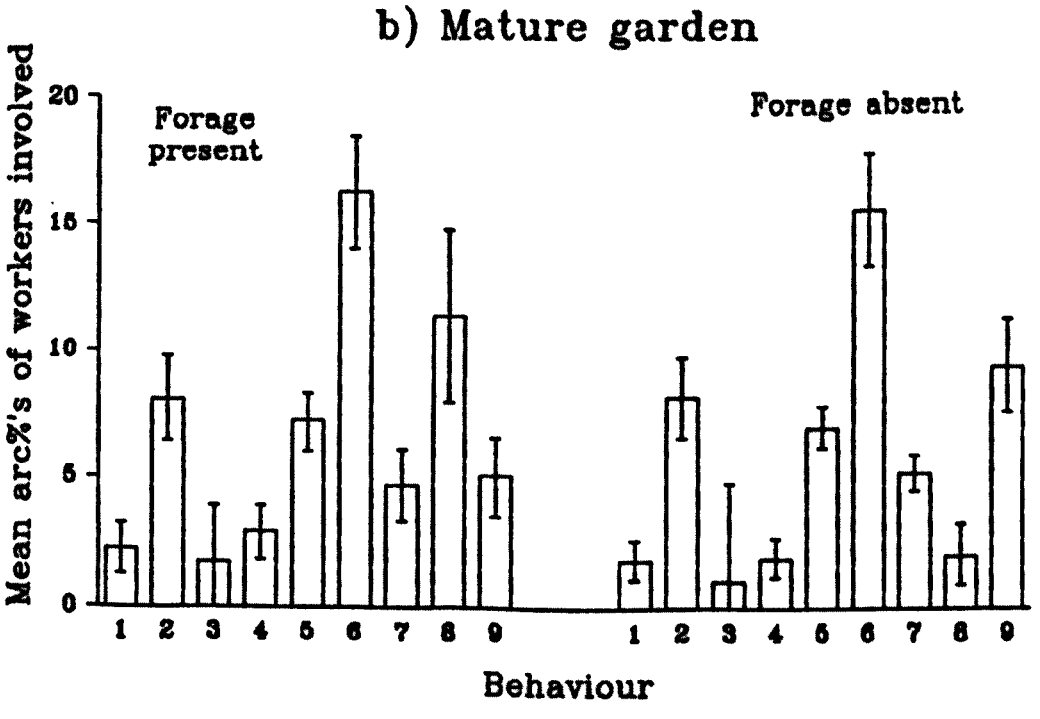
Relative frequencies of activities were also compared between foraging and non-foraging periods (see page 83 for definitions). Arcsine-transformed percentages of workers engaged in different types of act were compared between foraging and non-foraging periods using ANOVAs (except for brood-care, which did not have a normal distribution and was examined using Mann-Whitney tests. This method was used rather than transformation, to ensure that the analysis was consistent with the rest of the tests performed).

Numbers eating staphylae ('alone' and 'mutually' combined) were significantly lower during the foraging period on young garden ($p<0.03$) but showed no significant differences on mature ($p>0.5$) or aging areas ($p>0.9$). However, significantly greater numbers were carrying staphylae on aging garden ($p<0.05$) during this period, although not on younger areas ($p>0.1$). Numbers grooming (pooling self and allogrooming) or caring for brood were not significantly different between non-foraging and foraging times on any area of the garden ($p>0.1$). Numbers licking or restlessly moving were significantly higher on young garden during the foraging period ($p<0.04$) but not on older areas ($p>0.3$). Numbers antennating garden were significantly lower on young and aging areas during the foraging period ($p<0.001$) but not on mature garden ($p>0.5$). Numbers resting were also significantly lower during the foraging period on all garden ages ($p<0.001$). However, although substrate preparation should theoretically have been higher during the foraging period, this was only significant on mature garden ($p<0.001$; $p>0.4$ for young garden and >0.1 for aging garden).

There were therefore major changes in activity on young garden when forage became available, with increases in the amount of restless movement and decreases in antennating garden and resting. There were fewer changes on mature garden, but substrate preparation increased while numbers resting decreased. This was also true for aging

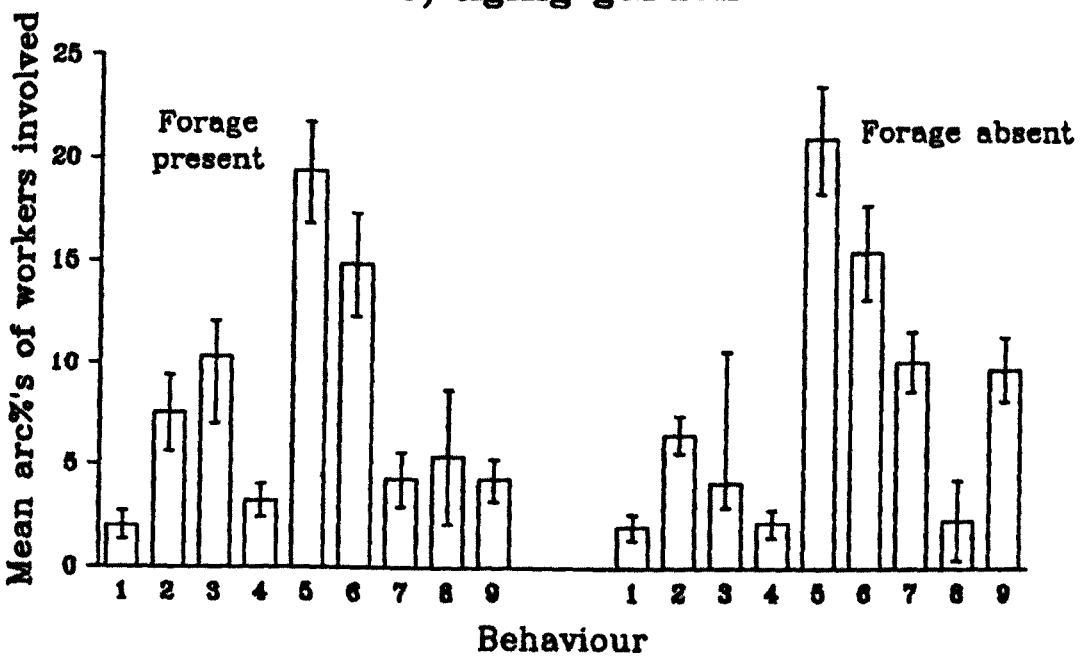
Figure 5.3: Mean arcsine-transformed percentages (± 95% confidence limits) of workers engaged in nine types of behaviour on three ages of fungus garden, when forage was either present or absent (25 replicates).





- continued overleaf

c) Aging garden



Key:

1 Consume staphyla
2 Groom
3 Care for brood
4 Carry staphyla
5 Lick garden
6 Move restlessly
7 Antennate garden
8 Prepare substrate
9 At rest

garden (Fig. 5.3). Fig. 5.3 also illustrates the differences in behaviour on different areas of the garden, with more brood-care and consumption or transport of staphylae on older areas.

(v) Numbers of types of act performed

Mean numbers of types of act occurring were highest on aging garden (12.5 acts ± SE 0.4) with fewer on mature (10.4 acts ± SE 0.3) and the fewest on young garden (8.1 acts ± SE 0.3). These means were all significantly different to each other ($p<0.001$, ANOVA, confirmed by Tukey's Multiple Comparison, $p<0.05$).

(vi) Numbers of brood present during the observations

Larvae were more common than pupae (Table 5.4), few of which were approaching maturity (shown by darkening colour). The numbers of eggs present were estimated rather than counted, since they were clumped together and difficult to observe. Because larvae were much more common than pupae, brood care acts involving larvae would be more common than those involving pupae and Table 5.2 shows that this was true.

Table 5.4: Total numbers of different ages of brood present in 180 replicate observation areas, each of 16 cm^2, on three ages of fungus garden.

GARDEN AGE	TOTAL NUMBERS PRESENT				
	EGGS	LARVAE	WHITE PUPAE	DARK PUPAE	TOTAL
Young	0	0	9	0	9
Mature	0	340	74	4	418
Aging	100	1131	334	24	1589
Total	100	1471	417	28	2016

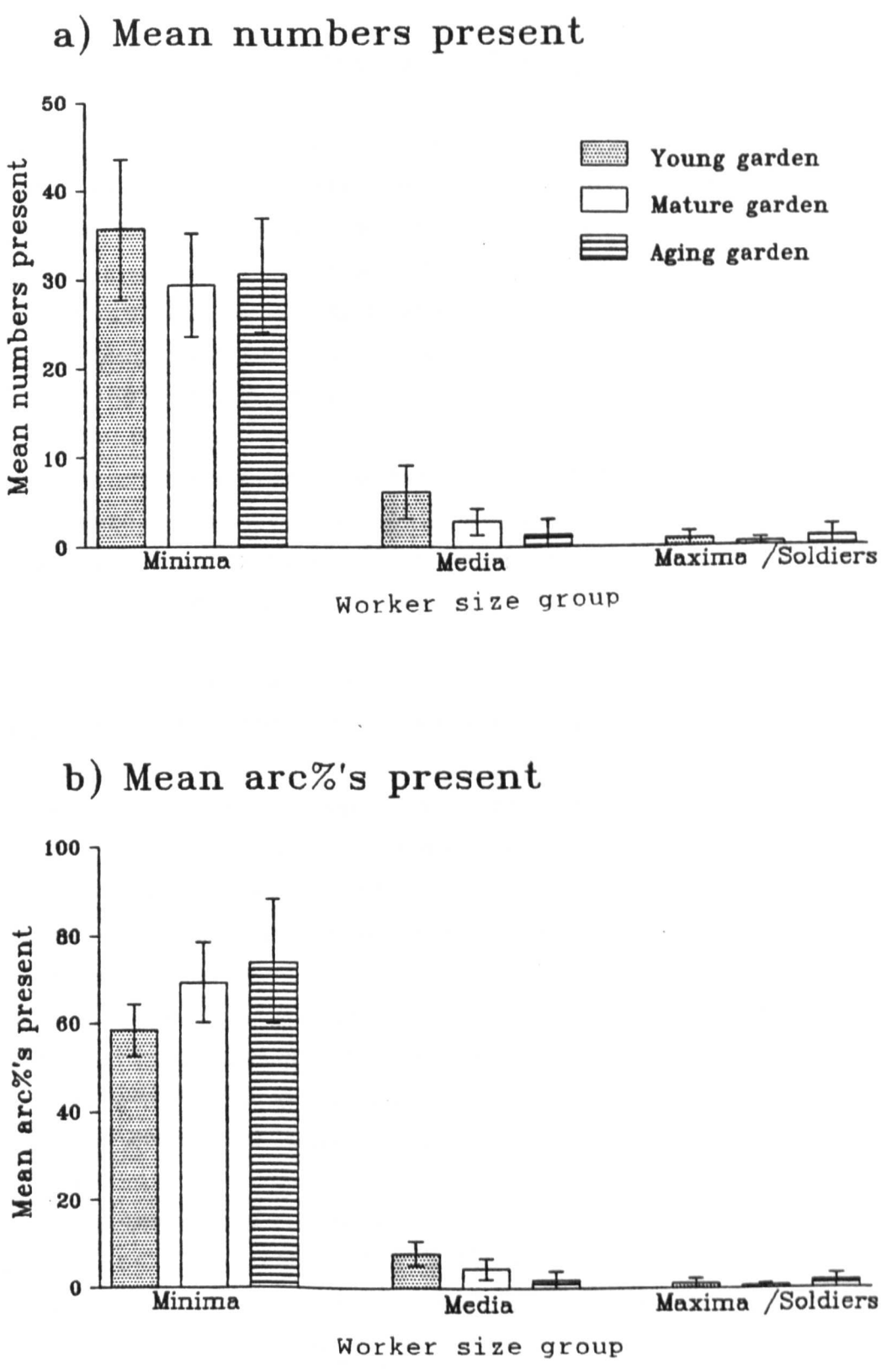


Figure 5.4: Numbers of workers of different size observed on the inner surfaces of three ages of fungus garden per 16 cm^2 area, expressed as, a) Mean numbers present and, b) Mean arcsine-transformed percentages (arc%'s) (± 95% confidence limits; 8, 10 and 6 replicates were used for young, mature and aging gardens respectively).

b) Worker behaviour on the inner surface of the fungus garden

The numbers of workers of the four size groups defined by Weber (1972) and their activities were examined on inner surfaces of young, mature and aging garden. Eight replicate observations were made on young garden; 10 on mature and 6 on aging. Replicate numbers differed due to availability of suitably positioned garden.

(i) Numbers of workers present on inner surfaces

In total, 852 workers were observed in 24 replicates, of which 88% were minima , 10% were media and only 2% were maxima . No soldiers were observed.

Mean total worker numbers did not differ significantly per replicate between the three garden ages ($p>0.9$, ANOVA), but overall, there were significantly more workers per replicate on outer than on inner surfaces of garden ($p<0.001$, Mann-Whitney). Overall, there was a mean of 36.0 (SE ± 2.2) workers per replicate (95% confidence limits 35.1, 36.9) on inner surfaces and a median of 46.0 workers (95% confidence limits 44.0, 49.0) on outer surfaces. Minima and maxima numbers on inner surfaces were not significantly different between garden ages ($p>0.4$, ANOVA), while media numbers were significantly higher on young than on mature or aging garden ($p<0.05$, ANOVA and Tukey, see Fig. 5.4a).

Arcsine-transformed percentages of different castes present were also examined and did not differ significantly between the three garden ages for minima and maxima ($p>0.05$, ANOVA and Tukey). However, arc%'s of media were significantly larger on young than on aging garden ($p<0.05$, ANOVA and Tukey), while arc%'s on mature garden were intermediate and not significantly different to numbers on other areas ($p>0.05$, ANOVA and Tukey, see Fig. 5.4b).

Comparing Fig. 5.4a with Fig. 5.1a showed that minima numbers tended to increase with garden age on outer surfaces, but stayed the same on inner ones. Similarly, media numbers tended to increase with garden age on outer

surfaces, while decreasing with garden age on inner ones. Maxima were more frequently seen on outer than on inner surfaces.

Caste ratios were also compared between outer and inner surfaces using 4 x 2 Heterogeneity Chisquare analyses. The total numbers of workers of each caste observed on outer surfaces (6,109 minima , 1,382 media and 1,157 maxima and soldiers) were compared with those on inner surfaces (750 minima , 85 media and 17 maxima). Significant differences were found ($p<0.005$), due to more than expected numbers of minima on young inner surfaces and fewer than expected media and maxima on mature and aging inner surfaces.

(ii) Behavioural repertoires of different worker castes

Relative frequencies of observed behaviours for each caste are shown in Table 5.5, which includes two acts not recorded in Tables 5.2 and 5.3 (refuse extraction and planting hyphae).

Minima performed 23 acts, media ; 15 and maxima ; 7, with 23 types of act recorded overall. Fewer acts were therefore performed than on outer surfaces, but fewer workers were observed. Good's (1953) sample coverage estimates were 0.99600, 0.96471 and 0.82353 respectively for the three castes.

The total numbers of workers engaged in each act were compared between inner and outer surfaces using 2 x 2 Heterogeneity Chisquare analyses on the numbers of workers engaged in each act versus the total numbers engaged in other acts. The major differences between Tables 5.5 and 5.2 are the relatively low frequency of restless movement and the relatively high relative frequencies of licking and antennating garden recorded on inner surfaces, compared to outer ones and these differences were all significant ($p<0.005$). Numbers of workers grooming (pooling numbers self and allogrooming) were also significantly higher on inner surfaces ($P<0.005$).

Table 5.5: Relative percentage frequencies of observed behaviours of different worker castes on inner surfaces of fungus garden (n = total number of acts recorded). No soldiers were observed.

BEHAVIOURAL ACT	RELATIVE FREQUENCY			
	MINIMA n=750	MEDIA n=85	MAXIMA n=17	TOTAL n=852
Resting	4.533	11.765	47.059	6.103
Restless movement	5.733	7.059	0	5.751
Self-groom	10.667	5.882	11.765	10.211
Allogroom -active	3.733	2.353	0	3.521
Allogroom -passive	2.133	1.177	0	1.995
Trophallaxis	0	0	0	0
Eat staphyla alone	2.133	2.353	5.882	2.230
Eat staphyla mutually	0.533	2.353	0	0.704
Defaecate	0.133	0	0	0.117
Antennate worker	0.533	0	0	0.470
Feed larva	0	0	0	0
Lick larva	0.933	1.177	0	0.939
Lick pupa	0	0	0	0
Carry larva	0	0	0	0
Carry pupa	1.200	5.882	0	1.643
Guard brood	2.533	8.235	5.882	3.169
Lick garden	33.600	7.059	0	30.282
Lick staphyla	0.133	0	0	0.117
Antennate garden	23.200	21.177	11.765	22.770
Carry staphyla	2.533	1.177	0	2.347
Extract refuse	0.267	0	0	0.235
Carry refuse	0	0	0	0
Carry hyphae	0.667	0	0	0.587
Drink droplets on garden surface	0	0	0	0
Cut leaf	0	0	0	0
Crimp leaf	2.000	9.412	11.765	2.934
Lick leaf	0.667	0	0	0.587
Rest on leaf	0	0	0	0
Carry leaf	1.600	12.941	5.882	2.817
Insert leaf fragment	0.400	0	0	0.352
Plant hyphae	0.133	0	0	0.117
TOTALS	100.0	100.0	100.0	100.0

c) The behavioural repertoire of callow workers

Only 48 of the 8,648 workers observed on outer surfaces were callows and of these, 68.8% were minima , 29.2% media and 2.1% maxima . No soldiers were present. The majority of callows (70.8%) were on aging garden while 2.1% were on young garden and 27.1% were on mature. Most were found in the proximity of brood.

The most common act for all castes was resting, followed by antennating garden and restless movement (Table 5.6). All other acts observed were seen less than four times each. Minima performed 9 acts, media ; 7 and only two maxima were seen. Good's (1953) estimate of sample coverage was 0.89583 (for callows as a whole).

Table 5.6: Relative frequencies of observed behaviours of different castes of callow workers (n = total number of acts observed).

BEHAVIOURAL ACT	RELATIVE FREQUENCY			
	MINIMA n=32	MEDIA n=14	MAXIMA n=2	TOTALS n=48
At rest	37.500	42.857	50.000	39.583
Move restlessly	9.375	14.286	0	10.417
Self-groom	6.250	0	0	4.167
Allogroom - active	0	0	0	0
Allogroom - passive	3.125	14.286	0	6.250
Eat staphyla alone	3.125	0	50.000	4.167
Eat staphyla mutually	0	7.143	0	2.083
Antennate worker	3.125	0	0	2.083
Feed larva	0	7.143	0	2.083
Carry larva	0	7.143	0	2.083
Guard brood	9.375	7.143	0	8.333
Lick garden	3.125	0	0	2.083
Antennate garden	25.000	0	0	16.667
TOTALS	100.0	100.0	100.0	100.0

2. A closer examination of 'licking'

The large numbers of workers engaged in 'licking' fungus garden have been noted by several authors (Weber 1972, Quinlan and Cherrett 1979) and such a common act is likely to have an important function. A licking worker rasps the substrate with its glossae (see Fig. 5.5).

More than 98% of licking workers were minima (calculated from Table 5.2) and individual minima licking each of the three ages of garden were therefore observed over short periods of time, to see how licking is related to other activities. On each garden age, sufficient workers were observed to make a total of at least 60 minutes of observation time.

Workers licking garden did so in short bursts, interspersed with other acts. Licking garden was usually associated with either self-grooming or antennating garden and workers frequently switched between these acts (Table 5.7).

Table 5.7: Percentage relative frequencies of licking and associated acts on three ages of garden, during 60 minutes of observation of individual minima , on each (n = total numbers of acts recorded).

BEHAVIOURAL ACT	RELATIVE FREQUENCY ON:			
	YOUNG GARDEN n=240	MATURE GARDEN n=178	AGING GARDEN n=133	TOTALS n=551
Lick garden	35.000	41.011	41.353	38.476
Antennate garden	25.833	28.652	29.323	27.586
Self-groom	33.333	28.652	27.068	30.309
Resting	0.417	1.685	1.504	1.089
Antennate worker	0.417	0	0	0.182
Pluck hyphae	2.500	0	0	1.089
Plant hyphae	2.500	0	0	1.089
Lick leaf	0	0	0.752	0.182
TOTALS	100.0	100.0	100.0	100.0

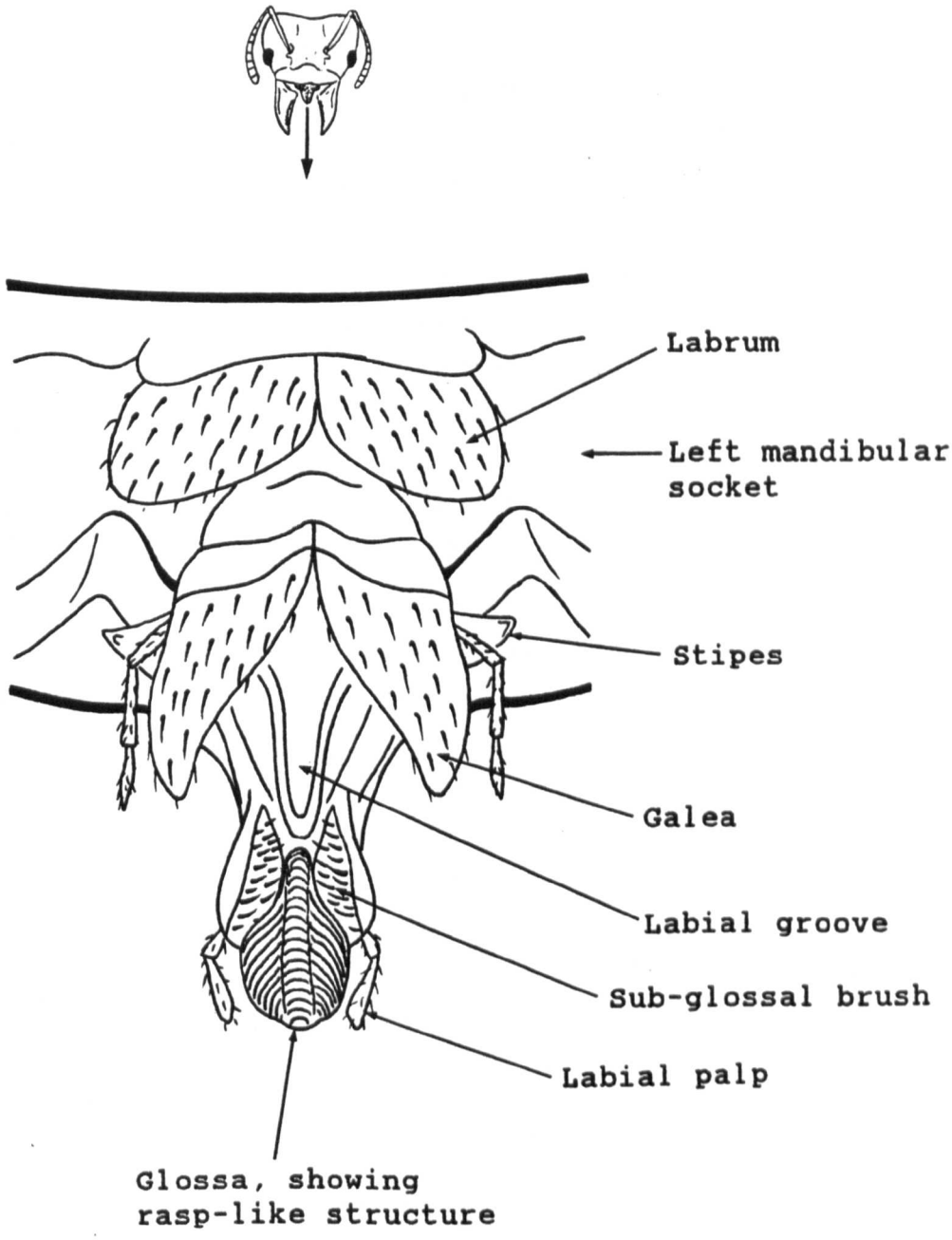


Figure 5.5: Anterior view of the mouthparts of *Atta*, maxillo-labial apparatus extended (the mandibles are not shown).

Fig. 5.6 shows the median durations of licking and associated acts on three ages of fungus garden (the data could not be transformed to produce normal distributions). Licking acts increased significantly in duration with garden age ($p<0.001$, Mood), with durations on aging garden being significantly higher than those on mature, which in turn were significantly higher than those on young garden ($p<0.001$, Mann-Whitney tests). Periods spent antennating garden were also significantly different on different garden ages ($p<0.001$, Mood), being significantly longer on young than on aging garden ($p<0.001$, Mann-Whitney) but not significantly different between young and mature garden or mature and aging ($p>0.1$, Mann-Whitney). Times spent self-grooming were not significantly different on different garden ages ($p>0.2$, Mood).

On all three garden ages, licking lasted significantly longer than other acts ($p<0.001$, Mann-Whitney tests) and periods spent antennating were longer than those spent self-grooming ($p<0.05$, Mann-Whitney tests). Licking took up most (74.8%) of the total observation time (11,095 seconds), followed by self-grooming (14.6%) and antennating garden (7.4%). Other activities took up only 3.1% of the time.

Transition frequencies between acts for workers initially licking garden were calculated. These were the relative frequencies with which one particular act was followed by another particular act, when compared with all following acts. Licking workers frequently paused to antennate garden or groom, before continuing to lick the garden surface (Fig. 5.7).

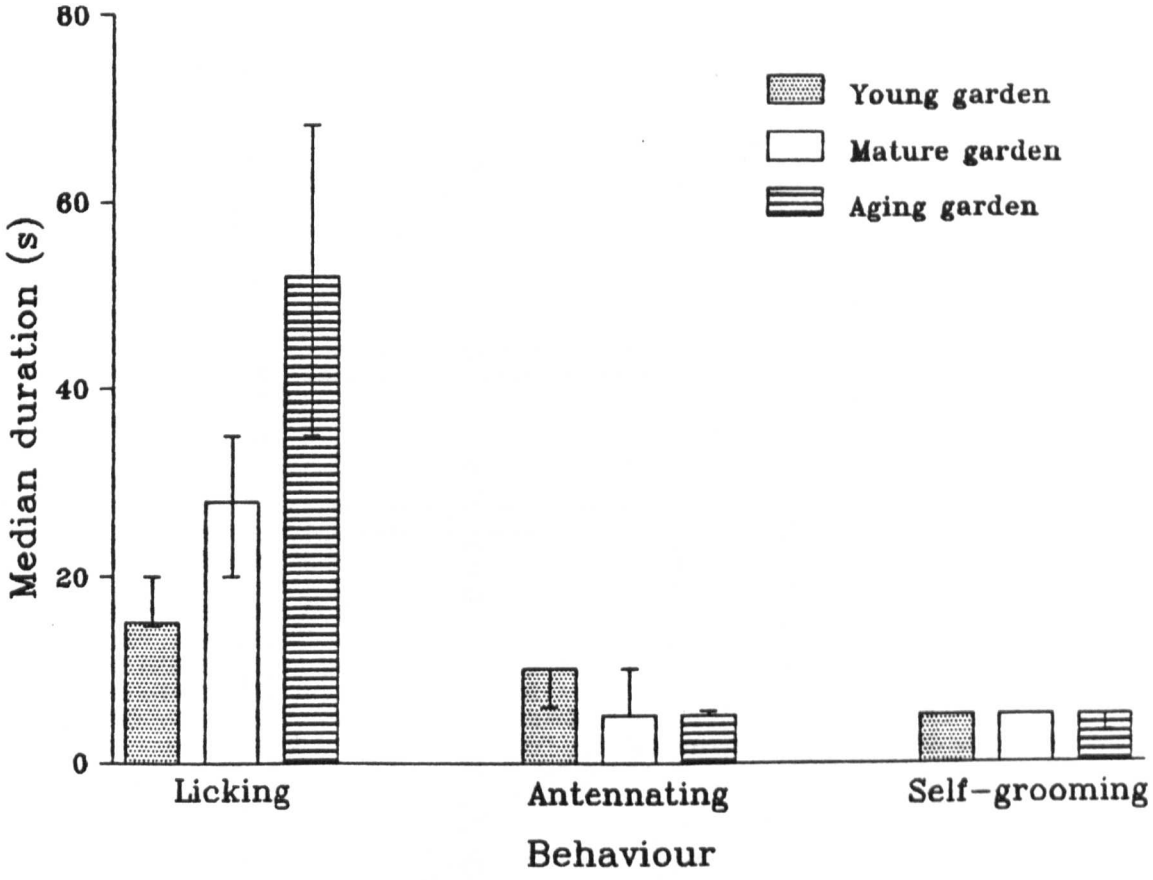


Figure 5.6: Median durations (seconds, ± 95% confidence limits) of licking and associated acts on three ages of fungus garden (36-84 replicates).

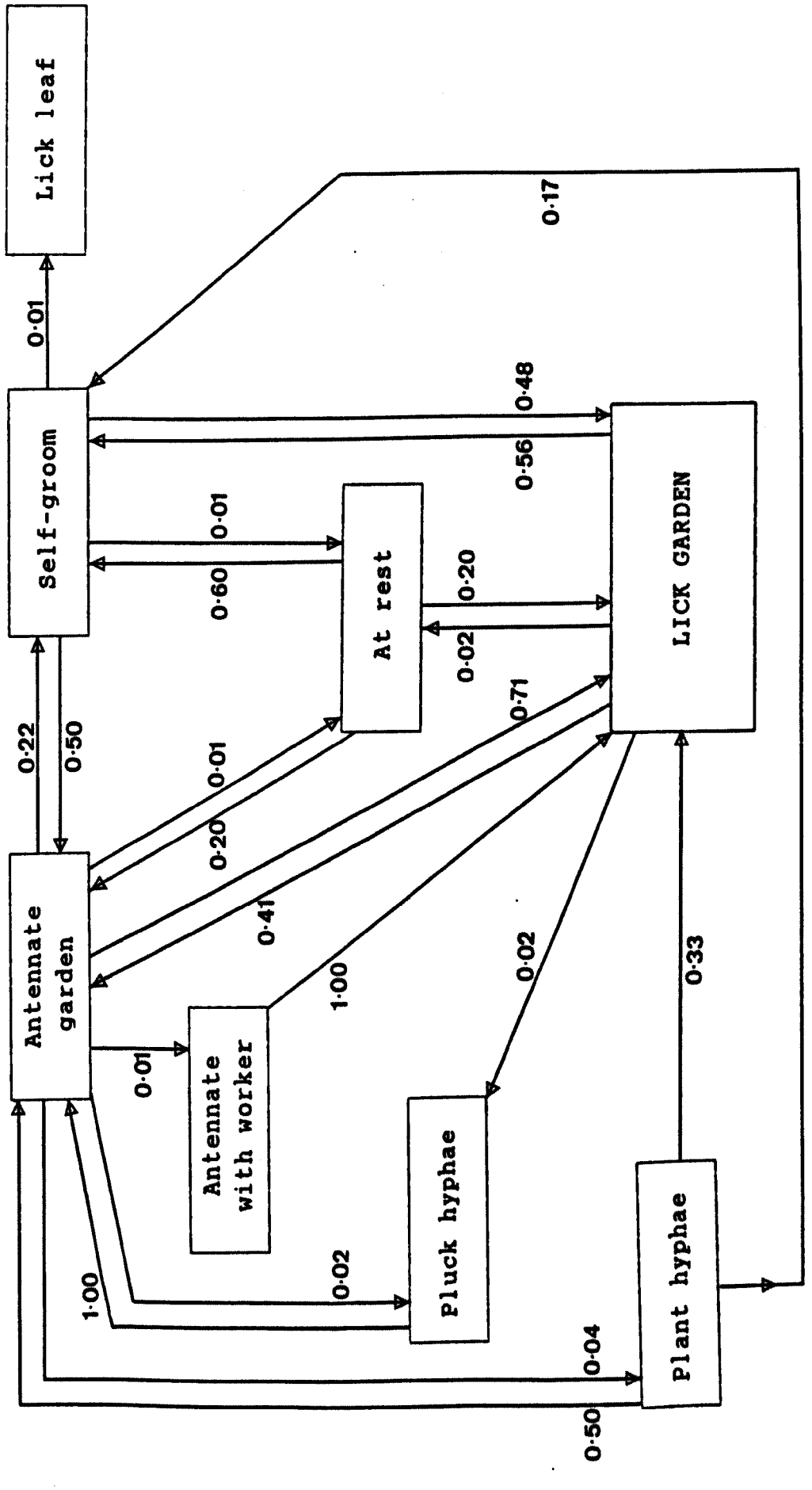


Figure 5.7: Transitions between acts for workers initially licking garden. Transition frequencies are shown for each act.

3. Temporal castes in *Atta sexdens*

No satisfactory marking method was found. Paint and glued-on labels were removed within 24 hrs by other workers, no matter what type of paint or glue was used. Workers either cut or pulled off fine wire used to make petiolar loops. Marked ants (of all types), when returned to their colony, were apparently accepted back but were assiduously groomed by groups of minima as they re-entered gardens and marked workers were not dumped with refuse. Again however, marks were removed after 24 hrs.

Unless an ant-proof marker is discovered, studies of individuality or temporal castes in *Atta* nests will be difficult except in incipient colonies with only a few workers. These are not directly comparable with mature nests since they possess different caste ratios (Wilson 1983a).

4. The behaviour of *Atta* sexuals

a) A fertile *Atta sexdens* queen

The interactions of a queen with workers and fungus garden were observed for five 15 minute periods during January 1990.

Typically, the queen stood on the garden surface, covered by a retinue of 10-15 workers. Occasionally, she walked slowly across the garden but did not interact with it. During the 75 minutes of initial observation, she received staphylae at a mean rate of 5 (SE ± 0.4) per 15 minutes, macerated remains being removed by workers. Trophallaxis between the queen and a media donor was recorded on one occasion but no feeding of trophic eggs was observed.

The queen laid a mean of 1.4 (SE ± 0.8) eggs per 15 minutes which were produced in short bursts and immediately removed by workers. She also produced a single faecal droplet which was immediately imbibed by three minima (abdominal trophallaxis).

Several months after these observations were made, the queen became visible again and was receiving large globular eggs, presumably trophic eggs produced by workers. Over a continuous period of 17 minutes, she received 5 eggs before moving out of sight. After another 13 minutes she reappeared and over the next 23 minutes received 7 eggs before moving out of sight again; rates of 17.7 and 18.3 eggs per hour. She received no staphylae during this time and produced no eggs herself. Four hours later, she was still receiving eggs, but fewer were visible and workers were offering her staphylae as well. Numbers of brood visible appeared normal for that nest and remained so for the next few months.

b) *Atta cephalotes* virgin alates

(i) The behavioural repertoire of alates

Alates performed a small range of acts which generally parallelled those performed by workers. Because alates are large and easy to observe, slight behavioural variations could be defined, as follows:

Receive/consume staphylae - alates may receive several staphylae at once from workers, whilst still ingesting previously received ones.

Pluck/consume staphylae - alates may pluck staphylae from the garden themselves.

'Steal' staphylae - workers usually offer staphylae to alates but sometimes alates may seize staphylae from passing workers without any antennal interaction.

Allow workers to remove remains of staphylae - alates cease other acts and allow workers to remove macerated remains.

Lick garden - alates (especially males) lick more slowly than workers and can be seen ingesting hyphae.

Pluck/ingest hyphal tufts - tufts of hyphae may be pulled from the garden and ingested during periods of licking.

Antennate garden - as for workers

Headwaggle - head, mouthpart and antennal movement, with the head waving from side to side.

Beg - intense antennation with workers, directed towards those approaching with staphylae

Walk across garden - often in order to follow workers with staphylae. Motion is slow and alates frequently continue to ingest staphylae as they walk.

At rest - small amounts of mouthpart and antennal movement, but no sideways head motion.

Self-groom - as for workers

Brood-care - as for workers

Alates, like fertile queens, were continually surrounded by worker retinues with which they remained in antennal contact. Females had significantly larger mean retinues than males ($p<0.02$, ANOVA) with 14.7 (SE ± 0.9) compared to 11.0 (SE ± 1.4) attendant workers, probably because they were larger.

Receiving/consuming staphylae was the most frequent act for both males and females. Females frequently antennated garden and headwaggled while males frequently rested or headwaggled but did not antennate garden. Females engaged in occasional garden and brood-care acts whereas males rarely interacted with the fungus garden. Six out of ten females licked garden at some time during the observations, compared to only one out of nine males and four females also ingested hyphal tufts, while no males did so (Table 5.8).

Good's (1953) estimate of sample coverage was 0.99435 for females and 0.99642 for males (good coverage for both).

Table 5.8: Relative percentage frequencies of behavioural acts of alates for ten females and nine males, observed for 285 and 228 minutes respectively (n = total number of observed acts).

BEHAVIOURAL ACT	RELATIVE FREQUENCY	
	FEMALES n=354	MALES n=279
Receive and eat staphyla	46.893	40.143
Pluck and eat staphyla	0.848	0.358
Steal and eat staphyla	0.283	0
Allow removal of staphyla remains	2.260	3.584
Lick garden	6.215	1.792
Pluck or eat hyphal tufts	3.107	0
Antennate garden	11.299	0
Beg	3.107	22.222
Headwaggle	13.842	13.978
Walk across garden	5.085	5.018
Self-groom	1.695	0.717
Lick larva	0.283	0
At rest	5.085	12.186
TOTALS	100.0	100.0

(ii) Time budgets and the durations of specific acts

For both alate males and females, receiving/consuming staphylae took up the largest proportion of time, although this activity did not have the longest duration (Table 5.9). Males spent almost five times as long begging as females, but spent little time in garden-care. Females spent relatively greater proportions of time walking or headwaggling, but males spent 2.6 times longer resting.

Different types of act had significantly different durations ($p<0.001$, Mood), for both females and males (the data could not be transformed to produce normal

distributions). Begging, antennating garden and self-grooming lasted only a few seconds, whereas receiving/consuming staphylae, headwaggling and resting lasted much longer. Times spent receiving/consuming staphylae, licking garden, begging, allowing the removal of staphyla remains or resting were not significantly different between males and females ($p>0.1$, Mann-Whitney). However, females spent significantly longer periods walking ($p<0.001$, Mann-Whitney) and headwaggling than males ($p<0.05$, Mann-Whitney).

(iii) Comparing staphylae intakes and ingestion times

When alates were fed by workers, they usually received bundles of two or three staphylae at a time. During the observations, numbers of staphylae received were recorded (Table 5.10) and these results compared between males and females but there was no significant difference between them ($p>0.2$, Mann-Whitney).

While staphylae were being ingested, alates often performed other acts like walking or begging. Total ingestion times were therefore longer than recorded in Table 5.9. However, these total ingestion times were also recorded so that overall ingestion times per single staphyla could be calculated (Table 5.10). Males took significantly longer to ingest individual staphylae than females ($p<0.001$, Mann-Whitney).

Alate males and females were weighed approximately 1 and 5 weeks after eclosion. After 1 week, females had a mean weight of 0.51 g (SE ± 0.03, 3 replicates), while males weighed 0.35 g (SE ± 0.01, 8 replicates). After 5 weeks, females weighed significantly more ($p<0.001$, ANOVA) but males weighed significantly less ($p<0.02$, ANOVA) indicating the enormous input of resources into alate females. Females now had a mean weight of 0.64 g (SE ± 0.01, 3 replicates) while males weighed only 0.32 g (SE ± 0.01, 6 replicates).

Table 5.9: Time parameters for alate behaviour: durations of specific acts (seconds) and time budgets (N = number of observations). Total observation times were 17,100 s for females and 13,680 s for males.

a) Females

BEHAVIOURAL ACT	MEDIAN DURATION (S)	95% CONFIDENCE LIMITS	N	PROPORTION OF TOTAL TIME
Eat staphylae	30.0	30.0,40.0	170	44.357
Beg	10.0	9.6,30.0	11	1.257
Lick garden	30.0	15.0,45.0	18	3.889
Antennate garden	15.0	15.0,25.9	40	7.281
Walk	55.0	37.8,144.1	18	11.667
Headwaggle	35.0	21.1,45.0	49	15.088
Allow removal of staphyla remains	17.5	14.4,57.3	8	1.433
Resting	45.0	27.6,194.5	18	14.415
Self-groom	15.0	6.8,24.6	6	0.526
Brood-care	-	-	1	0.088

b) Males

Consume staphylae	40.0	35.0,50.0	113	42.288
Beg	10.0	5.0,15.0	62	5.738
Lick garden	35.0	20.0,95.0	5	1.791
Antennate garden	-	-	0	0
Walk	20.0	14.7,35.3	14	3.253
Headwaggle	20.0	15.0,25.3	39	7.018
Allow removal of staphyla remains	17.5	10.0,46.7	10	1.864
Resting	75.0	39.4,121.9	34	37.756
Self-groom	-	-	2	0.292
Brood-care	-	-	0	0

Table 5.10: Median numbers of staphylae consumed per 30 minutes and median ingestion times for ten alate females and nine alate males.

	MEDIAN STAPHYLAE INTAKE	N	95% CONFIDENCE LIMITS	MEDIAN INGESTION TIME PER STAPHYLA	N	95% CONFIDENCE LIMITS
FEMALES	21.0	10	8.3,33.9	33.6	9	26.6,44.4
MALES	15.0	9	2.4,21.8	54.3	8	47.0,90.2

DISCUSSION

1. The behaviour of workers of *Atta sexdens* on the fungus garden

The majority of workers observed were minima (70.6%) and Weber (1972) estimated that they make up 60% of the total worker population. When numbers of workers on the garden surface are examined, foraging media and maxima are not present, which biases the estimate towards minima . Soldiers were the least common caste but each individual is very large, requiring a lot of resource input and relatively few are produced.

On outer surfaces, minima , media and maxima performed a much greater variety of acts compared to soldiers, which were rare and consequently had a poorer sample coverage and smaller observed repertoire than other castes. With more observations, more rare acts would therefore be likely to have been seen. However, in polymorphic species majors tend to be specialists, often with limited repertoires and Attine soldiers are specialised for defence (Oster and Wilson 1978). The majority were either resting or moving restlessly, rather

than contributing to nest-care. In *Atta*, media are the primitive caste (Oster and Wilson 1978), so should perform the widest range of tasks. In these observations they performed fewer acts than minima , but activities away from the garden such as foraging and defending were not included in this study. In *Atta*, minima perform a wide range of acts and specialise in brood and garden-care, both of which involve complex series of behaviours. Some minima are however, found on forage trails.

Fewer replicate observations were used on inner surfaces than on outer ones, so fewer workers were observed, resulting in poorer sample coverages. Few inner surfaces were available and some had to be created artificially to get a reasonable number of replicates. On these surfaces, a higher percentage of workers were minimas, compared with outer surfaces and no soldiers were observed, suggesting that worker access to inner areas is restricted by the size of the cavities within the garden, as indicated in Chapter 4.

The two dominating acts on outer surfaces were licking garden and restless movement. Quinlan and Cherrett (1979) reported that up to 30% of workers on the garden surface were licking it and this was certainly true on young garden in this study. Most licking workers were minima which are, as stated earlier, garden-care specialists. Licking became more common on inner surfaces but this may have been a reflection of the castes present; minima were also relatively more common on inner areas. Many authors (Herbers and Cunningham 1983, Wilson 1980a) have not counted restless movement as an act. However it engaged a quarter of all workers on the garden surface, although its purpose, if any, remained unclear. Little restless movement was recorded on inner surfaces, suggesting that outer surfaces act as a highway for workers travelling between tasks, to obtain staphylae to feed brood, or to seek forage. Restlessly moving workers may also be important for spreading pheromones around the nest. The social cohesion

in ant colonies depends upon pheromones produced by the queen and brood and in *Atta* the loss of the brood results in the disintegration of social organisation (Powell 1984). Media , which are within-nest generalists (Wilson 1980a), were the most common restless movers, followed by maxima .

Another common act was antennating garden, which was usually performed by minima and decreased in percentage relative frequency with increasing caste size. The antennae are highly sensitive organs, essential for social behaviour, with olfactory, gustatory, tactile, thermal, vibratory and other receptors (Delabie *et al.* 1986). Antennating workers may be checking the garden surface for alien contaminants, staphylae or the degree of hyphal growth.

The popular idea of ants is that they are perpetually busy and industrious, hence the famous biblical quotation; 'go to the ant, thou sluggard; consider her ways and be wise' (Proverbs Ch.6-8). However, 11% of workers observed on outer surfaces were at rest, although fewer rested on inner surfaces. Both Otto (1958), studying *Formica* and Cole (1986), studying *Leptothorax*, showed that workers may spend up to 70% of their time resting. In *Atta*, the frequency of resting increased with caste size; only 6% of minima were resting compared with 51% of soldiers. However, numbers resting changed with time of day, fewer being at rest during the foraging period. Resting workers may therefore form a reservoir labour pool, available for foraging when leaves are being exploited.

Acts like grooming, eating/transporting staphylae and guarding brood were all of intermediate relative frequency (on outer surfaces). Self-grooming was performed everywhere by every caste and usually involved the use of the tibio-tarsal combs to clean the antennae and legs. Allogrooming, in contrast, is a social act and small workers tended to groom larger workers. Similarly, small workers harvested and carried staphylae around more than large ones. This

emphasizes how minima are garden-care specialists and suggests that they extend decontamination and cleaning of the garden surface to large workers as well. Guarding brood however, was really a form of resting since workers remained motionless and all castes guarded brood at similar relative frequencies.

Different types of behaviour occurred at different frequencies, some acts being very common while others were seldom observed. However, apparently rare acts may simply take less time than 'common' acts. Herbers and Cunningham (1983) showed that acts performed by *Leptothorax longispinosus* ranged from 6-600 seconds in mean duration. Rare acts may therefore not be uncommon; Wilson and Fagen (1974) stated that ant behavioural acts tend to be common, since the small size of the brain precludes the storage of responses that are not commonly used. Wilson (1971) also pointed out that social insects make repeated use of the same communicative signals and responses in different contexts to achieve various purposes. However, if ants do not possess 'rare' behavioural acts, then this raises the question of why acts like queen-care, refuse removal, and assistance with ecdysis and eclosion were not observed in this study. Wilson and Fagen (1974) considered that 1,962 separate acts recorded showed almost the complete repertory of *Leptothorax curvispinosus* workers; in this study, 8,648 separate acts were recorded for *Atta sexdens* workers. However the *Atta* colony used in the observations had 80 fungus gardens, therefore the chances of seeing the queen were slim. Queen-care acts are likely to be extremely common in her vicinity, but nowhere else and many workers in a large nest may never encounter the queen.

Little refuse-handling or removal was observed. Refuse is usually removed from garden bases and the observations made in this study were on garden sides. In the laboratory, gardens are constructed with the bases resting on a ceramic tile and therefore unobservable. This caused a bias towards observing behaviour on garden sides. Similarly, although planting hyphal tufts on to freshly inserted substrate is

recorded in the literature (Weber 1972), none was seen in the study on outer surfaces, although some observations were made of this act on inner ones. This again was because observations were made on the sides of garden and the upper surfaces of the garden (young garden) where such acts occur, were not observed. In one separate observation of 15 workers on the new edge of a cell of a young garden, five were inserting substrate fragments and in another case, a single worker continually removed tufts of hyphae and planted them on to newly-inserted substrate only 2 mm away.

Assistance in eclosion and ecdysis are recorded by Wilson (1980a) but were not observed in this study. However, numbers of pupae were quite low during the observation period, therefore few individuals were at the stage of ecdysis or eclosion (see Table 5.4).

Each worker consumes a mean of 0.3 staphylae per hour (Quinlan and Cherrett 1979) and this corresponds with the low levels recorded during this study. However, from Table 5.2, only 0.451% were seen to feed larvae (39 workers). According to Quinlan and Cherrett (1979) larvae each receive 0.55 staphylae per hour, but there were 1,014 larvae present; a mean of 0.03 staphylae per larva. However, larvae already consuming staphylae were not considered and the time taken to receive a staphyla is probably much shorter than the time taken to consume one.

a) Worker numbers and behaviour on different ages of garden

Workers, particularly soldiers, were more common on aging garden than on young. Young garden has much larger cells than aging (see Fig. 2.1, Chapter 2) and consequently, the 16 cm^2 'window' covered a smaller actual surface area on young garden than on aging. Greater surface area would therefore mean larger numbers of workers present. Alternatively, aging garden, being at the bottom, is closer to the entrance hole (situated at the base of the containing dome) and may therefore have acted as the nearest convenient place for workers to go after entering or before exiting the chamber. This may have been

the case for soldiers, which play a defensive role and rarely assist in nest-care acts such as substrate preparation (although soldiers of *Atta sexdens* frequently forage). Both staphylae and brood are present on mature and aging garden, but not on young, so providing extra tasks in these areas (caring for brood, harvesting and transporting staphylae). In fact, the number of types of behaviour was higher on aging than on young garden and more types of behaviour would be likely to involve more ants. However, this leads to the question of whether more ants are present because a wider range of tasks must be performed, or whether more tasks are performed because the workers are there? The former is probably correct, because staphylae only begin to develop after a period of mycelial growth, i.e. on mature garden. Allogrooming and resting were possibly more common on older garden because young garden is an 'active' place, where fresh material is prepared and added. Antennation and licking garden both decreased on older garden however and may be important on young garden for reasons such as 'mopping up' leaf sap, fungal exudates and contaminants brought in on imported forage or detecting the presence of young, palatable hyphae. In contrast to these acts, some, such as self-grooming and restless movement, remained constant over the whole garden.

b) The calculation of behavioural repertoires

Some authors have calculated total behavioural repertoires by fitting behavioural abundance data to frequency distributions (Wilson and Fagen 1974, Fagen and Goldman 1977). However, behavioural repertoires and frequencies change in different contexts and observing *Atta* soldiers both outside and inside the nest shows completely different behaviours. Outside, a soldier might be defending the nest while inside the nest, it would probably be resting or wandering aimlessly. Wilson and Fagen (1974) pointed out that the greatest part of an ant's life is spent inside the nest, therefore a behavioural repertoire calculated from observations of behaviour inside the nest would show what workers do for most of their lives; a high

'lifetime coverage'. Foraging is a very dangerous occupation and typically, only 2 out of 200 workers in a laboratory colony of *Lasius niger* were foraging at any one time (pers.obs.). However, a behavioural repertoire calculated from data collected from the fungus garden surface might show what workers do for most of their lives but would also be wildly inaccurate in terms of the total behavioural repertoire. It is also difficult to see how Wilson and Fagen can justify ignoring foraging, since it is an integral part of the nest economy.

Another problem with calculating repertoires is that if, for example, 30 acts are observed and it is calculated that there are an extra 2-4 undiscovered acts, then the nature of these undiscovered acts remains unknown and they may not even exist. Predicting the existence of such 'phantom' acts is therefore not useful in this context. Similarly, Good's (1953) estimate for sample coverage is only useful as a rough guide to how complete data obtained is, for that context alone.

In total, 32 acts were observed on inner and outer surfaces (see Tables 5.2 and 5.5), allowing for the fact that 'carry staphylae' included both harvesting and carrying staphylae. These were combined with other acts reported in the literature (Autuori 1941, Febvay and Kermarrec 1981b, Quinlan and Cherrett 1979, Stradling 1978, Weber 1972, Wilson 1980a, 1983a) or in other observations, to give a more complete repertoire. The entire repertoire is listed in Table 5.11.

Table 5.11: The behavioural repertoire of *Atta sexdens* workers, compiled from acts recorded in Tables 5.2 and 5.5, personal observation and the literature. Acts underlined are those not recorded in Tables 5.2 and 5.5.

Personal behaviours	At rest
	Move restlessly
	Self-groom
	Eat staphyla alone
	Defaecate
	Lay eggs
	Egest infrabuccal pellet
Social behaviours with other workers	Allogroom - active participation
	Allogroom - passive
	Trophallaxis
	Eat staphylae mutually
	Antennate worker
	Abdominal trophallaxis with worker
	Carry worker
Foraging behaviours	Explore
	Lay pheromone trails
	Recruit
	Cut leaf petioles
	Cut section from leaf
	Carry leaf sections from forage site
	'Hitch-hike'
	Drink sap *in situ*
	Drink water *in situ*
	Carry leaf sections inside garden chamber
	Rest on leaf pieces inside garden chamber
	Cut up leaf section
	Crimp leaf fragments
	Lick leaf pieces
	Insert leaf fragments into garden
Nest and garden-care behaviours	Excavate chamber
	Lick nest wall
	Carry garden fragments to found new garden
	Reconstruct garden
	Pluck hyphal tufts
	Carry hyphal tufts
	Plant hyphal tufts
	Antennate garden
	Lick garden
	Lick staphyla
	Pluck staphyla
	Carry staphyla
	Drink droplets from garden surface

continued overleaf -

Manipulation of refuse	Extract refuse from garden
	Carry refuse
	Carry dead worker
	Work in refuse chambers
Brood-care	Feed larva
	Lick larva
	Lick pupa
	Carry eggs
	Carry larva
	Carry pupa
	Carry immature callow worker
	Assist ecdysis
	Assist eclosion
	Guard brood
Queen-care	Antennate with queen
	Groom queen
	Feed queen staphylae
	Remove staphylae remains from mouthparts
	Feed queen trophic eggs
	Trophallaxis with queen
	Abdominal trophallaxis with queen
Alate-care	Antennate with males
	Antennate with females
	Groom males
	Groom females
	Feed males staphylae
	Feed females staphylae
	Remove staphylae remains from mouthparts
	Move out of nest for mating flight
Alarm responses	Stridulate
	Threaten - 'stilting'
	Fight/defend
	Produce alarm pheromones
	Run, head raised and mandibles gaping

There are therefore at least another 44 acts which were not seen during the observations because conditions were not suitable, because, for example the queen, alates and enemies were not present. The total behavioural repertoire for *Atta* is therefore in the region of 76 acts, including resting and restless movement. This contrasts with *Zacryptocerus varians,* which performs 40-42 acts (Wilson 1976b, Cole 1980). Wilson's description of this ant's repertoire is highly detailed and although it excludes resting, it divides many acts into separate ones dealing with minors and majors. If this were repeated for *Atta*, then the estimated repertoire would be much larger.

