Defining Quality in a Wood Ant Aphid Tree Foraging Network

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Abstract

Quality distance trade-offs shape foraging dynamics, but defining quality can be complex. Ants ‘farm’ aphids to collect honeydew, a significant food source for *Formica lugubris* wood ant colonies. Previous modelling and empirical data indicate that ant colonies do not invest equally in all trees where honeydew is foraged: they treat different trees and their associated aphid populations as being higher or lower value food sources. Honeydew composition varies based on tree and aphid species. Ants could be looking for specific sugars, amino acids, plant defence compounds, or other components of aphid honeydew. Using worker investment as an indicator of a site’s value to the colony, we aim to define “quality” based on the chemical composition of the food retrieved. We collected samples from trees (phloem), aphids (material), and ants (crop contents) on low and high investment sites. Through metabolomic analysis, we investigated the metabolites flowing over the tree-aphid-ant processing pipeline and the final composition of crop contents. We found distinct metabolomic profiles of low and high investment sites in all three sample types (Phloem Sap, Aphid Material, and Crop Contents). Our results provide the first data about what components of the resources provided by trees are treated by ants as high quality, and suggest future work to further elucidate this line of inquiry into resource use by Red Wood Ants.

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Author's declaration

I declare that this thesis is a presentation of original work, and I am the sole author (excluding sections disclosed in the Contributions Statement). This work has not previously been presented for a degree or other qualification at this University or elsewhere. All sources are acknowledged as references.

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1. Introduction

1.1 Foraging Trade-offs

1.1.1 Foraging and trade-offs

Securing adequate food is fundamental to survival and reproduction, and so foraging behaviour is under strong selection to ensure an organism acquires both the volume and type of nutrients it requires for success. Animals must draw from a limited time and energy budget for all their activities, and so constantly face trade-offs in how they invest their resources.

Optimal Foraging Theory (Charnov, 1976; MacArthur & Pianka, 1966) states that animals should maximise the energy input (the food they collect) per unit of energy used (for travelling, sampling, handling, etc). Studies into foraging behaviour using the Optimal Foraging Theory framework revolve around trade-offs between different currencies, the essential being what to forage on, and for how long (Pyke et al., 1977). Foraging incurs different costs depending on the ecology of the study species, but ultimately for all species foraging has an energetic and time cost.

1.1.2 Time costs

Foraging takes time, and time is often used as a foraging currency in model systems, the assumption being that time-constrained animals will optimise the rate of energy gain, i.e. energy input per unit time, rather than energy per unit energy expended (Schoener, 1971). Time costs can be split into three categories. Firstly, time can be a proxy for energy, as the more time spent foraging, both in searching and handling, the more energy used (Mead, 2004). Secondly, time can be valuable if other activities could increase an animal's fitness, like reproduction, territory defence, social behaviour, etc.. This is sometimes referred to as the "missed opportunity" cost (Eccard & Liesenjohann, 2014). Finally, foraging can lead to increased exposure risks (predation, parasites, or environmental risks), either due to spending time in more dangerous locations, or simply spending more time exposed whilst engaged in foraging and thus less vigilant (Brown & Kotler, 2004). It can be challenging to empirically demonstrate which time cost an animal is incurring, but in some study systems some of these costs can be absent, for example if foraging does not increase the risk of predation of an animal, as seen in bank voles (Ferreira et al., 2023).

1.1.3 Energy costs

Travelling has an energetic cost, and that cost should increase with distance. The cost differs considerably by species, and individual, as well as the mode of transport; walking, flying, and swimming will have relatively higher or lower costs, and again different species will experience different relative costs (Bautista et al., 2001). The choice of route will also influence the costs. For walking species, climbing upwards or moving through loose substrate may be more energetically costly than travelling on flat ground (Holt & Askew, 2012). For flying and swimming species, moving against air or water currents, or through turbulent areas, will likewise increase energetic costs (Godin & Rangeley, 1989). Under Optimal Foraging Theory, animals would be predicted to choose the least energetically costly route between two known locations, unless there are other risks to take into account.

For many animals, another energetic cost to travelling when foraging is the cost of carrying food back to a place of residence, to eat it in safety, feed young or kin, or store it; this is referred to as Central Place Foraging. Central place theory (Orians & Pearson, 1979; Wetterer, 1989) states that if load mass does not affect travel time, the load carried should increase with distance from the nest. Metabolic costs do not increase much with loads in ants (Nielsen et al., 1982), unlike in flying social insects like bees (Schmid-Hempel et al., 1985). Animals can make minute adjustments to foraging strategies to arrive at the most efficient process.

1.1.4 The quality problem

Energy inputs and outputs may allow for an easy model, but do not reflect the complexities of most foraging systems where multiple types of resources may be collected and their values may differ between individuals and vary over time. Foraging models often assume that quantity of energy available is equivalent to patch quality (Charnov, 1976; Davidson & El Hady, 2019; McNamara & Houston, 1985). For animals foraging in a complex world, and with complex and varying nutritional needs, the nature of ‘quality’ may be much less straightforward to define. Animals may prioritise specific nutrients, proteins, fats, or minerals, over the overall energy provided by a food source, for example parrots in sodium-poor habitats forage for this mineral in clay licks and palm trees (Brightsmith & Cáceres, 2017). As it is unrealistic to know the exact nutritional needs of every individual at all times, much less a colony, the perceived quality could be characterised by the amount of effort individuals invest in extracting a food source.

1.1.5 Social foraging

When animals forage in groups, theories of social foraging come into play. In an idealised group, an individual will find a food source (a producer) and can then choose to alert others or keep it to themselves. If group foraging increases the fitness of group members, then it should be adopted by a majority of the members of a population, as an evolutionarily stable strategy (Maynard Smith, 1972). Nevertheless, group foraging creates an opportunity for certain individuals (scroungers) to put in less effort and rely on producers to find all the food.

The theories around these behaviours are often based on the choice to join, stay in, or leave a group, which depends on fitness trade-offs and competition between members of the group. Groups with stable membership are more likely to make foraging decisions as a group than as individuals, therefore the framework of the Optimal Foraging Theory is more applicable (Davis et al., 2022).

In contrast, in the eusocial insects, the interests of the group members (members of the same colony) are much more strongly aligned, as this life history strategy involves individuals supporting the production and care of relatives, driven by the inclusive fitness benefits of supporting the indirect transmission of their genes (Hamilton 1964). Leaving the group is usually not an option for social insects such as ants, and the producers-scroungers model does not apply in the same way when all food collected goes to the colony, although we still see a division of labour between scouts (producers, finding new food sources) and recruits (interpreting producers' signals to assist in exploiting these food sources) in many species (Dreller, 1998).

Viewing the colony as a (super)organism, investment of workers in a particular task could be likened to an investment of energy from the colony's energy budget, or an investment of time, as these workers could otherwise be performing other tasks (Hou et al., 2010).

In a system where ant colonies have reliable and renewable energy sources, how do they choose which to invest foragers into? Optimal Foraging Theory would predict the colonies to create the most efficient trails possible, whether in terms of energy or time costs. Long-term trails can be transformed to be more energy efficient (more pheromones, removal of debris) (Middleton et al., 2019). Higher quality food sources are worth more cost expenditure. In a system with food patches that vary in quality through time, we would expect presence at many of these patches to maintain information on their quality, and a plastic system to reassign workers based on quality.

In order to study the effect of resource quality on foraging trade-offs, an ideal study system is an ant species with fairly long-lasting and conspicuous nests and trails for ease of recording, that forages on reliable and renewable food sources.

1.2 Red Wood Ants

1.2.1 *Formica rufa* group

The *Formica rufa* group, also known as the Red Wood Ants, is composed of approximately six species in Europe (Stockan & Robinson, 2016). Many can produce fertile hybrids so both speciation and remixing blur the species definition, and can make it hard to identify individuals based on morphology (Seifert, 1999, 2004). Thus, we often refer to the *Formica rufa* group more generally when discussing behaviours and diet, which are mostly common amongst all the species (Stockan & Robinson, 2016).

1.2.1.1 Nests

Wood ants are perhaps best known for the large, thatched mounds they construct as part of their nest structure, which are mainly composed of plant material: coniferous needles, leaves, and twigs, and provide them with protection and environmental stability (Gösswald, 1989). These nests can be long-lasting, even estimated to reach up to 30 years of age (J. Breen, 1979). Red wood ants use a variety of nest-founding strategies, which vary between, but also within, species (Stockan & Robinson, 2016). These are broadly split into monogyny, which includes nuptial flights and parasitic founding, and polygyny, which can include nuptial flights, secondary adoption, and budding. These polygynous colonies can be polydomous, with queens split into multiple nests within a colony network (Ellis & Robinson, 2014; Fortelius et al., 1993; Stockan & Robinson, 2016).

1. 2.1.2 Trails

Wood ants are omnivorous, they collect aphid honeydew as well as hunting and scavenging invertebrate prey (see ant-aphid association section). They create stable networks of trails between trees (where they forage honeydew) and nests (Ellis et al., 2017).

Wood ants are central place foragers, with foragers leaving the nest to hunt and scavenge in the nearby area, and also forming and following long-lasting trails to trees, in which they tend hemipterans and hunt invertebrates (Stockan & Robinson, 2016). Wood ants have high site fidelity; trails can last for decades if they are productive (Stockan and Robinson 2016). It can take them weeks to completely abandon a food source if it is suddenly removed, for example a felled tree (Stockan and Robinson 2016), but this is likely due to them evolving in ecosystems of stable food sources, rather than the human-managed plantations they inhabit regularly today. Although they do not abandon food sources easily, they show plasticity in their investment into different resources (see trail switching in section 3.3). Trails to aphid galleries require maintenance, a constant supply of workers to retain, clean, and defend the aphids in order to keep those resources for the future. Additionally, a longer trail requires more memory capacity, especially over the winter, limiting the colony's total repertoire of trails (Burns et al., 2020; Rosengren, 1971, 1977). This demonstrates that wood ant colonies invest a lot into their trails, but are able to adjust their levels of investment throughout the network they maintain.

1.2.1.3 Foraging trade-offs on wood ant trails

Compared to many study systems, the semi-permanent trails of wood ants provide the opportunity to measure distance (between nest and food source) and investment (workers on a trail) relatively simply, but resource quality is not well understood.

It can be difficult to separate the cost of distance in terms of time and energy; e.g. a trail that takes longer to traverse is costly because that extra time walking takes energy, or because that time could be spent doing something else. Denny et al. (2001) attempt to decipher this in *Formica rufa* by using a choice of two obstacles: thin and vertical bridges. The idea is that vertical walking is more costly than horizontal walking for an ant, which is quantifiable. The thin dowel bridges are horizontal but only allow one ant to cross at a time, making them more time consuming. By measuring the ants’ walking speeds on different obstacles, and comparing this to the energetic cost of climbing up, they determined that wood ants prefer to conserve energy over time. This is contrary to findings in other ant species (Fewell, 1988; Fewell et al., 1996; Weier & Feener, 1995).

It has been shown empirically that when red wood ants are foraging further from the nest, they are more likely to transport heavier loads (Gibb et al., 2016; Gordon et al., 1992). These kinds of studies also show that the foragers that travel further are larger (McIver & Loomis, 1993; Rosengren et al., 1987; Tanner, 2008; Wright et al., 2000). Rosengren and Sundstrom (1987) suggest three explanations 1) Foragers travelling further are more likely to encounter competitors, and therefore larger individuals would do better 2) larger ants have larger brains and eyes and would be better navigators 3) larger ants will be able to carry more food back. The first explanation does not apply to the polydomous network of colonies at our field site where no aggression has been recorded between colonies. The second has some support -

larger ants are better navigators (Bernstein & Bernstein, 1969) and wood ants travelling along long trails must memorise and look for many landmarks. Finally the third suggestion is demonstrated in *F. rufa* by Wright, Bonser and Chukwu (2000), where ants travelling further were both larger and carrying more food.

Other than energy and time costs, red wood ants do not meet many other obstacles; they are seldom predated on trails, as their (rare) predators target nest mounds (Hughes & Broome, 2007; Otto, 2005; Rolstad et al., 2000). There is also no evidence of parasitism on trails.

1.2.2 *Formica lugubris*

Formica lugubris (Zetterstedt 1838), also known as the Hairy Wood Ant, is one of three species in the *F. rufa group* native to the UK (the other two being the Southern Red Wood Ant *F. rufa*, and the Scottish Wood Ant, *F. aquilonia*). The range of *F. lugubris* overlaps with both other species, and in some areas, hybrids of *F. lugubris* and *F. aquilonia* are present (Monaghan, 2022). *Formica lugubris* is monodomous and monogynous in Ireland (Mäki-Petäys & Breen, 2007) and Fennoscandia (Pamilo et al., 1992), but polydomous and polygynous in Britain (Ellis & Robinson, 2014) and strategies vary throughout the rest of Europe (Bernasconi et al., 2005). Nests are conspicuous thatched mounds, present in sunlit areas (often clearings, paths, and edges), in coniferous or mixed forests. *Formica lugubris* does well in managed forests, with even-aged trees, little understory cover, and high leaf litter (Arnan et al., 2009).

. Based on long-term empirical data , modelling of a quality-distance trade-off indicates that there are food sources near to nests that the ants could exploit, but they often persist in travelling long distances to more distant resources (Piross et al. 2025). A gap in our knowledge of what makes up ‘quality’ for a wood ant seems more likely than prolonged and profound inefficiencies across multiple colonies. This study aims to provide empirical evidence of the nature of ‘quality’, to inform future modelling of these foraging networks and to increase our understanding of the foraging system of this species.

1.2.3 Wood ants’ Role in Ecosystem

Not only are wood ants a highly tractable study system, they are also ecologically important in many woodland habitats in their range, as ecosystem engineers, nutrient cyclers, predators, and indirectly, significant herbivores (aphid enablers).

Over their geographic range, wood ants forage a wide variety of tree species, deciduous and coniferous, but can show context dependent preferences (Robinson et al 2008), and prefer larger trees in general (Sondej et al., 2021). In ant navigation, the width of a landmark is most important visually (Harris et al., 2007); this may allow them to select for larger more mature trees.

