COLONY-MATE RECOGNITION IN THE WEAVER ANT

OECOPHYLLA SMARAGDINA

Thesis submitted by

Philip Simon NEWEY Ph.D. (Flinders University), B.Sc (Hons.) (JCU)

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Statement on the Contribution of Others

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Abstract

Understanding recognition systems is at the heart of a range of evolutionary, biological and social processes, including the immune response, reproductive barriers, mate choice, kin selection and the evolution of parasitism. Among social insects, nestmate or colony-mate recognition may evolve as a proxy for kin recognition, as social insect colonies usually consist of a family group. I sought to advance our understanding of recognition systems by studying colony-mate recognition in the arboreal weaver ant, *Oecophylla smaragdina*. In particular, I explored the effects of spatial and temporal variation in recognition cues, and variability in the capacity of individual ants to recognise non-colonial conspecifics, on the effectiveness of recognition systems. I used a novel technique for studying colony odour: near-infrared reflectance spectroscopy (NIRS). I found that colonies of weaver ants had distinctive spectral profiles, but that there were also significant differences in the profiles of nests within colonies. Significantly, the spectral characteristics differentiating colonies from each other and nests from each other were different. The level of aggression between colonies was positively correlated with the spectral distance between colonies, especially when only those spectral characteristics that differentiated between colonies were used to calculate spectral distance. I also found that the spectral characteristics of colonies changed over time. However, the spectra of a colony and an isolated nest from that colony did not diverge significantly over time, suggesting that these spectral changes may reflect genetically programmed seasonal changes. I detected no increase in aggression over time between colonies and their corresponding isolated fragment; however, the level of trophallaxis did increase. Neither spatial nor temporal variation in colony odour appears to impair the effectiveness of colony-mate recognition in weaver ants. I also explored the effect of spatial relationships on the levels of aggression expressed by weaver ant colonies. Specifically, I tested whether weaver ants were more aggressive towards intruders from distant colonies or from neighbouring colonies. I found that weaver ants were better at identifying intruders from neighbouring colonies as non-colony-mates than intruders from distant, unfamiliar colonies. They were also significantly more aggressive towards neighbours. Thus weaver ants conform to the "nasty neighbour" model rather than the "dear enemy" model. Finally, I sought to determine whether the variability in the response of individual workers towards intruders could be attributed to the recipients or the intruders. I found that most of the variability could be attributed to differences between recipients. I further demonstrated that this variability was sometimes due to differences in the response of workers but also, in some case, due to differences in the perception of workers. I hypothesise that workers do not use the colony odour as the template against which intruders are assessed, but, rather, their individual odour prior to any mixing. This is the first study to explore colony odour using NIRS, and the first to demonstrate a behavioural response by any insect to the information contained in NIRS. This

has the potential to significantly advance social insect studies, and the study of insect behaviour in general. This is the first study to demonstrate parallel changes in odour in nests isolated from their colony of origin, contributing significantly to our understanding of how very large colonies, spread over a wide geographic area, can maintain a single colony identity. The identification of different spectral elements that differentiate between nests and colonies also contributes to this understanding, and indicates that NIR spectra carry multiple signals. Also important in this regard is the finding that differences in some aspects of spectra provoke a stronger response than others. This is also the first study to demonstrate that different individuals within a colony vary in their perceptions, and not just their response, when encountering an unknown individual. Further work remains to be done in determining how weaver ants learn to identify neighbours as a serious threat and how the behaviour of the colony is modified accordingly. Research also needs to be undertaken into the genetic basis of colony spectra, and the relationship between spectra and cuticular hydrocarbons.

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1 Introduction

1.1 Recognition Systems

Distinguishing self from non-self (Tsutsui 2004) is at the heart of a range of evolutionary, biological and social processes, including the immune response, reproductive barriers, mate choice, kin selection and the evolution of parasitism (reviewed in Mateo 2004). When one individual encounters another, it is faced with the task of identifying the other, assessing its status and developing an appropriate response. In the context of immunity, this involves the body's defence system differentiating between the self and dangerous pathogens. In the context of reproduction, this requires the ability to recognise another individual as self, in the sense of belonging to the same species, but non-self in the sense of belonging to the opposite gender and, to avoid inbreeding, of not being close kin. Kin selection also presupposes the ability to differentiate kin (self) from non-kin (non-self). Finally, parasitism involves the ability of non-self to disguise itself as self. Thus the inclusiveness of selfhood may vary with the context: it may refer to the individual organism, the kin group or the species. Among human beings, selfhood may at various times refer to the individual, the family, the tribe, the nation, the race or the species.

Recognition systems involve the use of a range of visual, auditory, chemical or tactile cues (Sherman et al. 1997). For example geographic variations in bird vocalization can generate reproductive barriers resulting in speciation (Brambilla et al. 2008). Closely related species of frogs can be differentiated on the basis of the acoustic parameters of their calls (Padial et al. 2008). Some butterfly species use both chemical and visual cues when selecting a mate (Costanzo and Monteiro 2007). The use of chemical or olfactory cues is particularly widespread among a range of taxa, including mammals (reviewed in Brennan and Kendrick 2006; Brown and Eklund 1994), fish (for example, Ward et al. 2007), lizards (for example, Hayward and Mouton 2007; Punzo 2008) and birds (reviewed in Rajchard 2007). Chemical cues play a crucial role in insect recognition systems (reviewed in Howard and Blomquist 2005; Singer 1998). For example, appropriate mate selection may depend on chemical differences between closely related species (Mullen et al. 2008), or between sexes within species (Dapporto 2007; Liimatainen and Jallon 2007). Differences in chemosignals are also important for maintaining the integrity of social insect colonies.

Among eusocial insects (some bees and wasps, and all ants and termites) the colony constitutes another level of self. The colony often consists of a family unit, although various levels of polygyny (several queens) and polyandry (several mates per queen) can significantly

reduce the degree of intra-colonial relatedness. In a colony with a single, once-mated queen, the workers are full sisters. If the queen has mated with more than one male, then workers will be a mixture of full- and half-sisters. With more than one queen (each possibly multiply-mated), average relatedness within the colony may be very low. Nevertheless, within a colony some individuals are more likely to be full- or half-sisters than between colonies, and most colonies work together as a unit, defending the colony from other conspecifics. In this context, colony-mate recognition may evolve as a proxy for kin recognition (Tsutsui 2004). I use the term "colony-mate" here rather than "nestmate" because ant colonies may consist of many nests (polydomy).

The odour, or chemical profile, of a colony generally consists of a single "gestalt" (sensu Crozier and Dix 1979), or holistic representation, that results from the exchange of chemicals throughout the colony. This exchange usually takes place via trophallaxis (Boulay et al. 2000), or allogrooming (Lenoir et al. 2001; Soroker et al. 1998). These chemical cues may have a genetic origin (Dronnet et al. 2006; Foitzik et al. 2007; Lahav et al. 2001; Stuart 1988), but they may also derive from dietary components (Buczkowski et al. 2005; Buczkowski and Silverman 2006; Liang and Silverman 2000; Richard et al. 2007), or environmental sources such as nesting materials (Couvillon et al. 2007; D'Ettorre et al. 2006). Furthermore, they may change over time, as has been demonstrated in several ant species (Dahbi and Lenoir 1998; Lenoir et al. 2001; Liu et al. 1998; Nielsen et al. 1999; Provost et al. 1993; Suarez et al. 2002; Vander Meer et al. 1989). This variability raises questions about their reliability as recognition cues.

The overall aim of this project was to advance our understanding of self/non-self recognition systems by exploring colony-mate recognition in the polydomous weaver ant *Oecophylla smaragdina* F. (Hymenoptera: Formicidae). I was particularly interested in the variability in time and space of potential recognition cues, and the variability in response to those cues among individuals. The former is concerned with the expression component of the recognition system, while the latter is concerned with the perception and response components of the recognition system. The specific objectives were:

1. to explore spatial variation in colony odour between and within weaver ant colonies;
2. to explore temporal variation in colony odour;
3. to determine the response of workers to variations in colony odour;
4. to determine whether spatial relationships between colonies have an impact on recognition; and
5. to explore the basis of individual variation in recognition skills.

1.2 Study Species

Oecophylla smaragdina is a formicine ant found throughout south and south-eastern Asia and northern Australia (Azuma et al. 2006). Its only living congener, *O. longinoda*, is restricted to tropical and subtropical Africa (Azuma et al. 2006). Both species are arboreal, constructing nests from leaves, using silk derived from larvae to bind leaves together. Colonies of *O. smaragdina* can be very large, spanning several trees and with reportedly up to 500,000 workers (Hölldobler and Wilson 1990).

Oecophylla smaragdina is important in both natural and agricultural systems. It constitutes a major component of the ant mosaic in primary rainforest in south-eastern Asia (Davidson et al. 2007), and is one of two dominant arboreal species in the wet tropics of northern Australia (Bluethgen and Stork 2007). It also plays an important role in mangrove ecosystems, protecting trees from herbivory (Offenberg et al. 2004; Offenberg et al. 2006). Further, it is also an important biological control agent, effective in controlling several pests in mango orchards (Peng and Christian 2007; Peng and Christian 2004, 2005, 2006), cashew plantations (Peng et al. 2005; Peng et al. 1999), citrus orchards (Van Mele and Cuc 2000; Van Mele et al. 2002), coconut plantations (Kumaresan 1996) and cocoa plantations (Way and Khoo 1989, 1991). Finally, in parts of south-east Asia, weaver ant queen larvae and pupae are an important source of food and income (Sribandit et al. 2008).

Within the study area in far northern Queensland, colonies are predominantly monogynous (Schlüns et al. 2008), although in the Northern Territory colonies are reported to be polygynous (Peng et al. 1998; Schlüns et al. 2008). I did not find any evidence for more than one nest foundress (pleometrosis) (cf. Peeters and Andersen 1989) within the study population: every newly dealate queen that I located during the course of this study was alone with her brood. Recent research has shown that queens mate with from one to five males (Schlüns et al. 2008).