However, a repertoire of 76 acts is still the largest recorded so far for any ant.

Adopting a fungus-culturing strategy has therefore led to the evolution of a much larger behavioural repertoire than in other ants. The benefits of the Attine strategy are obvious; the fungus is capable of growth on a wide variety of plant substrates, enabling the ants to become polyphagous and Cramer (1967) stated that they are one of only five groups of polyphagous pests. Colonies can therefore utilise a wide range of available plant resources and the problems experienced by farmers in South and Central America testify to this fact. Cherrett (1968) in Guyana, found that *Atta cephalotes* took 50% of woody plant species available. However, there will have been costs involved in developing the complex specialised behaviour and physiology required for fungus culturing. The fungus garden is easily overcome by alien fungi and bacteria in the absence of the ants (Weber 1972), therefore workers must invest a lot of time and effort in maintaining the monoculture. Some authors (Weber 1972) have suggested that this is the purpose of the large numbers of licking workers.

c) The behaviour of callow workers

Only 0.6% of workers observed were callows (Table 5.4) 73% of the brood observed were present as larvae, with only 1.4% present as dark maturing pupae. Few pupae were therefore maturing into callows at this time.

The majority of callows observed were minima and the proportions of castes present reflected those of adult workers (Fig. 5.1). Most callows were resting or moving restlessly, although they also antennated garden. Useful acts like brood-care or garden interactions did occur at low relative frequencies, at least in minima and media callows. This agreed with Wilson's (1980a) results. However, callows were so scarce that no real conclusions could be made about their behaviour; the sample coverage estimate of 0.89583 was poor.

2. A closer examination of 'licking'

Licking was one of the most commonly seen acts on the garden surface (Table 5.2), suggesting that it has some important function. During these observations when light conditions were right, licking workers could be seen actually ingesting hyphae, although Quinlan and Cherrett (1979) and Wilson (1980a) claimed that this did not take place. The infrabuccal filter traps solid material in the infrabuccal pocket and chitin digestion occurs here (Febvay *et al.* 1984). Ingested hyphae might therefore provide a source of food and this possibility is examined in Chapter 10.

Licking workers paused frequently to groom themselves or to antennate garden and occasionally diverged into other acts; a worker cannot lick garden indefinitely. However, licking was obviously the main act and took up more time than the other acts performed. Workers may have antennated garden to search for the best place to lick, using their antennae to examine the garden surface. Grooming is a common act in most insects and in Attines, probably plays a significant part in inactivating alien fungi and bacteria picked up during foraging which might contaminate the garden. The metathoracic glands secrete fungistatic chemicals (Schildknecht and Koob 1970, 1971) which may be spread by grooming. This however, does not explain why licking workers frequently pause to groom. It is possible that fragments of hyphae stick to the antennae and legs, stimulating the worker to groom itself. Alternatively, self-grooming may be a reflexive action which takes place every so often, regardless of how clean the ant is.

It is difficult to explain why the duration of licking acts increased with garden age (Fig. 5.6). It may take longer for workers to ingest the same relative amount of hyphae on aging than on young garden. Workers on young garden, with its luxuriant hyphal growth may be able to ingest hyphae more quickly, or may have to remove fragments of hyphae from their antennae and legs more often,

shortening the licking period. Alternatively, hyphae on aging garden may be more attractive, although this seems unlikely since the substrate is becoming exhausted. Hyphae might therefore be less nutritious. However, workers may be removing hyphae from aging garden in preparation for discarding it.

3. The behaviour of *Atta* sexuals

Fertile queens are capable of carrying out all acts required to establish the colony but once workers are present, they become primarily egg-layers (Weber 1960). During these observations, the queen observed behaved in this classic manner, doing nothing but consume staphylae or trophic eggs, laying eggs and producing the occasional faecal droplet, which was imbibed by workers (abdominal trophallaxis). Quinlan and Cherrett (1979) did not see queens receiving trophic eggs, although they did observe the feeding of staphylae (21.6 per hour) and trophallaxis. Numbers of staphylae received during the current study were similar to their figures, which were not sufficient to meet respiratory requirements. The feeding of trophic eggs to the queen appeared to be sporadic. During the first set of observations the queen received only staphylae and during the second set, she received only eggs. However after several hours, numbers of eggs offered tailed off and staphylae were offered instead. Trophic eggs may stimulate egg-laying, as they would provide an ideal source of nutrients. However there was no evidence for this, although brood production in Attines is apparently cyclic (see Chapter 3).

In this study, the queen laid very small numbers of eggs; less than 6 per hour. If this were continued at a steady rate for the prospective 15 year queen lifespan, less than 800,000 workers would be produced, while a typical *Atta sexdens* nest contains around 2.2 million workers (Weber 1966). This implies that egg-laying is a sporadic event, or that the observed queen was laying eggs

at an abnormally slow rate. The nest did in fact begin to decline and die 1 year after this study was made (see records for Nest 3, Chapter 11). However, Weber (1982) observed a queen over 1 hour and saw only ten eggs produced, in the space of 26 minutes.

There remains an anomaly in the number of eggs and workers produced. Weber (1972) suggested that *Atta* workers may live 1-2 years, therefore if a typical nest contains 2.2 million workers and persists for 10-20 years with one queen, a minimum of 11 million workers must be produced. Kerr (1961) found that newly-fecundated *Atta sexdens* queens contained a mean of 254 million spermatozoa in their spermathecae. Consequently, to produce 11 million workers, only 23 spermatozoa must be used per egg. If worker lifespan is only 1 year and the colony persists for 20 years, then 44 million workers will be produced, with each egg using up only 6 spermatozoa during fertilisation. In both cases, this is an almost unbelievable level of efficiency. In some wasp species eggs divide after fertilisation to produce clones of several hundred individuals ('polyembryony'; Gould 1983). If this occurred in *Atta*, such levels of efficiency of sperm conservation would not be required.

In contrast to the fertile queen, alate females and males received only staphylae and some hyphae, which they plucked from the garden surface themselves. Some authors have implied that alates deliberately pack hyphae into their infrabuccal pockets before the mating flight (Mariconi 1970, Wilson 1971). Cherrett *et al.* (1989) considered this to be unlikely, stating that Wheeler's (1910) view that the infrabuccal pellet used by the newly-fecundated queen to found a fungus garden is the unexpelled refuse of her last meal, was more likely to be the correct one. However both ideas appear to be correct since alate females do pluck hyphae from the garden, but apparently for food rather than in preparation for the mating flight. The sexual brood observed did not actually 'fly' and individuals left the nest at intervals over several weeks.

Both alate males and females received more staphylae per hour on average than fertile queens. Since they are effectively the 'future' of the colony, it is worth investing these resources in them but alate numbers are limited. When the sexual brood was produced, one of the first signs was the presence of large, flaccid, apparently starved larvae. When a colony produces a sexual brood, more individuals may begin to develop than can be supported. A compromise must be reached between brood size and the risk of over-stretching resources, since too many sexual larvae may mean that none are successfully raised and the nest declines. If too few are produced, the colony is not maximising its inclusive fitness; a poor evolutionary strategy. It is likely that once a certain number have reached a critical size, the rest are allowed to starve and either die or develop into workers. Workers of *Myrmica rubra* deal with overproduction of sexual larvae by biting them and inhibiting further growth, which promotes worker production (Brian 1973). The drain on colony resources was very obvious in this study when the sexual brood was present, as the parent nest subsequently declined for several months.

Unfortunately, no records were kept of begging or other types of behaviour, therefore fertile queen behaviour could not be directly compared with that of alates. Alates did not perform a wide range of acts, but estimated sample coverage was good for both males and females. Further observations would have been desirable, but the sexual brood produced was not very large and alates were not always situated in ideal places for observation. However some conclusions could be drawn. Alate females interacted more with the garden than males while males seemed to have to 'work harder' than females to obtain staphylae. 'Begging' more often preceded receiving/consuming staphylae in males than in females. Females therefore have more resources invested in them than males, which must solicit food and are effectively just 'flying sperm dispensers' (Wilson 1971).

SUMMARY

Combining the results of this study with information recorded in the literature showed that *Atta sexdens* workers perform 76 acts and this is the largest recorded repertoire for any ant species. Alates of *Atta* performed a much smaller range of acts than the workers and acted as colony resource sinks.

Up to 25% of workers observed on the outer surface of the garden were moving restlessly but few did so on inner surfaces, indicating that outer surfaces are used as a highway by workers. Large workers were rarely seen on inner surfaces; worker access to the inner garden is restricted by sizes of garden cavities.

'Licking garden', performed mostly by minima , was the most common worker act on both outer and inner garden surfaces, engaging 20% of workers on outer surfaces and 30% on inner ones. Licking workers ingested hyphae and frequently paused to antennate garden or self-groom. Alate females also ingested hyphae when licking garden. The purpose of licking remained unclear and Chapters 6-10 are devoted to investigating this activity.

A fertile queen of *Atta sexdens* produced eggs sporadically and received a diet of staphylae, supplemented by trophic eggs, presumably produced by workers. Some trophallaxis between workers and queen also took place.

Chapter 6 : The nature and production of infrabuccal pellets

INTRODUCTION

In ants, only liquids can enter the crop , while solid particles collect in a blind evagination below the anterior part of the pharynx, known as the infrabuccal pocket (Fig. 6.1). These are then ejected as a solid pellet (Janet 1905, Weber 1972). An infrabuccal filter prevents solid material from passing out of the pocket and this prevents particulate material from blocking the proventriculus, which acts as a 'dam', allowing liquid storage in the crop (Eisner and Happ 1962). This may not however, be so important in the Myrmicines as in other groups (Eisner 1957) and trophallaxis (the transfer of liquid food between individuals) is uncommon in *Atta* (Weber 1972, see also Chapter 5). Weber (1972) said that this was because they feed on fungus cultured within the nest, which Eisner (1957) considered as an external storage mechanism.

In *Camponotus pennsylvanicus*, the infrabuccal filter can trap 150 µm particles but finer particles are removed by trophallaxis, which ensures several passes through the filter (Eisner and Happ 1962). Since food exchange by trophallaxis is uncommon in leafcutting ants, it cannot be used as an important method of filtering. However, this has been offset by the development of a highly efficient infrabuccal filter. Quinlan and Cherrett (1978b) showed that minima of *Acromyrmex octospinosus* could filter out particles of 10 µm, while media and maxima could filter out particles of 30 µm diameter. Ant fungal hyphae measure 6-10 µm in diameter (Weber 1972) and are therefore trapped in minima pockets and probably those of larger castes too.

The infrabuccal pocket receives detritus from grooming (Bailey 1920) and Weber (1972) considered that this activity, coupled with the pocket structure, is used by Attines to remove pathogens that might inhibit or kill the

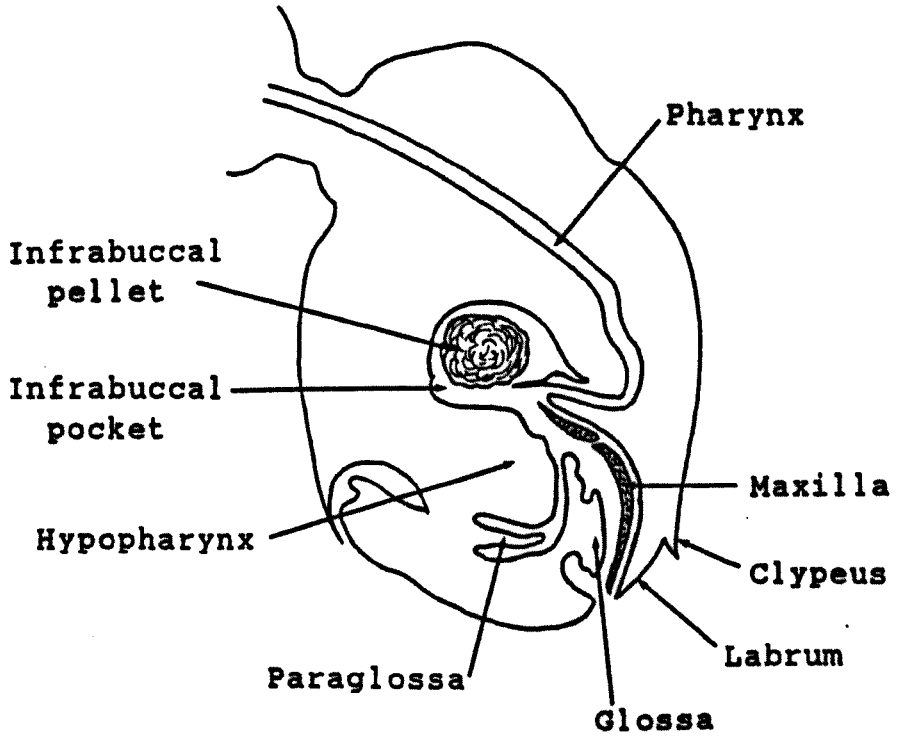


Figure 6.1: Structure of the ant head (adapted from Febvay and Kermarrec 1981b).

ant fungus. Weber (1972) stated that infrabuccal pocket contents of Attines included unidentifiable debris, soil particles and spores, while Febvay and Kermarrec (1981b) recorded the presence of hyphae, plant material and nematodes. The infrabuccal pocket also acts as a receptacle for material licked from fungus garden, worker bodies and leaf wax (Quinlan and Cherrett 1978b).

Fungal spores are likely to be found in Attine infrabuccal pellets because the workers assiduously lick all material brought into the nest (Stahel and Geijskes 1939, Quinlan and Cherrett 1977). Zygospores of the Mucorales have diameters of 200 µm, but the majority of spores are around 10 µm (Hawker and Madelin 1974) and are therefore likely to be filtered out by workers. Infrabuccal pellets produced by workers are discarded with refuse (Quinlan and Cherrett 1978b) thus removing these spores from the vicinity of the garden. In fact, the pellets produced by many ant genera contain fungal spores or hyphae (although obviously not of ant fungus). Bailey (1920) examined pellets from 38 species and fungal material formed a 'considerable fraction' of total pellet volume in many of them. Bailey concluded that this was material taken in during grooming but does not give any figures for his 'considerable fraction'.

The infrabuccal pocket is also used to transmit the ant fungus from the parent nest to new colonies (von Ihering 1898). A newly-fecundated queen excavates a nest chamber and regurgitates her infrabuccal pellet, which acts as a basis for a new garden (Huber 1905, Weber 1972). Wheeler (1910) considered the queen's pellet to be the unexpelled refuse of her last meal but some authors have implied that gynes deliberately pick fungal hyphae from parent nest gardens and pack them into their infrabuccal pockets before the mating flight (Mariconi 1970, Wilson 1971).

Workers regurgitate infrabuccal pellets at regular intervals at rates of 13.4 (SE ± 1.0) per ten workers of

Acromyrmex octospinosus isolated with fungus garden over 24 hrs (Febvay and Kermarrec 1981b). Rates of production are lower when workers are isolated without garden and pellets are probably regurgitated after repletion is reached (Febvay and Kermarrec 1981b). These authors also pointed out that the period spent by material in the infrabuccal pocket is too short to allow microbial degradation, although they suggested that enzymatic degradation might take place.

Febvay and Kermarrec (1981a) demonstrated the presence of ß-N-acetylglucosaminidase activity in the labial glands of *Acromyrmex octospinosus*, which is often found in association with chitinolytic activity (Jeuniaux 1966). The labial glands open between the paraglossae and food is bathed with gland secretions before entering the infrabuccal pocket. Febvay *et al.* (1984) showed that the labial glands possess a chitinolytic enzymatic system and demonstrated chitin digestion in the infrabuccal pocket in *Acromyrmex octospinosus*. However, they stated that this digestion is probably restricted to a small proportion of total wall chitin. The digestion of additional polysaccharides, like glycogen from fungal cytoplasm and starch from plant sap, also takes place in the pocket by enzymes produced in the labial glands (Febvay and Kermarrec 1986).

Febvay *et al.* (1984) pointed out that chitin digestion contradicts viability of the fungus inoculum carried by foundress queens. However, they also said that the first stages of chitin digestion occur on the inner surfaces of fungal walls; the outer sides are masked by non-chitinase-degradable pectic compounds. Living hyphae, containing cytoplasm, are therefore not attacked.

Chitin digestion in the infrabuccal pocket may be important for the inhibition of microflora ingested during substrate preparation or grooming. Febvay *et al.* (1984) said that microorganism growth was inhibited in regurgitated pellets and suggested that cell wall chitin digestion might be of secondary importance.

Observations made in Chapter 5 confirmed Quinlan and Cherretts' (1979) observation that around 30% of workers on the garden surface were licking it and also showed that licking workers, 98% of which are minima , ingest hyphae from the garden surface. Since solid material is trapped in the infrabuccal pocket, it was likely that these ingested hyphae were being incorporated into infrabuccal pellets. A brief examination of these pellets in nest refuse revealed that there are three clear types, possibly originating from different activities.

In this chapter, the origins and rates of production of these three different types of pellet are examined, along with the production of pellets by sexuals and callow workers. Attempts are also made to trace material through fungus gardens via infrabuccal pellets, using dye.

MATERIALS AND METHODS

Unless otherwise specified, fungus garden, refuse and workers came from an *Atta sexdens* nest (>80 gardens).

1. Definitions of the types of infrabuccal pellet

When refuse from nests of *Atta* and *Acromyrmex* is examined under a binocular microscope, three types of infrabuccal pellet are visible and were defined as follows:

Type 1 (T1) - black, opaque, variable size
Type 2 (T2) - amber, translucent, consistently small size
Type 3 (T3) - reddish, semi-translucent, variable size

Mounting these pellets in cotton-blue stain in lactophenol and examining under a microscope showed that T1 pellets were heterogeneous, containing plant fragments like xylem elements, amorphous brown material (possibly leaf wax), small amounts of amorphous blue-stained material, opaque black fragments of unknown origin and blue-stained fungal spores of species like *Penicillium* and *Aspergillus*. In contrast, T2 pellets were relatively homogeneous,

consisting of amorphous blue-staining material with hyphae and fungal spores sometimes visible. T3 pellets were intermediate between T1 and T2 pellets and contained mixtures of blue-staining material, amorphous brown material and dark opaque fragments of unknown origin.

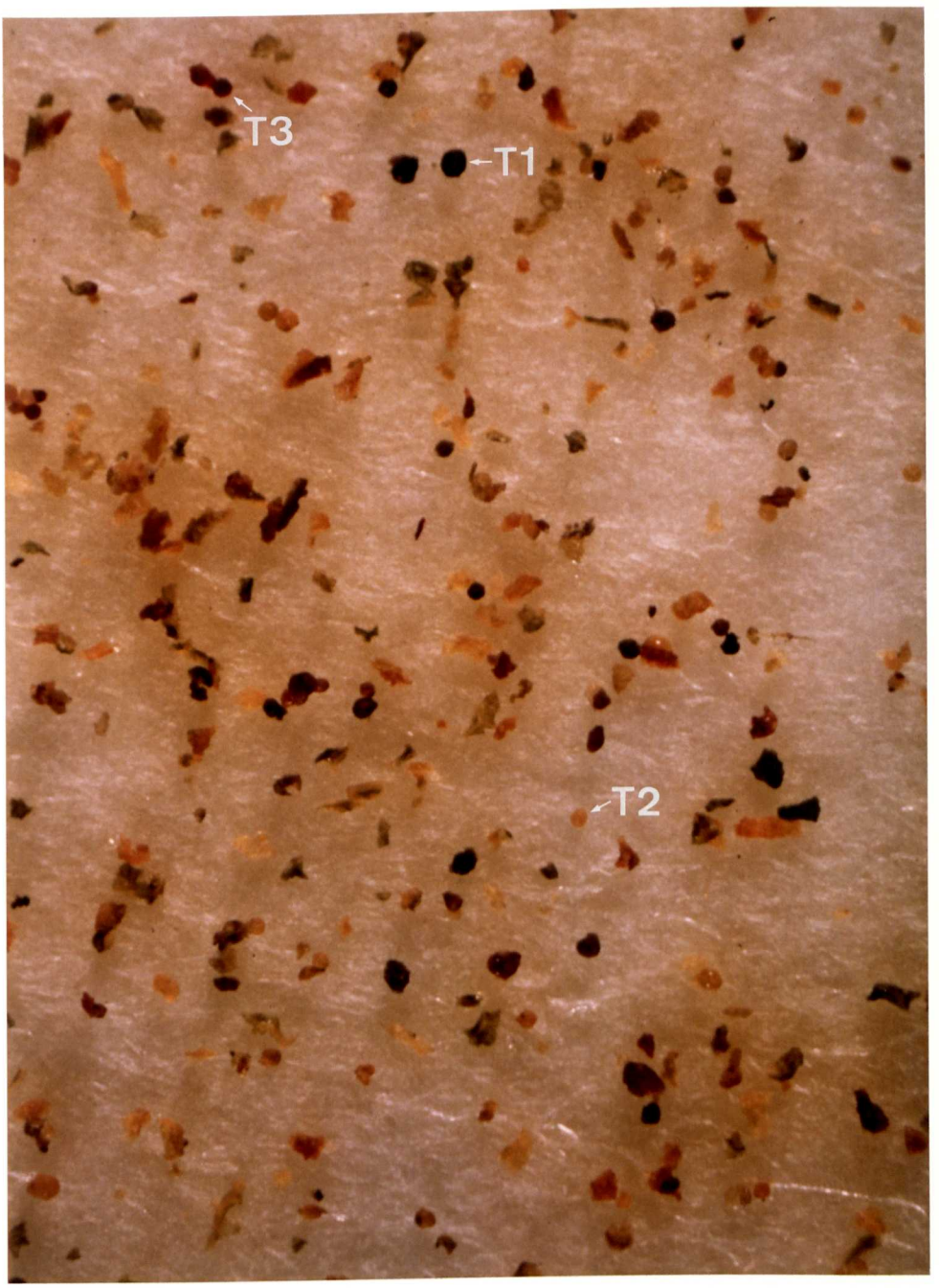
These definitions of pellet types are used throughout this chapter and Plate 2 shows a sample of dried, powdered refuse containing infrabuccal pellets, visible as small ovoids or spheres (refuse was gently ground using a pestle and mortar).

2. Examining infrabuccal pellets from nest refuse

Infrabuccal pellets in nest refuse were examined by sprinkling 0.1 g samples of dried refuse, gently ground with a pestle and mortar, on to damp filter paper. This paper rested on a rigid plastic sheet marked with a grid. When illuminated from beneath, this grid could be used to scan the whole sample with a binocular microscope, to examine all infrabuccal pellets present. The dampness of the paper held the infrabuccal pellets still, so that they could be picked up on the point of a mounted needle for further examination.

Pellet sizes were assessed by mounting pellets in cotton-blue in lactophenol and measuring them using an eyepiece graticule. Diameters were measured because most pellets were semi-spherical. Weights were measured by collecting 1,000 pellets from refuse and weighing them collectively.

Plate 2: Sample of dried, ground nest refuse containing three types of infrabuccal pellet (x14). T1 pellets are opaque, black and contain detritus, T2 pellets are translucent, amber coloured and contain material which is stained by cotton-blue stain in lactophenol. T3 pellets are reddish and are intermediate in nature.


T3
T1
T2

2. Examining infrabuccal pellet production by workers

a) Workers removed from sources of fungus

Groups of workers were placed into closed containers with damp filter paper for 24 hrs, after which the infrabuccal pellets produced could be examined.

b) Workers maintained on fungus garden

Workers were placed in 9 cm petri-dishes with damp filter paper and 5 cm^3 of fungus garden. After 48 hrs, they were transferred to fresh material in clean petri-dishes for a further 5 days. Pellet production was assessed after this 5 day period. All pellets produced consisted of material ingested during the period of confinement.

c) Workers receiving different fungal diets

Minima workers (headwidth <1.2 mm) were placed in a chamber with damp filter paper for 24 hrs, to allow them to egest infrabuccal pellets. These workers were easily handled using a mounted needle (which they clung to) and required less food than larger ones. Groups of ten were then placed in petri-dishes (5 cm) with damp filter paper and received different diets;

(1) Bundles of staphylae (approximately 50; Quinlan and Cherrett (1979) recorded intakes of 0.3 staphylae per hour for workers, but this was for mixed sizes).
(2) Tufts of hyphae (of similar total mass to staphylae supplied).
(3) 1 cm^3 of fungus garden bearing staphylae.
(4) Water only; controls.

Staphylae and hyphae used were removed from mature fungus garden with a mounted needle and placed on to petri-dish lids, which were then placed on to petri-dishes containing workers (by rapidly switching lids, to prevent workers from escaping). This garden had been kept ant-free in petri-dishes for 24 hrs (in a humid chamber in the ant culture room) to allow the build-up of fungal growth.

The worker groups received these different diets for 3 days. Then, numbers and types of infrabuccal pellet produced were assessed.

4. Examining infrabuccal pellet production by alates

Alates of *Acromyrmex octospinosus* were used initially because none of *Atta* were available. A declining nest of *Atta sexdens* (Chapter 11's 'Nest 1') then produced a small brood of males and these were used, along with a small mixed sexual brood produced by a healthy nest of *Atta cephalotes* (40 gardens).

Infrabuccal pellets were obtained by confining sexuals in containers with damp filter paper for 48 hrs. A 48 hr period was used to ensure that all pellets were egested and collected, since few insects were available for study.

5. Examining infrabuccal pellet production over 24 hrs from a single fungus garden

A single dome containing a fungus garden was placed on a table separated from the main nest, to which it was connected by means of a 'vertical bridge' (see Fig. 4.5, Chapter 4). Workers had to climb upwards to walk in either direction between the main nest and single garden and since they do not usually carry refuse loads upwards, this prevented refuse traffic across the bridge. However, a number of short (5 minute) observations were made to confirm that no refuse was exchanged across the bridge. Refuse from the single garden was discarded via a 'dumping string', as in Fig. 4.5.

Refuse was collected every hour for 24 hrs and numbers and types of pellets present were then counted for each sample. An estimate of worker population inside the garden was also made. Carbon dioxide gas was used to anaesthetize all workers within the dome and all ants present on approximately 10% of the garden volume were divided into

Weber's (1972) size classes and counted. The garden volume was first estimated by direct measurement, then by breaking it up and placing the pieces in a large measuring cylinder.

6. Observing the flow of materials through colonies using infrabuccal pellets

Quinlan and Cherrett (1978b) used the presence of carborundum powder or dye in infrabuccal pockets to show that the ants ingest material from fungus garden, their own bodies and from substrate leaves. They then dump the infrabuccal pellets with refuse. It was believed that extending their methods would provide information about the flow of materials through colonies, which could be important for testing insecticides.

Neutral Red dye powder (see Gurr 1960) was used because of availability, lack of toxicity and detectability. Twenty maxima workers were dusted with dye powder and confined in a 9 cm petri-dish for 24 hrs with damp filter paper. Many large infrabuccal pellets were produced, which could be easily handled using a mounted needle. Individual pellets were placed on to damp filter paper and dyed pellets developed red haloes around them.

To see if dyed pellets could be produced and detected in a nest, a small closed system was set up. A piece of fungus garden (10 cm^3) with attendant workers was placed in a 9 cm petri-dish with damp filter paper. Five dye-dusted maxima were introduced. After 24 hrs, infrabuccal pellets were collected and ten were examined for dye content using the above method.

Five pellets contained high concentrations of dye, three had lower concentrations and two contained no dye. Dye applied to large ants was therefore detectable in refuse, in a small enclosed system.

To test this method in a system approximating normal nest conditions, single domes containing fungus gardens were isolated on tables with collection trays for refuse. Since a dome has a maximum capacity of 2.5 litres, approximate garden volumes could be calculated. The worker population estimate described on page 127 could also be applied to estimate worker populations for each garden, once garden volume was known. Sets of three isolated gardens were then subjected to five treatments:

(1) The available soldier population was removed, anaesthetized with carbon dioxide gas, dusted with Neutral Red dye with a fine paint brush (on the back of the thorax) and returned to the garden. This available population was around 70 soldiers per garden.
(2) 100 large, foraging workers were removed, dusted with dye as above and returned. (Similar amounts of dye were used in both (1) and (2)).
(3) 10 g of sycamore leaves (*Acer pseudoplatanus*) were dusted with dye by shaking them in a closed container with 20 mg of dye powder and offered to gardens.
(4) 15 mg of dye were dusted on to the garden surface with a fine paint brush, avoiding workers.
(5) No treatment; control.

After 24 hrs, refuse discarded from each garden was collected, dried and weighed. Numbers of pellets per 0.05 g sample and numbers and sizes of dyed pellets produced were examined, by sprinkling ground samples on to damp filter paper and examining with a binocular microscope and eyepiece graticule.

RESULTS

1. Confirming that blue-staining material in infrabuccal pellets is fungal in origin

Cotton-blue stain shows the presence of chitin, which is present in both insect exoskeletons and fungal material. Insect chitin might appear in pellets through grooming of workers or brood, whereas fungal chitin would originate from the fungus garden, through consumption of staphylae and hyphal ingestion during licking. Chapter 5 showed that grooming and particularly garden licking are common acts on the garden surface.

The epicuticle of the insect exoskeleton (see Fig. 6.2) contains no chitin and provides a waxy waterproof layer, consisting of long chain hydrocarbons and esters of fatty acids and alcohols. However, chitin makes up 25-50% of the dry weight of exo and endocuticle, the remainder being protein, with which it is always associated as a water-soluble heterogeneous glycoprotein, 'arthropodin' (Chapman 1969). It is therefore unlikely that chitin is removed from the exoskeleton during grooming. However, two tests were carried out to check this:

a) Production of T2 pellets by workers confined without fungus garden

Fifty minima and media workers were isolated in a closed container for 24 hrs with damp filter paper, then were transferred to a fresh container, where they proceeded to lick themselves and the container walls. After a further 24 hrs, the pellets regurgitated during both periods were examined. If blue-staining material was fungal in origin, none should be produced during the second period when no fungus garden was available.

During the first period, the workers produced 18 T1, 18 T2 and 11 T3 pellets. However, during the second period they produced 28 heterogeneous T1 pellets, containing dust fragments, filter paper fibres and fragments of Fluon, but no T2 or T3 pellets were produced at all.

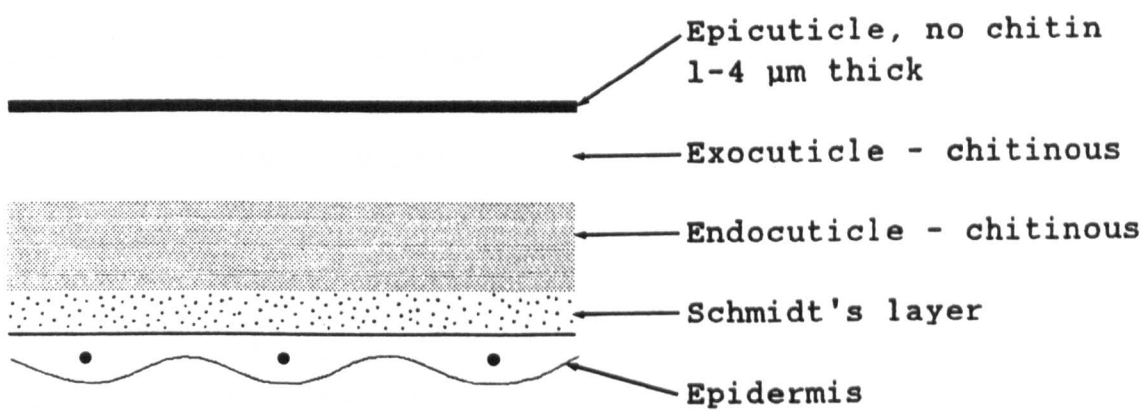


Figure 6.2: The structure of insect exoskeleton (from Chapman 1969).

b) Production of T2 pellets by non-Attines

If blue-staining material is fungal in nature, then non-Attines (non-fungus-culturing ants) should produce few blue-staining pellets, since they are not exposed to concentrated sources of fungus like fungus garden. Infrabuccal pellets produced by 50 *Myrmica ruginodis* workers (see Chapter 2, page 13) confined for 24 hrs were collected, mounted and examined microscopically.

They produced 58 T1 pellets containing soil fragments, unidentifiable debris and fungal spores but no blue-staining T2 pellets were produced.

T2 and T3 pellets were therefore produced only by workers which had been in contact with a source of fungus. Consequently, the blue-staining material was fungal in origin.

2. <u>The frequency of pellets in refuse</u>

The numbers of pellets present in 0.1 g samples of refuse from all the laboratory nests were examined. Large numbers of pellets were present in refuse (Table 6.1). Total numbers of the pellet types produced were compared using a 3 x 6 Heterogeneity Chisquare analysis on the numbers of pellets of each type versus ant species and different pellet types occurred at significantly different rates ($p<0.001$). These relative rates did not vary between nests; the ratio of Types 1, 2 and 3 remained constant, at approximately 5:4:1.

Table 6.1: Numbers of infrabuccal pellets of the three types, found in 0.1 g samples of dried refuse from six laboratory nests of four different species.

SPECIES	NUMBERS AND TYPES OF PELLETS PER 0.1g OF REFUSE			
	TYPE 1	TYPE 2	TYPE 3	TOTAL
Acromyrmex octospinosus	410	284	98	792
Atta cephalotes	654	493	132	1279
Atta laevigata	736	496	208	1440
Atta sexdens (80 gardens)	525	465	118	1108
Atta sexdens (22 gardens)	960	700	264	1924
Atta sexdens (7 gardens)	930	760	218	1908

3. The dimensions of pellets found in refuse

One hundred pellets of each type were measured for *Atta sexdens* and *Acromyrmex octospinosus*. Previous work (Quinlan and Cherrett 1978b, Febvay and Kermarrec 1981b) has been carried out only on the latter species.

Infrabuccal pellet sizes were not normally distributed, being biased towards smaller sizes. Normal distributions were obtained by transforming data to the $\log_{10}$ values, for statistical analysis. In *Atta sexdens*, T1 pellets had a wide size distribution, with a peak at 0.18 mm (Fig. 6.3). T3 pellets also had a wide distribution, but T2 pellets had a small size range, peaking below 0.16 mm. T1, T2 and T3 pellets were all significantly larger in *Acromyrmex* than in *Atta* ($p<0.001$, ANOVA) but showed similar distribution patterns (Fig. 6.4).

Comparing the sizes of different types of infrabuccal pellet for *Atta sexdens*, showed significant differences

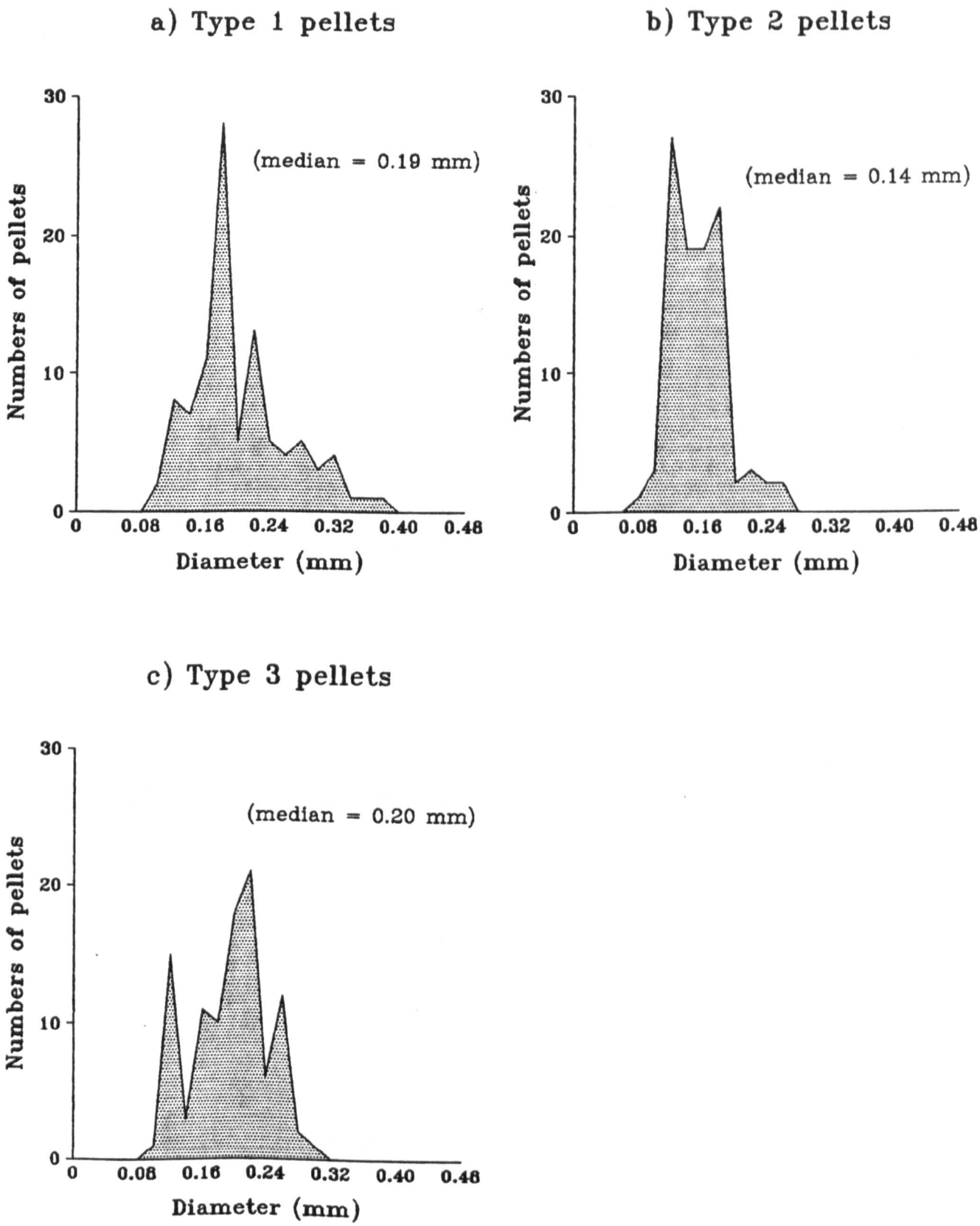


Figure 6.3: Size distributions (diameters, mm) of three types of infrabuccal pellet produced by *Atta sexdens* workers.

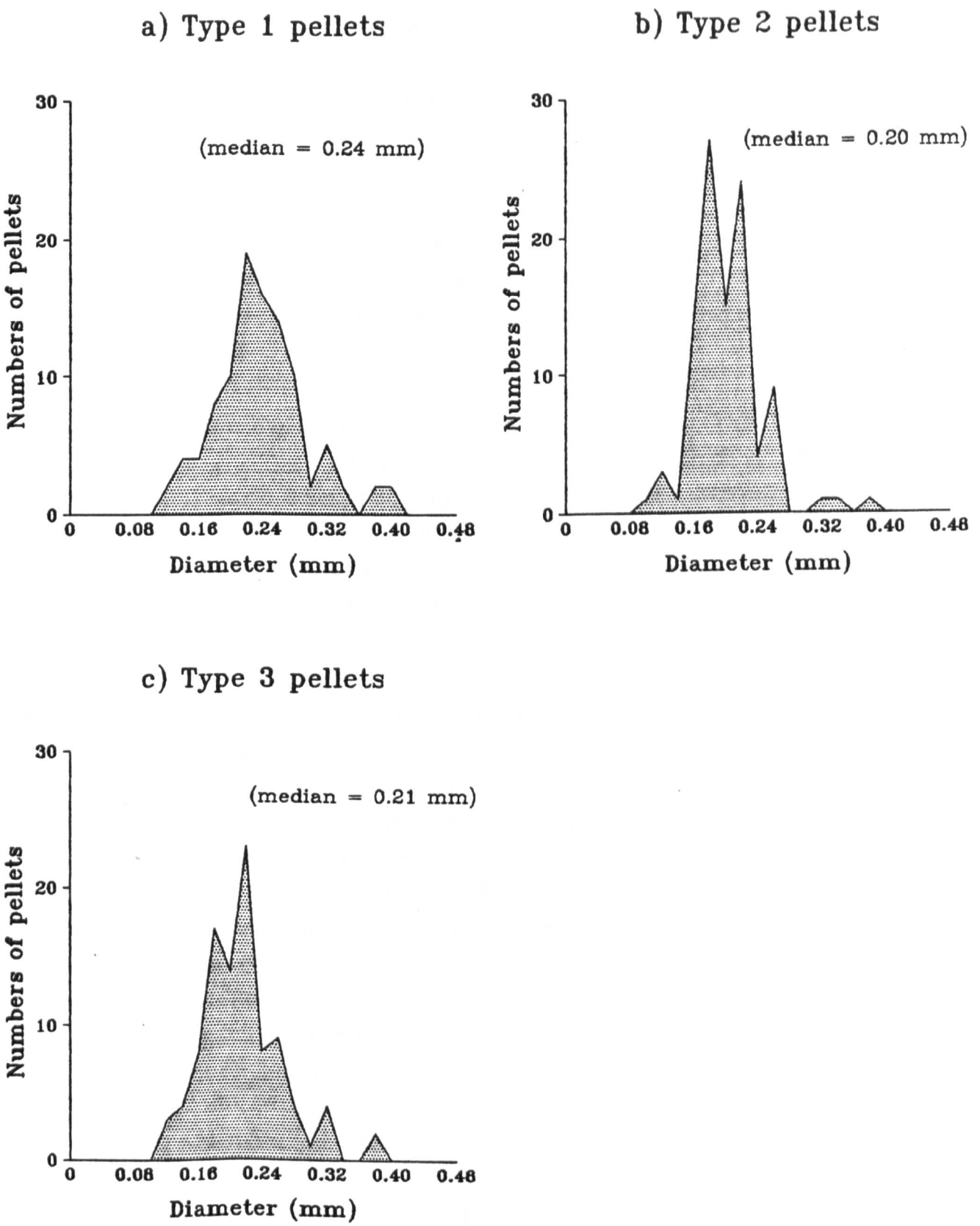


Figure 6.4: Size distributions (diameters, mm) of three types of infrabuccal pellet produced by *Acromyrmex octospinosus* workers.

($p<0.001$, ANOVA). Tukey's multiple comparison showed that both T1 and T3 pellets were significantly larger than T2 pellets ($p<0.05$), but not different from each other ($p>0.05$). Similarly, pellets of different types were also significantly different in size for *Acromyrmex octospinosus* ($p<0.001$, ANOVA), following a similar pattern, although T3 pellets were significantly larger than T1 pellets ($p<0.05$, Tukey).

The mean weights of T1, T2 and T3 pellets were 3.4 µg, 1.5 µg and 3.5 µg, respectively, for *Atta sexdens*. T1 and T3 pellets were therefore similar in weight, but both were heavier than T2 pellets (on average). However, since pellet sizes were not normally distributed, these values for weights may be slightly inaccurate.

4. Infrabuccal pellet production by different worker castes

a) Workers removed from fungus garden and foraging trails

The pellet productions of workers originating from fungus garden or from forage trails and confined without a source of fungus, were compared. Seven groups of 50 workers of Weber's (1972) four arbitrary size classes were examined. These included two groups each of minima , media and maxima and a single group of soldiers. One group of each of the first three castes was removed from fungus garden and the second from forage trails. Soldiers were obtained from both areas.

Only minima workers produced T2 or T3 pellets in large numbers, whatever their origin (Table 6.2). There were no differences in the types of pellet produced by workers removed from garden or forage trails.

Table 6.2: Numbers and types of pellets produced by different worker castes (groups of 50) taken from fungus garden or forage trails and confined in containers for 24 hrs.

WORKER CASTE	ORIGIN	NO'S OF PELLETS PRODUCED			
		TYPE 1	TYPE 2	TYPE 3	TOTAL
Minima	Garden	21	19	11	51
Minima	Trails	33	15	2	50
Media	Garden	60	0	0	60
Media	Trails	55	1	0	56
Maxima	Garden	56	0	0	56
Maxima	Trails	76	0	0	76
Soldier	Both	52	0	0	52

b) Workers maintained on fungus garden

Groups of ten workers were used, each consisting of minima , media or maxima . One group of each caste was given young, staphylae-free garden, while a second received mature staphylae-bearing garden, to see if there were any differences between pellets produced by workers on different areas of the garden. Five replicates of each group were used.

Total numbers of pellets produced by workers of three castes on young and mature garden were compared using a two-way analysis of variance. There were no significant differences between numbers of pellets produced on young and mature garden ($p>0.05$), but different castes produced significantly different numbers of pellets ($p<0.005$, see Table 6.3). There was no significant interaction between garden age and worker caste ($p>0.05$).

Numbers of the three different types of pellet produced by each caste were also compared using this method. There were significant differences between the numbers of pellets produced by each caste, between the numbers of

different types of pellet and there was also a significant interaction between caste and pellet types ($p<0.001$; see Table 6.4).

Minima produced the largest numbers of pellets (Table 6.5). T1 pellets were the most common type for all three castes, while only minima produced large numbers of T2 pellets. All three castes produced relatively small numbers of T3 pellets.

Table 6.3: Two-way analysis of variance table comparing the effects of worker caste and age of fungus garden provided, on the numbers of infrabuccal pellets subsequently produced.

SOURCE OF EFFECT	DF	SS	MS	F	P
Worker caste	1	241	241	<1	>0.05
Garden age	2	5271	2635	7.75	<0.005
Interaction	2	712	356	1.05	>0.05
Error	24	8167	340		
Total	29	14390			

Table 6.4: Two-way analysis of variance table comparing worker castes and the numbers of the three types of infrabuccal pellet they produced.

SOURCE OF EFFECT	DF	SS	MS	F	P
Worker caste	2	1756.9	878.4	12.4	<0.001
Type of pellet	2	16497.9	8248.9	116.2	<0.001
Interaction	4	5796.5	1449.1	20.4	<0.001
Error	81	5754.9	71.0		
Total	89	29806.1			

Table 6.5: Mean numbers (± SE) of pellets of the three types produced per worker per day by groups of ten minima, media or maxima workers maintained on fungus garden for 5 days (10 replicates).

WORKER CASTE	MEAN NO'S OF PELLETS OF THREE TYPES (± SE) PRODUCED PER WORKER PER 24 HOURS			
	TYPE 1	TYPE 2	TYPE 3	TOTAL
Minima	0.67±0.06	0.63±0.07	0.27±0.03	1.57±0.14
Media	0.71±0.06	0.05±0.02	0.17±0.04	0.93±0.07
Maxima	0.99±0.10	0.02±0.01	0.18±0.03	1.18±0.13

c) Infrabuccal pellet production by workers receiving different diets

Five replicate groups of ten workers were used for each of the four fungal diets. In dishes containing only damp filter paper, most workers had died by the end of the 3 day period. Surviving workers produced means of 2.4 (SE ± 0.4) T1, no T2 and 0.6 (SE ± 0.4) T3 pellets. Mortalities in other treatments were less than one or two workers per dish.

Numbers of the three types of pellet produced were compared between the three groups fed on whole garden, hyphae or staphylae using a two-way analysis of variance. There were significant differences between diets and between numbers of the three types of pellet produced ($p<0.001$; see Table 6.6). There was also a significant interaction between diet and numbers of pellets produced ($p<0.025$). Mean numbers of pellets of the three types produced by groups receiving different diets are shown in Table 6.7.

When numbers of T1 pellets produced in these three treatments were compared with a one-way analysis of variance, no significant differences were found ($p>0.5$).

Similarly, there were no significant differences between numbers of T3 pellets produced by different treatments ($p>0.1$). However, there were significant differences between the numbers of T2 pellets produced ($p<0.05$, ANOVA and Tukey), more being produced by workers fed on hyphae than on staphylae while numbers produced by workers receiving whole garden were intermediate and not significantly different to those produced in the other two treatments ($p>0.05$, ANOVA and Tukey).

Table 6.6: Two-way analysis of variance table comparing different diets and the numbers of the three types of infrabuccal pellet subsequently produced by groups of workers.

SOURCE OF EFFECT	DF	SS	MS	F	P
Type of diet	2	356.8	178.4	9.2	<0.001
Type of pellet	2	616.7	308.4	16.0	<0.001
Interaction	4	258.5	64.6	3.3	<0.025
Error	36	695.2	19.3		
Total	44	1927.2			

Table 6.7: Mean numbers (± SE) of infrabuccal pellets produced per group of ten workers receiving three different diets during 3 days (5 replicates).

DIET	MEAN NO'S (± SE) OF PELLETS PRODUCED PER GROUP OF TEN WORKERS			
	TYPE 1	TYPE 2	TYPE 3	TOTAL
Whole garden	3.6 ± 0.4	11.8 ± 1.3	7.0 ± 0.5	22.4 ± 1.6
Hyphal tufts	4.6 ± 0.8	21.0 ± 4.9	11.4 ± 2.4	37.0 ± 6.0
Staphylae	3.8 ± 0.9	6.4 ± 0.8	6.8 ± 1.3	17.0 ± 2.1

d) Infrabuccal pellet production by callow workers

Immature callow workers have not usually left the fungus garden, so should produce only fungus-containing pellets. To investigate this, 20 callow workers (mostly minima and media) were placed in a 9 cm petri-dish with damp filter paper for 24 hrs. Pellets produced were mounted and examined microscopically.

No T1 pellets were produced, but 8 T2 and 9 T3 pellets were found. Callow workers therefore produced only pellets containing large amounts of fungus.

e) The relationship between worker headwidth and infrabuccal pellet size

Approximately 400 individual workers of varying size were maintained on 1 cm^3 of fungus garden, in 5 cm petri-dishes. After 24 hrs, pellets produced were collected and measured. Corresponding worker headwidths were measured using a binocular microscope and eyepiece graticule.

Regressing worker headwidths against sizes of infrabuccal pellets produced showed a significant relationship, with the equation:

Pellet size (mm) = 0.0436 + 0.115 Headwidth (mm)

($p<0.001$, $Rsq_{(adj)}$ 86.0%, df 406, see Fig. 6.5)

If the maximum headwidth for minima workers is 1.2 mm, then the maximum pellet size produced will be 0.18 mm, from this equation. Since workers tend to produce pellets of constant size, then all pellets of less than 0.18 mm diameter can be considered to have been produced by minima , whilst those above this range are produced by larger castes. A media worker with a headwidth of 1.4 mm will produce pellets 0.20 mm in diameter and a maxima worker of headwidth 2.2 mm, will produce pellets of 0.30 mm diameter. These ranges can be used to see what proportions of the different pellet types are produced by different

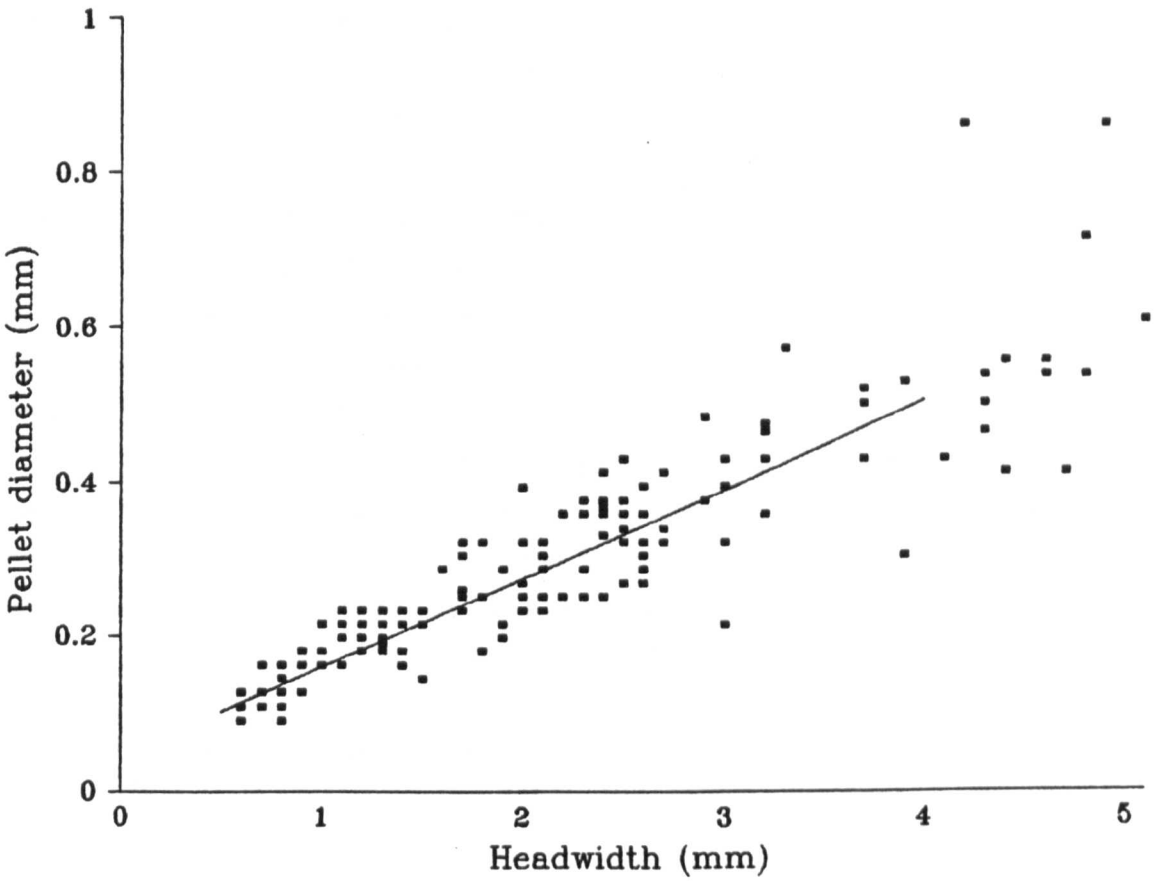


Figure 6.5: Increases in infrabuccal pellet sizes (mm) produced by workers of increasing headwidth (mm), shown with the regression line ($p<0.001$, $Rsq_{(adj)}$ 86.0%, df 406).

castes. Referring back to Fig. 6.2 and applying the regression equation shows that peak T1 pellet production was by workers of headwidth 1.2 mm (minima) and a smaller peak was produced by those of headwidth 1.4 mm (media). However, the wide size range was produced by workers with headwidths ranging from 0.3-3.1 mm. Pellets smaller than 0.1 mm were not produced by workers of headwidth 0.3 mm, since these do not exist. Some workers therefore produce under-sized pellets.