Ant-tended aphids make up an important portion of the herbivory in forests where they are present (Styrsky & Eubanks, 2006). This often leads to aphids outcompeting other herbivores within an ecosystem, as shown in *F. lugubris* in woodland on the North York Moors (Fowler & Macgarvin, 1985). Wood ants also prey on other herbivores, which can have a significant effect on tree health (Whittaker & Warrington, 1985), and kill aphid predators, meaning they have a significant effect on the invertebrate populations within an ecosystem. Their role in nutrient cycling secures their place as a keystone species; total nitrogen and carbon are significantly higher in nest soil relative to non-nest soil, which creates heterogeneity in the habitat (Podesta, 2023; Stockan & Robinson, 2016).

Wood ants also tend some other hemiptera (treehoppers, scale insects, psyllids and adelgids), but the majority of their interactions are with members of the Aphidae (Domisch et al., 2009; Stockan & Robinson, 2016). Wood ants also depend on their aphid mutualists, honeydew fuels the workers to the point that they are not energy limited (Denny et al., 2001).

Many species depend on wood ants, not only their aphid mutualists; many myrmecophiles which live in, on, or around their nest (for example the Shining Guest Ant, many species of Coleoptera, etc. (Parmentier et al., 2015; Stockan & Robinson, 2016). Wood ants have few natural predators due to their defences - large numbers of biting and acid spraying individuals dissuade most natural predators of invertebrates. In Britain, Woodpeckers (Otto, 2005; Rolstad et al., 2000) and Capercaillie (Hughes & Broome, 2007) are known to predate the nests, while in Europe bears and boar can destroy mounds. There are surprisingly few parasites and pathogens of Red Wood Ants given their localised abundances, however mites (Weber, 1935) and parasitic wasps (Otto, 2005; Poinar, 2004) have been recorded, as well as fungal pathogens such as *Pandora* (Espadaler & Santamaria, 2012; Roy et al., 2006).

1.2.4 Hygiene and pathogen defence

Insect colonies are especially susceptible to the spread of pathogens: they consist of dense groups of highly related individuals and participate in fluid sharing as an essential part of colony life. Their nests are also generally stable environments in terms of humidity and temperature. In response to this evolutionary pressure, social insects have evolved many preventative and active defences against pathogens. While each ant has an internal immune system, the colony as a whole, which can be viewed as an organism, has strategies which are known as “Social immunity” (Cremer et al., 2007). They have separation between foragers (who are most likely to encounter disease) and subterranean nest members (Stroeymeyt et al., 2018). Ants partake in allogrooming as well as nest hygiene (Fefferman et al., 2007; Hughes et al., 2002; Tragust et al., 2013): they remove dead insects and other ants, faeces, and many species even produce antimicrobial compounds (Inwood & Morgan, 2008; Poulsen et al., 2002). When infected with a fungal pathogen, ants prefer food with a higher proportion of amino acids to carbohydrates, to support their personal immune defences (Csata et al., 2024).

Wood ants are likely to be particularly susceptible to the spread of disease due to the high density and longevity of populations, and high relatedness between species. Red Wood Ants prophylactically collect large amounts of conifer resin and place pieces in and on their nest mounds (Brütsch & Chapuisat, 2014; Castella et al., 2008b; Christe et al., 2003). Conifer sap contains a mixture of terpenes which can be antibacterial, antifungal, and antiviral (Phillips & Croteau, 1999). The presence of resin increases the survival of adult and larval wood ants when exposed to entomopathogenic bacteria and fungus (Brütsch & Chapuisat, 2014; Chapuisat et al., 2007), while reducing their use of personal immunity (Castella, Chapuisat, Moret, et al., 2008a). Wood ants collect more resin when brood are present (Brütsch and Chapuiset 2014), as brood is more vulnerable to disease and valuable to the colony. Finally, wood ants can even increase the potency of the antimicrobial compounds in the resin by applying acid (Brütsch et al., 2017)).

1.3 Ants and Aphids

1.3.1 Ecology of aphids

Aphids are small sap-sucking hemiptera of the superfamily Aphidoidea. There are around 5110 described species (Remaudiere & Remaudiere, 1997, Singh & Singh, 2021), which live on a wide variety of plants, as specialists or generalists. Due to their extremely fast reproductive cycle, which involves both asexual and sexual reproduction, they can colonise areas quickly, and become significant herbivores in an ecosystem. Phloem sap can be variable in its composition, but is generally high in simple sugars and low in nitrogen. To exploit this, aphids have formed a symbiotic relationship with the bacteria genus *Buchnera*, which provides essential amino acids to the aphid (Douglas, 1998). As a by-product of processing the phloem sap, aphids produce honeydew, a sugary liquid. Most aphids simply excrete this and move on, and some other animals forage it, as has been recorded in bumblebees (Batra, 1993), houseflies (Hung *et al*., 2015) honeybees, hoverflies, and earwigs (Harris-Shultz *et al*., 2022).

1.3.2 Honeydew composition

Broadly speaking, honeydew is made up of sugars and water, as the aphid's gut bacteria extract most of the nitrogen from the phloem sap. The concentration of sugars in honeydew varies heavily based on the environmental conditions, and that of the phloem sap. Honeydew differs from phloem sap in the type of sugars, with the aphid's processing turning monosaccharides (predominantly glucose) and disaccharides (predominantly sucrose), into oligosaccharides. Honeydew also contains low amounts of amino acids and inorganic ions, with dry weight being up to 98% sugars (Völkl et al., 1999).

Honeydew composition is complex not only in its chemical analyses, but also in the factors contributing to its variation: aphid species, genotype, and symbionts; tree species and rhizobia associations; environmental conditions; and ant-tending behaviour.

The composition of honeydew is an understudied area, partially due to the difficulty of analysing small sample sizes of solutions containing many different sugars. Often, studies into honeydew composition come from research into honeydew honey (Iglesias et al., 2004; Seeburger et al., 2022; Shaaban et al., 2020, 2021). This substance is made by bees from honeydew collected from around aphid colonies. The sugar composition depends on the aphid species, whereas the amino acid and inorganic ions make up distinct chemical profiles of plant species (Iglesias et al. 2004, Shaaban et al. 2020, Shaaban et al. 2021).

Bacterial mutualists also play a big part in the composition of honeydews. Plant-rhizobia interactions affect honeydew sugar composition, concentration, and amount (Whitaker et al., 2014). Aphids with certain facultative endosymbionts had fewer amino acids in their honeydew, the same total amount of sugar, but a different composition of sugars, while aphid genotype still had an effect (Schillewaert et al., 2017).

Aphids produce more melezitose in droughts - this is thought to combat dehydration caused by the change in osmolarity of the phloem sap (Rhodes et al., 1997; Seeburger et al., 2022). Melezitose is a trisaccharide unique to honeydew which aphids also produce to attract ants (Fischer & Shingleton, 2001; Kiss, 1981).

1.3.3 Ant-Aphid association

Some species forage excreted honeydew from where it pools on vegetation around aphid colonies. Over time, this has led to associations forming between aphids and ants. Ants will guard and tend aphids, and the aphids retain their honeydew until ants come to collect it. The main benefits to the aphids are hygienic services and protection from predators (Nixon, 1951; Stadler & Dixon, 2005); their presence can even repel parasitoids of aphids (Beltrà et al., 2015; Fanani et al., 2020; Mouratidis et al., 2021). However, in some cases the conspicuous ants actually attract parasitoids (Völkl, 2000). Phloem feeding aphids have a specialised stylet mouthpart to pierce bark, which is slow to retract if threatened (Dixon, 1997), this increased sedentary behaviour explains why ant protection is particularly beneficial to phloem feeders (Dixon, 1997). While overall, ant attendance is uncommon throughout the aphid families (Bristow 1991 as cited in Stadler & Dixon, 2005), some aphid species have become obligately ant-tended, meaning they cannot have significant reproductive success without ants, as they often lose defensive strategies in the course of the evolution of the association (Depa et al., 2020). Aphids can attract or retain the attention of ants by producing melezitose (Fischer & Shingleton, 2001; Kiss, 1981). Aphid genotype also influences ant attendance intensity (Katayama et al., 2013).

While the associations between ants and aphids are widely used examples for illustrating Mutualism, some consider this more of an agricultural relationship akin to the domestication of animals and plants by humans (Bronstein, 1994, 2001; Bronstein et al., 2006; Offenberg, 2001). Ants sometimes consume tended aphids, as a protein source (Hölldobler & Wilson, 1990; Ivens et al., 2012) or to mediate honeydew production by preying on adults (Stadler &

Dixon, 1998). They prey on their aphid ‘partners' that are not producing enough honeydew when it is plentiful overall (Edinger, 1985; Sakata, 1994, 1995). Anecdotally, ants will remove the wings from or kill aphid alates. With their general lack of defence, mobility, and existence in groups, aphids are highly predated, mainly by other arthropods like coccinelid beetle larvae (Frazer & Gilbert, 1976; Dixon, 2000), lacewing larvae (Dixon, 2000), spiders (Birkhofer & Wolters 2012), and, as stated above, ants (mutualists or not). This predation pressure likely drove the development of a ‘mutualism’ (Sakata, 1995) with aphids ‘paying’ for protection with honeydew, despite this being very costly for them (Stadler & Dixon, 1998; Yao & Akimoto, 2002). With ants often controlling the distribution and dispersal of their aphids (more significant in root aphids) (Pontin, 1978), they appear to do better as they produce bigger clonal colonies (Fowler & Macgarvin, 1985). However, they produce fewer or no alates and have a low dispersal rate (Ivens et al. 2012, Goidanich 1958 as cited in Stadler and Dixon 2005). This seems more akin to animal husbandry than a mutualism (Ivens et al. 2012) as obligately ant tended aphids may not actually be receiving fitness benefits which outweigh the costs of honeydew production. These aphid species could be at an evolutionary dead end. Since ants do not (generally) depend on a single species of aphid, they have the choice to switch their association to a better-producing species.

This reliable sugar supply can fuel huge colonies of ants, which protect their aphid ‘farms’ from predation, rivals, and disease (Nixon 1951, Stadler and Dixon 2005). While honeydew is fairly poor in nutrients, it is a good fuel for workers to use for other foraging purposes, like hunting invertebrate prey. Ants are morphologically suited to a liquid diet. They have a social stomach, and the ability to move food around their digestive system depending on whether it needs to be used, stored, or passed on to other colony members (Horstmann, 1970; Lange, 1967; Otto, 2005). They are capable of regurgitating food to feed others; this is known as trophallaxis and can be used as a form of communication rather than just food transfer (Meurville & Leboeuf, 2021). In this way, honeydew is ideal for sharing throughout a colony as well as storing through the winter. Some species of ants even have workers known as repletes whose gasters can expand dramatically with the sole purpose of food storage. These ‘honeypot’ ants have evolved convergently in deserts to preserve liquid foods (Hölldobler, 1981).

1.3.4 Ant diet and preferences

In order to grow, ant colonies need protein - nitrogen, mainly to feed their larvae; adult workers mainly need sugars to fuel their work (Stadler & Dixon, 2008). This dichotomy is a generalisation: in some invasive ant species the brood need more sugars than workers (Shik et al., 2014; Wilder et al., 2011) and some species of ants are primarily predatory.

Across many studies of ant preference, ants (including red wood ants) appear to prefer sucrose as opposed to other sugars (Blüthgen & Fiedler, 2004; Detrain et al., 2010; Madsen & Offenberg, 2020; J. Sudd & Sudd, 1985). Boevé & Wäckers, 2003 measured worker survival of *M. rubra* on 13 different sugar solutions and found that ants that fed on sucrose had the lowest mortality rate over 60 days. Some ants, such as *Lasius niger*, are attracted to melezitose more than other sugars (Detrain et al., 2010; Duckett, 1974; Fischer & Shingleton, 2001) but this was not shown in the *F. rufa* group (Madsen and Offenberg, 2020).

Ants also prefer food sources with amino acids present, but which amino acid depends on the species, season, and colony requirements. It seems ants can adapt foraging preferences to meet current colony requirements, even to the level of individual amino acids (Madsen and Offenberg 2020). Ants will particularly forage foods with higher amino acid concentrations when infected with a pathogen (Csata et al., 2024). In this vein, it is possible that ants seek out not only individual amino acids but also other compounds. Ants are known to collect antimicrobial substances, such as resin, and these could be present in honeydew from the plant defence metabolites in the phloem sap (Pearce, 1996). When under threat from a pathogen, ants will also consume aphids to obtain vital nutrients (Rissanen et al., 2023). All these factors put into question the concept of ‘value’ to an ant, which will depend on the species, season, and colony needs.

Task specialisation is well documented across a wide range ant species; tasks outside the nest are generally carried out by a subgroup of workers, whose identity may be flexible and context dependent (Gordon, 1996). In Wood Ants, there are “out-of-nest” workers, hereby known as foragers’, which are generally older than “in-nest” workers and can even be loyal to a certain kind of foraging (honeydew collection, invertebrate hunting) or to a particular trail or area (Parmentier, 2012; Rosengren, 1971, 1977). This strategy can allow for colony-wide trail loyalty over years, where veteran workers hold their established trails in their memories throughout the winter (Yao, 2012). Job specificity also applies to productive honeydew foraging sites. At the beginning of the year, aphid colonies start with a single founder, who must gather resources to produce clones. Aphids are most vulnerable at this stage, so it is advantageous for ants to remember where productive areas will be and protect them. In *F. rufa*, workers exhibit micro-site fidelity, returning to the same leaf even when disturbed (Parmentier, 2012). This is hypothesised to prevent intra-colony competition, to ensure an even spread of the workforce, and a constant supply of sugar (Parmentier, 2012). Micro-site fidelity is less rigid than trail fidelity, allowing workers to abandon an unproductive resource and find another one nearby (over time) (Burns et al., 2020; Parmentier, 2012).

It is possible that different aphids offer not only different sugar productivity levels, but also other compounds like amino acids or antimicrobial compounds. This honeydew specificity would most likely depend on the species of aphid (which could also require different tending skills) but also tree species, individual tree location, resources, age, health, maybe even genotypes of the same species of aphids (Fischer and Shingleton 2001). Then the fidelity of workers could demonstrate a further splitting down of jobs, from forager, to honeydew tender vs hunter, to different types of honeydew, responding to different colony needs.