Weaver ants provide an ideal subject for studying recognition systems for several reasons. First, they form very large polydomous colonies, in which maintaining a single colony odour may be problematic if this depends on the continuous exchange of chemicals between workers. Second, their monogynous status means that maintaining colony integrity is essential for the survival of the colony. Colony budding and queen replacement do not occur in this species, so the breakdown of the colony gestalt and fragmentation of the colony would seriously threaten its survival. Third, queen mating frequency is variable, resulting in different numbers of patrilines within colonies. If individual odour has a genetic basis this may have an impact on the ability of different colonies to maintain a single odour. Finally, weaver ants are aggressively territorial, so recognising conspecific aliens is crucial for colony defence.

1.3 Thesis Structure

The thesis is divided broadly into three sections. The first section consists of introductory material, including this Introduction, a brief chapter (Chapter 2) reviewing research into nestmate or colony-mate recognition in social insects, and a chapter (Chapter 3) reviewing the application of near-infrared reflectance spectroscopy (NIRS) to questions in ecology and evolutionary biology. This chapter is included because I make extensive use of this methodology throughout the study, but many of those involved in the research of social insects may be unfamiliar with it. The current thesis involves a novel application of this methodology, and Chapter 3 places this usage within a broader context.

The second section, the bulk of the thesis (Chapters 4 to 9), consists of a series of research projects, each of which is relatively self-contained, but progresses fairly naturally to the next. Chapters 4 and 6 relate to my first objective: to explore spatial variation in colony odour between and within colonies. In these chapters I identify the spectral characteristics that differentiate between colonies, and between nests within colonies, of weaver ants. Chapter 5 relates to my third objective: to determine the response of workers to variations in colony odour. I determine the relationship between the level of aggression between colonies and the spectral distance between them. In Chapter 7 I address my second objective: to explore temporal variation in colony odour. This also touches on my third objective as I examine the way in which these temporal changes impact on behaviour. I address my fourth objective in Chapter 8: to determine whether spatial relationships between colonies have an impact on recognition. Specifically, I test the "dear enemy" hypothesis, which suggests that levels of aggression might be greater between strangers than neighbours. I also investigate the potential of workers to become familiar with the odour of previously unknown colonies. Finally, in Chapter 9, I address the fifth objective: to explore the basis of individual variation in recognition skills. I was particularly interested in determining whether the variation observed in the behaviour of weaver ants towards conspecific intruders could be attributed primarily to the recipient or the intruder.

The final section of the thesis consists of a general discussion (Chapter 10), in which I present a review of the thesis as a whole and suggest some directions for future study, the References and the Appendices.

1.4 Note on Methods

Most of the studies within the thesis involve the acquisition and analysis of NIR spectra, and behavioural bioassays. Although the methods employed for each chapter are similar, there are differences as I refined and modified these techniques over the course of the

study. Thus, for example, there are differences in the way spectra were analysed between Chapters 4 and 6, and there are variations in the behavioural bioassays employed in Chapters 5 and 9. The chapters are not presented in precisely chronological order, so that a later methodology may appear in an earlier chapter.

Although I will not go into detail here concerning the methods employed in the study, one particular technique requires some discussion. The conventional method for exploring colony odour and related behavioural issues is to extract CHCs from the cuticles of individuals and analyse them using Gas Chromatography/Mass Spectroscopy (GCMS). This is a lengthy and relatively expensive procedure. Throughout this study I use an alternative method for determining colony odour, namely NIRS. This involves generating absorption spectra of wavelength and intensity in the near-infrared, from about 800 to 2500 nm (4000 to 12500 wavenumber per cm). Functional groups in molecules have characteristic vibration frequencies within certain sections of this range (Scarff et al. 2006), and this provides a broad picture of the chemical "signature" of any scanned sample. The method lacks the chemical specificity of GCMS, but is attractive because it is rapid and inexpensive: a specimen can be scanned in less than a minute. This makes behavioural and ecological studies of the kind undertaken here much easier and more affordable.

Does NIRS actually detect colony odour? The term "colony odour" is itself somewhat inexact in that odour refers to airborne particles rather than to the relatively non-volatile compounds on the insect cuticle. The term should probably be replaced with a term such as "colony chemical profile". However, Dowell et al. (1999) showed that cuticular hydrocarbons (CHCs) and other cuticular lipids and compounds could be linked to the spectral characteristics of grain beetles. Aldrich et al. (2007) also demonstrated that CHCs could account for most of the spectral variation between termites in the genus *Zootermopsis*. It is reasonable to assume that NIRS is detecting chemicals on the weaver ant cuticle that are commonly associated with the term "colony odour". While these almost certainly include compounds other than CHCs, it is far from certain that only CHCs are involved in the recognition process. More volatile compounds, adsorbed in the cuticular hydrocarbon layer, have been implicated in the recognition process of some leaf-cutter ants (Atta spp.; Hernandez et al. 2006). Oxygenated compounds may be involved as recognition cues in some bees and wasps (Dani 2006). It is worth noting that the use of non-polar solvents in the extraction process is more effective at removing hydrocarbons than more polar compounds (Dani 2006). This may result in a bias towards CHCs in the study of potential recognition cues. Throughout this thesis I use the term "odour" as shorthand for "chemical profile". NIR spectra are clearly related to this chemical profile, although the precise relationship in the case of weaver ants is yet to be determined.

I frequently used the terms "information" and "signal" when referring to spectra or to CHCs. By this I mean identifiable patterns in spectra or identifiable relationships between

spectra and other phenomena or entities. In this context, a signal is something that carries information. In the first instance, a signal is perceived by the researcher, and this does not imply that it is also perceived as such by the organism under investigation. Only additional research can determine that.

1.5 Note on Publications

Several chapters of this thesis have already been published. They are:

- Chapter 4 as:
  Newey, P.S., Robson, S.K.A. & Crozier, R.H. (2008) Near-infrared spectroscopy identifies the colony and nest of origin of weaver ants, *Oecophylla smaragdina, Insectes Sociaux* 55(2): 171-175. doi: 10.1007/s00040-008-0985-6
- Chapter 5 as:
  Newey P.S., Robson S.K.A. and Crozier R.H. (2008). Near-infrared spectroscopy as a tool in behavioural ecology: a case study of the weaver ant, *Oecophylla smaragdina*. *Anim. Behav.* **76:** 1727-1733. doi:10.1016/j.anbehav.2008.07.025
- Chapter 6 as:
  Newey, P.S., Robson, S.K.A. & Crozier, R.H. (2009) Nest and colony specific signals in the weaver ant *Oecophylla smaragdina, Insectes Sociaux* 56(3): 261-269. doi: 10.1007/s00040-009-0019-z
- Chapter 7 as:
  Newey P.S., Robson S.K.A. and Crozier R.H. (2009). Temporal variation in recognition cues: implications for the social life of weaver ants *Oecophylla smaragdina*. *Anim. Behav.* **77:** 481-488. doi: 10.1016/j.anbehav.2008.11.003

These publications have been modified only to minimise unnecessary repetition, and to ensure a consistent style throughout the thesis. The pronoun “we” in the co-authored papers has been replaced with “I”, and Figures and Tables have been renumbered throughout. References within any of these publications to the others have been replaced with references to the appropriate chapters of the thesis.

2 Colony-mate recognition in ants

Among eusocial insects, including ants, the ability to recognise colony-mates is essential for maintaining the integrity of the colony. It is usually indicated by a behavioural response, generally agonistic, towards conspecific intruders, although this is not always the case (Steiner et al. 2007). The lack of such a response does not necessarily imply a lack of recognition (Holmes 2004); there may be circumstances when attacking a perceived intruder is not worth the cost, particularly if this involves the death of the defender. Therefore, it is important to distinguish between recognition and discrimination, the latter being a differential behavioural response directed towards non-colonial conspecifics.

In this chapter I will review some of the research into nest- or colony-mate discrimination in the eusocial Hymenoptera, with a particular focus on ants. I will concentrate on research that has been conducted since ca. 1970. For reviews of earlier research see Wilson (1971) and Breed and Bennett (1987).

2.1 Colony Odour

A colony-mate recognition system can be broken down into three basic elements: expression, perception and action (Tsutsui 2004). Among eusocial insects it is generally acknowledged that the expression component consists of an odour that is particular to each colony (Crozier and Pamilo 1996; Hölldobler and Wilson 1990; Wilson 1971). Furthermore, it is increasingly evident that cuticular hydrocarbons (CHCs) constitute a key component of this (Akino et al. 2004; Dani 2006; Denis et al. 2006; Dronnet et al. 2006; Errard et al. 2006; Howard and Blomquist 2005; Lahav et al. 1999; Van Wilgenburg et al. 2006). These chemicals may derive from dietary components (Buczkowski et al. 2005; Buczkowski and Silverman 2006; Liang and Silverman 2000; Richard et al. 2004; Richard et al. 2007), or environmental sources such as nesting materials (Couvillon et al. 2007; D'Ettorre et al. 2006; Singer and Espelie 1996); or they may have a genetic origin (Dronnet et al. 2006; Foitzik et al. 2007; Lahav et al. 2001; Stuart 1988). They may arise from a combination of these factors: in the Argentine ant, *Linepithema humile*, for example, there appears to be both a dietary and genetic component to colony odour, although the influence of the former may be fairly weak compared to that of the latter (Suarez et al. 2002). In some cases, the queen may also play a significant role in determining colony odour (Provost 1989).

Two main models have been proposed for understanding colony odour: the “gestalt” model, and the “individualistic” model (Crozier and Dix 1979). According to the gestalt model, individuals continually exchange chemical cues with other individuals, usually via trophallaxis

(Boulay et al. 2000) or allogrooming (Lenoir et al. 2001; Soroker et al. 1998). This results in a more or less uniform odour across the colony, consisting of a blend of individual odours. According to the individualistic model, each individual retains its own odour, with little or no exchange taking place. The colony odour therefore consists of a greater or lesser variety of odours depending on the level of genetic and/or environmental diversity within the colony.