T2 pellets were mostly produced by workers between headwidths 0.8 and 1.2 mm (minima) with a few being produced by workers of headwidths 1.4-2.1 mm. However, T3 pellets were produced by a peak headwidth of 1.5 mm (large media) and by smaller peak headwidths of 0.7 and 0.9 mm.

Referring back to the raw data for Fig. 6.2 showed that 41% of T1, 83% of T2 and 33% of T3 pellets were produced by minimas.

5. Infrabuccal pellet production by alates

a) Alates of *Acromyrmex octospinosus*

Forty winged females and twenty males were collected from fungus garden and placed into containers with damp filter paper. After 24 hrs, the pellets produced were examined. In addition, two groups of ten winged females were maintained on fungus garden. One group received young, staphylae-free garden, while the other received mature garden. After 48 hrs, when all the ants had regurgitated infrabuccal pellets, they were transferred to clean dishes containing fresh fungus garden. Pellet production was then assessed after a further 5 days. This was repeated for a group of ten males but not enough insects were available for further replication.

Alate females and males produced all three kinds of pellet, the majority being T3 while T2 pellets were rarely produced. Almost identical numbers of pellets were produced by females and males (Table 6.8).

Table 6.8: Numbers and types of infrabuccal pellets produced by 40 alate females and 20 males confined in containers with damp filter paper for 24 hrs.

	NUMBERS OF PELLETS PRODUCED:			
	TYPE 1	TYPE 2	TYPE 3	TOTAL
FEMALES	10	5	15	30
MALES	11	3	15	29

Females produced pellets with a mean diameter of 0.38 (SE ± 0.01) mm, compared to 0.28 (SE ± 0.02) mm for males and this difference was significant ($p<0.001$, ANOVA), a reflection of greater female head size. However, the figure for female pellet size was somewhat higher than that obtained by Quinlan and Cherrett (1978b), which was 0.339 (SE ± 0.016) mm. Females also made attempts to culture gardens by defaecating on regurgitated pellets and placing torn pieces of filter paper on to them, whereas males showed no such behaviour.

Alate females confined with young or mature fungus garden, each produced several pellets during the 5 day period and most of these were either T2 or T3, which were produced on both staphylae-free young garden and on mature garden (Table 6.9). There were no differences in numbers of pellets produced by females on young or mature garden, suggesting that the presence or absence of staphylae did not affect pellet production.

Table 6.9: Numbers of pellets of the three types produced by groups of ten alate females confined on either young (staphylae-free) or mature garden for 5 days.

AGE OF GARDEN SUPPLIED	NUMBERS OF PELLETS PRODUCED:			
	TYPE 1	TYPE 2	TYPE 3	TOTAL
Young	4	18	12	34
Mature	4	12	16	32

A group of ten males confined on mature garden for 5 days, produced 33 pellets (3 T1, 16 T2 and 14 T3 pellets) and this was similar to the numbers produced by females (Table 6.9). Both males and females therefore seemed to produce mostly fungus-containing pellets.

Females and males differed in their treatment of regurgitated pellets. Females placed pellets on or near the fungus garden, whereas those produced by males were scattered at random.

b) Alates of *Atta*

Twenty *Atta sexdens* males produced 23 pellets (10 T1, 0 T2 and 13 T3) when confined for 48 hrs without fungus garden. These had a mean diameter of 0.45 (SE ± 0.02) mm. Ten *Atta cephalotes* males produced 10 pellets (4 T1, 2 T2 and 4 T3) with a mean diameter of 0.39 (SE ± 0.02) mm, while 13 females produced 12 pellets (2 T1, 7 T2 and 3 T3) with a mean diameter of 0.53 (SE ± 0.07) mm. Females therefore appeared to produce more pellets containing fungus than males but these figures were too low to draw any definite conclusions.

6. Infrabuccal pellet production over 24 hrs from a single fungus garden

A mean of 0.084 (SE ± 0.02) g of refuse (dry weight) was produced per hour from the isolated garden during the first 24 hr collection period. Refuse was also collected over a further two successive 24 hr periods, to confirm the initial result. Five samples, each of 0.05 g, were removed from these two collections and assessed for pellet content as before. The total numbers of pellets produced per hour were not significantly different between the three 24 hr collection periods ($p>0.1$, ANOVA) and the ratio between numbers of types of pellet produced reflected that shown on page 131.

The garden had an approximate volume of 1,400 cm^3 (± 50 cm^3) and 10% of this contained 514 minima , 116 media , 46 maxima and 8 soldiers (6,840 workers in 1,400 cm^3). Pellet numbers produced by each worker in 24 hrs could therefore be calculated (Table 6.10).

Table 6.10: Mean numbers (± SE) of infrabuccal pellets of three types produced per hour from a single garden (34 replicates), during three 24 hr collection periods, shown with calculated mean pellet productions per worker over 24 hrs, assuming a population of 6,840.

PELLET TYPE	MEAN NO'S (± SE) OF PELLETS PRODUCED PER HOUR FROM GARDEN	MEAN NO'S OF PELLETS PRODUCED PER WORKER PER 24 HRS
1	217 ± 5.8	0.76
2	141 ± 5.7	0.50
3	51 ± 1.7	0.18
TOTAL	409 ± 12.3	1.44

Earlier calculations (page 139) showed that 41% of T1, 83% of T2 and 33% of T3 pellets in refuse samples were produced by minima . Applying this to the mean numbers of pellets produced per garden (Table 6.10) showed the differences in relative production by minima and non-minima (Table 6.11). Non-minima produced more T1, fewer T2 and more T3 pellets than minima .

Comparing these results with those shown in Table 6.5 showed that minima produced fewer of each type of pellet when in a fungus garden than when confined with fragments of garden in petri-dishes. Non-minima produced more pellets in the garden than when in petri-dishes (comparing with pooled media and maxima pellet productions in Table 6.5).

Table 6.11: Estimated mean values for infrabuccal pellet production (three types) by minima and non-minima workers per 24 hrs, obtained by applying the calculations made on page 139 to the data presented in Table 6.10.

WORKER CASTE	MEAN NO'S OF PELLETS OF EACH TYPE PRODUCED PER WORKER PER 24 HOURS			
	TYPE 1	TYPE 2	TYPE 3	TOTAL
Minima	0.42	0.55	0.08	1.04
Non-minima	1.81	0.34	0.48	2.63

Numbers of each type of pellet produced per hour during the first 24 hr period were compared between foraging and non-foraging periods (foraging occurred between 1000 and 2100 hrs). A two-way analysis of variance showed that there were significant differences between numbers of different types of pellet produced and that numbers varied between foraging and non-foraging periods ($p<0.001$; see Table 6.12). All three types of pellet were

produced in lower numbers when forage was available (Table 6.13).

Table 6.12: Twoway analysis of variance table comparing the numbers of pellets of three types produced during foraging and non-foraging periods, from a single fungus garden over 24 hrs.

SOURCE OF EFFECT	DF	SS	MS	F	P
Period	1	29161	29161	67.8	<0.001
Type of pellet	2	336671	168335	391.5	<0.001
Interaction	2	7036	3518	8.2	<0.001
Error	66	28376	430		
Total	71	401244			

Table 6.13: Mean rates (± SE) of production per hour of three types of infrabuccal pellet, by workers in 1,400 cm^3 garden when forage was either present or absent (12 replicates).

FORAGE STATUS	NO'S OF PELLETS OF EACH TYPE PRODUCED PER HOUR BY WORKERS IN 1400 cm^3 OF GARDEN			
	TYPE 1	TYPE 2	TYPE 3	TOTAL
Present	196.4±9.8	118.6±7.0	48.0±2.3	363.0±15.5
Absent	246.5±7.2	176.6±3.2	60.7±1.5	483.8±8.5

7. Observing the flow of materials through colonies using infrabuccal pellets

The mean worker population of the gardens used was 6,150 (SE ± 300), calculated from estimated garden volumes.

ANOVA and Tukey's multiple comparison showed that the total numbers of pellets produced by workers in gardens receiving forage dusted with dye were significantly greater than those produced in untreated control gardens ($p<0.05$), but no other significant differences were recorded between workers receiving the other dye treatments, compared with control gardens ($p>0.05$, see Table 6.14).

The percentages of dye-containing pellets produced by workers with access to different dye sources differed significantly ($p<0.001$, ANOVA), dye-dusted leaf sources causing the greatest percentage of dyed pellets, followed by dye-dusted garden (these were not significantly different from one another; $p>0.05$, Tukey's multiple comparison). Tukey's multiple comparison showed that dye-dusted soldiers caused the production of significantly lower percentages of dyed pellets than these two treatments ($p<0.05$), while dye-dusted foragers led to the production of intermediate percentages ($p>0.05$, see Table 6.14).

Combining the above results showed that there were significant differences in the numbers of dyed pellets produced by workers exposed to different sources of dye ($p<0.01$, ANOVA), with those exposed to dye-dusted leaves producing significantly higher numbers than other treatments ($p<0.05$, ANOVA and Tukey, see Table 6.14). Workers in control gardens receiving no dye produced 1.9 pellets each per 24 hrs, which is larger than the figure shown in Table 6.10 (1.44 pellets per worker per 24 hrs). Calculating pellet productions from estimates of population and numbers of pellets present in refuse samples may therefore give variable results.

Table 6.14: Mean numbers (± SE) of infrabuccal pellets and dyed pellets produced by workers over 24 hrs in gardens receiving dye-dusted soldiers, foragers, leaves or dye placed directly on to garden, compared to controls which received no dye (3 replicates).

METHOD USED TO INTRODUCE DYE TO WORKERS	MEAN NO'S OF PELLETS PRODUCED PER WORKER	MEAN % OF PELLETS CONTAINING DYE	MEAN NO'S OF DYED PELLETS PER WORKER
Soldiers	3.0 ± 0.5	26.1 ± 1.9	0.8 ± 0.1
Foragers	2.6 ± 0.1	30.3 ± 0.1	0.8 ± 0.01
Leaves	4.0 ± 0.4	41.5 ± 1.6	1.7 ± 0.2
Garden	3.1 ± 0.4	35.4 ± 1.9	1.1 ± 0.1
Control	1.9 ± 0.2	NONE	NONE

The ratios of different types of pellet produced were also examined for each treatment, since pellets could be differentiated by their different translucences. Examining samples of 200 pellets (the first 200 dyed or undyed pellets observed in refuse samples) showed that the ratio of production had changed from 5:4:1 (see page 131) to 5:3:2 for Types 1, 2 and 3 respectively. There had therefore been a change in favour of T3 pellets. A sample of 258 dyed pellets was also examined to find out how many were of each of the three types. Most were T1 (72.2%) or T3 (25.9%) but few were T2 pellets (1.9%).

Measuring dyed pellets produced by each of the treatments and plotting the size distributions (Fig. 6.6) showed that they were all similar. However, large numbers of pellets were recorded which were smaller than 0.08 mm in diameter. Applying the regression equation (page 138) shows that these were produced by workers with headwidths of less than 0.3 mm, which do not exist. These pellets were therefore due to errors in measurement or the regression

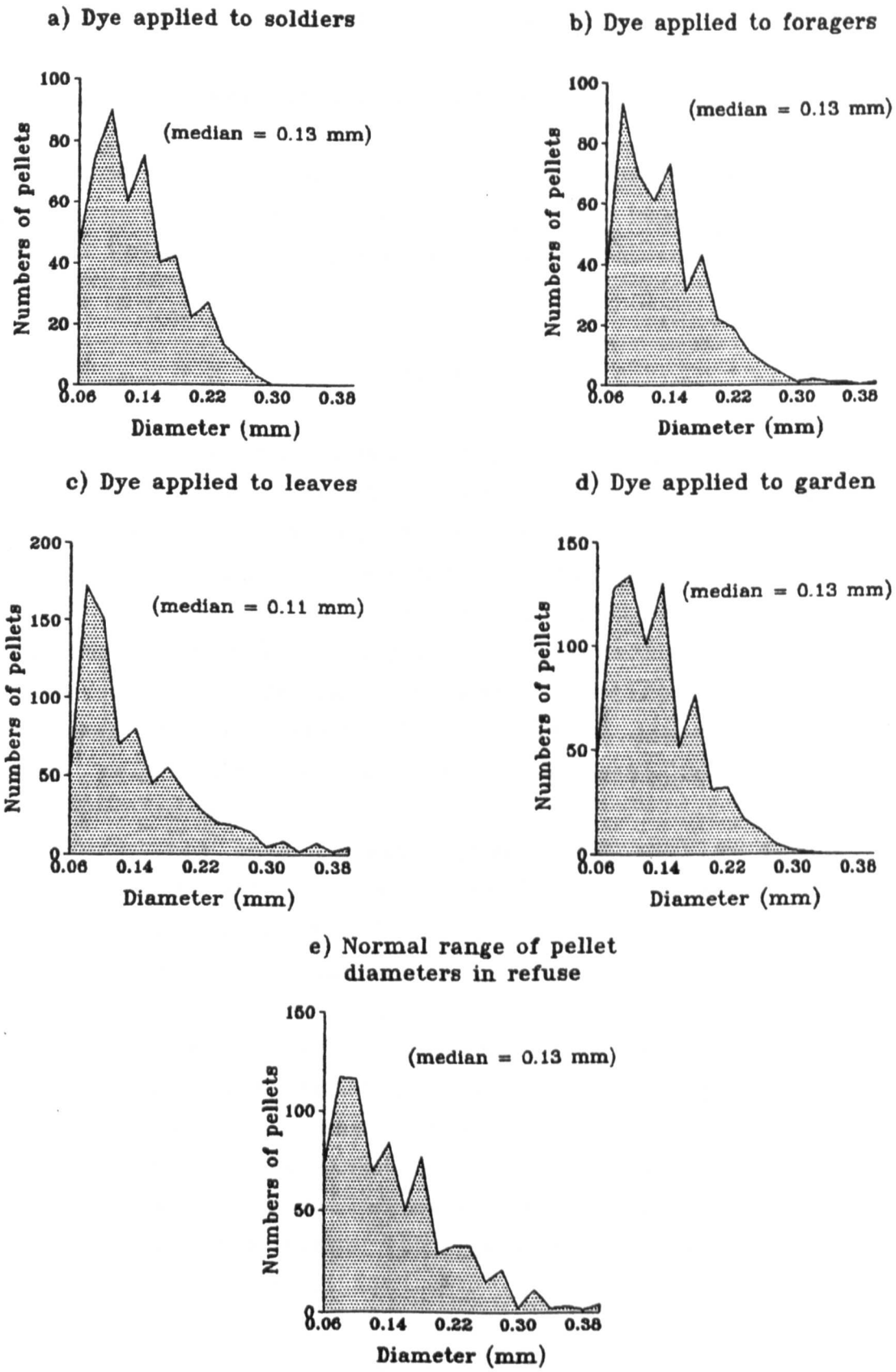


Figure 6.6: Size distributions (diameters, mm) of dye-containing infrabuccal pellets, from five samples of refuse (each of 0.05 g) produced by workers in gardens receiving dye via four treatments (a-d), compared with the distributions of pellet sizes in refuse from a garden receiving no dye (e).

equation, or workers producing smaller than normal pellets.

The largest numbers of pellets were produced by workers in the minima size range, with smaller numbers produced by media and occasional maxima .

DISCUSSION

1. The production of infrabuccal pellets by workers

Previous authors (Bailey 1920, Weber 1972, Quinlan and Cherrett 1978b, Febvay and Kermarrec 1981b) reported the heterogeneous nature of infrabuccal pellets produced by workers, but failed to note the production of discrete types, which are best observed in dried nest refuse. Pellets are found in large numbers, ranging from 8,000-19,000 per gram of refuse. These figures depend upon the rate of refuse dumping; greater rates mean lower pellet numbers per gram.

Each worker in a fungus garden produced 1.44 pellets per 24 hrs and this was similar to Febvay and Kermarrecs' (1981b) figure of 13.4 pellets per group of ten workers (*Acromyrmex octospinosus*).

The size ranges of pellets measured were all biased towards smaller sizes and pellet size was related to worker headwidth. However, caste ratios in a colony were also biased towards smaller sizes. Weber (1972) said that around 60% of workers in a garden were minima and the present population estimate (page 142) showed that up to 75% of workers present might be minima .

Heterogeneous T1 pellets, the most common type, are the result of grooming and substrate preparation, containing general detritus, plant debris and fungal spores. Those measured had a wide size range and were therefore produced by all worker castes. Some contained small amounts of blue-staining material, probably the result of ingesting either fungi growing on collected leaves

or staphylae from the fungus garden. Workers consume only staphyla juices (Quinlan and Cherrett 1979) but it is likely that some fungal wall fragments enter the infrabuccal pocket.

T1 pellets reflect the amount and type of material entering the nest. Introducing forage dusted with dye therefore led to more pellets being produced, 72.2% of which were T1 pellets, compared to less than 2% T2 pellets. Introducing dye dust to worker populations also showed that material can be traced through the colony by examining infrabuccal pellet production. Any insecticide present in the infrabuccal pocket would probably kill the ant and an assay where 30% of resulting pellets contained dye suggests that if insecticide had been used, 30% of ants would have been killed. However, many *Atta* species dump nest refuse in underground chambers (Weber 1972), therefore such an experiment could not be carried out in the field.

More pellets were produced when dye was introduced and many small pellets were also produced. It is possible that workers found the dye irritating and regurgitated pellets earlier than usual.

T2 pellets were produced mainly by minima and only by workers which had been in recent contact with fungus garden. Workers of *Myrmica ruginodis* produced no such pellets. Since T2 pellets consisted almost entirely of fungus, with some fungal spores and were produced by workers on garden with or without staphylae, it can be concluded that they were produced by workers tending fungus garden. Workers confined in petri-dishes and fed on staphylae also produce T2 pellets but this is probably because they ingest the whole staphyla through pure hunger in this artificial situation. In the fungus garden, a worker sucks juices from a staphyla then feeds the macerated remains to a larva.

T3 pellets contained fungus, but mixed with plant debris and other material. They were produced by a wide range of worker sizes, on garden with or without staphylae,

which suggests that at least some of them resulted from the ingestion of hyphae during licking. However, some may have been due to the ingestion of staphylae, particularly by larger workers which seldom lick garden (see Chapter 5). These pellets may also have been produced by workers 'switching' tasks. For example, a licking worker might respond to an influx of forage by 'switching' to substrate preparation, or lack of forage might cause the reverse. The decrease in pellet production when forage was available indicated that this probably occurs.

Because numbers and weights of T2 and T3 pellets produced were estimated, the total amount of fungal material represented in these pellets could be calculated, assuming that T3 pellets were 50% fungus (hyphae). If T2 pellets and T3 pellets have approximate weights of 1.5 and 3.5 µg respectively, with worker production rates of 0.50 T2 and 0.18 T3 pellets per 24 hrs (Table 6.10), then:

0.50 T2 pellets per 24 hrs x 1.5 µg = 0.8 µg of fungus
0.17 T3 pellets per 24 hrs x ½(3.5 µg) = 0.3 µg of " "

This gives a total of 1.1 µg of fungal material per worker per 24 hrs. However, this figure is difficult to interpret. Theoretically, 1.1 µg is a measure of the dry weight of fungus ingested in 24 hrs.

Repeating the calculations for pellet productions by workers fed on different diets (Table 6.7) showed that workers fed on whole garden produced 1.0 µg of fungal material per worker per 24 hrs in T2 and T3 pellets; a close figure to that obtained above. In contrast, workers fed on hyphae produced 1.8 µg and those fed on staphylae produced only 0.7 µg of fungal material per worker per 24 hrs. Where staphylae were available, workers therefore produced fewer fungus-containing pellets. Workers given only hyphae may have ingested more in an attempt to obtain nutrients, since staphylae are juicier than hyphae and workers can only swallow liquids.

These calculations can be taken further. If ten workers received 150 staphylae over 3 days and the mean dry weight of a staphyla is 2.07 µg (Quinlan and Cherrett 1979), then they received 310.5 µg of fungal material. Workers regurgitated means of 6.4 T2 and 6.8 T3 pellets per group over 3 days (Table 6.7) and calculating from these figures as before, gives a mean of 21.5 µg of fungal material regurgitated per group per 3 days, or per 310.5 µg of received staphylae. Approximately 7% of the staphylae received were therefore found later in regurgitated pellets.

Quinlan and Cherrett (1979) found that workers consumed a mean of 0.3 staphylae per hr, or 7.2 per 24 hrs and 7% of the dry weight of these staphylae would be 1.0 µg per day, regurgitated as infrabuccal pellets. This suggests that almost all the fungus regurgitated in infrabuccal pellets originates from staphylae. However, T2 fungal pellets were produced mainly by smaller workers, which indicates that they are not solely the result of consuming staphylae, since all worker castes do this. In addition, keeping workers in petri-dishes and feeding them solely on staphylae is a very artificial situation. In the nest, workers rely mainly on leaf juices for their energy requirements (Quinlan and Cherrett 1979) and usually consume only staphyla juices, the macerated remains of staphylae being fed to larvae. Confined workers may consume a larger amount of staphyla material than normal, leading to unnaturally high levels of fungal pellet production.

It is therefore likely that although much of the fungal material in T2 and T3 pellets originates from staphylae, a large proportion also originates from hyphae ingested by workers licking the garden surface.

Infrabuccal pellet material is regurgitated and discarded, although some digestion takes place in the infrabuccal pocket (Febvay *et al.* 1984). This makes it difficult to decide how much material is actually utilised by workers as food.

Licking workers must continue to ingest hyphae for long periods in order for pure T2 pellets to be produced and less than one T2 pellet is produced per day by each worker, supporting this idea. Porter and Bowers (1982) showed that workers dealing with foraging or refuse dumping showed task fidelity and this may also occur with minima which lick garden or forage. If minima continually switched tasks, infrabuccal pellets would reflect this by being more uniform. Instead, two clear types are produced, with small numbers of intermediates produced by workers which do switch tasks. Some role switching must occur, however, since there was a decrease in pellet production when forage was available, indicating that workers caring for the garden switched to preparing substrate.

Young workers may tend to care for the garden or brood, while older workers switch to substrate preparation. The oldest workers would then forage, possibly as a protection mechanism for larger foragers at risk from parasitism by Phorid flies (Eibl-Eibesfeldt and Eibl-Eibesfeldt 1967, Feener and Moss 1990). Such temporal systems are common and are present in *Atta sexdens* (Wilson 1980a) whereby a media or maxima worker engages in garden care as a callow, then forages when it is older. Since foraging is a dangerous occupation, it makes sense for workers that are about to die anyway to engage in it.

Callow workers are in constant contact with the garden and are seldom found away from it so should, in theory, produce only T2 pellets, with occasional T3 pellets resulting from grooming. In fact, the callow workers examined produced roughly equal numbers of T2 and T3 pellets, although no T1 pellets were produced. These T3 pellets probably resulted from grooming, or helping in substrate preparation, which has been recorded by Wilson (1980a).

2. Production of infrabuccal pellets by alates

Alate sexuals, like callow workers, are in constant contact with the garden before the mating flight, but produced all three types of pellet. Numbers produced were low unfortunately, due to the limited numbers of insects available. However, pellets obtained did represent the infrabuccal pellet contents just before the mating flight.

Hyphae in the infrabuccal pocket are enzymatically degraded from the inside outwards, therefore live hyphae tend not to be degraded, otherwise the female could not transmit viable fungus (Febvay *et al.* 1984). Licking females ingest large tufts of hyphae (see Chapter 5), while the much smaller workers tear up tiny fragments of mycelium. The former is easy to observe, but hyphal ingestion in workers is difficult to see and its occurrence has previously been denied (Wilson 1980a). The large tufts ingested by females will contain many undamaged hyphae and little enzymatic degradation will therefore occur in the infrabuccal pocket. The fragments of hyphae ingested by workers will however, be considerably damaged and degradation by enzymes would be possible and likely. If females were smaller, fungal transmission via the infrabuccal pocket might be more difficult because the hyphae would be more damaged. Fungus transmission might therefore be one of the reasons why Attine queens, particularly those of *Atta*, are so large. Larger fungal pellets will also mean that a larger fungal inoculum is available to found a new garden. This is important because the fungus competes poorly with other fungi (Weber 1972).

A crucial part of colony foundation is the successful growth of the fungus from the infrabuccal pellet regurgitated by the queen and Cherrett *et al.* (1989) estimated that 25% of queens lose their pellets at this stage. Females usually produce T2 or T3 pellets but if they were to try to found gardens from T1 or T3 pellets, lack of fungus and contaminant spores present would reduce their chances of success. Quinlan (1977) managed to culture ant fungus from worker infrabuccal pellets, but found that most were too contaminated. Queens which succeed in founding new

gardens will therefore be those which carried pellets containing large fungal inocula, i.e. T2 pellets. Non-fungus containing pellets were probably produced because of grooming and licking of container walls.

Males confined with fungus garden produced relatively more T1 pellets than females, indicating their lower level of commitment to garden-care (see Chapter 5).

SUMMARY

Leafcutting ants produced two distinct types of infrabuccal pellet, with a third intermediate type. The most common type (T1) consisted of detritus from grooming and substrate preparation and was produced by all worker castes. They could be used to trace the flow of dye through a colony.

The second distinct type of pellet (T2) was fungal in origin and produced mainly by minima workers, but only if these had been exposed to fungus garden.

Workers produced pellets at rates of 1.57 (minima), 0.93 (media) and 1.18 (maxima) each per 24 hrs when confined with fungus garden in petri-dishes, but at rates of 1.04 (minima) and 2.63 (non-minima) per worker per 24 hrs in fungus gardens. Each worker regurgitated a mean of 1.1 µg of fungal material in infrabuccal pellets per 24 hrs, which originated either from the ingestion of staphylae or hyphae.

Alate females and males produced all three kinds of pellet, but females produced more fungus-containing pellets than males. This is important for the foundation of new gardens.

Chapter 7: The results of restricting worker access to the fungus garden surface

INTRODUCTION

In the past, licking of fungus garden by workers has been considered to be a decontamination mechanism for the removal of alien spores (Quinlan and Cherrett 1977). The ant fungus is found only in Attine nests and is rapidly overcome by alien organisms in the absence of the ants (Cherrett *et al.* 1989). Wheeler (1937) believed that workers 'weeded' the garden, but Weber (1947) refuted this and later (1966) suggested that antibiotics might be present in the ant saliva. Although Martin *et al.* (1969) found no such activity in the ants, several authors have since demonstrated it in the metathoracic (metapleural) glands (Schildknecht and Koob 1970, Maschwitz *et al.* 1970, Sihanoth *et al.* 1973). Febvay *et al.* (1984) showed the presence of a chitinolytic enzyme system in the labial glands and suggested that this was important for suppressing microorganism growth.

Chapter 6 showed how large amounts of fungal material enter the infrabuccal pocket, probably as a result of licking the garden. Wilson (1980a) denied that licking workers ingested hyphae and Quinlan and Cherrett (1979) considered it unlikely that workers obtain nutrients from ingested hyphae due to the filtering action of the infrabuccal pocket, although Febvay *et al.* (1984) demonstrated chitin digestion here.

Workers apply faecal droplets to fungus garden substrate during its preparation and these faeces contain enzymes which accelerate the digestion of fresh substrate and hence fungus growth rate (Martin and Martin 1970a, Martin *et al.* 1973). They originate from the ant fungus (Martin 1974, Martin and Boyd 1974) and are therefore taken in by workers either by consuming staphylae or hyphae, or

possibly by licking mycelium to obtain exudates. It is therefore likely that fungus garden licking is important for this.

Licking may therefore be important for removing contaminants from garden or for obtaining nutrients or enzymes. The role of the ants in caring for fungus garden can be studied in part by removing them and seeing what happens subsequently and this is examined in this chapter. For example, if workers lick garden to obtain something, then will it become plentiful when ant access to an area of garden is temporarily prevented? Will this then lead to an increase in numbers licking when access is resumed? This 'something' might be either 'nice' or 'nasty'. The former might include resources that workers find desirable, like fungal exudates or the hyphae themselves, whereas the latter might include competitor fungi and bacteria, which workers remove from the garden surface.

MATERIALS AND METHODS

Workers and fungus garden from an *Atta sexdens* nest (80 gardens) and an *Atta cephalotes* nest (40 gardens) were used.

1. Assessing worker reaction to areas of fungus garden temporarily restricted from them

In the laboratory, gardens were built in 2.5 litre capacity plastic domes and to allow access to a fungus garden, one of these in the *Atta sexdens* nest was replaced by a glass box of similar size, with a removable lid. A 'cage', 20 x 15 mm and 10 mm deep, was made from clear plastic and fine wire mesh, through which workers could not pass (Fig. 7.1). This was put in the nest area for several days to allow the ants to become used to it. It was then placed on to an area of young fungus garden within the glass box, enclosing the area along with a few workers

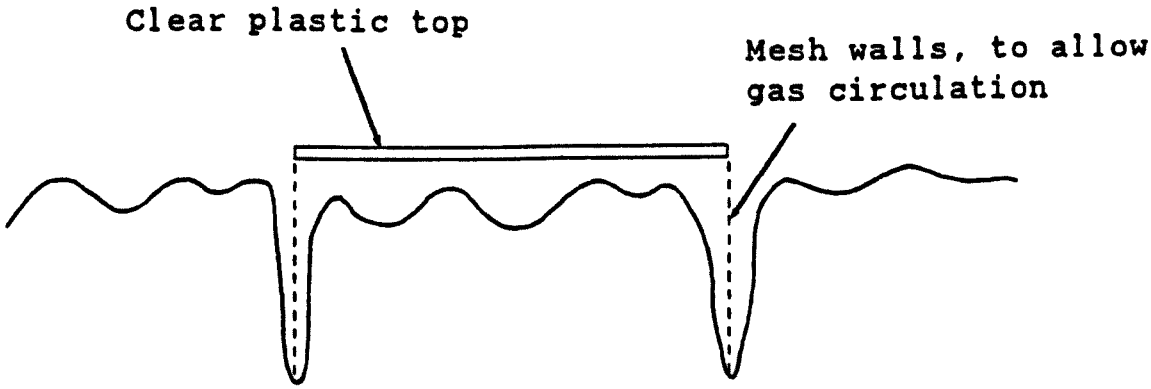


Figure 7.1: Method used to prevent workers from licking small areas of fungus garden *in situ*, inside a dome. The 'cage' sides were 10 mm high and the clear plastic top measured 20 x 15 mm.

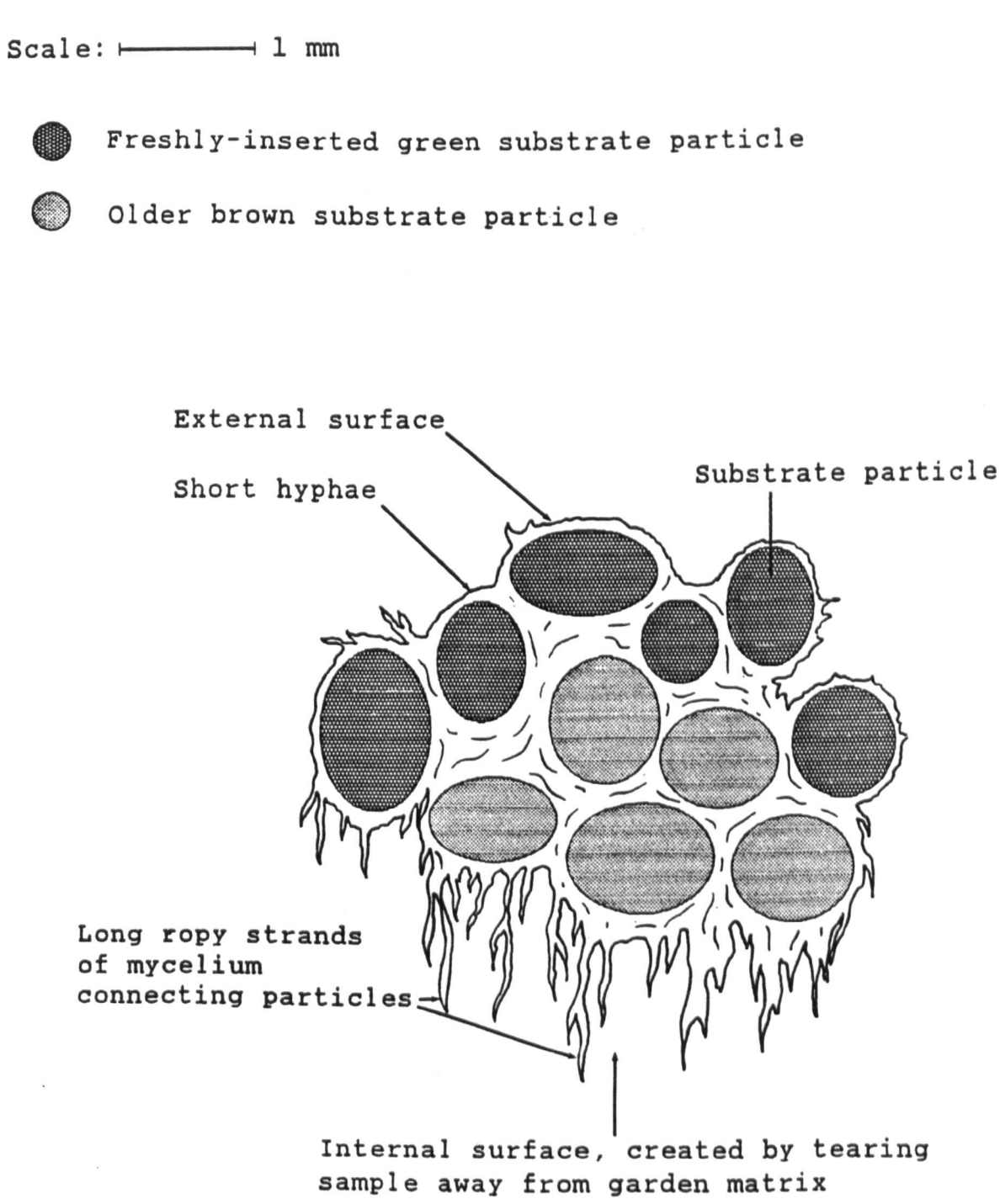


Figure 7.2: The differences between surfaces of substrate particles on the exterior and interior of fungus garden.

(total ant numbers and numbers licking in the test area were previously noted). Gently pressing the cage on to the garden prevented workers from licking the garden surface.

After 24 hrs, the cage was removed and the numbers of workers present and licking on the previously restricted area were assessed at intervals, using a binocular microscope and cold fibreoptic light source with a red filter (see Chapter 5, pages 78-80). When this process was repeated, different areas of the garden were used.

Fragments of fungus garden removed before caging, after caging and 90 minutes after re-exposure to workers were mounted in cotton-blue stain in lactophenol and examined microscopically. Fragments of garden from uncaged areas were also mounted at the same times. Only exposed surfaces of garden were examined (surfaces accessible to workers). Non-exposed surfaces have thick 'ropy' growths of mycelium connecting them to other particles and workers cannot reach these. Young garden was used, therefore exposed surfaces had greener substrate fragments than non-exposed surfaces, since new substrate is added to the outside of the garden (Fig. 7.2). Hyphal depths on these fragments were measured using an eyepiece graticule at regular intervals on substrate particle surfaces.

2. Assessing worker reaction to fungus garden temporarily removed from them

To examine the effects of 'caging' garden without the problem of cage removal by the ants, a perspex box was constructed, consisting of a large chamber with ten sub-chambers, in which fungus garden could be maintained in isolation (Fig. 7.3). Each sub-chamber was 23 mm deep and had a removable glass lid (a microscope slide) through which observations could be made, using a binocular microscope and cold fibreoptic light source with a red filter. An area of 19 cm^2 of garden could be observed. Barriers could be placed between the two perspex sheets to restrict access to sub-chambers. The whole box was covered

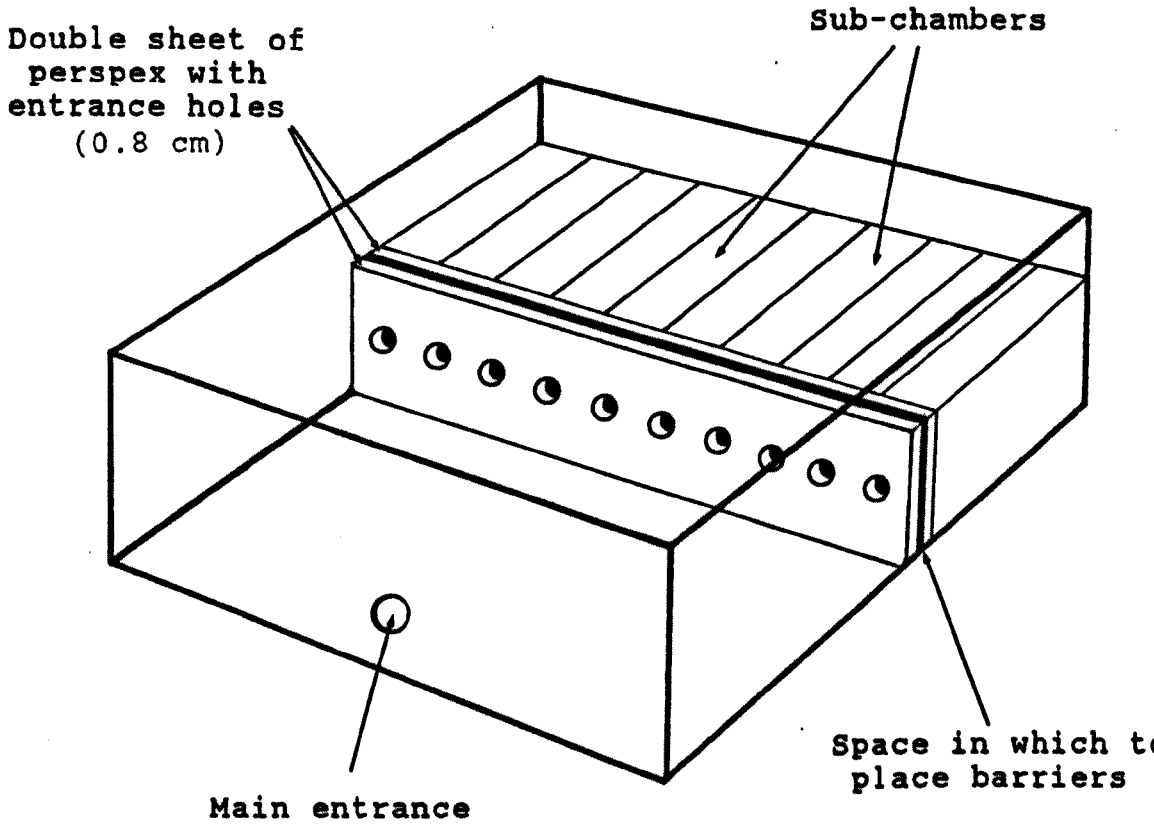


Figure 7.3: Container with ten sub-chambers used to maintain fungus garden away from workers and to allow selective re-exposure of individual chambers containing garden to workers (shown without lid). The visible upper surface of the garden in each sub-chamber had a surface area of 19 cm^2.

by a clear perspex lid which could be removed and sprayed on the inner surface with water, to maintain high humidity in the box. This box could then be used to examine worker reactions to garden isolated from them for different periods.

RESULTS

1. Worker reaction to areas of fungus garden temporarily restricted from them, '*in situ*'

After garden had been caged *in situ* for 24 hrs, workers rushed on to it and immediately began to lick it. The experiment was also carried out using a caging period of 2 minutes, to see if effects were due to disturbance. However, workers did not rush on to garden caged for 2 minutes and were so alarmed by the disturbance that they initially left the area (Fig. 7.4). Caging the garden for 24 hrs rendered it so attractive that this alarm effect was overcome. Unfortunately, only 3 replicates were carried out for each of the two caging periods because the ants became adept at dragging the cage away, but replicates of 24 hrs and 2 minutes caging were alternated to reduce any effects of time.

Comparing numbers licking the garden after the two caging periods showed that significantly more workers licked garden caged for 24 hrs than that caged for only 2 minutes. This was true from 2-20 minutes after exposure ($p<0.05$, ANOVA). Clearer results would probably have been obtained with more replicates, therefore an easier way of obtaining them was used (see part 2). After 90 minutes the effect appeared to be declining.

Microscopic examination of the fungus garden showed that usually, hyphae were short and remained close to the substrate surface, whereas beneath the surface, particles were connected by more vigorous fungal growth.

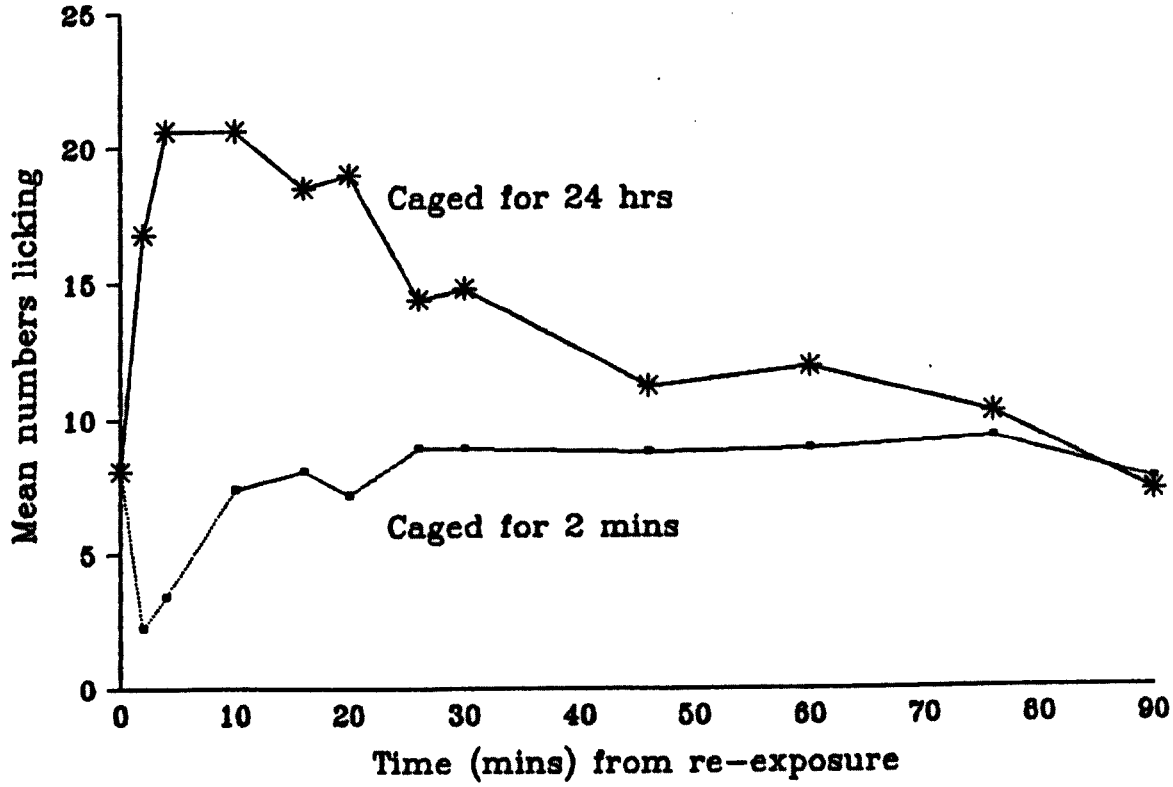


Figure 7.4: Mean numbers of workers licking 300 mm^2 areas of fungus garden caged (*in situ*) for 24 hours or 2 minutes, to prevent ant access. Significant differences were present between 2 and 20 minutes after garden re-exposure ($p<0.05$, ANOVA, 3 replicates). Errors are shown in Appendix 2.1.

The data obtained for hyphal depths were not normally distributed (see Table 7.1 for median values) and were transformed to the $\log_{10}$ values to achieve normality, before analysis. There were no significant differences between $\log_{10}$ hyphal depths on uncaged control areas taken at different sampling times ($p>0.8$, ANOVA). However, $\log_{10}$ depths on garden caged for 24 hrs were significantly greater than those on garden exposed to workers for 90 minutes, which in turn were significantly greater than depths on uncaged control areas and depths before caging ($p<0.05$, ANOVA and Tukey). Caging for 24 hrs therefore led to luxuriant hyphal growth on the garden surface, but workers quickly cut this back to nearly normal levels when it was exposed to them.

The numbers of hyphae growing on the edges of substrate fragments were also recorded for control garden and garden isolated for 24 hrs. On the former, there was a mean of 21 (SE ± 2.0) visible hyphae per 0.3 mm length of substrate particle edge and on garden caged for 24 hrs, there were 62 (SE ± 3.6) hyphae per 0.3 mm length (10 replicate counts). Numbers of hyphae, as well as depths, therefore increased during isolation from workers.

Table 7.1: Median hyphal depths (± 95% confidence limits) of uncaged garden, of garden caged for 24 hrs and of similarly caged garden re-exposed to workers for 90 minutes (50 replicates).

TREATMENT	MEDIAN HYPHAL DEPTH (mm)	95% CONFIDENCE LIMITS
Uncaged garden	0.08	0.06,0.11
24 hrs caging	0.25	0.19,0.26
24 hrs caging + 90 mins re-exposure to workers	0.10	0.08,0.14

2. Worker reaction to garden temporarily removed from them

a) Garden isolated for 24 hrs

Ant-free garden (see Chapter 2, page 15) was placed in five alternate sub-chambers of the box shown in Fig. 7.3. The other five received garden which had been similarly handled and dissected, but with the ants remaining.

Chambers with ant-free garden were closed off for 24 hrs while the other five remained open. The box was placed on a forage table connected to the nest, so that workers could enter or leave the box at will. After 24 hrs, the numbers of workers licking and the total numbers present were assessed in each chamber ('ant-free' garden contained some small workers which had escaped attention in the original clearing). The chambers were then opened, allowing the ants to enter and the numbers of workers present or licking in each of the chambers were assessed at intervals. Further counts were also made after 24 hrs.

This was carried out for both *Atta cephalotes* and *Atta sexdens*. The box was carefully cleaned between these two experiments, to remove all traces of fungus garden and ants and was exposed to the relevant nest for 2 days before the experiment to allow the ants to mark it and become familiar with it. All ants were removed before isolated garden was introduced.

The first workers to enter the five closed chambers were mostly maxima and media and little licking occurred while they rearranged the disrupted garden. As more ants entered, the numbers licking increased until they overtook the control levels (Fig. 7.5). This happened more slowly than on caged garden *in situ*, therefore observations were continued over 3 hrs.