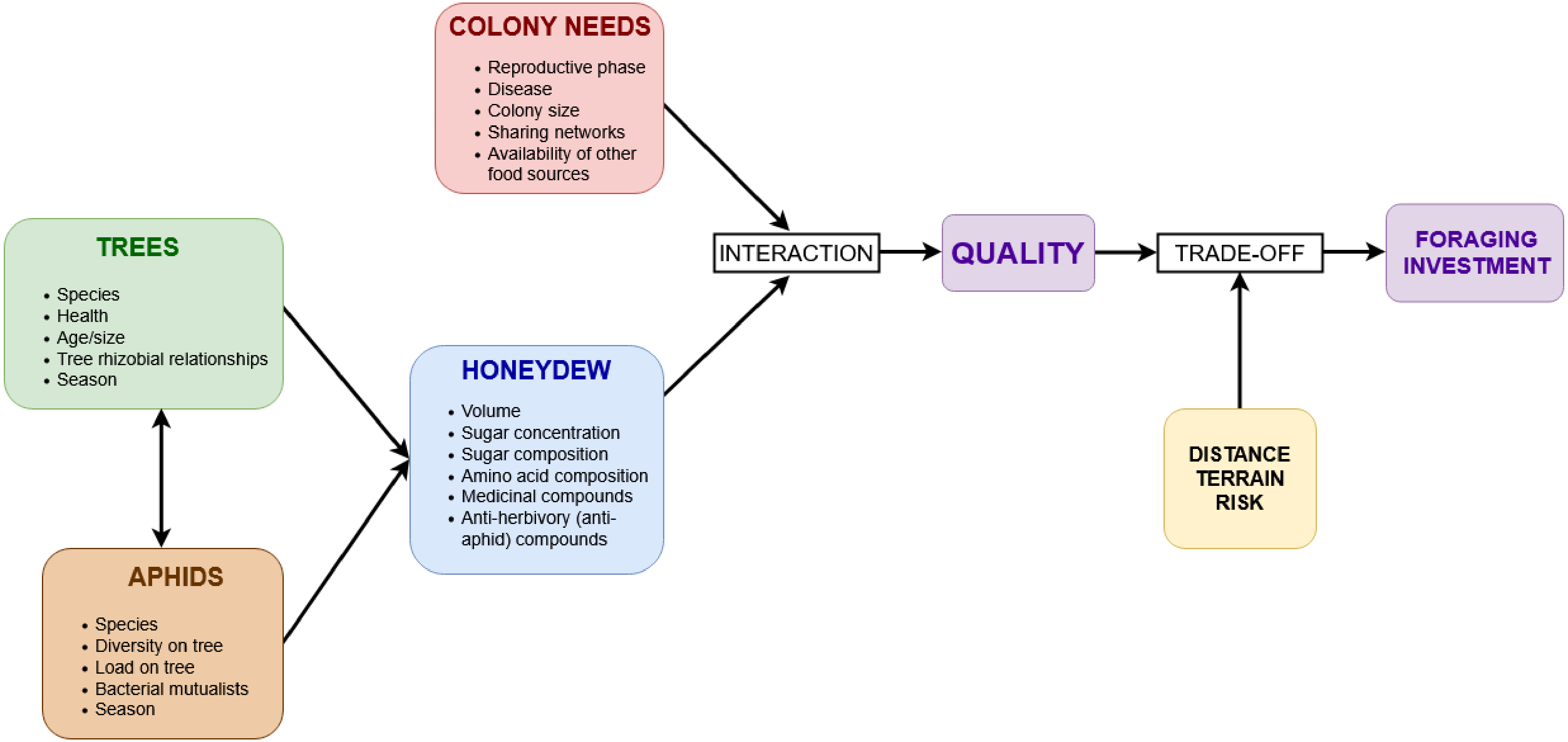


Figure 1: Representation of interactions of potential variables impacting foraging trade-offs and the definition of ‘quality’ in Red Wood Ants. This figure is not a comprehensive guide but rather an illustration of the complexities involved in defining quality. Most properties are variable over short or long timeframes.

1.4. Aims

To explore the effect of quality in the trade-offs of a foraging network, we will investigate the composition of resources into which Red Wood Ants invest more effort. This network is made up of the primary producers: trees, the processors: aphids, and the foragers: *Formica lugubris*. The individual foragers are representatives of the colony, as they detect any differences between food sources and make decisions about what to collect, but the food collected reflects the needs of the colony (Kay, 2004; Rissanen, 2023; Iakovlev, 2017) and foragers may be prompted by brood or other workers to bring back specific foods (Dussutour and Simpson 2008, and 2009). "Quality" to a wood ant colony could encompass sugars, proteins, secondary metabolites, immune active components and much more, so we will use a Metabolomics approach to characterise the composition of three fluid types: Phloem Sap (from the trees), Aphid Material (from the aphids), and Crop Content (from the ants), with the aim of identifying signatures of resources into which the ants invest heavily.

Hypotheses:

1) Sample metabolomic content will vary based on tree species
2) Sample metabolomic content will vary based on aphid species
3) Changes in metabolomic content will occur from tree to aphid to ant
4) High investment trails and low investment trails will have different components in samples

2. Methods

2.1 Field Methods: Pre-sampling

2.1.1 Site and species

A long-term study population of *Formica lugubris* was used for this study (Burns et al., 2020; Ellis et al., 2017; Ellis & Robinson, 2014, 2015, 2016; Piross et al., 2025), allowing the choice of study nests and trails to be informed by long-term knowledge of the species interactions with the resource environment at this site. The population consists of over 900 *Formica lugubris* nests forming polydomous networks (Chen & Robinson, 2013).

The study was carried out at the Longshaw Estate in the Peak District, UK, a $3km^2$ mixed woodland pasture grazed by sheep and cattle and managed by the National Trust since 1931. The site being grazed by sheep makes the wood ant trails easier to find and record. The wood ants forage on most of the tree species present at the site, with the four principal taxa being Scots Pine (*Pinus sylvestris*), Oaks (*Quercus* spp.), Birches (*Betula* spp.), and European Larch (*Larix decidua*). They have also been observed foraging at this site on Rowan (*Sorbus aucuparia*), Hawthorn (*Crataegus monogyna*), Alder (*Alnus glutinosa*), Sycamore (*Acer pseudoplatanus*), and Elder (*Sambucus nigra*); however these other trees are not numerous at this site. We therefore focussed on the four principal tree taxa listed above.

Aphid Species

To identify suitable candidate aphid species that we would predict to a) be tended by wood ants; b) be present on these four tree taxa; and c) be present at this site, we started with the Aphid Mutualists table from Stockan and Robinson 2016 (Chapter 7, Table 7.2). We filtered this table by the four tree taxa to be included in the study and carried out a literature search to determine which aphid species are present in the UK as well as to verify that they are ant tended (Updated Checklist of Aphids in Britain. Baker, Dransfield & Brightwell, 2024) (Table 1).

Table 1. Overview of aphid species associated with the 4 focal tree taxa to be used in this study, and also associated with *F. rufa* group ant species, and found in the UK.

Tree Species	**Aphid Species**	**Association with *F. rufa* group - sources**
Pinus sylvestris Scots Pine	*Cinara nuda* Shiny Pine Aphid	(Zoebelein, 1957) (Rosengren and Sundström 1991)
Pinus sylvestris Scots Pine	*Cinara pinea* Large Pine Aphid	(Sudd 1983) (Rosengren and Sundström 1991)
Pinus sylvestris Scots Pine	*Cinara pini* Scots Pine Aphid	(Wellenstein 1952) (Zoebelein 1957) (Breen 1976) (Sudd 1983) (Rosengren and Sundström 1991)
Quercus spp. Oak	*Lachnus roboris* Variegated Oak Aphid	(Wellenstein 1952) (Zoebelein 1957) (Breen 1976) (Skinner and Whittaker 1981) (Driessen et al. 1984)
Quercus spp. Oak	*Thelaxes dryophila* Common Oak Thelaxid	(Wellenstein 1952) (Zoebelein 1957) (Breen 1976)
Betula spp. Birch	*Symydobius oblongus* Shiny Birch Aphid	(Wellenstein 1952) (Breen 1976) (Sudd 1983) (Cosens and Toussaint 1986) (Rosengren and Sundström 1991)
Betula spp. Birch	*Glyphina betulae* Green Birch Thelaxid	(Wellenstein 1952) (Novgorodova 2005)
Larix decidua European Larch	*Cinara cuneomaculata* Brown Larch Aphid	(Brightwell and Dransfield 2015)
Larix decidua European Larch	*Cinara kochiana* Giant Larch Aphid	(Breen 1976) (Breen 2014) (Brightwell and Dransfield 2015)
Larix decidua European Larch	*Cinara laricis* Speckled Larch Aphid	(Wellenstein 1952) (Zoebelein 1957) (Brightwell and Dransfield 2015)

2.1.2 Ant colony mapping

The mapping of 10 *Formica lugubris* colony networks (networks ranging from 1-52 nests) at this site has been carried out for 13 consecutive years, with nest locations and sizes, tree locations and sizes, trail lengths (metres) and strengths (ants/cm) being recorded. Trails included both trails between nests, defining the polydomous colony network, and also trails from nests to trees. These strength measurements provide a measure of relative trail use, comparing with trails within the same colony mapped at the same time, but absolute level of trail usage is strongly linked to temperature, and so the values of the strength measurements from separate mapping periods should not be compared. Length measurements are the true length of the trail, which can be longer than the straight line distance between the start and end points, as trails are often indirect. These existing maps provided a starting point to identify suitable trails to sample for my study. To supplement these maps, in order to achieve sufficient replicates of paired trails per tree species, we mapped one additional colony while remapping the known colony networks in June 2024.

2.1.3 Trail selection

After remapping in June 2024, we used these colony network maps to select paired trails (strong and weaker) to the same tree species of the most similar lengths available within a colony network (Fig 2). To identify "overinvested" and "underinvested" trails, we first calculated a trail investment measure, based on strength but taking into account nest size, as smaller nests will always have smaller trails, even if those trails are very strong given the size of the nest. This was calculated by fitting models using trail length and strength, tree species, other trees visited by the same colony, number of nests visiting the same tree, nest volume, and colony network identity. The best fitting model (determined by a step function (RStudio)) was trail strength predicted by trail length, species, and nest volume. Using this model, we then grouped trails with the largest residuals into positive and negative groups based on the 1st and 3rd quartiles (Fig 3B), and paired these trails within colony networks. A trail pair consisted of one weak (residual < -0.7) and one strong (residual > 0.7) trail of the most similar lengths available, within the same colony network, to the same tree species. Most trees used by the ants were mature, but two of these replicates were pairs of saplings, as they had very strong residuals. Trails to saplings were not paired with trails to mature trees. Each tree species we included has 3 replicates of trail pairs. We therefore ended up with 24 unique nest to tree trails, divided into 12 pairs (as stated above). These trails were taken from 8 colony networks, with 1 to 3 pairs per network. Colony network identity was not found to be a significant variable in preliminary data analysis.

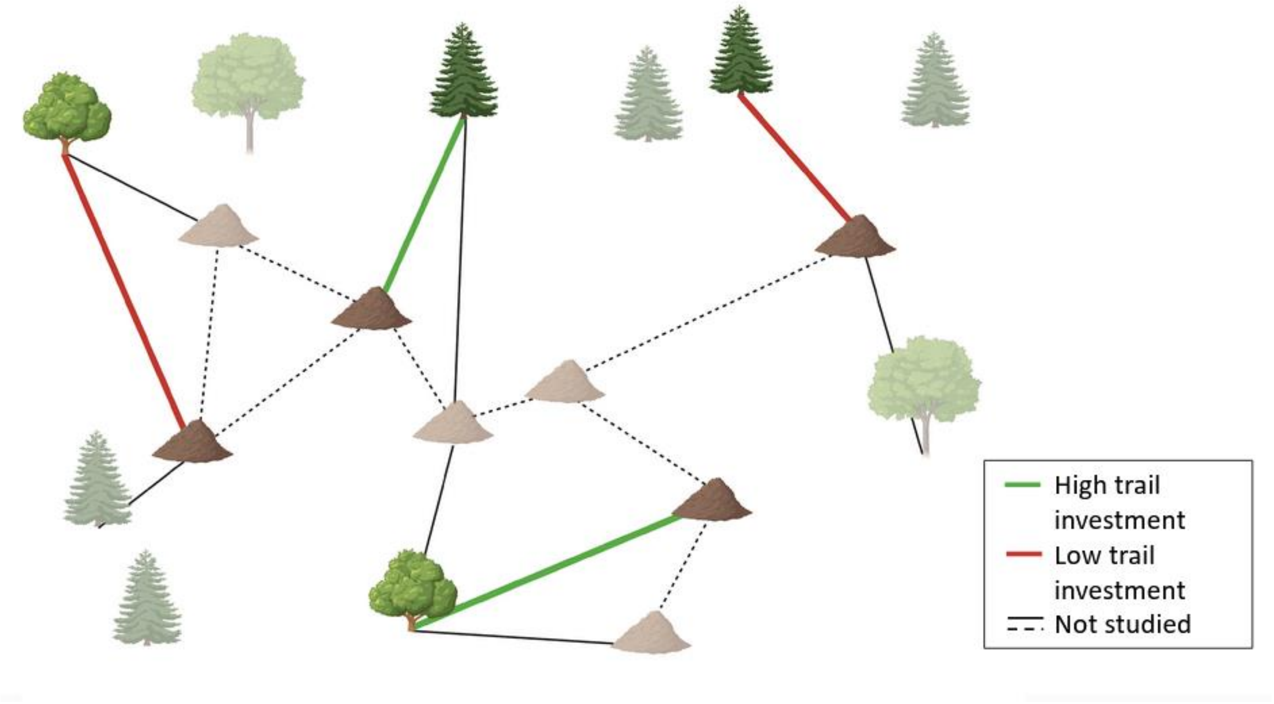
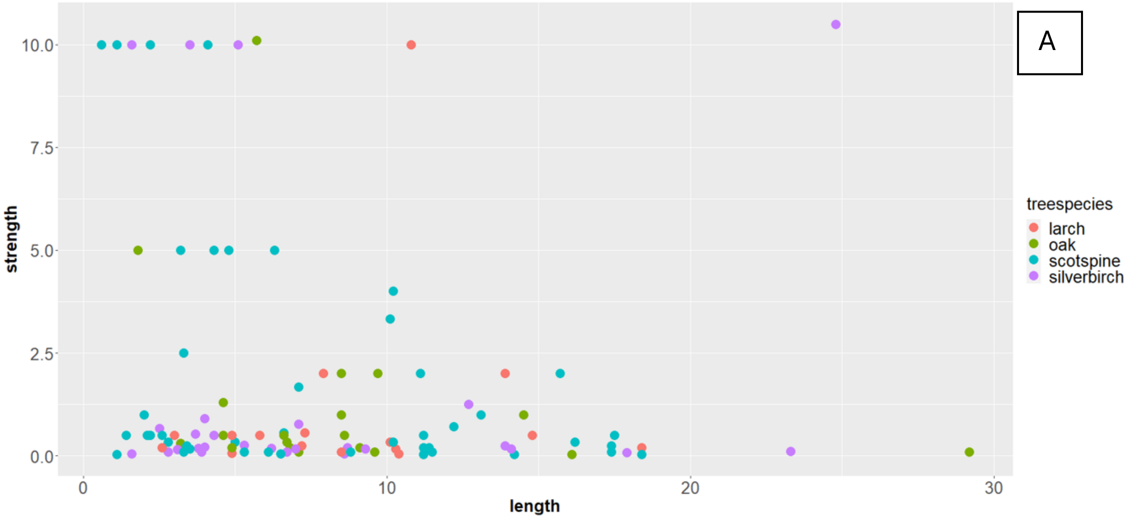


Figure 2: Idealised colony network of inter-nest and foraging trails. Two paired trails to a conifer tree species and two paired trails to a broadleaf tree species are shown, with trails coloured by investment level.