The gestalt model is now widely accepted as the general rule among eusocial insects (Lenoir et al. 1999). Crosland (1989), however, argued that the individualistic component was more important in colonies of *Rhytidoponera confusa*. Furthermore, many aggression bioassays reveal a range of responses by individuals towards the same intruder. Boulay et al. (2000), for example, argued in favour of the gestalt model for *Camponotus fellah*, despite reporting that upon re-introduction to their colony of origin, workers that had been isolated from their colony for up to 40 days could be attacked by one worker and simultaneously solicited for trophallaxis by another. Some individuals appear to make recognition errors (see below) when confronted with an intruder, while others do not, suggesting some degree of variation in the templates against which intruders are assessed. Furthermore, some non-self individuals from another colony are incorrectly recognised as self while others are not. This suggests some degree of variation in the signals borne by colony members, as well in the templates against which they are assessed.

In large colonies, perhaps with multiple queens and/or multiple queen-mating, or with heterogeneous microhabitat conditions across the range of the colony, complete mixing of the colony odour might be difficult to maintain. In the polydomous Australian meat ant, *Iridomyrmex purpureus,* there appear to both colony specific and nest (within colony) specific chemical signals (Van Wilgenburg et al. 2006). There is need for additional studies on other species with large, polydomous colonies to determine whether this phenomenon is widespread.

It is becoming increasingly evident that colony odour is not fixed, but changes over time. This has been demonstrated in several ant species, including *Solenopsis invicta* (Vander Meer et al. 1989), *Temnothorax lichtensteini* (Provost et al. 1993), *Cataglyphis iberica* (Dahbi and Lenoir 1998), *Formica truncorum* (Nielsen et al. 1999), *Aphaenogaster senilis* (Lenoir et al. 2001), and *Linepithema humile* (Suarez et al. 2002). If there is imperfect exchange of CHCs between nests within a polydomous colony, complete isolation could eventually lead to incompatibility between nests if the odours of separated segments diverge sufficiently. In polygynous colonies, this could result in colony budding, but in monogynous colonies this is not possible. Either sufficient exchange takes place between nests to prevent this, or there is a significant genetic component maintaining sufficient similarity between odours. This also requires further study of both monogynous and polygynous polydomous colonies.

2.2 Recognition Template

The recognition template involves the template itself, and the referent upon which it is based. The latter may be the self, the gestalt colony odour (in the gestalt model), or the diverse odours of individuals (in the individualistic model). In the gestalt model, self odour and colony odour amount to the same thing.

It is often hypothesised that colonies with greater genetic diversity will possess a broader colony odour, and a correspondingly broader recognition template (Breed and Bennett 1987), which in turn will make them more tolerant towards alien conspecifics. In support of this hypothesis, Pirk et al. (2001) found that aggression was positively correlated with the intra-nest relatedness of recipient colonies in *Formica pratensis*. Starks et al. (1998) reported that monogynous colonies of *Pseudomyrmex pallidus* were more aggressive than polygynous colonies. Monogynous species of acacia ant (also *Pseudomyrmex* spp.) in Mexico were found to be aggressive towards conspecifics from other colonies, whereas polygynous species were not (Janzen 1973). In contrast to this, Rosset et al. (2007), found that aggression did not vary significantly with queen number in colonies of the ant *Formica selysi*; and the wasp *Polybia paulista* was found to be very aggressive towards intruders despite a high degree of polygyny (Kudo et al. 2007).

Another factor thought likely to contribute to a broad recognition template is polydomy (Van Wilgenburg et al. 2006). There are at least two reasons for this. First, individual nests within colonies may be subject to variations in microenvironment and/or diet. Secondly, exchange of hydrocarbons between nests may be less efficient than within nests. A third factor, rarely considered, is the possibility that nests may constitute small subfamily groups (differentiated, for example, along matrilines or patrilines) within the colony. Van Wilgenberg et al. (2006) demonstrated that ants from polydomous colonies of the Australian meat ant, *I. purpureus*, could be correctly assigned to one of two nests within the colony on the basis of their CHC profile, and, in addition, that workers from polydomous colonies were less aggressive towards intruders than ants from oligodomous colonies. In contrast, Pirk et al. (2001) detected no difference in the level of aggression expressed by polydomous and monodomous colonies of *Formica pratensis*, although they did not determine CHC profiles.

Something that has not been considered, as far as I am aware, but which would seem reasonable, is that a broader recognition template may result in a more variable, rather than a uniformly lower, aggressive response. With a narrow template, intruders are likely to fall consistently outside acceptable parameters. With a broad template, intruders may fall within or outside acceptable parameters; but there is no particular reason to believe that those that fall outside will be treated less aggressively by a colony with a broad template than one with a narrow template.

2.3 Recognition Errors

It is apparent that colony members do not always respond to alien conspecifics in an aggressive manner, but it is rarely clear whether this is due to a failure of recognition or a lack of discrimination. However, within an evolutionary context this distinction may not be crucial, as the resulting behaviour, and therefore the fitness benefits or costs, are likely to be the same. Therefore I will use the term "recognition errors" to cover both eventualities.

Inappropriately accepting non-self as self is, according to Reeve (1989), analogous to a Type II statistical error. While this appears to be quite common among Hymenoptera, Type I errors (inappropriately rejecting self as non-self) seem to be quite rare. Both types of error presumably incur fitness costs; but it appears that selection has generally favoured an acceptance threshold (Reeve 1989) that permits relatively high levels of Type II errors, but almost no Type I errors. A high threshold makes sense, particularly for large colonies, as encounters with self are much more likely than encounters with non-self (Reeve 1989). However, this would differ for individuals at the edge of the colony, compared to those at the centre, and for foraging workers, compared to brood workers. It may also vary seasonally if colonies expand outwards at certain times of the year, increasing the probability of encountering workers from other colonies.

Selection for an acceptance threshold favouring Type II over Type I errors seems to be favoured under some conditions. The amazing success of the introduced Argentine ant, *Linepithema humile*, seems to be attributable in part to the formation of large supercolonies, which can only occur because very little aggression occurs between introduced colonies, even when they are widely separated both geographically and genetically (Giraud et al. 2002). A lack of intraspecific aggression appears to give this ant a distinct competitive advantage (Holway et al. 1998). Nestmate recognition clearly occurs in another unicolonial species, *Formica paralugubris*, but without giving rise to aggression (Chapuisat et al. 2005). Steiner et al. (2007) demonstrated that *Lasius austriacus* workers could distinguish between self and non-self (as indicated by lengthier periods of antennation of non-self compared to self) but exhibited no aggressive behaviour towards non-self. Workers of the leaf cutter ant *Acromyrmex subterraneus molestans* did not appear to discriminate between workers from their own or another colony (de Souza et al. 2006). In this instance there seemed to be little variation between the CHC profiles of colonies (de Souza et al. 2006). Selection against discriminatory behaviour might follow three paths: (i) loss of the capacity to differentiate self from non-self; (ii) loss of the signal carrying such information; or (iii) loss of a differential response to individuals carrying different signals. Only in the first case is there, strictly speaking, selection in favour of Type II errors, but the result is the same in each case: the absence of discriminatory behaviour.

It has been argued (Steiner et al. 2007) that selection for the maintenance of clear boundaries between self and non-self may be weakened when there is little competition for resources between colonies. *Lasius austriacus* tends mealy bugs inside the nest, and these can be tapped as a permanent food source, reducing the need for long distance foraging, and limiting intercolonial contact (Steiner et al. 2007). However, it is not clear whether this hypothesis would apply to supercolonies such as those of *Linepithema humile*, and additional research is required in this area.

2.4 Dear Enemy

Aggressive behaviour is sometimes reduced between neighbour colonies (Heinze et al. 1996; Langen et al. 2000), due to a phenomenon known as the "dear enemy" effect. This avoids costly battles between neighbours. Some apparent examples of this phenomenon may not be quite what they seem. *Acromyrmex octospinosus*, for example, shows more aggression towards intruders from distant colonies than towards intruders from neighbour colonies, but this might be attributable to neighbours foraging for similar plant material, resulting in a similar colony odour, rather than the dear enemy effect *per se* (Jutsum et al. 1979). The dear enemy effect may also be difficult to distinguish from the effects of genetic relatedness if near neighbour colonies are closely related through colony budding (Pirk et al. 2001). It is likely that the dear enemy effect is a result of habituation (Langen et al. 2000), indicating that mature workers are able to learn new chemical signatures when exposed to them.

Sometimes more aggression is shown towards neighbours than towards more distant conspecifics, the opposite of the dear enemy effect (Gordon 1989; Knaden and Wehner 2003; Sanada-Morimura et al. 2003; Thomas et al. 1999; Thomas et al. 2007). It could be argued that neighbours pose a more real and immediate threat than others, particularly if there is severe competition for resources. It should be noted that aggression between neighbours can become ritualised in order to avoid series casualties on either side, as in the case of the honeypot ant *Myrmecocystus mimicus*: during encounters with alien conspecifics, workers walk on stilt legs while raising their abdomens and heads (Lumsden and Hölldobler 1983; cf. Thomas et al. 1999), although sometimes pitched battles can occur between neighbouring colonies of *O. smaragdina*, resulting in large numbers of casualties (Hölldobler 1983b). In some species, such as *Camponotus cruentatus*, intruders attract the same level of aggression regardless of the proximity of their colony of origin to the recipient colony (Boulay et al. 2007; cf. Dahbi et al. 1996).

2.5 Conclusion

There are many questions concerning colony-mate recognition that remain unanswered, or are only partially answered, particularly with regard to extensive, polydomous colonies. These include: the extent to which colony odour is shared among different nests; the extent to which a single colony identity can be maintained despite intra-colonial variation in odour; how colony identity can be maintained in spite of temporal variations in odour; whether workers are able to differentiate between, not only self and non-self, but also different categories of non-self; the extent to which intra-colonial aggression is a learnt behaviour. In what follows, I will be exploring some of these questions with regard to colony-mate recognition in *O. smaragdina.*

3 NIRS as a tool in ecology and evolutionary biology

Abstract

Near-infrared spectroscopy (NIRS) is widely used in the food and agricultural industry to ascertain food quality, to assess the quality of pasture and animal feed, to detect diseases and pests, to identify pest species, and to determine the geographic origin of food products. Here I review the application of NIRS to studies in the field of ecology and evolutionary biology. I find that its application to the analysis of plant nutrients and secondary metabolites, and to the analysis of soil and sediment samples, is now almost routine. However, there are many other potential applications that have only just begun to be explored, and others that are in their infancy. Two applications showing great promise are: 1) measuring or predicting "holistic" qualities, functions or characteristics that arise from the complex interplay of the underlying chemical and physical features of the sample under consideration; and 2) classifying or discriminating between samples. I argue that NIRS has enormous potential to facilitate a range of studies in behavioural ecology, and particularly in the study of insects and other organisms in which chemical communication plays a central role. Finally, I encourage investigation into the capacity of NIRS to serve as an instrument for the rapid identification of species. NIRS has the potential to become a standard tool in the armoury of ecologists and evolutionary biologists.