Comparing replicates at each time of observation between test and control chambers showed significant differences ($p<0.01$, ANOVA) for both species between 60 and 180 minutes post-exposure. However, significantly more ants were present in isolated chambers than in control ones

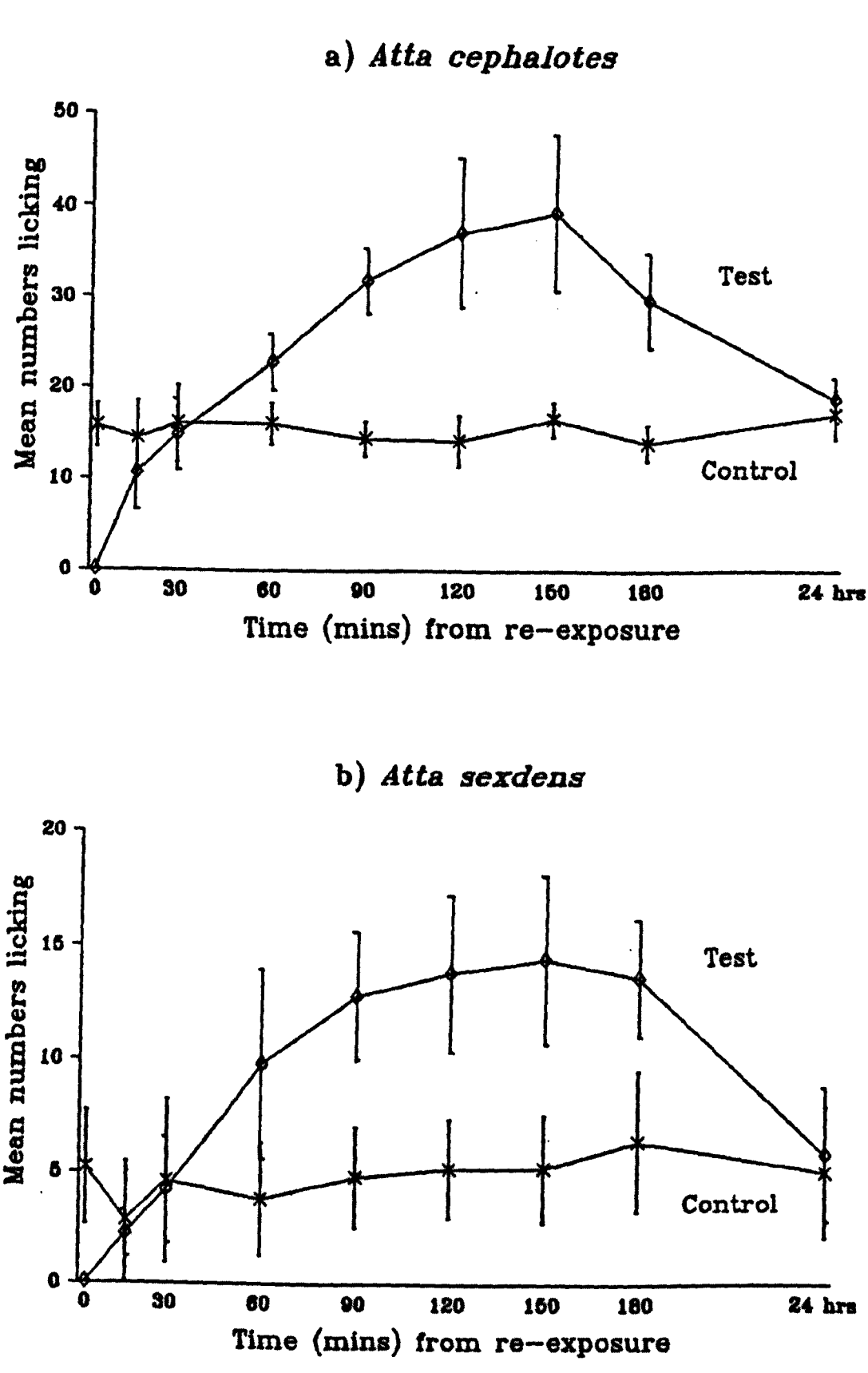
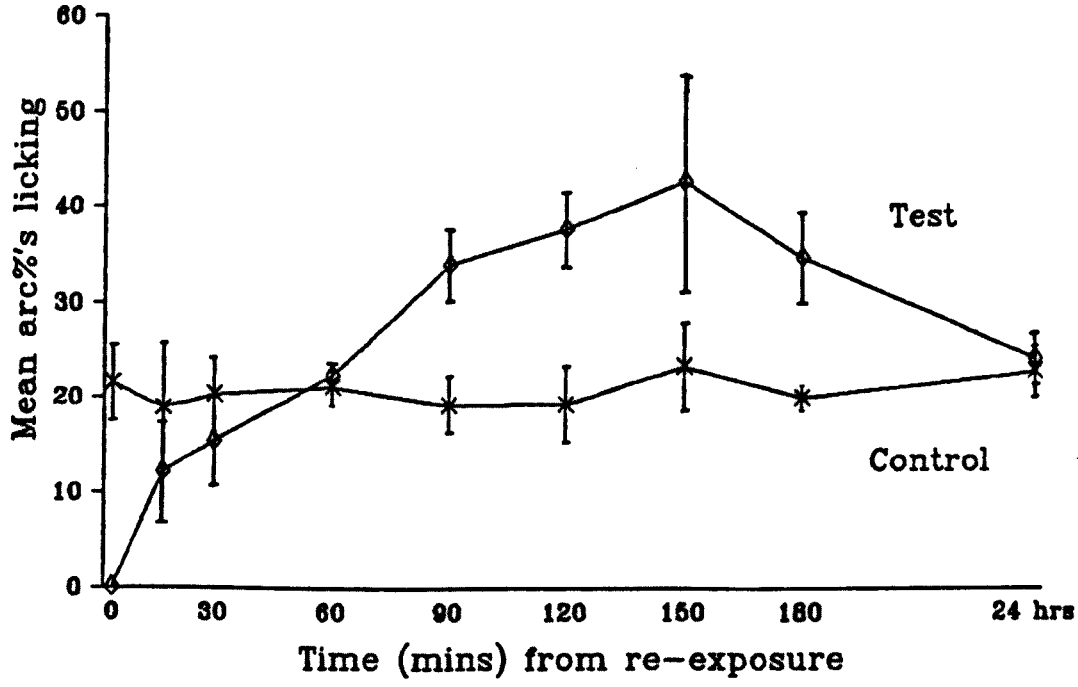


Figure 7.5: Mean numbers of workers per 19 cm^2 of two species of *Atta* licking in chambers containing fungus garden previously isolated from them for 24 hrs, compared with numbers licking non-isolated control garden. Significant differences were found from 60-180 minutes after re-exposure of the garden for both *Atta cephalotes* and *Atta sexdens* (5 replicates).

a) *Atta cephalotes*



b) *Atta sexdens*

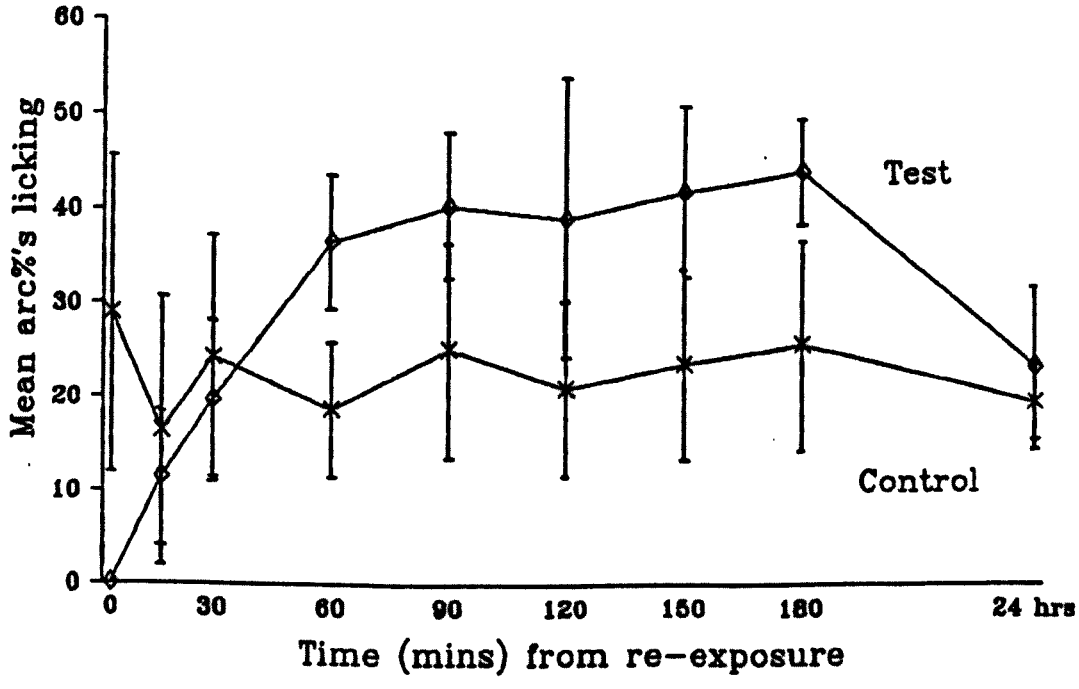


Figure 7.6: Mean arcsine-transformed percentages of workers per 19 cm^2 of two species of *Atta* licking in chambers containing fungus garden previously isolated from them for 24 hrs, compared with numbers licking non-isolated control garden. Significant differences were present from 90-180 minutes after re-exposure of the garden for *Atta cephalotes* and from 60-180 minutes after re-exposure for *Atta sexdens* (5 replicates).

($p<0.05$, ANOVA) from 60 to 180 minutes for *Atta sexdens* and from 15 minutes for *Atta cephalotes*. Because of this, arcsine-transformed percentages of worker numbers licking were examined (Fig. 7.6). Comparing replicates at each observation period showed that arc%'s licking isolated garden were significantly larger than the control levels from 90-180 minutes post-exposure for for *Atta cephalotes* and from 60-180 minutes post-exposure for *Atta sexdens* ($p<0.05$, ANOVA).

After 24 hrs of exposure to workers there were no significant differences between numbers licking in either species ($p>0.2$ for *Atta cephalotes* and $p>0.6$ for *Atta sexdens*, ANOVA). This was also true for arc%'s licking ($p>0.3$ for both species).

b) Garden isolated for different periods

Garden was isolated for 1-4 days to see if different isolation periods had any effect on subsequent worker response. Since the box only contained ten sub-chambers, seven 'runs' were used, with chambers containing garden isolated for different periods being opened simultaneously. Each run consisted of four chambers containing garden isolated for 1-4 days, achieved by placing ant-free garden into individual chambers every day for 4 days, so that on the fourth day garden samples isolated for all four periods were present simultaneously. Chambers containing control garden were filled at the same times. These contained garden which had been similarly treated to isolated garden, but with attendant workers present.

After re-exposure, total numbers of workers present and numbers licking were recorded at 15 and 30 minutes and then every 30 minutes until 3 hrs post-exposure. Further counts were made after 24 hrs.

After re-exposure of the isolated garden, the mean numbers and arc%'s of workers licking the garden reached their highest levels in chambers closed for 2 or 3 days, followed by those closed for 1 day (Figs. 7.7a and b). Comparing replicates at each observation time showed that

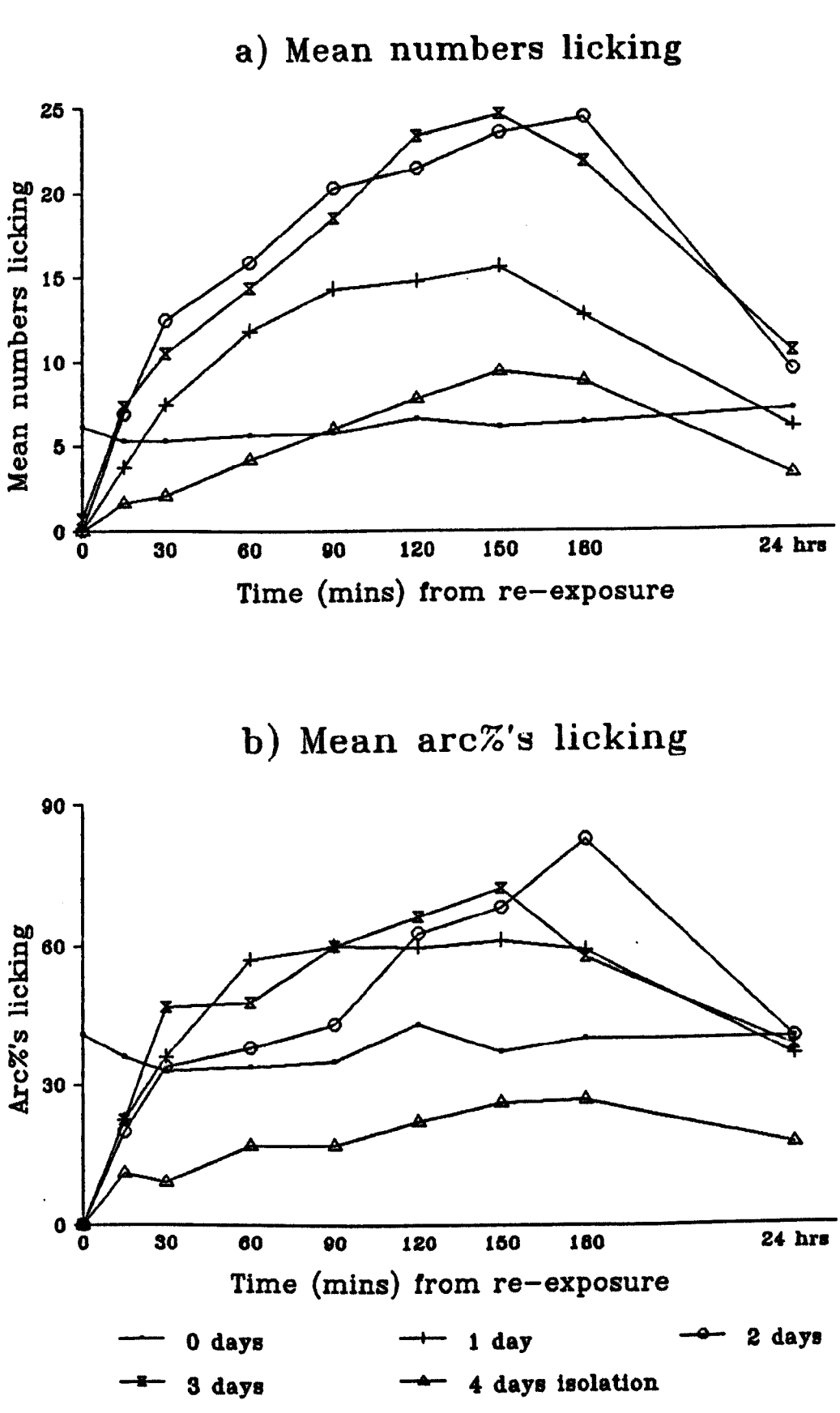


Figure 7.7: Numbers of *Atta sexdens* workers licking 19 cm^2 areas of fungus garden in chambers previously isolated from them for 1-4 days, compared with numbers licking non-isolated control garden. Numbers are expressed as, a) Mean numbers licking and, b) Mean arcsine-transformed percentages licking (arc%'s). Errors are shown in Appendix 2.2 (7 replicates).

after 90 minutes of exposure, numbers and arc%'s licking in these chambers were all significantly higher than those in control chambers where worker access had been continuous ($p<0.05$, ANOVA and Tukey). This continued until 180 minutes post-exposure, when numbers and arc%'s licking decreased to levels which were not significantly different from those in control chambers ($p>0.05$). Numbers and arc%'s licking in chambers containing garden isolated for 1, 2 or 3 days were not significantly different from each other at any time and numbers and arc%'s licking in chambers closed for 4 days were never significantly different to those in control chambers ($p>0.05$).

However, arc%'s licking garden isolated for 4 days were significantly less than on control garden between 30 and 180 minutes post-exposure ($p<0.05$, ANOVA and Tukey).

Garden isolated for 1-3 days was therefore highly attractive to workers. On garden isolated for 4 days, however, large numbers of staphylae were present and workers could be seen consuming them. Some 4 days-isolation replicates had small areas of contaminant growth and affected garden was rapidly removed and discarded, leaving healthy garden behind. No licking occurred on garden with contaminant mycelium growing on it.

c) The effect of a large number of staphylae on numbers of workers licking garden

After 3-4 days of isolation from workers, large numbers of staphylae are present on garden. These are highly attractive and may distract workers from licking, or may reduce the area available for hyphal growth, thus reducing the numbers licking.

To examine this, petri-dishes (5 cm) were filled with ant-free garden at intervals, so that replicates containing garden isolated for 1, 4, 6 and 7 days were available simultaneously. Controls, with attendant workers present were also set up at each time. Petri-dishes were maintained in boxes containing damp filter paper in the culture room

during the isolation periods. Five replicates were used for each group.

Approximately 50% of the staphylae present were removed from five dishes of garden isolated for 4,6 and 7 days, using fine forceps and a binocular microscope. (This was why petri-dishes were used rather than the ten-chambered box, since they were more easily handled). The numbers of staphylae present were counted for each petri-dish to check that removing staphylae significantly reduced the numbers present. Staphylae numbers per dish were counted by scanning through a clear lid with a grid marked on to it, which ensured that areas of garden were only assessed once.

The lids of petri-dishes containing isolated garden and controls were then removed and replaced by lids with 0.5 cm entrance holes. These dishes were then placed in the nest area to allow worker access. Total numbers of workers present, numbers licking garden and numbers handling staphylae were all recorded at the same times as before.

Replicates were compared at each observation time using ANOVA and Tukey's multiple comparison ($p=0.05$). Reducing the numbers of staphylae present on garden isolated for 4 days significantly increased the numbers and arc%'s of workers licking the garden after re-exposure to the same level as that on garden isolated for only 1 day from 30-180 minutes after exposure (Figs. 7.8a and b). A similar significant, though less drastic effect also occurred on garden isolated for 6 days with staphylae numbers reduced, between the same times (Fig. 7.8c). However, removal of staphylae on garden isolated for 7 days caused no significant increase in numbers or arc%'s licking after re-exposure to workers (Fig. 7.8d).

Numbers and arc%'s licking garden isolated for 4, 6 or 7 days without removal of staphylae were not significantly different from those on non-isolated controls and numbers licking garden in the four sets of controls were not significantly different from each other at any time

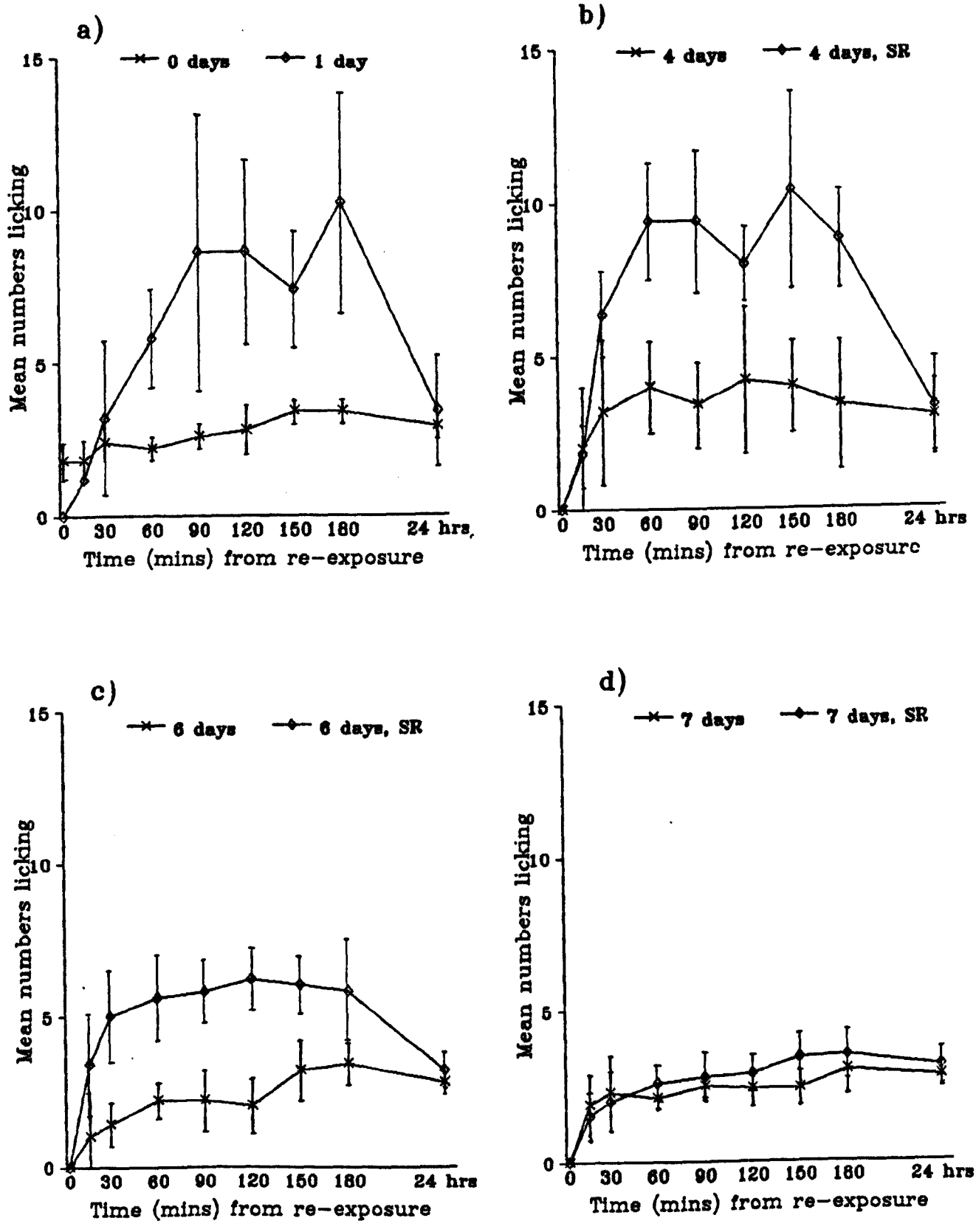


Figure 7.8: Mean numbers of *Atta sexdens* workers licking 19 cm^2 areas of fungus garden in chambers previously isolated from them for 4, 6 or 7 days, when staphyla numbers in each replicate were either reduced ('SR')or left untouched. Mean numbers of workers licking garden previously isolated from them for 0 and 1 days without staphyla numbers being reduced are also plotted (7 replicates).

($p>0.05$). Fig. 7.8 therefore has only one line for 0 days isolation, which is a grand mean of the four.

Mean numbers of workers present were highest on garden isolated for 6 or 7 days (Figs. 7.9a-d), followed by those on garden isolated for 4 days (without removal of staphylae). ANOVA and Tukey's multiple comparison showed that numbers present on 6 or 7 days-isolated garden were significantly larger than numbers on 0 days isolated controls, from 15 minutes to 180 minutes post-exposure ($p<0.05$). They were also significantly larger than numbers on 1 or 4 days isolated garden at 30 and 60 minutes post-exposure ($p<0.05$). Numbers of workers present on garden isolated for 4 days, without removal of staphylae, were not significantly different to numbers on garden isolated for 6 or 7 days between 90 and 180 minutes post-exposure. Neither were they significantly different to numbers on garden isolated for 1 or 4 days (with staphyla removal) after 30 or 60 minutes post-exposure, respectively ($p>0.05$). Garden isolated for 1 day had significantly larger numbers of workers present than non-isolated control garden from 120 to 180 minutes post-exposure. Similarly, numbers of workers on 4 days isolated garden (with staphyla removal) were significantly larger than numbers on non-isolated control garden from 60 to 180 minutes post-exposure ($p<0.05$). Numbers on garden isolated for 1 and 4 days (with staphyla removal) were not significantly different from each other at 15 and 30 minutes post-exposure ($p>0.05$).

Numbers of workers eating or carrying staphylae ('handling staphylae') also varied between treatments. Reducing staphyla numbers on garden isolated for 4, 6 or 7 days caused a slight decrease in the numbers handling staphylae (Figs. 7.10a-d). The largest numbers of staphylae were handled by workers entering dishes containing garden isolated for 6 or 7 days and numbers and arc%'s were significantly higher in these than in other treatments from 15 minutes after re-exposure ($p<0.05$, ANOVA and Tukey).

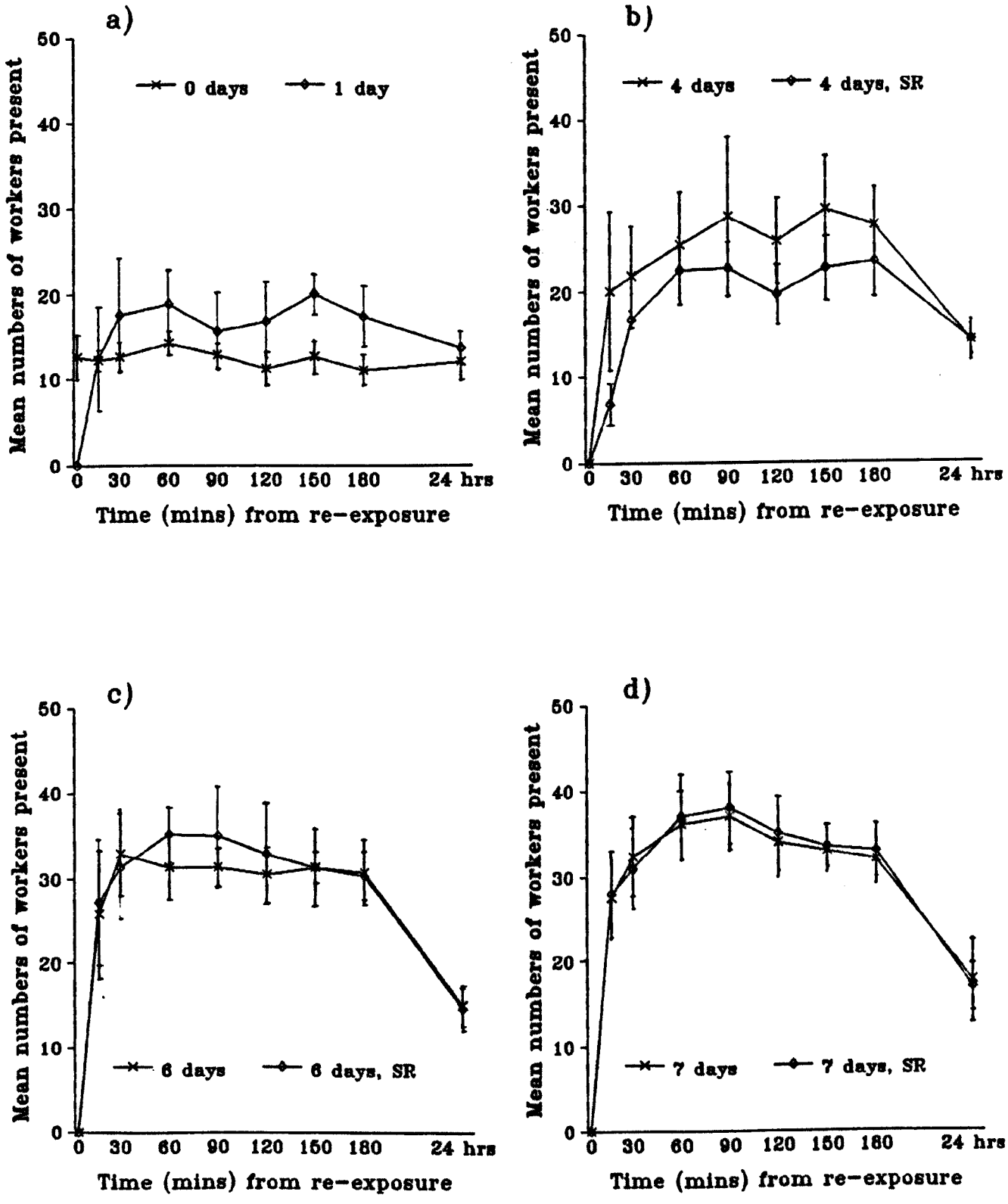


Figure 7.9: Mean numbers of *Atta sexdens* workers present on 19 cm^2 areas of fungus garden in chambers previously isolated from them for 4, 6 or 7 days, when staphyla numbers in each replicate were either reduced ('SR')or left untouched. Mean numbers of workers present on garden previously isolated from them for 0 and 1 days without staphyla numbers being reduced are also plotted (7 replicates).

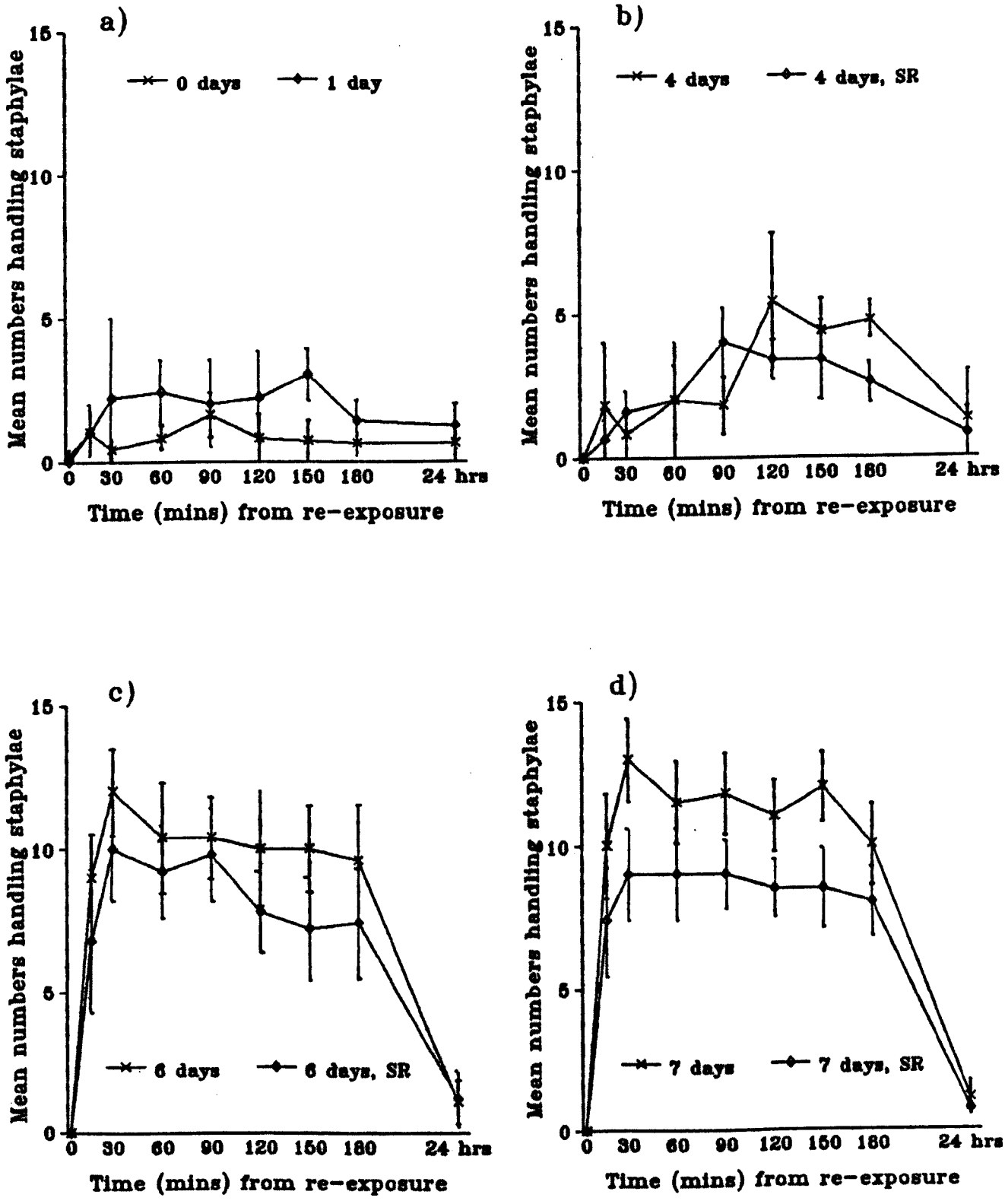


Figure 7.10: Mean numbers of *Atta sexdens* workers handling staphylae on 19 cm^2 areas of fungus garden in chambers previously isolated from them for 4, 6 or 7 days, when staphyla numbers in each replicate were either reduced ('SR')or left untouched. Mean numbers of workers handling staphylae on garden previously isolated from them for 0 and 1 days without staphyla numbers being reduced are also plotted (7 replicates).

Significantly lower numbers and arc%'s handled staphylae on garden isolated for 6 or 7 days with staphyla numbers reduced after 150 minutes of re-exposure ($p<0.05$), due to the smaller number of staphylae available.

After 3 hrs of re-exposure, the numbers of staphylae present in all test and control petri-dishes were similar ($p>0.05$, ANOVA), but no significant increases in numbers of workers licking occurred at this time on garden isolated for 6 or 7 days without staphylae removal ($p>0.05$, ANOVA and Tukey). The 'licking effect' therefore occurred on garden isolated for up to 6 days, but not on garden isolated for longer periods.

DISCUSSION

Temporarily 'caging' or isolating garden from workers caused an increase in the number of workers subsequently licking it once access was resumed. Temporary worker exclusion therefore increased garden attractiveness to workers and this was not the result of artificial experimental conditions, since the first experiment was carried out *in situ*, inside a nest. Neither was this phenomenon the result of disturbance, since 2 minutes of caging *in situ* caused an initial drop in numbers licking. In addition, this effect was not an isolated phenomenon in a single nest, since the same response also occurred in a nest of a second species.

The major visible change in garden isolated from workers was the increase in hyphal depth which made the garden appear white. This was especially visible on garden caged *in situ*, where the white caged area contrasted with the normal grey/green of surrounding uncaged garden. The fact that these hyphae were cut back when workers were allowed to return suggests that the caging effect was a response to hyphae. On young garden, hyphae grow in fluffy white masses, but on older areas they are less profuse and appear 'ropy'. The decline of the 'isolation effect' on

garden isolated for more than a few days may be related to this, since workers may prefer to lick young hyphae. Chapter 5 showed how licking by workers is relatively more common on young than on older garden and in the experiments, young and early-mature fungus garden were used, with short 'fluffy' hyphae. However, after a few days of isolation, when staphylae were beginning to proliferate, the hyphae became less profuse and 'ropy'; the isolated garden was aging. The older hyphae available on maturing garden may be less attractive to workers.

If the 'isolation effect' is a response to the extra growth of hyphae, then licking fungus garden is also likely to be a response to hyphae. Hyphal depths increased during the caging period, but were quickly reduced back to nearly normal levels when the workers were allowed back on to the garden; they were removing hyphae.

Chapter 4 showed how worker access to the internal areas of the garden is restricted and how this is reflected by the larger numbers of staphylae present here. These internal areas also provide large available fields of hyphae for workers to lick. In Chapter 4, it was calculated that a 2 litre garden would have a total surface area of 5,220 cm^2 (internal and external; see page 70). Hyphae normally had a maximum median depth of 0.08 mm, while after caging for 24 hrs, they had a median depth of 0.25 mm (Table 7.1). This increase in depth represents the volume of hyphae ingested by workers over 24 hrs; 0.17 mm of hyphae, over the whole garden surface. Hyphae do not however, form a uniform mass. On control garden, there were 21 hyphae per 0.3 mm of substrate particle edge, while on garden caged for 24 hrs, there were 62 hyphae; a three-fold increase. Assuming that these figures reflect the numbers of hyphae growing per unit area, then control garden would have 4.9×10^5 hyphae per cm^2 and garden caged for 24 hrs would have 4.3×10^6 hyphae per cm^2. If it is assumed that hyphae are cylindrical, with diameter 6 µm (Weber 1972) and that hyphae are, on average, half the length of the median

depths recorded in Table 7.1, then an estimate of hyphal volumes can be made. The volume of a cylinder is $\pi r^2 l$ (where r is the radius and l is the length of the cylinder). On control garden (median depth 0.08 mm), the volume of a single hypha will therefore be 1.13×10^{-4} cm^3 and hyphal volumes in a 1 cm^2 area will be 5.5×10^{-4} cm^3. Similarly, on garden isolated for 24 hrs, each hypha will have a volume of 3.5×10^{-9} cm^3 and total hyphal volumes per cm^2 will be 0.015 cm^3. Over 24 hrs, there will therefore be an increase in hyphal volume per cm^2 of 0.014 cm^3. Over the whole garden, with a volume of 2 litres and a surface area of 5,220 cm^2, this will be a hyphal volume of 73.1 cm^3.

These figures are based on estimates of hyphal depths on young or early-mature garden, which have luxuriant growths of hyphae, while older garden produces more staphylae and fewer hyphae (see Fig. 2.1, Chapter 2). If for example, it is assumed that only 50% of the garden produces suitable hyphae for workers to lick and 50% of the hyphae produced are used to inoculate newly-inserted substrate fragments, then this leaves a hyphal volume increase over the whole garden over 24 hrs, of 18.3 cm^3. In Chapter 6, a 1,400 cm^3 garden contained 6,840 workers (see page 142), therefore a 2 litre garden would contain in the region of 10,000 workers. Each worker would therefore have 1.8 mm^3 of hyphae available to it every day.

This figure is probably an over-estimate. In Chapter 6, it was estimated that workers each regurgitate 1.1 µg of fungal material in their infrabuccal pellets (see page 149), but this is a residue of ingested material, rather than a true measure of intakes. When initial hyphal depths were being measured, the length of the longest hypha at that point was used. Hyphal lengths on substrate may well follow a skewed distribution, whereby the majority are short and grow close to the substrate. However, these calculations do demonstrate that large amounts of hyphal material are available to workers and are removed from the garden by them.

Workers might ingest hyphae for several reasons. Since digestion occurs in the infrabuccal pocket (Febvay *et al.* 1984) they might be obtaining nutrients. Some authors (Moeller 1893, Stahel and Geijskes 1941) believed that if workers were removed from the garden, the ant fungus produced conidia. If this were so, cutting back hyphae would prevent this. However, Weber (1972) pointed out that conidial growths could sometimes be identified as unusual species of *Aspergillus*, but such pruning of hyphae might be important for another reason, namely to encourage the ant fungus to produce more staphylae.

Both the nutritional and the pruning hypotheses can explain the 'isolation effect'. In the first case, the abundant hyphal growth on young garden would be attractive as food for licking workers, whereas the sparser hyphae on older garden would not. Similarly, pruning would be most frequent on garden producing profuse hyphae; again, young garden.

However, the 'isolation effect' was affected by the length of the isolation period; unless proliferating staphylae were removed from isolated garden, the effect upon numbers of workers licking declined after 3 days of isolation. By removing staphylae, the effective period could be doubled. Garden isolated for 6 or 7 days was not usually visibly contaminated with alien growths, suggesting that the effect was not a response to contaminants. Any small areas of visible alien contaminant growth on fungus garden were not tolerated by workers and were rapidly removed. However, in areas where alien spores were germinating, workers might be stimulated to lick them and hence remove them, in an attempt to salvage the valuable garden. Chapter 6 showed that fungal spores are present in infrabuccal pellets, which contain material licked up by workers.

If this is so, then why did the effect not increase proportionately with increasing isolation period? Garden isolated from workers for 6 or 7 days was not rejected or ignored by workers. On the contrary, they rushed on to it and consumed or removed the large quantities of available

staphylae, then continued to tend it even after these were gone, although numbers of workers licking it remained low, even when staphylae numbers present were artificially reduced (Fig. 7.7).

The relationship between licking by workers and garden contamination is examined in the next chapter.

SUMMARY

Temporarily preventing worker access to fungus garden increased its subsequent attractiveness, expressed by an increase in numbers of workers licking it. The maximum effect was obtained by preventing access for 1 to 3 days after which the effect declined, but removing staphylae from garden kept ant-free for 4 to 6 days restored the effect. Garden kept ant-free for longer than this was no more attractive than non-isolated control garden.

The major visible change on garden kept ant-free was the increase in depth of mycelia, which was reduced again when workers were allowed to return. This suggests that the two are related, either for nutritional reasons, or because workers 'prune' hyphae.

Chapter 8: The removal of alien contaminants as a reason for 'licking' fungus garden

INTRODUCTION

The ant fungus is continually subjected to contamination by spores of a wide range of potential competitors, brought in on substrate materials collected by foragers. The fungus is easily overrun by contaminant fungi if workers are removed (Cherrett *et al.* 1989) but may produce some antibiotics (Hervey and Nair 1979, Angeli-Papa 1984). However, workers help its competitive ability by planting large inocula of ant fungus on new substrate, so that it is colonised quickly. They also physically remove alien contaminants from substrate entering the nest (Quinlan and Cherrett 1977) and from the surfaces of chambers in which gardens are built (Autuori 1941). Weber (1972) described how workers responded to alien fungi introduced near or on to their garden by quickly removing them to a refuse heap and if contaminant colonies did develop on the garden, workers weeded them out. Attine faeces contain chitinase (Martin *et al.* 1973) and this may provide a chemical means of protecting the fungus garden by lysing competitor fungi on new substrate, when workers defaecate on it.

Contaminant spores are actually present in fungus garden. Cazin *et al.* (1989) had difficulties in making isolates of ant fungus due to the growth of contaminant fungi and bacteria present on the garden surface. Moeller (1893) and Eidmann (1935) isolated filamentous fungi from fungus garden and Powell (1984) reported that removing workers from garden led to the growth of *Phialocladus zsoltii*, which was present as spores in the garden. He also recorded *Aspergillus*, *Fusarium*, *Mucor* and *Penicillium*, but pointed out that 75% of contaminant fungi found in the garden were yeasts, previously reported by Craven *et al.* (1970).

Bacteria are also found in the fungus garden (Stoppel 1940, Goetsch and Stoppel 1940, Weber 1956, 1957, 1966, Papa and Papa 1982a) but its acidity limits their growth (Papa and Papa 1982b). Powell and Stradling (1986) reported that the garden becomes increasingly acidic with age, falling from pH 4.8 in young areas to pH 4.5 in older areas in spite of the varying pH of substrate materials.

Contaminants actually present in the fungus garden may be controlled by worker secretions. Sihanoth *et al.* (1973) found that Deuteromycete contaminants of the garden were all more inhibited by worker secretions than was the ant fungus. The presence of ß-hydroxydecanoic acid (myrmicacin), indole-acetic acid (IAA) and phenylacetic acid have been demonstrated in Attine metathoracic glands by several authors (Maschwitz *et al.* 1970, Schildknecht and Koob, 1970, 1971). Iwanami (1978) found that myrmicacin inhibited mitosis in pollen grains and Powell (1984) found that it also affected spore germination and production in common garden contaminant fungi like *Aspergillus* and *Penicillium*, although it had little effect on yeasts.

The potency of myrmicacin is governed by acidity and it is effective in very low concentrations (Iwanami and Iwadare 1979), but these concentrations are still higher than those recorded in Attine fungus gardens (Powell 1984). However, myrmicacin activity may be enhanced by synergists under acidic conditions (Osberghaus *et al.* 1974, Koppensteiner and Bansemir 1975, Powell 1984).

Chapter 5 showed how licking fungus garden occupies a large proportion of the work force on the garden surface and in the past, this has been considered as a decontamination mechanism (Quinlan and Cherrett 1977). However, Chapter 6 showed that workers ingest large quantities of hyphae, probably as they lick garden and the results of Chapter 7 suggested that workers lick garden specifically to obtain hyphae (or something associated with hyphae). In this chapter, the view of licking as a decontamination mechanism is examined.

MATERIALS AND METHODS

All experiments were carried out using workers and/or fungus garden from a large *Atta sexdens* nest (>80 gardens). Petri-dishes (5 cm) filled with ant-free young to early mature garden (see Chapter 2, page 15) were used, unless otherwise stated. These were maintained in boxes with close-fitting lids containing filter paper (Whatman No.1) moistened with distilled water, for high humidity. These boxes were placed in the ant culture rooms to ensure a constant high temperature.

1. Methods for studying the origin of contaminants developing on the garden surface

Ten petri-dishes were filled with garden with carbon dioxide-anaesthetized ants still attached (ants were anaesthetized to facilitate handling of the garden). The dishes were then sealed using wax film. Each had a 0.5 cm entrance hole in the lid through which workers could pass. The dishes were then placed in a sterilised wooden rack which held them in a vertical position, in a sterile sandwich box, to reduce the risk of contaminant spores entering through the entrance hole by gravity (Fig. 8.1). This box also contained filter paper moistened with distilled water to maintain a high humidity.

The ants were given 24 hrs to recover from anaesthesia, to rearrange disrupted garden and to clean it thoroughly. They were then treated with pirimiphos-methyl dust (a broad spectrum contact and fumigant insecticide, formulated as ICI 'Antkiller Dust'). This was introduced into the sandwich box using a syringe and coated workers wandering outside the petri-dishes. Workers reentering dishes introduced insecticide to those remaining inside (via allogrooming). Presumably, the insecticidal dust was not contaminated with fungal spores.

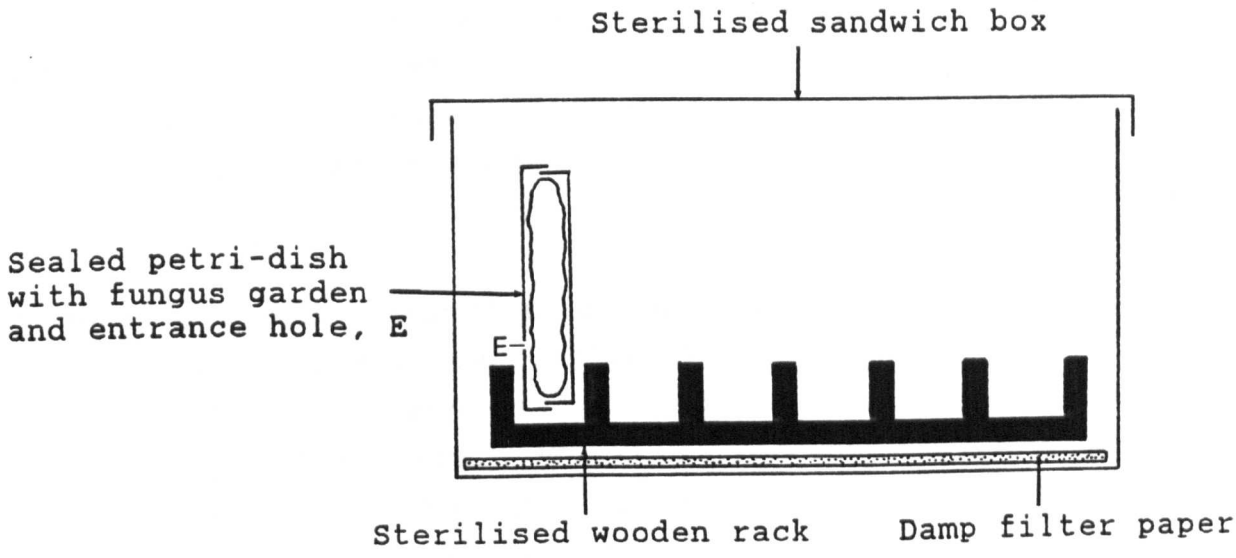


Figure 8.1: Apparatus used to examine the origins of contaminant fungal spores found in the fungus garden.

When all workers had died, the petri-dishes were carefully removed from the box and placed, entrance-hole downwards, in similar sterile boxes, exposing them to air for as little time as possible. Subsequent growth of contaminant organisms was recorded. Samples of such organisms were removed, mounted in cotton-blue in lactophenol and identified.

2. **Measuring growth-rates of an alien fungus colony on garden isolated from workers for different periods**

Petri-dishes were filled with ant-free garden at intervals, so that garden isolated from workers for 0, 10 and 15 days were available simultaneously. Those with contaminant growth were discarded. Five replicates for each time period were inoculated in the centre with a Zygomycete fungus, *Mucor*. A fine needle was used to pick up small amounts of mycelium and sporangia and transfer them from the parent colony. This was a single colony growing in a dish of previously isolated fungus garden. Using a single colony meant that there was little variation in growth rates between samples used for each replicate. The times taken for the introduced contaminant to develop and grow were then recorded.

3. **Methods for deliberately introducing contaminants into fungus garden**

A dome with sliding side panels was used to gain access to the fungus garden. A variety of contaminants were then introduced to small areas.

(1) Fungal spores were dusted over 1 cm^2 areas using a fine paint-brush. Two species were used; rose mildew (*Sphaerotheca pannosa*), a harmless biotrophic parasite of living leaves and *Penicillium*, a general saprophyte and one of the contaminant fungi found growing on garden isolated from workers.

(2) 1 µl fungicidal benomyl droplets (Benlate, 0.77 gl^{-1}) were placed on to fungus garden to kill small areas of mycelium, mimicking the action of a capsulated fungicide brought into fungus garden in baits. Droplets were dispensed using a fine syringe.

(3) Freshly collected worker loads of nest refuse were placed on young, mature and aging garden.

Each treatment was repeated ten times and the worker response and time taken to respond were recorded.

4. Studying the precision of removal of contaminants from the fungus garden

a) Introducing refuse into fungus garden

Nest refuse is distinctively yellow/brown in colour and can be easily seen when mixed with the grey/green young garden. Ten freshly-discarded worker loads of refuse were stirred into approximately 2 cm^3 of young garden *in situ* and the subsequent worker response recorded.

b) Examining weight loss of contaminated garden after exposure to workers

Central 0.5 cm^3 plugs of garden were removed from 10 petri-dishes of ant-free garden, which had been isolated for 24 hrs. The dishes were then weighed, using a Unimatic SN1 balance and the central holes filled with pieces of garden contaminated with sporulating *Penicillium* mycelium. The petri-dishes were then exposed to workers for 24 hrs, after which workers were removed and the dishes reweighed. The remaining garden within the dishes was then removed so that the weights of the actual dishes could be found. Weights of garden exposed to workers and the amount of garden lost could then be calculated. Ten petri-dishes containing garden isolated for 24 hrs but with no introduced contaminant were also exposed to workers to act as controls. Workers gained access to dishes via 0.5 cm entrance holes in the lids.

RESULTS

1. The reaction of workers to garden isolated from them for different periods

Workers rejected garden contaminated with alien fungi and the amount of garden thrown away after periods of isolation from workers was examined.

Petri-dishes containing ant-free garden were weighed using a Unimatic SN1 balance and maintained in humid boxes for 0-10, 12, 15 and 20 days. Ten replicates were used for each period. Dishes were then reweighed and the lids exchanged for ones with 0.5 cm entrance holes. These were placed in humid chambers which were freely accessible to workers (the high humidity prevented weight loss due to drying out). After 24 hrs of exposure to workers, the dishes were re-weighed, with the original lids. Then, any remaining garden was removed and the empty dishes weighed, so that the weights of garden samples used could be calculated.

During the isolation periods, garden samples in petri-dishes lost 1-4% of their original weight, probably due to water loss. In many replicates, garden was kept isolated from workers for up to 12 days without developing any signs of contaminant growth and during this time, it became covered with thick growths of hyphae and staphylae which were rapidly removed when workers were again allowed to enter dishes. In the majority of replicates isolated from workers for up to around 10 days, the small amount of weight loss which occurred over the subsequent 24 hrs of exposure to workers could be attributed to this (Fig. 8.2). Workers continued to tend uncontaminated garden, even after they had removed most of the visible staphylae.

Where alien contaminant growth was visible, areas of garden were subsequently removed by workers and discarded with nest refuse. Workers which came into contact with such contaminated areas showed great excitement, with very rapid

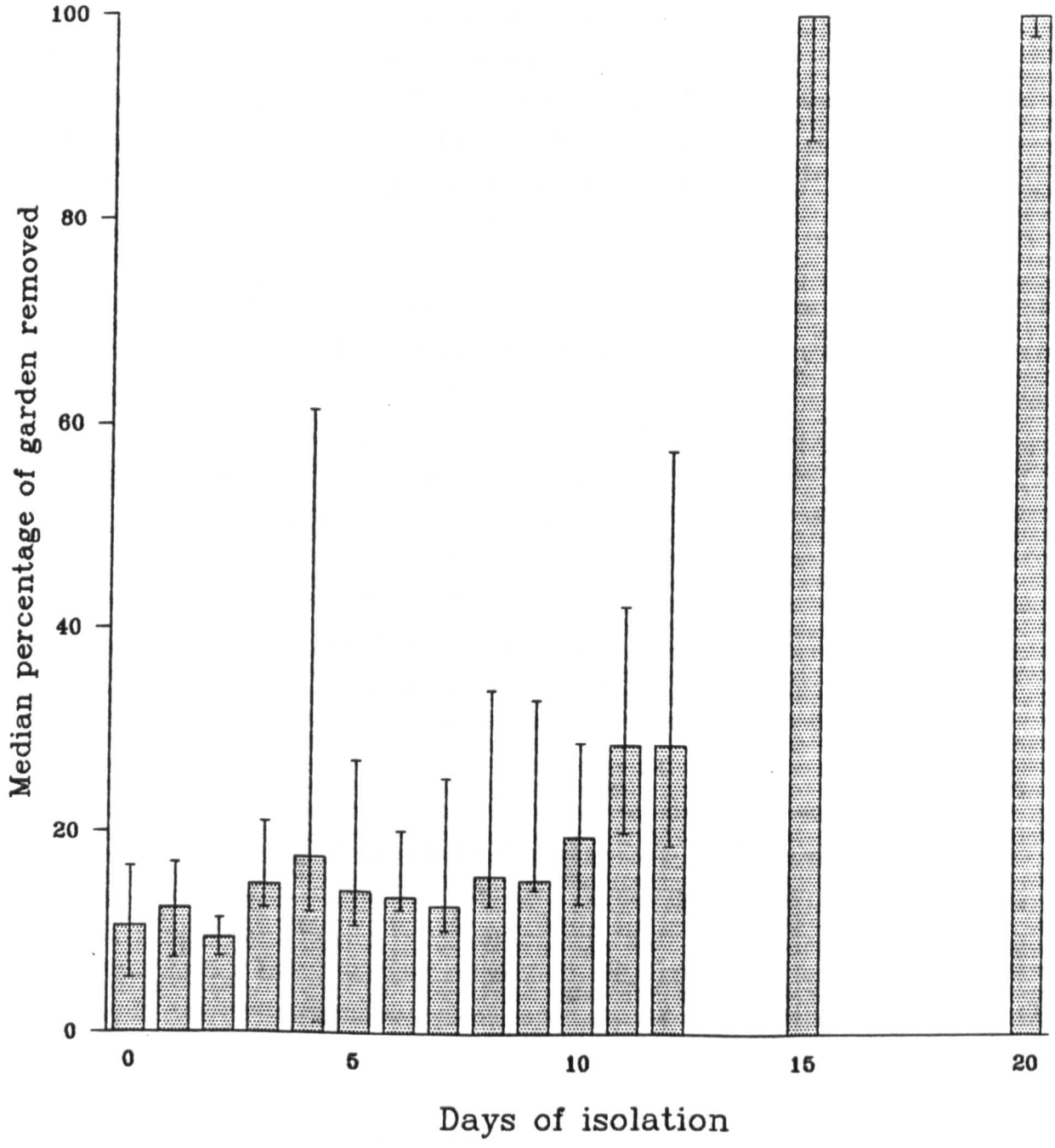


Figure 8.2: Median percentage weights (± 95% confidence limits) of fungus garden removed per petri-dish, after garden isolated from workers for between 0 and 20 days was re-exposed to them for 24 hrs (10 replicates).

antennal movement. With mouthparts retracted, they pulled out fragments of garden with their mandibles and carried them away. A few licked contaminant growths briefly and all workers encountering them paused frequently to groom their antennae and legs. Frequently, there were visible concentrations of workers on contaminated areas until these had been completely removed.

On garden samples isolated from workers for 12-15 days, the amount of contamination developing increased with time, leading to rejection by workers and to more than 90% subsequent weight loss of garden. Small areas of garden remained healthy in a few replicates even after 20 days of isolation although in the majority, all garden was contaminated by day 15 (Fig. 8.2).

Two replicates isolated for only 4 days were unfortunately completely contaminated and discarded by workers, but such total contamination was not found again until after 8 days of isolation.

2. The origin of contaminants developing on the garden

The insecticide killed all workers within 24 hrs and 10 days after this, 40% of the garden in one dish was contaminated with an alien mycelium. After 12 days, 70% of the garden in this dish was covered by a green fluffy mycelium, another was 50% contaminated and six other dishes also contained small contaminant colonies, covering less than 5% of the garden area. After 15 days, all ten dishes showed some contamination; two were 100% contaminated, one was 30% contaminated, four were between 5 and 20% contaminated and three were less than 5% contaminated.

Some contaminant fungus colonies developed around worker corpses but many appeared directly on fungus garden where no dead workers were present, indicating that the parent spores were present in the original garden. Colonies of contaminant fungi usually spread all over the garden

within the affected dish very quickly (within 24 hrs), but some spread more slowly.

These results were similar to those obtained in the previous experiment. They also indicated that either suppression of alien fungal growth occurs in the garden or that germination is a very slow process. The former is more likely since fungal spores germinate rapidly under suitable conditions. These results also suggested that contaminants developing on fungus garden isolated from workers were not necessarily introduced during handling, assuming that the insecticide was not contaminated.

Contaminant colonies were identified and included yeasts, *Aspergillus, Cladosporium, Fusarium, Mucor and Penicillium.* Bacterial growth was also likely since some samples of garden isolated from workers for several days smelled strongly of ammonia.