Using previous data collected across 10 colony networks, we saw that neither trail length nor trail strength (two axes of trail investment) was predicted by tree genus, and that long and strong trails were seen to all four tree genera (Fig 3A). Because these results make clear that neither tree species nor distance (length) fully explain the level of investment (strength), we decided to investigate the composition of the Phloem, Honeydew, and Crop Contents.



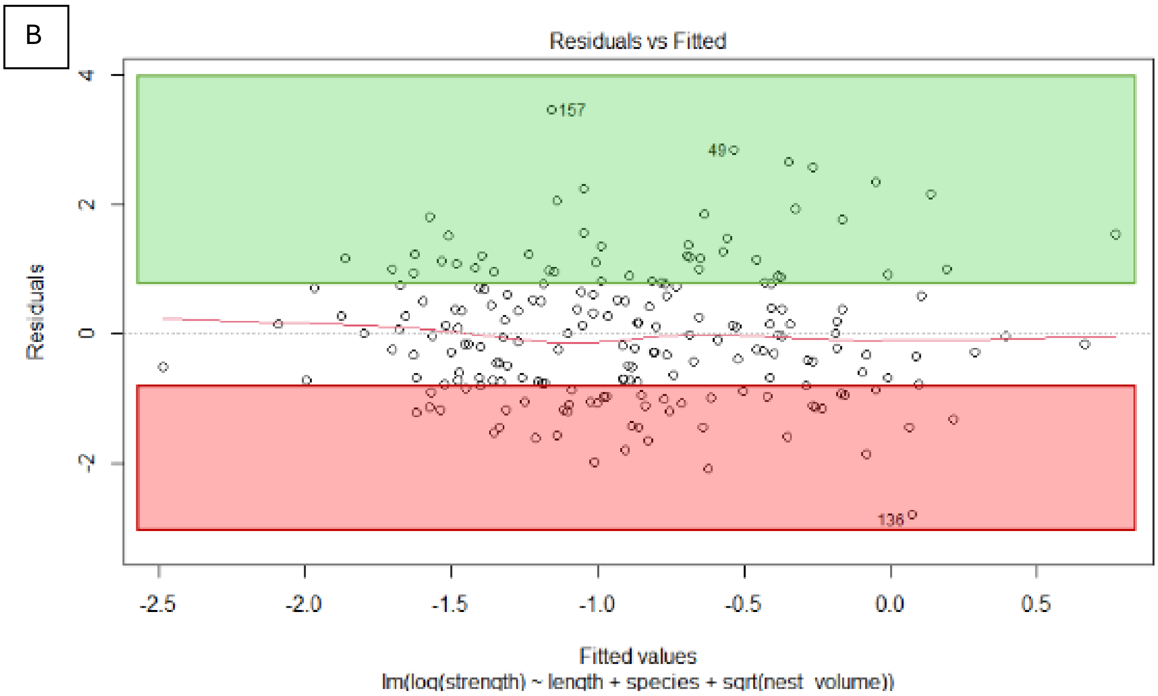


Figure 3: A) Foraging trail lengths (metres) and strength (ants/cm), by tree taxonomic group (colours), from previous data from *Formica lugubris* at the Longshaw Estate, Derbyshire UK (Robinson 2023 unpublished). N=10 colonies, N=175 trails. B) Representation of the best fitting model, using residual value (first and last quartiles) to determine high and low trail investment groups

2.2 Field Methods: Collecting samples

Figure 4 gives an overview of the sampling method for each trail, which involved collecting Phloem Sap, Aphid Material and ant Crop Contents for metabolite analysis, collecting updated measure of trail investment, and taking specimens for ID.

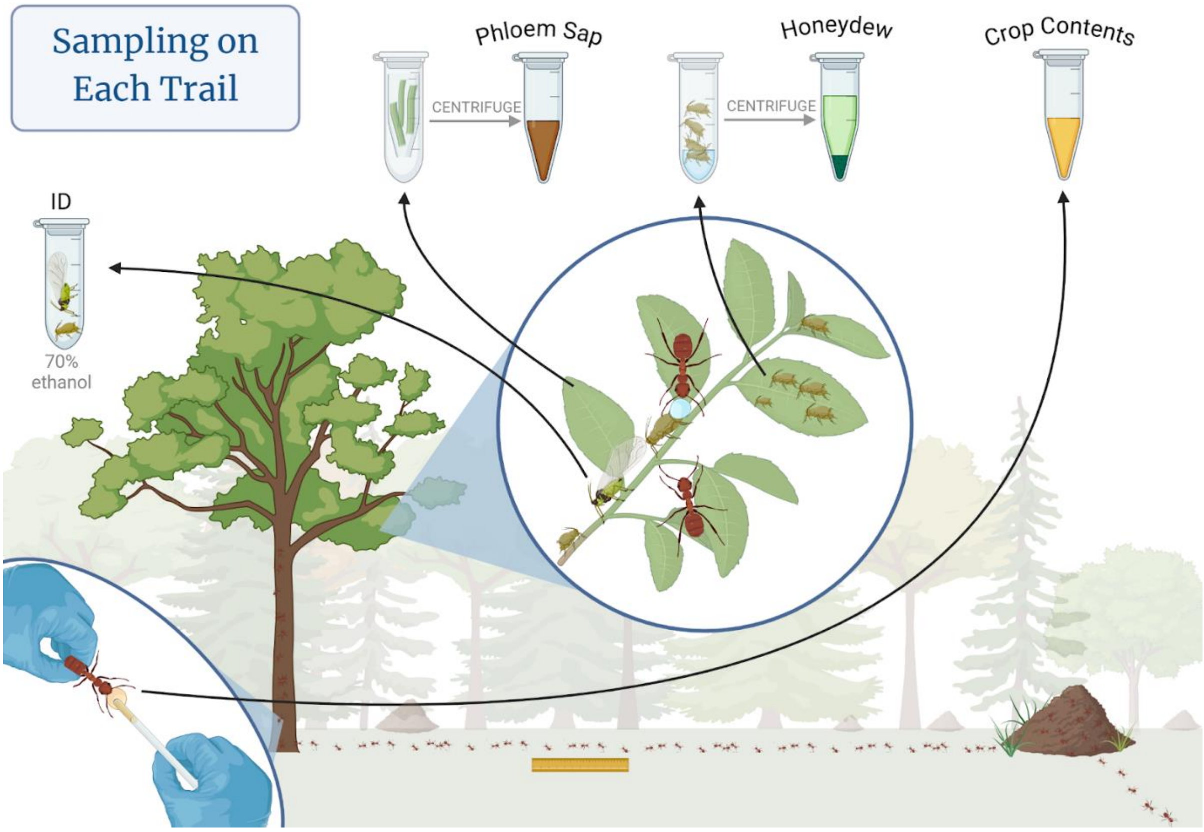


Figure 4. Schematic overview of sampling protocol for each ant trail included in the study.

2.2.1 Trail details

During sampling in July 2024, each trail length and strength (ants/cm) was re-measured, as over a month had passed since the initial trail mapping and the trails might have changed. Both length and strength differed from the original mapping, and the values collected at the time of sampling were the ones used for later analyses.

2.2.2 Phloem extraction

In order to sample the phloem of the food trees, we used the method laid out in (Killiny et al 2015). Roughly 3 twigs of 5 mm diameter and 50 mm length were pruned from the newest accessible growth of the tree and placed in the freezer at -20°C.

After 8-14 days they were defrosted at room temperature (within the collecting tubes) for 3-4 hours. The stems were then trimmed to segments 3 cm in length and slit twice lengthwise using a scalpel. The bark and phloem were peeled from the xylem, and then rinsed with distilled water and dried with KIM wipes, to remove any contamination of compounds released from the xylem being cut. The segments were then trimmed to 1 cm pieces. The scalpel was cleaned between each sample and stage.

For each sample, we removed the lid and tip from a 0.5 ml Eppendorf tube, and entirely filled this with the segments of phloem. This modified tube was placed in a 1.5ml Eppendorf tube and placed in a benchtop centrifuge for 20 minutes at 12,000 rpm. The inner tube was removed and discarded, and the tube with the extracted phloem sap was placed in the freezer at -20°C.

2.2.3 Honeydew

We aimed to sample honeydew directly from the aphids. Many methods were attempted to harvest honeydew. A pulled capillary tube was used to try to harvest honeydew droplets as the ants tended the aphids; however in ant-tended colonies the ants responded to these attempts by defending the aphids and moving them, making this method impossible, and when ants were excluded from the aphid colonies, the aphids could not be stimulated to produce a honeydew droplet. Squeezing live aphids does not release honeydew, and eventually crushes the body. Therefore, we sampled the whole aphids, rather than extracting just the honeydew. Centrifuging whole aphids allows for all the honeydew compounds to be present, although these samples will also include other compounds found in the aphid. Rather than a specific snapshot of the contents of honeydew, these samples act as a reference for comparison with compounds from the phloem and crop contents, to confirm whether compounds in the crop originated from the phloem or from the aphids.

With a standard 30 minute search time for aphids on the lower branches of each tree, 1 to 20 aphids from colonies tended by ants were collected dry into Eppendorf tubes and placed on ice in the field, before being stored in a freezer at -20°C. 2-3 weeks later they were thawed at room temperature. We added 15 µl of distilled water to each tube, which was then centrifuged at 14,000 rpm for 10 minutes. The supernatant was pipetted off into fresh tubes, and formed our “Aphid Material” sample.

2.2.4 Crop contents

On each trail 4-17 foragers were collected from those travelling down the tree with distended gasters, and their gasters squeezed manually, using a 25 µl capillary tube to collect the regurgitated liquid. We aimed to sample over 10 individuals to account for individual variation, but a few trails were so weak that this was not feasible within the time scale. The sample volume collected ranged from 10 µl to 25 µl. The ants were then returned to the trail. The capillary tube was immediately emptied into a PCR tube which was kept on ice in the field, before being transferred to the freezer at -20°C.

2.2.5 Processing of fluid samples

All fluid samples (Phloem Sap, Aphid Material and Crop Contents) were thawed and split, with 3 µl of each being put into a fresh tube with 3 µl of 1x Protease Inhibitor (https://www.sigmaaldrich.com/GB/en/product/sigma/p8340). These samples were kept for possible future proteomics analysis. The other half of each sample was used for metabolomics analysis. All samples were sent on dry ice to the Metabolomics Unit (MAPP) in Fribourg.

2.2.6 Specimen collection

Five ants from each trail were collected into 95% ethanol for possible future analysis of DNA, presence of disease, or individual ages. From each sampled aphid colony, 1-5 individuals were collected into 95% ethanol for potential future DNA analysis and a mix of adults, juveniles, and alates (1-10 individuals) were collected into 70% ethanol for specimen ID. Aphid specimens were identified to species using (Updated Checklist of Aphids in Britain. Baker, Dransfield & Brightwell, 2024) and uncertain identifications were confirmed by aphid experts Dransfield, R.D. and Brightwell, R..

2.3 Laboratory Technical Methods

Liquid chromatography-mass spectrometry (LC-MS) parameters Chromatographic separation was performed on a Vanquish Flex UPLC system (Thermo Fisher Scientific) interfaced with a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific), using a heated electrospray ionization (HESI-II) source. Thermo Scientific Xcalibur 3.1 software was used for instrument control. The LC conditions were as follows: column, Waters BEH C18 100 × 2.1 mm, 1.7 µm; mobile phase, (A) water with 0.1% formic acid; (B) acetonitrile with 0.1% formic acid; flow rate, 600 µL/min; injection volume, 2 µL; gradient, linear gradient of 2%−100% B over 10 min and isocratic at 100% B for 2 min. The optimized HESI-II parameters were as follows: source voltage, 3.5 kV (pos); sheath gas flow rate (N2), 55 units; auxiliary gas flow rate, 15 units; spare gas flow rate, 3.0; capillary temperature, 350°C, S-Lens RF Level, 50. The mass analyzer was calibrated using a mixture of caffeine, methionine–arginine–phenylalanine–alanine–acetate, sodium dodecyl sulfate, sodium taurocholate, and Ultramark 1621 in an acetonitrile/methanol/water solution containing 1% formic acid by direct injection. The data-dependent tandem mass spectrometry (MS/MS) events were performed on the three most intense ions detected in full scan MS (Top3 experiment). The MS/MS isolation window width was 1 Da, and the stepped normalized collision energy was set to 15, 30, and 45 units. In data-dependent MS/MS experiments, full scans were acquired at a resolution of 35,000 Full Width at Half Maximum (FWHM) (at m/z 200) and MS/MS scans at 17,500 FWHM both with an automatically determined maximum injection time. After being acquired in an MS/MS scan, parent ions were placed in a dynamic exclusion list for 3.0 s.

