THE POTENTIAL OF COLONY WASTE MATERIAL AS AN EFFECTIVE

BIOLOGICAL CONTROL OF LEAFCUTTING ANT HERBIVORY:

AN OLFACTORY APPROACH

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Abstract

With an increasing human population comes a greater demand for food. Those involved in food production are under pressure to increase agricultural output. A major threat to crop yield is the loss incurred by pests, specifically insects. Modern farming methods are subject to regulations that forbid the use of once deadly chemicals to control these threats rendering management more difficult.

In the Neotropics, the major agricultural pest threat are leafcutting ants (*Atta* and *Acromyrmex*). These ants exhibit a well-structured social organisation, fungus cultivation, colony hygiene and a complex nest structure that allows them to overcome most pest management control methods. Living in such large colonies creates substantial amounts of waste material within the nest, which leafcutting ants often avoid contact with because of the pathogenic microorganisms that it harbours. The use of this waste material has previously been proposed as a natural deterrent method against leafcutting ant attack.

Previous studies in this area tested the repellent effects using solid waste material collected from nests, I propose that the same repellent effects are present in waste odour. I tested experimentally whether the leafcutting ant *Atta cephalotes* would exhibit similar behaviour to waste odour as they do with solid waste material. I also tested the residual effectiveness of ant waste odour as a repellent over a period of time.

In laboratory-based tests using a Y-shaped olfactometer, ants were subjected to several treatments to determine the repellent properties of any waste odour. When presented with clean air or air polluted with: (a) nest waste from any leafcutting any colony, they significantly chose to avoid the waste ($P < 0.0001$); (b) nest waste from their own colony, they significantly chose to avoid the waste ($P = 0.0093$) and (c) nest waste from a donor colony, they also significantly avoided the waste ($P < 0.0001$).

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However, this deterrent effect was only found to be effective for up to five days before the behavioural responses to it were not considered to be significant. However, as the deterrent effects offered by the solid organic nest waste material are also present in the chemical properties emanating from the odour of the waste, it has the potential to be used as a short-term method of natural, biological control in the management of leafcutting ants.

Declaration of Own Work

I declare that the work in this thesis was carried out in accordance with the regulations of the University of Gloucestershire and is original except where indicated by specific reference in the text. No part of the thesis has been submitted as part of any other academic award. The thesis has not been presented to any other education institution in the United Kingdom or overseas.

Any views expressed in the thesis are those of the author and in no way represent those of the University.

Signed …………………………………….. Date ………………………..

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Contents

Index of figures

1. Introduction

1.1 The challenges facing modern agriculture

With human population projections expected to reach 9.7 billion by the year 2050 (United-Nations, 2015) and a greater demand for superior quality foods, it is estimated that food production will need to increase by 70% to satisfy the demand (Alexandratos and Bruinsma, 2012). As a result of this necessary growth in agricultural output and because of associated changes in agricultural methods (such as the growing of monoculture rather than mixed crops) food producers are faced with increased challenges (Tilman *et al.*, 2001; Godfray *et al.*, 2010). Biotic threats, which include pathogens, weeds and arthropods, have a significant effect on food productivity with Oerke (2006) suggesting a global losses of between 29-37% across a variety of crop plants. Whilst these estimates also include pathogenic threats, the crop damage caused by insects is considerable. Agricultural losses caused by insects are difficult to quantify but the losses from a reduction in yield combined with increased overheads to control them do offer an insight into the extent of damage they cause. For example, in the United States, corn rootworms (*Diabrotica* spp.) are considered a major pest in the production of maize with estimated annual yield losses and prevention expenditure exceeding $1 billion per year (Metcalf *et al.*, 2012).

1.1.1 A most serious pest?

Pest threats differ greatly in different parts of the world. In the American tropics, with their voracious and polyphagous appetite, it is the leafcutting ants (Hymenoptera: Formicidae: Attini) that are arguably the most serious insect pest in agricultural and forest ecosystems (Cherrett, 1986a).

Leafcutting ants are obligate agriculturists. They are unique among ants in that they forage for a wide variety of fresh plant material, not for consumption but to cultivate a mutualistic fungus that provides the sole source of food for their larvae and an important food source for adults (Weber, 1972). As their primary food source, maintaining the integrity of the fungus garden is paramount and any threat to the garden could lead to the collapse of the colony.

Leafcutting ants live in large, subterranean colonies (Buhl *et al.*, 2006), with the number of workers in a single colony estimated as high as 8 million (Fowler *et al.*, 1986b). They exhibit a complex division of labour, where tasks including queen attendance and brood care, foraging, and waste management are all performed by members of distinct castes (Wilson, 1971; Wetterer, 1999; Hart *et al.*, 2002).

The status of leafcutting ants as a serious pest is a view shared not only by those within the agricultural industry but also local communities who rely financially on forest plantations (Serna and Correa, 2003). Their predominantly Neotropical distribution means that the most frequently attacked crops include coffee (*Coffea Arabica* L.), cocoa (*Theobroma cacao* L.), citrus (*Citrus* spp.), maize (*Zea mays* L.) and cotton (*Gossypium hirsutum*), resulting in conservative estimates of damage running in to the billions of dollars (Hölldobler and Wilson, 2010; Montoya-Lerma *et al.*, 2012). Crops are not the only plant material that are affected; Rockwood (1976) reported that in some cases as much as 77% of all plant species within the foraging range of leafcutting ants were victim to defoliation. It comes as no surprise that the control of leafcutting ants could be responsible for as much as 75% of reforestation costs in Brazil alone (Vilela, 1986). Examples of the damage caused by leafcutting ants are found throughout the region. In cocoa plantations of Brazil, several leafcutting ant species strip the plants of not only

the leaves, but also bark and small pods, with young plants being the most susceptible (Delabie, 1990). The vulnerability of young plants is not uncommon as other Brazilian studies show that leafcutting ants forage more intensely on pine seedlings within the first 30 days of their growth (Nickele *et al.*, 2012), and *Eucalyptus* plants under three years old (Matrangolo *et al.*, 2010). Further evidence is found in Argentina, where a significant reduction in tree development was observed in pine (*Pinus taeda*) if they were subject to attack by leafcutting ants within two years of planting (Cantarelli *et al.*, 2008). In pine forests in Venezuela, a single colony of leafcutting ants caused 48% seedling mortality and reduced growth in 40% of those seedlings that remained (Jaffe, 1986).

Herbivory isn't restricted only to young plants as older trees are also attacked, with those up to 10 years old having reduced wood productivity of up to 50% and trees that are victims of successive defoliations suffering in growth, shape and yield (Hernández and Jaffé, 1995). Land owners in Panama also identify leafcutting ants as causing serious problems in reforestation areas (Garen *et al.*, 2009). Wirth *et al.* (2003) report that leafcutting ants destroy up to 13% of standing leaf crop within a single colony's territory per year and Cherrett (1986b) estimating that they are responsible for up to as much as 80% of all apparent leaf damage in some Panamanian rainforests. As well as these direct losses, they also cause indirect losses by decreasing the crop's resistance to pathogens as well as root damage caused by the presence of leafcutting ant nests, as observed in coffee shrub plantations in Costa Rica (Varón *et al.*, 2007). To make matters worse, several studies have documented that the population sizes of some leafcutting ant species benefit significantly from anthropogenic–driven habitat alterations such as increasing agricultural land use and deforestation (Jonkman, 1978; Blanton and Ewel, 1985; Fowler *et al.*, 1986a; Jaffe and Vilela, 1989).

1.2 The leafcutting ants

Leafcutting ants belong to tribe Attini. The attines are a unique group of ants, in that they cultivate fungus as their dominant food source (Weber, 1972). They are a monophyletic group, consisting of 16 genera and include 257 species including the recently described *Cyatta abscondita* (Schultz and Brady, 2008; Klingenberg and Brandao, 2009; Sosa-Calvo *et al.*, 2013). The history of agriculture in Attine ants most likely originates some 50 million years ago when South America separated from Africa (Schultz and Brady, 2008), but modern phylogenetic methods suggest that this symbiosis may have originated 67 million years ago (Ward *et al.*, 2015). Subdivided into two further groups, the 'lower' subgroup rely on detritus as the primary source of their fungal substrate, whilst the 'higher' subgroup (*Atta* and *Acromyrmex*) use freshly cut foliage, thus the common name of leafcutting ants (Belt, 1874; Weber, 1972). *Atta* and *Acromyrmex*, are almost exclusively limited to the Neotropics (Fowler, 1985), although some species occupy the most southern areas of the United States (Hölldobler and Wilson, 2010). Their use of agriculture is often described as being one of the most complex and efficient biological systems known (Schiøtt *et al.*, 2010).

1.2.1 Complex colonies

The population of leafcutting ant colonies can be enormous and differ considerably between species. *Atta colombica* is considered to be a species that contains a relatively modest number of workers yet whose numbers can vary between 1 to 2.5 million; however species such as *Atta sexdens* have been reported as having colonies consisting of in excess of 8 million individuals (Wilson, 1971; Hölldobler and Wilson, 2010). Leafcutting ants also display a social organisation which entomologists describe as 'eusocial' or 'truly social'. Eusociality is described as a group of individuals that are characterised by three distinct traits: (1) the presence of cooperative brood care of

4

immature adults; (2) an overlap of at least two generations that contribute to brood care and (3) a reproductive division of labour where sterile individuals assist with work on behalf of those individuals engaged in reproduction (Batra, 1966; Wilson and Hölldobler, 2005).

To house the large populations within a leafcutting ant colony, the ants construct large, deep and intricate nests, with numerous chambers varying in size, all interconnected by a complex network of underground tunnels and trails opening out on to the soil surface (Weber, 1972; Buhl *et al.*, 2006; da Silva *et al.*, 2012). To put the size and complexities of *Atta* nests into context, researchers in Brazil formed casts of mature nests using liquid cement poured directly into the nest openings. Once excavated, they found in excess of 7000 chambers as deep as 7 metres, all connected by tunnels extending up to 70 metres away from the soil openings (Moreira *et al.*, 2004a; Moreira *et al.*, 2004b). The high level of complexity of these nests is commensurate with their social organisation and their agricultural habit. The nest construction and management of leafcutting ants is dynamic and can be adapted by workers to overcome threats to the colony (Farji-Brener and Tadey, 2012). Natural examples of this include raising the underground chambers to minimise water infiltration should the risk of flooding occur during excessively wet weather; the opening of new ventilation holes to counterbalance any increase in CO_2 levels and the capacity to reconstruct damaged areas of the nest using recycled materials (Jiménez and Decaëns, 2006; Bollazzi *et al.*, 2008; Farji-Brener and Tadey, 2012).

1.2.2 The ancient symbiosis

The symbiotic relationship between the Attine ants and their cultivated fungus has been present since the separation of South America from Africa approximately 50 million years ago (Schultz and Brady, 2008). The ancient interaction between ants of the genera *Atta* and *Acromyrmex* and fungus dates back some 30 million years (Hölldobler and Wilson, 2010). This relationship is often described as a 'model system' of symbiosis (Herre *et al.*, 1999; Wirth *et al.*, 2003). Leafcutting ants are amongst the most polyphagous and voracious insects known, defoliating vast quantities of plants of varying species (Rockwood, 1976; Vasconcelos and Cherrett, 1997). Workers forage for fresh leaves, not for direct consumption, but to be macerated to a pulp to generate degraded material, which is then used as the substrate upon which a symbiotic fungus is cultivated (Schultz, 1999). It is this fungus, belonging to the two genera of basidiomycetes: *Leucoagaricus* and *Leucocoprinus*, that the colony primarily consumes for food and therefore has a binding dependency upon (Chapela *et al.*, 1994; Fisher *et al.*, 1994; Mueller *et al.*, 1998; Schultz and Brady, 2008).

1.2.3 The 'Superorganism'

Social organisation is a key contributor to the ecological success of not only leafcutting ants but social insects in general (Oster and Wilson, 1978). Often described as a 'superorganism' (Wheeler, 1911), the general structure and viability of a leafcutting ant colony is sustained by the reproductive output of a single, long-lived queen. The organisational efficiency is a consequence of the adoption of a complex division of labour to accomplish the numerous tasks associated with living within a eusocial group. Within the ant societies, leafcutting ants are considered to be the foremost example of how an organised division of labour improves the overall colony performance and efficiency (Hölldobler, 1990; Bonabeau *et al.*, 1998; Hart and Ratnieks, 2001; Hart and

Ratnieks, 2002). Within leafcutting ant colonies, workers engage in tasks that enable the colony to function such as feeding and grooming the queen of parasites and fungus (Wilson, 1983), the tasks associated with caring for the ever-increasing brood including distribution of eggs across the nest and the feeding and cleaning of larvae (Lopes *et al.*, 2005), the assembly line associated with food production including foraging and cultivation of the fungus (Hart *et al.*, 2002) colony defence against parasites (Feener Jr and Moss, 1990), and waste management process (Hart and Ratnieks, 2001). There is a flexibility regarding the recruitment of individuals for specific tasks within social insect societies, with the number of workers recruited for the various tasks being adjustable as conditions demand (Robinson, 1992; Gordon, 1996).

In *Atta* there is also caste polyethism, where tasks are divided into smaller subtasks and performed by different workers. This is evident in all forms of work organisation from foraging to waste management (Hart *et al.*, 2002). Some tasks require different individual characteristics and are therefore performed by particular subsets of workers (Wilson, 1971). Examples of factors that determine task allocation are age, physiology and morphology of each worker. In species among the lower Attines, worker size is relatively small with very little task-related variation. However the higher Attines, specifically those of the genera *Atta* and *Acromyrmex* are polymorphic, with workers displaying an extremely broad distribution for both size and morphological proportion, allowing for an efficient and effective division of labour. Workers also exhibit alloethism, where differences in size and morphology result in different behaviours allowing for further efficiency, in particular with tasks such as foraging; where the larger individuals complete the task of cutting the leaf for a smaller worker to transport it to the nest (Röschard and Roces, 2003). The size of a leafcutting ant colony understandably produces large amounts of organic waste consisting of dead workers,

decaying food remains, and other unwanted debris, that act as a reservoir for parasites and pathogens unless successfully managed (Bot *et al.*, 2001; Hart and Ratnieks, 2002). In species such as *At. colombica*, the workers concerned with waste management display quite an extreme level of polymorphism, where larger and heavier 'bulldozer' workers are employed to manipulate the waste in external waste heaps, whereas those that transport the waste to the heaps are smaller, lighter and faster-moving individuals (Hart and Ratnieks, 2002). An example of age-related task allocation is shown in the location of where workers perform their tasks. Younger workers have a tendency to be employed within the security of the nest, undertaking tasks such as attending the queen or brood or maintaining the fungus garden, whereas older workers are recruited for the more dangerous tasks undertaken outside of the nest, such as foraging and waste management (Wilson, 1971). This is possibly because the older workers have a shorter remaining lifespan and are therefore more dispensable to the colony. However, not all studies agree; there is evidence that colony size is a more important attribute in determining worker allocation than those characteristics listed above and that younger, and therefore smaller, colonies exhibit increased flexibility in relation to task allocation (Wilson, 1983). As a consequence of this social structure, leafcutting ants are considered to be a model system of work organisation (Wilson, 1983; Wetterer, 1999) with Wilson (1980) stating that *At. sexdens* are only operating within 10% of their theoretical maximum level of performance and efficiency with reference to their leafcutting.