3. <u>The role of airborne contaminants</u>

Ten petri-dishes of ant-free garden were left to stand open for 1 hr in the high humidity of the ant-culture room. This allowed spores of alien organisms to fall on to the garden. A further ten dishes were sealed at once. The times taken for 100% contamination to develop on the gardens were then recorded.

Contaminant colonies developed significantly later on hygienically isolated garden than on garden exposed to air for 1 hour ($p<0.01$, ANOVA). Contaminants developed after a mean of 14.4 (SE ± 1.0) days on the former, but after only 8.1 (SE ± 1.3) days on the latter. Airborne spores were therefore an important factor in the development of contaminants on ant-free garden, but colonies still took at least 8 days to develop, indicating that there was suppression of alien growth on the garden. However, the suppression was more effective on hygienically isolated garden which contained fewer spores, introduced while the

ants were present. On exposed garden, there were more spores, which were introduced while the ants were absent. This suggested that the suppression effect acted most strongly when workers were present on garden when the alien spores were introduced; the suppression effect originated from the workers.

4. Growth rates of an alien fungus colony on garden isolated from workers for different periods

When isolated garden in petri-dishes was contaminated with *Mucor*, all dishes contained small sporangia-producing colonies within 24 hrs, no matter how long garden had been isolated. After 48 hrs, these colonies covered up to half of the fungus garden in each petri-dish and after 72 hrs, all dishes were 100% contaminated. There were no visible differences between garden isolated from workers for different periods, suggesting that the suppression effect acts only on spores introduced to the garden when workers are present.

5. The results of deliberately contaminating fungus garden

Introducing spores of both biotrophic and saprophytic fungi caused great excitement among workers and visible clusters of workers licking the treated areas developed during the first 2 minutes after spore introduction. These clusters had a mean size of 11 (SE ± 0.6) workers and covered areas of around 1 cm^2. They disappeared after about 8 minutes. Swabbing one of the treated areas with a clean paint-brush and mounting the material picked up in cotton-blue in lactophenol showed that very few fungal spores remained. No spores were found when an untreated area was examined similarly, although no quantitative observations were made.

Ten minutes after the application of benomyl droplets, workers were imbibing them. The ants appeared to find the

solution distasteful, backing or running away with rapid antennal movement after initial contact, then returning.

Introducing refuse loads also elicited a response from workers. They carefully examined pieces of refuse with their antennae, then carried them away, down through the garden, presumably to be dumped with the rest of the refuse. On young garden, mean time to removal was 6.7 (SE ± 0.8) minutes, on mature garden it was 6.4 (SE ± 0.7) minutes and on aging it was only 1.7 (SE ± 0.2) minutes; this was a significantly shorter time than on young areas ($p<0.05$, ANOVA and Tukey).

6. The precision of removal of contaminants from the fungus garden

a) When refuse is introduced into the fungus garden

When refuse was mixed with young garden, workers rushed around in panic for approximately 5 minutes. Then, they began to rearrange the fragments of young garden and separate out the refuse. Garden fragments were licked assiduously and placed in surrounding areas of undisturbed garden while refuse was carried down through the garden, presumably to be dumped. However, no fungus garden was carried out of sight. After 15 minutes, all that remained was a crater where the mixing had taken place.

b) Weight loss of contaminated garden after exposure to workers

After re-exposure to workers, there were no significant differences between the amounts of healthy garden removed in the *Penicillium*-contaminated and uncontaminated control replicates ($p>0.05$, ANOVA). The former lost a mean of 12.1% (SE ± 1.7) of their original weight while the latter lost a mean of 16.9% (SE ± 2.9). This was comparable with the amount of weight loss from garden isolated from workers for 24 hrs in Fig. 8.2. Contaminated garden was completely removed from the test replicates.

These two experiments therefore showed that workers were extremely efficient at removing contaminants from their fungus gardens.

DISCUSSION

Earlier results (Chapter 7) showed that if garden is isolated from workers for several days, it is subsequently very attractive to them. This attractiveness declines after 6 days isolation but only contaminated garden is removed; workers continue to tend garden isolated from them for up to 12 days. However, Fig. 8.2 suggests that major contaminant growth does not develop until after 12 days. If the 'isolation effect' described in Chapter 7 were due to a response to contaminant growth, then it should theoretically increase to a maximum just before garden is completely contaminated (after 12 days), which it does not. However, this does not mean that licking is not important for hygiene. Leaf material entering the nest is scrupulously licked clean to remove waxes and contaminant spores (Quinlan 1977). Ejected infrabuccal pellets contain detritus licked from worker bodies during grooming, along with fungal spores (see Chapter 6). Fungus garden licking may fulfil more than one role, which would be an efficient way of performing more than one task at once.

It was surprising that the development of contaminant fungi took so long on garden separated from workers. The opportunist saprophytes found as contaminants are usually quick to develop to take advantage of resources. Powell (1984) found contamination by *Phialocladus zsoltii* within a few days and *Mucor*, a typical contaminant, germinates within hours and will completely colonise a 10 cm culture plate within 3 days (Ingold 1973).

This suggests that there is a suppressive effect on spore germination in the garden, particularly since many of the spores which eventually germinated and produced colonies were initially present in the garden, rather than

being introduced during the handling process. Isolating fungus garden unhygienically showed that contaminant colonies overwhelmed garden after 8 days, compared to more than 12 days for hygienically isolated garden. This is still quite a long time for a contaminant spore to take to germinate and colonise the garden. However, it does suggest that the suppressive effect works best on spores which entered the garden when workers were present.

This indicates that spores are suppressed by the myrmicacin and other substances produced by the metathoracic glands of workers (Schildknecht and Koob 1970, 1971, Schildknecht *et al.* 1973) or by antibiotics produced by the ant fungus itself (Hervey and Nair 1979, Angeli-Papa, 1984). Once the ants are removed, this suppression effect seems to last for a while, but eventually declines.

The contaminant fungi found were all common saprophytes recorded by previous authors (Weber 1972, Sihanoth *et al.* 1973, Powell 1984), found ubiquitously in soil and in the 'phylloplane'; on leaf surfaces (Cooke and Rayner 1984). These fungi are therefore likely to be brought into Attine nests with leaf substrate and although licking removes significant amounts of contaminant spores from leaf surfaces (Quinlan 1977), a few are likely to be missed.

When fungus garden was deliberately contaminated, the workers showed great precision in removing it and almost no healthy fungus garden was discarded. Workers were able to recognise nest refuse placed on to garden very rapidly, suggesting that they respond to chemical cues. Jaffe (1986) suggested that volatile chemicals are important in refuse recognition.

In the field, gardens are constructed in underground chambers in soil and contaminant spores might be more common than under laboratory conditions. Fungus garden is a valuable resource and the ability to remove alien material without losing uncontaminated material would be beneficial. Fungus garden represents the result of a

long chain of physiologically expensive behavioural acts, including initial foraging, substrate preparation and care of the garden. Workers have deposited large amounts of nitrogen-rich faeces (Martin and Martin 1970b) plus salivary secretions on it (Weber 1972). In the field, forage may not be freely available at all times of the year, such as during the dry season, therefore if a small area of garden becoming contaminated led to the ants dumping large quantities of garden, the fitness of the nest would suffer. Natural selection would therefore favour nests which could deal efficiently with contaminants. However, this precision of removal means that any attempt to control leafcutting ants by destroying their garden with pathogens would be very difficult. Any agent used would have to be unrecognisable to workers as 'something undesirable' or repulsive. Furthermore, it would have to be introduced into all fungus gardens in the nest simultaneously, or workers would simply remove dying areas before contamination could spread. However, a combination of a pathogenic agent to kill the garden and an insecticide to knock down the worker population and reduce the level of garden care would probably be effective.

SUMMARY

Fungus garden could be isolated from workers for up to 12 days before major contaminant growth occurred, which contrasted with the 6 days isolation maximum for the 'isolation effect' (Chapter 7). Contaminant fungal spores were present in fungus garden before workers were removed but did not germinate, suggesting that they were inhibited by chemicals produced by workers.

When fungus garden was deliberately contaminated, workers rapidly and precisely removed alien materials, with very little loss of garden.

Chapter 9: Pruning hyphae as a reason for workers licking the fungus garden

INTRODUCTION

Pruning is a common horticultural strategy for diverting resource allocation in a plant, which is usually used to manipulate or improve fruit or flower production. Similarly, in *Phytophthora infestans*, an Oomycete Pseudofungus, successive crops of spores can be induced by harvesting (Ferguson 1986). Stimulation of growth may also occur when hyphae are lightly grazed by, for example, Collembola. The pruned hyphal tips branch and grow at a faster rate (Hanlon 1981). Van der Drift and Jansen (1977) also concluded that Collembola grazing on fungi growing on nematode faeces stimulated fungal growth and respiration. Pruning the ant fungus might induce it to produce more nutritive staphylae, which are bundles of swollen hyphae or 'gongylidia'.

In the Eumycota, which includes the Basidiomycotina and hence the ant fungus (since it possesses characteristic dolipore septation; Powell 1984), branches arise at a considerable distance behind the hyphal apex, suggesting some form of apical dominance (Webster 1970). Cutting or pruning hyphae may therefore remove this apical dominance and stimulate branching, and this in turn may lead to differentiation and the production of more staphylae.

This chapter examines the licking of garden by workers as possible pruning to remove excess hyphal growth and to encourage the production of more staphylae.

MATERIALS AND METHODS

All experiments were carried out using workers and fungus garden from a large *Atta sexdens* nest (>80 gardens). Petri-dishes (5 cm) filled with ant-free garden (see Chapter 2, page 15) were used and were maintained in boxes with close-fitting lids, which contained filter paper moistened with distilled water, for high humidity. These were placed in the culture rooms to ensure constant high temperature.

1. Method used to count staphylae numbers in petri-dishes of garden

The numbers of staphylae present on the garden surface in petri-dishes were assessed by drawing grids on the dish lids and counting staphylae within them, using a binocular microscope. The grid ensured that different parts of the petri-dish were not missed or examined twice. When more than one dish was being assessed, the dishes were mixed up and their labels covered to prevent unconscious bias.

2. Methods used to examine the persistence of staphylae on gardens

The relationship between 'total' crop production and 'standing' crop was unclear, since staphylae are continually removed by workers.

A sterile cellulose acetate sheet, with five 1 cm^2 holes cut in it, was placed over the ant-free garden within a petri-dish and fixed into position using adhesive tape attached to the edge of the dish. Every day until contaminant growth occurred, the staphylae present in these 1 cm^2 areas were mapped on to acetate sheets using fine marker pens. These 1 cm^2 'maps' were then transferred on to 5 x 5 cm areas of graph paper, which enlarged them and made following individual staphylae over time easier. The lifespans of individual staphylae could therefore be found.

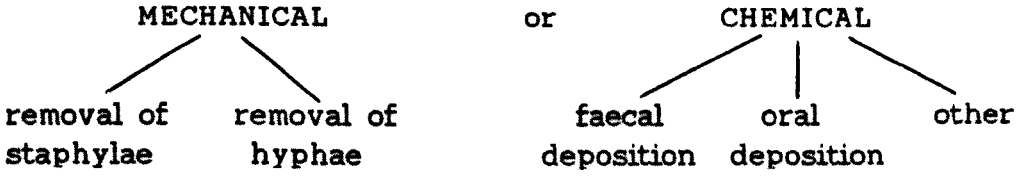
A second method of estimating staphyla persistence was also used; individual staphylae in a petri-dish of ant-free garden were marked by placing fragments of aluminium foil next to them. Their persistences were then recorded.

3. Examining individual staphylae

Staphylae were mounted in cotton-blue stain in lactophenol for microscopic examination. Diameters were measured using an eyepiece graticule and numbers of gongylidia per staphyla were assessed by counting the numbers visible in the outer circumference of each staphyla, since counting total numbers was impractical. The ratio of diameter to gongylidia numbers was then used as a measure of compactness.

4. Methods for simulating the effects of ants on garden

Several possible factors could affect staphyla production:



Attempts were made to simulate these factors. Twenty-one petri-dishes were filled with ant-free garden (see page 15) and sterile acetate sheets with five 1 cm^2 holes cut in them, were placed on top of this garden and held in place by adhesive tape, as before. These dishes were isolated in humid chambers for 2 days before treatment, after which seven different treatments were applied through the 1 cm^2 'windows', as follows:

(1) Staphylae were removed, using a mounted needle.
(2) Hyphae were cut by gently stroking the garden surface with the side of a mounted needle. Some staphylae were removed by this process.
(3) Faecal droplets were added to the garden by squeezing living worker gasters (media and maxima) on to it, at rates of 5 droplets per treatment area.
(4) Faecal droplets were applied at rates of 10 per treatment area.
(5) Worker heads (media) were crushed in 0.1 ml distilled water on a microscope slide and the resulting suspension was applied to garden, at the rate of 1 head per treatment area, using a fine syringe.
(6) Extracts of 3 heads per treatment area were applied.
(7) No treatment; control.

Fifteen replicates were used for each treatment, distributed at random through the petri-dishes. Standing crops of staphylae per dish were counted at the start, on day 2 before and after 'pruning' in treatments 1 and 2 and 2 days after treatment.

5. A method for assessing the number of faecal droplets on fungus garden

The presence of faecal droplets on the fungus garden surface was examined by gently pressing a circle of filter paper (Whatman No.1) on to it. Droplets were detected as brown spots on the filter paper, which could be counted.

6. A method of preventing faecal deposition by workers

When a worker is anaesthetized with carbon dioxide and held carefully by the thorax with forceps, gently squeezing the gaster with the side of a mounted needle expels the rectal contents without injury. The droplets can be collected on filter paper, where they produce brown spots varying in size, depending upon droplet volume. Once a worker has been treated in this way, it cannot immediately

produce more rectal fluid. Faecal droplet production by workers could therefore be examined by measuring the diameters of faecal spots produced on the filter paper, with a binocular microscope and eyepiece graticule and calculating total spot area.

RESULTS

1. Staphyla production on garden with and without workers

Twenty numbered petri-dishes were filled with ant-free garden and the numbers of staphylae present per dish counted. They were then placed in humid chambers for 2 days. Staphyla numbers per dish were then recounted and the lids of ten randomly selected dishes replaced by ones with 0.5 cm entrance holes. These were exposed to the parent nest for 3 hrs. They were then removed and the workers present anaesthetized with carbon dioxide and removed with forceps. The original lids were replaced and staphyla numbers per dish were recounted. The numbers of staphylae present per dish (standing crops) were recounted 2 and 4 days after treatment. By adding the numbers of staphylae lost during the exposure period, total crops could be calculated for the exposed dishes.

There were no significant differences between the numbers of staphylae present in test and control replicates at the start of the experiment ($p>0.7$, ANOVA), or on day 2 ($p>0.9$, ANOVA) but after test dishes had been exposed to workers for 3 hrs, numbers of staphylae were significantly lower in the exposed dishes than in the controls ($p<0.01$, ANOVA). However 2 days after exposure, there was a significantly higher standing crop in exposed dishes than in controls ($p<0.01$, ANOVA) and total crops were even higher (Fig. 9.1). Four days after exposure, there were no longer significant differences between standing crops on test and control replicates ($p>0.05$, ANOVA), but total crops were significantly higher on exposed replicates than on controls ($p<0.01$, ANOVA).

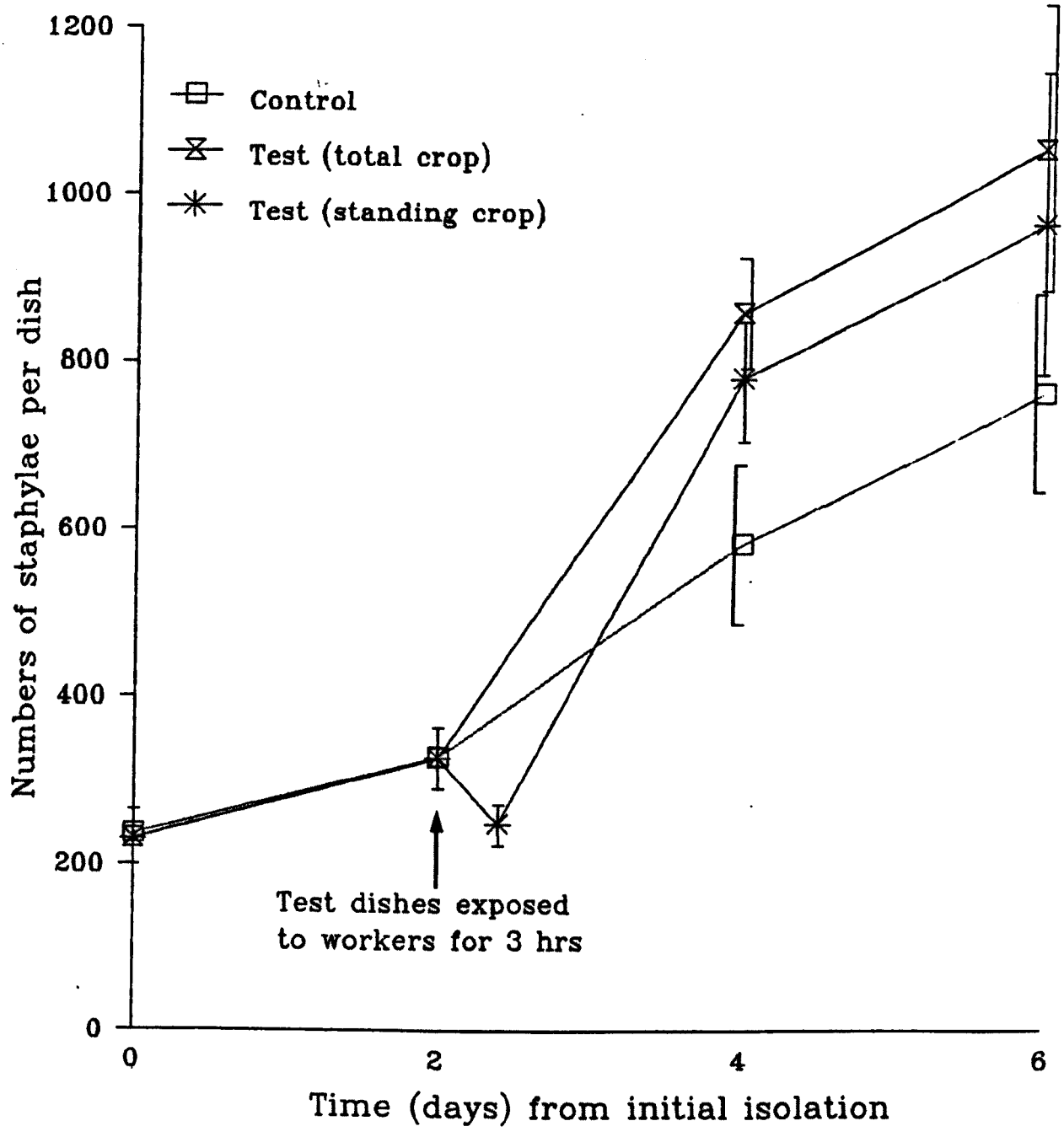


Figure 9.1: Mean standing and total crops of staphylae produced per 5 cm petri-dish of fungus garden isolated from workers, in dishes re-exposed to workers for 3 hrs after 2 days of isolation, compared with non-exposed control dishes (10 replicates).

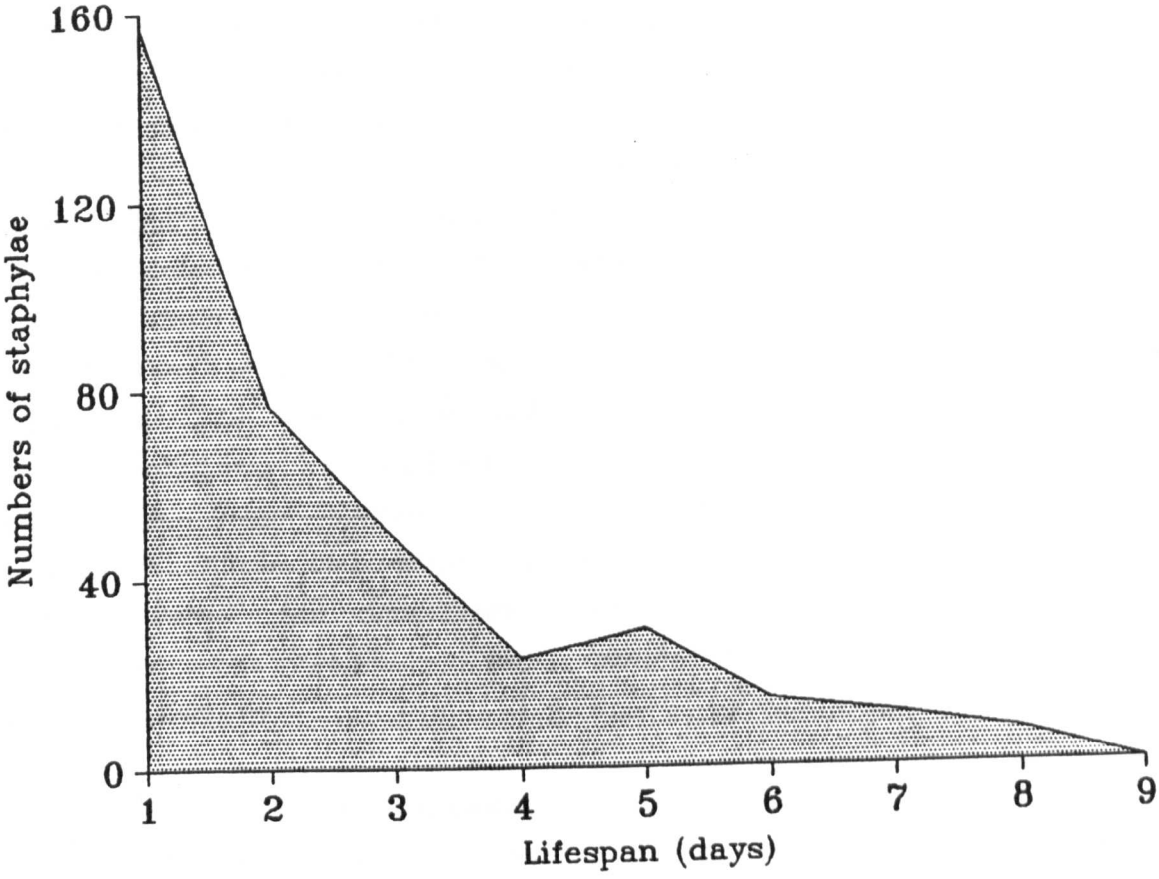


Figure 9.2: Distributions of the life-spans of staphylae (days) in a petri-dish of ant-free fungus garden (367 replicates).

2. The persistence of staphylae on the fungus garden

Mapping staphylae over time showed that they had a median persistence of 2 days, but small numbers persisted for up to 8 days (Fig. 9.2). Marking individual staphylae and observing them over time also showed a median persistence of 2 days. The data were not transformable, hence a Mann-Whitney test was used to show that there were no significant differences between the persistences obtained from the two methods ($p>0.1$).

Individual staphylae often arose very quickly, sometimes from large tufts of hyphae. However, many appeared where the mycelium was not particularly dense. They tended to disappear more gradually, frequently becoming yellowish, then water-soaked and finally disintegrating.

Some staphylae present on the garden surface may therefore persist for another 2 days, but those that decay may release resources back to the mycelium, fuelling further staphylae production. If staphylae are removed, these resources are lost. Both standing and total crop were therefore used to express the experimental results.

3. The effect of different periods of ant access on the yield of staphylae

When ant-free garden, isolated for 24 hrs, is re-introduced to workers, they rush on to it and lick it. After 3 hrs, numbers licking begin to decrease and hyphal depths have been significantly reduced (see Chapter 7). If pruning hyphae is important for staphyla production, then the major effect should take place within the first 3 hrs of re-exposure to workers. Longer periods of re-exposure should produce similar crops to 3 hr periods.

This hypothesis was tested using the same procedure as before. Thirty petri-dishes filled with ant-free garden were isolated for 2 days, numbers of staphylae per petri-dish being recorded initially and at the end of this time. Then, ten dishes were exposed to workers for 3 hrs, ten

were exposed for 6 hrs and the remaining ten were not exposed at all. Workers were removed, staphyla numbers recounted and again recorded 2 and 4 days after treatment.

There were no significant differences between the standing crops of staphylae in the two treatments after 2 days of garden isolation ($p>0.8$, ANOVA) and there was a collective mean of 365 (SE ± 6.0) staphylae present in each of the dishes. Staphyla numbers were however significantly reduced by 3 or 6 hrs of exposure to workers ($p<0.01$, ANOVA) compared with the unexposed controls. Three hrs of exposure reduced staphyla numbers to a mean of 279.1 (SE ± 9.0) while 6 hrs exposure reduced them to 284.4 (SE ± 9.2), while the unexposed controls had 380 (SE ± 8.1) per dish. Two days after exposure, standing crops were significantly higher on all exposed replicates compared to the controls ($p<0.05$, ANOVA) as were total crops ($p<0.01$, ANOVA). Tukey's multiple comparison showed that standing or total crops in replicates exposed to workers for 3 or 6 hrs were not significantly different ($p>0.05$). The majority of the effect therefore took place during the first 3 hrs of exposure to workers (Table 9.1).

Table 9.1: Mean numbers of staphylae present per petri-dish (± SE) 4 days after the removal of workers. After 2 days, the gardens were re-exposed to workers for either 0, 3 or 6 hours, (10 replicates).

HOURS OF RE-EXPOSURE TO WORKERS AFTER 2 DAYS	MEAN NO'S OF STAPHYLAE PER DISH (± SE) AFTER 4 DAYS	
	STANDING CROP	TOTAL CROP
0	943.0 ± 44.1	943.0 ± 44.1
3	1190.7 ± 35.0	1264.2 ± 44.5
6	1216.8 ± 38.6	1282.4 ± 50.3

4. Simulating the effects of ant access to gardens on the yield of staphylae

Some replicate areas became contaminated but at least ten remained for each of the seven treatments. There were no significant differences in staphyla numbers between replicate areas at the start and there was a collective mean of 7.9 (SE ± 0.3) staphylae per treatment area ($p>0.8$). Similarly, there were no significant differences after 2 days, before treatment ($p>0.8$, ANOVA), while mean numbers of staphylae per treatment area had risen to 11.9 (SE ± 0.3). Treatment 1 (removing staphylae) significantly reduced staphyla numbers present ($p<0.01$, ANOVA), down to a mean of 0.3 (SE ± 0.1) per treatment area. Treatment 2 (cutting hyphae) also significantly reduced the numbers of staphylae present ($p<0.01$, ANOVA), down to 8.2 (SE ± 0.6).

Two days after treatment, significant differences were present between treatment areas ($p<0.05$, ANOVA and Scheffe's test). Standing crops of staphylae were higher on areas where hyphae were cut, faecal droplets applied (5 per cm^2) and in control areas, compared with other treatments (Table 9.2). When total crops were compared, a similar relationship was obtained ($p<0.05$, ANOVA), with these three treatments producing the largest subsequent crops, along with treatment 1 (removing staphylae).

These results suggested that large amounts of oral or faecal material may retard fungal growth rather than promote it.

Table 9.2: Mean standing and total crops of staphylae (± SE) on 1 cm^2 areas of garden, 2 days after the application of seven treatments. Non-significantly different means are joined by vertical lines ($p>0.05$, ANOVA and Scheffe's multiple comparison, 10-12 replicates).

TREATMENT NO.*	MEAN STANDING CROP ± SE	TREATMENT NO.	MEAN TOTAL CROP ± SE
3	40.1 ± 3.3	2	40.5 ± 2.2
7	37.8 ± 1.7	3	40.1 ± 3.3
2	35.5 ± 2.0	7	37.8 ± 1.7
5	24.7 ± 3.4	1	33.5 ± 1.2
1	22.9 ± 1.2	5	24.7 ± 3.4
4	20.2 ± 1.6	4	20.2 ± 1.6
6	15.1 ± 1.6	6	15.1 ± 1.6

* Treatment 1 - Removal of staphylae
2 - Hyphae cut
3 - Faecal droplets, 5 per treatment area
4 - Faecal droplets, 10 per treatment area
5 - Head extract, 1 head per treatment area
6 - Head extract, 3 heads per treatment area
7 - No treatment; control

Because of doubts about the validity of such small treatment areas, the experiment was repeated on larger areas. Petri-dishes containing garden were divided into quarters by placing two 1 mm wide strips of cellulose acetate on to the garden surface, at 90^0 to each other. Each quarter had an area of 4.9 cm^2 and acted as a single treatment area. Twelve replicates were used, distributed at random through dishes. Treatments 1, 2, 3, 5 and 7 (above) were applied and staphyla numbers per treatment area were counted at the same times as before.

A few replicate areas again became contaminated, but at least ten remained for each treatment. There were no significant differences in staphyla numbers between

different treatment areas at the start ($p>0.8$, ANOVA) and each area had a mean of 36.8 (SE ± 3.9) staphylae. Similarly, there were no significant differences after 2 days isolation ($p>0.4$, ANOVA), while each area now had a mean of 134.1 (SE ± 85.8) staphylae. Treatment 1 significantly reduced the numbers of staphylae present per replicate area ($p<0.01$, ANOVA) down to 7.0 (SE ± 0.9). Treatment 2 also significantly reduced the numbers of staphylae present ($p<0.01$, ANOVA), down to 26.6 (SE ±1.7).

Three days after treatment, there were significant differences between standing crops and between total crops($p<0.05$, ANOVA and Scheffe's multiple comparison). Removal of hyphae and application of faecal droplets had the most effect (Table 9.3) whereas for total crops, removal of staphylae and hyphae had the most effect.

Table 9.3: Mean standing and total crops of staphylae (± SE) per petri-dish (5 cm), 3 days after the application of five treatments. Non-significantly different means are joined by vertical lines ($p>0.05$, ANOVA and Scheffe's multiple comparison, 10-12 replicates).

TREATMENT NO. †	MEAN STANDING CROP ± SE	TREATMENT NO.	MEAN TOTAL CROP ± SE
2	201.4 ± 8.6	2	222.8 ± 8.5
3	179.4 ± 4.2	1	212.3 ± 6.3
1	172.0 ± 5.9	3	179.4 ± 4.2
5	169.0 ± 6.3	5	169.0 ± 6.3
4	163.3 ± 7.0	4	163.0 ± 7.0

† Treatment 1 - Removal of staphylae
2 - Hyphae cut
3 - Faecal droplets, 5 per cm^2
4 - Head extract, 1 per cm^2
5 - No treatment; control

In the first experiment (page 186) 3 hrs ant-access led to a 1.3 times increase in standing crop by day 4, compared with control areas. In this simulation experiment, the standing crop in replicates where hyphae and staphylae were mechanically removed were 1.2 times the control yield by day 5. However, mechanical hyphal pruning may cause more damage, initially retarding rather than stimulating growth.

5. The effect of faecal material on the yield of staphylae on ant-free garden

a) The deposition of faecal droplets on garden isolated for 2 days, after re-exposure to workers

If faecal droplets are important in stimulating staphyla production, then workers presented with garden which had been kept free of ants for the previous 2 days might attempt to remedy the lack of fresh faecal material by defaecating freely on it. The subsequent number of faecal drops on the garden would then be greater than similar ant-free garden to which the ants had not subsequently been given access.

Twenty petri-dishes (5 cm) were filled with ant-free garden and isolated in humid chambers for 2 days. Then, standing crops of staphylae in each dish were counted, along with the numbers of brown spots picked up on filter paper applied to each. Ten dishes were then exposed to the parent nest for 3 hrs. The presence of faecal droplets and the numbers of staphylae remaining were then assessed. Staphyla standing crops were also assessed 2 days later.

After 2 days of isolation, the numbers of staphylae on test and control gardens were similar ($p>0.7$, ANOVA), with means of 184.4 (SE ± 12.9) and 190.0 (SE ± 7.5) each respectively. Two days after treatment, both the standing and the total crops of staphylae were greater in the treated gardens exposed to workers for 3 hours, than in the unexposed controls ($p<0.05$, ANOVA). The mean standing and total crops in the treated areas were 553.2 (SE ± 36.6) and

610.4 (SE ± 37.1) respectively, while the standing crop in the controls was only 435.6 (SE ± 93.8) This confirmed the earlier results.

The number of faecal spots present as detected by the filter-paper pressing technique, did not differ significantly between test and control gardens before exposure. A mean of 86.1 (SE ± 7.7) were present on test gardens and 84.6 (SE ± 3.8) were present on control gardens. After test dishes had been exposed to the ants, there was no significant increase in the numbers of faecal spots detected ($p>0.6$, ANOVA) and test dishes now had a mean of 89.3 (SE ± 7.1) spots. This was borne out by the observation that during the 3 hour exposure period, no workers were seen to defaecate on the garden.

b) The result of preventing faecal deposition by workers on fungus garden

To find out how quickly the evacuated rectum of a worker refilled, groups of 30 media workers (headwidths 1.4-1.6 mm) were evacuated on to filter paper and then placed in petri-dishes (9 cm) with 10 cm^3 of fungus garden and damp filter paper. Different groups were left in these dishes for nine different periods; 0 to 8 and 24 hrs. Workers of constant size were used to promote uniformity between groups and this size group was used for ease of handling. These workers were then re-anaesthetized and their rectal contents collected again. Five groups of workers were used for each isolation period and all groups had their recta evacuated on to separate pieces of filter paper. Faecal droplet production was examined by measuring the diameters of faecal spots on filter paper, to calculate total spot areas produced by 30 workers.

Groups of 30 workers each produced 29 or 30 faecal spots with a mean total area of 62.3 (SE ± 6.5) mm^2. Immediately afterwards, the amounts of faecal material which could be squeezed out of worker gasters were very small. After 6 hrs however, faecal production by squeezing

gasters began to approach the normal pre-evacuation level (Fig. 9.3). After 24 hrs, faecal droplet production was similar to this normal level, in spite of possible trauma and injury to some workers. At least 6 hrs were therefore required for a worker rectum to refill after evacuation.

This technique was used to examine the effects of exposing isolated garden to workers without their introducing faecal material. Thirty petri-dishes were filled with ant-free garden and maintained for 2 days in a humid chamber. Then, groups of 50 media and minima workers were removed from fungus garden, anaesthetized with carbon dioxide and the rectum of each was gently evacuated. Ten groups of workers were placed in petri-dishes of garden with known numbers of staphylae. A further ten dishes of garden were left ant-free to act as controls. Groups of 50 intact anaesthetized workers were placed in the remaining ten dishes. One hour was allowed for workers to recover from anaesthesia and disturbance followed by 3 hrs to allow them to lick the isolated garden. Then they were removed from the petri-dishes and staphylae were recounted. Standing and total crops were assessed 2 days after treatment.

When intact and evacuated workers were introduced to petri-dishes of fungus garden, the majority initially groomed themselves and each other, but after about 30 minutes many settled down and either licked garden or ate staphylae. There were no significant differences in the number of staphylae in the three sets of treatment areas on day 2 before the exposure period ($p>0.2$, ANOVA) and each area had a mean of 188.2 (SE ± 22.2) staphylae. Exposure to both intact and evacuated workers caused a significant decrease in staphyla numbers per dish ($p<0.01$, ANOVA), although there was a significantly greater reduction with intact ants (down to 115.5 (SE ± 8.1) staphylae per area) than with evacuated ones (down to 138.7 (SE ± 10.6) staphylae per area, $p<0.05$, ANOVA), reflecting trauma and possible injury in the latter.

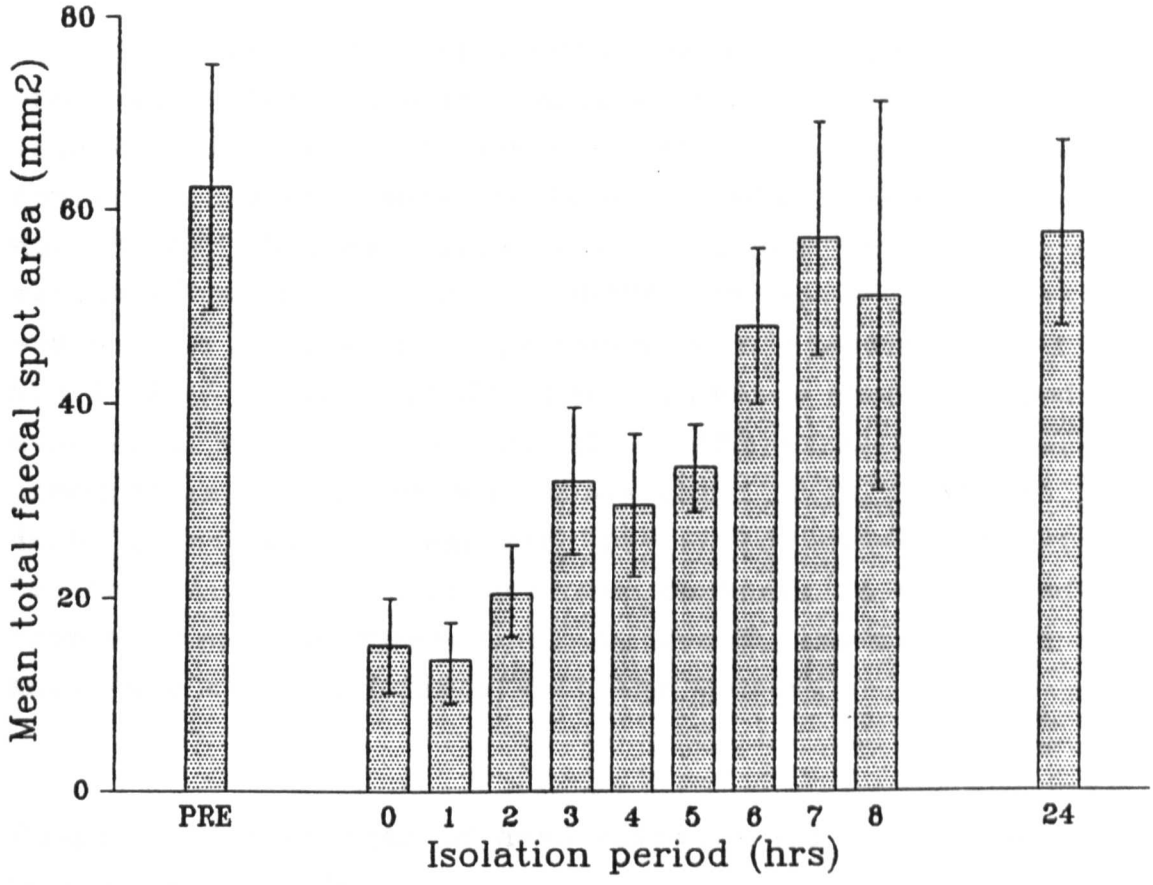


Figure 9.3: Mean production of faeces expressed as the total area (mm^2) of spots on a filter paper (± 95% confidence limits). These were squeezed from groups of 30 media workers which had previously had their rectums evacuated and the original faecal production is shown as 'PRE'. Groups of workers were isolated in petri-dishes for 0-24 hrs (5 replicates).

Two days after treatment, mean standing and total crops were significantly higher in petri-dishes exposed to intact and evacuated workers, compared to non-exposed controls ($p<0.05$, ANOVA and Tukey). However, mean crops did not differ between replicates exposed to intact and evacuated workers ($p>0.05$, ANOVA and Tukey). Replicates exposed to intact ants produced mean standing crops of 523.8 (SE ± 33.8) staphylae per dish while those exposed to evacuated workers produced 507.6 (SE ± 39.8). Controls produced mean crops of only 404.4 (SE ± 17.2) staphylae per dish. Temporarily re-exposing garden to workers therefore led to a 1.3 times increase over controls in the standing crop of staphylae subsequently produced, whether or not the ants were able to defaecate on the fungus.

6. Comparing the effects of head extracts and water droplets on staphyla yield

When the fungus garden is treated with liquid extracts in distilled water, any effects may be due to water-logging and this possibility was examined. Ten petri-dishes of ant-free garden were divided into quarters using sterile cellulose acetate cross-pieces, each quarter being used as a treatment area (4.9 cm^2). After 2 days of garden isolation, the following treatments were applied, with 10 replicates for each, distributed at random through the dishes.

(1) Head extract, at a rate of 1 head per cm^2.
(2) Head extract, at a rate of 5 heads per cm^2.
(3) Distilled water, at a rate of 0.6 ml per test area, applied with a fine syringe as 10 µl droplets.
(4) No treatment; control.

Head extracts were made by grinding 50 and 250 heads respectively for the two concentrations in a small glass mortar with distilled water. This produced 0.6 ml and 0.8 ml of the two concentrations and these liquids were drawn into fine syringes and divided equally between the 10

replicate areas. Staphyla standing crops were assessed on day 2 before treatment and 2 days after treatment.

There were no significant differences between the four sets of treatment areas after 2 days of isolation ($p>0.7$, ANOVA) and replicate areas had a mean of 88.2 (SE ± 1.7) staphylae. Two days after treatment, there were still no significant differences between treatment areas, but p was 0.051 (ANOVA), indicating that a significant difference was almost present. Applying water droplets did appear to depress staphylae production slightly (Table 9.4).

Table 9.4: Mean numbers of staphylae (± SE) per treatment area (4.9 cm^2), 2 days after the application of four different treatments. Means were not significantly different ($p>0.05$, ANOVA, 10 replicates).

TREATMENT	MEAN NO'S OF STAPHYLAE PER TREATMENT AREA (± SE)
Head extract, 1 head per cm^2	192.4 ± 4.6
Head extract, 5 heads per cm^2	189.5 ± 6.0
10 µl water droplets	168.5 ± 7.3
No treatment; control	189.0 ± 7.7

7. <u>Differences between staphylae on garden exposed or not exposed to workers</u>

Garden which has been isolated from workers for several days appears whiter than normal and staphylae sometimes appear 'diffuse'. Staphylae from non-isolated mature garden were compared with staphylae from garden isolated from workers for 7 days and with garden isolated from workers for 4 days with and without 3 hrs re-exposure to workers after 2 days. Biomasses of staphylae produced on different garden types were not compared, since collecting staphylae from the garden surface is a slow process and

many staphylae may be lost or missed. More than 30 staphylae were examined for each garden type.

Staphyla diameters were significantly larger on garden isolated from workers for 7 days than on garden isolated for 4 days (with or without 3 hrs ant access) or on non-isolated mature garden ($p<0.05$, ANOVA and Scheffe) (Table 9.5). Staphyla diameters were not significantly different between these other garden samples ($p>0.05$, ANOVA and Scheffe). Comparing the numbers of gongylidia in the circumference of each staphyla showed similar relationships ($p<0.05$, ANOVA and Scheffe, see Table 9.5).

Ratios of staphyla diameters to gongylidia numbers provided a measure of compactness. However, the rate at which diameter increases will be slower than the rate at which gongylidia numbers increase, when staphyla size increases. This is because diameter is a two-dimensional measure, while the number of gongylidia in the circumference is related to staphyla volume, a three-dimensional measure.

These ratios were significantly larger for staphylae on garden isolated for 4 days (but not exposed to workers for 3 hrs) than for staphylae on other garden samples ($p<0.05$, ANOVA and Scheffe) which were not significantly different to each other ($p>0.05$, ANOVA and Scheffe, see Table 9.5).

Table 9.5: Mean diameters and numbers (± SE) of gongylidia present in the outer circumference of staphylae removed from four samples of differently treated garden. The ratios of these two measures are also shown (30-40 replicates).

GARDEN STATUS[†]	MEAN STAPHYLA DIAMETER (mm) (± SE)	MEAN NO'S OF GONGYLIDIA PER CIRCUMFERENCE (± SE)	MEAN RATIO OF DIAMETER TO GONGYLIDIA NO'S (± SE)
1	0.45 ± 0.01[a]	37.7 ± 1.1[a]	0.95 ± 0.03[a]
2	0.44 ± 0.02[a]	28.5 ± 1.7[b]	1.35 ± 0.11
3	0.39 ± 0.02[a]	33.2 ± 1.8[a,b]	0.98 ± 0.04[a]
4	0.79 ± 0.04	61.9 ± 2.8	1.04 ± 0.03[a]

† 1 - Mature garden (not isolated)
2 - Garden isolated for 4 days
3 - As above, with 3 hrs of worker exposure after 2 days
4 - Garden isolated for 7 days

[a], [b] Statistically non-significant means ($p>0.05$, ANOVA and Scheffe).

DISCUSSION

The presence of workers on fungus garden appeared to stimulate the production of staphylae. This might have been due to faecal deposition, since worker faeces contain nitrogenous material such as allantoin, ammonia and amino acids, plus proteolytic enzymes (Martin and Martin 1970b). However workers allowed on to fungus garden were not seen to defaecate on it and experiments with workers unable to defaecate showed that the increases in staphyla yields compared with controls were not due to faecal deposition.

Substrate incorporated into the garden receives faecal applications from workers (Quinlan and Cherrett 1977). The ant fungus lacks the enzymes necessary to degrade substrate

proteins efficiently and the application of faecal material compensates for this metabolic deficiency. The short term needs of the newly-planted hyphae are supplied by the ammonia and mixture of amino acids in the faeces (Martin and Martin 1970b). Hence, the deposition of faecal fluid is vital for the establishment and growth of the mycelium on young garden, but has little effect on the numbers of staphylae produced on mature garden.

Weber (1947, 1966) believed that substances present in the salivary glands and anal secretions probably serve to create environmental conditions favourable to the growth of the ant fungus. A number of glands, including the labial, mandibular, maxillary and postpharyngeal glands are associated with ant mouthparts and these might produce growth-promoting substances. In leafcutting ants, the labial glands produce chitinase (Febvay *et al.* 1984), while the mandibular glands produce alarm substances like citral and 5-methyl-3-heptanone (Holldobler and Wilson 1990). In many ant species, the postpharyngeal gland is the source of larval food and in *Atta sexdens* this gland is disproportionately large in the smallest workers, which suggests that workers either feed larvae by regurgitation or produce something which they apply to fungus garden (Holldobler and Wilson 1990). However there is no evidence in the literature for any growth-promoting substances produced in Attine head glands and crude head extracts in experiments described in this chapter failed to have any effect upon staphyla production. However, if growth-promoting substances are present in glandular secretions, their effects may have been cancelled out by other substances, since no attempts were made to separate the contents of the different glands. Artificial 'pruning' of hyphae and staphylae with a mounted needle produced almost as many staphylae as when garden was briefly exposed to workers, which might have applied salivary growth-promoters. This suggests that the effect was purely mechanical rather then chemical, although it must be stressed that chemical aspects of worker exposure to garden

may still have important roles, such as protecting the garden against contamination by alien microorganisms.

If workers prune the mycelium to encourage the growth of staphylae, then there remains the problem of how they recognise incipient staphylae and allow them to develop, since they grow from tufts of hyphae. Similarly, hyphal tufts are required for use as inocula on freshly-inserted substrate. Is there some stimulus that tells a licking worker to avoid licking a tuft of hyphae which will, given time, develop into a staphyla? This could be explained by the presence of repellents in the early stages of staphylae. However, this seems unlikely, since the ants would probably react to a repellent area of mycelium in the same way as they do to contaminants and remove it. Also, it is unlikely that the fungus could localize its repellency into particular areas or ages of mycelium. However, the 'mapping' experiment showed that staphylae often arose overnight and frequently in places where no large hyphal tufts had been previously. They may therefore 'escape' worker attention by rapid development, plus hyphae growing close to the substrate surface will shield incipient stages of staphylae.

Staphylae do arise without any pruning taking place and in artificial culture on agar media, the presence of gongylidia is the most important identifying feature of the ant fungus. This production of staphylae by fungus which has never been exposed to workers illustrates the high degree of coevolution that has occurred. The experiments described in this chapter were carried out on naturally-produced fungus garden, to more closely reflect the true relationship between the workers and their fungus. However it would be also useful to test the effect of hyphal pruning on ant fungus cultures grown on agar.

In the fungus garden, hyphae have to penetrate plant tissues and are continually being consumed by the workers. In an agar medium there are no such problems for the fungus and in an ideal medium, it may be less stressed than when

growing in a garden. In the garden, pruning of hyphae may stimulate staphylae production, since the fungus, in an attempt to avoid being completely eaten, might provide more staphylae to distract workers from consuming hyphae needed to penetrate fresh substrate. Superficially, this appears anomalous; the largest numbers of licking workers (which are mostly minima) are on young garden (see Table 5.3a, Chapter 5, page 88) which has few staphylae compared to older areas (see Fig. 2.1, Chapter 2). However, young garden matures in a few days and the growth of staphylae on mature garden may therefore be a response to previous intense licking of young garden. Licking continues on mature garden, but declines on aging garden and staphyla numbers also decrease here. However, this is probably due to exhaustion of the substrate just before it is discarded (see Chapter 4).

There may therefore be conflict between the ants and their fungus. The ants 'want' to eat the fungus, but are more likely to take staphylae than hyphae. Staphylae, as discrete packages, are easier to harvest than hyphae, which grow close to the substrate surface, particularly for larger workers (which seldom lick the garden; see Table 5.3a-d, Chapter 5, pages 89-91). The fungus 'wants' to escape and will produce more hyphae and fewer staphylae when the workers are absent. The ants, by continually harvesting staphylae and hyphae, therefore stimulate more staphyla production.

When the ants were absent, larger staphylae were produced, suggesting that they are usually harvested before they reach their maximum size. This may be because productivity per day per unit area is higher when staphylae are harvested at a small size. Because a staphyla will take longer to grow to a large size, it will 'consume' more substrate than one which is harvested whilst still small, and the extra size may not be worth the extra time and substrate taken. Alternatively, young staphylae may be more nutritious than older ones, which may in turn be repellent.

The removal of hyphae from the garden surface also ensures that the ant fungus cannot reproduce either sexually or asexually, via conidia. Muchovej *et al.* (1990) believed that they had found a sporophore of the ant fungus growing from a fungus garden, but they did not culture recognisable ant fungus mycelium from the basidiospores produced. Their sporophore might equally have been a parasite of the ant fungus which had become unrecognisable as such to the ants, which usually respond strongly to alien contaminants (see Chapter 7). Similarly, the conidia of the contaminant *Phialocladus zsoltii* have frequently been mistaken for ant fungus conidia (Powell 1984).

This prevention of reproduction by the ants might have evolved for two reasons. Hungry workers eating hyphae would restrict the growth of the fungus and prevent it from maturing. Secondly, the reproductive stages of the fungus may be unpalatable and workers would therefore strive to remove them. Fungal reproduction might lead to colony disorganisation and failure to produce as many sexual broods as colonies which possessed non-reproducing fungal cultures. Selection would therefore favour colonies with non-reproducing strains of fungus or those colonies whose workers were successful at keeping the fungus in an immature state and preventing its reproduction.

SUMMARY

Ant-free fungus garden which was exposed to workers for 3 hrs produced a 1.3 times larger standing crop of staphylae 2 days later, than did garden which remained ant-free. Simulating the possible chemical and mechanical reasons for this effect showed that crude head extracts and worker faeces did not affect staphyla production, but artificially pruning hyphae with a mounted needle increased subsequent staphyla production (standing crop) by 1.2 times. Removing staphylae had no effect upon subsequent standing crops, but total crops, which included the staphylae removed, were subsequently significantly greater

than on control garden. The removal of hyphae by licking may also have been important in the early stages of evolution of the mutualism for preventing fungal reproduction.

The staphylae produced by ant-free garden were larger than those produced when the ants were present.

Chapter 10: Licking fungus garden as a source of nutrients

INTRODUCTION

The fungus garden provides the ants and their larvae with a source of food in the form of staphylae. However, the large numbers of small workers constantly licking the fungus garden also ingest hyphae, which accumulate in their infrabuccal pockets (see Chapters 5 and 6). Digestion of chitin takes place in the infrabuccal pocket (Febvay *et al.* 1984), therefore these ingested hyphae may provide an extra source of nutrients and Littledyke and Cherrett (1976) found that workers picked up radiolabel from labelled fungus garden before appreciable numbers of 'hot' staphylae had arisen. The fungal mycelium may also produce exudates which the workers ingest, probably along with the hyphae.

Larvae fed artificially preferred staphylae to hyphae, which were richer in lipid and carbohydrate but poorer in protein than hyphae (Quinlan and Cherrett 1979). Similarly, *Acromyrmex octospinosus* workers also preferred staphylae to hyphae, although homogenized staphylae and hyphae were equally acceptable liquid food (Quinlan and Cherrett 1978a). Workers obtain only 4.8% of their respiratory requirements from staphylae, the rest of their needs presumably being met by plant sap. In contrast, staphylae make up 100% of the larval diet (Quinlan and Cherrett 1979).

The ants do have some dietary flexibility and a greater percentage of workers in forage-starved nests ate staphylae than in actively foraging nests, indicating a change in diet when plant sap is unavailable (Quinlan 1977). Hodgson (1955) also showed that a regular daily intake of plant sap is not necessary since after heavy rain, when foraging ceased, the ants did not subsequently take more material to make up the deficit. The fungus

garden therefore acts as a food-store or buffer, when workers are unable to forage for fresh leaves (Powell 1984).

This chapter examines the effects of comparative diets of hyphae and staphylae on workers. Material from ingested fungal material was traced into the worker crop using fluorescence microscopy and comparative weight gains of workers fed on hyphae or staphylae were examined, to assess the nutritional benefit of each to workers.

