Mushrooms: Oviposition substrate selection in sciarid and phorid fly pests

Project Number M 36

Project Staff	L Tibbles (CASE student)
HRI-Wellesbourne	P F White (supervisor) T J Elliott
Cardiff University	L Boddy (supervisor) M A Jervis (supervisor) C Muller (supervisor)

Project Leader: P F White

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Project Co-ordinator: Mr George Pointing

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Practical Section for Growers

Fungus gnats (Sciaridae: *Lycoriella* and *Bradysia* spp.) and scuttle flies (Phoridae: *Megaselia* spp.) are important pests in commercial mushroom production. The cost of pest infestations to the UK mushroom industry probably amounts to about £11-12 million per annum in terms of direct loss to insect attack, transmission of pathogens and ongoing insecticide costs.

Aerial control of adult mushroom flies during cropping relies on only two similar-acting products (containing either synthetic or natural pyrethrins) due to product withdrawal, crop intolerance and insecticide resistance: Darmycel Agarifume smokes (active ingredient permethrin) and Pynosect 30 (active ingredient synthetic pyrethrins and resmethrin). There is a similar dearth of approved larvicides. Reliance on so few active compounds leaves pest control extremely vulnerable to further resistance or product withdrawal problems. Pest control will in the future be increasingly difficult and, without adequate protection, the mushroom crop is extremely susceptible to pest attack.

Whilst a reasonable amount is know about the substrate distribution and 'preferences' of the larvae of these pests, very little is known about the foraging behaviour of female flies, especially the chemical cues employed during oviposition substrate selection.

The aims of this project are: to identify which olfactory and contact chemical stimuli, associated with mushrooms and their cultivation, female flies respond to; and to produce non-insecticidal aids to mushroom fly control. The project will determine the identity of the volatiles present in compost, actively growing mycelium or mushroom sporophores, that are active in attracting and eliciting oviposition by female sciarids and phorids. The commercial aim of the project is two-fold: (a) to devise cheap, effective target technology for killing female flies (*e.g.* mass or diversion trapping techniques); and (b) to devise cheap, effective technology for monitoring flies in mushroom houses. By such methods a grower would be able to optimise or reduce the use of chemical insecticides, thereby improving the environmental acceptability of waste compost/casing. There could also be a positive effect on mushroom consumer acceptability. Thus, the project aims to improve crop protection strategies, reduce the use of chemicals, improve environmental acceptability and consumer preferences for production and product marketability.

Objectives

1. To determine which substrates in mushroom houses (specifically: compost, actively growing mycelium, mushroom sporophores) attract females and elicit oviposition
2. To determine which component chemicals in the aforementioned substrates can *potentially* elicit attraction and oviposition
3. To determine which volatiles *actually* are bioactive
4. To apply knowledge gained from (1-3) to the development of targets/traps/ monitoring devices
5. To test the devices in the laboratory and mushroom house environment

A static air olfactometer was designed to investigate the behavioural response of sciarid (*Lycoriella castanescens*) and phorid (*Megaselia halterata*) flies to mushroom cultivation substrates. The substrates within mushroom houses which are active in attraction of female flies and elicit oviposition could, thus be determined. Preliminary experiments were undertaken to assess the suitability of the olfactometer for testing the response of flies to different, potential oviposition substrates. Within a controlled temperature room the olfactometers were tested for internal bias and the effect of olfactometer position on fly distribution. Results were analysed using chi-squared and G tests. The results suggest that the position of the olfactometers within the controlled temperature room was not a factor influencing the distribution of flies in the test pots of the olfactometer. The results also suggested that the olfactometer designed is suitable for use to test the behavioural response of *M. halterata* flies. However the results gained for the sciarid fly, *L. castanescens*, were inconclusive. The statistical tests, applied to the data, were deemed inadequate for the experiments undertaken. As a result, the preliminary experiments were redesigned and a log-linear model will be used to test the uniformity of the olfactometers and the controlled temperature room in which experiments are carried out. Once this has been completed, the behavioural response of sciarid and phorid flies to mushroom cultivation substrates, compost, actively growing mycelium and mushroom sporophores, will be determined and volatiles from attractive substrates identified. Putative olfactory sensilla were observed on the antennae of *M. halterata* using scanning electron microscopy. Pit sensilla were observed on the antennae of *L. castanescens*. These sensilla may enable the use of EAG/GCMS to directly assess the behavioural response of the flies to potentially biologically active compounds.

Ultimately the results of this work are expected to provide additional or improved methods of monitoring and trapping sciarid and phorid flies of commercial importance on all mushroom farms. These methods will help the industry dispense with, or a least reduce reliance on, pesticide application for pest control that currently costs the UK mushroom industry about £6-7 million per annum. It may even provide superior control compared with existing methods. It is feasible that such compounds could be used in critical areas of a mushroom farm away from the growing houses. For example, non-insecticidal removal of flies from packing sheds would be extremely beneficial with regard to pre-packs.

A knowledge of the attractive components produced by growing mycelium would also provide a useful additional screening agent in the search for commercial mushroom strains that are less susceptible to mushroom pests.

Such compounds have the potential for worldwide application as the number of mushroom pests species that infest commercial mushroom production are few. Commercial exploitation of such attractive compounds would be required.

Science Section

Introduction

History of mushroom cultivation

Mushroom consumption by man, in all probability, predates our recorded history. Chang & Miles (1987) estimated that the first mushroom to be intentionally cultivated by man was *Hirneola auricula-judae* Quel. in 600AD. The cultivation of *Agaricus bisporus* (Lange) Imbach began more recently and was first documented by Bonnefons (1650; cited by Spencer, 1985). Unsurprisingly, early mushroom cultivation was relatively crude. Mushrooms were observed to grow from horse manure, so this was collected but was not inoculated with fungal spores. Therefore successful cropping was reliant on the chance presence of fungal spores within the manure. During the 17th and 18th centuries mushrooms were cultivated in open ground. It was not until 1810, when mushrooms were first grown in underground quarries in France, that a year-round cultivation system was developed (Spencer, 1985). New methods quickly spread to the UK and throughout Europe, as well as to North America, Australia and South East Asia. However, it was only during the 20th century that mushroom cultivation became a more precise science. Investigations into the biology of *A. bisporus* led to the understanding of the development of mycelium from spores. Spawn, fungal mycelium inoculated onto an inert substrate, was first produced in 1846 by the British based company Hamlin & Co. (Cayley, 1938, cited by Elliott, 1985). Cereal grains are the most commonly used substrate for spawn and this method was first patented in 1932 by Sinden (Elliott, 1985). Since then, knowledge of fungi and their cultivation has increased. Mushroom cultivation is now a world-wide industry, with constantly improving techniques for compost production, increasing yield and reducing disease and pest infestations.

Economic importance of mushroom cultivation

Although 69,000 species of fungi have been described, it has been estimated that 1.5 million species exist (Hawksworth, 1991). Of known fungal species 10,000 are fleshy macro-fungi, 2,000 species from 30 genera are edible and 6 species are commercially cultivated on an industrial scale (Chang, 1993). *Agaricus bisporus* accounted for 38% of world mushroom market in 1989/90 (Chang & Miles, 1991) and it is the predominant mushroom species grown in the USA and Europe.

North America dominates the world market for mushroom production, whilst France and the Netherlands, followed by the UK, are the biggest producers of mushrooms in the EU. The value of the mushroom crop in the UK was £169,339,000 in 1997, accounting for 55% of the value of protected vegetables in that year (MAFF, 1998). The mushroom industry is therefore of considerable economic importance to British horticulture. The total value of world-wide mushroom production was estimated at US$7,485,058,500 for 1991 (Chang, 1993).

Insects as mushroom pests

Naturally occuring fungal fruiting bodies provide a patchily distributed, unpredictable resource for any species dependent upon them for a breeding or feeding resource (Fäldt *et al*.,1999). However, the commercial cultivation of mushrooms provides a year-round source of breeding and feeding sites for many pest species and pathogens.

Diptera are the most serious pests of mushrooms and, of these, the most common and economically important are species of Sciaridae and Phoridae flies (White, 1985). Of these the mushroom sciarids *Lycoriella ingenua* (Dufour) (*L. mali* (Fitch)) and *L. castanescens* (Lengersdorf) (*L. auripila* (Winnertz)) (Menzel & Mohrig, 1997) and the mushroom phorid *Megaselia halterata* (Wood) cause the most economic damage. For pests to successfully colonise a mushroom crop, they must infest the compost after the pasteurisation process (Fletcher, White & Gaze, 1989).

Phorids

Morphology

Megaselia halterata adults are small, hump-backed flies, 2-3 mm in length, that possess short antennae which are visible at low magnification. There is no obvious sexual dimorphism, although males can be distinguished under a dissecting microscope by a black capsule at the base of their abdomen. The female abdomen is paler and has a more pointed apex. Eggs are oblong, opaque and white and laid in small groups (Scheepmaker, 1999). The larvae are between 1-6 mm in length and creamy white in colour. They possess a pointed head that is not coloured and a blunt rear end. Approximately two thirds of their immature life is spent as an immobile, non-feeding pupae (Fletcher, *et al.*, 1989).

Life cycle

Gravid females are attracted to growing mycelium (Hussey, 1959) and only oviposit where mycelium is actively growing (Hussey & Wyatt, 1962; Richardson & Hesling, 1978). Consequently, compost or casing which have fungal mycelium actively growing through it, are the only stages of mushroom cultivation at which phorid infestations are likely to occur. Each female lays about 40-60 eggs, normally at an active mycelial front. Larvae may remain *in situ* throughout development (Hussey, 1959). Eggs hatch rapidly and larvae complete their three instars in 9-10 days (Hussey, 1959; no temperature details given). Pupae are off-white in colour when newly formed but as the pupae mature they darken, becoming yellow-brown (Hussey, 1959; Hussey, Read & Hesling, 1969). At 20ºC adults emerge 24 days after oviposition. Female flies are sexually receptive 24 hours after emergence, though males copulate when they are four days old (Ondraschek, 1953, cited by Hussey, 1959). Adults require a period of flight prior to mating (Davies, 1941, cited by Hussey, 1959) and females require carbohydrate before egg maturity (Hussey, 1959). Oviposition occurs 2-3 days after insemination (Ondraschek, 1953, cited by Hussey, 1959).