1.2.4 Colony hygiene

The macerated organic material that forms the substrate for the fungus gardens, as well as other debris present in the nest, are known to harbour numerous microorganisms that offer a threat to both the ants directly and the symbiotic fungus upon which the colony

relies for survival (Bot *et al.*, 2001; Hart and Ratnieks, 2002). The process associated with fungus cultivation is contaminated with numerous parasites and fungal competitors (Haeder *et al.*, 2009; Farji-Brener and Tadey, 2012). One such parasite is a pathogenic fungus of the genus *Escovopsis* (Currie *et al.*, 1999b; Bot *et al.*, 2001). This specialised parasitic fungus does not cause any known physical harm to the ants but has adapted to attack the fungus garden itself, thus reducing the growth of the garden and occasionally causing the death of the colony (Currie *et al.*, 1999a). Whilst *Escovopsis* has so far only been found in association with fungus growing ants, it is not the only threat to the gardens, with other pathogens attacking the fungus to compete with space and resources and also the ants directly (Currie and Stuart, 2001). Leafcutting ant nests are subterranean and require stability in terms of temperature and humidity for the fungus to be successfully cultivated which provides the optimal conditions for parasites to thrive. It is therefore imperative that colonies are able to maintain extremely high levels of hygiene to minimise the risks associated with the cultivation of fungus.

As with many social animals, to overcome the pressures caused by pathogens and parasites requires social defence systems, both physiological and possibly more importantly, behavioural (Elliot and Hart, 2010). These behaviours are of a collective and altruistic nature benefitting the colony as a whole rather than just the individual (Wang and Moeller, 1970; Müller and Schmid-Hempel, 1993). Examples of anti-parasitic behaviour within social groups are found throughout the animal kingdom. These include allogrooming, where one individual grooms another individual, that has been studied in a variety of mammals including primates (Nunn and Altizer, 2006) and ungulates (Hart and Hart, 1992); and the avian equivalent involving the mutual preening of feathers or allopreening (Cullen, 1963; Radford and Du Plessis, 2006). In social insects, there are many more examples. Honeybees *Apis mellifera* are able to detect and

remove parasitised or diseased larvae before they reach an infectious stage preventing further spread (Rothenbuhler, 1964; Spivak and Gilliam, 1998); the immune system of bumblebees *Bombus terrestris* to fight off infections but at a cost to workers' survival (Moret and Schmid-Hempel, 2000); allogrooming in the dampwood termite *Zootermopsis angusticollis* to remove potentially infectious spores from another nestmate's cuticle (Rosengaus *et al.*, 1998) and wood ants *Formica paralugubris*, that collect and deposit solidified conifer resin in to their nests in an attempt to inhibit the growth of microorganisms (Christe *et al.*, 2003). The ability of leafcutting ants to maintain continuous monocultural fungus cultivation is almost certainly owing to the culmination of various hygienic related strategies. Such strategies include chemical production from the exocrine glands, the use of antimicrobials such as the mutualistic bacteria *Pseudonocardia,* a fungus that is active against *Escovopsis*, and possibly most importantly, maintaining a compartmentalised system of work in relation to waste disposal and management (Currie *et al.*, 1999a). The removal, disposal and isolation of waste to specified locations that reduce exposure to it share similarities with human communities and comprise an effective waste management solution.

1.2.5 Waste production

It is generally considered that living in a social group has its advantages over solitary living because of the support and assistance available from other members of the group. This is something that also applies to ants. The division of labour mentioned previously in leafcutting ants could quite easily have been describing that which exists within human society. There is no coincidence that many group-living insect taxa, in particular ants, termites, and some species of bees and wasps, go on to become the dominant species within their habitats (Wilson, 1971). However, living within social groups, notably those that maintain high levels of interactions between individuals such as

reptiles, primates and the social insects, increases the risk of disease transmission and the spread of infections (Hamilton, 1987; Shykoff and Schmid-Hempel, 1991; Schmid-Hempel, 1998; Godfrey *et al.*, 2006; Nunn and Altizer, 2006). To minimise the risk of disease transmission, adopting hygienic behaviours is an important aspect of social organisation. One such hygienic behaviour is the processing and management of waste material. Waste of any kind has the potential to be hazardous and accumulation problems are always exacerbated within large societies, this is true for humans as well as insects. The larger the society, the greater the volume of waste produced therefore increasing the challenges associated with waste disposal; this is particularly recognised in human societies where good sanitation and hygienic behaviours to minimise contact with human waste have proven to reduce the prevalence of disease (Esrey *et al.*, 1985; Esrey *et al.*, 1991; Curtis *et al.*, 2000; Hunter *et al.*, 2002).

In human societies, pathogens transmitted through poor waste management include *Salmonella* spp*, Escherichia coli* and *Vibrio cholerae* (Curtis and Biran, 2001). The Cholera pandemic of London in 1849 is one of particular importance as it was during this time that English physician John Snow connected the spread of disease to the consumption of mixed drinking water and raw sewage (Okun, 1996; Paneth *et al.*, 1998). From this discovery, the improvements in water supply and sanitation started the decline of previously high mortality rates from water-borne infectious diseases. The waste produced by leafcutting ants is also hazardous to the colony due to the pathogens contained within it and also the potential to act as a reservoir for disease (Weber, 1972; Bot *et al.*, 2001; Hart and Ratnieks, 2002). With large colonies and the cultivation of the symbiotic fungus, leafcutting ants can create large quantities of waste comprising of decaying food remains, old fungus garden and the corpses of dead workers. All these

contain abundant pathogenic microorganisms, including *Escovopsis*, with the potential to cause disastrous consequences to the colony (Weber, 1972; Bot *et al.*, 2001).

1.2.6 Waste management

The adoption of an effective waste management system has the potential to minimise the spread of disease and reduce the threat to the colony. The system employed by *Atta* species of leafcutting ants is the removal of harmful, microorganism-contaminated waste from the fungus gardens, relocating it in areas away from not only the fungus gardens but also other key areas of the nest, such as the reproductive areas (Farji-Brener and Medina, 2000). Most *Atta* species isolate this waste in middens or waste dumps that are situated internal to the nest structure, but at least two species, *At. colombica* and *At. mexicana* dispose of their waste in refuse dumps externally, above ground and away from the nest entirely (Weber, 1972; Hölldobler and Wilson, 2010). Studies have shown that these waste dumps, regardless of location, are contaminated with harmful pathogens, fungal competitors, specialised parasites and other microorganisms hazardous to the colony (Fisher *et al.*, 1996; Bot *et al.*, 2001).

A successful waste management system, such as that adopted by leafcutting ants is determined by two important features: (1) the waste is not scattered around the nest and left to degrade and cause harm, but is isolated from reproductive areas and the fungus garden in one or several locations and (2) the removal of waste material from the nest and subsequent manipulation, is subject to task partitioning. The recruitment of workers to perform separate roles within a task such as waste management, prevents contaminated workers coming into contact with more sterile areas of the nest and thus minimises the risk of potential spread of pathogens (Hart and Ratnieks, 2001). Bot *et al.* (2001) discovered that this task partitioning was managed according to the risk

associated with each task when studying the waste management of *Atta cephalotes*. The least hazardous part of the task, transporting the fresh waste to the dumpsite, employed more workers than the more precarious role of rearranging and manipulating the refuse on the waste heap. During this study, and also a similar study by Hart and Ratnieks (2001), it was discovered that the most hazardous roles, such as waste manipulation, were performed by ants approaching the latter stages of life, thus reducing the risk of contaminating the younger workers whose longer remaining life-span offers more value to the colony. Additionally, in a study by the same authors focussing on the waste management of *At. colombica*, of all workers recruited to external work, i.e. those tasks performed outside the nest itself, 11.2% were engaged in waste management, with the number of waste transporters outnumbering the waste heap workers by 55:1 (Hart and Ratnieks, 2002). This study also reported some interesting insights into the possibility of ants switching between tasks. They found that in *At. colombica*, there was only a negligible role change from forager to heap worker, with no evidence to suggest that foragers ever switched to that of waste transporter. There was also no evidence to show that those recruited to perform waste worker duties ever undertook foraging work indicating that once an individual was contaminated by waste, the risk it posed of coming in to contact with non-contaminated workers was high.

To further highlight the repellent properties of ant waste, Hart and Ratnieks (2001) demonstrated that non-contaminated nest mates displayed increased aggressive behaviour towards those that had been subjected to waste when studying *At. cephalotes*. It was also reported that the position of the waste heap has a profound effect on the location of foraging trails, creating an exclusion zone of at least 5 metres, through which no foraging trails were present. This positive avoidance of the waste dumps resulted in an additional 6% increase to the length of the foraging trails (Hart and

Ratnieks, 2002). This increase suggests that the harmful properties of waste are to be avoided even at the expense of increased energy usage. The harmful properties that waste contains and the importance of its removal from the nest is supported by Herz *et al.* (2007) when studying herbivory rates by *At. colombica*. They found that although foraging activity alters with weather conditions, the rates at which refuse is deposited on waste dumps vary little throughout 24 hours. With all this considered, leafcutting ants have clearly developed a variety of behavioural adaptations to avoid unnecessary contact with the waste produced within a colony.

1.2.7 Ecosystem engineers

Leafcutting ants play an important role in natural ecosystems (Haines, 1975). They are often regarded as 'ecosystem engineers' because of their nesting and foraging habits and the impact these have on the physical state of the neighbouring biotic and abiotic materials (Jones *et al.*, 1996; Rico-Gray and Oliveira, 2007; Farji-Brener *et al.*, 2010). The construction of subterranean nests changes the surrounding soil properties by improving aeration, drainage and root penetration. Their foraging behaviour and fungus cultivation, as well as waste disposal, increases organic matter and nutrient availability (Farji-Brener and Medina, 2000; Moutinho *et al.*, 2003; Sousa-Souto *et al.*, 2008). Their contribution towards productivity, nutrient cycling and their energy flow as well as environmental diversity, mean that leafcutting ants are considered to be a keystone species within any ecosystem in which they occupy (Fowler, 1985; Perfecto and Vandermeer, 1993). This creates a dilemma; while extermination of leafcutting ant nest may well increase productivity and revenue for the growers that are affected by their presence, their value to the ecosystem means that this could have detrimental effects to the surrounding habitat. This results in a trade-off between their value to the ecosystem,

in terms of their contribution to the future quality of the land being farmed, and the costs associated with their presence such as yield loss and pesticide purchase.

1.3 Popular control methods – past and present

The control methods employed to date to reduce defoliation by leafcutting ants vary considerably and involve a broad array of approaches including chemical control (pesticides), physical techniques, biological methods such as the introduction of predator species, or a combination of all three referred to as Integrated Pest Management. Regardless of the method adopted, they all achieve only minor success as a result of the biology and both behavioural and physical mechanisms that leafcutting ants possess.

1.3.1 Chemical control

In most agro-ecosystems where leafcutting ants are pests, the use of chemical pesticides as a means to reduce or eliminate the damage caused by their herbivory have been commonly employed (Cherrett and Russell, 1986). These are often presented in a variety of formulations such as powder, granular or liquid forms (Kermarrec *et al.*, 1986; Boaretto and Forti, 1997). Powder application involves the use of chemical compounds, and whilst early formulations such as dodecachlor were used for decades prior to being identified as a persistent organic pollutant and subsequently banned in many countries (RAPAL, 2004; USEPA, 2011), current compounds such as organophosphates, carbonates and pyrethroids have all been widely used (Boaretto and Forti, 1997). These pesticides are applied by pumping directly into the active entrances of the nest attempting to not only directly poison the workers, but to kill the queen and therefore bring reproduction to a standstill. This method has shown to offer reasonable success for those species that build small, shallow nests, but for those of the genera *Atta,*

whose nests are often deeper and more complex, the powder is unable to reach the lowest chambers, specifically those protected, reproductive areas, which house the queen, her brood and the minor workers that attend them. In an attempt to combat this, fumigants offer a more suitable option. Similar to powder applications in that they are applied directly to the nest via the active openings, these are presented in a gaseous state offering a greater spread throughout the chambers. Gases such as the colourless, odourless methyl bromide were used for this method for over 50 years with estimates of almost 70 tonnes being used annually worldwide (Miller, 1999). Stored as a liquid when under pressure, the harmful gas quickly spreads throughout the nest causing colony suppression. However, it has long been suspected that the residue left behind by methyl bromide causes harm to mammals (Danse *et al.*, 1984). In addition, the gases used in this application are now considered to be ozone depleting and were phased out in industrialised countries in 2005 and in 2015 by the less-developed countries as part of the Montreal Protocol (UNEP, 2014).

Thermal fogging employs the use of heated diesel or mineral oil such as kerosene to generate a 'fog' in which an insecticide is suspended and applied directly to the nest (Zanetti *et al.*, 2008). Granular toxic baits are used in this application because of their low cost and high efficacy and unlike fumigants, their comparatively low environmental impact. As with the use of fumigants, the limitations of this method are that it is relatively slow, giving the ants the opportunity to block off tunnels to prevent the insecticide from reaching key nest chambers and the cost of equipment for thermal fogging makes it more expensive that using traditional toxic baits (Santos *et al.*, 2007).

The direct use of toxic baits is extremely common for both their practical and economical purposes. Pellets containing active compounds such as sulfluramid and

fipronil, mixed with dehydrated citrus pulp, are positioned close to ant foraging trails with the quantity of baits dependant on the colony size. These baits are collected by the foragers and taken back to the nest (Lima *et al.*, 2003; Teixeira and Santos, 2008). Although these baits are not considered to be effective at killing the ants quickly, the leafcutting activity of the colony is dramatically reduced within only a few days (Zanetti *et al.*, 2014). It is also very rare for this method to achieve colony suppression, as in addition to the defence mechanisms of the ants themselves; the symbiotic fungus is able to detoxify toxic compounds and thereby maintain colony reproduction (Dowd, 1992; Montoya-Lerma *et al.*, 2012).