2.4 Data analysis

2.4.1 Data pre-treatment (MAPP)

The MS data were converted from .RAW (Thermo) standard data format to .mzML format using the MSConvert software, part of the ProteoWizard package (Chambers et al., 2012). The converted files were treated using the MZmine software suite v. 2.53 (Pluskal et al., 2010). The parameters were adjusted as follows: the centroid mass detector was used for mass detection with the noise level set to 2.0E4 for the MS level set to 1, and to 0 for the MS level set to 2. The ADAP chromatogram builder was used and set to a minimum group size of scans of 5, minimum group intensity threshold of 2.0E4, minimum highest intensity of 2.0E4, and m/z tolerance of 12 ppm (Myers et al., 2017). For chromatogram deconvolution, the algorithm used was the wavelets (ADAP). The intensity window signal-to-noise (S/N) was used as an S/N estimator with a S/N ratio set at 15, a minimum feature height at 2.0E4, a coefficient area threshold at 80, a peak duration range from 0.02 to 1.00 min and the retention time (RT) wavelet range from 0.02 to 0.05 min. Corresponding MS2 was paired with the following parameters (0.025 Da and 0.15 min). Isotopes were detected using the isotope peaks grouper with a m/z tolerance of 8 ppm, an RT tolerance of 0.08 min (absolute), the maximum charge set at 4, and the lowest m/z was used as the representative isotope. Peak alignment was performed using the join aligner method (m/z tolerance at 12 ppm), absolute RT tolerance 0.08 min, weight for m/z at 30, and weight for RT at 30. The aligned feature list (XXXX features) was exported using the export to Global Natural Product Social Molecular Networking (GNPS) Feature-Based Molecular Network (FBMN) module. The MZmine parameters used for the data treatment are available at XXXXX

2.4.2 Molecular networking (MAPP)

To analyze the spectral diversity of the profile collection, a molecular network (MN) was created on the GNPS website (http://gnps.ucsd.edu) using the .mgf spectra file generated at the previous step (Wang et al., 2016). The precursor ion mass tolerance was set to 0.02 Da and an MS/MS fragment ion tolerance of 0.02 Da. A network was then created where edges were filtered to have a cosine score above 0.7 and more than six matched peaks. Further, edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. Finally, the maximum size of a spectral family was set to 100, and the lowest-scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the network were then searched against GNPS spectral libraries. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least six matched peaks. The resulting MN is available online at https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=XXXXXXX. The converted mass spectrometry data files, the corresponding metadata table, and the metabolite annotation results table, together with a Cytoscape file corresponding to the full MN annotated with the experimental and theoretical spectral matches are available under the MassIVE ID MSVXXXXXXXX.

2.4.3 Metabolite annotation

Experimental spectral libraries search

The full spectral data set corresponding to the extracts collection has been uploaded on the MassIVE repository https://doi.org/XXXX, and a continuous identification workflow is automatically carried against the Global Natural Products Social Molecular Networking (GNPS) experimental spectral libraries: https://gnps.ucsd.edu/ProteoSAFe/result.jsp?task=XXXXXview=advanced_view.

Theoretical spectral libraries search

In addition to experimental spectral libraries search we have shown that spectral matching against theoretical spectral libraries of natural products was an efficient way to cover a much wider, yet relevant, spectral space (Allard et al., 2016). Furthermore, we showed that taking into account the taxonomical distance between the biological source of the candidate structure and the biological source of the annotated extracts greatly improved the overall quality of the annotation results (Rutz et al., 2019). Thus, in addition to the spectral search performed at the molecular networking step against publicly available spectral libraries (see previous section) a taxonomically informed metabolite annotation was performed. For this, we first established a large theoretical spectral database of natural products following a previously established metabolite annotation workflow. This spectral database and associated biological sources metadata were constructed using chemical structure and information compiled during the LOTUS Initiative's first project aiming to establish an open and evolutive resource compiling natural products and biological occurrences (Rutz, Bisson, et al., 2022; Rutz, Sorokina, et al., 2022). The theoretical spectral database is publicly available (Allard et al., 2022). The biological sources metadata are available online (Rutz, Bisson, et al., 2022; Rutz, Sorokina, et al., 2022). The taxonomically informed metabolite annotation was performed using the met_annot_enhancer scripts version v0.1: https://github.com/mandelbrot-project/met_annot_enhancer/releases/tag/v0.1. Sirius (Dührkop et al., 2019) (v.5.5.7) and CANOPUS (Djoumbou Feunang et al., 2016; Dührkop et al., 2021; Kim et al., 2021) were also employed to proceed to metabolite annotation and attribution of chemical classes to MSMS spectra.

2.4.4 Hypothesis testing and plotting

PCA

Principal Component Analysis (PCA) allows for the reduction of dimensionality and the visualisation of clustering within a dataset that has many variables. The analysis combines several variables into components (seen graphically as axes) which maximise the variance of the data.

PLS-DA

Partial Least-Squares Discriminant Analysis (PLS-DA) reduces dimensionality, as a Principal Component Analysis (PCA) would; however, the advantage of this method is its ability to incorporate class labels, allowing the analysis to use variables (components) that best separate the groups set (here the classes are the investment levels “high” and “low”) rather than total variance within the data. In metabolomics this is particularly useful as not only do the PLS-DA plots allow us to observe clustering based on conditions, but also highlights which features are the most important for driving this clustering. Variable Importance Projection (VIP) scores measure the relative importance of each component (here, each feature) to the class separation of the PLS-DA.

2.4.5 Manual feature identification

Once the most important features to the class separation were identified, we manually determined their identification based on the four automatic labelling tools: GNPS (a library of real, experimental spectra, which is therefore more limited), CANOPUS (a tool relying on molecular structure to assign chemical classes, without comparing to a database), Sirius (similar to CANOPUS but goes further), and ISDB (which gives context on the taxons compounds have been recorded in in the past).

By excluding features found in the blank samples, and those where the different identification tools disagree, we can get a more reliable picture of the features. In the results we use the terms “identified” to describe metabolites identified by multiple tools. GNPS being an experimental library makes its identifications more reliable. It assigns labels to identification to indicate the level of confidence: bronze, silver, and gold (increasing confidence). Where a bronze label is given we have referred to a metabolite as “tentatively identified”, where a gold label is given we have described the metabolite as “identified” or “matched”.

3. Results

In total, 67 fluid samples were analysed, comprising 24 Ant Crop Content samples, 24 Phloem Sap samples, and 19 Aphid Material samples, extracted from whole aphids. Across all sample types, 2587 features (molecules, including those that could not be fully identified) were isolated, and by matching against experimental and theoretical libraries, 844 metabolites were identified to chemical or chemical grouping.

Illustrating this full sample set using a PCA (Figure 5) we see separation between ant samples, aphid samples, and tree samples. The aphids cluster together and are not separated by species. The trees are separated into 2 clusters, conifers and broadleaves.

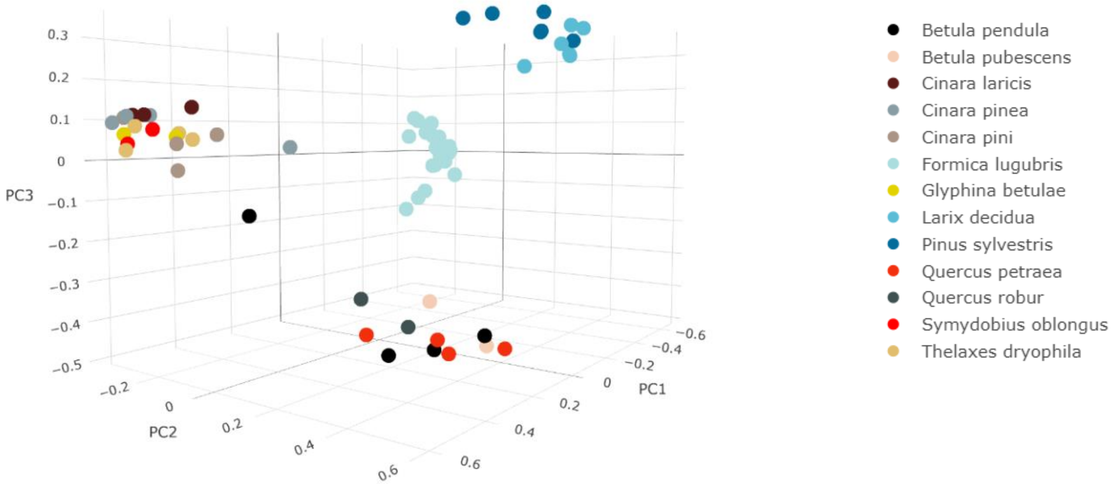


Figure 5: 3D-PCA comparison of all species using all samples (Phloem Sap, Aphid Material, and Crop Content) *Formica lugubris* is central (pale blue); *Pinus* and *Larix* are top right (dark and mid blue); *Quercus* and *Betula* species are bottom central (dark, orange, and peach), and all Aphid species are clustered together mid left.

3.1 Does metabolomic content vary based on tree species?

The PCA results show strong clustering by tree species, particularly for Phloem Sap and Ant Crop Content samples (Figure 6). PCA is an unsupervised method to reduce dimensionality while showing the most variance, so the metabolomic differences between samples linked to different tree species are fundamental.

For Phloem Sap samples (Figure 6A), PC1 & PC2 together explain 38.4% of the variation, which is a high result for this method of analysis. We can see that the broadleaf species (oaks and birches) group closer to each other than to the conifers (pine and larch), and while PC1 is more important for separating the oaks and the birches, PC2 separates the larch and pine samples.

The Aphid Material samples (Figure 6B) are less obviously separated by tree species, although the PC1 and PC2 together still explain 31.1% of variance. These samples contain aphid body material, and the metabolomic profiles of this appear quite conserved across species, reducing the relative contribution of the plant-derived honeydew to the overall sample profiles.

The metabolomic profiles of Ant Crop Content differ markedly depending on the tree species on which they have foraged (Figure 6C). PC1 and PC2 together explain 36.7% of the variation, which remains almost as high as for the Phloem Sap, meaning the discrimination between the tree species has been retained. In this plot we can see that the Crop Contents originating from conifers are separated by PC2 only. The distinction between broadleaf and conifer species is less clear than in Figure 6A; however, within the tree genera, the distinction between the two species of birch (and oak) is made clear by PC1.

We can view the distribution of broad categories of metabolites in different groups by species, helping illustrate their differences (Figure 7). Overall across all tree species, the Phloem Sap samples contained many flavonoids, terpenoids, amino acids, and fatty acids; these were the most prevalent four groups in almost all cases, although in *Larix* phloem the fatty acids are less prevalent, and the fourth most prevalent group is instead open chain polyketides (sesquiterpenoids). Overall, terpenoids were relatively more dominant in the larch phloem than in the phloem of other tree species. The two oak species differ in the relative dominance of fatty acids and terpenoids.

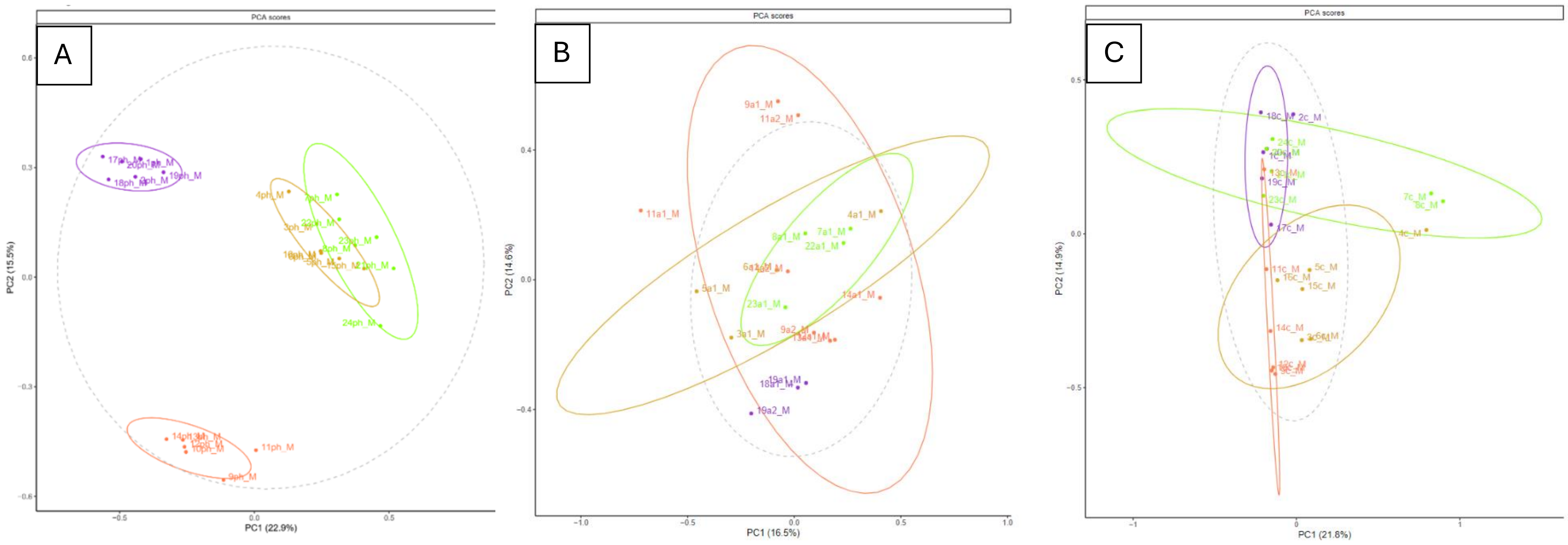


Figure 6: PCA comparison (unsupervised) of the samples from the four tree groups: Birch (*Betula pubescens* and *Betula pendula*), Larch (*Larix decidua*), Pine (*Pinus sylvestris*), and Oak (*Quercus robur* and *Quercus petraea*) for A) Phloem Sap samples; B) Aphid Material samples; C) Ant Crop Content samples