Sciarids

Morphology

Sciarid flies are 3-4 mm long with thread-like antennae, held characteristically erect. There is obvious sexual dimorphism. Females have a larger abdomen that is pointed at the apex and males have a more slender abdomen, terminating in prominent claspers. Larvae are between 1-8 mm in length, white, leggless and fairly active. They have a distinct, shiny black head, with powerful mouthparts (Fletcher *et al.*, 1989). Each of the four larval instars can be determined according to the size of the head capsule (Cantelo, 1988).

Life cycle

Many sciarid species have been associated with mushrooms, though only two are common pests in the UK, *Lycoriella. castanescens* and *L. ingenua*. Almost all known sciarid species are saprophagous, although *L. castanescens* and *L. ingenua* can attack healthy mushrooms (Hussey & Gurney, 1968).

The eggs of *L. castanescens* are blunt, oval and translucent and either laid singly or in groups of up to ten in compost (Hussey & Gurney, 1968). Larvae develop through four instars which can be differentiated by the size of the black head capsule (0.07, 0.11, 0.17 and 0.24 mm; 1st-4th instars respectively). The fully grown larvae are 5-8 mm long. The transparent body has twelve segments and the dark alimentary canal and white body fat are clearly visible. Prior to pupation larvae construct a cocoon from silken threads and compost fragments, taking up to 2 days.

Female flies are attracted to compost at the end of Phase II composting, by the fermentation odours that are given off during the cool down period (Hussey *et al.,* 1969; Binns, 1975). Maximum female fecundity is estimated to be 170 eggs, and since spawn-run conditions are also favourable for sciarid development, the next generation may emerge in 2-3 weeks. By this time the crop has been cased and females lay their eggs in the casing layer, where subsequent generations will develop. It is also in the casing layer where damage is caused to the crop, affecting the yield of mushrooms.

Sensory structures

The most obvious sensory structures of insects are present on the head. The main structures are a pair of compound eyes, a pair of antennae and, typically, three ocelli or simple eyes. Antennae are present on all insects, though they may be greatly reduced or absent in larval stages. They are mobile, segmented paired appendages and though their structure and function vary widely across the insect classes, antennae have three main divisions. The first antennal segment is known as the scape, it may be larger than the other segments and acts as a basal stalk. The second segment, or pedicel, usually contains the Johnston's organ, a sensory structure that responds to movement of the distal portion of the antennae relative to the pedicel. The remaining segments of the antennae are known, collectively, as the flagellum. The flagellum is often filamentous and multi-segmented however, it may be reduced or modified according to species. Numerous sensory structures, or sensilla, are found on the antennae, which vary in structure and function, dependent on the life history strategy of the species. Sensilla may be in the form of hairs, pegs, pits and cones and may function as chemoreceptors, mechanoreceptors, thermoreceptors and hygroreceptors. Usually there are several common types of sensilla present on antennae, including trichoid hairs, basiconic and coeloconic pegs, plate organs and campaniform sensilla (Chapman, 1982). Sensilla may also be concentrated in different regions of the antenna.

Effect on mushroom yield

Phorid larvae feed on actively growing fungal mycelium; however they are rarely seen, as they do not burrow into the mushroom fruit bodies. Phorid adults, in common with sciarids, are important vectors of disease, such as *Verticillium fungicola* (Preuss), and the effect of these on the crop may be greater than the effect of feeding larvae (White, 1981). Females oviposit adjacent to growing mycelium and larvae

tend to remain *in situ* throughout development. Therefore, heavy oviposition after spawning, when mycelium is first colonising compost, would have a more severe effect on subsequent mushroom crops than a later infestation, where the mycelium is vast (Hussey, 1959).

Sciarids affect all stages of mushroom production after pasteurisation of compost. Sciarid larvae may tunnel into stalks and caps of mushrooms, although this is generally only associated with high larval densities and while this damage is easily detected it does not, in itself, cause a significant drop in yield. Compost containing larval frass cannot be colonised by mycelium and if there is a high density of larvae in the compost, spawn run will be inhibited (Hussey & Gurney, 1968).

The most economically significant damage from sciarid infestation occurs where larvae sever mycelial attachments to pinheads (immature mushroom fruit bodies) and sporophore primordia thus preventing their further development into mushrooms. Such damage may go unnoticed by growers but can cause a substantial drop in crop yield (Fletcher, *et al.*, 1989).

Adult sciarid flies may be carriers of mites - often associated with bacterial disease - and other pathogens and diseases, such as *V. fungicola* spores (Fletcher, *et al.*, 1989). These may be spread by adults from contaminated to clean houses, causing a new infestation of diseases

Lycoriella castanescens larvae cause a reduction in mushroom yield that is proportional to the number of larvae present in the casing layer. For example, White (1986, 1987) found that 10 larvae present in a 125g sample of casing caused an 8% reduction in yield. A linear relationship was found between yield reduction and larval number, indicating that there is no injury threshold for this sciarid species, since at any larval density a yield reduction will be experienced. Kielbasa & Snetsinger (1980), working on the other mushroom sciarid, *L. ingenua*, found that a population density equivalent to 13 larvae per 125g sample of casing did not cause any reduction in crop yield, and could therefore be regarded as the injury threshold. Whilst there is no injury threshold for *L. castanescens* there is an economic threshold for the pest. The economic threshold can be defined as the larval density, above which the cost of control methods outweighs the losses that would be encountered through yield reduction. For *L. castanescens* a mean number of one larva per 125g sample of casing layer has been determined as the economic threshold (White, 1986, 1987).

Megaselia halterata does not account for a similar level of crop yield reduction as *Lycoriella* spp. The economic threshold of *M. halterata* was estimated as 100 larvae per 30 g compost (Hussey, 1961). However, Rinker & Snetsinger (1984) estimate the damage threshold of *M. halterata* to be between 9,800-12,400 females/m^2 in spawning houses, equivalent to 14-17 larvae/30 g samples of compost (White, 1987) If larval densities exceed this, crop yields will become affected. However, while phorids usually do not induce a direct reduction in crop yield, unless present in high numbers, they can indirectly affect crops by reducing quality and quantity of mushrooms by acting as vectors for fungal, bacterial and viral pathogens, nematodes and mites (Rinker & Snetsinger, 1984).

Chemical control

Sciarid larvae can be effectively controlled using diflubenzuron (White, 1981, 1997) (Table 1). However, this insecticide may induce yield losses due to phytotoxic side-effects (White, 1997). The application of 30 ppm after casing is recommended (Scheepmaker, 1999).

Table 1. Approved insecticides for use in the UK mushroom industry. Taken from http://www.greenmount.ac.uk/hort/techinfo/mushchem.htm Using *The UK Pesticide Guide 1998* and issues of *Pesticide Register* for updated approvals.

Active ingredient	Trade Name	Use	MAFF No.	Manufacturer
Dichlorvos	Darlingtons Dichlorvos	Mushroom flies (Avoid spraying mushroom beds)	05699	Darmycel
Diflubenzuron	Dimilin Flo	Sciarid flies	07151	(Zeneca)
Methoprene	Apex 5E	Sciarid flies	05730	(Sandox Speciality)
Permethrin	Turbair Permethrin	Phorid & Sciarid Flies	02246	(Fargro)
Pyrethrin & Resmethrin	Pynosect 30 Water miscible	Mushroom flies	01653	(Mitchell Cotts) (off-label approval)
Resmethrin	Turbair Resmethrin Extra	Sciarid Flies	02247	(Fargro)

Pyrethrins are plant derived products, from the flowers of *Tanacetum cinerariifolium*. This insecticide has been in use since the early nineteenth century (Gullan & Cranston, 1994). Pyrethrins, and related synthetic pyrethroids. are effective against lepidopteran larvae, kill on contact even at low doses and have low environmental persistence (Gullan & Cranston, 1994). Pyrethrins have a lower mammalian and avian toxicity, when compared with synthetic insecticides, however resistance among pest species is increasing (Gullan & Cranston, 1994).

Most synthetic insecticides have non-specific, broad spectrum activity and generally act on the nervous system of insects.

Insect growth regulators act to halt development and insects either fail to reach maturity or result in sterile malformed adults. Methoprene, an insect growth regulator, acts through interference of the maturation hormones. Diflubenzuron is a strong chitin synthesis inhibitor and prevents formation of the insect cuticle, causing insect mortality prior to or immediately after ecdysis (the final stage of moulting).

Olfactometer design

In order to determine the behavioural response of female sciarid and phorid flies to mushroom cultivation substrates, suitable apparatus had to be designed and tested. In previous studies on sciarid and phorid flies various types of bioassays were used. Burrage (1981) used a wind tunnel to determine the attraction of *M. halterata* to

spawned compost. Furley (1998) used a static air olfactometer to investigate the behavioural response of *L. castanescens* to fruiting bodies of *A. bisporus* and case run peat (casing material with full mycelial growth). Pfeil (1990) used a different design of static air olfactometer to investigate the attraction of *M. halterata* to spawned compost. Air flow olfactometers have been used to determine the behavioural preference of insects to test substances. For example, a Y-tube olfactometer was first used by Read, Feeny & Root (1970) for investigations into habitat selection by aphid parasitoids.