1.3.2 Physical methods

One method that has been used for some time in an attempt to control leafcutting ants is the utilisation of 'barriers', where plastic cylinders, coated in grease, are fastened around the base of the tree trunks. This method requires constant inspections and regular repairs. The removal of the queen is another effective way of controlling leafcutting ants in smaller areas. Soon after the queen's nuptial flight, the newly formed nests, or mounds, are dug up and the queen removed, resulting in suppression of the colony (Giraldo, 2007). However, this method is really only used on a small scale such as in small orchards rather than on a large agricultural scale as not only is it extremely labour intensive but access to the nests often proves difficult (Montoya-Lerma *et al.*, 2012).

1.3.3 Biological methods

While studies show that chemical pesticides such as sulfluramid are more efficient at controlling leafcutting ants than some suggested alternatives (Ferreira-Filho *et al.*, 2015), this comes at a cost. With chemical residue lasting in soil and water systems they

are considered highly toxic to non-target animals (Newsom, 1967; Gunasekara *et al.*, 2007). This has lead to the use of biological control such as the use of predators, pathogens and alternative land management finding favour because they have less broad environmental impacts (Della Lucia *et al.*, 2014). The use of Phoridae parasitic flies (Diptera) has been proposed as one such biological control because of their position as natural enemies of the ants (Elizalde and Folgarait, 2012). These flies lay eggs in the thorax of foraging workers along the trail resulting in the larvae consuming the ant worker's internal tissue (Orr, 1992; Porter, 1998). However, the success of this method is hampered by two issues: (1) infection of each ant is an extremely slow process and is highly unlikely to result in colony suppression, especially of a mature colony and (2) the employment of 'hitchhiking' ants, those of small size that are carried on leaf fragments are a natural defensive behaviour employed by leafcutting ants (Feener Jr and Moss, 1990). The use of pathogens is considered to be a biological control that has shown promise. Methods include the use of two different species of fungal pathogens, one that directly infects the ants, and a second to antagonise the symbiotic fungus within the nest and therefore cutting off food supply. Whilst successful laboratory results have been achieved, they have not been translated to the field with total success (Lopez and Orduz, 2003; de Souza Loureiro and Monteiro, 2005). Montoya-Lerma *et al.* (2012) suggests that this is in consequence of the "*remarkable ability of the leafcutting ants to detect, defend themselves against, and recover from pathogens under natural conditions*", possibly in relation to the morphological and behavioural characteristics that leafcutting ants possess in order to eliminate any pathogenic threat that has the potential to harm the colony (Schultz, 1999; Currie and Stuart, 2001; Mueller *et al.*, 2001; Poulsen *et al.*, 2002).

A modern approach to protecting plants from leafcutting ants is the employment of natural biological methods. As they eliminate the risks associated with chemicals and poisons, they are now generally favoured (Zeh *et al.*, 1999). The use of natural compounds found in the leaves of some *Eucalyptus* species have been found to increase aggression within colonies of *At. sexdens rubropilosa* resulting in mutilation and death of co-workers, by possibly interfering with colony nest mate recognition (Marinho *et al.*, 2005). Crop protection from leafcutting ants is also possible through the manipulation of agricultural environments. By combining plants such as sesame, sweet potato and mixed grass varieties, either alongside the crop or as maintenance strips surrounding the main plant species, it offers an alternative food source and has shown to reduce herbivory rates and ultimately the costs associated with their control (Zanetti *et al.*, 2000). This not only minimises the risk to the main plant species, but also improves the diversity of the habitat itself (Urbas *et al.*, 2007). Careful timing of agricultural practices can also play their part. Ploughing within four months of the queens' nuptial flights, whilst nests are still relatively small and shallow, has also been used effectively (LaPointe *et al.*, 1990).

Almost all of the methods employed in the control of leafcutting herbivory have something in common; when used on their own, the ants have the capability to by-pass them through adaptations, behavioural controls, social organisation and a complex nest structure that offers little success and renders their management extremely difficult. To improve the chances of successful control of leafcutting ants, rather than relying on a single method, a combination of methods could be employed. However, although considered the most efficient and practical method, chemical pesticides have come under criticism and increased regulation, resulting in more natural biological controls to be favoured (Della Lucia *et al.*, 2014). The use of alternatives to synthetic chemical

pesticides is the mainstay of Integrated Pest Management (IPM). By simultaneously using a range of sustainable biological-based tactics such as habitat design and the use of natural predators, the impact in pest management success as a result of the loss of chemical controls may not only be limited but perhaps become more successful. The implementation of IPM also reduces the cost of pest management with the removal of expensive chemical pesticides. Recent legislation in the use of sulfluriamid and fipronil as active ingredients of solid toxic baits has threatened the use of this almost exclusively used control method (Isenring and Neumeister, 2010). To find natural biological alternatives to chemical pesticides, several studies have attempted to investigate the possibility of harnessing the aversion that the ants have to the waste matter produced by the colony (Zeh *et al.*, 1999; Farji-Brener and Sasal, 2003; Ballari and Farji-Brener, 2006). Whilst these results were mixed, the exploitation of the hygienic behaviour associated with colony cleanliness is certainly an area to explore as a possible replacement to synthetic chemical pesticides.

1.4 The potential of colony waste as a natural repellent?

With evidence suggesting that leafcutting ants will avoid being in the presence of their own waste wherever possible (see Chapter 1, section 2.6), it is not surprising that its potential as a natural biological control method has been previously investigated. During time spent working in Panama, Zeh *et al.* (1999) claim they heard many stories from local people indicating that ant waste collected from *At. cephalotes* colony waste dumps is often used as a successful method of protecting their plants from attack. These anecdotal, but unsubstantiated, accounts led them to investigate the possibility of using ant waste as a small-scale deterrent to ant herbivory. They state that investigating ant refuse as a natural deterrent was the first of its kind and although their study presented quite remarkable results, there are only a few instances of further research in the

literature. Using plant seedlings surrounded by one of three treatments: (1) a mound of ant waste dug up from the internal midden of a nearby colony, (2) a mound of soil and (3) no mound at all, their results show that none of the plants surrounded by the ant waste were subject to defoliation within the study period of 5 days, compared to complete defoliation of all other plants subjected to the non-waste treatments. They therefore conclude that ant waste could potentially be used as a highly effective natural control method to prevent defoliation in *A. cephalotes.*

Another study, by Farji-Brener and Sasal (2003), attempted to replicate these results but with another species which deposits the colony waste in internal middens and has a wider latitudinal range than those used in the previous study. This study focused on *Acromyrmex lobicornis* in Argentina and employed very similar methods to Zeh *et al.* (1999) but with one additional treatment – they used ant waste from different colonies of the same species to investigate the possibility that the deterrent properties of waste were nest-specific rather than conspecific. This was considered important due to the practicality of using waste as a replacement not only to pesticides but also other management methods. They also increased the length of the study from 5 days to 28 days to determine the effectiveness over an increased period of time. The results from this study did not entirely support those from the previous study. They found that mounds of refuse material could not be considered an effective deterrent, as all plants, regardless of treatment, were eventually attacked. However, they did report that the presence of ant waste offered a short stay of execution for plants surrounded by both their own and foreign waste, which supports the study by Zeh *et al.* (1999). They also discovered that the deterrent effect was enhanced when using the colony's own waste rather than that collected from another colony. They concluded that ant waste could not be used as an effective natural deterrent to ant herbivory because of the laborious nature

of having to replenish the waste every few days to maintain its efficiency but also obtaining the quantities required for effective management would be almost impossible. However, they did recognise its potential for further investigation.

1.5 Chemical cues and olfactory sensitivity

Studies highlight the possibility of leafcutting ant herbivory being affected by the presence of waste, even if only for a short time. Rather than resulting from visual cues, the behavioural changes that bring about the avoidance of waste are possibly triggered by the chemical cues in the waste rather than physical contact.

Sensitivity to olfactory cues varies greatly amongst different taxonomic groups and is more highly developed in animals than it is in humans**.** For example, spotted hyenas *Crocuta crocuta*, found in sub-Saharan Africa use two forms of chemical signal to mark their territories. In addition to depositing faeces at latrines, they also smear the grass with paste produced in the sub-caudal glands (Mills and Gorman, 1987). The long-term effect of this ensures that even when individuals are not physically present, the scent indicates the territory is occupied and to be avoided. Invertebrates have a particularly high sensitivity to olfactory chemical signals such as volatiles from plants to assist with pollination, interspecific odours from parasites or predators, and pheromones - a single or blend of chemicals used to elicit a behavioural response in one or more conspecific individuals (Karlson, 1959), which they use for communication and aggregation; although olfaction is primarily used as a method to locate various resources within their environment (Whittaker and Feeny, 1971; Tumlinson *et al.*, 1993). This highly sensitive adaptation is regarded as being of pivotal importance to insect survival (Dethier, 1948). The ability of the insect olfactory system to evolve over relatively short time scales is

considered to be one of the reasons why they are such successful organisms and have occupied almost every ecological niche on the planet (Hansson and Stensmyr, 2011).

Leafcutting ants are also reliant on their olfactory senses. They depend heavily on their extended foraging trails to lead workers towards sources of harvest. These foraging trails are chemically marked with a trail pheromone produced from the ants' poison gland sacs (Moser and Blum, 1963). This pheromone compound includes an ephemeral, highly volatile recruitment component identified as either methyl 4-methylpyrrole-2-carboxylate (MMPC) in most *Atta* species or 3-ethyl-2,5-dimethylpyrazine (EDMP) in *At. sexdens* (Cross *et al.*, 1979). So potent is MMPC, that 1 milligram is theoretically sufficient to lay an ant-detectable trail several times around the world (Riley *et al.*, 1974; Kleineidam *et al.*, 2007). Another component of the trail pheromone is a less volatile substance used for long-lasting orientation purposes (Hölldobler and Wilson, 2010). As with many of the tasks within a leafcutting ant colony, polymorphism is also present in foraging, with the minims playing a key part in the creation and maintenance of the trails and the larger workers being responsible for the leaf carrying back to the nest (Evison *et al.*, 2008). With so much to understand regarding the importance and complexity of chemical signals and environmental cues within all taxonomic groups, it is easy to establish why these are a studied so frequently.

1.6 Olfaction and scientific studies

One particular taxonomic group in which olfaction experiments are commonplace are the arthropods, where individuals or groups of individuals are given the choice between two or more odour sources and the behavioural responses resulting from that choice are recorded. These experiments are commonly used in chemical ecology research to study host location (Heil, 2004; Bruce *et al.*, 2005). In the laboratory, insect responses to

chemical stimuli are usually determined using specialised choice chambers of various designs, known as olfactometers, that allow direct observation of the study subjects without them being in direct contact with the treatment itself (Hare, 1998). The use of this equipment has been used for almost a century with one of the earliest designs of olfactometers used by McIndoo (1926) when studying the attractiveness of host-plant odours to potato beetles. Using an olfactometer constructed of a Y-shaped tube, the beetle was introduced at the base of the 'Y' within a darkened section. The beetle progressed towards the fork of the apparatus (the point at which the arms meet), attracted towards it by a light source. Once at this fork, it was faced with a choice: one arm would have the stimuli odour emanating from it in the form of either an attractant or repellent, whilst the other would be used as the control treatment and would contain no chemical stimulus. The arm 'chosen' by the beetle was considered to be an olfactory-induced behavioural response to the chemical stimuli either emanating from said arm in the form of an attractant, or as a repellent from the odour emitting from the opposite arm.

Almost a century later, Y-shaped olfactometers or T-shaped linear tracked olfactometers (Fig. 1) are still commonly used to test the behavioural responses of various arthropods such as the cabbage sand weevil (Bartlet et al., 1997) and the bark beetle (Sullivan et al., 2000). Although simple by design, they do have their disadvantages, as described by Vet et al. (1983). When using two odour sources, the shape of Y and T olfactometers does not permit the treatments to be in close enough proximity for the walking insects to enter, sample the odour, leave and then re-enter without some degree of treatment mixture. They also suggest that turbulence is usually evident at the junction of the arms resulting in yet a further mix of odours, thus preventing the insects from making their choice based solely on the chemical signals being presented to them.
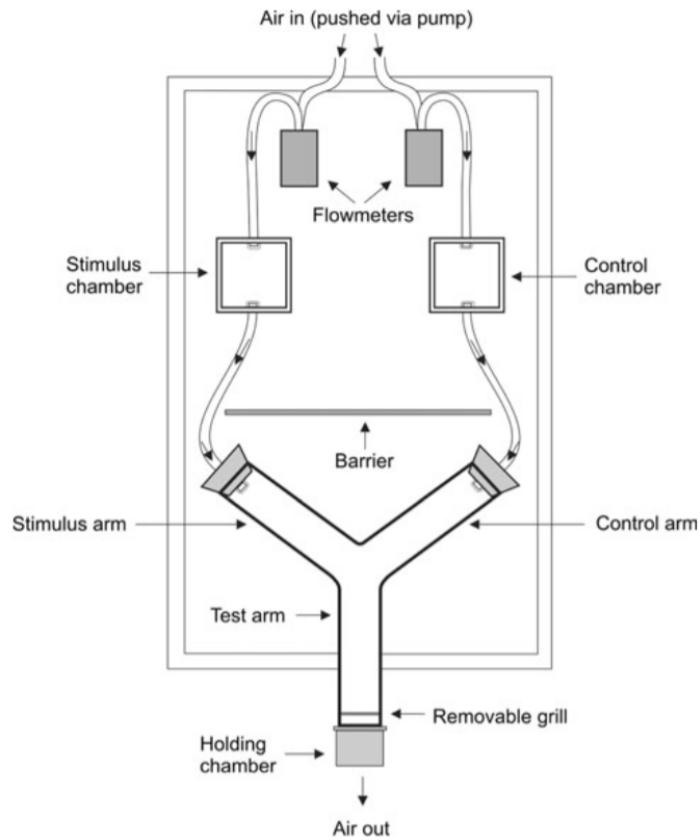
Figure 1. Example of a simple Y-shaped olfactometer used for investigating the ability of spiders to discriminate between mate and non-mate odour. Air was pushed via the pump independently through the stimulus and control chambers. An odour source was present in the stimulus chamber only (Cerveira and Jackson, 2013).