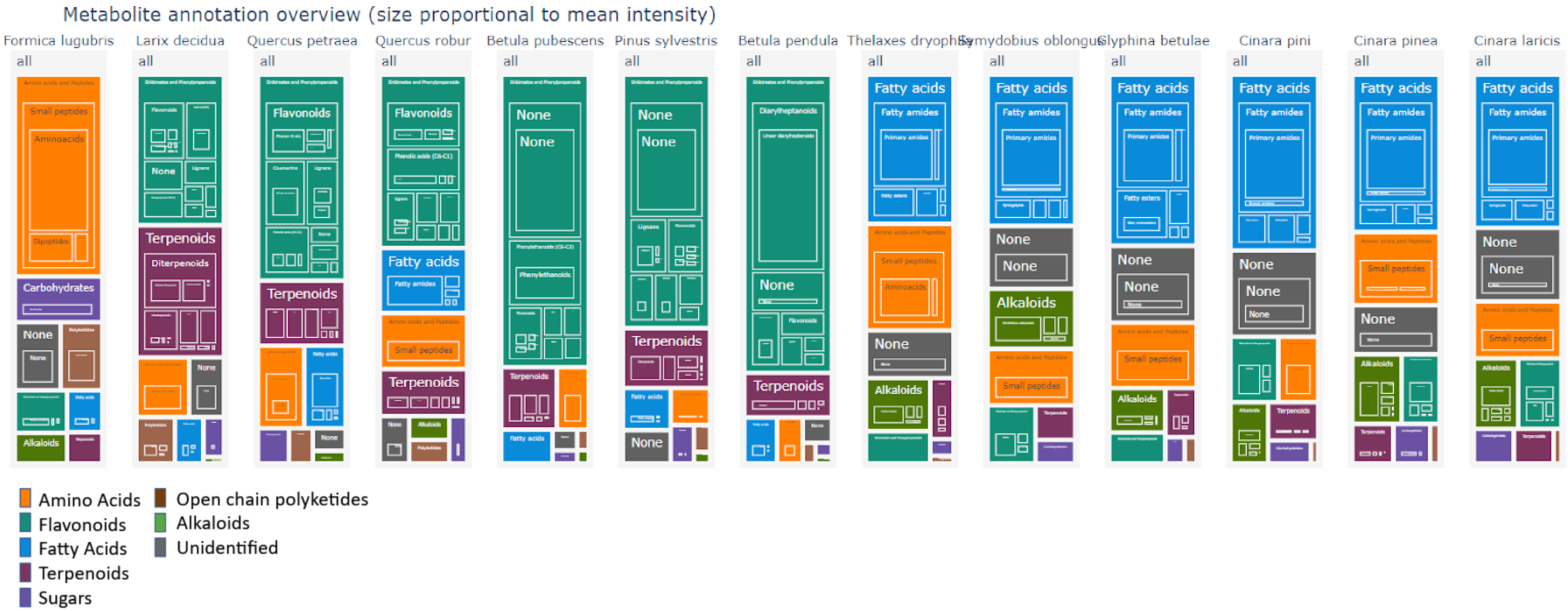


Figure 7: Qualitative treemap of metabolites present in samples based on their source species. Boxes labelled ‘none’ indicate unidentified compounds. This treemap provides an overall impression of the categories of metabolites within each species, but does include features identified with low certainty, so should be interpreted cautiously. Note that the *Cinara pinea* were parasitised, likely by *Pauesia picta.*

3.2 Which aphids were used by the ants?

Aphids were accessible for sampling on 16 out of the 24 trees used for the Phloem Sap and Ant Crop Contents samples. Of these 16, enough individuals were available for metabolomic analysis on 15 trees, 4 of which had enough of two aphid species, resulting in a final sample size of 19 aphid metabolomics samples Table 2). We found six species of aphids overall, *Thelaxes dryophila, Symydobius oblongus, Glyphina betulae, Cinara laricis, Cinara pinea*, and *Cinara pini.* These six species were within the candidate set of 10 species we had identified in advance (Table 1). All individuals of *Cinara pinea* that we collected were later determined to have been parasitised (B. Brightwell, personal communication) due to their swollen, brown bodies, which mostly consisted of a hardened hollow cuticle. They were most likely parasitised by *Pauesia picta* (B. Brightwell, personal communication), a specialized parasitoid on *Cinara pinea* (Völkl, 2000; Völkl & Kroupa, 1997). *Symydobius oblongus* and *Glyphina betulae* were found on both *Betula pubescens* and *Betula pendula.* Aphid species showed no clear separation on the combined PCA plot (Figure 5). Across all six species, samples were dominated by fatty acids, amino acids, flavonoids, and alkaloids (Figure 7).

Table 2: Aphid species table

Trees	**Aphids**	**Number of trails with which the aphid species was associated**	**Final number of samples**
Quercus robur and *Quercus petraea*	*Thelaxes dryophila*	4	4
Betula pubescens and *Betula pendula*	*Symydobius oblongus*	2	2
	Glyphina betulae	3	2
Larix decidua	*Cinara laricis*	2	3
Pinus sylvestris	*Cinara pinea*	4	4
	Cinara pini	5	4
Totals	6 species	20 trails	19 samples

3.3 Is trail investment driven by tree species?

Although the tree species have very clearly different metabolomic profiles, which carry through to the Ant Crop Contents (Figure 6), the differences in trail investment are not driven by tree taxonomic group (Figure 8).

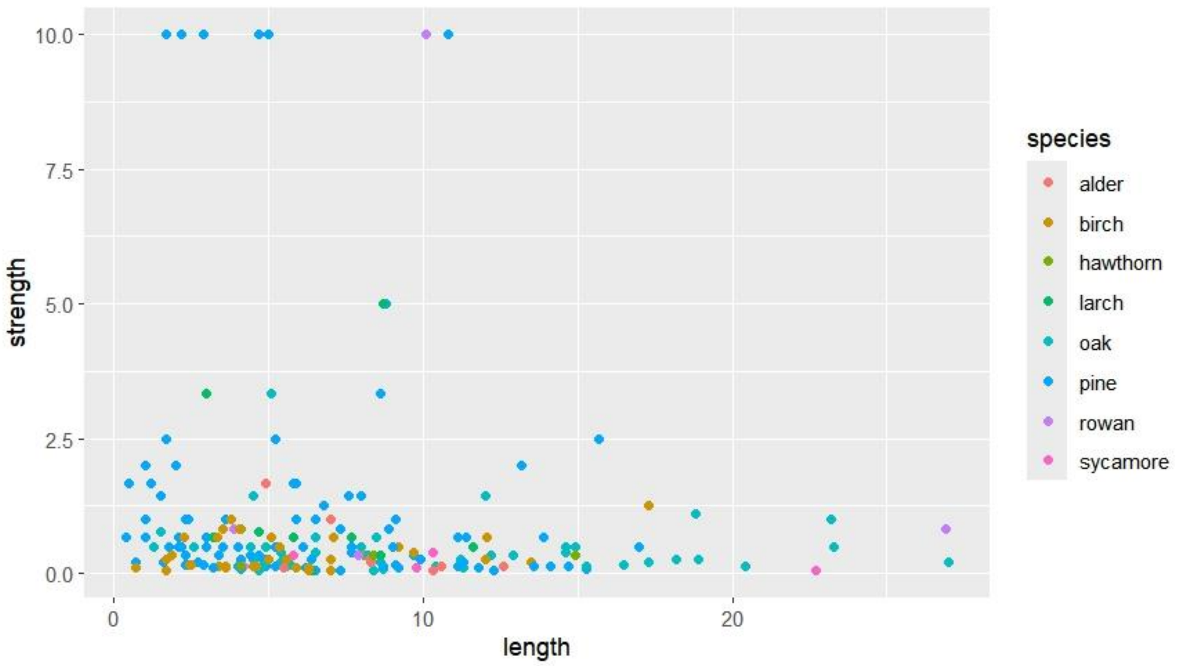


Figure 8: Foraging trail lengths (metres) and strength (ants/cm), by tree taxonomic group (colours), from *Formica lugubris* at the Longshaw Estate, Derbyshire UK N=10 colonies, N=175 trails

For the trail pairs selected for use in this metabolite study, between initial trail mapping (in June 2024) and re-mapping with sampling (in July 2024) trail strengths changed somewhat, In 4 cases, the trail pairs switched investment levels (the planned low investment trail became the high, and the planned high investment trail became the low) and so for these 4 pairs the later (re-mapping) investment level was used in the analysis; in the other cases the original classifications were used.

3.4 Do high investment trails and low investment trails differ in metabolites?

We compared the metabolomic profiles of samples from “low” and “high” investment trails. In the Ant Crop Content and Phloem Sap sample sets, samples from trails of “low” and “high” investment differ in their metabolomic profiles, based on the presence of certain key metabolites in each group, as shown in the PLS-DA plots (Figure 9 Ai, Ci), and associated heatmaps (Figure 9 Aiii, Ciii). The separation of the two trail types in the Aphid Material samples is less clear (Figure 9 Bi), with the low investment trail metabolite profile mostly nested within the high trail investment cluster. The Aphid Material samples include aphid body material which is likely diluting the signal of the honeydew present within the samples.

The consistency of the difference in metabolomic profiles by level of trail investment across both the Phloem Sap data (Figure 9 Ai) and the Ant Crop Content data (Figure 9 Ci) indicates that the differences between the materials in the ant crops are derived, at least in part, from differences in the original plant material collected by the ants from the aphids, rather than from intrinsic differences between the ants involved in the "low" and "high" investment trails. It is also notable that these data include all six tree species and that, despite the strong differences between tree species also observed in the PCAs (Fig 6), using the classification of the PLS-DA, we can still see clear separation based on trail investment level.

We can further investigate these metabolite profiles using VIP scores and heatmaps to illustrate the importance of certain types of metabolites to the characterisation of each group (Figure 9 Aii & Aiii, Bii & Biii, Cii & Ciii).

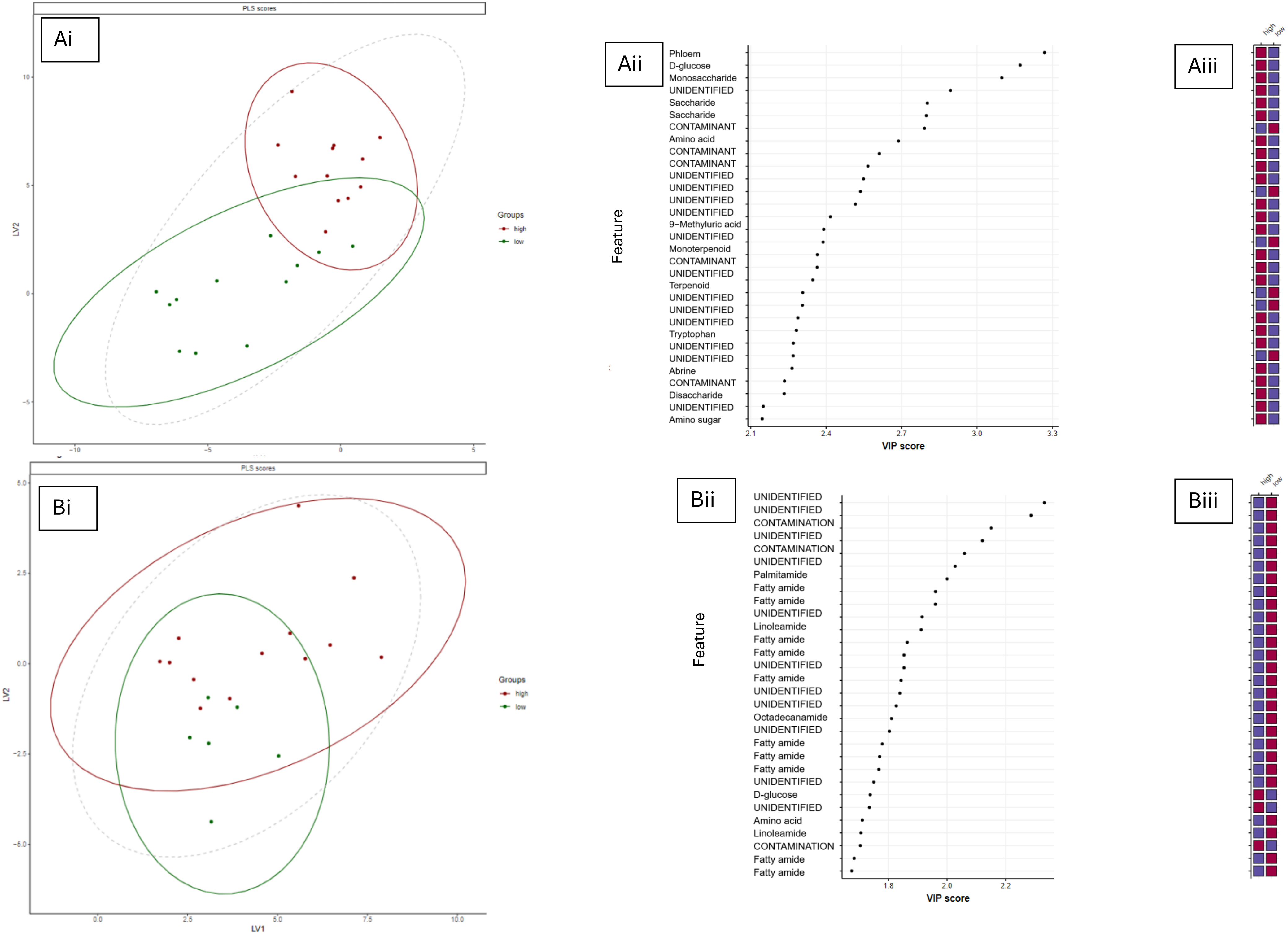
Only one feature appears in the top 30 VIP features of all 3 sample types (the Phloem Sap, the Aphid Material, and the Crop Content). This feature has been identified by GNPS as D-Glucose, and has a VIP score of 3.27 for Phloem Sap, 1.74 for Aphid Material, and 1.89 for Crop Content, making it a driving factor in the separate clustering of the high and low trail investment profiles, due its consistent presence in the high trail investment samples and absence from low trail investment samples. Four other saccharides are associated with high investment trails in the top 30 VIPs of Phloem Sap metabolites, 3 of which are likely to be monosaccharides (Features 169, 185, and 191, VIPs= 3.17, 2.89, and 2.80, respectively) and one disaccharide (Feature 188 , VIP=2.23).