General Methods

Mushroom cultivation

The pre-cropping stages of mushroom cultivation can be split into 3 distinct phases. Phase I & II are composting stages concerned with conversion of wheat straw and organic supplements into a medium suitable for growth of mushroom mycelium. Phase III is concerned with actual growth of the mycelium through compost. The following is a description of the processes involved in a typical crop grown at the Mushroom Unit, Horticulture Research International (HRI).

Phase I composting

During Phase I bales of straw are broken up and mixed with water and piled into stacks at least 1.5 m in height. This enables temperatures within the stack to reach in excess of 70ºC. Temperatures of this magnitude ensure rapid completion of Phase I composting by encouraging the growth of thermophilic decomposers which break down the raw materials forming a selective medium for mushroom growth. To obviate the production of deleterious anaerobic conditions, the compost is turned daily and wetted as necessary, ensuring a moisture content of approximately 75%. As Phase I composting proceeds the volume of the stack will decrease, due to biodegradation of the raw materials by microflora. As the stack height decreases the temperatures reached within the stack will also decrease, thus slowing down the process. To prevent this, stacks are 'punched up', compost from one end is piled onto the top of the stack prior to turning, thus ensuring that the stack will be at least 1.5 m high after the turn.

Horse manure was traditionally the only supplement added to compost; however as mushroom cultivation has become increasingly commercialised, research into all aspects of their growth has increased. Consequently, various compost supplements are commercially available. Chicken litter is now preferred over horse manure as an organic supplement for compost, probably due to its increased availability. Various compost activators are commonly used on commercial mushroom farms, such as Sporovite, a molassed fibrous meal containing 26% sugar (w/w DM) (ADCO-Rumenco Ltd, Burton, Staffs, UK) (Noble & Gaze, 1994). This has a relatively stable nitrogen content and reduces the variation in nitrogen content of compost caused by different batches of poultry manure and straw (Noble, Gaze & Willoughby, 1998). Gypsum and calcium sulphate can also be added to compost, in the latter stages of Phase I, to prevent anaerobic conditions developing within the stack, which would cause the compost to become sour.

In a typical crop on the Mushroom Unit, HRI, 470 kg chicken litter, 100 kg Sporovite and 50 kg gypsum are added to each tonne of straw in a compost stacks. Stack size may vary with different crops, but the average stack is about 16-20 bales of straw (4.4-5.5 tonnes). The addition of chicken litter and Sporovite occurs in two applications, half 3 days after the start of Phase I composting and the remainder 5 days later. Gypsum is added approximately five days prior to Phase II composting. The duration of Phase I varies, dependant on straw quality and weather conditions, but normally lasts between 14-19 days.

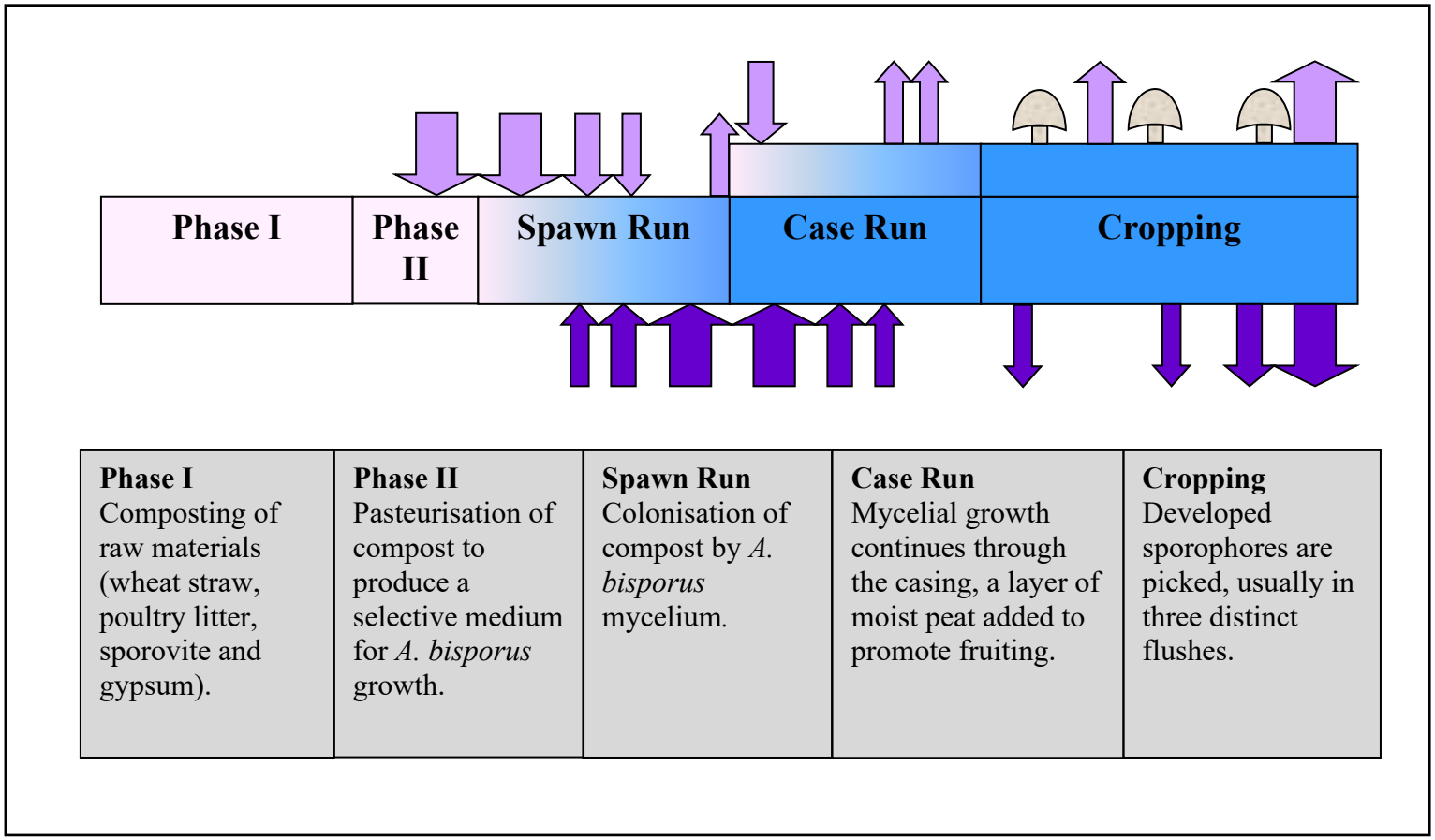

Figure 1. Commercial production of *Agaricus bisporus*, showing timing of insect attack and adult emergence. Key: Fully colonised compost; Uncolonised compost; Sciarid infestation; Phorid infestation. Scale one week.

Phase II composting

The second phase of composting occurs when the compost is well and evenly degraded and has a moisture content of 75-76% (oven dry weight) and a nitrogen content of 2.1-2.3% dry matter (Willoughby & Gaze, 1999). Phase I composting normally occurs in a covered, outside yard. For Phase II, compost is transferred to a bulk pasteurisation tunnel. Phase II composting continues the decomposition of the straw and supplements, but in a controlled environment. Phase II also involves a period of pasteurisation where the temperature of the compost and surrounding air is increased to and held at 57ºC for 6 hours. Pasteurisation ensures that any pests or pathogens and any micro-organisms that would compete with *A. bisporus* in the compost are destroyed. During Phase II the moisture content of the compost drops to between 71-73% (oven dry weight). After Phase II the compost is then prone to contamination by potential pest species.

Phase III

After Phase II the compost is left to cool to approximately 18ºC prior to inoculation with mycelium. A compost temperature of 26ºC is the upper temperature limit for mycelial growth, above this mycelium will not develop through the compost. Spawn, which consists of rye or millet grains that have been inoculated with *A. bisporus* mycelium, are mixed into compost, 0.5% by fresh weight. The spawned compost is then packed into trays (50 kg/tray) and stored at 25ºC, with a relative humidity of 95-97% for 15-17 days, enabling mycelium to colonise the compost, a process known as the 'spawn-run'.

When the mycelium has fully colonised the compost a nutrient-deficient casing layer is added to initiate formation of fruit body primordia. The addition of a casing layer is essential to induce mushroom growth. The casing layer consists of peat, mixed with lime and either finely ground spawned compost or mycelium inoculated onto another substrate, such as vermiculite. The casing layer forms a neutral medium that stimulates fruiting and provides anchorage for the developing sporophores. Cased trays are then transferred to a mushroom house, where humidity, compost temperature, air temperature and CO_2 concentration are monitored. The cased compost is then left for 7 days at 25ºC for the mycelium to colonise the casing layer. The air temperature is then decreased to 18ºC and CO_2 concentration is reduced for three days to provide optimum conditions for sporophore development.

Fruiting then occurs in well defined flushes, starting two weeks after the casing layer has been added and continuing at approximately weekly intervals. After the last flush has been picked the mushroom house, compost and trays are normally 'cooked-out' with steam to eradicate any pests and diseases that may be present by this time. The compost is then disposed of.

Insect culture

Insects for initiating cultures were kindly supplied by Jane Smith, HRI, Wellesbourne.

Sciarid culture

Sciarid larvae were reared on a mixture of moss or sphagnum peat and Soya flour. Soya flour was autoclaved at 121ºC for 15 minutes and mixed thoroughly into saturated peat (5% Soya by weight). The peat and Soya mix was pressed firmly into

the base of a propagator (Ward 215 x 150 mm seed tray, Darlston, Staffordshire). The propagator lid (Ward 215 x 150 mm seed tray lid, Darlston, Staffordshire) was sealed around the rim with foam insulation tape (0.5 cm draught seal, Woolworth's PLC, London) to ensure a fly-proof seal. Two 1 cm air holes were burnt into the top of the propagator lid and two larger, 3 cm, air holes were burnt into the sides. The smaller holes were sealed with a cotton wool dental plug, whilst the larger holes were sealed with larger cotton wool plugs, providing the propagator with ventilation that prevented flies escaping. The lid of the propagator was attached to the base, containing the peat and Soya mix, and held firmly with elastic bands.