MATERIALS AND METHODS

The workers, larvae and fungus garden used were from an *Atta sexdens* nest (>80 gardens). Petri-dishes containing starved workers or fungus garden were maintained in boxes with closely-fitting lids. These contained filter paper (Whatman No.1) moistened with distilled water for high humidity and were kept in the ant culture room.

1. Comparing the longevities of workers maintained on different diets

Few staphylae are found on young garden, therefore only hyphae will be available to workers maintained on it. Workers maintained on mature garden will however, have access to both hyphae and staphylae.

Groups of 30 workers were confined in closed containers with damp filter paper and received five different diets:

(1) Sucrose solution (1 Molar) in a small test-tube with a cotton wool plug from which workers could drink.
(2) 10 cm^3 of ant-free, staphylae-free young garden.
(3) 10 cm^3 of ant-free, staphylae-producing mature garden.
(4) 10 cm^3 of mature garden, plus 1 Molar sucrose solution.
(5) No food; control.

Young garden was replaced every second day to ensure that few staphylae were available. Mature garden was replaced whenever it became contaminated, or approximately every fourth day, whichever was the sooner. This was carried out by preparing a clean container with fresh fungus garden, cooling the soiled container and workers in a refrigerator to pacify them and then transferring the workers into the new container with forceps. Longevities were assessed by recording daily worker deaths, to find the time taken for 50% mortality to occur.

Worker longevities on different diets were also examined by providing groups of workers (maintained in 5 cm petri-dishes with damp filter paper) with hyphae and staphylae removed from fungus garden. Staphylae were obtained from mature garden which had been isolated in a petri-dish with very few workers for 24 hrs. Hyphal tufts were obtained from young garden which had been similarly treated. Hyphae and staphylae were removed from garden using a mounted needle and placed on the inner surfaces of petri-dish lids, which were then given to the worker groups by rapidly exchanging the lids.

2. Culturing the ant fungus on agar

The fungus of *Atta sexdens* was cultured on Sabauraud's dextrose agar, enriched with 1% yeast extract, as recommended by Cazin *et al.* (1989). This medium ensured that the fungus would grow, but did not optimise its growth, no attempts being made to control pH, although Powell and Stradling (1986) reported that the fungus grows best between pH 4.5 and 5.0. Optimal growth was not desirable because more rapid growth of the fungus would have reduced the time available for experiments using colonies which had not yet produced gongylidia.

The agar was autoclaved at 15 psi and 121^{0}C for 20 minutes, then poured into sterile petri-dishes (9 cm) in a laminar flow cabinet. The plates were inoculated (in the laminar flow cabinet) with individual staphylae picked from

a piece of fungus garden with a sterile mounted needle. The culture plates were kept in a dark room at a constant temperature of 23^{0}C.

After 28 days, all plates were uncontaminated and had produced colonies of gongylidia-producing fungus which were acceptable to the ants. The culture plates used in the experiments were then set up, using the same medium and inoculating from the colonies obtained (which acted as a stock).

3. Tracing nutrients into the worker crop using fluorescence microscopy

Once ingested material passes down the oesophagus and into the crop, it is likely to be digested to provide food. Crop contents were therefore used to examine the amount of material obtained from comparative diets of hyphae and staphylae. Fluorescence microscopy was used because fluorochrome dyes can be detected at low concentrations, they are simple to use and preparations can be ready for examination within a few hours (Nairn 1976), in contrast to radio-labelling.

Fluorochromes are dyes which emit visible radiation when stimulated by visible or ultraviolet light. They have been used for many years in medicine, cell biology, industry and in mycological research. In fluorescence microscopy, ultraviolet light from a high pressure mercury lamp is shone on to the sample slide, causing the dye present to fluoresce. Filters are used to produce high intensity monochromatic light to provide maximum excitation of the fluorochrome (Butt *et al.* 1989). Quantitative measurements of fluorescence can be made by diverting light from the fluorescing sample through a photomultiplier and then to a fluorometer (Fig. 10.1). This has an arbitrary scale which can be adjusted for different sensitivities, depending upon how brightly samples fluoresce. The amount of fluorescence present is then recorded by a pen plotter.

Measurements must be made in a dark room because of the sensitivity of the fluorometer.

Materials which autofluoresce must be avoided and dyed samples must be mounted in non-autofluorescing media such as water or glycerol. Some chemicals, like iodide and excess fluorochrome, can quench fluorescence and most fluorochromes fade rapidly, so dyed samples must be examined quickly (Butt *et al.* 1989).

a) The fluorochrome used

The fluorochrome fluorescein diacetate (FDA) was used and this is a vital stain, often used as an indicator of cell viability in mycobacteria (Jarnagin and Luchsinger 1980), fungi (Soderstrom 1977) and plants (Widholm 1972). The FDA molecule crosses the plasma membrane into the cell and is hydrolysed in the cytoplasm to release fluorescein, which accumulates in the cell and fluoresces yellowish-green when illuminated with blue light. FDA stock must be kept desiccated at -20^{0}C to prevent deterioration, although working solutions may be kept for several days if refrigerated. To stain the ant fungus, 0.1 g of FDA powder was dissolved in 10 ml of acetone. This stock solution was then kept at -20^{0}C and 1 ml samples were removed and made up to 100 ml with distilled water to make the working solution (a 0.01% w/v solution).

b) Staining the ant fungus with a fluorochrome

Whole colonies of ant fungus growing on agar were stained using fluorescein diacetate. Placing droplets of stain solution on to an 8 week old fungus colony failed to stain large areas due to surface tension and the entrapment of an air layer by the mycelium. To counteract this, 20 ml of stain solution was poured on to the culture plate to cover the fungus and left for 30 minutes, after which the fungus was stained yellow. The residual stain solution was then poured off and the plate rinsed with distilled water. As much liquid as possible was then removed from the plate using filter paper. Samples of stained fungus were removed, mounted in distilled water on slides and examined

microscopically, using the apparatus shown in Fig. 10.1. Fluorescing hyphae and gongylidia were readily visible.

c) Detecting fluorochrome in worker head and crop contents

To check that stained fungus was acceptable to workers and that fluorescence could be detected in worker crop and head contents, a group of 20 media workers (headwidths 1.4-1.6 mm), which had previously been starved for 3 days, were placed in a petri-dish containing the stained fungal colony. For the first hour, the workers explored the culture plate, drank fluid from the fungus colony surface and then began to lick the fungus. After 3 hrs, workers began to attack the agar too. Five workers were then removed and their head and crop contents squeezed gently on to microscope slides and covered with coverslips. These were immediately examined for fluorescence and compared with the head and crop contents of non-exposed ants, with rectal fluid (a potential contaminant) and with pure stain solution, since traces of this may have been available on the garden surface. Crop contents were obtained by severing the gaster and squeezing it gently towards the petiole with fine flexible forceps. Head contents were obtained by squeezing the severed head to force out the contents of the infrabuccal pocket. These were examined because this pocket receives detritus from feeding and grooming.

The crop contents of workers exposed to dyed fungus fluoresced brightly whereas those of workers not exposed to dyed fungus did not fluoresce at all. Stain solution did not fluoresce because the fluorescein diacetate molecule must first be hydrolysed to produce fluorescein, which occurs most rapidly within cytoplasm. Rectal fluid however, autofluoresced. Fortunately it was easy to keep crop and rectal fluids separate. Crop contents, which were generally clear and colourless, were extracted by gently squeezing the severed gaster towards the petiole but too much pressure ruptured the proventriculus, releasing rectal fluids forwards into the crop contents sample. Rectal fluids are usually yellowish brown in colour and could

easily be distinguished from crop contents, enabling contaminated samples to be recognised and discarded.

This method was used to trace material from stained hyphae and staphylae into the worker gut. Measurements of fluorescence were obtained for each sample using the fluorometer and background fluorescences were obtained for every measurement made (using the fluorescence of the glass slide and coverslip). To make the arbitrary figures obtained more meaningful, the fluorescence of each sample was divided by the background fluorescence, to obtain a 'number of backgrounds' figure which could be used for comparisons.

4. Comparing weight gains of workers fed on staphylae and hyphae

Groups of 50 media workers (headwidths 1.4-1.6 mm) were starved in petri-dishes (9 cm) with damp filter paper for 0 or 24 hrs. Each group was then weighed using an Oertling R20 balance. Weight gains per group when receiving fungus could then be assessed.

a) Workers receiving natural fungus garden

Groups of workers were introduced into 5 cm petri-dishes containing ant-free young garden (staphylae-free) or mature garden (staphylae-producing), previously isolated for 24 hrs. After 3 hrs the dishes were cooled in a refrigerator to allow the removal of workers, which were then reweighed. Groups of workers which did not receive access to fungus garden acted as controls. Changes in garden weights were not recorded because the moist garden rapidly loses water when handled and exposed to air and fragments may also be lost when workers are being separated from it.

b) Workers receiving fungus cultured on agar

Cellulose acetate sheets with holes cut in them to allow access to fungal colonies, were placed over culture

plates to prevent the workers from licking the surrounding agar. Groups of workers (previously starved for 24 hrs) were introduced for 3 hrs into culture plates with either colonies of hyphae only or colonies also producing staphylae. Each group was then removed from the plate and reweighed. Groups of 50 starved and unstarved workers were weighed, left for 3 hrs in petri-dishes with damp filter paper and reweighed to act as controls.

5. Examining the larval diet

Larvae were observed on the fungus garden surface using a binocular microscope and cold fibreoptic light source, with a red filter (see Chapter 5, page 78). They were classified into three size groups on the basis of body length; small (<2 mm), medium (2 - 5 mm) and large (>5 mm). This was not a measure of age, since small larvae may develop into minima workers or grow into large larvae which produce soldiers.

Groups of brood present on the fungus garden surface were scanned over time to observe individuals which were being fed or which had food in their mouthparts. The size of each larva and the type of food it received were recorded.

RESULTS

1. Worker longevities on different diets

a) Five different diets

For each of the five diets provided, five groups of 30 workers of each of the four arbitrary groups defined by Weber (1972) were used to compare the longevities of different castes. Minima and media were confined in 9 cm petri-dishes, while larger castes were placed in plastic boxes (17 x 11 x 6 cm) and received twice the amount of each diet, to compensate for their larger size.

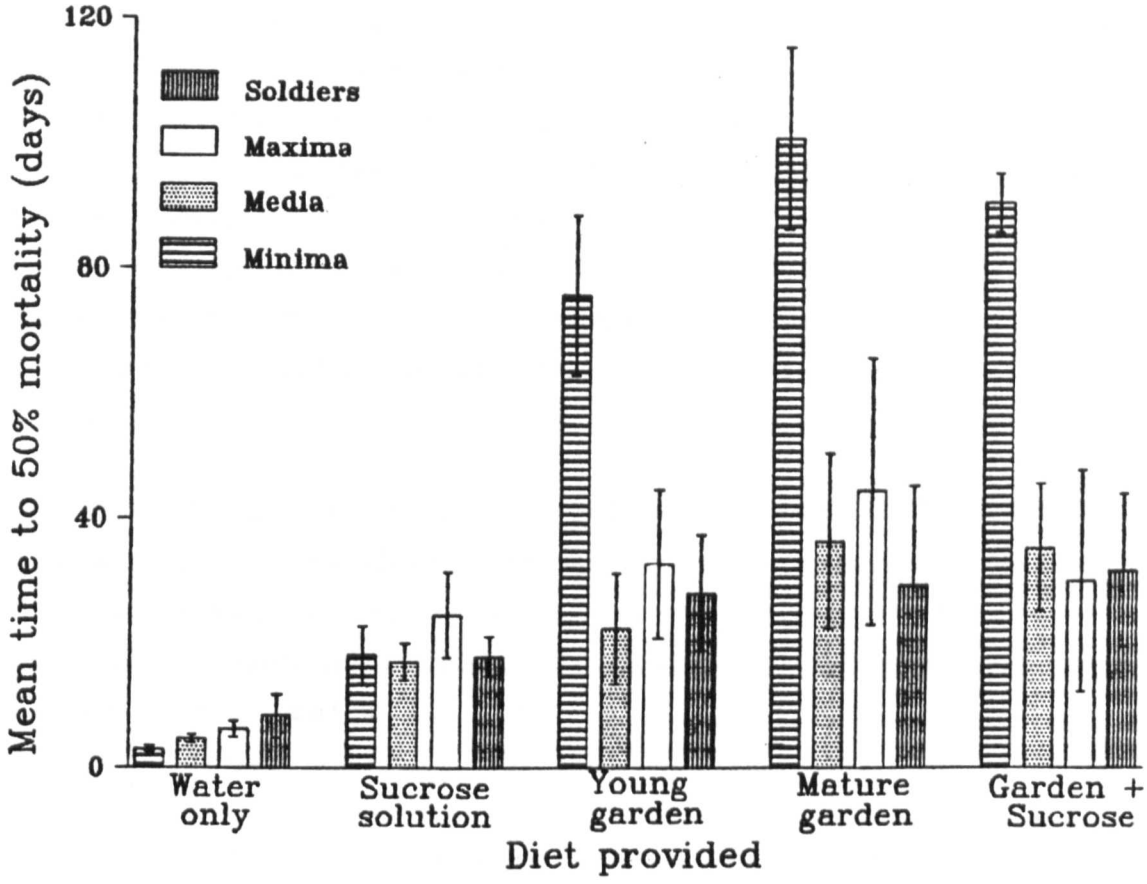


Figure 10.2: Mean times to 50% mortality (± 95% confidence limits) for groups of 30 workers of four castes, maintained on five different diets (5 replicates).

The fungus garden provided for maxima and soldier castes became contaminated with alien fungi much more frequently than that provided for minima and media workers and had to be replaced more often. This meant that larger workers underwent the stress of being moved into fresh containers more often than small ones. To reduce this, after the first garden replacement, a few small minima were included with the garden to care for it and prevent contamination.

Mean longevities (± 95% confidence limits) of the four castes fed on five different diets are summarised in Fig. 10.2 and a two-way analysis of variance showed that there were significant differences in longevities between castes and between treatments, with a significant interaction ($p<0.001$, Table 10.1).

Table 10.1: Two-way analysis of variance table comparing the effects of caste and diet on worker longevity.

SOURCE OF EFFECT	DF	SS	MS	F	P
Caste	3	20597.9	6866.0	93.8	<0.001
Diet	4	31267.1	7816.8	106.8	<0.001
Interaction	12	16951.6	1412.6	19.3	<0.001
Error	80	5856.2	73.2		
Total	99	74672.8			

One-way analyses of variance showed that longevities for workers receiving water only increased significantly with worker size ($p<0.001$), while maxima workers on a diet of sucrose solution lived significantly longer than any other caste ($p<0.05$). Similarly, minima receiving diets including fungus garden lived significantly longer than larger workers ($p<0.001$). Tukey's multiple comparison was

carried out for each and confirmed these relationships ($p<0.05$; see Table 10.2).

Table 10.2: Mean longevities (days to 50% mortality) of four worker castes fed on five different diets. Non-statistically significant means are joined by lines ($p>0.05$, ANOVA and Tukey's multiple comparison, 5 replicates).

DIET 1: WATER ONLY		DIET 2: SUCROSE SOLUTION		DIET 3: YOUNG FUNGUS GARDEN	
CASTE	MEAN LONGEVITY (DAYS)	CASTE	MEAN LONGEVITY (DAYS)	CASTE	MEAN LONGEVITY (DAYS)
Soldier	8.4	Maxima	24.6	Minima	75.6
Maxima	6.3	Minima	18.3	Maxima	32.8
Media	4.7	Soldier	17.9	Soldier	28.2
Minima	2.9	Media	17.0	Media	22.5

DIET 4: MATURE FUNGUS GARDEN		DIET 5: MATURE GARDEN + SUCROSE SOLUTION	
CASTE	MEAN LONGEVITY (DAYS)	CASTE	MEAN LONGEVITY (DAYS)
Minima	100.5	Minima	90.3
Maxima	44.5	Media	35.4
Media	36.4	Soldier	31.8
Soldier	29.5	Maxima	30.0

Workers of each caste lived for significantly different periods on different diets ($p<0.001$, ANOVA). Minimas lived longest on mature garden with or without sucrose solution, next longest on young garden, then on

sucrose solution and survived for the shortest period when provided with water only. Longevities on the latter three diets were all significantly different to each other ($p<0.05$, Tukey's multiple comparison).

Media workers lived longest on diets including mature garden. Longevities on sucrose solution and water only were significantly shorter ($p<0.05$, Tukey) and not significantly different to each other, while those on young garden were intermediate ($p>0.05$, Tukey).

Maxima and soldiers showed less obvious differences between longevities on different diets. Those receiving fungus garden all survived significantly longer than those receiving only water. Longevities on sucrose solution were intermediate and not significantly different to either ($p>0.05$, Tukey).

b) Diets of staphylae and hyphae

Groups of ten media workers (headwidths 1.2-1.4 mm) were placed into petri-dishes with damp filter paper. Medias were used for ease of handling. Ten groups received no food, ten received approximately 80 staphylae per day (Quinlan and Cherrett (1979) quoted intakes of 0.3 staphylae per hour, or 7.2 per day) and a further ten groups received tufts of hyphae, of similar mass to 80 staphylae.

For the first 1-6 days, workers often showed culturing behaviour when receiving hyphae or staphylae, cutting up pieces of filter paper and defaecating on the fungus provided. However, at least some fungal material was consumed.

Comparing the times taken for 50% mortality to occur showed that workers on the three diets lived for significantly different times ($p<0.001$, ANOVA). Tukey's multiple comparison confirmed that these times were all significantly different from each other ($p<0.05$). Those receiving staphylae survived for a mean of 9.4 (SE ± 0.7) days, those receiving hyphae; for 6.9 (SE ± 0.5) days and those not supplied with any fungus; for only 4.5 (SE ± 0.2)

days. Staphylae therefore provided a better diet than hyphae, which in turn, were better than nothing.

c) Comparing the longevity of *Atta sexdens* with that of a non-Attine species

The longevities of workers of *Atta sexdens* were compared with those of workers of *Myrmica ruginodis* (see Chapter 2, page 13). Large minima and small media *Atta* workers were used, since these were of comparable size to the *Myrmica* workers.

Groups of 50 workers were maintained in closed containers with damp filter paper. Five groups of each species received tubes of 1 Molar sucrose solution with cotton wool plugs, while another five acted as controls. The *Myrmica* containers were placed in an incubator at 20°C, while those containing *Atta* workers were placed in the ant culture room, at 27°C. Mortalities were assessed daily, to find the time taken for 50% mortality to occur.

The times to 50% mortality of the two species used, when fed on diets of water or sucrose solution were compared using a two-way analysis of variance. There were significant differences between both species and diet and *Myrmica* workers lived longer than those of *Atta* on both diets (Table 10.3). When receiving water only, *Myrmica* workers had a mean time to 50% mortality of 8.5 (SE ± 0.7) days while *Atta* workers lived only 3.9 (SE ± 0.1) days. Most of the *Myrmica* workers in the groups receiving sucrose solution were still alive after 30 days, when the experiment was stopped, indicating that they can survive much longer than this. *Atta* workers however, had a mean time to 50% mortality of only 14.2 (SE ± 1.5) days.

Table 10.3: Twoway analysis of variance table comparing the effects of species and diet on the longevities (days to 50% mortality) of workers of *Atta sexdens* and *Myrmica ruginodis*.

SOURCE OF EFFECT	DF	SS	MS	F	P
Species	1	519.18	519.18	146.7	<0.001
Diet	1	1268.82	1268.82	358.4	<0.001
Interaction	1	156.24	156.24	44.1	<0.001
Error	16	56.58	3.54		
Total	19	2000.83			

2. Tracing nutrients into the worker crop using fluorescence microscopy

An attempt was made to trace dye from hyphae and staphylae into worker crops. For hyphae, 18 day old colonies on agar were used, which had not yet produced staphylae. Each culture plate contained five colonies, each of approximately 1 cm diameter (and weighing approximately 0.05 g). For staphylae, 26 day old colonies were used, which were producing large amounts of gongylidia. Unfortunately, since all fungal cultures were set up simultaneously, runs using hyphae and staphylae were not carried out at the same time.

Groups of 30 media and maxima workers (used for ease of handling), previously starved for 24 hrs in containers with damp filter paper, were introduced into culture plates containing stained hyphal or staphylae-producing colonies. Starving them ensured that workers were hungry enough to eat the fungus. Each culture plate was stained with a separate batch of stain solution and the agar was protected from the workers using a cellulose acetate sheet with holes cut in it to allow access to the fungal colonies. Five groups of workers were used for both hyphal and mature

colonies and were left for 5 hrs. This ensured that most workers would have taken in at least some fungus. The culture plates and workers were then frozen at -20°C.

The fluorescences of the head and crop contents of 20 workers from each culture plate were examined quantitatively within 24 hrs. This allowed for up to ten 'accidents' per plate, where crop contents became contaminated with rectal fluid, or dried up before they could be examined. In total therefore, 100 workers were examined for both hyphae and staphylae-producing plates. In addition, to act as controls, the following were examined:

(1) The head and crop contents of 50 workers removed at random from the nest.
(2) 50 faecal droplets, squeezed from the gasters of workers randomly removed from the nest.
(3) The head and crop contents of 50 workers fed on unstained hyphal colonies on culture plates.
(4) The head and crop contents of 50 workers fed on unstained colonies producing gongylidia on culture plates.
(5) The head and crop contents of 50 workers allowed to drink diluted stain from the surfaces of undried stained colonies (which might include the contents of burst hyphae and gongylidia).
(6) The fluorescences of stained and unstained hyphae and staphylae from the fungal colonies used.

The data were not normally distributed and transforming them failed to produce normal distributions. The fluorescences of head and crop samples (expressed as numbers of backgrounds) were compared between replicate groups of workers receiving hyphal or mature fungus colonies, to check that they were homogeneous. Having ascertained that they were homogeneous ($p>0.08$, Mood), the replicates were pooled to give four sets of data; head and crop contents of workers fed on hyphae and of those fed on

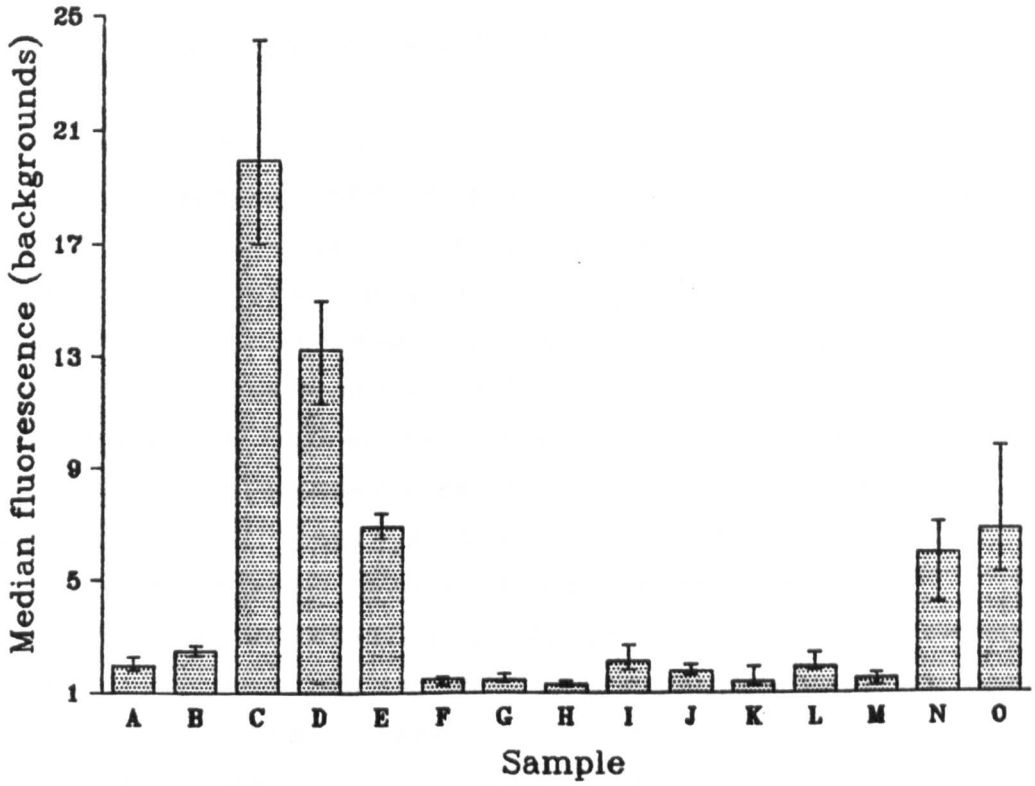
mature fungus. These were compared with the controls and significant differences were found ($p<0.001$, Mood).

The most brightly fluorescing samples were those of stained fungal material (Fig. 10.3; Samples C and D). Stained hyphae and staphylae both fluoresced significantly more than unstained hyphae and staphylae ($p<0.001$, Mann-Whitney tests). Unstained staphylae fluoresced more than unstained hyphae, but stained hyphae fluoresced more than stained staphylae ($p<0.001$, Mann-Whitney tests).

Rectal fluid and the crop contents of workers receiving stained hyphae or staphylae also fluoresced brightly (Fig. 10.3; Samples E, N and O) but comparing these data showed no significant differences ($p>0.49$, Mood). Rectal fluid and crop contents did, however, fluoresce significantly more than the head contents of workers receiving stained fungus ($p<0.001$, Mood). Crop contents of workers receiving stained fungus fluoresced significantly more brightly than those of workers receiving stain solution, unstained fungus or those which were removed from the nest at random ($p<0.001$, Mann-Whitney tests). Head contents of workers receiving stained fungus also fluoresced significantly more than those of the controls ($p<0.05$, Mann-Whitney tests).

Comparing the crop contents of control workers removed from the nest or fed on stain solution or fungus, showed that significant differences were present ($p<0.001$, Mood), due to significantly higher fluorescences in the crop contents of workers receiving stain solution, compared to other groups ($p<0.01$, Mann-Whitney tests). Comparing head contents of these workers showed no significant differences, however ($p>0.1$, Mann-Whitney tests).

Not all the test workers actually consumed stained fungus, hence the non-normal distribution of crop content fluorescences. Some were no more fluorescent than the controls, but some fluoresced very brightly and these were presumably from those ants which had fed. A level of fluorescence for crop contents was therefore defined which



Key:

A	Unstained hyphae
B	Unstained staphylae
C	Stained hyphae
D	Stained staphylae
E	Rectal fluid
F	Head contents of workers removed from the nest at random
G	Head contents of workers allowed to drink dilute stain
H	Head contents of workers fed on unstained fungus
I	Head contents of workers fed on stained hyphae
J	Head contents of workers fed on stained staphylae
K	Crop contents of workers removed from the nest at random
L	Crop contents of workers allowed to drink dilute stain
M	Crop contents of workers fed on unstained fungus
N	Crop contents of workers fed on stained hyphae
O	Crop contents of workers fed on stained staphylae

Figure 10.3: Median fluorescences (number of backgrounds-worth, ± 95% confidence limits) of worker head and crop contents after the workers had consumed hyphae or staphylae stained with a fluorochrome. The fluorescences of head and crop contents of workers not having access to stained fungus, worker rectal fluid and stained or unstained fungus are shown for comparison (50 replicates for A-E and 100 replicates for F-O).

was not exceeded by rectal fluid or the crop contents of any control workers and this was fixed at 13 backgrounds-worth of fluorescence for crop contents. In total, 24 and 30 samples of crop contents exceeded this figure for workers fed on stained hyphae and stained staphylae-producing colonies respectively (Samples N and O, in Fig. 10.3). There was therefore little difference between them. Comparing the fluorescences of these high-intake workers showed no significant difference between those fed on staphylae and hyphae ($p>0.1$, Mann-Whitney test).

Similarly, a level of fluorescence for head contents was also defined, which was not exceeded by the head contents of control workers and this was fixed at 9.5 backgrounds. However, only 5 and 2 samples of head contents exceeded this figure for workers fed on stained hyphae and staphylae-producing colonies respectively (Samples I and J in Fig. 10.3). Again, there was little difference and comparing their fluorescences showed no significant difference between them ($p>0.8$, Mann-Whitney test).

Stained hyphae tended to fluoresce more strongly than stained staphylae (Fig. 10.3; Samples C and D) and this may have affected the subsequent fluorescences of crop and head contents of workers fed on the two types of fungus. If stained hyphae had a median fluorescence of 19.9 backgrounds and stained staphylae, a median of 13.3 backgrounds, then adjusting crop contents of workers fed on stained fungus (with medians of 6.0 backgrounds on hyphae and 6.8 on staphylae) gives medians of 6.0 backgrounds for crop contents of workers fed on hyphae, compared with 10.2 for workers fed on staphylae. When the data sets were adjusted in this way and compared, significant differences were found between crop contents of workers fed on staphylae and hyphae ($p<0.001$, Mann-Whitney test) and also between their head contents ($p<0.01$, Mann-Whitney test). In both cases, staphylae caused more fluorescence than hyphae.

3. Weight gains of workers fed on staphylae and hyphae

a) Workers receiving fungus garden

Groups of 50 workers had mean weights of 0.116 (SE ± 0.005) g before exposure to fungus garden. The arcsine-transformed percentage changes in weight of groups of 50 workers placed on garden with or without staphylae, after 0 or 24 hrs of starvation were compared by a two-way analysis of variance. This showed that significant differences were present between the weight changes of workers with or without staphylae and between workers starved for different periods ($p<0.001$, Table 10.4). There was also a significant interaction between the two factors ($p<0.001$). Workers gained weight when starved for 24 hrs and then introduced to garden and gained more weight on garden where staphylae were present, compared to when only hyphae were available (Table 10.5). However, workers lost weight when they were introduced to garden without previously being starved. Control workers starved for 24 hrs or not at all also lost weight during the 3 hr period, more being lost by those which were not starved.

Table 10.4: Twoway analysis of variance table comparing the effects of diet and previous length of time without food on arcsine-transformed percentage weight gains of workers.

SOURCE OF EFFECT	DF	SS	MS	F	P
Diet	2	158.6	79.3	112.3	<0.001
Previous time without food	1	132.9	132.9	188.3	<0.001
Interaction	2	52.6	26.3	37.3	<0.001
Error	54	38.1	0.7		
Total	59	382.3			

Table 10.5: Mean arcsine-transformed percentage changes in weight (arc%'s, ± SE) of groups of 50 workers starved for 0 or 24 hrs, when introduced to young staphylae-free garden, mature staphylae-bearing garden or no garden (controls) (10 replicates).

TYPE OF GARDEN RECEIVED	MEAN ARC% WEIGHT CHANGES (± SE) OVER 3 HRS FOR GROUPS OF WORKERS STARVED FOR:	
	0 HRS	24 HRS
Young (hyphae, no staphylae)	-0.15 ± 0.33	0.73 ± 0.12
Mature (hyphae and staphylae)	-0.07 ± 0.15	5.36 ± 0.48
No garden (control)	-2.63 ± 0.18	0 ± 0.12

b) Workers receiving fungus cultured on agar

Groups of 50 workers had mean weights of 0.142 (SE ± 0.005) g before they were exposed to fungus. Arcsine-transformed percentage weight changes of groups of workers starved for 24 hrs and then fed on cultures of fungus consisting of pure hyphae, or with gongylidia present were significantly different from each other and also from the control ($p<0.001$, ANOVA). Workers receiving mature colonies had a mean arc% weight increase of 5.76 (SE ± 0.27), those on hyphal colonies had a mean arc% increase of 3.95 (SE ± 0.27) and control groups had an arc% decrease in weight of 0.18 (SE ± 0.11). Workers receiving hyphal or mature colonies therefore gained much more weight than control groups, the largest gains being made by workers receiving mature gongylidia-producing colonies.

These weight gains were not due to ingestion of agar, since the cellulose acetate sheets placed on to the culture plate surface prevented the ants from licking the agar around the fungal colonies. Also, the 3 hr exposure period was not long enough to allow the workers to dig through the fungal colonies and reach the agar beneath.

4. The larval diet

Fifty larvae of each of the three size groups were observed being fed. They received either staphylae or macerated staphylae, from which workers had drunk juices. No hyphae were given and this was confirmed by removing 30 larvae after they had been fed and mounting the food they had received in cotton-blue in lactophenol, then examining microscopically. Small larvae were given 34 staphylae and 16 macerated staphylae, as were medium larvae, while large larvae were given 39 staphylae and 11 macerated ones. Approximately 66% of the larval diet was therefore whole staphylae, with the remainder being macerated ones. However, very small larvae were difficult to observe and may have received other sources of food.

DISCUSSION

Larger workers lived longer than smaller ones on diets of water only (Fig. 10.2) and the majors of ant species often live longer than the minors, so that if the colony is deprived of water, food or larvae, the ratio of majors to minors increases, since larger ants can withstand stress longer than their smaller nestmates. This has been documented in *Camponotus* and *Formica* by Kondoh (1977).

Workers receiving diets of sucrose solution all lived longer than those on water, but not as long as those receiving mature fungus garden. Workers can therefore utilise sucrose, which is the main substance transported in the phloem of most plants (Kursanov 1984). Sucrose may be present in phloem sap at concentrations of 0.4-0.6 Molar (Ziegler 1956, Zimmerman 1958). Maxima workers lived longer on sucrose solution than other workers, but the reasons for this were unclear.

Minima lived much longer when receiving fungus garden than any other caste. Minima have evolved as specialists in garden and brood care (Oster and Wilson 1978). They lived longer when provided with mature staphylae-producing garden than when receiving young garden with only hyphae

available. However, the fact that they were able to survive on the latter for long periods (longer than larger castes could survive when provided with mature garden) indicated that these workers can survive on a diet of hyphae alone. This was in spite of the fact that minima maintained on young garden underwent more stressful changing-over of garden than those on mature garden, as young garden had to be changed every 2 days to prevent staphylae becoming accessible to workers, while mature garden could be left for more than 4 days. Larger workers could also utilise the hyphae on young garden, but not to the same degree. Different castes can therefore exploit the garden at different levels of efficiency.

There were few differences between worker longevities on diets of mature garden and mature garden plus sucrose solution, which was unexpected. Theoretically, workers on the latter diet should have lived longer, since it was an attempt to simulate the natural situation, with fungus garden and substitute plant sap available. However, the sucrose solution may not have been a good replacement for plant sap.

The stress of being separated from the nest and larvae may have affected larger workers more strongly than small ones. Without larvae, social disorganisation occurs rapidly and foraging levels decline (Powell 1984). Minima workers have the stimulus of the fungus garden and may therefore be more resistant to this disorganisation, but larger workers are not specialised for garden care and may therefore 'give up'. Larger workers may then consume less food and gradually die of starvation.

A further alternative is that different castes have different natural life spans. When members of a caste are subject to a high accidental death rate, such as through predation during foraging trips, then relatively early senescence should occur in members of that caste, when protected in a predator-free laboratory environment (Oster and Wilson 1978). With a short life expectancy, there will be no selection pressure on foraging workers for long physiological life. If the majority of individuals die

before a certain age due to external causes, then senescence after this age will have little influence on individual contributions to the next generation. Genes providing benefits before this age will be selected for, even if they subsequently cause senescence. Honeybee workers for example, are usually killed during foraging by the age of 40 days and if not, die of senescence by 60 days (Rockstein 1950, Sakagami and Fukuda 1968).

Foraging ant workers may therefore senesce at an early age, while those that remain safely in the nest will live longer. Foragers of *Pogonomyrmex owyheei* survive only 15 days on average (Porter and Jorgensen 1981) and foraging workers are frequently the oldest in a colony (Oster and Wilson 1978). In *Atta sexdens*, most minima workers remain in the nest, although some will forage, while larger workers, including the soldiers, all forage to some degree. Minima should therefore have the longest natural life span, while larger workers senesce relatively more rapidly.

The biggest investment in maintaining a worker population is during the larval stage, therefore extending the working life of the individual would offset this cost. An individual with a longer lifespan would spend a smaller percentage of its life as a larva and this would be beneficial for the colony. Colonies with the most economical workers might be more successful at producing sexual broods since they would have more resources available to produce them. In *Atta*, this might therefore give rise to long-lived small workers, but there would be no point in larger workers potentially having a longer physiological lifespan, because they would be likely to die while foraging or defending the nest. There are no obvious genetic differences between worker castes and individual caste is probably determined by the amount of food it receives as a larva, where large larvae receive more food and develop into large workers (Oster and Wilson 1978). However, genetic material may be expressed differently in different castes.

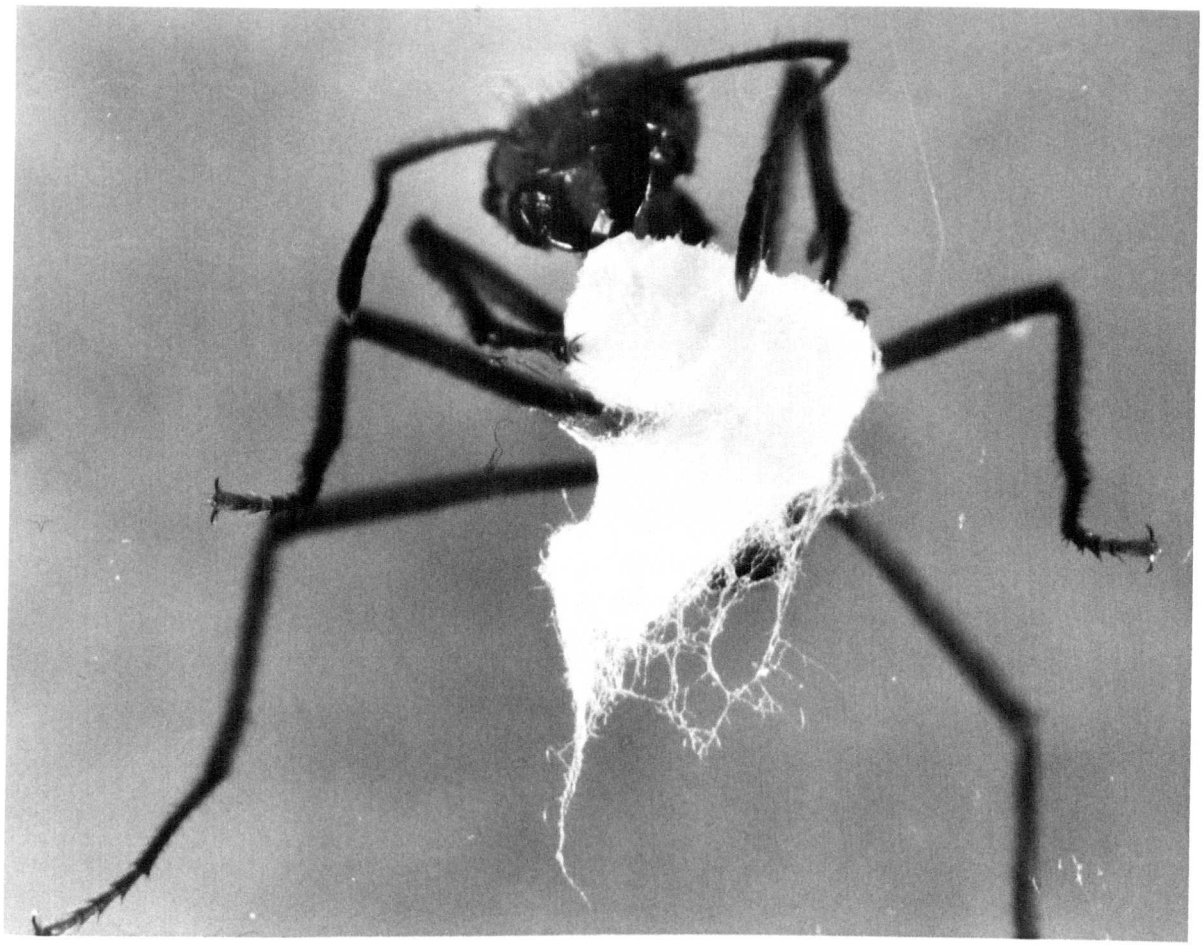
Maxima and soldiers proved inept at keeping the fungus garden uncontaminated and frequently required fresh

garden. Consequently, it became necessary to introduce a few minima to improve hygiene. Turner (1974) observed that larger workers lived longer in subcultures with minima present than without. This meant that maxima and soldiers on diets including fungus garden may have been surviving for longer than expected, because of this minima presence.

The longevities shown by confining workers under artificial conditions are likely to be shorter than actual longevities, due to the stress of handling, confinement and lack of brood. Weber (1972) observed that the first workers produced by new *Atta sexdens* colonies lived from 6-9 months and these figures are comparable to the longevities of minima workers receiving mature garden. Wilson (1983b) found that there was a 4 month turnover of workers and Powell (1984) reported that worker mortality is up to 4.45 times greater in the absence of brood. The longevities exhibited by *Atta* workers were much shorter than those exhibited by *Myrmica ruginodis*. The latter is a relatively primitive species and very robust. This suggests that Attines are physiologically delicate and completely dependent upon the fungus garden. When deprived of it, they cannot survive for long.

Workers fed on diets of staphylae or hyphae separated from the fungus garden survived for very short periods, although they lived longer than groups receiving water only. This was probably due to stress, since a petri-dish with a piece of damp filter paper is a very harsh environment. The staphylae and hyphae provided may not have been of optimum palatability or nutritional value and were frequently damaged or partially desiccated by the transfer from garden to ants. However, workers again showed that they could utilise hyphae as food, although they lived longer on staphylae. It is, of course, unlikely that the ant-fungus mutualism could have evolved if the fungus mycelium was not attractive to the ants. Plate 3 shows a worker ingesting hyphae from ant fungus cultured on agar.

Plate 3: Maxima worker of *Atta sexdens* ingesting hyphae from a colony of ant fungus cultured on agar.



Tracing a fluorescent dye into the ant gut provided further evidence that workers could utilise hyphae as food, as did the work on weight gains on different diets. Fluorescence microscopy suggested that there was little difference between intakes of hyphae and staphylae, but the problem with using fungus cultured on agar is that it has been free to grow in any direction and is much easier for the ants to harvest and ingest. It is a concentrated source of hyphae rather than a thin layer of available mycelium on the outer surfaces of leaf fragments in the fungus garden. Since hyphae are more easily available, hungry workers can ingest more, which will be detected as greater levels of crop fluorescence, comparable with those of workers fed on staphylae. When the figures were adjusted to allow for differences in the relative fluorescences of stained hyphae and staphylae, crop content fluorescences for workers fed on staphylae were 1.7 times higher than those for workers fed on hyphae.

In contrast to crop contents, head contents had relatively low fluorescences. However, squeezing the infrabuccal pocket contents on to a slide also forced out saliva and possibly the contents of other glands. This would have tended to mask or dilute the fluorescent fragments of hyphae. Alternatively, the workers were managing to extract juices from hyphae and staphylae without filling their infrabuccal pockets with debris. It is likely that the relatively short period allowed for ingestion of fungus was not long enough to allow a large infrabuccal pellet to develop. Chapter 6 showed that under normal conditions, workers produce just over one pellet per day. This would therefore have been difficult to detect.

The easy availability of hyphae on culture plates of fungus also affected the weight gains on different diets. The results shown on page 221 indicate that workers fed on cultured hyphae gained more than 5 times as much weight as those fed on staphylae-free garden. In contrast, workers fed on cultured staphylae gained only 1.1 times as much weight as those fed on mature fungus garden. This suggested

that the workers find hyphae attractive but difficult to obtain in the fungus garden. Workers gained 7.3 times as much weight when receiving mature garden with staphylae than when they were given young garden. In contrast, when they received cultured fungus, they only gained 1.5 times as much weight on staphylae compared to hyphae.

Workers may have gained more weight when receiving staphylae because of the different structures of gongylidia and hyphae. The former are semi-spherical and will therefore have a high volume to wall ratio, therefore the majority of ingested material can pass quickly into the crop while the few ingested fragments of wall will collect in the infrabuccal pocket. In contrast, hyphae are narrow tubes and will therefore have a low volume to wall ratio. To obtain the same amount of liquid food as when feeding on gongylidia, a worker will have to ingest large amounts of hyphal wall material, which will quickly fill up the infrabuccal pocket. Since infrabuccal pellets weigh up to 3.5 µg (see Chapter 6, page 133), this would have been detectable as a weight increase of 175 µg for a group of 50 workers. If groups weighed 0.116 g (as they did for the experiment using fungus garden), then this would have been a weight increase of 0.15% (an arcsine-transformed percentage of 0.09). Groups fed on young garden actually had an arc% weight gain of 0.73 (see Table 10.5). Similarly, if groups weighed 0.142 g (as in the experiment using fungus cultures, page 221), this infrabuccal material would have been detected as a weight increase of 0.12% (arc% of 0.07). Clearly, the weight increases were due to relative intakes of liquid. Workers receiving fungus garden would not have had time to find enough hyphae to put on a lot of weight, while those receiving cultured hyphae could consume more in the same time. Also, for reasons already discussed, the exposure period was probably not long enough to allow workers to fill their infrabuccal pockets.

These results all indicate that workers can utilise hyphae as food, but the degree to which they actually do this in the nest remains in doubt. Staphylae provide only

4.8% of the respiratory needs of workers (Quinlan and Cherrett 1979) and hyphae appear to be less available and perhaps poorer as a diet than staphylae. If the relative adjusted levels of fluorescence in the crops of workers fed on hyphae and staphylae are examined, the latter are 1.7 times higher than the hyphal fluorescences. If this is an indication of the relative importances of hyphae and staphylae in the ant diet, then hyphae may provide approximately 2.8% of worker respiratory requirements, while staphylae provide 4.8%. This assumes that hyphae and staphylae provide similar amounts of energy. It is difficult to calculate energy values for hyphal intake, because it remains unclear how much material can be digested or extracted in the infrabuccal pocket. However, staphylae contain more carbohydrate and lipid, but less protein than hyphae (Quinlan and Cherrett 1979).

If weight gains of workers receiving fungus garden are used to consider the relative importance of hyphae and staphylae in the diet, then staphylae will cause 7.3 times more weight increase than hyphae. In this case, hyphae might provide 0.7% of the worker respiratory requirements. However, if weight gains on fungus cultures are considered, hyphae will provide 3.3% of the respiratory requirements. For reasons already discussed, this would be an overestimate.

It is probable that hyphae provide a small extra source of nutrients for workers licking the garden, more than 98% of which are minima (see Chapter 5), but larger workers are unlikely to obtain much of their energy requirements from them.

It was suggested in Chapter 6 that workers exhibit a degree of role fidelity, either caring for the garden or performing other tasks. Such specialisations in Attines, whether as individual idiosyncrasies or temporal castes would mean that some workers obtain almost all their food from the fungus garden as both hyphae and staphylae, while others obtain their energy requirements from plant sap and staphylae. To resolve this problem, either workers must be

individually marked in the nest and observed over time, or the gut contents of randomly taken workers analyzed. Marking *Atta* workers is however, difficult, since the workers are adept at removing paint or glue (see Chapter 5, page 100).

Worker rectal fluids contain proteinases which originate from the fungus and are vital for successful growth of the fungus on new substrate (Martin *et al.* 1975). It has been tacitly assumed that these enzymes are ingested in staphylae juices. This is probably true to a degree, however, small workers have enlarged recta (Holldobler and Wilson 1990) and are also responsible for the latter stages of substrate preparation. If workers ingest large amounts of hyphae, they will therefore obtain large amounts of enzymes which will pass into their faeces. Defaecation takes place all over the garden surface and brown droplets can be seen shining on the surface. Whether or not role fidelity occurs for licking workers, they will defaecate all over the garden and thus return the enzymes to the fungus. This may occur especially on young garden, because licking is more frequent here. Substrate-preparing minima also defaecate on to freshly inserted substrate, but even if they have not been ingesting hyphae, they will have eaten staphylae. Rectal fluids also contain allantoin, allantoic acid and a wide variety of amino acids (Martin and Martin 1970b) which are ideal for nurturing fresh fungal inocula until they can begin to penetrate the new substrate. By this time, another (licking) worker might also have defaecated in the region. Rectal fluids are much more likely to be important for this establishment of the fungus on new substrate than for fungal growth on older garden. This was borne out by the fact that rectal fluids had no appreciable effect on staphyla production (see Chapter 9). Of course, comparing colonies of ant fungus grown in culture with or without nitrogeneous sources and enzymes will show that these have a major impact on fungal growth. However, in mature fungus garden, they are already

present and additional rectal fluids cannot make much difference in this respect.

No larvae were ever seen to receive hyphae and were given a mixture of staphylae and macerated staphylae, which had previously been chewed by workers. This was expected, since Quinlan and Cherrett (1979) found that larvae prefer staphylae to hyphae and gain more weight on the former. They also found no evidence of feeding on fungal hyphae or of worker-larvae trophallaxis.

SUMMARY

Workers of all castes lived longer on diets including mature garden than when receiving water only. Sucrose solution (1 Molar) and staphylae-free garden were also better diets than plain water. Minima lived longer than larger castes when receiving garden, probably because they are specialised to care for garden and can exploit it. On water-only diets, longevity increased with worker size since larger workers are more resistant to stress than small ones. Workers also lived longer on diets of staphylae (separated from fungus garden) than on hyphae, both diets being preferable to water only.

Attine workers survived on water or sucrose solution for shorter periods than *Myrmica ruginodis*. They were therefore comparatively delicate, physiologically.

Material from both fluorochrome-stained hyphae and staphylae were detected in worker head and crop contents. Adjusting the figures obtained, to allow for the different fluorescences of stained hyphae and staphylae, showed that the crop content fluorescences of workers receiving staphylae were 1.7 times greater than those of workers receiving hyphae.

Workers gained 7.3 times more weight on garden with staphylae than on garden without, but only 1.5 times as much when on cultured colonies with staphylae than on those with hyphae. Hyphae were therefore more available on

culture plates than fungus garden, while staphylae were easily available on both.

Calculations showed that hyphae may provide between 0.7 and 2.8% of worker respiratory requirements. Minima are likely to benefit from this more than larger workers, because they most commonly lick garden.

There was no evidence that larvae ever receive hyphae as food. Instead, they received a diet of mixed fresh and macerated staphylae.

Chapter 11: Insights into the ant-fungus mutualism provided by nest decline and death

INTRODUCTION

Interrupting a balanced system may provide insights into its internal mechanics. When an ant nest is deprived of brood, social disintegration occurs. The workers become disorganised, cease to forage and the nest eventually dies (Powell 1984). The breakdown of interactions can therefore shed light on the mutualism. Loss of brood occurs naturally when the queen either ceases to lay eggs or dies. Nests can therefore decline and die even if the queen remains alive and in colonies without brood for long periods, the queen ceases to have any effect on workers and may subsequently be killed by them (Powell 1984).

When lack of brood leads to nest decline, the fungus garden may be abandoned by the workers and then overrun by large populations of bacteria, yeasts and other organisms, including Collembola, mites and nematodes (Weber 1972).

This effect of brood appears to be due to larvae rather than pupae. Robinson and Cherrett (1974) found that larvae offered to nests were recognised by workers while pupae were discarded. The workers may require substances from the larvae to remain healthy and in wasps, *Vespula* spp., the adults obtain enzymes from larvae by exchanging liquids during oral contact (Maschwitz 1966). In the Oriental hornet, *Vespa orientalis*, the adults are incapable of gluconeogenesis and depend on the larvae for sugars (Ishay and Ikan 1969).

The presence of brood may directly stimulate foraging as in *Myrmica*, where the larvae beg food from workers (Brian and Abbott 1977). Similarly, in *Myrmecia* and *Promyrmecia*, the larvae stimulate prey collection (Haskins and Haskins 1950). Attine larvae are however, immobile (Wheeler and Wheeler 1960, Weber 1972), so behavioural signals are limited, although they do 'pout' (Weber 1972). Foraging might therefore be directly stimulated by the

presence of brood, since larvae continually consume staphylae (Quinlan and Cherrett 1979). The number of staphylae produced would depend upon the quality and quantity of vegetal substrate.

Larvae may secrete a pheromone which stimulates worker activity. Powell (1984) found that levels of foraging increased as the numbers of larvae increased, corresponding with the increasing concentration of brood pheromone in the colony.

The providential decline and subsequent deaths of three laboratory nests of *Atta sexdens* allowed observations on the breakdown of the mutualism to be recorded, concentrating on gardening behaviour. It was considered likely that social disorganisation would lead to less licking of the fungus garden by workers and that changes might take place in infrabuccal pellet production as foraging declined (because Type 1 pellets probably result from foraging activity - see Chapter 6). It also seemed likely that the standing crop of staphylae would increase in declining nests since larvae, the main consumers of staphylae, are no longer present. All of these factors are examined in this chapter.

MATERIALS AND METHODS

Observations were made on three declining *Atta sexdens* nests. Nest 1 was 11 years old with 22 gardens while the other two had 7 and 8 gardens respectively and were only 2 years old. Nest 1 declined between July 1990 and May 1991, Nest 2 between November 1990 and June 1991 and Nest 3 between January and November 1991. Detailed observations were made only on Nests 1 and 2. In Nest 3, the queen remained alive and visible in Nest 3 and at first, it seemed likely that the nest was merely in a short-term decline.