To test for a reaction to multiple odour sources, the employment of four and six-arm olfactometers is common. The four-armed olfactometer (Fig. 2) was originally developed for the study of sex pheromones by Pettersson (1970), but modified by Vet *et al.* (1983), possibly due to the aforementioned shortcomings of Y-shaped devices. These were designed to test simultaneous treatments by introducing the stimulus through each arm, with the insect moving freely in a central chamber. Vet *et al.* (1983) stated this was to offer an important advantage over the Y-shaped tube as it allowed the organisms to sample all of the 'attractants' before making a choice. A major disadvantage of this system, as stated by the author, was that it does not allow a steady stream of odour to the test subjects without the need for expensive flow meters and other volumetric equipment.
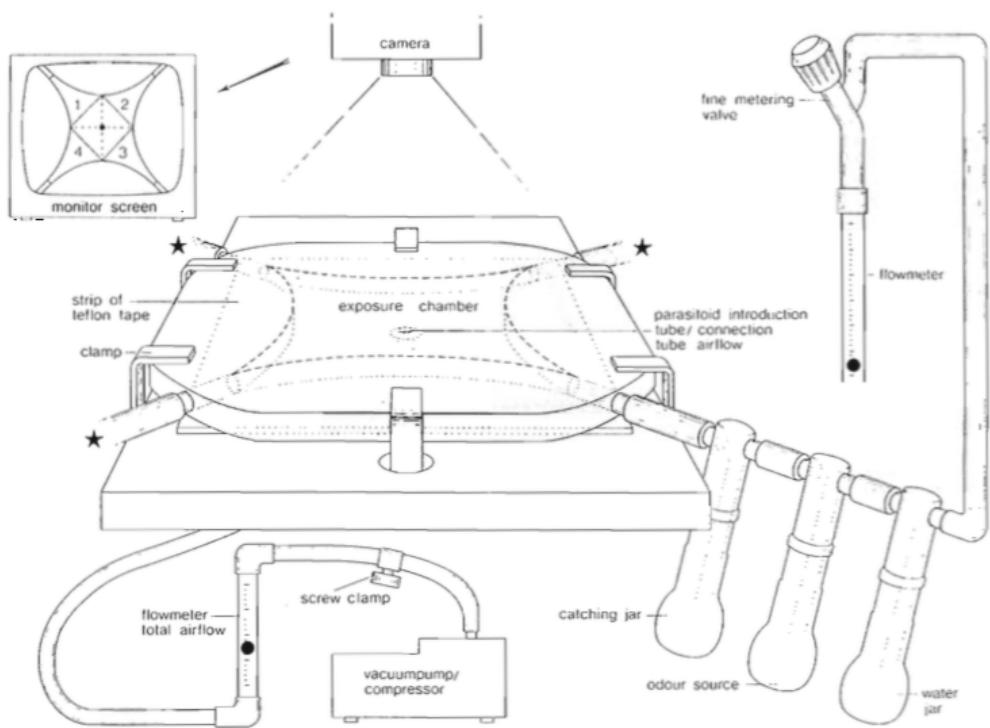
Figure 2. Diagram of a four-arm olfactometer used to test multiple odour sources simultaneously (Vet *et al.*, 1983). Whilst testing more than one odour offers some advantage, the construction of multi-treatment devices is considerably more expensive to construct than a simple two-way olfactometer.

The use of six-arm olfactometers also allows the testing of multiple odours with yet a further advantage. With an increase in the size of the apparatus, in particular the decision chamber, multiple insects can be tested thus dramatically saving experimental time (Fig 3). Turlings *et al.* (2004) reports, when studying the responses of solitary wasps to volatiles from maize plants, that the six-arm olfactometer is highly efficient and practical although, in support of comments by Vet *et al.* (1983), the manufacturing costs are relatively high as is the risk from using glass as a construction material. They also comment on the inconvenience of having to clean the device between experiments to avoid any traces of previous tests behind left behind, but that is a concern for all studies using olfactometers.
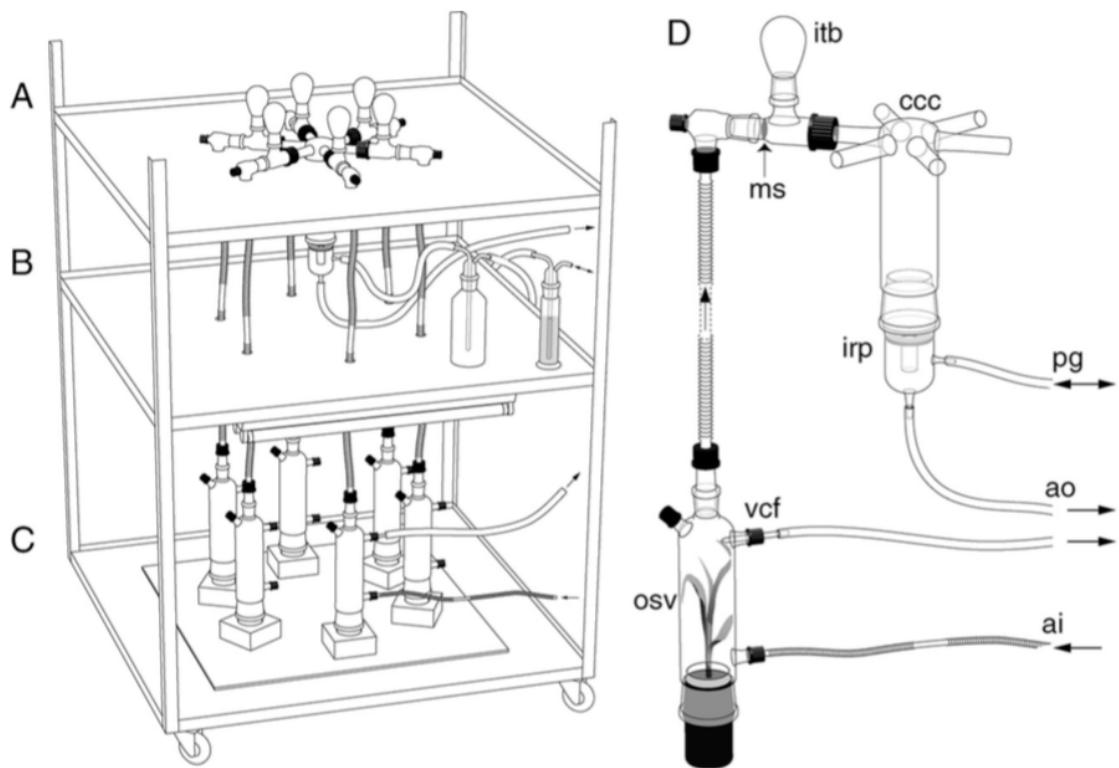
Figure 3. A more complex six-arm olfactometer used to offer a choice of six odours to flying insects simultaneously (Turlings *et al.*, 2004). The use of multiple insects and multiple odour sources allows for a faster collection of data although manufacturing costs are considerably more expensive.

Regardless of the design of olfactometer used, it is evident that there are common disadvantages with all olfaction studies. In addition to time involved, if each test-subject is introduced to the odour separately, there is also evidence that pseudoreplication is common in many studies. Pseudoreplication, as defined by Hurlbert (1984), occurs when multiple samples from one experimental unit are treated as multiple experimental units. Ramírez *et al.* (2000) investigated the methods used in 60 published studies using olfactometers. They concluded that over half of the studies show evidence that pseudoreplication had taken place and reported that fifteen per cent of these cases made no mention of changing the chemical source after a given number of tests or the changing or cleaning of the apparatus. To remove pseudoreplication, they strongly suggested that "devices should be thoroughly cleaned, and test insects and chemical

sources changed between observations". A further criticism of laboratory olfaction experiments is whether the behavioural responses shown by the test subjects correspond to their behaviour in nature.

1.7 Aims of the study

Previous studies suggesting the use of ant nest waste as a method of deterring ants from defoliation of crops and other plants have focused entirely on the waste being collected directly from the nest and presented, in solid form, around the base of each plant for the ants to come in to contact with. Farji-Brener and Sasal (2003), although reporting short-term effects from their study, make the point in their concluding remarks that "*repeating the dump treatment every two weeks….. might be too laborious*" for it to be considered a realistic method of control. Therefore, although evidence suggests that contact with ant waste, whether direct or not, does cause behavioural changes in leafcutting ants, it is the method of application that needs further investigation. A high number of pesticides used today are applied by spraying the crops with a solution containing an active ingredient. If the repellent properties of ant waste are also present in the chemical cues given off by the solid ant waste, there is the potential for spray application to be effective. The collection of the vast amounts of waste required to surround the crops would incur high labour costs which would affect its potential as a replacement for synthetic chemicals. However, in spray form, the application costs would be greatly reduced. The use of chemical cues in pest control is not a new concept. For example, sex pheromones have been widely used across the world to control pests such as the olive fruit fly *Bactrocera oleae* throughout Europe, the red palm weevil *Rynchophorus ferrugineus* in Asia and the gypsy moth *Lymantria dispar* in the United States (Witzgall *et al.*, 2010).

This study will use laboratory olfaction experiments to determine if the presence of the chemical signals contained within ant waste odour, whether colony-specific or conspecific, causes similar behavioural responses to that of the presence of solid waste and whether the use of odour alone could be used as a method of natural, biological control, therefore reducing the labour required for the collecting and presenting of solid waste. It will also determine if the repellency of ant waste odour reduces over time. The use of waste is definitely a potential solution for the reduction in ant herbivory and through further investigation, it may be possible to find not only a practical method of application to reduce the labour costs involved with its use, but also a solution into its, as yet, ephemeral effectiveness.

2 Materials and Methods

2.1 Study organism

Four colonies of the leafcutting ant species *Atta cephalotes* were used in this study and all were kept in the same conditions in the laboratory at the University of Gloucestershire, UK. Colonies 1 and 2 were larger, more established colonies with approximately 30,000 workers, whilst the workers of colonies 3 and 4 numbered approximately 15,000. All colonies were housed in climate-controlled tanks regulated to approximately 25° Celsius and 70% humidity. The ants were fed exclusively fresh privet leaves (genus: *Ligustrum*) daily throughout the duration of the experiments.

At. cephalotes is a widespread species of leafcutting ants within the Neotropics with a distribution including Mexico, Costa Rica, Ecuador and Brazil (Hölldobler, 1990; Perfecto and Vandermeer, 1993; Meyer *et al.*, 2011) and are considered agricultural pests owing to their varied appetite and resulting losses in crops such as coffee, cocoa, maize and damage caused to forest systems (Urbas *et al.*, 2007). They are considered a particular nuisance to agricultural stakeholders as they adapt their behaviour as the seasons change by foraging diurnally during the dry season and nocturnally during the wet (Lutz, 1929; Cherrett, 1968; Lewis *et al.*, 1974). They display a highly polymorphic caste system (Fig 4C) with variation in head widths ranging from 0.6mm to 4.5 mm amongst workers (Wilson, 1983). These morphological differences relate to the tasks performed by the workers. *At. cephalotes* colonies are relatively long-lived with a life expectancy of 8 years (Meyer *et al.*, 2009). The waste management behaviour of *At. cephalotes* is common amongst most of the genera in that they remove and isolate the organic waste material in waste dumps that are internal to the nest structure rather than external middens (Hölldobler and Wilson, 2010).
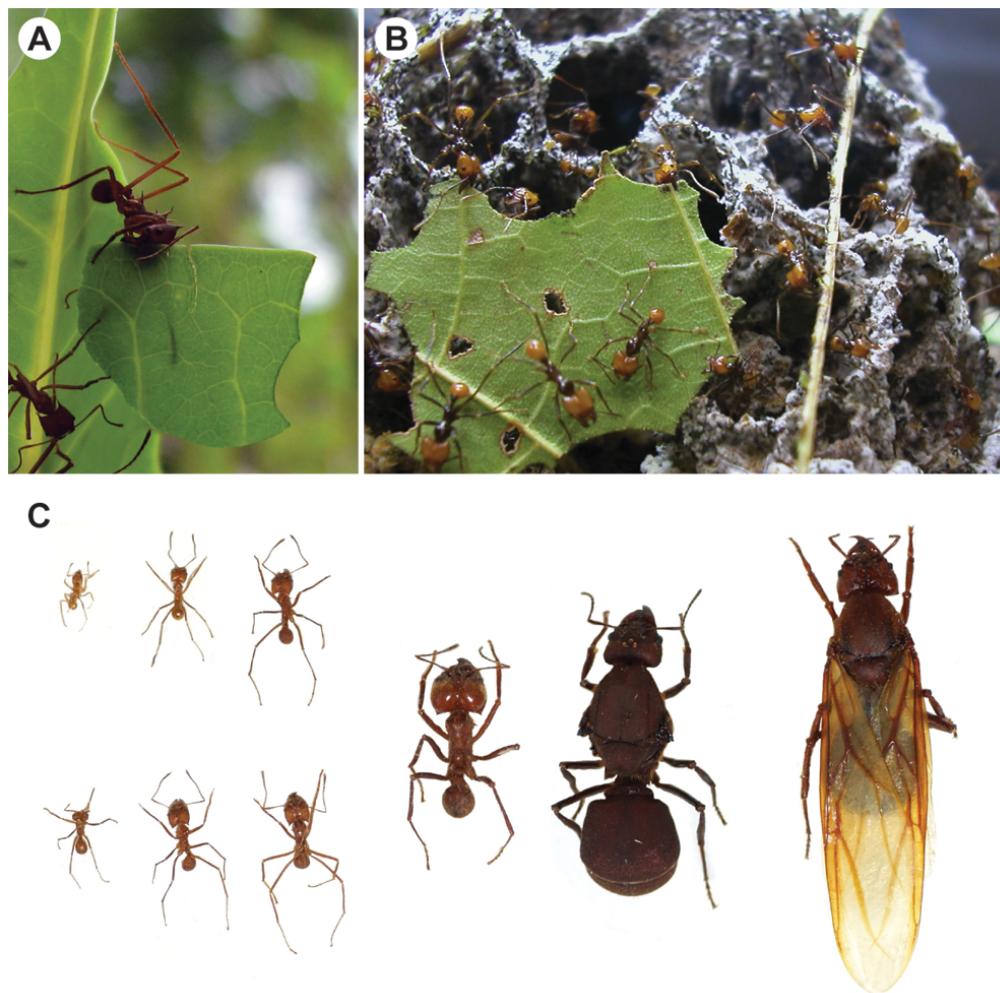
Figure 4. The social organisation and use of agriculture contribute to their ecological success. *Atta cephalotes* forage for fresh leaf material (A) to provide fresh material for their symbiotic fungus to grow within their subterranean nests (B). They display a morphologically diverse caste system that reflects a complex social organisation (C). (Photo credit: A, Jarrod J Scott; B, Austin D Lynch; C, Sara E Foster)

For standardisation, workers of the same caste, or at least those individuals employed for the same task, were used throughout the experiment. To ensure this, each colony was fed immediately prior to testing and only individuals carrying leaf segments back to the nest were selected. These individuals were collected in small numbers, between 5 and 10 at a time, and kept away from the host colony for a maximum of ten minutes, to reduce any potential stresses they may encounter prior to testing, and consequently any possible changes in behaviour, when removed from the conditions of the colony for an

extended period of time. This was decided upon during pilot trials when all ants due for testing were removed and kept separate from the colony until all testing had taken place. During this time, the behaviour of those ants yet to be tested visibly altered and they started to aggregate together within the holding container. This could possibly have been as a result of them being removed from the stable climatic conditions of the tanks in which they inhabit. Once an ant had been subject to testing, it was kept in a separate holding container until all testing had been carried out on individuals from that colony. Once testing was complete, all ants were returned to the host colony.