Newly emerged adult flies, from a previous culture, were introduced into the propagator (approximately 40 female and 10 males flies in a 215 x 150 mm propagator). Propagators were stored at room temperature within the lab for five days, ensuring mating, egg maturation and oviposition had occurred. Propagators where then stored at either 15ºC or 25ºC. Larvae developed within the peat and Soya mix and on emergence of adults, approximately 2-3 weeks later, a new culture was started.

Sciarid pupae could be collected directly from the rearing substrate. A culture of newly emerged adults was narcotised using CO_2. A sample of compost was then removed from the propagator and placed into a 2 litre capacity, tall, glass jar (Fisherbrand, Fisher Scientific, UK). This was carefully filled with tap water, ensuring that no turbulence was created. This was briefly left to settle. The substrate should float to the surface whilst the pupae and any remaining larvae should sink. The compost and most of the water was decanted off and the remaining water observed under a dissecting microscope. Pupae could be clearly observed under a low magnification and were removed from the water using a paint brush.

Phorid culture.

Phorid larvae were reared on actively growing fungal mycelium. Twenty grammes of fully spawned compost (1% spawn, by weight Phase II compost incubated at 25ºC for 3 weeks) were mixed with 80 g of Phase II compost (Mushroom Unit, HRI, Wellesbourne). The compost mixture was then pressed firmly into plastic pots (No 16 clear pots, with white polythene lids, AW Gregory & Co. Ltd., London). Pots that had emerging adults were then paired with the new compost mixtures within large propagators (36 cm seed tray, Richard Sankey & Sons Ltd., Nottingham), modified with air holes (1 cm diameter) burnt into the top, sealed with dental plugs and a strip of foam insulation tape (0.5 cm draught seal, Woolworth's PLC, London) around the rim. The base of the propagator was covered in dampened absorbent paper in ensure that the compost did not dry out. Syrup (Lyles Golden Syrup, Tate & Lyle, London) was dabbed onto the inside surface of the propagator lid, providing the necessary carbohydrate source for the female flies. The pot lids from the emerging fly cultures were removed and the propagator lid and base sealed and held firmly in place with elastic bands. Propagators were stored at room temperature within the lab for five days, ensuring mating, egg maturation and oviposition had occurred. After this time CO_2 was introduced to the propagators to narcotise any remaining flies and the pots of new compost were sealed with lids containing a 1 cm ventilation hole in the middle sealed with a cotton wool dental bung. Pots where then stored at either 15ºC or 25ºC. Larvae developed within the mycelium seeded compost and on emergence of adults, approximately 2-3 weeks later, a new culture was started.

Narcotising chamber

An narcotising chamber (Figure 2) was constructed to enable observation and sexing of flies under a dissecting microscope. The base of a 10 x 10 cm square Petri dish was filled with a 0.5 mm deep layer of plaster of Paris. The base of a 9 cm diameter, round Petri dish was removed and a corresponding area was removed from the lid of the square Petri dish. The base of the round Petri dish and the lid of the square tray were glued together with a layer of mesh between them. A ring of plastic tubing was formed above the layer of plaster, in the base of the square Petri dish, and linked to a CO_2 supply via a Y-tube connector. The Y-tube connector was passed through a hole in the lid of the square tray. The circle of tubing had slits cut into it, enabling the uniform release of CO_2 into the Petri dish. The lid and base of the square tray were sealed using parafilm, as was the hole through which the CO_2 supply was fed. Flies could then be placed in the Petri dish and would remain narcotised due to the CO_2 supply. This enabled flies to be easily sexed prior to use in bioassays.

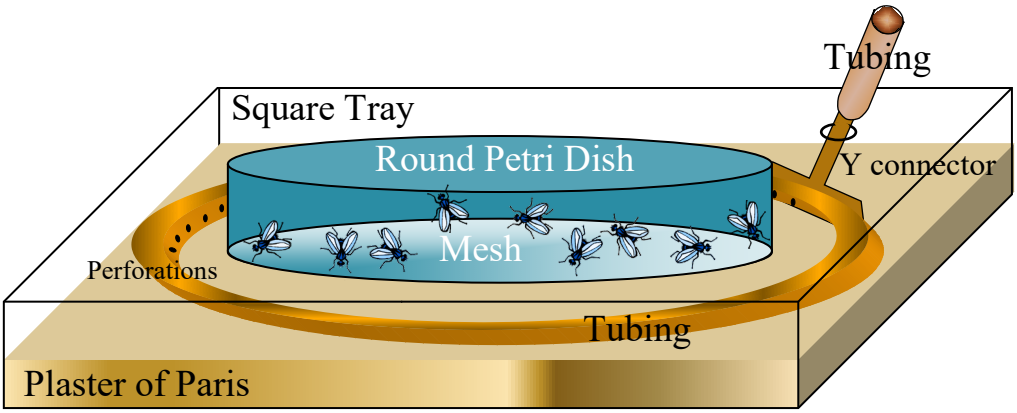

Figure 2. Narcotising Chamber.

Olfactometer design

A static air olfactometer was employed to determine the attraction of male and female, *L. castanescens* and *M. halterata* to cultivation substrates. The design was based upon that used by Pfeil (1990). This involved a release chamber, Petri dish release device and three test cups attached to the base of the release chamber via glass tubes. Samples could be put into two of the test pots while the third acted as a blank. Flies could then be released into the release chamber and left for 12 hours in total darkness. After this time the number of flies within the test pots and the release chamber were counted. For the purpose of statistical analysis Pfeil (1990) determined that the chance of finding flies within the release chamber and the three test pots would be equal and a chi-squared test was applied to this hypothesis. However, this approach was flawed in a number of ways. If flies remained within the release arena, this would not necessarily indicate a choice by the fly. Only flies that had made a choice to move to one of the test pots should have been included in the statistical analysis. Also, the presence of two test pots and just one control pot introduced a bias within the experimental design towards the test substrates: there were 6 glass tubes leading to the test samples and only 3 to the control. For an unbiased experimental design there should be an equal chance of the flies encountering a control pot as there would be to a test pot. For these reasons the static air olfactometer, as used by Pfeil (1990), was modified to incorporate a more robust experimental design.

The static air olfactometer designed for these experiments consisted of a 20 x 12 x 8 cm release arena with two 2 cm diameter ventilation holes, covered with fine mesh (Figure 3). A fly release device, consisting of a 9 cm diameter Petri dish, was centrally mounted on a rubber stopper (No 25, Fisher scientific, UK) within the arena. A 15 cm length of string was attached to the lid of the Petri dish and ran through an aperture in the lid of the release arena. Flies were placed within the Petri dish and released by pulling the string through the aperture. The string was secured, with the Petri dish lid raised above the base, by taping it to the external surface of the release arena lid with adhesive tape. This not only ensured that flies were released into the arena in an unbiased fashion but it also prevented flies from escaping from the small aperture.

The test pots (No 8 clear pots, with white polythene lids, AW Gregory & Co. Ltd., London) were positioned underneath the release arena and connected by glass tubes (6 cm x 0.6 cm i.d.) (four to each test pot). Holes were burnt into the plastic base of the release arena and glass tubes were positioned flush to the arena floor and held in place with an all purpose glue (for glue guns, Evo-stik, Evode Ltd, Stafford), creating a pit fall trap from which flies could not easily escape. The lids of the test pots also had corresponding holes burnt into them and then the lids were pushed onto the glass tubes, which protrude from the base of the release arena. The two test pots were positioned equidistant from each other and centrally with respect to the arena floor. The four holes, for the glass tubes, were positioned centrally about the lid of each test pot. This created a uniform olfactometer design that was easily reproduced and did not contain any bias within it, in relation to either release of the flies or preference to test pot. A 1 cm layer of plaster of Paris (Boots PLC, Nottingham) was added to all test pots. The plaster was made using water and plaster in the ratio of 3:4 (w/w). Thirty grammes were poured into each of the test pots and left to air dry. This layer, when moistened, would then act as a humidity buffer in the test pots and would protect the test samples from desiccation. Twenty static air olfactometers were constructed.