One feature appears in the top 30 VIP features of both the Phloem Sap, and the Crop Content: Feature 220 is an amino sugar that is indicative of high trail investment in Phloem Sap samples (VIP=2.15) and low trail investment in Crop Content samples (VIP=1.78). Two amino acids appear in the top 30 VIPs of Phloem Sap metabolites: Feature 30 (VIP=2.80) is associated with low investment trails, while Feature 661 (VIP=2.28) was identified by GNPS as Tryptophan and is associated with high investment trails.

High investment trails are associated with Phloem Sap containing two terpenoids: Feature 1207 (VIP=2.35), and Feature 663 (VIP=2.26) tentatively identified as Abrine. However a different terpenoid in the Phloem Sap is associated with low investment trails: Feature 1615 (VIP=2.39), a monoterpenoid tentatively identified as p-cymene. No terpenoids appear in the top 30 VIP features for the Aphid Material or Ant Crop Content.

In the top 30 VIPs of Aphid Material metabolites, the only identified feature associated with high investment trails is Feature 163, D-Glucose (VIP=1.74). All other identified features are associated with low investment trails, and are fatty amides, four of which are more specifically identified: Feature 2330 (VIP=1.91) and Feature 2330 (VIP=1.71) may be Linoleamides, Feature 2532 (VIP=2.00) is tentatively identified by GNPS as Palmitamide, and Feature 2573 (VIP=1.81) is tentatively identified by GNPS as Octadecanamide.

The top 30 VIPs of Crop Content metabolites are made up of sugars, amino acids, and amino sugars. As mentioned above, D-glucose (Feature 163 (VIP=1.89)) is important across all sample types. Feature 202 (VIP=1.82), which GNPS has matched as difructose anhydride III, is associated with high investment trails. Feature 496 (VIP=2.36) is a monosaccharide tentatively identified by GNPS as Melezitose, and is associated with low investment trails. Feature 220. Another amino sugar, or glycosylamine, is Feature 219 (VIP=2.19), which is associated with high investment trails, as are two amino acids, Features 256 (VIP=1.80) and 20 (1.77). Finally, an alkaloid (Feature 40, VIP=2.25), spermidine (Feature 44, VIP=2.08), and gamma−glutamylphenylalanine (Feature 888, VIP=1.75) are all associated with high investment trails within the Crop Contents.


Ai
PLS scores
LV2
10
5
0
-5
-10
-5
0
5
Groups
high
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Aii
Phloem
D-glucose
Monosaccharide
UNIDENTIFIED
Saccharide
Saccharide
CONTAMINANT
Amino acid
CONTAMINANT
CONTAMINANT
UNIDENTIFIED
UNIDENTIFIED
UNIDENTIFIED
UNIDENTIFIED
9−Methyluric acid
UNIDENTIFIED
Monoterpenoid
CONTAMINANT
UNIDENTIFIED
Terpenoid
UNIDENTIFIED
UNIDENTIFIED
UNIDENTIFIED
Tryptophan
UNIDENTIFIED
UNIDENTIFIED
Abrine
CONTAMINANT
Disaccharide
UNIDENTIFIED
Amino sugar
Feature
2.1
2.4
2.7
3.0
3.3
VIP score
Aiii
high
low
Bi
PLS scores
LV2
5.0
2.5
0.0
-2.5
-5.0
0.0
2.5
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7.5
10.0
LV1
Groups
high
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Bii
UNIDENTIFIED
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CONTAMINATION
UNIDENTIFIED
CONTAMINATION
UNIDENTIFIED
Palmitamide
Fatty amide
Fatty amide
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Linoleamide
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Fatty amide
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UNIDENTIFIED
Octadecanamide
UNIDENTIFIED
Fatty amide
Fatty amide
Fatty amide
UNIDENTIFIED
D-glucose
UNIDENTIFIED
Amino acid
Linoleamide
CONTAMINATION
Fatty amide
Fatty amide
Feature
1.8
2.0
2.2
VIP score
Biii
high
low

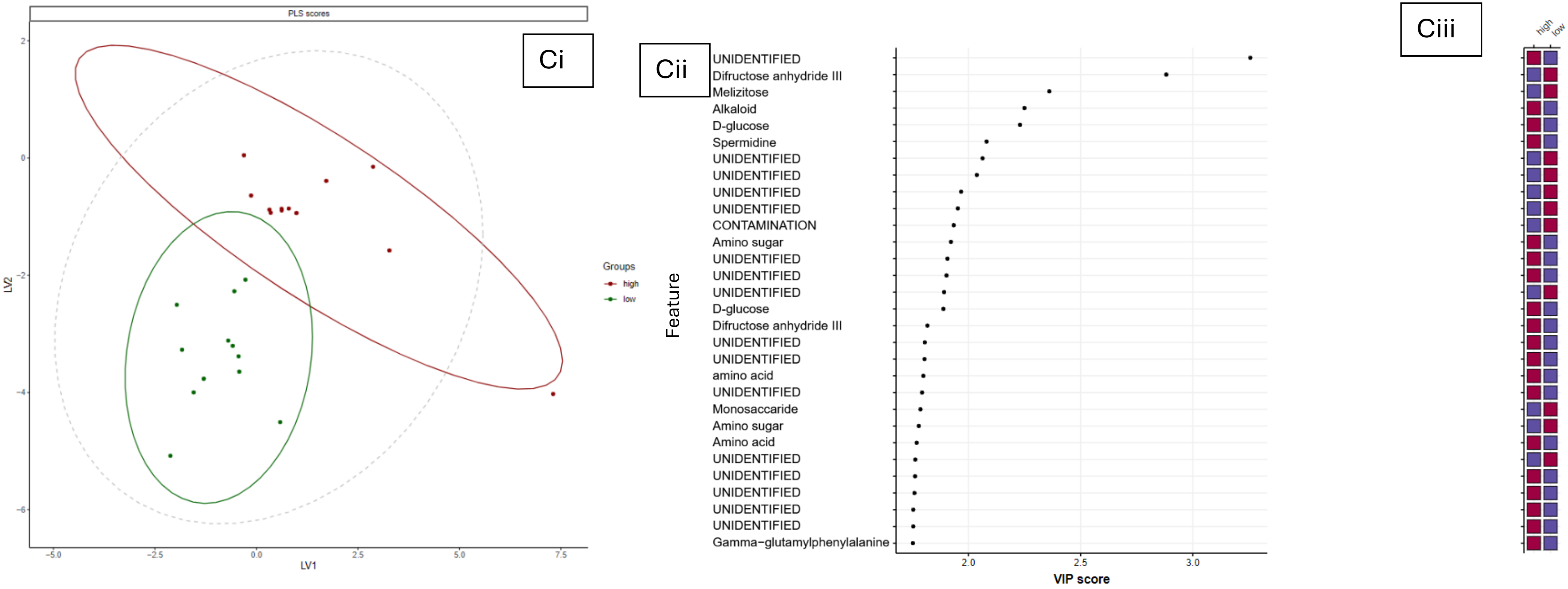

Figure 9: PLS-DA plots of metabolites from the 3 sample types: Ai) Phloem Sap, Bi) Aphid Material, and Ci) Ant Crop Content. Groups are classified by high and low trail investment. Feature Importance plots (Aii, Bii, Cii) show the top 30 features from each sample type, based on Variable Importance Projection (VIP) scores. The heatmaps (Aiii, Biii, Ciii) illustrate the presence (red) or absence (blue) of these VIP features in high or low investment trails.

Unidentified features: Seven of the top 30 VIP features of the Phloem Sap were unidentified, meaning that they were recognisable across samples and therefore statistically usable, however we don’t know what chemical groups the belong to / what structures the have

QC feature duplication: Feature 163 has a duplicate in the Crop Content metabolites due to the Quality Control method of Mass Spectrometry. A similar situation occurred with Feature 303, a copy of Feature 202.

4 Discussion

Overview of findings

Our results clearly show that different metabolomic contents of both phloem and wood ant crop content are predicted not only by tree species but also by the level of ant foraging investment in a trail. Previous work (Piross et al., 2025) indicates that distance and tree species alone do not drive foraging investment in wood ants; our LC-MS metabolomics approach has allowed a broad look into the composition of Phloem Sap, Aphid Material, and Crop Content within these foraging networks. Our results provide the first data about what components of the resources provided by trees are treated by ants as high quality, and suggest future work to further elucidate this line of inquiry into resource use by Red Wood Ants.

4.1 Metabolomics

4.1.1 How does ant Crop Content differ with tree species?

Using metabolomics, we can see differences between tree species in the Phloem Sap, supporting Hypothesis 1 “Sample metabolomic content will vary based on tree species”. The major categories of components remain the same across the four tree genera we included: flavonoids, terpenoids, alkaloids, fatty acids, and open chain polyketides (sesqueterpenoids) but their relative proportions in the sap varied somewhat between species (Figure 7), including between species of the same genera, for example *Quercus petraea* and *Q. robur.* This matches with previous studies comparing phloem composition between species (Garg & Kühn, 2020; Kehr & Kragler, 2018), what is interesting for us to note is that we found unique metabolomic tree species profiles also within the Aphid Material and the Crop Content samples. Different species of trees provide different resources to the ants, even after these resources have passed through the processing by the aphids into honeydew, as we predicted based on past studies (Iglesias et al. 2004, Shaaban et al. 2020, Shaaban et al. 2021).

We did not find evidence for Hypothesis 2 “Sample metabolomic content will vary based on aphid species”. Tree species appear to be much stronger as a predictor of metabolomic content than aphid species, as seen in Figure 6. This is particularly noticeable in *Thelaxes dryophila*, the single aphid species found on both species of oak - the ant Crop Content derived from these two species of Oak clusters separately, even though both have passed through the same species of aphid. However, the use of whole aphids in this part of the analysis may limit our ability to detect aphid species level differences; these limitations are discussed further below (Section 4.1.2.1).

4.1.2 Investment

Hypothesis 4, "High investment trails and low investment trails will have different components in samples" is the critical hypothesis for detecting signatures of 'quality' in wood ant foraging. Our data support this hypothesis; specifically, we observe separation of clusters based on the "high" and "low" investment classes assigned to the Crop Content samples. The PLS-DA approach means that we can move past the primary metabolomic clustering by tree species that is observed in the unsupervised PCAs, to identify these differing high and low investment trail metabolomic profiles across tree species. This suggests that there are true differences between food sources treated by the ants, via their investment, as high or low 'quality', or at least that there are characteristic signatures of certain trees, irrespective of species, into which the ants are concentrating their foraging effort.

Crucially, we also see these high and low investment trail metabolomic profile differences in the Phloem Sap samples. If the differences were found only in the Crop Content, then that could simply signify that different sets of workers forage on high and low investment trails, and that these groups also differ in crop contents, independent of the tree species. Division of labour in ants, even among the foragers, is well studied (Gordon et al., 1992; Rosengren & Sundström, 1987). Site fidelity in wood ants (Parmentier, 2012; Rosengren, 1971, 1977) means that it is indeed likely that different sets of workers forage on each trail; however because we found clearly differentiated high and low investment profiles in the Phloem Sap too, it is clear that the differences in metabolomic profiles originate, at least in part, from the trees (the primary producers in this foraging network), and we can hypothesise that it is these differences in the Phloem, that drive the level of investment by the ants.

4.1.2.1 What is associated with high investment trails?

Overall, certain saccharides, amino acids, and amino sugars are characteristic of the crop contents of ants foraging on high investment trails.

The association of saccharides with high investment trails is to be expected, given that honeydew is the primary source of energy for wood ants. Ants prefer some sugars over others (Blüthgen & Fiedler, 2004; Boevé & Wäckers, 2003; Detrain et al., 2010). These preferences vary by species and may depend on physiological suitability for different tasks (energy, storage, exoskeleton production). D-Glucose is the chemical most consistently and strongly associated with high trail investment in our samples, from Phloem Sap, to Aphid Material, to ant Crop Contents. This is the most common form of naturally occurring glucose, and known to be used by ants, although not preferred over sucrose (Blüthgen & Fiedler, 2004; Detrain et al., 2010; Madsen & Offenberg, 2020; J. Sudd & Sudd, 1985).

As discussed in Section 1.3.4, ants generally prefer food sources with amino acids present (Blüthgen and Fiedler, 2004), although preferences to specific amino acids vary (Madsen and Offenberg, 2020). Wood ants, like most species that feed primarily on homopteran exudates, are not typically sugar limited, due to honeydew being primarily composed of concentrated sugars (Cook & Davidson, 2006). They may seek out honeydew sources that are rich in amino acids if these are a more limiting resource to colony growth (Madsen and Offenberg, 2020). We found several amino acids and amino sugars to be key determinants of high investment level in the crop contents; however this was not the case for separating the phloem samples. This would be consistent with an aphid origin for these compounds, supporting the idea that aphids produce amino acids which the ants then harvest. Aphids use symbiotic gut bacteria to transform phloem sap (amino acid poor) and synthesise amino acids for their own use (Douglas, 1998), some of which are excreted in honeydew. With the aphid material being composed of whole individuals, it is higher in amino acids than pure honeydew would be, and this may account for why we do not see the same amino acids strongly defining high investment profiles in the aphid and crop content samples.

In the Phloem Sap, certain terpenoids are strongly associated with high investment trails. These secondary metabolites are produced by trees and usually have a role in defence against pathogens or herbivores (Singh & Sharma, 2015), or are used for signalling between plants and insect partners (Kappers et al., 2005). Ants are known to be attracted to herbivore-induced plant volatiles, which may help them detect aphid presence (Schettino et al., 2017). If we had seen terpenoids among the most important metabolites for separating high and low investment levels in Crop Content, and not only the Phloem Sap, this might have indicated that ants were attracted to terpenoids directly or attracted to the terpenoid as a signal of some other feature of quality, or of aphid presence. Infection control is highly important for colony-living species, where infections can spread rapidly. Wood ants collect resin, which originates from tree sap, and benefit from the antimicrobial compounds that the tree has produced (Castella, Chapuisat, & Christe, 2008, 2008; Chapuisat et al., 2007). We did not find evidence that wood ants are attracted to any plant defence compounds, as they are known to collect resin, they may not need to obtain these from honeydew.

Terpenoids are also not in the most important metabolites for separating high and low investment levels in Aphid Material. Aphid material is dominated by fatty amides due the presence of aphid tissue. If, with pure honeydew samples, we saw terpenoids separating high and low investment levels, this might have indicated that the aphids were processing these compounds before they reached the ants. As it is, higher investment trails are defined by more foraging ants, which would be expected to correspond with higher aphid load (although this has not been shown empirically as aphid load is difficult to measure (Hull & Grimm, 1983), more herbivory, and therefore we would expect heavily foraged trees to produce more anti-herbivory compounds.