2.2 Olfactometer construction

The aim of this study was to observe the behaviour of freely moving ants when confronted with waste odour. Therefore, a two-arm olfactometer was chosen as this allowed the recording of the decision made by each individual ant when presented with a simple two-way choice (Fig. 5). A Y-shaped maze was constructed from 3mm acrylic sheet to the same dimensions of the olfactometers used in related studies by Dupuy *et al.* (2006) and Carcaud *et al.* (2009). The cross-section of the maze measured 1.9 cm wide by 1.9 cm high; the entrance channel to the decision area was 8 cm long with each choice arm measuring 6 cm in length. The arms were separated from each other by 90° and from the entrance channel by 135°. The decision area, the centre of the Y where all arms met, was reduced in size by acrylic inserts to allow for a more directed flow of odour and also to encourage the individual being tested to make a definite choice. In addition, by shaping the decision area this way, an attempt was made to reduce the amount of turbulence created at the fork section, a criticism of Y-shaped olfactometers as highlighted by Vet *et al.* (1983). The internal walls to the maze were covered with Fluon®, a flouropolymer resin (PTFE) that creates a slick barrier preventing the ants from gaining a foothold on the walls and therefore limiting the area in which they can

roam within the maze. The roof of the maze was sealed to prevent air leaks using standard odourless aquarium sealant (Gold Label One Shot Pond and Aquarium Sealer), while remaining open at the base to allow for the removal of the ants and also for the resetting and cleaning of the equipment after each test. The cleaning of the apparatus after each treatment was of particular importance as it was emphasised by both Vet *et al.* (1983) and Ramírez *et al.* (2000) when being critical of using olfactometers to prevent odours from previous treatments remaining within the apparatus. Holes were drilled at the end of each choice arm (diameter 9 mm) and at the base of the entrance channel (diameter 12 mm) with rubber sealing grommets fitted to eliminate leakage of odour from the maze. This allowed air to enter and leave the apparatus thereby creating a flow and minimising odour build up within the olfactometer.
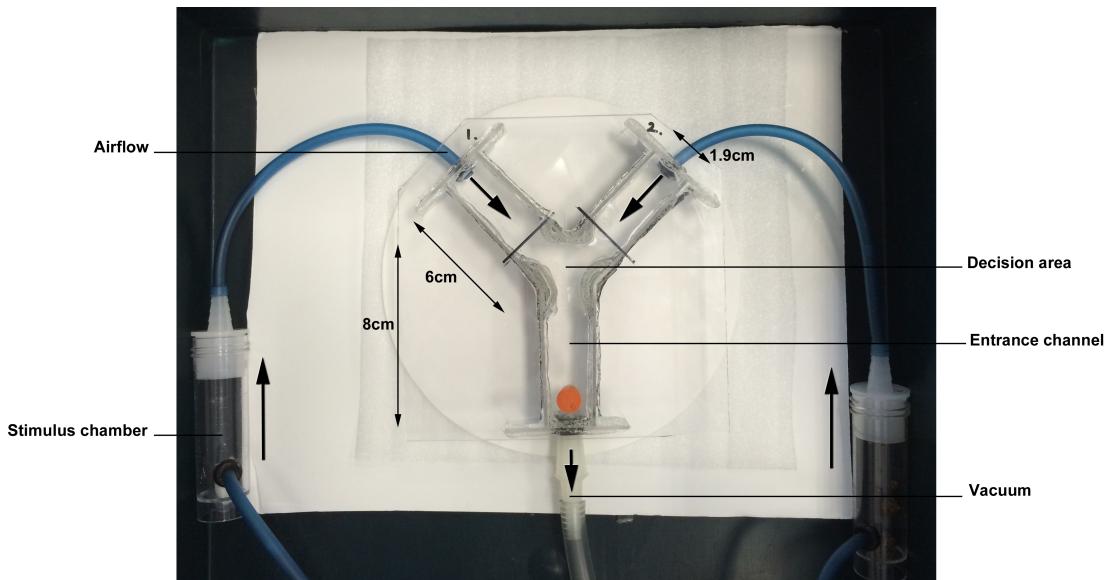
Figure 5. Top view of the acrylic Y-shaped maze used to determine the behavioural response of leafcutting ants in an olfactory experiment. Bold arrows indicate direction of airflow. Each ant was placed into the maze at the base of the entrance channel by removing a rubber bung (orange circle) to expose an entry hole, and moved towards the decision area where it had to choose between the two arms containing the odours created by the airflow through the stimuli chambers (delimited by the solid marked lines drawn on to the external face of the roof of the device). The airflow ensured odour diffusion. The initial choice of each individual ant was recorded.

To ensure the odour from the stimuli chambers was delivered to the decision area of the maze, airflow was created using an aquarium air pump manufactured by Aqua Air

(model AP3). This delivered charcoal-filtered air at a rate of 180 litres per hour, via silicone tubing, to each stimuli chamber and through to the maze. During pilot testing of the olfactometer, smoke was drawn through the device to make certain that the air was mixing and flowing through the decision area and entrance channel before leaving the olfactometer via the exit hole. There was no visual evidence to suggest that this airflow was so great that it impaired the locomotion of the ants or affected their behaviour or that the force of the air was too weak to allow for a controlled flow through the device. To maintain a continuous and unrestricted stream of air and to reduce pressure build-up within the olfactometer, air was drawn from the base of the entrance arm using a low-strength vacuum created by an aspirator pump fitted to a laboratory water tap. For confining the ant within the olfactometer, a fine mesh grill was placed over the exit hole.

To prevent the ants from using external cues that may have affected their choices, black electrical insulation tape was placed externally to all walls of the maze. To ensure light levels were symmetrical from all directions during the experiments, the entire apparatus was placed within a black PVC tray and positioned directly beneath the laboratory lights. Inside this tray, the maze was placed onto a sheet of filter paper, which was replaced after each ant visit to avoid the possibility of pheromone trails influencing the decision of the following ant.

2.3 Stimuli

With the laboratory colonies being of modest size in relation to those in the natural environment, the volume of waste created by the colonies was of small quantities, and was estimated at between 1 and 3 grams of fresh waste per colony per day. Workers deposited fresh waste from the nest onto the legs of the supports within the tanks housing them. Fresh waste is visually identifiable by its lighter colour and moisture

levels whereas waste that remains since the last time the colony was cleaned out is dry and much darker. To ensure there was an equal amount of fresh waste material for all experiments, a measured amount (1 gram) of ant waste was placed in the treatment stimulus chamber with cotton wool, dampened by distilled water, of the same weight and size in the control stimulus chamber. The presence of the dampened cotton wool was to prevent the volume of the waste in the stimulus chamber affecting the airflow compared to an empty 'control' chamber. It was found during trials that both the waste and cotton wool dried out gradually as both stimulus chambers were subject to constant airflow. To minimise the drying of the treatments during the experiment, both the stimulus and control chambers were weighed prior to testing and then again every ten minutes of olfactometer runtime. Any drop in weight during this period was replenished with distilled water to its original weight.

The position of the stimulus chamber, whether on the left or the right, was determined at random by using a simple random number generator set to generate 1:2 with 0.5 probability; this corresponded to the labelled choice arms on the maze (Fig. 5). Changing the treatment arm at irregular intervals reduced the possibility of the ants choosing either 'left' or 'right' by preference and focussed on them making decisions based on odour only – a point highlighted by Ramírez *et al.* (2000). To avoid the possibility of any remnants of odour material being left in the stimulus chambers, each chamber was wiped clean with 80% ethanol, followed by distilled water and then left to dry after each treatment.

2.4 Procedure

The test procedure remained the same for all experiments. The air pump was allowed to run for 30 seconds to allow the maze to fill with odour. An individual ant was then placed in the olfactometer through the entrance hole on the top of the entrance channel. The entrance hole was then sealed using a rubber stopper (Fig. 5 – orange circular object at base of entrance channel). As the walls of the maze were painted with Fluon®, the only direction for the ant to go was towards the decision area and the source of the odour or odours. A 'choice' was recorded when the entire body of the ant, including legs, crossed the black marked line. This signalled an end to the test and the ant was removed from the maze and returned to the colony as no individuals were subjected to the same treatment more than once. During pilot testing, no time limit was allotted for the ant to 'choose' which arm to travel, which resulted in some individuals remaining stationary within the entrance channel for extended periods of time, sometimes in excess of ten minutes, without making a decision at all. Possible explanations for this behaviour could have been high sensitivity to the harmful volatiles in the mixed air flowing through the device resulting in the ant choosing not to move towards the source, or, although not documented in ants, the possibility of sensory fatigue. This is where reduced antennae sensitivity through prolonged exposure has occurred as found in some moths (Judd *et al.*, 2005; Stelinski *et al.*, 2005). In order to avoid this, it was decided that the purpose of this study would be to measure the initial behavioural response to waste odour and if no 'choice' was made within 30 seconds, the ant was removed and exempt from testing.

3 Ant waste: is odour an effective repellent?

3.1 Introduction

The leafcutting ants of the genera *Atta* and *Acromyrmex*, are voracious herbivores in the Neotropics. They are polyphagous, capable of stripping plants of sizeable quantities of material, which they take back to their nest to form the substrate upon which their primary food source, a symbiotic fungus, grows (Rockwood, 1976; Vasconcelos and Cherrett, 1997; Schultz, 1999). Leafcutting ants will harvest material from a variety of plant species, both agricultural such as citrus, coffee and cocoa; and forest species like pine. They are often described as the most serious pest to any ecosystem in which they inhabit (Wirth *et al.*, 2003; Urbas *et al.*, 2007). The damage caused by defoliation alone is estimated to be billions of dollars (Hölldobler, 1990).

A range of control methods are currently used in an attempt to reduce the economic impact caused by leafcutting ants (see Chapter 1, section 1.3). Historically, chemical treatments have been used, although biological methods such as the use of pathogens and natural predators are also common (Cherrett, 1986a). However, despite varying degrees of success, they often pose a risk to human health as well as the environment (Cherrett, 1986a; Vilela, 1986). As the focus to minimise environmental damage is paramount, it is unsurprising that more eco-friendly, biological control methods are currently favoured and are also the subject of much scientific research (Della Lucia *et al.*, 2014). One such area of research involves harnessing the repellent properties of organic waste produced by leafcutting ant colonies to protect plants from herbivory. Ant waste has been found to contain harmful microorganisms, and due to the hygienic behaviour of the ants in order to protect their fungus gardens, is removed and transported to specific areas away from nest to avoid contamination (Bot *et al.*, 2001;

Hart and Ratnieks, 2001). A pioneering study by Zeh *et al.* (1999), after being inspired by local anecdotal accounts of waste being used to protect crops, investigated this further with *Atta cephalotes* in Panama. Using plant cuttings from a species frequently attacked by *At. cephalotes*, they tested several treatments and reported significant results. During their five-day study, they found that the plants surrounded by ant waste were unaffected despite the complete decimation of the seedlings surrounded by the control treatments. They concluded that the use of ant waste as a natural protection against defoliation by ants is something that should be seriously considered due to its effectiveness during their field experiments. Despite these results and recommendations, very little follow-up research was published until a study using *Acromyrmex lobicornis* in Argentina was undertaken by Farji-Brener and Sasal (2003). Encouraged by the results of Zeh *et al.*, they attempted to replicate them but with limited success. They increased the length of the study to 28 days rather than the previous five in order to assess the length of time that waste remains a deterrent and used conspecific ant waste from other colonies to test if the deterrent properties found by Zeh *et al.* were nest or species specific. Whilst they found that ant waste was effective for the first few days, the repellent aspects were only short-lived. As a result, they dismissed the idea of ant waste having potential as an effective natural deterrent because of the laborious nature of replenishing the waste every few days and to obtain the quantities required for effective management would be almost impossible. While these studies indicate that ants will avoid the presence of their own waste, they have only focused on using the waste in its solid form. As ants will reroute foraging trails in order to avoid waste heaps without coming into physical contact with them (Hart and Ratnieks, 2002), it is apparent that the ants detect the chemical cues emitting from the waste resulting in behavioural changes to avoid it. Therefore it is possible that the repellent characteristics emitting from ant waste are detected by the sensitive olfactory system that ants possess.

With the use of solid waste material being labour intensive and time-consuming because of the large amounts of material that would need to be collected and also constant replenishment of the treatment, its use as a control method to reduce the herbivory caused by leafcutting ants cannot be considered economic or practical. In an attempt to find an alternative to this laborious and long-drawn-out application process, this study attempts to determine if the repellency of ant waste is limited to ants coming in to direct contact with the material or whether the presence of waste odour will prompt the same behavioural responses. The effectiveness of odour as a repellent offers possibilities into its use as a natural deterrent that could be used as an active component in a pesticide spray.

3.2 Experimental treatment

3.2.1 Study insects

Atta cephalotes are one of the more common leafcutting ant species in the Neotropics (See Chapter 2, section 1 for more detailed description). They dispose of their colony-generated organic waste material in purposely-excavated underground chambers. This study used four colonies, all kept in the identical conditions at the University of Gloucestershire. Colonies 1 and 2 were larger and more established whilst 3 and 4 were younger, with fewer workers. Both were kept in climate-controlled tanks with a stable temperature of 25° Celsius and a relative humidity of 70%. All colonies were fed privet leaves daily and for standardisation, only workers that were actively transporting leaf segments from the feeding area to the nest were used for testing.

3.2.2 Experimental set up

An acrylic Y-shaped maze was constructed, allowing the observation and recording of ant behaviour when presented with a choice between two odours (see Chapter 2, section 2.3 for dimensions). The external walls of the device were darkened to avoid light spill affecting the behaviour, with the internal walls coated with Fluon® to reduce the surface area on which the ant has to roam, therefore encouraging them to make a decision. The roof of the maze was sealed to prevent odours from escaping, while the base was left open to allow for it to be positioned on easily replaceable filter paper. A solid black line on the roof of the maze was used as a reference point for when a 'choice' had been made. An aquarium air pump created airflow via the stimulus chambers to the olfactometer allowing for the treatment odour to enter the apparatus. A vacuum pipe formed an exit at the base of the Y allowing for a steady, continuous flow. The entire apparatus was housed within a dark PVC tray and placed directly beneath laboratory lights to ensure even light distribution. For all tests, the position of the odour source was changed between the left and right fork of the maze using a random number generator (set to generate 1:2 with 0.5 probability). This acted to counteract other influences that may determine a natural direction for the ants to walk. Ant waste was collected from the relevant colony being tested, whether from the same (own waste) or a different colony (donor waste), immediately prior to the start of each experiment, hereby referred to as 'fresh' waste. As the aim of this study was to determine the initial behavioural response of the ants when presented with ant waste odour, only those that made a choice within 30 seconds were recorded. Any ants that failed to 'choose' within 30 seconds were removed from the apparatus, kept in a holding tank and returned to the colony with the other test ants once testing had been completed. Following the completion of each experiment and change of treatment, both stimulus chambers were cleaned with an ethanol solution to remove any traces of previous treatments.