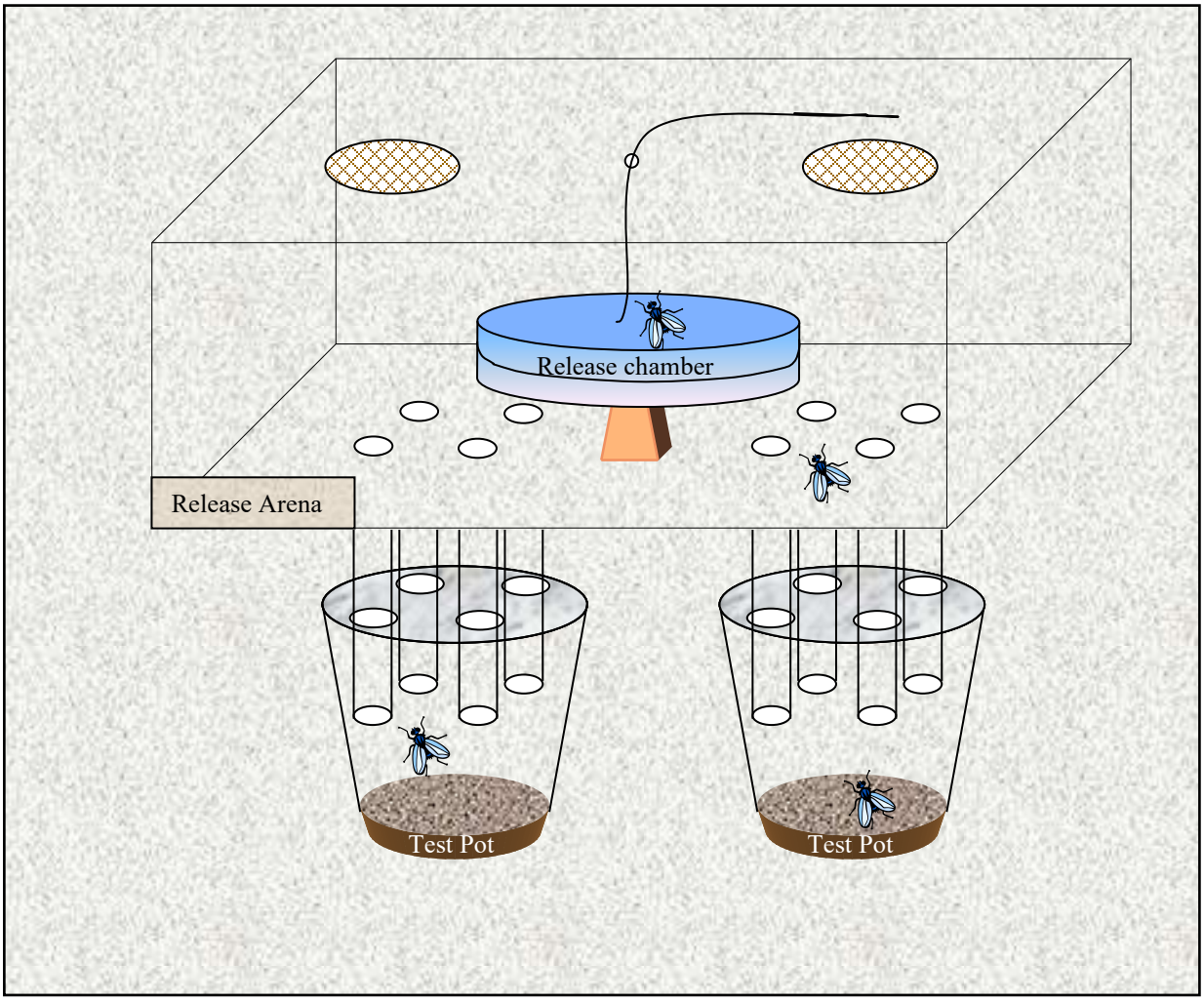

Figure 3. Static air olfactometer, comprising: Petri dish chamber for release of flies ( ), supported on a rubber bung ( ). String attached to lid of dish passes through aperture in top of release arena, forming an unbiased release mechanism. Glass tubes, arranged flush with the floor of the release arena, form pit-fall traps, through which flies drop into the test pots beneath. Test pots contain a layer of moistened plaster of Paris ( ), to maintain humidity of test substrates. Two small apertures on the lid of release arena, covered with fine gauze ( ) provide air exchange.

Scanning electron microscope

Scanning electron microscopes (SEM) operate in a similar way to light microscopes, though with some restrictions. When using a light microscope the specimen is illuminated using a light bulb, magnified using glass lenses and viewed through an eye piece. SEM uses a beam of electrons, instead of a light source, which are magnified using electromagnets as lenses. The electron beam scans the surface of the specimen and the number of backscattered, secondary and absorbed electrons are recorded. The interaction of the electron beam with the surface of the specimen is

determined by the specimens topography and enables an image to be built up of the surface. SEM allows high resolution at high magnification, enabling fine surface details to be determined. SEM can be used with high or low vacuum. Low vacuum SEM enables the observation of live specimens, though resolution at high magnification is compromised. High vacuum SEM enables high resolution at high magnifications, such that the fine surface structure of the specimen can be observed clearly. However, specimen preparation may involve dehydration of specimens and coating the specimens, if necessary, with a conductive layer, for example a thin layer of gold.

Low Vacuum SEM

No specimen preparation was necessary for low vacuum SEM. Double-sided sticky carbon discs were applied to SEM stubs. Insects were then positioned onto the sticky carbon discs. Carbon was used due to its inert nature: it would not cause interference with the electron beam or scattered electrons. The SEM stub was then positioned within the specimen chamber and the specimens morphology investigated.

High Vacuum SEM

To ensure specimens do not collapse under high vacuum conditions, biological material is fully dehydrated. This ensures that the morphology of the specimen remains as true as possible. Specimens were first fixed in 3% glutaraldehyde in phosphate buffer for one hour. To make up the fixative, 6 ml of stock solution (25%) were added to 44 ml M/10 phosphate buffer, pH 7.4. For M/10 phosphate buffer at pH 7.4, 80 ml of Na_2HPO_4 (14.196 g/litre) were added to 20 ml KH_2PO_4 (13.6 g/litre) and stored at 4°C. The specimen was then washed in phosphate buffer overnight. The sample was then rinsed in distilled water for 15 minutes before being taken through an alcohol dehydration series. This involved 15 minute washes in increasing concentrations of ethanol, starting with 30% ethanol and finishing with two 15 minute washes in 100% ethanol. Dehydrated specimens were then critical point dried, a process that uses liquid CO_2 to displace any liquid within the specimen. The CO_2 forms a gas at room temperature which diffuses from the specimen. The specimen was then completely dry with all the fine structures intact. The specimen was then coated with a very thin layer (56 nm) of gold using a sputter-coater. The specimens were then positioned within the specimen chamber of the SEM and their morphology investigated.

Results

Preliminary assessment of static air olfactometer

Before the olfactometers could be used to assess the behavioural response of sciarid and phorid flies to different mushroom cultivation substrates, they needed to be tested to determine if there was any internal bias present within them. This would then ensure that results pertaining to behavioural response of flies were accurate and not influenced by flaws in the experimental design. This also allowed the opportunity to assess the uniformity of the controlled temperature room, to be used in all experiments. This could be achieved by determining whether the position of the olfactometers within the room had any influence on the outcome of the experiment. Also it was necessary to establish that the olfactometer design was appropriate for use with both sexes of phorid and sciarid flies.

Sixteen olfactometers were set up (Figure 3) each with two empty test pots, both acting as a control. Flies were collected from 2-3 day old emerging cultures, thus ensuring that they were mated. Flies were sexed under a dissecting microscope utilising a narcotising chamber. Male and female flies were tested in separate olfactometers, with 12, 20 or 30 flies per release device. Assembled olfactometers were placed on a 4 x 4 grid within a control temperature (CT) room. Their position within the grid was randomised using a 4 x 4 Latin square, ensuring that each sex appeared twice in each row and column of the grid. The temperature was kept constant (25ºC) and all experiments were conducted in total darkness. The olfactometers were left for 16 hours and the number of flies within the right and left test pots and the release arena were recorded. Though the numbers of flies within the release arena would not be used for statistical analysis it was important to record if any of the flies had escaped from the olfactometers during the experimental period. After each experiment the olfactometers were dismantled, rinsed in warm water and left to air dry. Prior to use in a new experiment the olfactometers were sprayed with ethanol and left to dry. This ensured that the olfactometers were thoroughly cleaned between experiments, eliminating the complications of cross contamination between experiments.

The aim of this preliminary experiment was to determine if there was a random movement of flies into the test pots and if there was heterogeneity within the CT room itself. Therefore the null hypothesis was that there would be no significant influence on fly distribution by olfactometer position within the grid and that there will be an even distribution of flies within the test pots.

Statistical analysis

To test for homogeneity within the CT room the results from each row (r) and each column (c) were analysed using a chi-squared contingency test. The degrees of freedom were calculated as (r-1)(c-1). The number of columns in the test was the number of olfactometers in each row or column of the 4 x 4 grid in the CT room, *i.e.* 4. The number of rows was the number observed categories (test pots, *i.e.* 2), thus giving 3 degrees of freedom for each of the tests. The contingency tests were carried out using Minitab, an example of which is shown in Figure 4.

To test for deviation from a 50:50 distribution of flies within the test pots two statistical tests were employed: the chi-squared and G tests. In the chi-squared test (Equation 1), the degrees of freedom were calculated as n-1 where n is the number of observed categories, *i.e.* the number of test pots per olfactometer. The value of n was constant at 2, so the degrees of freedom were also constant throughout all tests , being 1. The value for chi-squared, as calculated from Equation 1 was compared to tables of chi-squared distribution. For 1 degree of freedom the critical chi-squared value, as derived from the distribution tables, was 3.841 at the 95% significance level. If the calculated value of chi-squared (derived from Equation 1) was less than the critical chi-squared value the null hypothesis was rejected.

$$\chi^2 = \sum \left[\frac{(O-E)^2}{E} \right]$$

Equation 1. The Chi-squared test.

Key to Equation 1: O is the observed frequency *i.e.* in this instance the number of flies in each test pot; E is the expected frequency *i.e.* in this instance the mean number of flies in both test pots; χ^2 is the calculated value of chi.

In the G test (Equation 2) the distribution of G has been shown to approximate to the distribution of chi-squared (Sokal & Rohlf, 1981). Where sample sizes are small (n<200), as in this instance, correction factors are used to produce a more conservative test

$$G = 2x\ \Sigma O \ln(O/E)$$

Correction factor, where n<200:

$O_{adj} = O + 0.5$ where O<E and O - 0.5 were O>E

$$G_{adj} = 2x\ \Sigma O_{adj} \ln(O_{adj}/E)$$

Equation 2. The G test.

Key to Equation 2: O is the observed frequency *i.e.* in this instance the number of flies in each test pot; E is the expected frequency *i.e.* in this instance the mean number of flies in both test pots; O_{adj} denotes the adjusted observed value; G_{adj} denotes the adjusted G statistic.

The degrees of freedom were calculated in the same way as for the chi-squared test and G_{adj} was compared to the chi-squared distribution, in the same way as for the calculated chi-squared. For 1 degree of freedom the critical chi-squared value, as derived from the distribution tables, was 3.841 at the 95% significance level. Where the G_{adj} was less than the critical chi-squared value the null hypothesis should be accepted.