3.1 Introduction

Many studies in ecology and evolutionary biology involve time-consuming and expensive compositional analyses. For example, studying foraging preference or mating choice, investigating the "arms race" between plant defence mechanisms and herbivores or parasites and hosts, studying recognition systems, and determining nutrient flow through an ecosystem, to give just a few examples, can all involve expensive, complicated and time-consuming wet chemical analyses. Any technique that eliminates or reduces dependence on this is certain to be welcome within the community of ecologists and evolutionary biologists. NIRS may provide such a technique.

NIRS uses reflected light in the near-infrared region of the electromagnetic spectrum (approximately 750 – 2500 nm) to generate spectra that are characteristic of the scanned sample. Sometimes the visible region is included (400 – 750 nm). Different chemical bonds vibrate, and thus absorb electromagnetic energy, at different frequencies within this region of the spectrum. Reflectance spectra (R) are converted into absorbance spectra (A) according to the formula: $A = \log(1/R)$. The chemical bonds forming the basis of most NIRS information are X – H bonds, including C – H, O – H, N – H and S – H (Foley et al. 1998; Scarff et al. 2006), which are primary constituents of organic matter. NIRS is therefore particularly suited to analysing organic samples.

The vibrational frequencies of different functional groups often overlap; furthermore, functional groups may absorb energy at two or more frequencies, generating various spectral overtones. It is therefore rarely possible to identify the components of a complex mixture directly from spectra. This requires a process of calibration, whereby reference spectra are generated for samples containing known proportions of the chemical compounds of interest, against which the spectra of unknown samples can then be compared. The method therefore depends on statistical models rather than exact identification of specific chemicals.

NIRS is very widely used within the food and agricultural industries. For example, it has been used to determine the quality of pasture plants (Duru 1997; Fonteneli et al. 2004; Santos et al. 2002; reviewed by Valenciaga and Saliba 2006) and animal feed (De la Haba et al. 2007; Gonzalez-Martin et al. 2006; Perez-Mendoza et al. 2005). It has also been used in the quality control of a range of products, including mangos (Saranwong et al. 2004), apples (Xing et al. 2006; Zhu et al. 2007), sorghum (Figueiredo et al. 2006), stored grain (Cassells et al. 2007) and meat (Price et al. 2008). It has been used to detect disease in corn kernels (Pearson and Wicklow 2006) and oilseed rape (Mert-Turk et al. 2008). NIRS has also been applied to the detection of insects and insect fragments in stored grain (Neethirajan et al. 2007; Perez-Mendoza et al. 2005; Toews et al. 2007), the assessment of tick infestation in horses and cattle via faecal analysis (Tolleson et al. 2007), and the assessment of leaf miner damage to tomato

plants (Xu et al. 2007). It has even been used to specify the geographic origin of samples of honey (Woodcock et al. 2007) and olive oil (Galtier et al. 2007).

Among the advantages of NIRS are: it is rapid and low cost; samples require minimal or no preparation; very small samples can be scanned; multiple analyses can be conducted on a single sample; it produces no chemical waste; and it is non-destructive (Foley et al. 1998). Because of these advantages, its use in ecological and evolutionary studies is on the increase. When Foley et al. (1998) reviewed this technology over a decade ago, the commonest applications were the quantification of plant nutrients and secondary metabolites, and the resolution of complex mixtures into their component parts. NIRS was also applied, but less extensively, to mineral analysis and soil analysis. NIRS was also increasingly used to predict what Foley et al. (1998) called "functional attributes", which are holistic qualities that are not easily reducible to the chemical constituents of the sample, but are nevertheless related to them. They include characteristics such as the susceptibility of plants to insect attack; the nutritional quality of foods; and the decomposability of leaf litter. The capacity to discriminate between groups on the basis of spectral characteristics was identified as one of the most significant emergent applications of NIRS (Foley et al. 1998).

The aim of this chapter is to review the progress and development of NIRS applications in ecology and evolutionary biology over the last decade, and to suggest additional areas of interest. Current applications will be discussed under three broad headings: chemical analysis, holistic analysis and discrimination. I will then go on to consider some further potential applications, as well as the advantages and disadvantages of using NIRS. I refer the reader to Foley et al. (1998) for more details of the technical aspects of NIRS.

3.2 Chemical Analysis

The most widespread application of NIRS in ecology and evolutionary biology is analysis of the chemical constituents of a sample. After appropriate calibration, NIRS can be used to estimate chemical composition by comparing spectra of unknown samples with those of known samples. Calibration is achieved by scanning a set of samples, some of which are used in training the model, and some of which are used to validate the model. Compounds of interest are then extracted from these samples by conventional means. Regression equations are determined between the spectral absorbances of the training samples and those of the chemicals extracted. Once these equations have been developed, the model is validated using the remaining spectra. Alternatively, a cross-validation method can be used, whereby the model is constructed leaving out one or a few of the known samples in turn, and the models are then validated using the samples omitted. Once the calibration equations have been determined, they can be used to estimate the composition of unknown samples, which eliminates the need for

Table 3-1: Selected studies using NIRS to predict chemical content.

Chemical	Sample Type	Source
Organic	Trees	(Amsellem and McKey 2006; Andrew et al. 2007; DeGabriel et al. 2008; Ebbers et al. 2002; Henery et al. 2007; Lawler et al. 2000; McIlwee et al. 2001; Moore and Foley 2005; Petisco et al. 2006; Stolter et al. 2006; Vourc'h et al. 2002; Woolnough and du Toit 2001).
	Other plants	(Aragones et al. 2006; Lawler et al. 2006; Woolnough and Foley 2002).
	Litter	(Couteaux et al. 1998; Gillon and David 2001; McTiernan et al. 2003; Quested et al. 2007; Terhoeven-Urselmans et al. 2006).
	Faeces	(Dorgeloh et al. 1998; Gillon and David 2001; Kamler et al. 2004).
	Lake sediments	(Das et al. 2005; Malley et al. 2000; Rosen 2005; Rosen and Hammarlund 2007).
	Marine sediments	(Chang et al. 2005).
	Soil	(Butkute and Slepetiene 2006; Chodak et al. 2007; Couteaux et al. 2003; Ludwig et al. 2002; Schimann et al. 2007; Terhoeven-Urselmans et al. 2006).
Inorganic	Soil	(Bogrekci and Lee 2005; Chodak et al. 2002; Chodak et al. 2003; Chodak et al. 2007; Ehsani et al. 1999; Ludwig et al. 2002; Moron and Cozzolino 2007)
	Trees	(Petisco et al. 2005)
	Other plants	(Font et al. 2004b; Miller and Thomas 2003; Moron and Cozzolino 2002)
	Animal	(Font et al. 2004a)
	Faeces	(Dorgeloh et al. 1998)
	Lake sediments	(Malley and Williams 1997; Malley et al. 1999; Malley et al. 2000)
	Marine sediments	(Chang et al. 2005)

further wet chemical analyses. NIRS has been used extensively in a variety of contexts to predict the proportions of organic compounds and inorganic minerals in a range of materials, including vegetation, leaf litter, faeces, soil, and sediments (Table 3-1).

3.2.1 Organic compounds

NIRS has been used widely to estimate a range of nutrients and secondary chemicals in the preferred foraging material of several herbivores (DeGabriel et al. 2008; Ebbers et al. 2002; Lawler et al. 2000; Lawler et al. 2006; McIlwee et al. 2001; Moore and Foley 2005; Wallis et al. 2002). The analysis of faecal material using NIRS has also been important in determining foraging preferences and food quality (Dorgeloh et al. 1998; Gillon and David 2001; Kamler et al. 2004). The coefficient of determination (r^2) for many substances, such as nitrogen, acid detergent fibre, neutral detergent fibre and tannins, is regularly greater than 0.90. However, some components cannot be predicted with this degree of accuracy. For example, the acid lignin content of seagrasses could be predicted with an r^2 value of only 0.73 (Lawler et al. 2006). This is almost certainly because acid lignin is a poorly defined and heterogeneous mixture of substances (Lawler et al. 2006).

NIRS has progressed beyond the exploratory stage to become an integral component in investigating the evolutionary interactions between plants and herbivores. For example, Andrew et al. (2005) used NIRS to determine the heritability of sideroxylonal and two other foliar defence chemicals in a population of *Eucalyptus melliodora*. In a subsequent study, Andrew et al. (2007) explored the spatial distribution of sideroxylonol in relation to selection pressures from foraging by the common brushtail possum, *Trichosurus vulpecula*. Henery et al. (2007) used NIRS to explore the molecular genetic basis of formylated phloroglucinol compounds in *Eucalyptus nitens*, while Amsellem and McKey (2006) estimated the lignin content in ant plant leaves to investigate the role of plant phenology in the evolution of anti-herbivore defence in symbiotic ant-plant mutualisms.