3.2.3 Behavioural response to own colony waste

To test the behavioural response of ants when given the option of choosing the odour of fresh waste collected from their own colony or no waste odour, 1 gram of fresh organic waste material was used as the olfactory stimulus. This was collected from the colony whose ants were subjected to testing, with the control treatment consisting of 1 gram of cotton wool, dampened by distilled water to avoid any potential chemical signals from tap water and was approximately the same dimensions as the waste to avoid any differences in airflow to that of the stimulus. With the olfactometer running for 30 seconds prior to the introduction of the ant to allow the olfactometer to fill with odour, an individual ant was placed within the base of the Y and its behaviour observed. A choice was recorded when the ant entered one of the decision arms and its entire body, including legs, passed the solid black line on the roof of the maze within a 30 second time period. Once a choice was made, or 30 seconds had passed without the ant making a choice, it was removed from the maze and returned directly to the colony. No ant was subjected to the same treatment more than once although as ants that had undergone testing were returned unharmed to the colony once all testing has been completed, there was a small chance that individual could be chosen for testing again in subsequent experiments. However, with the test colonies containing between 15,000 and 30,000 individuals, the odds of selecting the same ant was small and the risk of pseudoreplication was considered minimal. On the subject of avoiding pseudoreplication, Ramírez *et al.* (2000) suggested "a test insect may be reused if its biology is sufficiently well known to ensure that the insect will respond consistently at every observation it is used". Leafcutting ants are well studied and as it was determined that the testing of individuals would not have any long-lasting effects to their physiology, returning them to the colony after all testing had been completed would not affect future testing with different treatments. At the end of each test, the filter paper

acting as the base of the olfactometer was replaced in preparation for the next ant. This was to avoid trail pheromones that may have been left behind by previous ants having an influence on the following test. The position of the stimuli chamber was switched between the left and right choice arms in accordance with the pre-determined schedule and each test was then repeated with a different individual until 25 workers from each colony had been subjected to waste from their own colony (replication $n = 100$). During testing, both the waste within the stimuli chamber and the cotton wool in the control chamber were weighed and any loss was replenished with distilled water. This was to prevent the waste from drying out and possibly reducing the strength of its odour.

3.2.4 Behavioural response to other colony waste (donor waste)

To compare the behavioural responses when given the choice between the odour of fresh waste collected from the nest of a different colony and no waste odour, the protocol used in the previous experiment was repeated for all four colonies with the only changes to the protocol being the source of waste treatment and the number of tests in total. Here, rather than the waste of their own colony in the stimulus chamber, each colony was subjected to the waste collected from the other three donor colonies independently. Testing was complete once individuals from all four colonies had been subjected to the odour of each of the three other colonies (replication $n = 300$).

3.2.5. Behavioural response to own versus other colony waste

To determine the behavioural responses when given the choice between the odour of their own colony waste and that collected from a donor colony of the same species, the previous experiment was repeated for a third time. In this experiment, one stimulus chamber contained a 1 gram of fresh waste collected from their own colony and a second stimulus chamber containing the same amount of fresh waste from a donor

colony of the same species. The donor waste for all tests was collected from another *At. cephalotes* colony, whose ants had not previously been subjected to testing. All other experimental set up and protocols were repeated as per the previous tests (replication *n* = 100).

3.2.6 Statistical analysis

All data were analysed using chi-square tests for goodness of fit, with a hypothesised response proportion of 50:50, meaning that the probability of choosing one arm has the same probability as that of choosing the other. Using this model would establish whether the frequency deviations between the choices made by the ant (observed) were large enough to distinguish from those expected by chance alone (expected). Statistical analysis was carried out using the programming language R (R Core Team, 2015).

3.3 Results

3.3.1 Odour versus no odour

When tested independently, all ants chose the arm containing no odour source significantly more often than the arm containing waste odour. As there was no difference in the behaviour of ants when exposed to the odour of waste from either their own colony or that of a donor colony, the choices made by the ants were combined to determine the ants' behaviour to waste generally. Ants chose the arm with no odour source significantly more often than the arm containing the odour of any organic waste, irrespective of colony origin ($\chi^2 = 31.360$, *df* = 1, *P* <0.0001, *N* = 400; no odour = 256 waste = 144).

When broken down per colony, ants from all colonies chose the arm with no odour source significantly more often than the arm containing the waste odour (colony 1 ($\chi^2 =$

9.000, $df = 1$, $P = 0.0027$, $N = 100$, no odour = 65 waste = 35); colony 2 ($\chi^2 = 9.000$, $df = 1$, $P = 0.0027$, $N = 100$, no odour = 65 waste = 35); colony 3 ($\chi^2 = 5.760$, $df = 1$, $P = 0.0164$, $N = 100$, no odour = 62 waste = 38); colony 4 ($\chi^2 = 7.840$, $df = 1$, $P = 0.0051$, $N = 100$, no odour = 64 waste = 36)).

3.3.2 Own colony odour versus no odour

All ants, when given a choice between the arm containing the odour of their own colony waste and no waste, chose the arm with no waste significantly more often (Fig. 6A). As the behaviour towards their own waste displayed the same pattern, the data were pooled to determine the combined choices of all colonies. Ants chose the arm with no odour source significantly more often than the arm containing the odour from their own nest waste ($\chi^2 = 6.760$, $df = 1$, $P = 0.0093$, $N = 100$, no odour = 63 waste = 37).

3.3.3 Donor colony waste versus no odour

All ants, when given a choice between the arm containing the odour of waste from a donor colony and no waste, mostly chose the arm with no waste (Fig. 6B). As with the behaviour towards their own waste, the behaviour towards the donor waste also displayed the same pattern, so these data were pooled to determine the combined choices of all colonies. Ants chose the arm with no odour source significantly more often than the arm containing the odour from the donor colony waste ($\chi^2 = 24.653$, $df = 1$, $P < 0.0001$, $N = 300$, no odour = 193 waste = 107).

3.3.4 Own waste versus donor waste

Ants from all colonies chose the arm with the odour source collected from their own colony more often than the arm containing waste collected from a donor colony (Fig. 6C). However, only colony 4 chose it significantly more often (colony 1 ($\chi^2 = 0.360$, df

= 1, $P = 0.5485$, $N = 25$, own waste = 14 donor waste = 11); colony 2 ($\chi^2 = 0.360$, $df = 1$, $P = 0.5485$, $N = 25$, own waste = 14 donor waste = 11); colony 3 ($\chi^2 = 1.000$, $df = 1$, $P = 0.3173$, $N = 25$, own waste = 15 donor waste = 10); colony 4 ($\chi^2 = 6.760$, $df = 1$, $P = 0.0093$, $N = 25$, own waste = 19 donor waste = 6)).

When the choices of all ants were combined (as the behaviour followed the same pattern in all colonies) ants chose the arm with the odour source collected from their own colony significantly more often than the arm containing waste collected from a donor colony (all colonies ($\chi^2 = 5.760$, $df = 1$, $P = 0.0164$, $N = 100$) Fig. 6C).
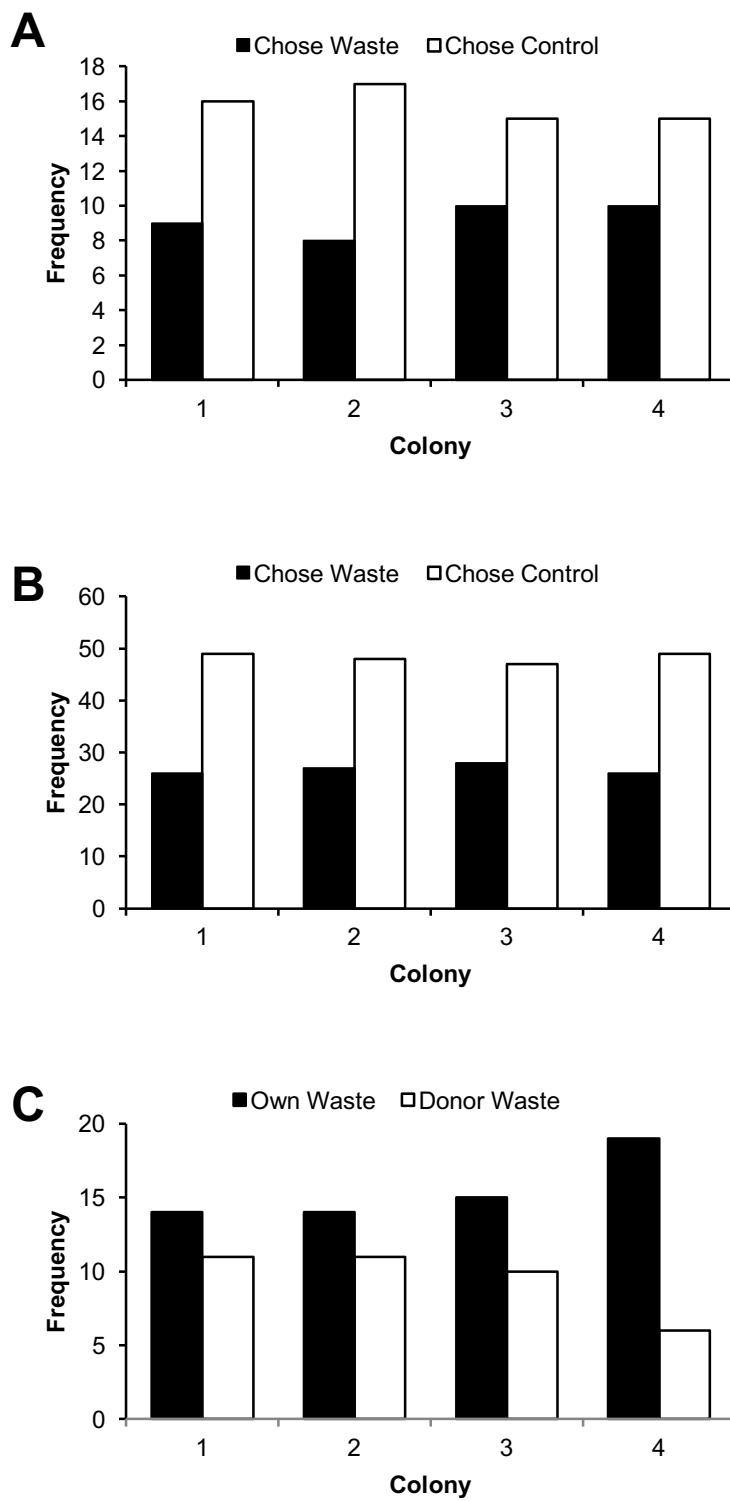
Figure 6. Results of initial behavioural responses of *Atta cephalotes* when presented with waste odour collected from (A) their own colony, (B) donor waste collected from other colonies (cumulative over all experiments) and (C) the choices made when presented with a choice between own and donor waste. In experiments (A) and (B), the control arm containing no odour source was chosen more often than one subject to the waste odour. When presented with two odours sources (C), a preference was made towards waste collected from their own colony than a donor colony.

46

3.4 Discussion

These results indicate that *At. cephalotes* avoid potentially encountering fresh organic nest waste material regardless of whether the waste originates from their own nest or that of a donor nest within the same species. They show that those behavioural avoidance responses that are initiated by physical contact with the waste material, as demonstrated in studies by both Zeh *et al.* (1999) and Farji-Brener and Sasal (2003), can also be seen when presented with only the odour of waste thus indicating that the repellent properties are chemoreceptive. This may explain why contact with ant waste is not required for foraging trails to be laid to avoid the sites of waste dumps as reported by Hart and Ratnieks (2002). In addition, these results identify that leafcutting ants are repelled by the presence of the waste of another colony of the same species significantly more than that of their own nest waste.

The initiation of a behavioural change in the presence of ant waste supports the findings of Hart and Ratnieks (2002) relating to nest-mate aggression when non-contaminated workers encounter those that have had contact with waste. This is not surprising considering the mortality rate of workers that are exposed to waste on a regular basis are higher than those that are not exposed (Brown *et al.*, 2006). When considered as a potential for pest control, these results also offer support to the study that initially trialled the use of waste as a repellent by Zeh *et al.* (1999) and also, but to a lesser degree, the follow up study by Farji-Brener and Sasal (2003), as it shows that fresh colony waste acts as a repellent to leafcutting ants. In contrast to the latter study, these results contradict their conclusion that the pathogens and competitors in waste heaps may be nest specific due to an enhanced deterrent effect towards waste collected from their own colony. However, the frequency in which ants in this study that chose the arm containing waste odour from their own nest over those from the donor colony nest was

negligible. The workers removed from colony 4 behaved substantially different to those from the other three, which may have affected the statistical results. This corresponds with what Farji-Brener and Sasal (2003) stated in their study that behavioural variation exists between nests, which could possibly have occurred in this study. Behavioural differences within species of leafcutting ant are not uncommon. An example of a marked difference between species behaviour is presented in the location of waste material dumps within leafcutting ants of the same genera: *At. cephalotes* have dump sites situated internally to the nest structure, whilst *At. colombica* remove the waste entirely from the nest and store it in external heaps (Weber, 1972).

Although little is known about whether there is a solitary component or a combination of components that make nest waste material something that ants prefer to avoid, there are examples where the presence of some individual components elicit a behavioural response.. Waste material, both in the field and found within the laboratory raised colonies used in this study, is comprised of old fungus garden, decaying leaf deposits and corpses of dead workers, all providing host to contaminants such as fungal competitors, parasites and other harmful microorganisms that are potential lethal not only to the fungus garden but the ants themselves (Fisher *et al.*, 1996; Currie *et al.*, 1999b; Poulsen *et al.*, 2002). The burial of dead colony members as a strategy to reduce the transmission of parasites is common amongst social insects with examples evident in termites (Crosland *et al.*, 1997) and some ant species (Renucci *et al.*, 2011); as is necrophoresis, where dead colony members are actively transported away from the nest because of the volatiles released by decaying corpses (Wilson *et al.*, 1958). In the desert leafcutting ant *Acromyrmex versicolor*, up to 30% of the entire colony perform the behaviour of corpse removal, indicating the importance of this strategy (Julian and Cahan, 1999).