Analysis of *Megaselia halterata* data

For the first preliminary experiment 12 *M. halterata* were used in each of the 16 olfactometers (Table 2). Eight replicates of both male and female flies were performed. The results were analysed for homogeneity within the CT room using a chi-squared contingency test (Figure 4) (Table 3) as previously described. The ***P*** values for the combined data for columns and rows were all found to be greater than 0.05. This indicated that the null hypothesis should be accepted and suggested that there was homogeneity within the 4 x 4 grid in the CT room.

Chi-squared tests were then performed on the data from individual olfactometers to determine if there was any left or right sided bias among fly distribution in test pots (Table 4). The expected value was calculated as the mean number of flies within the test pots, and this was calculated separately for each olfactometer. Any significant deviation from a 50:50 expected distribution would indicate that fly distribution within the olfactometers was being influenced by factors other than the contents of the test pots. For example the flies may be aggregating within a test pot due to trail following.

From the first experiment, *M. halterata*, where there were only 12 flies per olfactometer (Table 4), it became clear that there were insufficient flies within each olfactometer. As some flies remained in the release chamber, the fly number within both test pots was consistently less than 10. Consequently the expected number of flies within the test pot, the mean number of flies in both test pots, was consistently less than five. For chi-squared statistics it is recommended that the expected value should be at least five. Therefore, the number of flies was increased and the test performed again using 20 or 30 flies. (Table 5). Due to a transient shortage of suitable flies only 8 olfactometers were tested.

As before, chi-squared tests were performed on the data from individual olfactometers, with the expected frequency calculated as the mean number of flies within both test pots (Table 6). Where 20 flies were used, the expected number of flies within test pots increased. In two instances the expected frequency was still less than 5. In one olfactometer containing 20 male *M. halterata*, the calculated χ^2 value was greater than the critical χ^2 so the null hypothesis of equal distribution of flies was, in this instance, rejected.

The G test was also applied to the data (Table 7). The results of the G test varied from that of the χ^2 test, in that the all G_{adj} were less than the critical χ^2 indicating that the null hypothesis should be accepted.

Chi-Square Test

MTB > ChiSquare 'r1c1'-'r1c4'.

```
Expected counts are printed below observed counts
          r1c1     r1c2     r1c3     r1c4    Total
    1        5        1        3        6       15
          3.53     3.09     4.41     3.97

    2        3        6        7        3       19
          4.47     3.91     5.59     5.03

Total        8        7       10        9       34

Chi-Sq =  0.613 +  1.412 +  0.452 +  1.037 +
     0.484 +  1.115 +  0.357 +  0.819 = 6.288
DF = 3, P-Value = 0.098
6 cells with expected counts less than 5.0
```

Figure 4. An example of the Chi-squared contingency test used to evaluate homogeneity within the rows and columns of the 4 x 4 grid in the CT room (Minitab Output).

Table 2. Preliminary experiment for *M. halterata* male and female flies; 12 flies were used per olfactometer; both test pots were empty (control). Key: ▢, Row and column of 4 x 4 grid; ▢, Number of flies remaining in release arena; ▢, Number of flies in the left and right hand test pots of olfactometer.

	1	2	3	4
1	4	5	2	3
	5 | 3	1 | 6	3 | 7	6 | 3
	male	female	female	male
2	6	1	7	2
	1 | 5	8 | 3	1 | 4	6 | 4
	male	male	female	female
3	3	4	5	4
	5 | 4	5 | 3	4 | 2	1 | 6
	female	female	male	male
4	2	4	9	1
	1 | 8	3 | 5	2 | 1	5 | 6
	female	male	male	female

Table 3. Chi-squared contingency test on row and column data for *Megaselia halterata*, to test for homogeneity within the CT room. Where $\boldsymbol{P} \leq 0.05$ reject H_o. Calculations made using Excel.

	Column									
Row	**1**		**2**		**3**		**4**		**X^2**	***P* value**
1	5	3	1	6	3	7	6	3	***6.288***	***0.098***
2	1	5	8	3	1	4	6	4	***7.139***	***0.068***
3	5	4	5	3	4	2	1	6	***4.849***	***0.183***
4	1	8	3	5	2	1	5	6	***4.101***	***0.251***
X²	***7.17***		***6.844***		***3.84***		***5.039***			
***P* value**	***0.067***		***0.077***		***0.279***		***0.169***			

Table 4. Chi-squared test on individual olfactometer data for *M. halterata*. At $\boldsymbol{P}$=0.05 significance level with 1 degree of freedom the critical χ^2=3.841. Where the calculated $\chi^2 \leq$ critical χ^2 the null hypothesis should be accepted. The expected value E should be at least 5. Key: ■, Reject null hypothesis;□, E≤ 5; O, Observed values; χ^2, Calculated chi-squared value. Calculations made using Excel.

		Column							
Row		1		2		3		4	
1	O	5	3	1	6	3	7	6	3
	E	4		3.5		5		4.5	
	χ^2	0.5		3.571		1.6		1	
	Sex	male		female		female		male	
2	O	1	5	8	3	1	4	6	4
	E	3		5.5		2.5		5	
	χ^2	2.667		2.273		1.8		0.4	
	Sex	male		male		female		female	
3	O	5	4	5	3	4	2	1	6
	E	4.5		4		3		3.5	
	χ^2	0.111		0.5		0.667		3.571	
	Sex	female		female		male		male	
4	O	1	8	3	5	2	1	5	6
	E	4.5		4		1.5		5.5	
	χ^2	5.444		0.5		0.333		0.09	
	Sex	female		male		male		female	

Table 5. Preliminary experiment for *M. halterata* male and female flies; 20 & 30 flies were used per olfactometer; both test pots were empty (control). Key: , Row and column of 4 x 4 grid; , Number of flies remaining in release arena; . Number of flies in the left and right hand test pots of olfactometer. Calculations made using Excel.

	1		2		3		4	
1	12		14		4		17	
	2	6	6	8	4	12	8	5
	20 female		30 male		20 male		30 female	
2	16		10		8		12	
	3	1	14	6	6	5	9	9
	20 male		30 female		20 female		30 male	

Table 6 Chi-squared test on individual olfactometer data for *M. halterata*. At ***P***=0.05 significance level with 1 degree of freedom the critical χ^2=3.841. Where the calculated $\chi^2 \leq$ critical χ^2 the null hypothesis should be accepted. The expected value E should be at least 5. Key: , Reject null hypothesis; , E≤ 5; O, Observed vales; χ^2,Calculated chi-squared value. Calculations made using Excel.

		Sex							
Fly No		female		female		male		male	
20	O	2	6	6	5	4	12	3	1
	E	4		5.5		8		2	
	χ^2	2		0.091		4		1	
30	O	8	5	14	6	6	8	9	9
	E	6.5		10		7		9	
	χ^2	0.692		3.2		0.286		0	

Table 7. G test on individual olfactometer data for *M. halterata*. At ***P***=0.05 significance level with 1 degree of freedom the critical χ^2=3.841. Where the calculated $G_{adj} \leq$ critical χ^2 the null hypothesis should be accepted. The expected value E should be at least 5. Key: , Reject null hypothesis; , E≤ 5; O,Observed vales; O_{adj},Adjusted observed value; G_{adj},Adjusted G value. Calculations made using Excel.

		Sex							
Fly No		female		female		male		male	
20	O	2	6	6	5	4	12	3	1
	O_{adj}	2.5	5.5	5.5	5.5	4.5	11.5	2.5	1.5
	E	4		5.5		8		2	
	G_{adj}	1.152		0		3.1690		0.253	
30	O	8	5	14	6	6	8	9	9
	O_{adj}	7.5	5.5	13.5	6.5	6.5	7.5	9	8.5
	E	6.5		10		7		9	
	G_{adj}	0.309		2.503		0.071		0.056	

Analysis of *Lycoriella castanescens* data

For the preliminary experiment for *L. castanescens* 20 flies were used in each of the 16 olfactometers, to ensure that for statistical analysis the expected number of flies within the test pots was at least 5 (Table 8). Eight replicates of male and eight replicates of female flies were performed. The results were analysed for homogeneity within the CT room using a chi-squared contingency test (Table 9) (Mintab software Figure 4). There are two columns, in Table 9 where heterogeneity was observed, columns 1 and 2. A chi-squared test was performed on the data from individual olfactometers, to determine the source of the heterogeneity (Table 10). The chi-squared test would also show if there was any significant deviation from a 50:50 distribution of flies in the test pots.

The source of heterogeneity was determined to be the olfactometers in row 2, column 4 and row 4, column 2 (Table 10), since both these olfactometers had calculated chi-squared values greater than the critical, tabulated chi-squared value. Both these olfactometers contained female flies. Two other olfactometers containing female flies also had fly distributions that varied from 50:50 ratios (row 1, column 4; row 4, column 4 (Table 10)). The adjusted G test was also applied to the data (Table 11). Values from G test were consistent with those from chi-squared test. There was only one olfactometer were the expected frequency of flies within the test pot was less that five.

Table 8. Preliminary experiment for *L. castanescens* male and female flies; 20 flies were used per olfactometer; both test pots were empty (control). Key: , Row and column of 4 x 4 grid; , Number of flies remaining in release arena; , Number of flies in the left and right hand test pots of olfactometer. Calculations made using Excel.

	1		2		3		4	
1	8		3		7		4	
	7	5	6	11	8	5	13	3
	male		*female*		*male*		*female*	
2	3		8		7		11	
	2	15	4	8	7	6	5	4
	female		*male*		*female*		*male*	
3	2		9		0		10	
	11	7	5	6	7	13	7	3
	female		*male*		*female*		*male*	
4	4		1		4		3	
	7	9	16	3	10	6	13	4
	male		*female*		*male*		*female*	

Table 9. Chi-squared contingency test on row and column data for *L. castanescens*, to test for homogeneity within the CT room. Where $\boldsymbol{P} \leq 0.05$ reject H_o. Key: , Reject null hypothesis. Calculations made using Excel.