4.1.2.2 What is associated with low investment trails?

P-cymene in the phloem is associated with low investment trails. This terpene is one of the most important aromatic compounds released by plants. It has antimicrobial properties (Marchese et al., 2017) and is associated with aphid resistance (Digilio et al., 2010). Its presence in phloem could result in reduced food value to the ants directly, if this terpenoid affects the aphids' ability to thrive and produce honeydew (Ahmed et al., 2021; Digilio et al., 2010), or indirectly, if this compound is an indicator of tree disease or other stress, which may cause the production of lower quality honeydew.

The only saccharide in the ant Crop Contents that was associated with *low* investment trails, was tentatively identified as melezitose. Aphids can upregulate melezitose to attract and retain ant-tending, affording them protection and hygienic services (Fischer & Shingleton, 2001; Kiss, 1981); however, compared to other species, *Formica rufa* group wood ants are not particularly attracted to melezitose (Madsen & Offenberg, 2020). In addition to ant attraction, melezitose production also has a drought protection function in aphids (Rhodes et al., 1997; Seeburger et al., 2022). Given that we found melezitose to be associated with low investment trails, this may indicate that the aphids on these trees are suffering from drought stress, which may signify that these are lower value trees overall, thus prompting low investment by the ants.

The presence of melezitose and certain amino acids and amino sugars, and the absence of terpenoids, in the crop content supports Hypothesis 3: “Changes in metabolomic content will occur from tree to aphid to ant” defining aphids as the processors in this foraging network.

4.1.3 Causation or attraction

Within this analysis framework, we must be cautious in our conclusions about metabolites associated with high or low investment trails. While the presence of a metabolite being characteristic of a high investment trail, the ‘over-grazing’ of high investment trails may cause tree stress responses (Rosengren & Sundstrom, 1991). As discussed above this is particularly likely for the secondary metabolites (terpenoids) found in Phloem Sap, which are often responses to stress (Salam et al., 2023).

4.1.4 The metabolomics method

The metabolomic approach has been very useful to open this area of study, allowing a broad picture of the contents of wood ant forager’s crops, and of the plant and aphid origin of these contents. However this approach is not without drawbacks. The metabolomic profiles include many unidentified features, even to a chemical class level. These unidentified features are unlikely to be a random subset of the profiles - they do not appear in reference databases due to database bias: what has been studied so far will have matches, but metabolites that are significant in the medical or agricultural fields are more likely to have been studied than those relevant to specific ecological systems like ours. Ecological metabolomics is a rich and complicated field with a lot to be discovered, so many molecules have no matches in any databases yet, and automatic identification tools are still in development (Alseekh & Fernie, 2018). Proteomics would be an appropriate follow-up method to allow for more precise identification, allowing the description of new metabolites to be added to databases. Metabolomics is also limited in quantification of each metabolite in different samples. Our results use presence and probabilities, but quantity of specific compounds is likely to be a vital part of these foraging network dynamics.

4.2 Future ideas

4.2.1 Snapshot problem- trail switching

Between mapping and sampling (5 weeks), four trail pairs had switched which were experiencing higher and lower investment in foraging by the ants. Although trail usage (in a binary sense) is highly consistent over the ants' active season (Burns et al., 2020), these switches suggest that the level of investment in a given trail can be highly plastic or seasonal (Domisch et al., 2011). This plasticity may be in response to a variation in resources provided by the tree (Sudd & Sudd, 1985) a change in colony needs, or a periodic upregulation of tree defence. This third hypothesis of tree switching to avoid upregulated plant defences is seen in leafcutter ants (*Atta*), where trees upregulate antifungal compounds to prevent total defoliation and the foraging leafcutter ants respond rapidly to the detection of these compounds by ceasing all foraging on this tree, and switching to alternative sources of leaves for their fungus farms (Kost et al., 2011). We may be seeing something similar here with wood ants, with a reduction in foraging on some previously heavily-used trees, either because the trees' defensive compounds are harmful to the ants themselves or to their tended aphid colonies.

In future a time series experiment would be valuable, to account for fluctuating trail investment, and repeating a time series over multiple years would enable us to detect whether the changes in investment result from predictable seasonal changes, e.g. in colony needs or from more short-term changes in environmental conditions, e.g. tree health, and aphid load. The upregulation of tree defence hypothesis could be tested by a more frequent time series tracking ant trail investment alongside measures of plant stress, such as visible tissue damage, reduction in growth, phloem sap sampling, and estimations of total aphid load. This would allow us to observe if the investment switch occurs gradually or immediately, and match investment changes to the other measures, such as levels of terpenoids within the Phloem Sap. Observations of aphid tending may also demonstrate the effect of upregulation of plant defences on ant-tended aphids - whether the ants respond to these changes, detectable in the honeydew or as airborne terpenoids, and relocate the aphids, or do the aphid colonies decline and the reduction in honeydew drives the ants to reduce investment in this food source.

4.2.2 Aphids

4.2.2.1 Aphid collection

The study of the ant tended aphids presented many challenges, the primary of which is that we know that all ant-visited trees from which honeydew is collected must have ant tended hemiptera; however we only found aphids on 16/24 of trees. This was mainly an accessibility issue - we were unable to access the canopies of mature trees (due to site limitations, budget, and safety concerns) where aphids often reside (Larsson, 1985; Straw et al., 2006); indeed we observed most of the ants travelling up and down the trunk from the canopy. The aphids are also inconspicuous: most ant-tended aphid colonies that were found, were located only after spotting ants tending the aphids. An important factor in the ant-tree foraging relationship is the aphid load on the tree. ‘Overgrazed’ trees, i.e. those with a high aphid load, may upregulate defence compounds which will modify their metabolomic profile. A measure of tree aphid-load would provide context for tree switching patterns. Although we have explored “quality”, it is possible that the quantity of honeydew also plays a role in ant trail investment, which total aphid-load may help predict. The assessment of aphid load in wild populations is challenging (Hull & Grimm, 1983). Better sampling could be achieved with the use of platforms (Platková et al., 2020) to access aphids higher in the trees, drones to take canopy cuttings (Charron et al., 2020), or aphid traps (A’brook, 1973). We are assuming that higher ant investment means higher aphid load, but this may not be the case. This should be tested.

We also assumed that the “quantity” of a given food source was broadly correlated to the size of tree (measured by diameter), but as techniques advance it would be good to have estimations of aphid abundance on each tree. As mentioned, aphid load is very difficult to estimate visually. The clonal nature of aphid colonies may present a unique challenge if trying to estimate this based on genetics. With better data on aphid population sizes, we could find that this drives ant investment.

4.2.2.2 Aphid species

Due to the bias in tree search location (lower branches only) it is very possible that we missed some aphid species that contributed to crop content, as different species of aphid reside on different parts of trees (Platková et al 2020). However our data suggests that aphid species is not as important as tree species, since our aphid samples are made up of whole aphids, and we did not see clear metabolomic separation by species (Figure 5).

Future work could use eDNA or proteomic approaches to study genetic diversity of aphids on a tree, based on the crop contents of descending ants. This would require genome sequencing of more aphid species than are available. There is a lack of aphid genomes within myrmecophilous genera, as genome sequencing efforts have been focussed more on agriculturally significant aphid species.

4.2.2.3 Honeydew methods

Despite trying multiple methods (see section 2.2.3 of methods), it was not possible to directly sample honeydew from the aphids in this study. Honeydew has been collected from non-ant-tended aphids by placing a collection tray under host plants to collect excreted honeydew as it drips down (Ammar & Hall, 2011; Pathak et al., 1982). In contrast, ant-attended aphid species retain their honeydew until stimulated by an ant, making collection much more difficult. To fully understand the processing of Phloem Sap provided by the Aphids and their gut bacteria, novel ways of honeydew extraction should be explored (e.g. extraction with a micro-syringe), allowing a more direct comparison of the ant crop contents with the honeydew they collect.

4.2.3 Experiments - feeding trials and translocations

In order to distinguish between causality of tree stress metabolites on highly grazed trails, and ants' preference for certain metabolites driving selection of certain trees for high investment, significant metabolites could be isolated for use in ant feeding trials. As a starting point we would recommend D-Glucose, as well as a range of amino acids and amino sugars. Further analysis and proteomics would help elucidate particular amino acids. Additionally, terpenoids and melezitose could be tested as deterrents.

As the translocation of Red Wood Ant colonies becomes popular as a land management strategy, studies of their success and effect on the environment should include comparisons of food quality from the donor and translocation sites. While tree and aphid species may remain the same, metabolomic profiles could be significantly different. This would also allow for measurements of the effect of new grazing by ant-tended aphids on the metabolomic profile of Phloem Saps.

4.2.4 Other tree species

We studied these species because they were the most common at our study site, but future studies should look at Norway spruce *Picea abies* and sitka spruce *Picea sitchensis* which are key forage trees for wood ants across much of Europe (Stockan & Robinson, 2016) but are not present at our study site. In particular comparing the food collected by the ants from the UK native spruce (*Picea abies*), to that which they collect from the species introduce for forestry *(P. sitchensis*) would be informative, especially as these two spruce species share several species of myrmecophilous aphids (*Cinara piceae, Cinara pruinose, Cinara pilicornis*) (Breen, 1976, 2013; Cherix et al., 2006; Johansson & Gibb, 2012; Novgorodova, 2004; Rosengren & Sundstrom, 1991; Wellenstein, 1952)

4.2.5 Other variables

Many other variables are likely to be affecting these ecological networks and the definition of quality (Figure 1).

Further study is needed into ant microbiomes - honeydew foraging ants have complex microbiomes to help digest sugars (Kautz et al., 2013), so Crop Content samples may already be somewhat processed by the ants. Obtaining pure honeydew samples would elucidate this processing question, as would targeted study of bacterial diversity within the foragers on trails of different investment levels.

Pollutants (e.g. agricultural pesticides, traffic pollution) may enter this network through trees (Li, 2025), and could have detrimental effects on the ant populations as well as their tended aphids. The polydomous nature of *Formica lugubris* would make it particularly susceptible to the spread of pollutants within a population, and the spread of a toxin into the soil. Checking for pollutants in Crop Contents and Honeydew would allow us to test whether pollutants come through this ecological network from the Phloem Sap to the ants.

4.3 Takeaways for management

Red Wood Ants are a keystone species group in both natural woodlands and managed forestry sites, therefore any change in their behaviour could have significant effects on the rest of the habitat, so understanding how they use different tree and aphid species should be a priority.

Our results show that different tree species provide different resources to honeydew foraging ants, even within tree genera and when the same aphid species is tended on two tree species (as seen in *Quercus robur* and *Q. petraea* with *Thelaxes dryophila*). Therefore, a mix of tree species may be beneficial to Red Wood Ant populations.

Phytophthora ramorum, a ‘water mould’ which infects *Larix* trees, is resulting in the removal of larch from many UK managed woodlands, as a biosecurity measure; our results suggest that, in general, Larch is not providing any uniquely significant resources to the ants, so providing that they have access to other suitable tree species, colonies would not be overly affected by the removal of Larches.
Certain individual trees, across species, seem more attractive to wood ants than others, and we see a signature of different metabolites associated with high levels of foraging. However, the observation that relative trail strength was quite variable suggests that a single time-point survey of which trees are 'most important' to a nest or colony might not give a full picture, and different individual trees may be more or less foraged over the course of the ants' active season (Burns et al., 2020). If deciding at a site which trees are most used by the wood ants, surveys should be carried out multiple times, to at least capture seasonal changes. Wood ants may use this investment plasticity to respond to environmental conditions that affect tree phloem composition (like droughts), or as part of a cycle of tree switching based on aphid exploitation and upregulation of defence compounds.

Within the trees included in our study were some young trees (<10 years); our data indicate that even young saplings can be the destination for high investment foraging trails, and thus that newly planted trees do not have to reach maturity before providing valuable resources to wood ant colonies.

Quantifying the trade-offs animals make is vital to furthering our understanding of how they use their environments and what effects future changes in the habitat may have on their behaviour and survival. This can help us mitigate the impact of management decisions and climate change on ecosystems.

4.4 What does this tell us about Quality

From these data, we can say that some trees are more attractive sources of honeydew than others to Red Wood Ant colonies. These attractive food sources have distinct metabolomic profiles that are consistent across four different tree species. Trails that colonies invest many foragers into are defined metabolomically by the presence of D-Glucose across the Phloem

Sap, Aphid Material, and Crop content, while other sugars are also present. This indicates a potential preference for glucose by *Formica lugubris* at this site. We found melezitose to be characteristic of low investment trails, which corresponds with the findings of Madsen and Offenberg (2020) that Red Wood Ants are not attracted to this aphid-produced sugar. We also found amino acids and amino sugars to be defining of high investment trails in the crop contents, which we predicted based on past literature of ant preference for foods rich in amino acids (Blüthgen & Fiedler, 2004; Madsen & Offenberg, 2020). We can conclude that specific sugars, and general amino acid presence, define quality for wood ants. The dynamics of this network require more investigation – why are some tree-aphid pairs producing different resources, across species? Does the plasticity in trail investment arise from changes in resources available, or changes in resource needs?

These results are likely applicable other homopteran-tending ant species, although other species are known to be attracted to melezitose (Detrain et al., 2010; Duckett, 1974), which aphids produce to retain tending behaviours. If other species are most attracted to melezitose, we would not expect to see the plasticity we have observed in *F.lugubris* foraging trails to be as pronounced in other species. The metabolomics method has proved an excellent tool for this area of study and should be applied to other ant foraging networks.

More broadly, we find that patch quality is extremely complex; ants investing more in certain trees was the only evidence that these trees provided different resources, we might have assumed that trees of the same species, age, location, and aphid mutualist would be similar patches. More empirical evidence is needed to define patch quality across species, as we move beyond a purely energetic approach to Optimal Foraging Theory. The behaviours we observe are shaped by multiple interacting factors, and even something as apparently ‘simple’ as choosing between two equidistant trees is full of complexity. With fast developing technologies and databases allowing more advanced and accessible analyses, such as the metabolomics tools we have used here, resource quality can be investigated in all study systems to better understand ecological networks.

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