Another component of ant waste which may be eliciting a repellent behavioural response is *Escovopsis,* a genus of parasitic micro-fungus exclusively found in the waste heaps of leafcutting ants, found to be an element of an ancient tripartite coevolution with leafcutting ants and their fungus gardens (Currie *et al.*, 2003). The presence of this parasitic fungus has the potential to cause colony death if overgrown (Currie *et al.*, 1999a; Bot *et al.*, 2001). As part of a defence mechanism against *Escovopsis*, leafcutting ants employ two behavioural responses, fungus grooming, the physical removal of alien spores from the cultivar; and weeding, the removal of the infected area of substrate hosting the spores (Currie and Stuart, 2001). When studying these defence mechanisms using behavioural observations with a stereomicroscope, Abramowski *et al.* (2011) found that the removal of *Escovopsis* from the fungus garden was primarily subject to caste specialisation, with minor workers responsible for the grooming of the fungus with the weeding performed by major workers. The important finding in relation to this study is that workers of both castes were able to sense *Escovopsis* spores, possibly due to the similarity in antenna structure of both castes. As invertebrates use antennae, along with the maxillary and labial palps, to detect odour (Schneider, 1964), it is quite possible that leafcutting ants, regardless of caste, are able to detect the presence of *Escovopsis* contained within the colony waste via their sensitive olfactory system resulting in behavioural responses to avoid contact, as demonstrasted in the results of this study. This assumption is based on *Escovopsis* being the primary component of waste that repels the ants. However, it is not the only potential repellent constituent present in the waste material of leafcutting ants. New species of *Escovopsis* are still being discovered (Meirelles *et al.*, 2015), and recently, even a new closely related genus *Escovopsiodes* was described during phylogenetic analyses although the parasitic nature of this fungus is yet to be determined (Augustin *et al.*, 2013). It is possible that the component or components that are responsible for the deterrent effect of waste have yet

to be discovered. Whilst many studies have demonstrated that fungus gardens are host to a wide variety of microorganisms such as yeast and bacterium (Craven *et al.*, 1970; Bacci *et al.*, 1995; Currie *et al.*, 1999a), further investigation of waste dumps is limited within the literature. In one such study, a comparative analysis of microbiota between fungus garden and waste heaps, Scott *et al.* (2010) found distinct differences between the two communities but as yet, no study into the repellent properties of this bacteria has been undertaken. This is one potential avenue for further investigation. If the repellent component is found to be bacteria or a combination of bacterium, it may be possible to isolate it from ant waste and incorporate it into a form that allows for its application on plants as a control method. This is especially pertinent with the application of solid waste matter being dismissed as a natural biological control due to the laborious task of regular collections and replenishment of treatment. An improved method of delivery, such as spraying the treatment directly to the plants, would certainly reduced the labour involved with spreading solid waste and with the results from this studying identifying that waste odour is as effective a repellent as solid material, it is certainly something for consideration for future studies. Another route worthy of further investigation is testing the effectiveness of ant waste odour over time. Whilst it is doubtful that the repellency of ant waste, certainly in the form used in this study, would be greater than that of solid waste, results showing a residual effectiveness equal to that found in previous studies would be considered successful and form the basis of its use as a natural control method.

4 Any waste odour repellency: ephemeral or long-lived?

4.1 Introduction

The use of chemicals in agriculture is integral to the success of producing high crop yields, and has therefore become an essential part of modern day farming. Alongside fertilisers (chemicals used to promote plant growth), chemical pesticides are commonly used. Pesticides, as defined by the EPA (United States Environmental Protection Agency), are substances or a mixture of substances that intend to prevent, destroy or repel the threat of an agricultural pest. They comprise of two types of ingredients; active compounds that act to control the threat of the pest directly and inert compounds, those added for purposes such as plant penetration, to improve application, protection from degradation and to extend the shelf life of the product. Pesticides are grouped together depending on the type of pest that they control; fungicides provide protection from fungus, herbicides kill weeds and other unwanted plants and insecticides are used to kill or manage insect pests.

Whilst the benefits of using pesticides are measured by crop return, the environmental costs associated with them are considerable. As pesticides are often applied directly to the crops being protected, their residue can easily find its way into food and drinking water. An example of the danger to human health can be found in insecticides such as organophosphates and carbamates, where direct contact can lead to penetration of the skin (Kangas *et al.*, 1993). Health effects resulting from long-term exposure also warrant concern. With examples including cancer (Bassil *et al.*, 2007), reproductive and development disorders (Hanke and Jurewicz, 2004) and respiratory effects such as asthma (Hernández *et al.*, 2011b) being linked with pesticide use, it can be argued that

the effect to human health is the ultimate price to pay although threats to the environment are also of paramount importance.

Most pesticides, with their limited species selectivity and intrinsic toxicity, exhibit harmful effects on surrounding wildlife populations (Hernández *et al.*, 2011a), in particular aquatic life found in rivers and streams (Laetz *et al.*, 2009). When testing streams for toxicity in the United States, Zeliger (2008) found that more than 50% contained residue from five or more pesticides. Their transmission to water courses is unsurprising as the fate of most pesticides are played out in the soil (Helling *et al.*, 1971), with an estimated 85% of the applied active ingredient finding its way there. In addition, Pimentel and Levitan (1986) found that up to 50% of applied pesticide quantities are carried from the field by wind.

There are alternative methods to control pests than using pesticides, in particular the adoption of Integrated Pest Management (IPM). Rather than limiting the options of pest control to just one method, IPM is described as "*an ecosystem approach to crop production and protection that combines different management strategies and practices to grow healthy crops and minimise the use of pesticides*" (FAO, 2015). The use of biological control is an important strategy adopted within IPM (Collier and Van Steenwyk, 2004). One such method is the push-pull strategy. This uses a combination of behaviour-altering stimuli to cause an effect on the distribution and abundance of pest species (Cook *et al.*, 2006). Whilst behaviour manipulation is not a new concept (Foster and and Harris, 1997), the push-pull strategy offers a two-prong approach. Firstly, insect pests are deterred away from the host plant (push) by using one or more repellent stimuli and secondly, they are simultaneously attracted (pull) to either traps or more desirable trap crops by attractive stimuli. By using non-toxic stimuli and habitat

diversification, this strategy is certainly something that could assist with the reduction in use of chemical pesticides.

It has already been mentioned (see Chapter 1, section 2) that leafcutting ants, despite being regarded as playing an important role within any ecosystem they inhabit (Haines, 1975), are a serious agricultural pest within the Neotropics and subjected to prolonged exposure to chemical insecticides in an attempt to control them (Cherrett and Russell, 1986). Studies have also shown that they have a natural aversion to the presence of organic waste produced by the colony (Zeh *et al.*, 1999), and exhibit behavioural changes in order to avoid contamination (Hart and Ratnieks, 2001). In tests during this study (see Chapter 3), it is apparent that even the odour of ant waste invokes a behavioural response. Therefore, it is possible that the use of ant waste could be utilised as a potential stimulus for the push-pull strategy of pest control due to the reaction of leafcutting ants when in close proximity to the chemical cues given off by the waste.

An important element of chemical pesticides is they tend to have long lasting residual effectiveness, resulting in fewer applications to the crops throughout the growing season (Schmutterer, 1988; Zacharda and Hluchý, 1991). If ant waste is to be seriously considered as a realistic replacement to chemical pesticides, it must also have a residual effectiveness that supports a reasonable application frequency. With ant waste in solid form offering only short-term advantages in respect to residual effectiveness (Zeh *et al.*, 1999; Farji-Brener and Sasal, 2003), this study attempts to establish if ant waste odour has a similar or improved residual effectiveness to that of solid waste.

4.2 Experimental treatment

4.2.1. Study insects

Atta cephalotes are one of the more common leafcutting ant species in the Neotropics (See Chapter 2, section 1 for more detailed description). They dispose of their colony-generated organic waste material in purposely-excavated underground chambers. This study used four colonies, all kept in the identical conditions at the University of Gloucestershire. Colonies 1 and 2 were larger and more established colonies whilst 3 and 4 were younger, with fewer workers. Both were kept in climate-controlled tanks with a stable temperature of 25° Celsius and a relative humidity of 70%. All colonies were fed privet leaves daily and for standardisation, only workers that were actively transporting leaf segments from the feeding area to the nest were used for testing.

4.2.2 Experimental set up

The acrylic Y-shaped maze used in Chapter 3 was used, allowing us to observe and record the behaviour of ants when presented with a choice between two treatments (see Chapter 2, section 2.2 for dimensions). As with the experimental set up used in Chapter 3, the position of the odour source was changed between the left and right fork of the maze using a random number generator (set to generate 1:2 with 0.5 probability). This acted to counteract other influences that may determine a natural direction for the ants to walk.

Fresh ant waste was collected from all four colonies immediately prior to the start of each experiment. As with Chapter 3, the aim was to determine the initial behavioural response of the ants when presented with nest waste odour. All apparatus was cleaned with an ethanol solution to remove any traces of previous treatments.

4.2.3 Does the residual effectiveness of ant waste odour decrease over time?

To test if waste odour retained its repellency over time once isolated from the colony to prevent manipulation, such as rearranging the material, as found in studies of *At. Cephalotes* workers (Bot *et al.*, 2001), the tests during this experiment were approached using the same protocol as that described in Chapter 3.2, with the exception of the origin of the waste material and the duration over which the study was undertaken. This experiment was conducted over a period of ten days with each colony subjected to testing each day throughout this period. The results of Chapter 3, section 3.3.1, indicate that fresh colony waste (that removed from the nest immediately prior to testing) has repellent properties to *At. cephalotes*, regardless of colony origin. Therefore, prior to testing, a total of 10g of fresh organic ant nest waste was collected from each of our four colonies and combined to form 40g of mixed colony material. For ant waste to be considered a serious alternative to chemical pesticides, its practical application would need to involve a combination of waste rather than colony specific material. This amalgam of waste was stored within the same climate-controlled tanks as the ant nests but was isolated to avoid any contact with waste workers and for this experiment, is referred to as 'old' waste.

For testing, 1 gram of old mixed waste was removed and used as the stimulus with 1 gram of cotton wool as the control. From each of the four colonies, 25 ants were individually subjected to the waste odour as described in Chapter 3, section 3.2.3. This was repeated for all four colonies, with the ant waste stimulus being replaced each time to avoid dehydration and potential loss of effectiveness. Once all ants from a colony had been tested, the old waste was discarded. This process was repeated for 10 subsequent days using the isolated, non-manipulated degraded mixed waste. As this study was to

determine the repellent properties of the waste rather the prolonged exposure of ants to waste, different ants were used for all experiments.

Only enough old waste material was removed from the tanks for the experiments undertaken on that particular day, leaving the remainder to degrade and used in tests throughout the experimental period.

4.2.4 Statistical analysis

As in Chapter 3, all data were analysed using chi-square tests for goodness of fit, with a hypothesised response proportion of 50:50, meaning that the probability of choosing one arm has the same probability as that of choosing the other. Using this model would establish whether the frequency deviations between the choices made by the ant (observed) were large enough to distinguish from those expected by chance alone (expected). Statistical analysis was carried out using the programming language R (R Core Team, 2015).

4.3 Results

The odour produced by the old ant waste provided a significant initial deterrent effect, but this decreased gradually over the ten-day period as the waste degraded (Fig 7). With the behaviour towards any waste following the same pattern (see Chapter 3), the choices from all four colonies were combined. The ants from all colonies chose the arm with no odour source significantly more often than the arm containing the old waste for the first three days only (Day 1 (χ^2 = 11.560, df = 1, P = 0.0007, N = 100; no odour = 67 waste = 33), Day 2 (χ^2 = 10.240, df = 1, P = 0.0014, N = 100; no odour = 66 waste = 34), Day 3 (χ^2 = 7.840, df = 1, P = 0.0051, N = 100; no odour = 64 waste = 36)). From Day 4 until the end of the experiment, the ants did not significantly chose either arm over the other.
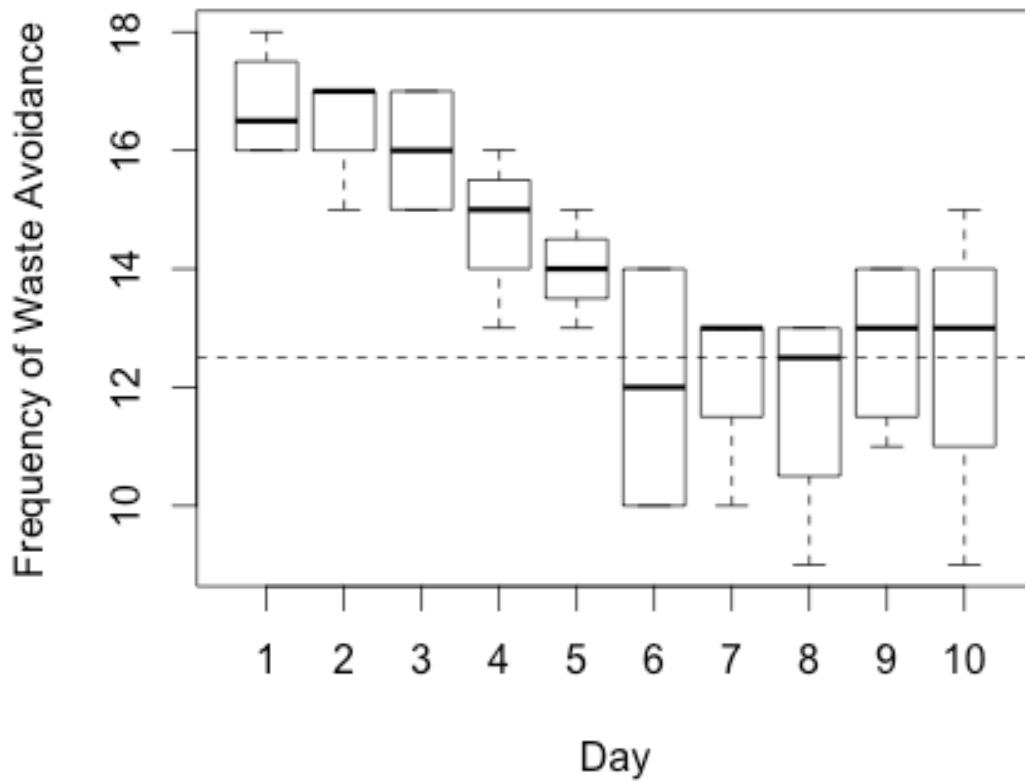
Figure 7. Effects of time and lack of waste worker manipulation on ant waste repellency for all colonies. Behavioural responses of *Atta cephalotes* when presented with mixed colony waste over 10 days. Ants that avoided the odour from old ant waste over ten days decreased steadily over the first 5 days before remaining ineffective for the remainder of the experiment. The horizontal dashed line indicates the 50% threshold where the choice of the ant is considered to be by chance, rather than the result of olfactory stimulation.