	Column									
Row	**1**		**2**		**3**		**4**		X^2	***P* value**
1	7	5	6	11	8	5	13	3	***7.237***	***0.065***
2	2	15	4	8	7	6	5	4	***7.718***	***0.052***
3	11	7	5	6	7	13	7	3	***4.364***	***0.225***
4	7	9	16	3	10	6	13	4	***7.355***	***0.061***
X^2	***10.339***		***11.667***		***3.508***		***2.095***			
***P* value**	***0.016***		***0.009***		***0.32***		***0.553***			

Table 10. Chi-squared test on individual olfactometer data for *L. castanescens*. At ***P***=0.05 significance level with 1 degree of freedom the critical χ^2=3.841. Where the calculated $\chi^2 \leq$ critical χ^2 the null hypothesis should be accepted. The expected value E should be at least 5. Key: , Reject null hypothesis; , E≤ 5; O, Observed vales; χ^2, Calculated chi-squared value. Calculations made using Excel.

		Column							
Row		1		2		3		4	
1	O	7	5	6	11	8	5	13	3
	E	6		8.5		6.5		8	
	χ^2	0.333		1.471		0.692		6.25	
	Sex	male		female		male		female	
2	O	2	15	4	8	7	6	5	4
	E	8.5		6		6.5		4.5	
	χ^2	9.941		1.333		0.077		0.111	
	Sex	female		male		female		male	
3	O	11	7	5	6	7	13	7	3
	E	9		5.5		10		5	
	χ^2	0.889		0.091		1.8		1.6	
	Sex	female		male		female		male	
4	O	7	9	16	3	10	6	13	4
	E	8		9.5		8		8.5	
	χ^2	0.25		8.895		1		4.765	
	Sex	male		female		male		female	

Table 11. G test on individual olfactometer data. At ***P***=0.05 significance level with 1 degree of freedom the critical χ^2=3.841. Where the calculated $G_{adj} \leq$ critical χ^2 the null hypothesis should be accepted. The expected value E should be at least 5. Key: ■ , Reject null hypothesis; □ , E≤ 5; O, Observed vales; O_{adj}, Adjusted observed value; G_{adj}, Adjusted G value. Calculations made using Excel.

		Column							
Row		1		2		3		4	
1	O	7	5	6	11	8	5	13	3
	O_{adj}	6.5	5.5	6.5	10.5	7.5	5.5	12.5	3.5
	E	6		8.5		6.5		8	
	G_{adj}	0.083		0.950		0.309		5.370	
	Sex	male		female		male		Female	
2	O	2	15	4	8	7	6	5	4
	O_{adj}	2.5	14.5	4.5	7.5	6.5	6.5	4.5	4.5
	E	8.5		6		6.5		4.5	
	G_{adj}	9.369		0.758		0		0	
	Sex	female		male		female		Male	
3	O	11	7	5	6	7	13	7	3
	O_{adj}	10.5	7.5	5.5	5.5	7.5	12.5	6.5	3.5
	E	9		5.5		10		5	
	G_{adj}	0.502		0		1.263		0.914	
	Sex	female		male		female		Male	
4	O	7	9	16	3	10	6	13	4
	O_{adj}	7.5	8.5	15.5	3.5	9.5	6.5	12.5	4.5
	E	8		9.5		8		8.5	
	G_{adj}	0.062		8.186		0.565		3.917	
	Sex	male		female		male		female	

Scanning electron microscopy

Low vacuum SEM was used to observe the surface structures of antennae of both *L. castanescens* and *M. halterata*. The use of low vacuum SEM did not provide sufficient resolution at high magnifications to observe the fine structures of antennae, so high vacuum SEM was used. From observation of the antennal structure, phorid adults were seen to have aristate antennae, where the third segment is enlarged and bears a bristle (Figure 5, A & F). Sciarid adults however posses long and slender filiform antennae (Gullan & Cranston, 1996). The micrographs of *M. halterata* clearly show the presence of bulbous pegs on the Johnstons organ (Figure 5, D & E). Numerous pits and hairs were observed on the apical antennal segment of *L. castanescens* (Figure 6, G & H).

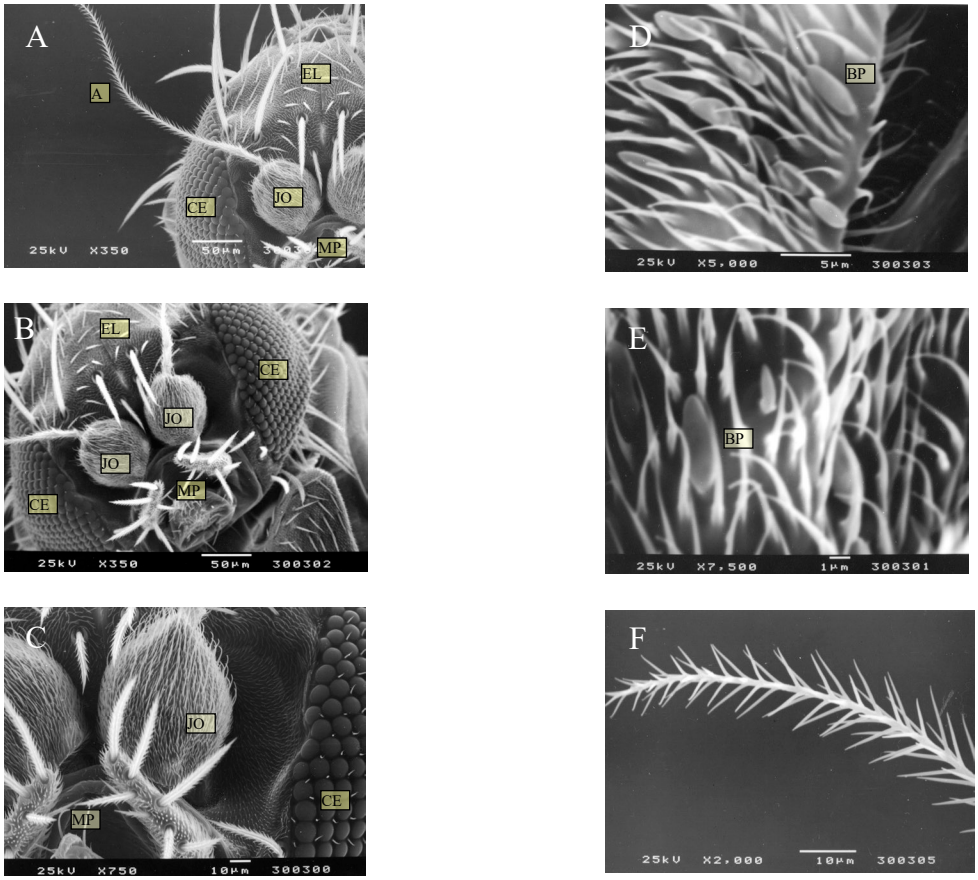

Figure 5. SEM micrographs of adult female *Megaselia halterata*. *A, B & C*: Head, showing compound eye, CE; mouth-parts, MP; ecdysial lines, EL; Johnstons organ JO; arista, A. *D & E*: Johnstons organ, showing bulbous pegs, BP. *F*: Arista,

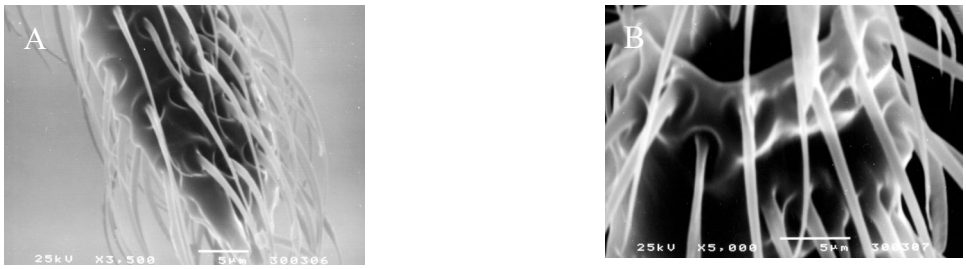

Figure 6. SEM micrographs of adult female *Lycoriella castaneascens*. *A*: Distal portion of apical antennal segment. *B*: Proximal portion of apical antennal segment.

Discussion

For *M. halterata* there appeared to be no positional contribution to the variation observed between olfactometers test pots. In only one olfactometer out of 16 was a significant variation from a 50:50 expected fly distribution shown (
Table 4, row 4, column 1).

In the preliminary experiments with *L. castanescens* 20 flies were tested in each olfactometer and in only one instance (Table 10, row 2, column 4) the expected number of flies in the test pots was less than 5. This indicated that 20 flies per olfactometer was adequate for results to be analysed using chi-squared statistics.

The chi-squared contingency test for *L. castanescens* (
Table 9) showed heterogeneity in columns 1 and 2. Further analysis of the individual olfactometers (χ^2 & G test) (Table 10, Table 11) showed that olfactometers in row 2, column 1 and row 4, column 2 had significant deviation from the 50:50 fly distribution expected. These olfactometers both contained *L. castanescens* females. Two of the other six replicates, of *L. castanescens* females, also had fly distributions, within test pots, that varied significantly from the 50:50 expected distribution. From the statistical tests used the significance of these results was unclear. The tests performed on the data gave no indication of whether these results were indicative of female aggregation in the test pots. In order to determine if this is a factor influencing the behaviour of female *L. castanescens* in the olfactometers further experiments will be necessary.

Due to the problems in interpretation of results it was clear that a different approach to the preliminary test was necessary. The use of a log-linear model will in future be used to determine the homogeneity of the CT room and the uniformity of the individual olfactometers.