A healthy non-declining nest of the same species (>80 gardens) was used for comparisons.

1. Assessing worker numbers and behaviour

Numbers and behaviours of workers present on the fungus garden surface were examined on 16 cm^2 areas using the method described in Chapter 5 (pages 78-80).

Worker populations and caste ratios were examined by removing samples of declining garden and anaesthetizing the workers present with carbon dioxide gas. The workers present could then be counted and their headwidths measured.

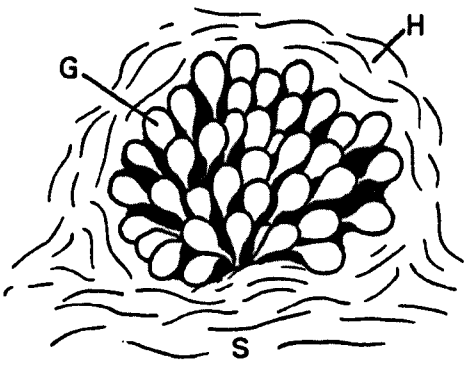
2. Measuring volumes of individual gardens

The volumes of individual gardens were measured by estimating the percentage 'fill' of each dome. Approximate garden volumes could then be estimated, since a dome has a maximum capacity of 2.5 litres. In Chapter 3, garden sizes were assessed by tracing silhouettes on to cellulose acetate sheets. However, many of the gardens in the declining nests were inaccessible and this method could not be used.

3. Assessing numbers and ages of staphylae

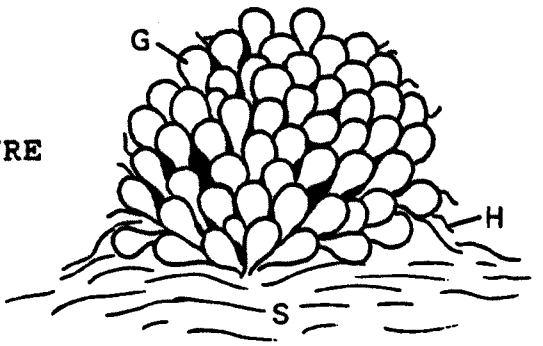
Staphylae were divided into young, mature and senescent stages using microscopic observation, gross visual appearance and by following the fates of individual staphylae on ant-free fungus garden from their origin to senescence (Fig. 11.1). Staphylae were marked and followed over time using the mapping methods described in Chapter 9 (pages 183-184). Numbers of staphylae of each age were assessed by counting the numbers present in 0.1 g of fungus garden, which was dissected open with mounted needles under a binocular microscope.

YOUNG



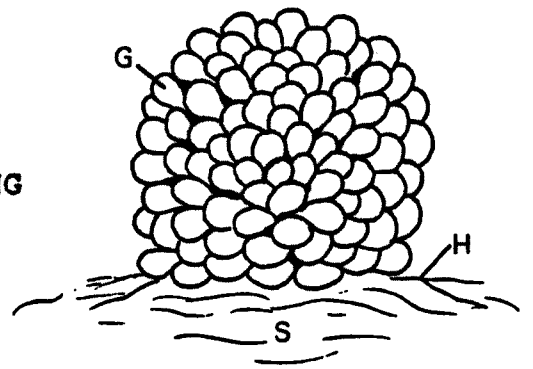
Halo of hyphae around or over staphyla. Gongylidia somewhat diffuse.

MATURE



Glistening white, no halo of hyphae, more compact than young staphyla.

AGING



Yellowish, dull in appearance rather than glistening, often appearing water-soaked.

Scale ⊢—⊣ 0.1 mm

G - Gongylidia
H - Hyphae
S - Substrate

Figure 11.1: Three stages in the development of staphylae. Young staphylae reach the mature stage within a few hours, while mature staphylae become over-mature after 1-2 days.

4. Testing worker preferences for different ages of staphylae

Staphylae of different ages were removed from fungus garden using a mounted needle and binocular microscope and placed in petri-dishes containing damp filter paper, to prevent desiccation. Twenty staphylae of each age were then placed at random on a black perspex sheet, previously exposed to the nest for 1 hour and the positions of each age noted. This was offered to the ants and the times taken for workers to remove each of the staphylae were then recorded. Workers swarmed all over the perspex sheet and encountered staphylae very rapidly, hence individual staphyla removal times were recorded, rather than numbers of each type removed after a given time.

5. Assessing infrabuccal pellet production

The numbers and types of infrabuccal pellets produced were examined using the method described in Chapter 6 (page 125) for looking at the pellet contents of dried refuse samples.

RESULTS

1. General symptoms of nest decline

In all three nests, a decrease in foraging activity became noticeable long before any obvious changes in nest appearance occurred. Workers foraged more sluggishly than those in healthy nests and took less forage. Some large workers did continue to forage until the nest eventually died but generally took only highly attractive or 'novelty' substrate, like citrus albedo or rose petals. Even when such material was cut, it was left to wilt for several hours or days before it was used to build fungus garden and piles of cut, wilted material built up around domes. Occasional 'recoveries' also took place in all three nests, where areas of new garden were built up for a few days. This extended the decline period considerably.

Another initial symptom was the lack of brood. This was less noticeable however, since the amount of brood present on the garden surfaces fluctuates even in healthy nests (see Chapter 3).

During its initial period of decline in July 1990, Nest 1 produced a sexual brood of approximately 60 alate males (see Plate 4a). No females were produced and no larvae or pupae were visible. Nests 2 and 3 produced no sexual forms.

In Nest 1, during October 1990, the hollowed-out exoskeleton of the dead queen, still covered with attendant workers, was discovered on the surface of a garden after several months of nest decline. In Nest 2, decline occurred after the apparently sudden death of the queen, which was carried dead but still intact and supple, out of a garden to be discarded with refuse. Brood was not visible, but worker activity had previously appeared normal and a new garden, less than 10 days old, was being constructed. In Nest 3 however, the queen remained alive throughout the period of decline, although producing no brood and eventually died just before the last garden disappeared completely.

In Nest 1 during August 1990, areas of garden began to appear pale and some parts grew 'out of control', particularly where they touched the dome wall, where worker access was restricted. They were particularly common on the upper surfaces of the garden, appearing white and rounded, until they encountered the plastic of the dome wall. Then, they became differentiated into a yellowish inner area and a white radiating fan of hyphae. Some of these growths reached a diameter of several centimetres (Plate 4b). When stained with cotton-blue in lactophenol and examined microscopically, these growths were seen to consist of tightly packed hyphae with a few gongylidia. Exposing fragments of these white growths to workers from healthy nests showed a positive response; fragments were licked and

some were carried into domes. These white growths were therefore ant fungus, rather than contaminant fungi.

The remainder of the garden also appeared whiter than normal, with large numbers of staphylae (Plate 4b). Counting staphylae per 0.1 g garden showed that there were significantly fewer staphylae on areas with white growths than in remaining areas ($p<0.001$, ANOVA, 10 replicates). Areas with white growths had a mean of 84.8 (SE ± 11.1) staphylae per 0.1 g of garden, while those without had 203.2 (SE ± 7.6) and samples of healthy non-declining garden had 74.6 (SE ± 2.5). Comparing these three sets of samples with a Tukey's multiple comparison showed that areas without white growths had significantly more staphylae than areas with white growths, or samples from healthy garden ($p<0.05$), which were not significantly different to each other ($p>0.05$).

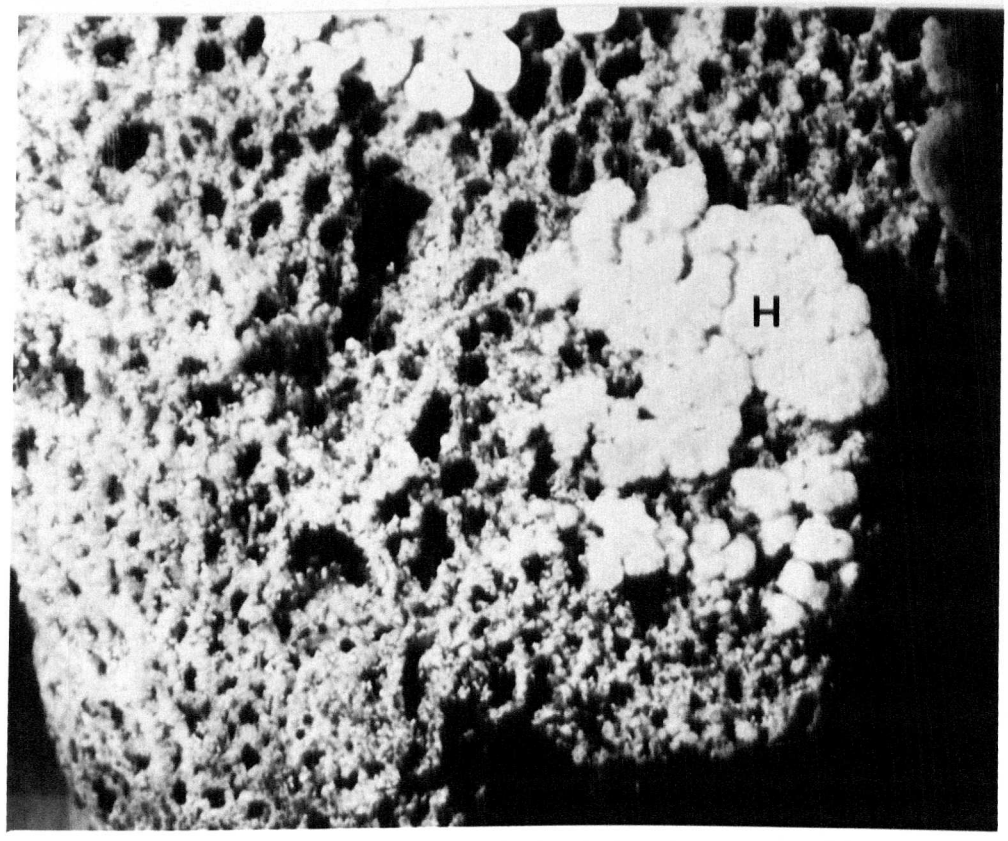
In Nests 2 and 3 the garden also became pale but no white growths were produced. Examining garden with a binocular microscope revealed that the paleness was due to extensive growths of hyphae over the garden surface, akin to the increases in hyphal depths described in Chapter 7, when ants were restricted from garden areas for short periods.

Many workers in all three nests were observed drinking water outside the domes, probably in an attempt to keep the gardens moist. A 25 cm^3 sample of garden was removed from Nest 1 during November 1990, along with a similarly-sized sample from a non-declining nest of the same species. Both samples were weighed, dried at 40^0C for 6 hrs in an incubator and then reweighed. The dry weight of declining garden was 49%, compared to 31% for healthy garden. Declining garden therefore tended to dry out. In all three nests, once gardens had shrunk to sizes which occupied less than 15% of domes, they often dried out completely and were abandoned or dismantled and dumped.

Plate 4:

a) Alate male on the surface of a fungus garden from a declining *Atta sexdens* nest.

b) Large white overgrowths of hyphae (H) on a fungus garden from a declining *Atta sexdens* nest (life-size). Few staphylae developed on the areas of garden around these growths, but the lower and left-hand areas of the garden shown had many staphylae, which are visible here as small white spots.


H

Contamination by alien fungi did not occur, although some abandoned gardens did smell of ammonia, indicating the presence of bacteria. One garden (Nest 1), occupying approximately 10% of a dome, was removed after the workers had abandoned it and placed in a closed chamber with damp filter paper. Within a few days, it was covered with fungal growth and samples were mounted in cotton-blue stain in lactophenol for microscopic examination. The fungi present included yeasts, *Aspergillus* and *Cladosporium*. The presence of bacteria was not examined.

Holes developed in some gardens, apparently where exhausted substrate was being removed to be dumped and some gardens also appeared to collapse or slump down. However, some gardens did remain almost normal, with small areas of grey, young garden at the top. These were the ones which persisted the longest and on which the surviving workers appeared to congregate.

In healthy laboratory nests, refuse is usually dumped from the table edges, although it is occasionally piled up along the route taken to the favoured dumping site. However, in all three declining nests, as domes were emptied or the gardens were abandoned, they were used to deposit nest refuse.

2. Worker numbers and behaviour in declining nests

Many individual gardens of Nest 1 were divisible into two regions; one with and one without white growths (see Plate 4b), therefore worker numbers and behaviour were examined on both, with 10 replicate observations each (made during August 1990). A second study was also carried out on Nest 2 (during January 1991). Nest 2 gardens did not have two distinct areas and did not develop white growths. Areas observed were also smaller (10 or 12 cm^2), because of the small size of the remaining garden.

There were significantly more workers present on areas without white growths than on areas with them, for Nest 1 garden ($p<0.001$, ANOVA), with a mean of 46.2 (SE ± 4.8) per 16 cm^2 on the former and 10.2 (SE ± 1.8) on the latter. On areas of Nest 1 garden with white growths, 94.0% of the workers were minima , while on areas without them 97.8% of workers were minima . Similarly, 96.6% of workers present on Nest 2 garden were minima , the remainder being media . Nest 2 garden had a mean of 26.3 (SE ± 3.6) workers per 16 cm^2 replicate area (multiplying up from 10 and 12 cm^2 replicate areas); intermediate between the two Nest 1 areas.

Twelve behavioural acts were recorded for workers (Table 11.1), the most common of which in Nest 1 was licking garden, followed by antennating garden. In Nest 2, resting and antennating garden were most common. This was a much lower number of acts than recorded for workers on healthy non-declining garden (see Table 5.2, Chapter 5, page 86).

The numbers of workers involved in each act on the two Nest 1 areas were compared using 2 x 2 Heterogeneity Chisquares where possible on the numbers of workers involved versus the total of workers involved in all other acts. Numbers grooming (pooling self and allogrooming figures) and licking were not significantly different ($p<0.25$), but numbers antennating garden, resting or restlessly moving were all significantly different ($p<0.025$, <0.01 and <0.005, respectively). Comparing behaviour on the three types of garden (two Nest 1 areas and Nest 2 garden) using 3 x 2 Heterogeneity Chisquares showed that numbers licking were not significantly different ($p>0.025$), but numbers grooming or antennating garden, resting and moving restlessly all varied significantly ($p<0.005$).

Table 11.1: Relative percentage frequencies of acts performed by workers in declining nests, on two distinct areas of Nest 1 fungus garden (with and without large white fungal growths) and on Nest 2 garden (10 replicates, n = number of acts observed).

TYPE OF ACT	RELATIVE % FREQUENCIES OF ACTS IN:		
	NEST 1		NEST 2 n=176
	AREAS WITH WHITE GROWTHS n=102	AREAS WITHOUT WHITE GROWTHS n=462	
At rest	7.843	2.381	45.565
Move restlessly	2.941	4.329	14.113
Self-groom	4.902	3.896	2.017
Allogroom - active	1.961	5.411	2.017
Allogroom - passive	1.961	4.329	1.210
Eat staphyla alone	0	3.896	0.403
Eat staphyla mutually	0	1.299	0
Antennate worker	0	0	3.226
Lick garden	53.922	55.844	1.210
Antennate garden	26.471	16.234	28.629
Carry staphyla	0	2.381	1.210
Remove refuse	0	0	0.403
Total	100.0	100.0	100.0

3. Rates of decrease in garden volume in declining nests

Nest 1 declined slowly, with a gradual loss of garden, while Nest 2 initially lost garden rapidly, then persisted with just one occupied dome for several months (Fig. 11.2).

Garden volumes in all domes usually decreased (Fig. 11.3), although some domes showed more activity than others. Some still contained areas of young garden, indicating recent building activity and these gardens persisted longer than others. When domes were less than

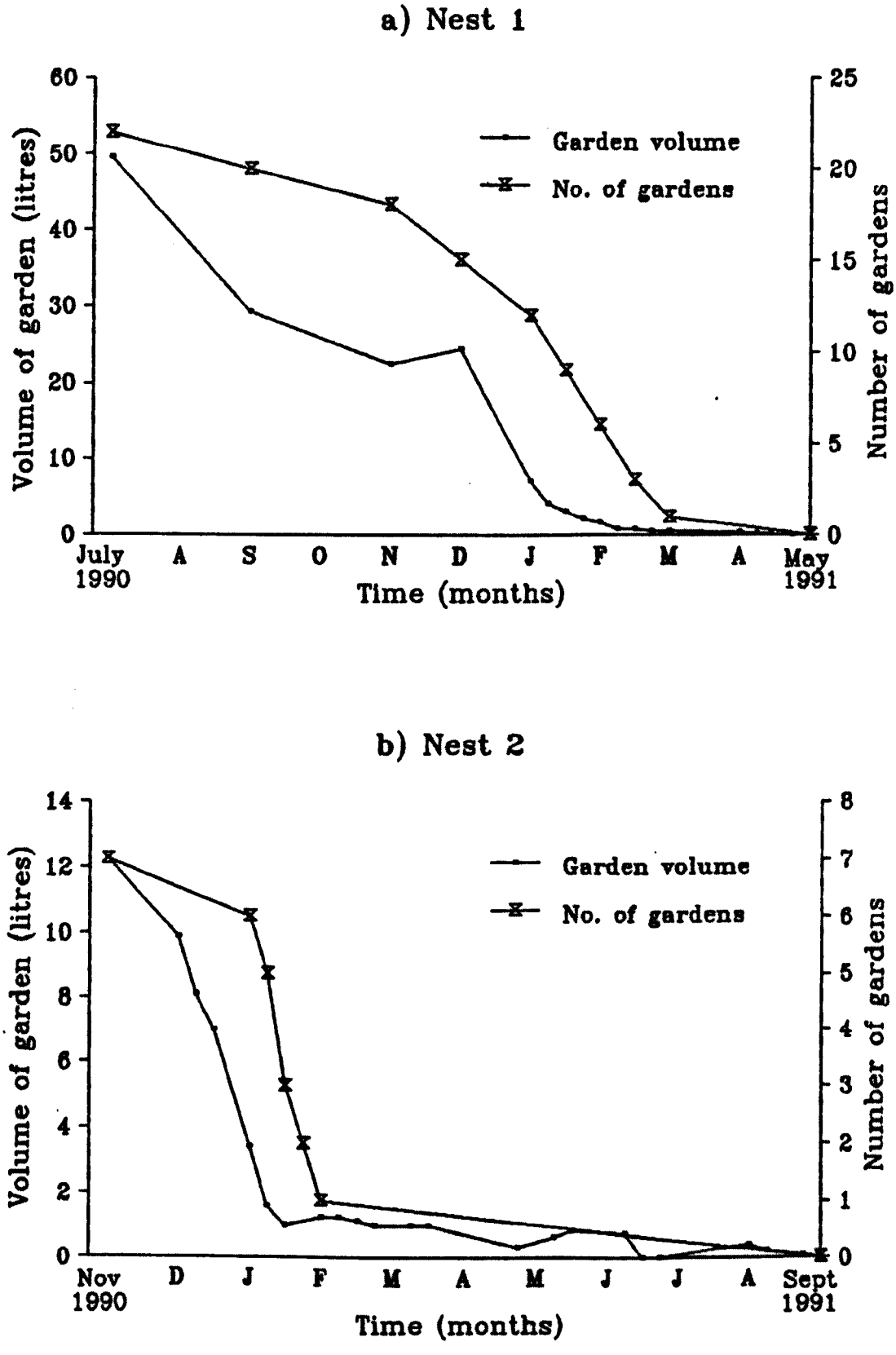


Figure 11.2: The rates of decrease in fungus garden volume (litres) in two dying *Atta sexdens* nests, from first symptoms of decline to complete death. The numbers of domes still containing garden and workers are also shown.

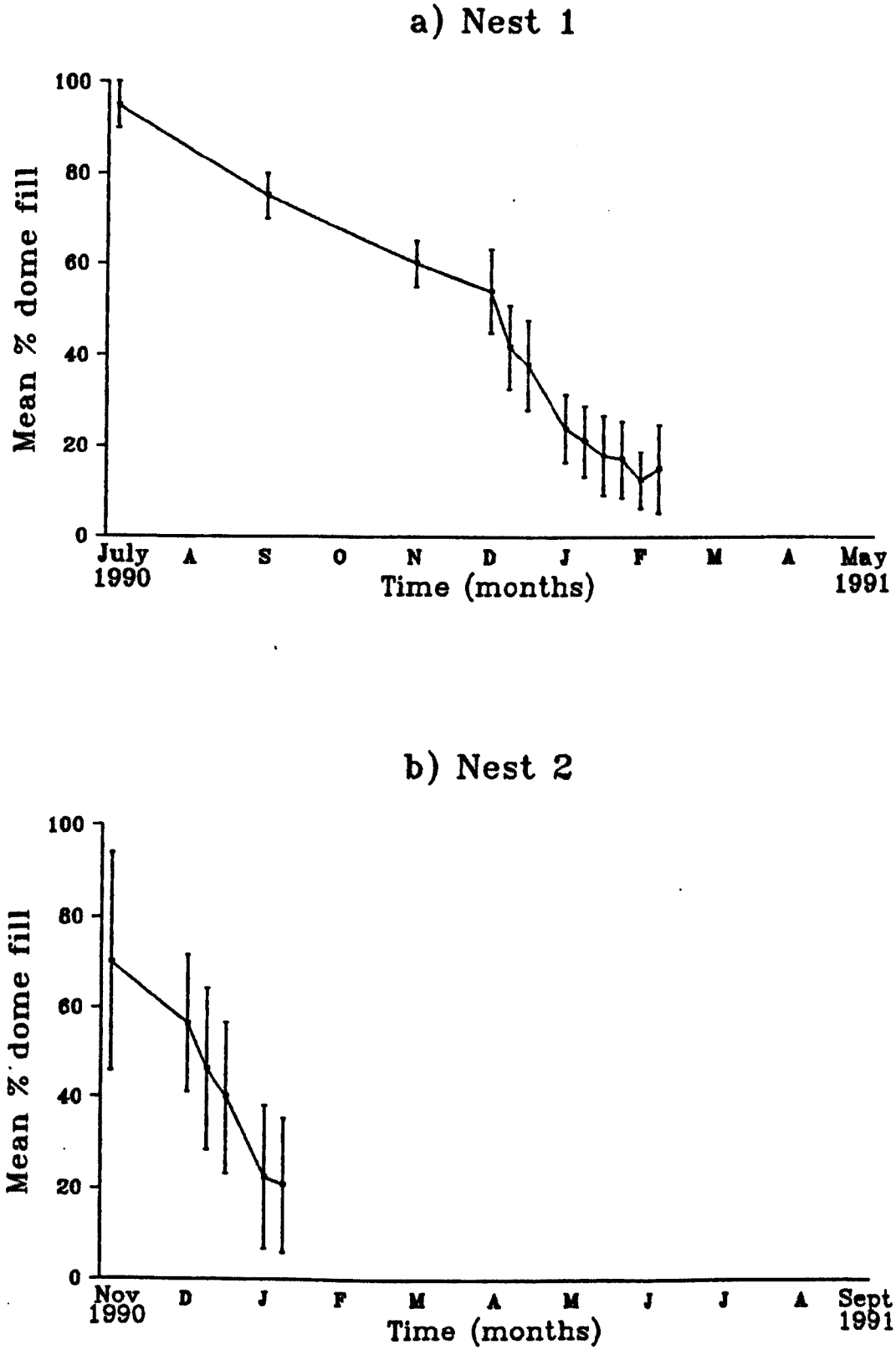


Figure 11.3: Mean percentages (± 95% confidence limits) of the 2.5 litre domes occupied by fungus garden in two declining *Atta sexdens* nests. Only those domes which contained garden and workers were included (3-18 replicates for Nest 1 and 3-7 for Nest 2).

around 15% full of garden, the workers frequently abandoned then or discarded the remaining garden. This was dry and shrivelled, although seldom contaminated by alien fungi.

4. The presence of staphylae in healthy and declining nests

a) Comparing staphyla age profiles

In theory, senescing staphylae should build up in a declining nest, since there are no larvae to consume them. Staphyla age profiles were therefore obtained from a healthy non-declining nest of *Atta sexdens* for comparison.

The numbers of different ages of staphylae present per 0.1 g sample of garden were compared between six different gardens in a healthy nest using a two-way analysis of variance. There were no significant differences between gardens ($p>0.05$, see Table 11.2) but there were significant differences between the numbers of different ages of staphylae ($p<0.001$). There was no significant interaction ($p>0.05$). The structure of the staphyla age population was therefore constant between gardens for a single nest.

Table 11.2: Two-way analysis of variance table comparing the numbers of different ages of staphylae present on six different gardens in a healthy nest.

SOURCE OF EFFECT	DF	SS	MS	F	P
Garden number	5	247	49	3	>0.05
Staphyla age	2	35555	17777	1091	<0.001
Interaction	10	317	32	2	>0.05
Error	72	1174	16		
Total	89	37292			

Samples of Nest 1 garden from areas with or without white growths were examined during November 1990. The numbers of different ages of staphylae present per sample were compared between garden ages and also with the numbers of staphylae present in samples of healthy non-declining garden examined at the same time, using a two-way analysis of variance (Table 11.3). Significant differences were present between the three types of garden and between the numbers of each staphyla age ($p<0.001$). There was also a significant interaction ($p<0.001$).

Table 11.3: Two-way analysis of variance table comparing the staphyla age profiles of fungus garden from a healthy nest with those from Nest 1 garden examined during November 1990, from areas with or without white growths.

SOURCE OF EFFECT	DF	SS	MS	F	P
Type of garden	2	19867	9934	220	<0.001
Staphyla age	2	34152	17076	379	<0.001
Interaction	4	42431	10608	235	<0.001
Error	81	3650	45		
Total	89	100100			

In the healthy nest, young staphylae were the most common type, followed by mature ones. Senescent staphylae were rare (Fig. 11.4). A similar pattern occurred on Nest 1 garden without white growths, although more staphylae were present in total (see page 237). However, on Nest 1 garden with white growths, young staphylae became rare and mature staphylae dominated, followed by aging ones.

Staphyla age profiles from Nest 2 were also compared with healthy nest profiles, using two-way analyses of variance (Table 11.4). Samples taken during November 1990

showed significant differences between the healthy nest and Nest 2, in the numbers of each staphyla age. There was also a significant interaction ($p<0.001$). This was also true for the two sets of samples taken during December 1990. Comparing the samples taken during November and December showed that significant differences were again present between sets of samples and numbers of each staphyla age, along with a significant interaction ($p<0.001$, Table 11.4).

The samples from Nest 2 were all obviously different from the healthy nest samples (Fig. 11.4). Numbers of young staphylae present decreased between November and the second December sample, while numbers of mature staphylae present increased. The numbers of aging staphylae present fluctuated with sampling time, but remained higher than those in the healthy nest.

Table 11.4: Two-way analyses of variance comparing the staphyla age profile on healthy garden with profiles from Nest 2 garden, examined at three different times during the decline period and also comparing between profiles on Nest 2 garden during the decline period.

Nest 2 garden, sampled 23rd November 1990, compared with healthy garden.

SOURCE OF EFFECT	DF	SS	MS	F	P
Type of garden	1	48792	48792	301	<0.001
Staphyla age	2	7761	3881	24	<0.001
Interaction	2	27799	13899	86	<0.001
Error	54	8728	162		
Total	59	93080			

continued overleaf -

Nest 2 garden, sampled 4th December 1990, compared with healthy garden.

SOURCE OF EFFECT	DF	SS	MS	F	P
Type of garden	1	50518	50518	2328	<0.001
Staphyla age	2	93351	46676	2151	<0.001
Interaction	2	93424	46712	2153	<0.001
Error	54	1173	22		
Total	59	238466			

Nest 2 garden, sampled 17th December 1990, compared with healthy garden.

SOURCE OF EFFECT	DF	SS	MS	F	P
Type of garden	1	92277	92277	2240	<0.001
Staphyla age	2	115997	57998	1408	<0.001
Interaction	2	143793	71897	1745	<0.001
Error	54	2224	41		
Total	59	354291			

Comparing Nest 2 garden sampled at different times.

SOURCE OF EFFECT	DF	SS	MS	F	P
Sampling date	2	8751	4376	33	<0.001
Staphyla age	2	368515	184258	1407	<0.001
Interaction	4	82357	20589	157	<0.001
Error	81	10584	131		
Total	89	470208			

b) Worker preferences for different ages of staphylae

When offered young, mature and aging staphylae, workers showed no preference and there were no significant differences between removal times ($P>0.7$, ANOVA, 20 replicates). The workers examined staphylae carefully as they came into contact with them by randomly walking over the perspex sheet and then removed them. Young staphylae were taken after a mean of 156.4 (SE ± 18.0) seconds, mature ones after 146.3 (SE ± 14.4) seconds and aging ones after 164.3 (SE ± 15.0) seconds.

5. Changes in infrabuccal pellet productions in declining nests

Refuse (0.1 g samples) was examined at intervals. Fortunately some samples were examined from these nests before decline began and could be used for comparison.

In Nest 1, before decline occurred, a 0.1 g sample of refuse, taken in June 1990, contained 960 T1 (detritus), 700 T2 (fungal) and 264 T3 (intermediate) pellets (see Chapter 6, page 124, for definitions). Another 0.1 g sample taken in December contained 206 T1, 232 T2 and 84 T3 pellets. In January 1991, a 0.1 g sample contained only 140 T1, 288 T2 and 108 T3 pellets. These figures were compared using a 3 x 3 Heterogeneity Chisquare analysis on the numbers of each pellet type produced at each sampling time. Significant differences were present ($p<0.005$). These occurred because higher than expected numbers of T1 and fewer than expected T2 pellets were found in June, compared to the January sample, which had fewer than expected T1 pellets and more than expected T2 and T3 pellets.

Similarly, in Nest 2 a pre-decline sample (June 1990) contained 930 T1, 760 T2 and 218 T3 pellets, whereas a sample taken in December 1990 contained 96 T1, 310 T2 and 52 T3 pellets. Another, taken in January 1991, contained 70 T1, 198 T2 and 56 T3 pellets. These figures were again compared using a 3 x 3 Heterogeneity Chisquare analysis and

significant differences were present ($p<0.005$), due to more than expected T1 and fewer than expected T2 pellets in June, compared to the December sample, with fewer than expected T1 and more T2 pellets. The January sample also had fewer than expected T1 and more T2 and T3 pellets.

Pellet production by Nest 3 was examined during December 1990, for comparison with Nests 1 and 2, since it appeared healthy at that time. A 0.1 g refuse sample contained 828 T1, 448 T2 and 180 T3 pellets; a fairly normal ratio of 57:31:12 (see Chapter 6, page 131). Another sample, taken in June 1991, after decline had become obvious, contained 358 T1, 344 T2 and 180 T3 pellets, while one taken in August contained 256 T1, 316 T2 and 150 T3 pellets. A final sample, taken during October, contained 116 T1, 218 T2 and 78 T3 pellets. Comparing these figures using a 3 x 3 Heterogeneity Chisquare analysis showed that significant differences occurred ($p<0.005$) with more T1 and fewer T2 and T3 pellets than expected in December 1990, compared with August 1991, which had fewer than expected T2 pellets. The October 1991 sample also contained fewer T1 and more T2 pellets than expected.

Nest decline therefore led to major changes in the ratios of the three types of pellet produced (Fig. 11.5). The proportion of T2 pellets increased, at the expense of T1 pellets. The proportion of T3 pellets either increased slightly or stayed the same.

Total numbers of pellets produced also decreased for each nest with time. Nest 1 produced 1,924 pellets per sample before decline, but after several months, produced only around 500 pellets per 0.1 g sample. Similarly, Nest 2 went from a pre-decline production of 1,908 pellets per sample to only 324 per sample after 2 months of decline. Nest 3, although similar to Nest 2 in size, declined more slowly. Initially producing 1,456 pellets per sample, by June 1991, it was producing 874 pellets per sample, then 722 pellets per sample in August. However, before complete death occurred, it was producing only 412 pellets per sample.

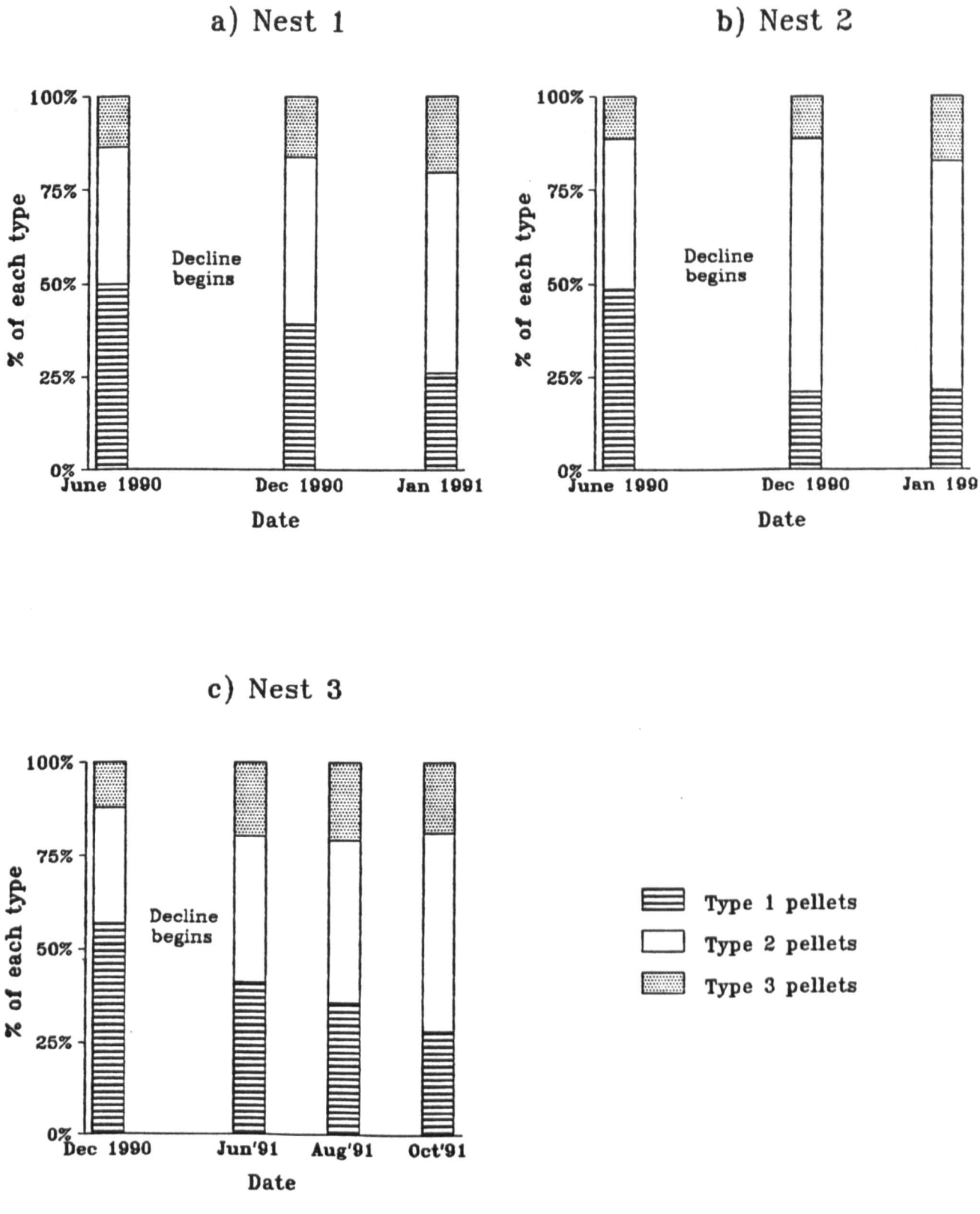


Figure 11.5: Decreases in the relative percentages of Type 1 pellets produced by three nests as they declined over periods of several months. For definitions of pellet types 1-3, see Chapter 6.

6. Changes in worker populations and caste ratios in declining nests

After several months of decline, workers appeared to congregate on to remaining gardens at very high densities in all three nests. Most of these workers were minima , although a few larger workers remained which foraged, dumped refuse or rested in or around domes.

During December 1990, 100 cm^3 of garden were removed from Nest 2 and treated with carbon dioxide gas to anaesthetize the workers, which were then counted. There were 224 minima , 3 media , 1 maxima and 1 soldier (Weber's 1972 size classes). Most of these workers were found on the outer surface of the garden. A second sample was taken in January 1991, of only 4 cm^3 of garden, since there was very little left. There were 157 minima present, only 12 of which were inside the garden sample. A few larger workers were still visible in the occupied dome, but were extremely rare compared to minima .

In February 1991 worker population and caste sizes in Nest 1 were compared with those from a healthy, non-declining nest. A similar volume of fungus garden was removed from both (approximately 40 cm^3) and weighed. The declining garden sample weighed 6.4 g, while that from healthy garden weighed 6.0 g. The declining sample yielded 139 workers, while that from healthy garden had 203. However, the declining sample had a population almost entirely of minima with very few larger workers, while that from healthy garden had fewer minima and more larger workers (Fig. 11.6).

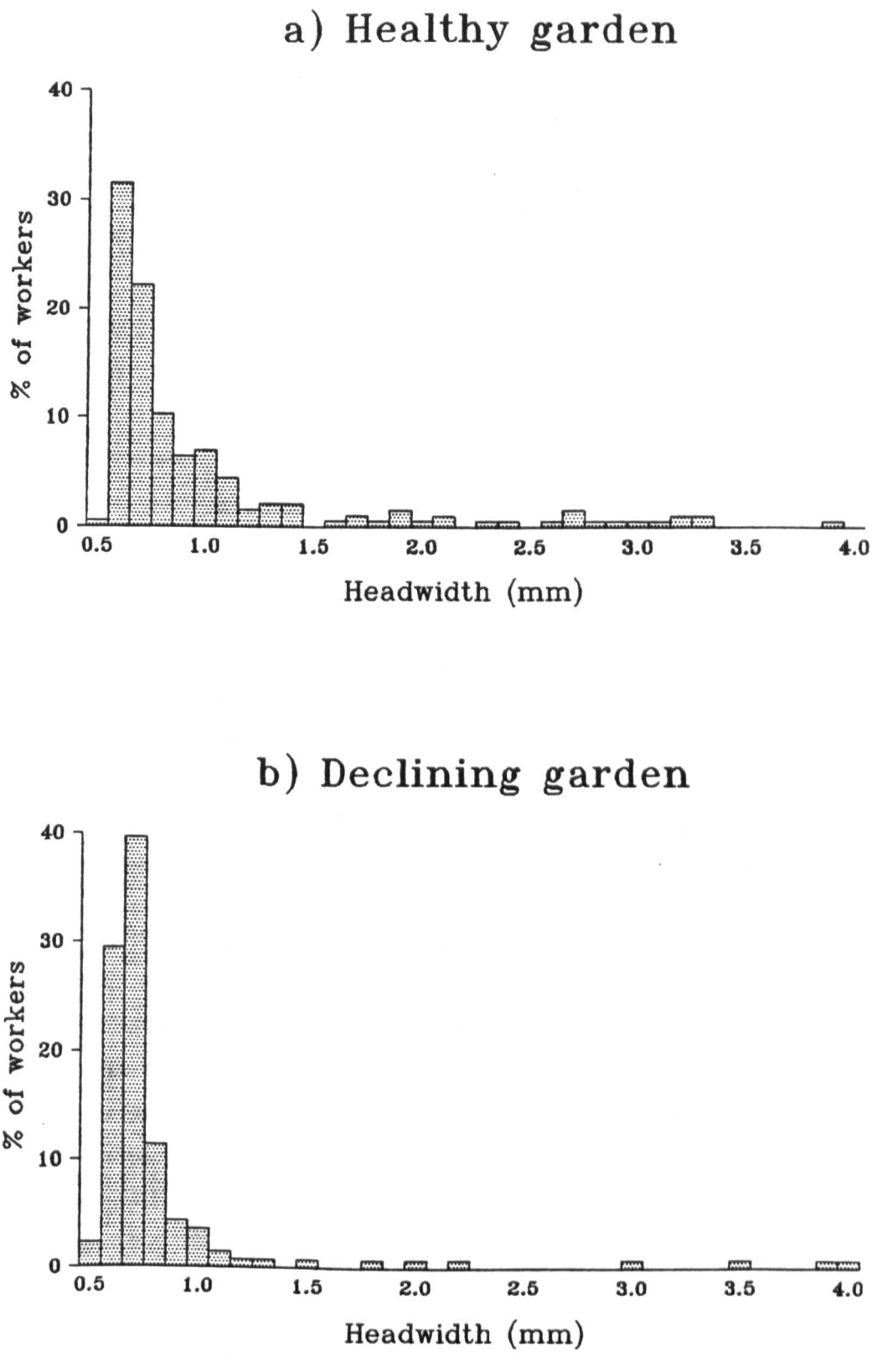


Figure 11.6: The percentages of workers of different headwidths present in samples of fungus garden from, a) a normal healthy nest (203 workers examined) and, b) Nest 1 (135 workers).

DISCUSSION

Most of the observations were made either rarely, at irregular intervals or were qualitative rather than quantitative. In particular, few observations were made on Nest 3, which declined while its queen remained alive. Nest 1 could not be directly compared with Nests 2 and 3 because it was older and larger than the other two, which were very similar to each other. However, some general conclusions can be drawn.

A decrease in the level of foraging was the first visible symptom of decline, coupled with absence of brood and Powell (1984) reported that foraging levels are related to the number of larvae present. The reduction in foraging led to a decrease in the amount of fresh leaf material being incorporated into the garden, which probably contributed to the increasing paleness of declining garden. The paleness was mainly due to the thick growth of hyphae on the garden surface and this was a symptom of the reduction in worker activity, although the actual proportion of workers licking and tending garden increased compared to healthy nests (comparing Tables 11.1 and 5.3, pages 240 and 88). Nest 1 showed the changes in worker activity most clearly, by developing two distinct zones; one 'out of control' area, with few ants, relatively few staphylae and enormous white growths of hyphae and a second zone with many ants, no large white growths and huge drifts of staphylae. These 'out of control' areas, with their large white growths, coinciding with low worker population, suggested that lack of hyphal pruning by licking workers had allowed the mycelium to 'escape' worker control. The second type of area, with many workers, many staphylae and no white growths, suggested that continuous pruning by workers was stimulating staphyla production and preventing the mycelium from becoming overgrown. However, in Nest 2 the numbers of young staphylae present decreased with time as mature ones built up (Fig. 11.4). In the normal healthy

nest, the largest numbers of staphylae were young, followed by mature, with very few senescent ones. However, in Nest 2 senescent staphylae became very common. This also happened in the out-of-control areas of Nest 1, although the second zone retained a more normal profile (Fig. 11.4). The changes in the staphyla standing crop were probably due to worker neglect (in the out-of-control areas of Nest 1) and a decrease in consumption rate, since no larvae were present (Nest 2). The highly populated second zone areas of Nest 1 retained a normal pattern, possibly because of the large worker population, which was approximately the same as in a healthy nest (see Fig. 5.1, Chapter 5). It is probable that this 'normal' profile later changed and resembled that of Nest 2, when disorganisation had progressed further.

The large white growths were only produced in Nest 1 gardens where the ants could not easily reach them, such as against the dome wall. They consisted of hyphae and some gongylidia, they did not produce conidia and were not representatives of the sexual stage of the fungus as reported by other authors (Moeller 1893, Stahel and Geijskes 1941, Muchovej *et al.* 1991). Similar, though smaller growths were also observed in a healthy laboratory nest of *Atta laevigata*. This colony built compact gardens which filled domes almost completely and fungus garden often came into contact with the dome wall, leading to the production of hyphal overgrowths.

Not all gardens in Nest 1 produced these growths; only 8 out of 22 gardens developed them. These were probably the largest ones, where garden most often came into contact with the dome walls.

Nest 1 also had a different rate of decline to Nest 2. Nest 1 lost garden volume slowly and steadily, while Nest 2 lost most of its garden very rapidly (Fig. 11.2). This may have been due to the buffering effect of a large initial garden volume in Nest 1 (22 gardens), compared to

the initially small volume in Nest 2 (7 gardens). Small systems tend to be more unstable than large ones. Although no quantitative observations were made on Nest 3, which also had a small garden volume initially, it lost garden volume much more slowly than Nest 2. This may have been due to a buffering effect caused by the presence of the queen. The queen alone cannot maintain social organisation since larvae are required for that, but her presence may have some effect.

Examining worker behaviour showed that although the relative percentages of workers licking and antennating garden increased compared to a healthy nest (see Tables 11.1 and 5.3, pages 240 and 88), levels of resting in Nest 2 increased dramatically, although it remained fairly normal in Nest 1 areas. However, the observations on Nest 1 garden were made relatively early in the decline period (August 1990) while a large volume of garden was still present. The observations on Nest 2 were made much later in the decline period (January 1991) and much of the garden volume had been lost. This may have led to less worker activity and hence greater numbers resting. The numbers of workers moving restlessly also decreased in both nests. Other activities, like grooming, consuming staphylae or carrying staphylae remained fairly normal, but changes would be difficult to detect since these acts are not particularly common even in healthy nests.

All three nests showed a decrease in the production rate of infrabuccal pellets, compared to normal pre-decline levels. This may have been due to decreased worker populations, decreased activity or an increase in the relative amounts of refuse discarded. All three reasons were probably true to some extent. Worker populations would decrease as losses through natural mortality were not replaced by maturing brood. Reductions in worker activity, with more resting and less foraging would also lead to a reduction in the pellet numbers produced by each worker and

it would have been interesting to have examined this more closely. Large amounts of shrivelled or abandoned garden were frequently discarded and this would have diluted the already depleted numbers of pellets.

The relative proportions of the three different types of pellet produced also changed in declining nests (Fig. 11.5). Type 1 pellets became scarcer and T2 pellets became relatively more common. T1 pellets contain plant debris and may result from foraging (see Chapter 6). In declining nests, decreased foraging would therefore lead to a reduction in T1 pellet production. T1 pellet production did not decrease to zero, but neither did foraging activity. T1 pellets may also contain debris from grooming. Workers continued to venture outside domes to discard refuse and debris from these activities may produced T1 pellets. T2 pellet production per worker may, in contrast, have remained relatively constant. However, with less foraging taking place, surviving workers may have consumed more staphylae, particularly since there was a large surplus of these. This in turn would have led to the production of more T2 relative to T1 pellets. Also, workers licking garden would have more opportunity to ingest hyphae, because the mycelium proliferated under the reduced licking pressure caused by the reduction in worker population. This again would have produced more T2 pellets.

T3 pellet production increased slightly or remained relatively constant and this may have been because these were the rarest type of pellet; any changes in relative production rates would have been less noticeable.

After several months of decline, more workers appeared to be present on the surface of declining garden than on normal healthy garden. However, counted per unit volume, there were fewer on Nest 1 garden than on healthy garden. This was because the declining garden became very compact, with small cavities that few workers could enter. Almost all the workers present therefore remained on the outer surface, giving the appearance of crowding. This reduction

in cavity size may have been related to the increasing dryness of declining garden. In healthy nests, the intake of fresh juicy substrate maintains a high moisture content. However, where foraging remains at a minimal level for long periods and only wilted material is used, as in the declining nests, the garden will gradually become drier, unless the workers can import large quantities of water. Large numbers frequently did drink water from petri-dishes on the foraging tables, but with the general reduction in activity, not enough liquid would have been taken in to maintain the high humidity. This would have led to garden shrinkage and hence reduced cavity sizes. Unfortunately no measurements were made of this.

After long periods of decline, few large workers remained (Fig. 11.6). In a normal nest, approximately 70% of the workers present on the garden surface are minima (see Chapter 6, page 142). However, well over 90% of the workers on garden that had declined for several months were of this caste. Large workers usually go out and forage (including many of the soldiers in *Atta sexdens*) and the few that remained alive in the declining nests also did so. However, the reduction in foraging activity may have taken place because the large workers were most sensitive to the lack of brood and became disorganised very quickly, perhaps becoming lost and falling off the tables. Minima workers, in contrast, are usually very closely involved with the fungus garden and are specialised for caring for it. They also live longer when isolated with fungus garden than larger workers (see Fig. 10.2, Chapter 10). The presence of garden might therefore act as a stimulus, buffering the effect of the lack of brood. Evidence for this can be seen in the continued care for the garden and the prevention of contaminant growth. Contaminant spores were present, since a piece of abandoned garden, placed into humid conditions rapidly developed a fungal flora, which was typical of the generalist saprophytes which usually colonise garden in the absence of workers (see Chapter 8, page 176).

Foragers may become so disorganised that they eventually starve to death, leaving only a few younger or less 'sensitive' survivors, which continue to forage and obtain plant sap, which provides most of the energy requirements of workers (Quinlan and Cherrett 1979). Genetic variability between workers might be manifested as differing sensitivity to lack of brood. In a nest deprived of brood, some workers would therefore die very quickly, while others continued to behave semi-normally. This would also explain why nests take so long to die. Nest 2 lost most of its initial garden volume very quickly and then persisted for several months with just one garden. However, under field conditions, such a nest would be rapidly invaded by other organisms, since the ants would be unable to defend it. Workers of some ant species, especially those belonging to the phylogenetically advanced subfamilies Dolichoderinae and Formicinae, vary in their readiness to work (Oster and Wilson 1978). Chen (1937) found that 'leader' workers of *Camponotus japonicus* sub sp. *aterrimus* began to dig sooner, moved more earth per individual, showed less variation in effort than other workers and stimulated their nest mates when placed in earth-filled jars. Similar elitisms have been observed in *Tapinoma erraticum* during brood transport (Meudec 1973) and *Formica fusca, Formica sanguinea* and *Camponotus sericeus* during adult transport (Moglich and Holldobler 1974, 1975). Workers of *Atta* show learning and particularisation of foraging behaviour (Lewis *et al.* 1974a) and tend to patrol certain portions of the foraging ground over long periods, becoming familiar with specific areas of the terrain.

Alternatively, different castes may have different rates of senescence, as discussed in Chapter 10.

Some declining nests might persist for longer periods by producing a brood of males, in a 'last-ditch' attempt to reproduce. Such males would originate from worker-laid eggs. In many ant species, such as *Eciton, Monomorium, Pheidole* and *Solenopsis*, the workers lack ovaries and all

males are produced by the queen (Oster and Wilson However, in a healthy Attine nest, workers produce trophic eggs which are fed to the queen (see Chapter 5) and this also occurs in other species, such as *Aphaenogaster rudis* (Crozier 1974). However, in the absence of the queen they may produce males since they possess functional ovaries.

Workers are actually more closely related to sons and nephews than they are to brothers and according to Hamilton's (1964) kin selection theory, should attempt to produce the colony's males, while the queen 'prefers' to produce sons rather than grandsons. There will therefore be conflict between the queen and workers. There are no known cases where workers are the exclusive parents of males when healthy queens are present, but mixed parentage is common and widespread in eusocial halictine and meliponine bees (Michener 1974) while *Myrmica* workers also produce most of the males in their colonies(Brian 1968).

This may have been the case in Nest 1, which produced approximately 60 adult males, just as a decline in the amount of foraging was taking place. Such a brood would, while in the larval stage, continue to stimulate normal behaviour. Alternatively, the queen may simply have run out of spermatozoa and laid unfertilised eggs.

SUMMARY

The first symptom of decline in colonies deprived of brood is a reduction in foraging activity, and alate males were produced in one nest. The fungus garden became pale and sometimes grew 'out of control', with large white growths of fungus. Standing crops of staphylae built up to high levels and relatively more mature and aging staphylae were present than in normal, non-declining garden.

A large nest declined slowly, whereas a small colony lost most of its garden volume very quickly. Another small colony declined relatively slowly, with its queen remaining alive.

Worker behaviour changed; there was relatively more licking garden and antennating garden and resting, while restless movement decreased. Worker populations decreased, although superficially, workers appeared to be crowded on the garden surface, due to a reduction in garden cavity size which prevented workers from entering the garden, possibly due to the garden drying out. Large workers disappeared more quickly than small workers, possibly because large workers suffered from social disorganisation more quickly than small workers and either died of starvation or fell out of the nest. Small workers had the stimulus of the fungus garden, which they are specialised to care for. Alternatively, different castes may senesce at different rates.

Infrabuccal pellet production decreased, compared to pre-decline levels, particularly that of Type 1 pellets, which are the result of foraging.

Chapter 12: General discussion - fungus culturing as an agricultural strategy

Growing a crop demands certain operations (cultivation, pest control) and inputs (fertiliser, suitable substrate, time and effort). The yield obtained from a crop depends upon the genotype of the crop and the environment in which it is grown. This applies to both human and insect agricultural systems. It is therefore interesting to compare how such very dissimilar animals as humans and ants have confronted and solved similar common problems. Human behaviour is very flexible since Man possesses insight and consciousness, so can plan ahead and devise strategies to achieve goals. Insect behaviour on the other hand is 'hard-wired'; insects have no plans or goals and their behaviour is regulated solely by the forces of natural selection. This favours the solution which transmits the most genes to the next generation. New agricultural systems might therefore be developed within a human lifetime, but new systems in insect societies can only evolve over many generations. The analogies between human and Attine agricultural systems are therefore approximate and their solutions result from different processes.