Most studies of vegetative material use dried, ground samples for spectral analysis. While this is not necessarily onerous, it may limit the applicability of NIRS in the field. Ebbers et al. (2002) compared the capacity of NIRS to predict concentrations of nitrogen, cineole and sideroxylonal in whole, fresh leaves and dried, ground leaves of *Eucalyptus melliodora* and nitrogen and cineole in *E. globulus*. They used cross-validation to determine r^2 values for the models. For *E. melliodora*, r^2 values for nitrogen, cineole and sideroxylonal in dried, ground samples were 0.98, 0.88 and 0.94 respectively; in whole, fresh samples these values were 0.92, 0.97 and 0.90. For *E. globulus*, r^2 values for nitrogen and cineole in dried, ground samples were 0.99 and 0.75 respectively, while in whole, fresh samples these values were 0.90 and 0.74. There was, therefore, only a slight loss of precision when using fresh leaves compared to dry, ground leaves, except in the case of cineole in *E. melliodora*, where there was actually an improvement using fresh material. This result suggests that it may be possible to obtain spectral information using portable or airborne equipment in the field.

Studies of nutrient flow through eco-systems have also benefited from the capacity of NIRS to detect a range of organic compounds in leaf litter (Couteaux et al. 1998; Gillon and David 2001; McTiernan et al. 2003; Quested et al. 2007; Terhoeven-Urselmans et al. 2006) and soil (Butkute and Slepetiene 2006; Chodak et al. 2007; Couteaux et al. 2003; Ludwig et al. 2002; Schimann et al. 2007; Terhoeven-Urselmans et al. 2006). NIRS has also been used successfully in the analysis of organic components of lake sediments (Das et al. 2005; Malley et al. 2000; Rosen 2005; Rosen and Hammarlund 2007) and marine sediments (Chang et al. 2005), which is potentially valuable for both palaeoecological studies and environmental monitoring.

3.2.2 Inorganic compounds

NIRS is generally considered less suitable for predicting levels of inorganic minerals, as most of these are not expected to produce absorption in the NIR region of the spectrum. As a result, their detection is largely dependent on their presence in organic or hydrated molecules (Foley et al. 1998). Despite this limitation, attempts have been made to use NIRS to detect inorganic minerals in a range of materials (Table 3-1). Petisco et al. (2005) were able to predict the nitrogen, calcium and phosphorous levels in a heterogeneous mix of Mediterranean tree species with r^2 values up to 0.94, 0.91 and 0.94 respectively, depending on the analytical method used. Moron and Cozzolino (2002) found that NIRS could predict calcium, nitrogen and potassium levels very successfully, and phosphorous levels with moderate success, in alfalfa (*Medicago sativa*) (r^2 = 0.93, 0.91, 0.87 and 0.81 respectively); however, in white clover (*Trifolium repens*), only nitrogen could be predicted with a high success rate (r^2 = 0.92), while potassium could be predicted with moderate success (r^2 = 0.80). Inorganic arsenic levels in prostrate amaranth (*Amaranthus blitoides*) growing in polluted areas could be predicted with an r^2 value of only 0.63, but this was considered sufficient to differentiate between high, medium and low levels (Font et al. 2004b). In contrast, Miller and Thomas (2003) found that NIRS could not satisfactorily predict phosporous, potassium, calcium, and magnesium concentrations in bermudagrass (*Cynodon dactylon* x *C. transvaalensis*). Thus, while results have been mixed, they have often been better than might have been expected.

More recent applications of NIRS to mineral analysis have focussed largely on their presence in soils. Ehsani et al. (1999) were able to detect nitrates in soil samples with high coefficients of determination ($r^2 > 0.90$), but only when soils from each specific location were used for calibration. When soil samples were pooled from different regions, r^2 often decreased to around 0.75 (Ehsani et al. 1999). This decrease is probably because the nitrates in each of these soils were associated with different combinations of organic compounds, which NIRS was actually detecting; combining soils weakened this association. A similar problem occurs when trying to predict phosphorous levels. Bogrekci et al. (2003) used spectra in the visible and NIR region to measure phosphorous levels in Lake Okeechobee drainage basins, with r^2 values of

0.78 and 0.89 for wet and dry samples respectively. When the UV range was also included, an r^2 value of 0.93 was obtained (Bogrekci and Lee 2005). However, Moron and Cozzolino (2007) showed that different calibrations were obtained for phosphorous, depending on the extraction method employed, as these tended to yield phosphorous from different sources. That is, calibrations were dependent on the organic compounds with which phosphorous was associated. Their calibrations enabled only rough prediction or classification of low, medium, and high phosphorous concentration in soils (Moron and Cozzolino 2007).

In addition to its application to the analysis of inorganic minerals in plants and soil, NIRS has also been used to analyse the mineral content of various peat-based growing media (Terhoeven-Urselmans et al. 2008), and to determine the heavy metal (Malley and Williams 1997), and carbon, nitrogen and phosphorous (Malley et al. 2000) concentrations in freshwater sediments. As far as I am aware, only a single animal study has been conducted to date: NIRS was able to satisfactorily predict levels of inorganic arsenic in the red crayfish, *Procambarus clarkia* (r^2 = 0.84) (Font et al. 2004a).

In summary, NIRS has the potential to replace traditional wet chemical analysis of organic chemicals, once the initial calibrations have been performed. The coefficient of determination for a range of compounds generally exceeds 0.90, although this is lower for less well-defined compounds. NIRS has also proved surprisingly effective at measuring concentrations of inorganic minerals. The best results are for plant materials, in which inorganic minerals are closely associated with organic compounds. Variable results have been obtained for other media, such as soil and sediments, in which the links between inorganic minerals and organic compounds are less clearly defined. The origin of samples will be important if the inorganic mineral under consideration has a different association with organic compounds in different locations. The reference method used may also have a profound effect on the calibration process. However, even an r^2 value in the range of 0.70 – 0.80 may be considered adequate if all that is required is a qualitative ranking of samples, or a rough estimate, such as low, medium or high. Finally, there is a paucity of studies relating to taxa outside the plant kingdom, although there is no immediately obvious reason why NIRS could not be applied to the analysis of both inorganic and organic chemicals in these taxa.

3.3 Holistic Analysis

Holistic analysis involves the use of NIRS to measure or predict qualities, functions or characteristics that arise from the complex interplay of the underlying chemical and physical features of the sample under consideration (Table 3-2). In one respect, all NIRS analysis is holistic, in that the spectra generated are a high level representation of the underlying physical components. Nevertheless, some holistic characteristics are less easily resolved into their

Table 3-2: Selected studies using NIRS to predict holistic characteristics.

Characteristic	Sample Type	Reference
Composition	Oesophageal extrusa	(Volesky and Coleman 1996)
	Stomach contents	(Andre and Lawler 2003)
	Above ground vegetation	(Smart et al. 1998)
	Root material	(Roumet et al. 2006)
	Faeces	(Kaneko and Lawler 2006)
Organic matter quality	Litter	(Joffre et al. 2001)
Decomposability	Litter	(Aldrich et al. 2007; Bouchard et al. 2003; Gillon et al. 1999; Stolter et al. 2006)
Foraging preference	Foliage	(Lawler et al. 2000; McIlwee et al. 2001; Wallis and Foley 2003)
Vegetation damage	Foliage and twigs	(Stolter et al. 2006)
Chronological age	Insects	(Perez-Mendoza et al. 2002; Perez-Mendoza et al. 2004)
Air temperature	Lake sediments	(Rosen et al. 2000)

component parts. In this approach, spectra are calibrated with the parameter under consideration, as determined for some samples by another method.

3.3.1 Resolving complex mixtures

One of the main holistic applications of NIRS is the resolution of complex mixtures into their component parts. In this context the purpose is to determine what proportion of a given mixture consists of *A*, *B* and *C*, where these are not chemical compounds, but complex entities. For example, Volesky and Coleman (1996) used NIRS to try to determine the proportion of several grasses, forbs and sedges in the oesophageal extrusa of sheep and cattle: the total grass content and forb content, as well as the proportion of one grass species, could be predicted with r^2 values greater than 0.70, while the proportion of sedges and other individual species could not be predicted. Better results were obtained for the stomach contents of the dugong, *Dugong dugon* (Andre and Lawler 2003): major components of the diet (total rhizome content plus three individual species of seagrass) could be predicted with r^2 values greater than 0.80. However, minor components could not be predicted satisfactorily ($r^2 \leq 0.50$) (Andre and Lawler 2003).

The leaf:stem ratio is an important characteristic of grasses, contributing to their nutritional quality. Smart et al. (1998) attempted to use NIRS to identify the relative proportions of leaf and stem material in ground samples of three grass species, with qualified success. They found that the leaf:stem ratio of two grass species, *Andropogon gerardii* and *Panicum virgatum*, could be predicted with moderate success, with r^2 values ranging from 0.60 to 0.75 and 0.69 to 0.75 respectively, depending on the number of samples used to establish the calibration equations; leaf:stem ratio could not be predicted for a third grass species, *Bromus inermis* (r^2 ranged from 0.06 to 0.14) (Smart et al. 1998).

Identifying and quantifying root material from soil samples can be particularly difficult, and Roumet et al. (2006) showed that NIRS was generally more accurate than extracting plant wax markers at determining relative proportions of root material from several species: correlation coefficients ranged from 0.89 to 0.99 using alkanes and alcohols, and 0.97 to 0.99 using NIRS. One disadvantage of NIRS in this context was that it required a relatively large quantity of root material for calibration (Roumet et al. 2006). On the other hand, whereas Foley et al. (1998) concluded that calibrations based on plant material from a narrow or closed population yielded more precise information than calibrations based on material from several areas, or grown under different conditions, Roumet et al. (2006) found that this was not the case for root material analysed using NIRS (although it was the case when analysed using wax markers).

By scanning the faeces of two captive seals, one California sea lion (*Zalophus californianus*) and one Australian fur seal (*Arctocephalus pusillus doriferus*), Kaneko and Lawler (2006) were able to quantify how much of a given diet item (consisting of two fish species and one squid) each seal had consumed the previous day. For *Z. californianus*, r^2 values were high for both species of fish and the squid species (0.89 – 0.98); for *A. pusillus doriferus*, r^2 values were high for one species of fish and the squid species (0.95 – 0.96), but low for the other fish species (0.25). It remains to be seen whether there might be significant variation within seal species (only a single male from each species was tested), and whether the technique can be extended to the field, where the diet of seals is likely to be more varied.