The design of the experiment was expanded to incorporate a number of factors relevant to the uniformity of the CT room and individual olfactometers. The influence of these factors on the variation of fly distributions within the olfactometer test pots could then be tested, using a log-linear model. The design for experiments were generated using the statistical software package Genstat 5 (Release 4.1).

The design enables the uniformity of all olfactometers to be tested, as well as any bias for the right or left hand side of olfactometers. The design also enables any variation due to position within the 4 x 4 grid to be highlighted. Flies will be tested in experiments where the distribution of the 16 olfactometers and orientation of the left or right hand end has been randomised. Twenty flies will be tested in each olfactometer. Olfactometers will be numbered (1-16) and each end will have an odd and even number. The position of each olfactometer will be randomised as well as the left side orientation, via the design generated from Genstat 5. These experiments will be repeated on three different occasions.

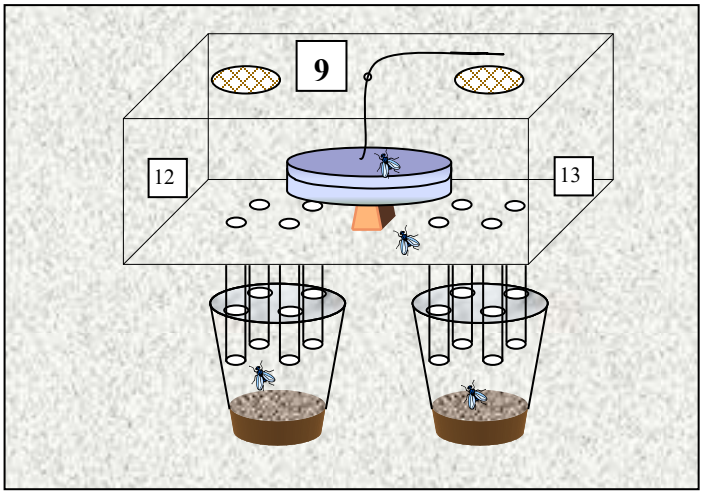

Figure 7. An example of the numbering of olfactometers, showing the olfactometer number (9) and the odd and even ends (13, 12).

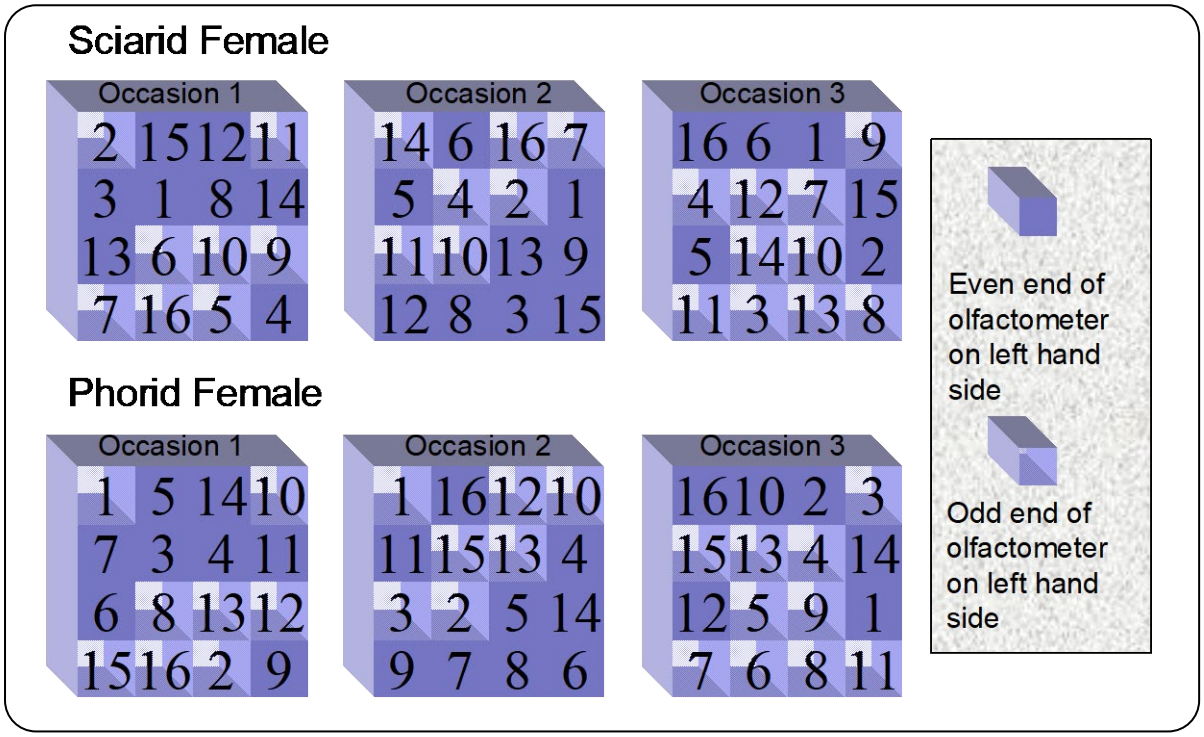

Figure 8. New design for preliminary experiments. The position of the olfactometer within the grid and the orientation of the left hand end were randomised, using Genstat 5.

Once all twelve experiments have been completed (3 replicates of male and female, sciarid and phorid flies) the data will be analysed using a log-linear model (Genstat 5). This test will highlight if there is any variation from a 50:50 distribution of flies within the test pots. It will also be able to determine what, if any, effect the position of olfactometers within the CT room has on the fly distribution. It will also highlight any individual olfactometers showing a consistent deviation form the expected

distribution and if there is any variation generated from the orientation of the olfactometer ends. It will also show if there is any deviation from a 50:50 distribution of flies in test pots caused by the sex or species of the flies.

This information will then be used to determine if the olfactometers are uniform and if any variation in abiotic conditions within the CT room are influencing fly distribution. The behavioural response experiments, determining preference to oviposition substrates (spawned and unspawned compost), can then be designed. The design of the next phase of tests will be dependent on the results of the uniformity tests.

The micrographs of *M. halterata* showed the presence of bulbous pegs which have been suggested as putative olfactory sensilla (Pfeil *et al.*, 1994). Other sensilla have not been detected as yet, but further investigations into olfactory structures are continuing. The presence of olfactory sensilla on the antennae will allow the use of coupled electroantennogram (EAG)/gas chromatography and mass spectroscopy (GCMS) to directly assess the behavioural response of insects to potentially biologically active compounds. This procedure introduces an electrode into the antenna to record any changes in electrical impulse due to the introduction of volatiles to the air surrounding the antenna. The GCMS allows the exact chemical nature of the volatiles to be determined. Further investigation of *L. castanescens* is required to determine the distribution of the pits throughout the antennae and the presence of other sensilla present on the flagellum or basal segments of the antennae. This information may also allow the use of EAG/GCMS on sciarid flies to determine their reponse to biologically active volatiles. The tarsi and ovipositors of both species will also be investigated for the presence of potential olfactory sensilla.

Future Research

The original milestones for future research have been altered to incorporate the progress to date. The main alterations to the original milestones include a necessary time-scale extension for the first behavioural response experiments: Air samples will no longer be collected and stored, instead they will be collected and analysed. This will ensure that sample integrity is maintained and that breakdown of potentially bioactive compounds is prevented: Investigations into the antennal and ovipositior sensilla will now occur in the third year in parallel with EAG analysis.

Revised Milestones

Year 1 (Objective 1)

1. To learn basic techniques in fly rearing, mushroom cultivation and mushroom ecology, including a literature search (HRI)(October 1999): Completed.
2. Set up and maintain fly cultures at Cardiff University (ongoing)
3. Set up olfactometers and other apparatus suitable for investigating behavioural responses in flies (Cardiff)(November 1999): Completed.
4. Carry out behavioural response experiments to determine the attractiveness to flies of different potential oviposition substrates (Cardiff)(December 2000)
5. Review year 2 milestones in light of year 1 results (June 2000)

Year 2 (Objective 2)

1. Devise a method for collection of air surrounding the various substrates (Cardiff)(December 2000)
2. Analyse the chemical composition of the air surrounding the various substrates (HRI/Cardiff)(February 2001)
3. Collect potentially bioactive fractions from the air surrounding the various substrates (Cardiff)(April 2001)
4. Carry out the second phase of behavioural response experiments (Cardiff)(June 2001)
5. Review year 3 milestones in light of year 2 results (June 2001)

Year 3 (Objective 3, 4 & 5)

1. Identify the chemical structures of the bioactive compounds (Cardiff)(September 2001)
2. Carry out the third phase of the behavioural response experiments: Assessing the behavioural responses to synthetic bioactive compounds (Cardiff)(December 2001)
3. Investigate antennal and ovipositor sensory structures of target pests and their possible relationships with cue recognition (Cardiff)(February 2002)
4. Correlate sensory perception with previously recorded behavioural responses using coupled EAG/GCMS and other electrophysiological techniques (Cardiff)(February 2002)
5. Design and test targets and/or traps in laboratory and mushroom house arenas (HRI)(June 2002)
6. To write the PhD thesis including a report on the research findings and any recommendations to the mushroom industry (October 2002)

The research milestones for year two are primarily concerned with the collection and analysis of air samples from potential oviposition substrates. A method for collection of the air samples will be devised. This will probably utilise an open system, by which air is drawn, via a vacuum pump, through a non selective filter which will absorb most volatiles present within the air. A suitable solvent will be determined and the volatiles from the air samples will be analysed using gas chromatography coupled with mass spectroscopy (GCMS). This method will allow the constituent compounds within the air to be identified. The second phase of behavioural response experiments will test the response of gravid females to fractions of air from the attractive oviposition substrates. These response tests may employ the same equipment as the first experiments or a different approach may be taken, for instance the use of Y-tube olfactometers. The milestones for year three will be reviewed after the completion of year two.

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