Agricultural systems, whether in human or insect cultures, require a sequence of events:

1. <u>Domesticating a crop</u>

Around 10,000 years ago, Man learned to cultivate primitive wild einkorn and emmer wheats and changed from a hunter-gatherer to a farmer. This probably occurred by accident as seeds harvested for food were scattered on middens. People then began to deliberately scatter seeds and grow crops (Boughey 1971). The majority of ants are hunter-gatherers, taking insect prey and honeydew or gathering seeds, as in harvesting species like *Messor* and *Pheidole*. The Attines however, shifted to eating a fungus.

In order to be domesticated, a crop must fulfil certain criteria. It must provide a worthwhile food source and its reproduction must be controllable. Once domesticated, selection by the grower, whether deliberate or not, will ensure that beneficial characters are exaggerated and undesirable ones lost, In this way, the aggressive aurochs was transformed into the docile domestic cow of today (Attenborough 1984) and there are now more than 17,000 varieties of wheat (Simmonds 1976). The ant fungus has also been changed by domestication. It seldom, if ever, produces reproductive structures, it is found only in Attine nests and it produces bundles of swollen hyphae, the staphylae, which are produced solely as food for the ants (Cherrett *et al.* 1989). Different species of Attines also appear to grow different strains of fungus, with different productivities (Stradling and Powell 1986). Thus the fungal strains of *Atta* species are much more productive than those of *Acromyrmex*, which in turn are more productive than those of more primitive species like *Trachymyrmex urichi*. Species with larger colony sizes have fungal strains with greater productivities. In mushroom growing, the farmer can select better strains using mutation and genetic recombination (Flegg *et al.* 1985) and has more control over his fungus than the Attines, in which selection is exerted on the colony rather than by the colony. Colonies with 'better' strains will have greater fitness and produce more daughter colonies than those with 'poorer' strains of fungus.

2. Productiveness of substrate

The early Attines probably resembled primitive species of today, such as *Apterostigma*, *Cyphomyrmex* and *Mycocepurus*, collecting insect frass and dead plant material on which to culture their fungus. Living leaves provide a much richer source of nutrients for the fungus and also contain sap, which the ants utilize as an energy source (Quinlan and Cherrett 1979). Hence today, leaf

cutting ants are the most complex and successful of the Attines.

In agriculture, most crops are grown in soil, but a few intensively grown crops such as mushrooms and many greenhouse crops are cultivated in specially prepared media and require specialised and controlled conditions (Hanan *et al.* 1978, Flegg *et al.* 1985). In mushroom growing, compost is brought in, sterilised, inoculated and after the crop has been harvested, the compost is thrown away and the culture room sterilised (Flegg *et al.* 1985). This is a similar situation to that found in Attines, where substrate is carefully prepared and licked to remove contaminant spores (Quinlan 1977) then inoculated with large amounts of mycelium. After the crop (staphylae) has been harvested and the substrate has been exhausted, it is discarded and very few staphylae are thrown away with it (see Chapter 4) . The length of time for which substrate can support fungal growth (the rate of turnover or crop rotation) is therefore important for Attines, particularly in seasonal habitats where leaves may not be available at all times of the year. Chapter 4 showed that rates of turnover in fungus gardens of a laboratory nest of *Atta sexdens* varied from 20-80 days, depending upon the time of year. Relatively slow turnover occurred during October and November, probably because of high-dry weight mature leaves received by the nest during the late summer and early autumn. Since in the laboratory, the nests received a constant volume of leaves all year round, this slow turnover led to a build-up in garden volume. This in turn produced a large crop of staphylae which supported a large brood.

3. Culture techniques

The relative merits of spatial and temporal monocultures are considered in Chapters 1 and 3, but any crops grown as monocultures require intensive care. Gardens of *Atta* are cultured in separate chambers, each of which has one or two entrance holes, usually in the lower half of

the chamber (Jonkman 1980). Thus *Atta* has a temporal but not a spatial monoculture and this may be important for preventing the spread of disease in large colonies.

The fungus garden of *Atta* is a dynamic structure which is maintained in continuous culture over several years (see Chapter 3) unless environmental conditions change or the nest declines. This is a similar situation to an apple orchard, whereby the trees continue to produce fruit over many years. Nest decline occurs when the queen either dies or ceases to lay eggs and the social cohesion of the colony is dependent upon the presence of larvae (Powell 1984). Larger nests probably decline more slowly than smaller ones and the rates of loss of garden volume were much slower in Chapter 11's 'Nest 1', which originally had 22 gardens than in the two other nests observed, which had only 7 and 8 gardens each originally. This was probably because large size tends to have a buffering effect. *Atta* nests, which usually have several hundred gardens (Jonkman 1980), are therefore likely to decline much more slowly than *Acromyrmex* nests, which usually have only a few gardens (Weber 1972). Such a slow rate of decline in *Atta* nests might enable them to make a 'last ditch' attempt to reproduce by raising males from worker-laid eggs, or to give an ailing queen time to recover and resume egg-laying. Adoption of newly-fecundated queens may also occur, although there is no evidence for this in Attines. Rapidly declining *Acromyrmex* nests would not have such opportunities. However, *Atta* colonies invest enormous amounts of resources in excavating their large nests, therefore any strategy which could salvage their inclusive fitness after the death of the queen would be likely to be selected for.

Mushroom growers maximise 'field size' by stacking the boxes in which the mushrooms are grown (Flegg *et al.* 1985). This saves space and increases the relative size of the crop produced in a given space. This is also energy efficient, since in mushroom growing environmental conditions must be carefully controlled and it is cheaper

to do this in a small room than a large one. Similarly, leafcutting ants build fungus gardens with large internal surface areas, which produce large numbers of staphylae (see Chapter 4). In a human population, access to mushroom culture rooms is restricted to the growers and similarly, in the leafcutting ant colony, only the minima workers, which are specialised for garden-care, can enter into all its internal cavities to harvest the staphylae found there. The demand for staphylae by larger workers is shown by the relatively fewer numbers of staphylae found on the outer surfaces of the garden and in large cavities.

For Attines, this strategy is also labour-saving. There is less chance of the internal 'fields' of the garden being contaminated by alien fungi or bacteria and humidity is maintained at a high level within the garden. Fungus gardens removed from their chambers dehydrate rapidly, but the rate of water loss would be drastically increased if the garden was constructed as, for example, a flat sheet.

4. Crop husbandry

There are several aspects of husbandry, including the use of fertilisers, control of pests and diseases and manipulating growth.

a) Fertilisers

A farmer sprays his field with fertiliser containing macronutrients (e.g. nitrogen, potassium, phosphorus) and micronutrients (e.g. boron, manganese) to improve crop yields (Halley 1982). In an intensive system, the farmer will apply large amounts of inorganic fertiliser, while the peasant farmer relies on organic methods like crop rotation, leys and manure. Leafcutting ants also apply 'fertiliser' to their fungus garden to encourage growth. Their faeces contain allantoin and allantoic acid, which provide a source of nitrogen and amino acids (Martin and Martin 1970b). When the fungal inoculum is planted on to a freshly inserted piece of substrate it is manured with faeces and the nutrients present sustain it until the

hyphae can penetrate and utilise the substrate. Worker faeces also contain enzymes which originate from the fungus (Martin *et al.* 1975). The majority of these enzymes are probably ingested and returned to the fungus via licking workers, which ingest hyphae.

b) The control of pests and diseases

Many domesticated crops are very susceptible to disease, partly because they are usually grown as monocultures and partly because selection by the grower for desirable characteristics like palatability and softness often leads to increasing dependence of the crop on the grower. High yielding crop strains often lose the ability to defend themselves against pests and diseases, or become more attractive to them. Leafcutting ants, for example, prefer to attack susceptible introduced crop plant species compared to native species, probably due to a combination of novelty, lack of defences directed against them and lack of defences against pests in general. Arthropods such as millipedes and springtails find the Attine fungus highly attractive and will invade the garden in the absence of workers (Quinlan and Cherrett 1978a).

Pests or weeds in a crop can be removed physically or by using pesticides. The former is labour intensive and in agriculture is usually only used in extensive systems. *Atta* uses both methods of control, since it has a large work force available, up to 30% of which may be licking the garden surface (Quinlan and Cherrett 1979). A licking worker removes alien contaminants from the garden surface with great precision, leaving the fungus garden intact. Licking therefore physically protects the garden from competitor fungi. Ingested material is taken into the infrabuccal pocket, where chitin-digestion occurs (Febvay *et al.* 1984) and this may denature fungal spores. Material collected into the worker infrabuccal pocket is discarded away from the garden with nest refuse.

Antibiotics like myrmicacin and phenylacetic acid are applied to the garden, possibly by licking workers. These

antibiotics are produced by the metathoracic glands (Schildknecht and Koob 1970, 1971) and it remains unclear how they travel from the back of the thorax to the fungus garden. Metathoracic gland secretions probably spread all over the exoskeleton and may therefore be picked up by social grooming and transferred via the mouthparts to the fungus garden. Alternatively, workers may have some mechanism for applying secretions from their own metathoracic glands to the garden surface.

The ant fungus is generally considered to be a poor competitor and fungus garden is said to be rapidly overrun by alien fungi and bacteria in the absence of the ants (Weber 1972, Powell 1984). However, Chapter 8 showed that fungus garden could be isolated from workers for up to 12 days before major contaminant growth occurred, in spite of the fact that contaminant fungal spores were present in it already. The antibiotics applied to the garden by workers may therefore have a residual effect, suppressing the germination of alien spores even in the absence of workers. In the same way, residual herbicides such as diuron suppress the growth of weeds for long periods after application (CABI 1988). The ant fungus may also produce antibiotics itself (Hervey and Nair 1979).

c) Growth manipulation

Pruning is a widely used horticultural technique for manipulating growth. For example, tea bushes are pruned to keep them at a manageable size (Simmonds 1976). Many plants are pruned in order to manipulate fruit or flower production, such as apple trees, chrysanthemums and tomatoes. *Atta* workers also control the growth of their fungus by pruning. The physical removal of hyphae from the garden surface by licking stimulates the production of more staphylae and also ensures that the ant fungus cannot reproduce and produce possibly unattractive reproductive structures.

Growth regulators are commonly used in intensive agricultural or horticultural systems, for example, chlormequat prevents cereal crops from growing too tall

(CABI 1988). Angeli-Papa and Eyme (1979) related the inhibition of growth of hyphal apices in the fungus garden of *Acromyrmex octospinosus* to the presence of virus-like particles. This may therefore act as a growth regulator in the ant-fungus mutualism.

5. Harvesting the crop

Modern intensive farming usually employs machinery to harvest crops but this option is obviously not open to Attines. Crops like strawberries and mushrooms are hand-picked and individual fruits do not ripen simultaneously. Such crops are expensive to harvest, requiring a large labour force. The production of staphylae by the fungus garden is similar. Different areas of garden have different numbers of staphylae and individual staphylae are at different stages of growth. There may be a 7 day rhythm in the production of gongylidia (Angeli-Papa and Eyme 1979) which is similar to the situation in cultivated mushrooms, whereby the mature mycelium produces several 'flushes' of mushrooms before it exhausts the substrate (Flegg *et al.* 1985).

If staphylae develop at random, 'escaping' worker attention, then they may also be harvested at random, as a hungry worker seizes the first staphyla it encounters. Staphyla productivity in the fungus garden may be higher if staphylae are harvested before they reach maturity and without the ants, staphylae grow much larger (see Chapter 7). As a staphyla develops, it uses resources from the forage harvested by the ants. Growth probably follows an 's-shaped' curve, therefore staphylae would grow quickly initially, then mature slowly. In pig production, as liveweight increases, more feed is used for each kilo of further weight gain, therefore it is more economical for the farmer to slaughter the pigs before they reach maturity (Whittemore 1980). A similar case may occur with staphylae on the fungus garden, although the ants, of course, will not consciously harvest them for this reason.

Hyphae may also be harvested as a source of nutrients, as another incidental factor of licking, since digestion occurs to a degree in the infrabuccal pocket (Febvay *et al.* 1984).

6. Managing changes in demand

Hunter-gatherer societies generally do not store food for long periods. The rise of agriculture however, meant that grain had to be stored to provide food between harvests. Storing food also enabled farmers to deal with over-production by storing it until times of shortage. However, some crops are difficult to store. Strawberries have a very short shelf-life (Hughes 1980) and garden (vining) peas must be frozen within 6 hrs of harvest. Similarly, staphylae persist on the fungus garden for only a few days. The ants cannot process them to store them for longer periods, but the garden provides a steady crop of new staphylae, rather than one large flush. The garden as a whole can act as a food store or buffer and if for some reason the ants cannot forage and obtain plant sap, they may consume more staphylae (Quinlan 1977). Once foraging is resumed, there are no attempts to make up the deficit of forage (Hodgson 1955).

In some Attine habitats, seasonal fluctuations lead to abundance of fresh leaves at one time and scarcity at others. Leafcutting ants respond to this by increasing their foraging rate at the time of leaf abundance. The fungus garden built from these leaves will then provide a steady crop over the next few months to feed the larvae and queen (Weber 1972).

Similarly, when preparing for a large brood, which places a great burden on colony resources, workers may increase their rates of foraging (see Chapter 3) and without larvae, foraging often ceases (Powell 1984). The ants therefore respond to food shortages by foraging more.

Within the nest, local shortages and gluts are dealt with by exporting excess staphylae from one region to another of high demand (see Chapter 3). Fungus gardens from different parts of the same *Atta* nest often contain

different proportions of each brood stage, hence creating differences in demand for staphylae (Lewis *et al.* 1974b). In agriculture, farmers producing large crops will sell to the places of greatest demand, in this case, for a higher price. The ants, by carrying staphylae to hungry larvae increase their inclusive fitness by ensuring the future prosperity of the colony.

7. Disposal of crop residues

After a farmer has grown and harvested a crop, before he sows the next he will have to remove residues of the old, to prevent the carry-over of pests and diseases. Crop residues may also be directly deleterious to the new crop. Straw stubble ploughed into the soil can cause increased soil acidity and the depletion of nitrogen resources, which may damage the new crop (Halley 1982). In intensive horticulture, the growing media used may be sterilised for re-use or discarded, as in mushroom production (Hanan *et al.* 1978, Flegg *et al.* 1985). This resembles the leaf cutting ant system, whereby fresh substrate is brought in, effectively sterilised by assiduous licking (Quinlan 1977), used to grow a crop, then discarded either in underground chambers, which are subsequently sealed, or in external dumps. As Powell (1984) pointed out, there is a spatial separation between nutrient-rich substrate which is mostly incorporated into the top of the garden and metabolite-rich debris which is removed from the garden base.

8. The profitability of agriculture, or 'agriculture's two-edged sword' (Diamond 1991)

Agriculture led to an increase in human populations because it provided more food. Leafcutting ant colonies are also very large, but so are colonies of army ants like *Eciton burchelli*. However, leafcutting ants can build large underground nests which are relatively safe from attack, while army ants must continually move into new areas, since they rapidly exhaust local supplies of insects. Agriculture

also led to the rise of well-defended cities like Uruk in Mesopotamia (Boughey 1971).

Foraging for food can be dangerous, both for ants and Man, hence activities that ensure that a stable food supply grows close to or within the settlement are advantageous. Leafcutting ants still have to forage for leaves, from which they obtain sap for their energy requirements and a substrate for their fungus. The majority of foraging takes place along well-defined trails by large groups of ants often with large defensive soldiers present (Weber 1972), since there is safety in numbers.

When agriculture first arose, it spread very slowly, at a rate of only 1000 yards per year (Diamond 1991). Only one group of ants, the Attines, cultivate a fungus and of these, only 2 out of 12 genera harvest fresh leaves as the main substrate for their fungus (Weber 1972). This suggests that there are constraints against the development of agriculture.

The rise of agriculture led to an average decrease in human height and health. Farmers concentrate on growing a few foods, whereas hunter-gatherers utilize a much wider range of foods, which ensures that they fulfil all their nutritional requirements. Farmers therefore obtain high-energy foods at the expense of nutritional diversity. Farming communities can also suffer periodic famines and infectious diseases and parasites persist in crowded societies of malnourished sedentary people who are constantly reinfected by each other and their own sewage. Measles, for example, needs a minimum population of several hundred thousand to maintain itself (Diamond 1991). Large crowded colonies of ants would be expected to suffer from similar problems, but do not. Ants practise rigorous hygiene and also secrete antibiotics from their metathoracic glands (Schildknecht and Koob 1970, 1971). Debris from grooming is ingested into the infrabuccal pocket and discarded with nest refuse along with the corpses of dead workers. Because of this, few infectious

diseases successfully attack ants (Holldobler and Wilson 1990).

Large populations can practise efficient division of labour. Farming communities can support full-time specialists and one of the temples in Uruk had a congregation which included 90 herdsmen, 80 soldier-labourers, 100 fishermen, 125 sailors, pilots and oarsmen, 25 scribes, 20-25 craftsmen and 250-300 slaves (Boughey 1971). Large communities of ants also have divisions of labour, although only 44 out of 263 genera have more than one physical caste and only three genera have more than two castes (Oster and Wilson 1978). The specialised army ant *Eciton burchelli* has four castes (Oster and Wilson 1978) but leafcutting ants of the genus *Atta* have a gradual size increase from 2-15 mm and four sets of behavioural roles; gardener-nurse, within-nest generalist, forager and soldier (Wilson 1980a). The less specialised Attines are monomorphic and resemble small farming villages compared to the cities of *Atta*.

However, large populations also led to exacerbated social and sexual inequality in Man (Diamond 1991). Although ants have dominance hierarchies (Cole 1981, Franks and Scovell 1983), it is unlikely that agriculture has led to any significant changes in the Attines in this respect.

Man probably did not adopt agriculture willingly. Bushmen spend only 12-19 hrs per week seeking food, while farmers must toil to cultivate the soil, protect the crop and subsequently harvest it. However, once agriculture has been adopted, Man cannot return to a hunter-gatherer way of life. Farming can support more people than hunting and a sedentary life style leads to increased rates of childbirth, since a woman does not have to wait until her child can walk before raising another. This in turn, leads to a greater population which is increasingly dependent on growing crops (Diamond 1991).

With the utilisation of fire and hafted weapons by Man in Africa, approximately 50,000 years ago, 40% of the

megafauna passed into extinction. Similarly, many large animals became extinct 15,000-20,000 years ago and this Pleistocene extinction coincided with new migrations or cultural advances in Man. This 'Pleistocene overkill' may have led to food shortages for hunter-gatherer societies which subsequently were forced to adopt agriculture (Martin 1967). It is unlikely that the ancestors of the Attines hunted their original food source to extinction, but they may have faced competition from other organisms. Weber (1958, 1972) believed that the leafcutting ants had evolved from more primitive ones resembling modern species like *Apterostigma* and *Cyphomyrmex*. The ancestral Attine probably found it expedient ecologically to take advantage of a new type of food source, since there would be little competition for it.

Primitive Attines have fungal strains with low productivity and this may be because of a historic lack of high yielding fungal clones or because of the ecological conditions of the niche they occupy, which dictates a lower yielding, less demanding and hardier fungus (Cherrett *et al.* 1989). They are subsistence farmers, with low input-low output systems. However, the intensified system of leaf-cutting ants has its costs. As Cherrett *et al.* (1989) stated, fungal competitive ability is poor and its susceptibility to pathogens is high, a rich substrate lacking fungal toxins is required and growing conditions are stringent. Powell and Stradling (1986) found that the fungus grows best over narrow ranges of temperature and pH. Adopting fresh leaves as a substrate has led to enormous behavioural complexity and *Atta sexdens* workers may perform 76 types of act or more, compared to those of *Zacryptocerus varians*, which perform 40-42 acts (Wilson 1976b, Cole 1980). Fagen and Goldman (1977) quote human children as having repertoires of 111 acts, Rhesus monkeys as having 120 acts and *Leptothorax curvispinosus* workers as performing 27 acts. Leafcutting ant repertoires are so large because of the complex process of collecting leaves, processing them to be used as substrate for the fungus and

then maintaining the fungus in pure culture. In addition, to cut leaves, workers must be large, while to care for the garden, they must be small (Wilson 1980b, 1986). Primitive monomorphic Attines like *Apterostigma*, which collects frass and dead plant material (Weber 1982) are likely to have much smaller repertoires. Leafcutting ants may well be close to the limit of behavioural complexity for ants and in this sense, resemble highly intensive farmers of today, growing crops like mushrooms, tomatoes or chrysanthemums.

However, the domestication of a fungus by the Attines has led to leafcutting ants becoming dominant invertebrates in South America, taking 17% of the leaf biomass produced in tropical rainforest (Cherrett 1989). They also play an important role in returning nutrients to the soil (Weber 1972). The mutualism between the ants and their fungus, described by Cherrett *et al.* (1989) as an 'unholy alliance' is beneficial for both parties, enabling both the ants and the fungus to exploit fresh leaves, which can normally be exploited only by specialised biotrophs (Agrios 1988, Strong *et al.* 1984).

10. Conclusions

There are similarities between agriculture and the culture of a fungus by Attines, whereby primitive Attines like *Apterostigma* are 'subsistence farmers' and the higher Attines are the 'intensive farmers', investing large amounts of resources in fungus-culturing for a high subsequent return. Fungus-culturing by leafcutting ants as an agricultural strategy is summarised in Fig. 12.1.

Unlike most other fungus-culturing arthropods, such as Xyleborine beetles and termites, the higher Attines culture their fungus on fresh leaf material, rather than wood or dead plant material. However, it could equally be said that the fungus had domesticated an ant.

The ant-fungus mutualism is very finely tuned and the ants care for their fungus intensively. One activity in particular, licking fungus garden, is very important, because licking workers simultaneously remove contaminant

spores from the garden, prune it to encourage staphylae production, obtain small amounts of nutrients from ingested hyphae and may also obtain fungal enzymes which are later returned to the fungus garden in its rectal fluids, to ensure the growth of freshly-planted inocula. Fungus garden licking is therefore a complex behaviour which forms a corner-stone of the ant-fungus mutualism.

Figure 12.1: Fungus culturing as an agricultural strategy.

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APPENDICES

APPENDIX 1 - SUPPLEMENTARY DATA FOR CHAPTER 3

1. The first 74 days of development of new gardens

a) Numbers of loads of forage carried into fungus gardens of two species during 15 minute observation periods.

TIME (DAYS)	NO'S OF LOADS OF FORAGE CARRIED INTO:									
	ATTA CEPHALOTES GARDEN NO'S:					*ATTA SEXDENS* GARDEN NO'S:				
	1	2	3	4	5	1	2	3	4	5
0	2	2	2	0	0	5	0	5	3	10
2	15	10	1	3	3	8	3	8	3	15
4	15	10	0	3	13	8	4	9	9	13
6	13	16	4	10	36	8	18	14	16	22
8	10	45	5	11	29	9	12	37	15	12
10	12	11	11	21	25	3	10	22	13	8
12	11	14	10	20	32	12	19	25	15	10
14	12	13	13	23	28	19	10	19	15	10
16	14	12	11	20	29	12	28	20	12	10
18	11	14	8	17	30	20	22	14	11	10
20	12	10	9	26	31	20	23	17	8	10
22	11	10	8	26	28	19	18	16	10	14
24	24	8	8	12	11	10	17	12	13	12
26	16	7	7	16	15	12	12	12	15	9
28	18	6	6	18	18	13	13	8	14	9
30	17	6	5	9	21	10	10	9	15	7
32	12	10	8	15	25	13	10	9	12	10
34	20	12	5	10	22	10	10	14	9	11
36	13	14	6	11	20	9	10	14	10	15
38	12	14	6	10	21	8	5	8	16	11
40	10	6	9	9	19	13	10	8	16	12
42	8	10	6	6	19	8	12	4	15	10
44	6	6	6	4	13	8	8	8	13	11
46	6	8	7	15	21	9	5	5	10	11
48	5	11	9	9	12	5	6	9	10	11
50	9	5	9	12	13	12	9	8	12	10
52	10	9	15	10	6	9	16	10	15	10
54	8	16	12	8	14	14	13	10	12	8
56	7	9	10	6	13	11	9	11	12	6
58	6	13	11	13	14	11	14	10	10	5
60	8	10	21	19	9	7	6	10	10	6
62	9	6	11	12	8	14	12	8	11	8
64	8	3	14	12	4	11	11	10	9	12
66	4	10	13	8	7	12	8	6	12	6
68	4	14	12	18	8	6	7	6	11	5
70	9	12	11	8	7	9	8	5	12	5
72	4	12	11	14	8	9	8	7	11	7
74	10	5	10	16	7	10	9	9	6	9

b) Numbers of loads of refuse carried out of fungus gardens during 15 minute observation periods.

TIME (DAYS)	NO'S OF LOADS OF REFUSE CARRIED OUT OF:									
	ATTA CEPHALOTES GARDEN NO'S:					*ATTA SEXDENS* GARDEN NO'S:				
	1	2	3	4	5	1	2	3	4	5
0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	1	0	0	0	0
4	0	0	0	0	0	1	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	1	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	1	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0	6
20	0	0	0	0	0	0	0	0	0	1
22	0	0	0	0	0	0	0	0	0	2
24	0	0	1	0	0	1	1	3	0	1
26	0	0	0	0	2	0	1	1	0	1
28	3	0	0	1	4	0	3	1	0	1
30	1	0	1	0	3	1	2	0	0	0
32	0	1	1	0	5	1	0	1	0	2
34	0	0	1	2	9	3	1	0	0	2
36	0	2	0	1	7	2	1	2	0	1
38	1	0	2	6	5	2	1	1	1	0
40	0	1	4	0	4	6	1	1	1	0
42	0	1	0	3	7	2	5	1	1	0
44	0	2	4	9	2	2	3	2	0	1
46	0	5	3	3	2	2	1	3	0	1
48	2	7	2	4	15	2	4	6	0	2
50	3	6	0	8	5	2	1	5	2	0
52	5	8	0	8	5	3	2	6	2	1
54	6	7	0	7	6	5	7	8	3	5
56	1	0	0	2	6	2	9	8	3	8
58	2	2	0	2	7	2	7	7	12	7
60	1	1	1	1	9	3	8	7	10	2
62	2	0	4	1	15	3	4	4	11	0
64	6	0	4	8	9	2	10	8	10	0
66	4	0	8	14	8	2	8	17	9	4
68	7	6	0	11	8	2	2	11	8	5
70	7	2	0	11	8	5	1	9	8	1
72	8	8	14	8	9	18	2	9	5	1
74	19	7	9	7	10	11	2	4	6	2

c) Numbers of loads of fungus carried into fungus gardens of two species during 15 minute observation periods, over the first 20 days of development (no fungus was carried in after day 14).

TIME (DAYS)	NO'S OF LOADS OF FUNGUS CARRIED INTO:									
	ATTA CEPHALOTES GARDEN NO'S:					*ATTA SEXDENS* GARDEN NO'S:				
	1	2	3	4	5	1	2	3	4	5
0	59	1	6	13	1	2	0	1	2	35
2	27	13	0	15	1	13	10	43	1	9
4	19	1	0	1	0	15	6	18	8	2
6	4	2	6	0	0	2	1	15	7	0
8	3	0	5	0	1	2	0	1	0	0
10	0	0	6	0	0	0	1	0	0	0
12	0	0	3	0	0	0	0	0	0	0
14	0	0	0	0	0	1	0	0	0	0
16	0	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0

d) Numbers of loads of staphylae carried into fungus gardens of two species during 15 minute observation periods.

TIME (DAYS)	NO'S OF STAPHYLAE CARRIED INTO:									
	ATTA CEPHALOTES GARDEN NO'S:					*ATTA SEXDENS* GARDEN NO'S:				
	1	2	3	4	5	1	2	3	4	5
0	0	0	2	0	1	2	0	0	0	0
2	1	1	0	2	0	3	0	0	0	1
4	0	0	0	1	0	2	1	2	1	0
6	1	1	1	2	0	1	4	1	6	4
8	0	0	0	1	0	5	9	3	2	1
10	0	0	0	2	0	1	2	5	0	1
12	0	0	0	4	0	1	1	1	0	2
14	1	0	0	8	0	3	0	1	0	1
16	0	0	0	0	1	0	0	0	0	1
18	0	0	0	0	0	0	0	1	0	0
20	0	0	0	0	0	0	0	0	0	0
22	0	0	0	1	0	0	0	0	0	0
24	0	1	0	0	0	0	0	0	1	0
26	0	0	1	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0	0
32	0	1	0	0	0	0	0	0	0	0
34	0	0	0	1	0	0	0	0	0	0
36	0	0	0	0	0	0	0	0	0	0
38	0	0	0	1	0	0	0	0	0	0
40	0	0	0	1	0	0	0	0	0	0
42	0	0	0	1	0	0	0	0	0	0
44	0	0	0	0	0	0	0	0	0	0
46	0	0	2	1	0	0	0	0	0	0
48	1	0	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0	0	0
52	0	0	0	0	0	0	0	0	0	0
54	0	1	0	0	0	0	0	0	0	0
56	0	0	0	0	0	1	0	0	0	1
58	0	0	0	0	0	0	1	0	0	0
60	0	0	0	2	0	0	0	0	0	0
62	0	3	0	1	9	0	0	0	1	0
64	1	0	0	1	2	0	0	1	0	0
66	0	1	0	3	0	0	0	0	0	0
68	0	0	0	2	0	0	0	0	0	0
70	0	0	0	0	0	0	0	0	1	0
72	5	0	0	2	0	1	0	1	0	0
74	1	0	0	1	0	0	0	0	0	1

e) Numbers of loads of staphylae carried out of fungus gardens of two species during 15 minute observation periods.

TIME (DAYS)	NO'S OF LOADS OF STAPHYLAE CARRIED OUT OF:									
	ATTA CEPHALOTES GARDEN NO'S:					*ATTA SEXDENS* GARDEN NO'S:				
	1	2	3	4	5	1	2	3	4	5
0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	4	0
8	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0
12	0	0	0	2	0	0	0	0	0	0
14	0	0	0	3	0	0	0	0	0	0
16	0	0	1	6	0	0	0	0	0	0
18	0	0	0	9	1	0	0	0	0	0
20	0	0	0	16	0	0	0	2	0	0
22	0	0	1	8	0	0	0	0	0	0
24	0	1	0	3	1	0	0	0	0	0
26	0	0	0	12	0	2	0	0	0	0
28	0	0	0	24	0	0	0	0	0	0
30	0	0	1	22	0	2	0	0	0	0
32	0	0	0	20	0	0	0	0	0	0
34	0	1	0	18	0	0	0	0	0	0
36	0	1	0	16	0	0	0	0	0	0
38	0	0	0	14	0	0	0	0	0	0
40	0	0	0	8	0	0	0	0	0	0
42	1	0	14	7	0	0	0	0	0	0
44	3	0	10	20	0	0	0	0	0	0
46	0	0	15	4	1	0	0	0	0	0
48	0	0	16	8	1	0	0	0	0	0
50	0	15	16	6	0	0	0	0	0	0
52	0	24	31	4	0	0	0	0	0	0
54	0	20	33	4	0	0	0	0	0	0
56	0	14	37	2	0	0	0	0	0	0
58	1	12	28	2	0	0	0	0	0	0
60	0	15	19	6	0	0	0	0	0	0
62	0	42	8	7	0	0	0	0	0	0
64	0	54	12	14	0	0	0	0	1	0
66	0	21	16	33	0	0	0	0	0	0
68	0	9	21	27	1	0	0	1	0	1
70	2	21	32	25	0	1	1	0	0	0
72	27	26	18	21	0	1	0	0	0	0
74	28	19	16	16	0	0	0	0	0	0

f) Numbers of brood groups present on the surfaces of fungus gardens of two species, over the first 74 days of development.

TIME (DAYS)	NO'S OF BROOD GROUPS PRESENT FOR:									
	ATTA CEPHALOTES GARDEN NO'S:					*ATTA SEXDENS* GARDEN NO'S:				
	1	2	3	4	5	1	2	3	4	5
0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	1	0	0	1	0
6	0	0	0	0	0	0	5	0	1	0
8	0	0	0	0	0	0	3	0	1	0
10	0	1	0	0	0	0	3	0	1	0
12	0	1	0	1	0	0	0	0	1	0
14	0	0	0	1	0	0	0	0	0	0
16	1	0	0	1	0	0	1	0	2	0
18	3	0	0	1	0	0	0	0	1	0
20	0	0	0	0	0	0	0	0	1	1
22	1	1	0	0	0	0	0	0	1	0
24	1	0	0	0	1	1	0	0	1	1
26	0	0	0	0	5	0	0	0	1	0
28	0	0	0	0	3	0	1	2	2	0
30	1	1	0	0	3	0	2	2	1	1
32	1	0	0	0	6	0	1	1	1	0
34	3	0	0	0	7	0	2	0	0	0
36	2	0	0	0	6	1	3	0	0	0
38	1	0	0	0	7	1	3	0	1	0
40	2	1	0	2	5	2	3	0	1	0
42	4	1	0	0	11	2	12	0	2	0
44	3	1	0	0	9	1	4	0	3	0
46	2	1	0	1	9	2	5	0	3	0
48	2	1	0	0	3	0	1	0	2	0
50	2	1	0	1	3	0	2	2	2	0
52	4	1	0	1	6	0	2	1	3	0
54	4	1	0	1	4	1	1	1	3	0
56	5	3	0	1	3	0	3	1	3	0
58	4	2	0	1	4	0	4	1	4	1
60	3	2	0	0	6	0	6	1	3	0
62	5	2	0	0	7	1	4	4	3	0
64	4	0	0	0	6	1	3	1	3	1
66	4	0	0	0	7	3	5	2	3	1
68	4	0	0	0	6	3	6	2	3	0
70	4	0	0	0	6	1	6	1	3	0
72	4	0	0	0	7	0	4	1	4	0
74	4	0	0	0	7	0	4	1	5	0

g) Sizes of fungus gardens over the first 74 days of development, measured as silhouette areas (cm^2).

TIME (DAYS)	MEAN SIZES (SILHOUETTE AREAS, cm^2) OF FUNGUS GARDENS FOR:									
	ATTA CEPHALOTES GARDEN NO'S:					*ATTA SEXDENS* GARDEN NO'S:				
	1	2	3	4	5	1	2	3	4	5
0	49.3	3.6	5.0	10.0	4.1	6.8	0.2	2.0	7.1	13.8
2	73.3	5.6	6.4	15.3	18.4	12.3	6.1	15.2	8.8	15
4	*	*	*	*	*	23.3	30.3	20.4	21.5	25.3
6	*	*	40.8	32.6	*	*	56.9	29.1	30.4	52.1
8	121.2	*	74.3	*	31.6	*	*	57.8	*	78.0
10	*	*	*	*	*	*	*	*	*	*
12	*	*	*	*	*	68.2	*	96.0	68.0	*
14	*	*	*	*	*	94.8	105.2	*	*	*
16	188.3	*	97.5	88.3	126.2	*	*	*	*	*
18	*	*	*	*	*	*	*	*	*	*
20	*	*	*	*	*	113.6	120.5	179.1	*	172.3
22	*	*	*	*	*	*	*	*	*	*
24	*	*	*	*	*	*	*	*	*	*
26	*	212.7	*	170.8	157.9	142.8	173.4	*	128.8	171.5
28	*	*	*	*	*	*	*	*	*	*
30	*	*	*	*	*	*	*	*	*	*
32	186.7	*	190.0	175.2	183.3	*	*	*	*	*
34	*	*	*	*	*	*	175.4	157.6	*	171.0
36	*	*	*	*	*	*	*	*	*	*
38	*	*	*	*	*	153.0	177.9	*	157.3	179.4
40	192.6	190.8	188.4	179.6	196.6	*	*	*	*	*
42	*	*	*	*	*	*	*	*	*	*
44	*	*	*	*	*	*	*	*	*	*
46	*	*	*	*	*	*	*	*	*	*
48	*	*	*	*	*	*	*	132.1	160.0	189.9
50	204.1	184.4	191.7	159.9	*	*	*	*	*	*
52	*	*	*	*	*	*	*	*	*	*
54	*	*	*	*	*	156.9	130.5	*	165.7	*
56	*	*	*	*	*	*	*	*	*	*
58	*	*	*	*	*	*	*	*	*	*
60	190.7	191.0	191.0	152	175.4	*	131.0	139.5	*	195.9
62	*	*	*	*	*	*	*	*	*	*
64	*	*	*	*	*	*	*	*	*	*
66	*	*	192.9	148.2	150.1	*	*	*	*	*
68	*	*	*	*	*	157.0	125.0	136.0	174.5	188.1
70	*	*	*	*	*	*	*	*	*	*
72	*	*	*	*	*	*	*	*	*	*
74	185.2	192.4	186.2	156.5	151.0	162.7	121.8	135.8	174.0	153.2

* Missing values.

2. Established gardens followed over 700 days

a) Mean numbers (± SD) of loads of forage carried into six established fungus gardens during 15 minute observation periods, averaged over 20 day observation periods (3-10 replicates).

TIME (DAYS)	MEAN NO'S (± SD) OF LOADS CARRIED INTO GARDEN NO'S:					
	1	2	3	4	5	6
0-20	20.4±8.6	31.4±9.8	12.3±9.5	12.4±8.9	14.0±5.5	9.3±4.3
20-40	8.9±3.3	17.1±6.3	6.2±2.8	3.1±1.8	6.1±2.0	7.4±3.4
40-60	5.8±3.2	7.1±1.9	8.4±2.9	9.1±4.4	10.4±4.9	5.6±3.6
60-80	9.1±2.2	9.8±1.3	8.6±3.1	6.1±2.9	7.2±1.6	7.1±1.7
80-100	8.5±4.3	8.4±3.6	5.4±1.7	7.4±3.8	9.4±3.9	4.3±2.7
100-120	16.5±5.2	7.1±2.6	13.3±6.1	4.7±1.5	17.2±3.3	11.8±6.2
120-140	12.2±2.2	16.3±5.4	12.5±3.7	15.1±8.1	12.0±2.9	8.6±3.0
140-160	11.6±4.0	12.1±6.3	11.3±7.7	13.7±6.3	15.6±5.3	14.7±6.5
160-180	18.8±6.6	12.5±5.0	18.3±4.9	14.3±6.7	9.0±3.7	14.8±6.6
180-200	8.0±0	17.0±0.5	13.0±0.5	9.0±1.5	14.0±1.0	6.0±0.7
200-220	26.3±4.9	27.8±6.7	14.5±1.3	28.0±7.1	35.8±9.7	23.8±4.7
220-240	22.3±6.8	26.0±8.7	19.3±7.9	17.5±3.0	20.8±5.7	15.8±5.4
240-260	23.0±6.0	20.8±6.8	15.5±7.0	17.3±3.0	24.3±9.9	23.5±4.7
260-280	20.5±6.9	20.0±5.9	13.0±2.2	16.0±5.2	16.8±6.2	13.0±2.4
280-300	16.8±7.8	13.5±2.1	8.3±3.7	7.3±4.6	9.3±3.0	9.3±4.4
300-320	12.0±5.2	11.0±2.3	9.5±5.8	7.3±2.0	9.3±3.0	6.3±3.6
320-340	15.0±0.8	10.3±4.3	7.8±3.3	7.5±0.8	10.8±3.9	10.5±4.0
340-360	21.0±0.1	8.0±0.6	11.0±0.7	6.0±0.8	7.0±1.2	4.0±0.5
360-380	12.0±5.4	12.8±5.0	14.3±2.2	10.5±3.1	9.0±1.2	6.8±3.2
380-400	5.8±2.1	19.5±8.2	15.3±4.4	12.0±4.2	17.5±3.9	12.3±5.9
480-500	18.4±8.0	16.8±4.1	10.8±5.0	12.0±1.7	16.0±9.3	15.6±5.4
580-600	26.4±3.3	23.8±7.4	19.2±3.0	22.4±3.6	29.2±4.4	19.4±3.5
680-700	17.6±5.5	15.6±2.5	12.0±2.8	7.6±3.0	12.8±3.7	10.2±4.8

b) Mean numbers (± SD) of loads of refuse carried out of six established fungus gardens during 15 minute observation periods, averaged over 20 day observation periods (3-10 replicates).

TIME (DAYS)	MEAN NO'S (± SD) OF LOADS CARRIED OUT OF GARDEN NO'S:					
	1	2	3	4	5	6
0-20	8.5±1.8	1.0±2.8	7.6±3.6	7.6±3.3	11.6±5.6	12.2±5.3
20-40	8.9±3.6	5.0±4.9	8.2±4.6	10.0±6.0	7.3±4.0	8.3±4.8
40-60	4.8±1.5	7.4±3.8	4.1±2.0	5.1±2.3	4.2±1.7	4.3±3.0
60-80	4.4±1.4	7.8±3.5	5.6±2.1	4.0±1.3	3.4±1.7	8.4±2.6
80-100	5.9±3.0	5.8±2.6	4.8±2.5	4.6±3.1	1.5±0.8	4.0±1.5
100-120	7.3±2.4	7.8±3.4	6.0±2.7	8.5±6.5	5.9±6.1	4.9±1.1
120-140	14.3±6.1	10.1±3.1	10.6±5.8	4.8±6.3	12.2±8.5	9.5±4.9
140-160	11.9±4.5	9.6±4.7	3.9±3.1	2.7±1.6	5.4±3.1	3.0±1.4
160-180	8.3±2.5	8.0±4.7	4.0±2.4	11.8±2.9	6.5±3.1	3.3±1.7
180-200	11.0±0.6	9.0±0.8	11.0±1.2	10.0±0.5	3.0±0.6	13.0±0.9
200-220	15.5±2.4	8.8±6.9	8.5±5.4	4.8±2.5	2.0±1.0	7.8±6.4
220-240	7.0±1.8	7.5±2.6	4.0±1.6	9.5±4.5	9.0±5.7	6.3±3.6
240-260	4.8±4.6	11.0±6.5	6.5±4.1	10.0±3.8	8.5±6.6	11.8±2.8
260-280	4.5±1.3	7.0±2.8	8.5±4.8	0.9±2.6	7.6±1.3	7.6±1.2
280-300	11.6±6.6	12.3±3.3	8.9±1.3	5.0±2.5	8.2±4.8	10.0±6.0
300-320	7.3±3.2	8.3±2.5	4.8±2.9	7.4±4.3	4.1±3.7	5.1±3.3
320-340	4.2±3.1	4.3±3.2	4.4±3.6	7.8±4.2	5.6±5.0	4.0±3.6
340-360	3.4±1.2	8.4±1.4	5.9±3.5	5.8±3.2	4.8±3.5	4.6±4.2
360-380	1.5±1.1	4.0±3.3	7.3±5.5	7.8±0.7	6.0±3.9	8.5±8.1
380-400	5.9±3.5	4.9±6.6	14.3±7.6	10.1±3.5	10.6±3.5	4.8±4.3
480-500	18.8±9.6	16.4±6.2	16.8±8.6	18.2±7.5	11.8±7.3	13.4±8.3
580-600	15.8±5.5	16.8±7.5	15.2±7.5	12.0±6.2	9.6±5.8	22.0±7.0
680-700	7.2±4.1	6.2±4.3	4.4±4.2	5.0±2.6	8.2±3.3	5.4±3.1

c) Mean numbers (± SD) of groups of brood present on the surfaces of six established fungus gardens, averaged over 20 day observation periods (3-10 replicates).

TIME (DAYS)	MEAN NO'S (± SD) OF BROOD GROUPS PRESENT IN GARDEN NO'S:					
	1	2	3	4	5	6
0-20	1.7±1.1	0.3±0.5	0.5±1.0	0.6±0.9	3.3±1.7	2.6±1.6
20-40	1.9±1.1	0.9±0.6	0.5±0.5	0.8±0.9	0.5±0.5	4.2±2.0
40-60	0.7±0.7	1.7±1.1	0.5±0.7	2.0±1.5	1.8±0.8	4.0±1.2
60-80	1.6±1.1	1.9±0.9	1.4±1.2	2.2±1.5	2.4±1.2	3.3±2.1
80-100	3.6±1.5	3.0±1.6	2.3±0.5	3.1±0.8	3.9±2.5	2.8±1.3
100-120	3.8±1.5	3.3±1.8	2.2±0.8	3.4±0.8	7.7±1.6	4.0±1.2
120-140	1.2±1.3	1.9±2.1	1.2±1.0	0.3±0.5	4.7±3.2	1.1±1.5
140-160	2.6±1.8	4.0±1.7	3.3±3.8	5.3±4.2	3.9±1.4	4.8±2.4
160-180	1.0±1.2	1.3±1.3	0	3.3±1.5	0.3±0.5	3.0±0.8
180-200	7.0±0.0	2.0±1.0	5.0±1.5	2.0±0.6	2.0±0.0	7.0±1.4
200-220	0.3±0.5	0	0	0.5±0.6	4.0±1.7	0.5±0.6
220-240	0.8±0.5	0.3±0.5	1.3±1.0	1.3±1.0	0.3±0.5	0.3±0.5
240-260	2.0±0.4	0.5±1.0	1.3±1.3	1.5±1.0	1.3±0.5	1.3±1.3
260-280	1.5±1.7	0.8±1.0	1.5±1.7	2.0±2.3	1.5±1.0	1.0±1.2
280-300	3.0±0.8	1.0±0.0	1.0±1.4	0.5±0.6	0.8±1.0	0.3±0.5
300-320	4.5±0.6	2.3±1.0	3.8±0.5	4.8±1.7	2.3±1.3	1.8±1.0
320-340	7.0±1.6	2.8±2.2	7.5±2.1	2.3±1.0	7.0±4.1	4.5±5.1
340-360	8.0±1.0	5.0±2.0	4.0±1.2	5.0±0.8	11.0±2.0	13.0±2.6
360-380	8.8±2.1	5.5±0.6	7.8±1.7	3.5±1.3	6.0±4.3	7.8±2.4
380-400	11.5±1.3	2.8±1.0	7.5±3.7	5.0±2.9	3.8±1.7	4.0±2.2
480-500	5.8±3.3	1.4±0.5	4.0±1.4	5.8±3.3	3.8±3.1	1.6±1.3
580-600	3.4±2.9	0.8±1.1	2.6±2.3	3.0±3.4	3.8±3.8	2.3±2.3
680-700	4.4±1.5	1.6±0.5	3.4±1.1	2.4±2.1	4.6±1.7	1.2±1.3

d) Mean sizes (± SD) of six established fungus gardens, measured as silhouette areas (cm^2), averaged over 20 day periods (2-3 replicates).

TIME (DAYS)	MEAN SIZES (±SD, cm^2) OF GARDEN NO'S:					
	1	2	3	4	5	6
0-20	160.1±4.0	80.4±43.8	116.7±16.3	154.1±19.0	138.8±25.3	187.1±8.0
20-40	125.2±14.4	165.5±16.0	104.3±23.2	115.1±2.2	90.3±19.7	129.3±1.1
40-60	126.3±7.4	128.5±23.5	107.5±26.4	144.4±5.5	127.1±11.8	157.5±20.6
60-80	138.7±10.2	147.9±0.0	100.2±1.8	146.9±1.0	140.6±3.2	182.5±5.9
80-100	133.9±12.8	151.3±0.7	145.8±12.2	165.9±4.3	160.3±12.9	169.7±16.3
100-120	162.2±22.0	142.6±26.1	162.6±29.3	107.4±69.3	150.0±5.1	172.5±0.1
120-140	153.2±48.4	128.5±7.1	93.4±36.7	79.4±64.3	116.0±35.6	129.9±46.0
140-160	115.0±5.9	126.0±6.9	123.6±14.6	163.8±8.6	142.4±3.8	94.7±27.4
160-180	141.3±2.2	116.4±20.2	142.6±11.9	167.4±1.2	154.0±7.4	150.6±26.5
180-200	185.8±1.5	151.9±0.0	158.1±2.0	156.4±3.2	131.4±3.4	153.5±1.9
200-220	180.1±7.8	170.2±2.1	163.8±23.4	144.7±43.8	162.5±10.7	179.8±2.5
220-240	117.6±6.6	148.4±20.6	154.5±0.6	183.9±0.4	165.9±6.0	156.8±0.4
240-260	162.8±7.7	168.4±17.2	151.5±4.5	191.4±2.1	155.1±21.2	163.4±1.3
260-280	169.9±8.3	169.2±5.5	173.4±2.0	186.0±3.5	162.4±4.7	173.7±1.3
280-300	184.3±0.4	185.7±4.2	174.1±5.8	187.1±3.0	168.8±0.5	176.2±8.3
300-320	186.8±1.7	196.0±3.1	181.3±4.3	220.2±45.3	183.7±7.6	188.1±11.0
320-340	158.9±3.7	196.2±2.1	184.5±9.0	189.0±4.5	186.8±0.0	190.7±5.4
340-360	193.4±1.5	194.1±1.5	185.8±1.6	182.2±2.0	187.7±2.6	194.3±3.7
360-380	190.9±2.0	192.3±1.5	179.5±0.0	222.0±0.4	152.1±3.9	190.4±2.6
380-400	191.5±16.5	181.5±1.4	179.0±10.0	171.2±2.0	152.6±14.3	165.8±12.8
480-500	132.7±23.8	144.4±19.5	122.1±25.7	166.4±11.7	141.8±11.9	146.0±21.6
580-600	157.7±15.4	175.7±18.7	172.6±3.9	179.2±20.7	166.7±15.4	164.7±3.8
680-700	192.1±1.5	199.4±6.6	184.2±4.1	186.0±8.2	184.5±7.9	182.3±12.4

APPENDIX 2 - SUPPLEMENTARY DATA FOR CHAPTER 7

1. Supplementary data for Figure 7.4.

Mean numbers of workers (± SE) licking 300 mm^2 areas of fungus garden previously caged (*in situ*) for 24 hours or for 2 minutes to prevent ant access (3 replicates).

TIME FROM RE-EXPOSURE (MINS)	MEAN NUMBERS OF WORKERS LICKING (± SE)	
	24 HRS HOURS CAGING	2 MINUTES CAGING
0	8.1 ± 0.9	8.3 ± 1.2
2	16.8 ± 3.1	2.2 ± 1.3
5	20.6 ± 1.8	3.4 ± 2.1
10	20.6 ± 1.8	7.4 ± 2.3
15	18.5 ± 1.0	8.1 ± 2.5
20	19.0 ± 2.0	7.2 ± 1.5
25	14.4 ± 1.4	8.9 ± 1.3
30	14.8 ± 2.3	8.9 ± 0.1
45	11.2 ± 1.9	8.8 ± 1.1
60	11.9 ± 1.8	8.9 ± 0.1
75	10.2 ± 2.4	9.3 ± 0.5
90	7.3 ± 1.5	7.7 ± 0.2

2. Supplementary data for Figure 7.7

Numbers of workers licking fungus garden previously isolated from them for different periods, with or without reductions in the numbers of staphylae present per chamber (containing 19 cm^2 of available garden area).

a) Mean numbers (± SE)

TIME FROM RE-EXPOSURE (MINS)	MEAN NO'S LICKING (± SE) ON 19 cm^2 AREAS OF GARDEN ISOLATED FOR DIFFERENT PERIODS:				
	0 DAYS	1 DAY	2 DAYS	3 DAYS	4 DAYS
0	6.1±0.8	0	0	0.7±0.7	0
15	5.3±1.4	3.7±0.9	6.9±1.2	7.3±1.7	1.6±0.5
30	5.3±1.1	7.4±0.9	12.4±2.1	10.5±2.1	2.0±0.4
60	5.6±1.1	11.9±0.9	15.9±2.4	14.3±1.4	4.2±0.8
90	5.8±0.9	14.3±0.6	20.3±2.8	18.5±2.4	6.0±1.0
120	6.6±1.1	14.7±0.9	21.4±1.8	23.3±3.1	7.8±0.9
150	6.1±2.9	15.6±1.1	23.6±2.2	24.7±3.8	9.4±1.0
180	6.4±1.1	12.7±0.7	24.4±5.3	21.8±2.8	8.8±0.7
24 hrs	7.1±1.4	6.0±0.8	9.4±1.8	10.5±0.8	3.2±0.4

b) Mean arcsine-transformed percentages (± SE)

TIME FROM RE-EXPOSURE (MINS)	MEAN ARC%'S LICKING (± SE) ON 19 cm^2 AREAS OF GARDEN ISOLATED FOR DIFFERENT PERIODS:				
	0 DAYS	1 DAY	2 DAYS	3 DAYS	4 DAYS
0	40.2±2.6	0	0	0	0
15	36.4±5.9	21.9±3.5	20.0±3.3	22.6±5.1	11.1±3.7
30	32.8±5.0	35.4±2.0	34.4±3.7	47.0±1.6	8.6±1.7
60	33.6±2.9	57.0±1.5	37.9±4.0	48.1±3.4	16.5±3.1
90	35.0±4.5	60.1±3.0	43.4±2.6	60.0±2.7	16.4±2.6
120	42.6±3.5	59.5±1.8	62.5±2.6	66.0±1.2	22.3±3.5
150	36.9±2.2	61.1±4.8	68.8±2.4	71.1±0.5	25.8±3.5
180	39.9±3.7	59.2±4.1	83.9±1.1	57.9±1.5	26.5±2.4
24 hrs	39.5±4.5	36.1±3.0	40.0±3.3	37.8±2.1	16.9±2.9