The effectiveness of NIRS for resolving mixtures of materials into their component parts remains largely at the exploratory stage. It is inevitable that r^2 values will be lower for this type of study than for those studying specific chemical compounds. Nevertheless, values above 0.90 are still achievable in some cases. This clearly depends on the strength of the correlation between the parameter under consideration and the actual chemical and physical characteristics that NIRS is measuring. Nevertheless, even a lack of success can raise important questions. For instance, why the leaf:stem ratio could be predicted for two grass species, but not a third, could be the starting point for further investigation. So too could the observation that the proportions of two fish species could be determined in the faeces of one seal, but only one fish species in

the faeces the other: is this indicative of different metabolic processes or is it, in this case, due to a lack of replication?

3.3.2 Other holistic characteristics

NIRS has frequently been used to measure holistic qualities of leaf litter. For example, Joffre et al. (2001) demonstrated that the organic matter quality of leaf litter could be predicted much more accurately using visible and NIR light (r^2 = 0.94), than when initial litter quality was derived from chemical fractions (correlation coefficients ≤ 0.56). Gillon et al. (1999) were able to show that NIRS could predict the decomposability (measured as the leaf mass loss after one week of incubation) of different leaf litters from a European forest ecosystem with a high degree of accuracy. In fact, predictions based on initial spectra were more accurate than predictions based on the initial characteristics of the litter (Gillon et al. 1999). Furthermore, decomposition could be accurately predicted for time periods well beyond that used to establish the initial calibrations (14 months compared to 8 weeks) and for litter decomposing under quite different conditions (field conditions compared to laboratory conditions) (Gillon et al. 1999). Bouchard et al. (2003) achieved similar results in a salt marsh ecosystem. Furthermore, there was evidence that NIRS could measure leaf mass loss more accurately than directly measuring mass loss from litter bags left in situ. This was based on the observation that NIRS accurately predicted leaf mass loss in bags above most high tides but underestimated loss in bags that were regularly inundated. This was attributed to the loss of particulate matter from litter bags due to tidal action (Bouchard et al. 2003). In contrast to these studies, Stolter et al. (2006) were able to predict the decomposition rate (mass loss over one year) of *Salix phylicifolia* litter only qualitatively: the slope of the regression line was only 0.23, indicating that NIRS consistently underestimated decomposition rate (r^2 = 0.49). The poorer result may reflect methodological differences. Stolter et al. (2006) used only 25 samples to establish their calibration, whereas Bouchard et al. (2003) used 48 and Gillon et al. (1999) used 34. Furthermore, in the latter two studies, the calibration was established using litter sampled at several time intervals, whereas the former study used litter sampled only after one year.

In addition to using NIRS to estimate the proportions of foliar nutrients in *Eucalyptus* spp., McIlwee et al. (2001) were able to accurately predict the foraging preferences of greater gliders (*Petauroides volans*) and common ringtail possums (*Pseudocheirus peregrinus*) feeding on these trees (r^2 = 0.94 and 0.95 respectively). Foraging preferences depend on a whole range of chemical and physical attributes of the foraging material, as well as interactions between them. It can therefore be difficult to single out one or a few factors and use them to predict foraging choices. Because NIRS spectra incorporate a broad range of information, they may be particularly useful in this regard. McIlwee et al. (2001) found that their predictions based on NIRS were as good or better than predictions based on direct chemical analysis of foliar

constituents. Lawler et al. (2000) established calibration equations for dry matter intake (DMI) of *P. peregrinus*, foraging on two species of *Eucalyptus*, with an r^2 value of 0.92. In an impressive validation of the predictive ability of NIRS, a few years later Wallis and Foley (2003) were able to use this model to accurately predict the DMI of different individuals from another area, with an r^2 value of 0.81.

In a novel application of NIRS, Stolter et al. (2006) sought to reconstruct the level of damage caused by moose browsing during winter of the previous year, to the willow *Salix phylicifolia* growing in the northern Taiga (Sweden). A previous study had demonstrated that willows exhibited a defensive chemical response to browsing in the following growing season (Stolter et al. 2005). Using an independent validation sample, the authors were able to reconstruct the level of browsing qualitatively, but not quantitatively: that is, predicted values deviated from actual values by as much as 50%, but the samples were correctly ranked (Stolter et al. 2006). The authors acknowledged that visually estimating the level of browsing for the purpose of calibration was somewhat error prone (Stolter et al. 2006). Furthermore, there is likely to be a complex interaction in this system, with browsing stimulating a response in the willows, and this in turn influencing the selection of browse material by moose. Under these circumstances, a qualitative result may be all that could be expected.

NIRS has also been employed as a rapid method for assessing the chronological age of insects. Perez-Mendoza et al. (2002) demonstrated that NIRS could predict the age of house flies (*Musca domestica*) with an accuracy of ± 1 week, regardless of the sex or size of the fly, or the temperature at which it was reared. The traditional method of estimating age (pteridine extraction) was more sensitive to each of these factors (Perez-Mendoza et al. 2002). In cross-validation, flies could be assigned to one of two age groups (≤ 6 days old or > 6 days old) with 83% accuracy (Perez-Mendoza et al. 2002). In a similar study of the rice weevil, *Sitophilus oryzae*, 84.7% could be assigned to the correct age group (first third of life or last third of life) regardless of sex or the temperature at which they were reared (Perez-Mendoza et al. 2004). In a preliminary study of the honey bee, *Apis mellifera*, 87.5% of bees could be assigned to the correct age group (brood bees or forager bees) using a leave-one-out cross-validation procedure (see Chapter 7 below).

In a holistic approach to studying lake sediments, Rosen at al. (2000) used surface sediments from 76 Swedish lakes to develop a model to predict the altitude of the lakes, as a surrogate for climatic conditions. In cross-validation, the model accounted for 86% of the variance in altitude (Rosen et al. 2000). They then applied the model to a sediment core from another lake to estimate the mean July air temperature based on the NIRS function, and found that it showed trends similar to inferences derived from chironomids, diatoms and pollen from the same core (Rosen et al. 2000).

NIRS is not the solution to all ecological questions involving holistic attributes. It is clear that it does not directly measure such qualities, but, rather, the underlying chemical components with which the target variable is correlated to some extent. The strength of this correlation will determine the extent to which the factor under consideration can be predicted by NIRS. There needs to be a plausible underlying rationale for expecting a relationship between NIRS and this factor. Care must also be taken to employ cross-validation methods and appropriate statistical analyses. Nevertheless, the application of NIRS to more holistic qualities is worthy of continued investigation.

3.4 Discrimination and Classification

In the applications of NIRS considered so far, the emphasis has been on measuring a parameter and fitting it to a regression line; but NIRS also has the capacity to place samples into categories, based on spectral characteristics. As for the applications discussed above, this analysis is preceded by a calibration process: samples known to belong to each category are scanned and a set of reference spectra is established for each category, with a mean and some estimate of confidence intervals around the mean. The model may be validated using either cross-validation or an independent data set. Unknown samples are then tested against these reference spectra and assigned to the appropriate category, based on a measure of spectral distance between the sample and the category means. This is preferably accompanied by a statistical procedure that determines whether the percentage of samples allocated to the correct category differs from what might be expected from random allocation.

In the food and agricultural industries, NIRS has been used to distinguish different species of wood (Adedipe et al. 2008; Brunner et al. 1996), to identify stored grain beetle pests to genus level (Dowell et al. 1999), to differentiate between insect pest species at immature stages of development (Jia et al. 2007), to specify the geographic origin of samples of honey (Woodcock et al. 2007) and olive oil (Galtier et al. 2007), and even to differentiate between varieties of wheat (Miralbes 2008).

Given this breadth of application, it is surprising that NIRS has not yet been used more widely by ecologists and evolutionary biologists for this purpose (Table 3-3). A number of studies have demonstrated the potential of NIRS for discriminating between closely related species. For example, Atkinson et al. (1997) used spectra in the visible and NIR range to tackle a long-standing problem, namely the ability to discriminate between two closely related birch species, *Betula pendula* and *B. pubescens*, and their interspecific hybrid. Richardson et al. (2004) also used visible and NIR light to discriminate between two montane conifer species, *Abies balsamea* and *Picea rubens*, using both discriminant analysis and partial least squares regression, the latter attaining an r^2 value of 0.99. It is important to note that these models were

Table 3-3: Selected studies using NIRS to classify samples into groups.

Sample Type	Grouping Variable	Reference
Trees	Species	(Atkinson et al. 1997; Richardson et al. 2004)
	Insect damage	(Cunningham and Floyd 2004)
Seeds	Geographical origin	(Tigabu et al. 2005)
	Parentage	(Tigabu et al. 2005)
Insects	Species/Genus	(Aldrich et al. 2007; Cole et al. 2003b)
	Colony of origin	(Chapter 4, this thesis)
Soil	Principal soil engineer	(Hedde et al. 2005)

not independently validated. Cole et al. (2003a) were able to distinguish between two morphologically similar parasitoids (*Cotesia flavipes* and *C. sesamiae*) of stemboring lepidopteran pests, by scanning cocoons: approximately 84% of unknown samples were correctly identified. Aldrich et al. (2007) used NIRS to differentiate between four species/subspecies of the termite genus, *Zootermopsis*. Using a partial least squares analysis, each of the species and subspecies of a validation set were identified with accuracy ranging from 85 to 100%, while a neural network analysis enabled the identification of the species and subspecies with 100% accuracy.

NIRS has also been used to differentiate between groups within a species. Using visible and NIR spectra, Tigabu et al. (2005) were able to identify the geographical source of *Pinus sylvestris* seeds, collected from three localities, with almost 100% accuracy. They were also able to identify the maternal parent with an accuracy of 93% for three maternal parents (only 60% of the fourth maternal parent was correctly classified) and the paternal parent with accuracy ranging from 70 to 100%.

Recently Hedde at al. (2005), adopting a more holistic approach to soil analysis, demonstrated that NIRS (including the visible range) could be used to differentiate between soils with different biogenic structures, that is, soils characterised by earthworm casts, termite sheathings and mound material, and ant deposits. NIRS was able to measure the "signature" resulting from this complex biogenic process. This potentially provides a method for functionally classifying soils in terms of the major ecosystem engineer responsible for their structure.