Overall, there was little variation between individual colonies (Fig 8). The ants of all colonies chose the non-odour arm less frequently over time. All colonies showed an initial aversion to the waste during the first few days but as the treatments were repeated, that reduced to an extent that was not significantly different from that of chance alone.
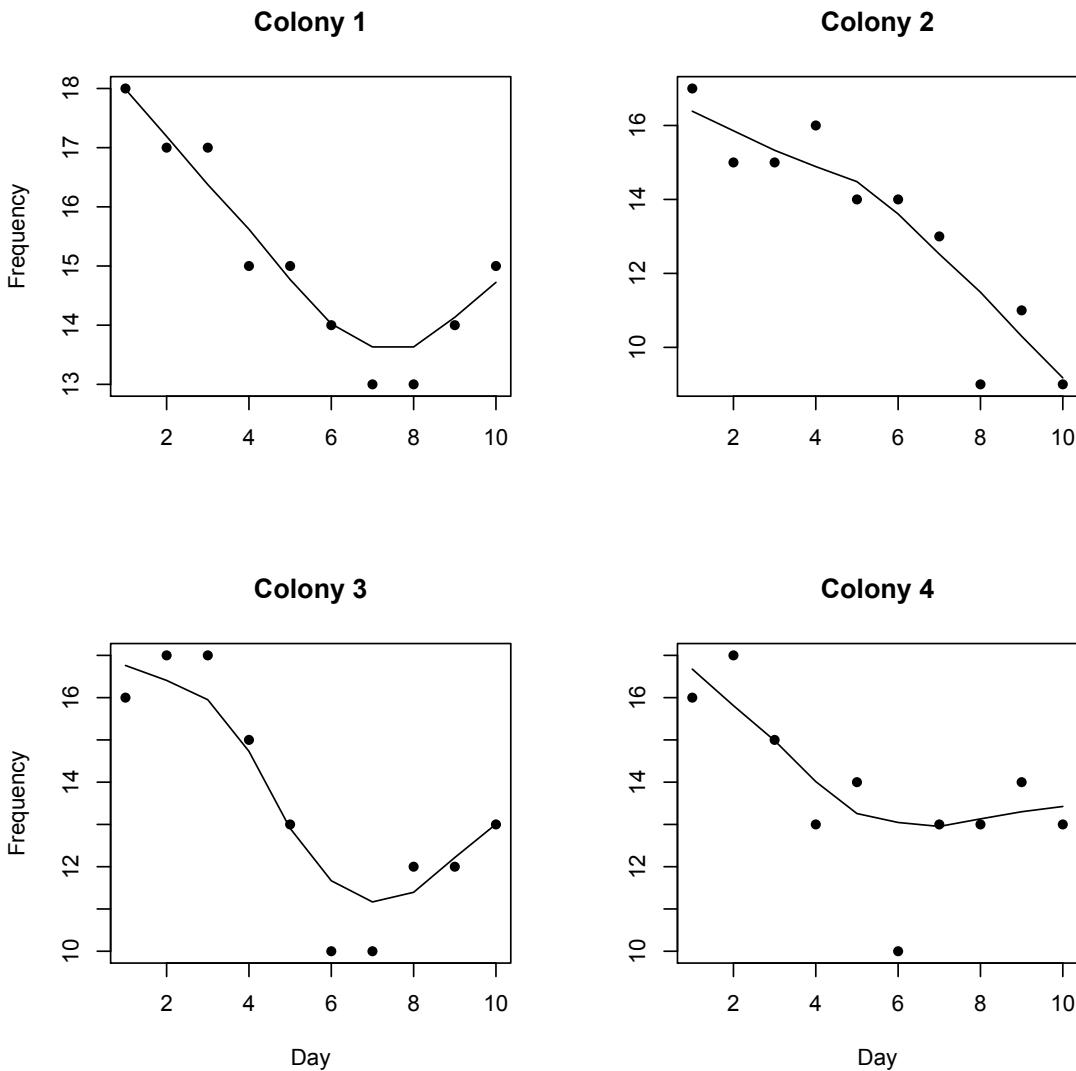
Figure 8. Effects of time and lack of waste worker manipulation on ant waste repellency for individual colonies. The number of ants avoiding the presence of old waste odour all decreased at a uniform rate over the first half of the experiment until the choices made by the ants were considered to be equal to that made at random.

4.4 Discussion

These results show that the odour from ant waste offered a good initial overall deterrent effect to leafcutting ants but this effect decreased rapidly over several days. The repellency of ant waste remained effective for the first three days but after five days, the recorded behaviour was unaffected by exposure, therefore offering no deterrent at all. After 10 days, all colonies were showing no preference between the arms containing the waste treatment and control treatment. During testing, there was some variation in the behaviour of individuals from different colonies but this was considered to be negligible.

All ants initially expressed an avoidance towards ant waste regardless of colony (further supporting the hypothesis that ant waste is something to be avoided. See Chapter 3), but were completely unaffected by its presence from day 6 until the end of the experiment. This decrease in effectiveness coincided with a clear degradation of the solid waste material, certainly in terms of physical appearance, when compared to the fresh waste used in Chapter 3 (personal observation).

The short lasting repellent characteristics of ant waste odour supports the findings of field studies involving the use of solid waste material. When testing the deterrent of waste material over time, Farji-Brener and Sasal (2003) reported that seedlings surrounded by a mound of ant waste material were attacked significantly less than seedlings not subject to treatment. After four days, the untreated plants were entirely defoliated whilst waste treated plants had as much as 68% of leaf material remaining. These treated plants were subject to continued attacks throughout the remaining sampling period as the deterrent effectiveness reduced. A later study by Ballari and Farji-Brener (2006), found that defoliation rates over time varied according to treatment. In addition to the initial treatments being either fresh or old waste, they used two further treatments, fresh and old waste but rather than left to degrade, both were replaced entirely every 3 days. Their results further supported the use of ant waste as a deterrent with untreated plants and those surrounded by soil being stripped of foliage after just one day. Whilst the presence of waste regardless of age also reduced attacks initially before losing most of their deterrent effect, the most successful treatments were those which were regularly replaced, supporting this study that ant waste is more effective within the first few days but decreases over time. This was something not found in the study by Zeh *et al.* (1999), who reported that no plants surrounded by ant waste were attacked during the 5 day sampling period. Farji-Brener and Sasal (2003), in their

attempt to justify the inconsistency between their results and that of Zeh *et al.* (1999) suggested that behavioural variation across species may be a factor. However, throughout their study, they state that Zeh *et al.* (1999) conducted experiments on *At. colombica*, a species known to build waste dumps external to the nest, however in the actual report by Zeh *et al.* (1999), they clearly state that the study organism was *At. cephalotes*, a species that houses their middens internally. If indeed, it was *At. colombica* that produced such conflicting results, it could be suggested that they may possess a lower tolerance to the presence of waste, resulting in the entire removal of waste material from the nest. As *At. cephalotes* are a species that store waste internally to the nest, it could also be suggested that they have a greater tolerance to the presence of ant waste, particularly the odour that emanates from it. This is a line of enquiry that deserves further study.

As for the short-lived effectiveness of waste odour, there are several theories that could be further explored. Firstly, there is a possibility that waste material isn't quite as harmful to the ants as studies have previously suggested. Whilst they do display defensive behaviours in order to avoid it, it could be argued that this is a natural initial behaviour of most social animals, including humans, when subjected to the presence of their own organic debris; but with greater exposure time, this avoidance behaviour is reduced. There is evidence of a higher mortality rate of workers involved with waste management (Brown *et al.*, 2006; Lacerda *et al.*, 2010), with the former study suggesting a combination of two factors that influence it: (1) the constant work involved in the waste environment results in an increase in metabolic activity and (2) these workers are subject to long-term contact with the pathogens commonly found in waste material. If, as suggested previously, that prolonged contact has little or no effect, then perhaps metabolic activity does have a more significant role to play. Adult leafcutting

ants rely on fungus cultivar as a source of food, but unlike the ant larvae, they are not entirely exclusive to it. They also feed on fruit and plant sap found during foraging (Bass and Cherrett, 1995). It can therefore be suggested that due to the rigid division of labour that not only prevents waste workers from entering the nest, the location of the fungal cultivar; but also removes them from foraging duties where fruit and plant sap are plentiful, an increase in metabolic activity as a result of the laborious task of waste management may result in poor nutritional intake rather than continued contact with supposedly harmful waste material. This is supported by Bot *et al.* (2001), whose study found that when nutritionally stressed, waste worker mortality significantly increased. If this were the case and increased mortality amongst waste workers was not related to prolonged exposure to waste but by nutrition deficiency, it could be argued that long-term contact is not as harmful as initially thought and that the short-lived deterrent effect found in this study may merely be an initial behavioural response to avoid contact with colony refuse as in many social animals.

Another explanation for the short-term repellent effect of waste may be the result of chemical defence systems that ants possess. Within the literature, *Escovopsis* is described as the primary pathogen that threatens the fungus gardens of leafcutting ants. With the co-evolution existing between leafcutting ants, fungus cultivar and *Escovopsis*, it is unsurprising that the ants have developed a method of defence from alien microbes. Ants produce antibiotics in secretory metapleural glands situated at the rear of mesosoma (Chapela *et al.*, 1994; North *et al.*, 1997). These antibiotics, of the genus *Streptomyces*, are produced to suppress the growth of *Escovopsis* and are present in all fungus-growing ants. It is proposed that physiological defences such as this, whilst costlier to produce; offer more towards immunity to disease than behavioural defences. While it may be behavioural defences such as avoiding contact that protects the ants

initially, the production of chemical defences may provide longer lasting protection when faced with a continued threat over time resulting in long-term exposure not being an issue.

5. Conclusion

A focus on the reduction of harmful chemical pesticide use is of economic and environmental importance on a global scale. Products and methods that were historically used are no longer appropriate with modern thinking. The use of alternative control methods to reduce threats to agricultural output whilst still remaining affordable is something that will continue as regulations only become ever more restrictive.

For control methods to remain within affordable limits, they must not only be relatively inexpensive to supply but also keep the labour time, and ultimately cost, to within reasonable limits. Any method that costs more to make and supply than will be saved in lost crop to pests is economically unfeasible.

Whist this study focused on the behaviour of leafcutting ants when presented with the odour of their own discarded, organic waste material, its overall aim was to explore its potential as an ecologically sound, natural deterrent to leafcutting ant herbivory. These experiments, alongside other studies, demonstrate that the presence of ant waste exhibits a behavioural response that leads to its avoidance. By doing so, in simple terms, it offers at least potential for it to be used in some capacity.

Unlike other studies into the repellency of ant waste, this study offered an alternative approach to the presentation of waste to ants. Where other studies have concentrated on small, localised field experiments using ant waste material in solid form, this study subjected the ants to the odour emitting from the waste within a laboratory environment to determine if those previous results could be replicated. The experiments and analysis undertaken have confirmed that odour is as effective as solid organic material in

deterring the ants and that the behavioural response to both material and odour are very similar.

This study also tested if the repellent properties of ant waste lasted long enough to be considered as a potential active ingredient for an alternative control method. As a naturally occurring by-product that is present in large amounts wherever the pest species exists, the supply costs associated with waste material are relatively minimal. This study found that the repellency effects of ant waste odour was possibly too short-lived to be considered as a long-term pesticide solution because of the need to apply it at least every five days, although there is certainly potential for it to be used in the short-term, perhaps as an emergency solution, given its short effective period. In studies into the defoliation caused by leafcutting ants, it was found that young plants were more susceptible to attack than more mature ones, so the use of ant waste during the early stages of growth is a possibility. The degradation of a pesticide before fulfilling its purpose is something that has been overcome by the use of 'carriers' or 'extenders'. These are chemicals that enhance the effective life of a pesticide in a variety of ways including screening out ultraviolet light or slowing down volatilisation (Marer, 2000). With ant waste providing the active component of a pesticide spray, coupled with an extender, the repellent effect could be extended and make it more viable as a longer-term solution.

A particular criticism of short-term chemical pesticides is that repeated application, as would need to be the case with ant waste, can cause pesticide resistance. This would not necessarily be the case with the use of ant waste as a short-term solution as the relationship between the ants, their symbiotic fungus and the pathogens that inhabit it, is

one with an evolutionary history of millions of years. To develop an habituation to the presence of their waste would seemingly take a considerably long time.

For longer-term use, or that on a larger scale, it does not offer much of a solution. However, as the deterrent effects of ant waste are apparent in both this and other studies, it may just require an improved method of delivery. This is certainly an idea that requires further investigation. The experiments in this study were of an olfactory approach, delivering odour via an air supply. The continuous airflow over the waste resulted in loss of moisture over time and needed replenishing throughout testing. The addition of water did not appear to affect the deterrent properties of the waste in any of these experiments and therefore suggests that there is a possibility, with further experimentation, that the repellent elements of the waste could be suspended in water. If found to be the case, it has potential to be made into a solution capable of spray application. Even with the short-term effect of waste, the ability to spray the repellent directly on to or around the plants dramatically reduces application time compared with digging up and forming piles of solid waste as is currently used. This is certainly an idea that deserves further study.

Although assessing the behavioural responses of leafcutting ants when presented with the odour of nest waste is something that is perfectly acceptable within laboratory conditions, the wider implications in relation to real-world environments is somewhat limited. Previous studies were carried out on site, in locations that were inhabited by numerous colonies of leafcutting ants where ant waste was plentiful. When performing similar experiments in the lab, there are obvious limitations. It is known that ant workers within typical, mature, wild colonies can number in excess of several million individuals, whereas lab reared colonies offer only a minute fraction of that number.

This doesn't restrict the number of ants available for experiments, but does prove difficult to collect enough waste material to replicate other studies undertaken in the field. This is was highlighted by Bot *et al.* (2001) as a particular problem when studying waste management of leafcutting ants in the lab. For example, the study undertaken by Ballari and Farji-Brener (2006) when looking at deterrent effects in relation to the age of waste, where mounds of material were replaced on a regular basis, is impossible to replicate under lab conditions.

Another criticism of laboratory experiments such as those included within this study is whether the behavioural responses shown by the test subjects correspond to their behaviour in nature. Research by Ballhorn and Kautz (2013) suggest that the use of olfactometer trials alone are not sufficient to be considered conclusive and that olfactory decisions may only provide a simplified picture of decision making in insects. As a result, it is suggested that for further studies involving an olfactory approach to the deterrent properties of ant waste, experiments are conducted within the field to determine if laboratory results are transferred to nature.

Concluding remarks

This study intended to address two questions: (1) does the odour of ant waste possess the same deterrent characteristics as solid waste heap material that leafcutting ants exhibit behavioural responses to avoid, and (2) does the odour of old waste replicate the repellency effects displayed by fresh waste and therefore offer a potential solution to be utilised within the control of leafcutting ants as agricultural pests? With the use of laboratory olfactory tests, the results offer answers to both questions. It is therefore concluded that ant waste odour does contain those chemical cues that deter ants from contact with waste and that due to the ephemeral repellency effect of old waste, it does

66

offer some potential as a short-term alternative to chemical pesticides. This study opens the area for further investigation into the use of ant waste material by suggesting that, with other methods of application, its use as a long-term control method offers possibilities and that more detailed analysis into the chemical structure of waste be conducted with a possibility of isolating the element or elements that ants choose to avoid.

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