Cunningham and Floyd (2004) attempted to use NIRS to classify leaflets of the rainforest tree, *Toona ciliata*, into categories of damage (low and high) caused by the shoot-boring moth, *Hypsipyla robusta*. Using discriminant analysis they were able to classify 78 of 98 trees correctly as having high damage and 37 of 55 trees correctly as having low damage. They

tested the statistical significance of this using a simple χ^2 test assuming equal probability of being assigned to each group, and found the result to be significantly different from random (Cunningham and Floyd 2004). However, they tested only the samples that were used to establish the discriminant functions, and this tends to overfit the data to the equation. Therefore a more robust statistical analysis, either using an independent validation set, or a randomisation process to determine the probability of achieving that result by chance, would be informative.

Although NIRS has enormous potential as a discriminant tool in ecology and evolutionary biology, its application is still in its infancy. Further experimentation in this direction is to be encouraged, while also emphasising the importance of conducting appropriate and rigorous statistical tests when carrying out any classification procedure.

3.5 Future Directions

3.5.1 Behavioural studies

NIRS has rarely been used in animal studies. McIlwee et al. (2001) showed that the foraging preferences of greater gliders and common ringtail possums feeding on leaves of *Eucalyptus* spp. were accurately predicted by NIRS. These choices are undoubtedly mediated by the chemical qualities of the foraging material that determine its nutritional value, its palatability and its texture. In this case, behaviour is strongly correlated with qualities that are directly amenable to analysis by NIRS. This makes NIRS potentially a very valuable tool in the study of foraging preferences, particularly of herbivores. This approach might easily be extended to the study of other areas of behaviour, such as the choice of oviposition sites by insects, based on the chemical characteristics of host plants.

As I have already observed, chemical cues play a major role among insects, and a growing body of evidence indicates that many insects are able to act upon the information contained in CHCs. For example, in hybridising zones of *Chrysochus* spp. (Coloeoptera: Chrysomelidae) male choice of conspecific females is governed by recognition of CHCs (Peterson et al. 2007). Male fruit flies (*Drosophilia melanogaster*) use CHCs on the female cuticle to assess how many times she has previously mated (Friberg 2006). Male ball-roller scarabs, *Canthon cyanellus cyanellus*, recognise females by their cuticular compounds (Ortiz-Dominguez et al. 2006b). Among social insects, the ability to detect and respond to CHCs as signals of fertility is well-established (reviewed by Monnin 2006), as is their use in differentiating between nestmates and other conspecifics (reviewed by Dani 2006).

Given the importance of chemical cues in the lives of insects, NIRS has the potential to greatly extend and simplify the range of potential behavioural and evolutionary studies that would otherwise depend on time-consuming and expensive wet chemical processes. In the

present thesis, for example, I demonstrate that the level of aggression displayed by colonies of weaver ants towards conspecific intruders from other colonies is directly related to the spectral distance between colonies, as measured using NIRS (Chapter 5). This method could easily be extended to a whole range of insect behaviours that are believed to have their basis in the discrimination of underlying chemicals.

3.5.2 Species identification

The ability to classify samples into groups rapidly would be a great advantage to ecologists and evolutionary biologists. Working with social insects, I recognise the significance of being able to rapidly determine colony of origin, to differentiate larvae at an early stage according to sex or caste, or to distinguish between reproductive and non-reproductive individuals. However, I believe that this application of NIRS will be of interest to a much broader audience, and here I highlight just one potential application.

Biodiversity surveys, the monitoring of indicator species, and the detection of invasive species, often require the rapid identification of species, some of which may be difficult to identify using morphological features, particularly by non-specialists. Several genetically based methods have been proposed, including DNA barcoding, the aim of which is to develop a reference library of suitable regions of DNA, against which unknown specimens can be compared (for recent reviews see Birky 2007; Darling and Blum 2007; Neigel et al. 2007). NIRS has demonstrated its capacity to differentiate between species, and even varieties within species. I propose exploring the possibility of extending this application to build spectral reference libraries analogous to those used for DNA barcoding. There are many issues to consider, some of which are shared with other methods. For example, intra-species variation needs to be lower than inter-species variation for the method to be effective. Databases would probably need to be established for specific taxonomic groups, although whether that would be at the level of genus, subfamily, or family remains to be seen, and may vary between groups. If there is significant geographical variation in spectra, calibrations may need to be based on local populations. An issue peculiar to NIRS is the method of preprocessing that spectra may require for the calibration procedure: the less preprocessing required, and the more uniform that method across taxa, the more widely applicable the method could be. Nevertheless, the potential savings in time and money make this a challenge worth undertaking. If portable instruments become more readily available, identification in the field may become a real possibility.

3.6 Advantages and Disadvantages of NIRS

The advantages of NIRS are clear. It has the potential to dramatically reduce the amount of conventional chemical analysis required for a range of studies, with concomitant

savings in time and money, and a reduction in chemical waste. Once the initial calibration has been established, no further conventional analysis may be required, although it may be advantageous from time to time to expand the set of spectra used to establish the calibration model as samples are collected from different areas or under different conditions.

Only minimal preparation of samples is required, and even some of this may be eliminated: Ebbers et al. (2002) showed that the use of fresh vegetative material in the analysis of nutrients and secondary compounds produced results that were only slightly inferior to those produced using dried and ground material. In some instances, insects can be scanned without any preparation at all (Perez-Mendoza et al. 2002), and even live insects can be scanned (Dowell et al. 1999).

NIRS spectra contain information on a whole range of chemical and holistic characteristics, and this makes it possible to carry out a range of analyses on the same samples. In general, only small samples are required for analysis, although these may still be considered too large if sample material is difficult to obtain (Roumet et al. 2006). Furthermore, because NIRS is non-destructive, additional analyses can always be conducted. For example, when determining the chronological age of insects, live samples can be repeatedly scanned at different time intervals in order to establish accurate calibrations.

Another advantage of NIRS is that it facilitates the analysis of holistic characteristics that may otherwise be intractable. For example, it may be very difficult to identify the individual chemical components or physical characteristics of a plant that determine its attractiveness to a potential consumer. NIRS generates a signature of the sample that is related to many if not all of these potential determinants, and spectra can therefore be used to predict foraging preferences. Similarly, in identifying nestmates as opposed to other conspecifics, social insects may be responding to a range of chemicals and their interactions that are not easily isolated: NIRS is capable of representing this information holistically.

At the same time, this might be regarded as one of the main shortcomings of NIRS. There could be a tendency to regard NIRS as a "black box" into which information is fed and, after some mysterious process, the answer is delivered. For that reason, I emphasise the need for a plausible rationale for expecting a relationship between the holistic characteristic being measured, and the underlying chemical and physical characteristics of the sample. When NIRS is able to accurately predict such a holistic quality, I expect this to stimulate further research into the precise causal relationships between holistic qualities and their chemical and physical foundations.

One potential drawback of NIRS is the degree of pre-processing that is required in order to extract the maximum quantity of information from spectra. These include various normalisation and corrective transformations, as well as the use of first and higher derivatives. While this in itself can be time consuming and complicated, a more critical issue is that very

different pre-processing methods may sometimes be required for very similar purposes. For example, if NIRS were to be used to differentiate between three closely related species of beetle, *A*, *B* and *C*, it may be that first derivative spectra are optimal for differentiating between *A* and *B*, but second derivative spectra are optimal for differentiating between *B* and *C*. Sometimes it may be necessary to settle for less than optimal results for the sake of greater consistency. Advances in statistical analysis may help to resolve this issue.

Another potential drawback is the fact that water is such a strong absorber of NIR radiation, and the large peaks generated may obscure smaller but information-rich peaks. Whether this is a major concern needs to be determined on a case by case basis. I have already noted that analysis of fresh leaf material can still yield very useful results.

Finally, NIRS may be less useful for identifying the precise chemicals present, for instance on the insect cuticle, than the traditional approach using GCMS. However, with appropriate calibration procedures even this may ultimately be possible. I would encourage the use of NIRS and GCMS together in order to explore this issue.

3.7 Conclusion

While the application of NIRS to ecological studies has expanded significantly during the last decade, many applications are still at the exploratory or evaluative stage. Only in the analysis of plant nutrients and secondary metabolites has the use of NIRS become virtually routine. The application of NIRS to soil and sediment analysis has advanced significantly in the last 10 years, and it has proven to be more successful at quantifying inorganic compounds than might have been expected. The application of NIRS to the analysis of holistic properties is rapidly increasing. It should be borne in mind that NIRS does not directly measure such qualities, but the underlying chemical components with which the target variable is correlated. The extent to which the factor under consideration can be predicted by NIRS depends on the strength of this correlation, and there needs to be a plausible rationale for expecting a relationship between NIRS and this factor. The use of NIRS as a discriminatory tool and its application to behavioural studies shows great potential. In particular I encourage further investigation into its ability to provide rapid identification of species, and to exploit the chemical world of insects. I believe that NIRS has the potential to become a standard tool in the armoury of ecologists and evolutionary biologists.

4 NIRS identifies the colony and nest of origin of weaver ants

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Abstract

The ability of social insects to differentiate between colony members and others is essential for the survival of the colony. It enables individuals to direct altruistic behaviour towards colony-mates, while protecting the colony from intruders. Colonies have a distinct chemical signature that facilitates colony-mate recognition. However, in large polydomous colonies, this signal is likely to be modified by factors unique to each nest. I demonstrate, using NIRS, that individual weaver ants can be differentiated with respect to their colony and nest of origin. Seventy-six point five percent of individuals from four colonies could be correctly assigned to their colony of origin and 79.6% of individuals could be assigned to the correct nest (of two) within their colony. Despite the differences between nests within colonies, in most cases individuals from one nest were more similar to individuals from the other nest within the colony than they were to individuals from any nest outside the colony. Therefore, a distinctive colony identity is maintained despite differences between nests within colonies. I discuss the advantages of using NIRS as a faster and less expensive alternative to the analysis of cuticular hydrocarbons following extraction and identification with GCMS.

4.1 Introduction

The evolution of co-operative behaviour has been a central focus of evolutionary biology since the time of Darwin himself, and gained considerable momentum from theoretical advances in the concepts of kin selection and inclusive fitness (Hamilton 1963, 1964; Trivers and Hare 1976). Social insects have played a major role in exploring these issues. The ability of social insects to discriminate between colony members and others is essential for the viability of the colony. It enables individuals to direct altruistic behaviour towards colony-mates, who are generally close kin, while protecting the colony from intruders.

Several studies have shown that CHCs can be used to differentiate between social insects from different colonies. By carrying out a discriminant analysis of CHCs, Denis et al. (2006) were able to classify 100% of workers from three colonies of *Pachycondyla goeldii* to the correct colony. Similarly, Heinze et al. (2002) were able to assign 86.4% of *Pachycondola cf. inversa* from four colonies to the correct colony. However, these were small, single nest colonies maintained in the laboratory. Van Wilgenburg et al. (2006) recently demonstrated that the technique also works for larger, polydomous colonies: they were able to assign 83.9% of workers from four colonies of the Australian meat ant, *Iridomyrmex purpureus*, to the correct colony. Interestingly, they were also able to assign 73.6% of individuals to the correct nest within the colony. This raises important questions about the way in which colony identity is maintained when there is such intra-colonial variation.

In the present study I sought to determine whether similar intra-colonial variation occurs in the weaver ant. Instead of GCMS, I used NIRS to differentiate between colonies and nests. As described in Chapters 1 and 3, this involves generating absorption spectra in the near-infrared, from about 4000 per cm to 12500 per cm (wavenumber), to provide a broad picture of the chemical "signature" of the scanned sample. For a discussion of recent applications of NIRS to the study of insects see Chapter 3. As far as I am aware, this is the first time it has been used to differentiate between social insects from different colonies of origin. It may prove to be a simpler, faster and less expensive method than GCMS.

4.2 Methods

I collected 120 weaver ant workers from each of four separate colonies on the campus of James Cook University, Cairns, Queensland (S 16° 49', E 145° 41'), with 60 ants from each of two nests per colony. I stored samples at -4°C before thawing and scanning with a Bruker Optics Multi Purpose Analyzer ®.

I obtained spectra by placing individuals on the surface of a fibre-optics probe so that scanning was restricted to the dorsal surface of the gaster. The probe was covered to minimize the impact of external light. The instrument scans each individual 16 times to generate a mean spectrum, which is then converted by Fourier transformation into an absorbance spectrum. I used baseline correction and smoothing algorithms available in OPUS 6.0 (Bruker Optics Inc., Build: 6,0,72, 1997-2006) to reduce spectral noise and distortion, and another algorithm to identify peaks, with sensitivity set at 0.44%. At this setting, very small peaks could be detected, down to an intensity of only 0.44% of the intensity of the largest peak, without too much spectral noise obscuring genuine peaks. I identified six peaks present in most spectra, and recorded their location (peak frequency), intensity, and width (at 50% intensity) resulting in 18 parameters. I replaced missing values with the mean for that peak, in the case of location, or with a value halfway between zero and the lowest recorded value in the case of intensity and width. I assumed that these peaks were not absent, but were below the threshold of detectability. This made it possible to include all individuals within the analysis. However, to determine whether this had any effect on the results I repeated some of the analyses using only those individuals with all peaks present.

I analysed the data using multinomial logistic regression, with backward stepwise variable entry. When logistic regression is used to reclassify a data set based on functions derived from that same data set, there is a risk of over-fitting the data and generating an inflated proportion of correct classifications (Fox 1997). Therefore, to determine if the proportion of individuals correctly assigned to their colony or nest of origin by the regression was significantly greater than would be the case with random groups, I carried out the following procedure: individuals were randomly assigned to one of four groups, with 120 members per group. The logistic regression was repeated for these random groups, and the randomization process was carried out 1000 times. For each colony I recorded the proportion of simulations in which the number of individuals correctly re-assigned to their random group was equal to or exceeded that of the original data, and used this as an estimate of the probability of obtaining that result by chance. If this value was less than 0.05, I considered the success rate for that colony to be significantly better than random. I repeated this procedure for each pair of nests, with 60 individuals per random group.

To determine the extent to which unknown individuals could be correctly identified I also performed a leave-one-out cross validation procedure. This involved omitting each individual in turn from the logistic regression and recalulating the logit functions. Thus the spectrum of the excluded individual was not used to determine the logit functions. I then assigned the omitted individual to the group with the highest probability, as calculated using these same logit functions. Leave-one-out cross-validation is an accepted method for reducing over-fitting when sample sizes are small (Brown 1993; Härdle 1990), and has been employed

extensively in other studies using NIRS (eg. Locher et al. 2005; Offer and Percival 1998; Wu et al. 2007), including studies of insects (Dowell et al. 2005). It has also been used in the analysis of cuticular hydrocarbon (CHC) profiles of social insects (Van Wilgenburg et al. 2006). This method of model validation has the advantage that all available individuals can be used to determine the calibration model without the need to maintain separate validation and calibration sets (Foley et al. 1998).

In a further analysis, I sought to measure the similarity between and within nests and colonies. I first used principal components analysis to reduce the 18 variables to a set of six orthogonal factors with an eigenvalue greater than one. Together these factors accounted for 79.56% of the total variance. Using these factors as new variables, I estimated the similarity (*S*) of individuals within and between nests and colonies by taking the inverse of the Euclidean distance between them and calculating the mean. The greater the value of *S*, the more similar to each other individuals were considered to be. I tested for significant differences in *S* among pairs of nests using permutation tests, with 10,000 permutations of the original data for each comparison (see Appendix A.1).

The analyses and simulations were carried out using SPSS 13.0 for MacIntosh and MS Excel 2007.

4.3 Results

Six peaks were selected for analysis, P1 to P6 (Figure 4-1), which fell between 8583-8699, 6987-7097, 5778-5801, 5623-5671, 5202-5255 and 4553-4651 cm^{-1} respectively. P2, P3, P4 and P5 were detected in all samples, while P1 was detected in 88.1% of samples and P6 in 77.2% of samples.

4.3.1 Colony allocation

When the logistic regression was conducted with individuals randomly assigned to groups, approximately 37% of individuals from each group were correctly re-assigned to that group. Among the actual colonies, the percentage of individuals assigned to their colony of origin was 69.2% for Colony 1, 76.7% for Colony 2, 82.5% for Colony 3, and 77.5% for Colony 4. Overall, 76.5% of individuals were correctly re-assigned, and in each case this was significantly better than random assignment (Table 4-1). Except for Colony 1, these figures improved when only individuals with all peaks present were included in the analysis (66.1%, 85.4%, 83.5%, 83.5% for Colonies 1 to 4 respectively, and 80.8% overall), although sample sizes were substantially reduced (59, 89, 85 and 79). Replacing missing values therefore biased the results in a conservative direction.

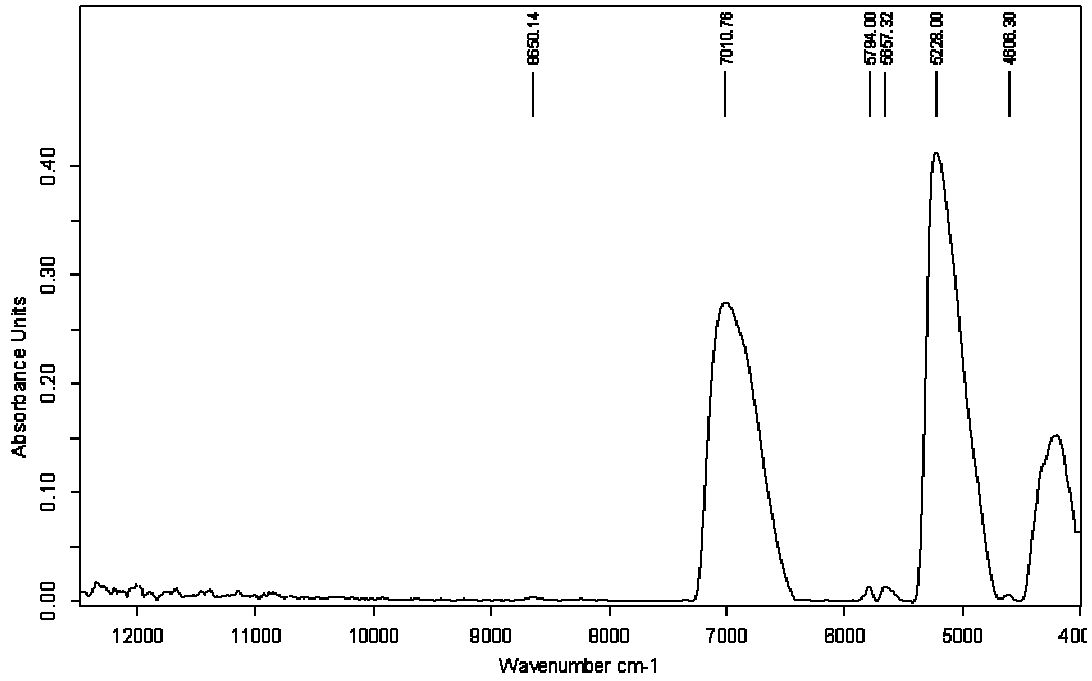

Figure 4-1: An example of a near infrared spectrum, after baseline correction and smoothing. The six peaks used in the multinomial logistic regression are indicated above the spectrum.

Table 4-1**:** Allocation of ants to colonies using multinomial logistic regression. The shaded cells indicate the individuals assigned to the correct colony.

	Assigned Colony					
Colony of Origin	**1**	**2**	**3**	**4**	**% Correct**	***p*** [a]
1	83	22	9	6	69.2	<0.001
2	15	92	6	7	76.7	<0.001
3	8	2	99	11	82.5	<0.001
4	5	8	14	93	77.5	<0.001
Total					76.5	<0.001

[a] The probability of achieving this result by chance

In the cross-validation procedure, 68.33% of unknown individuals were correctly assigned to Colony 1, 70.8% to Colony 2, 81.7% to Colony 3, and 77.5% to Colony 4, with an overall success rate of 74.6%.

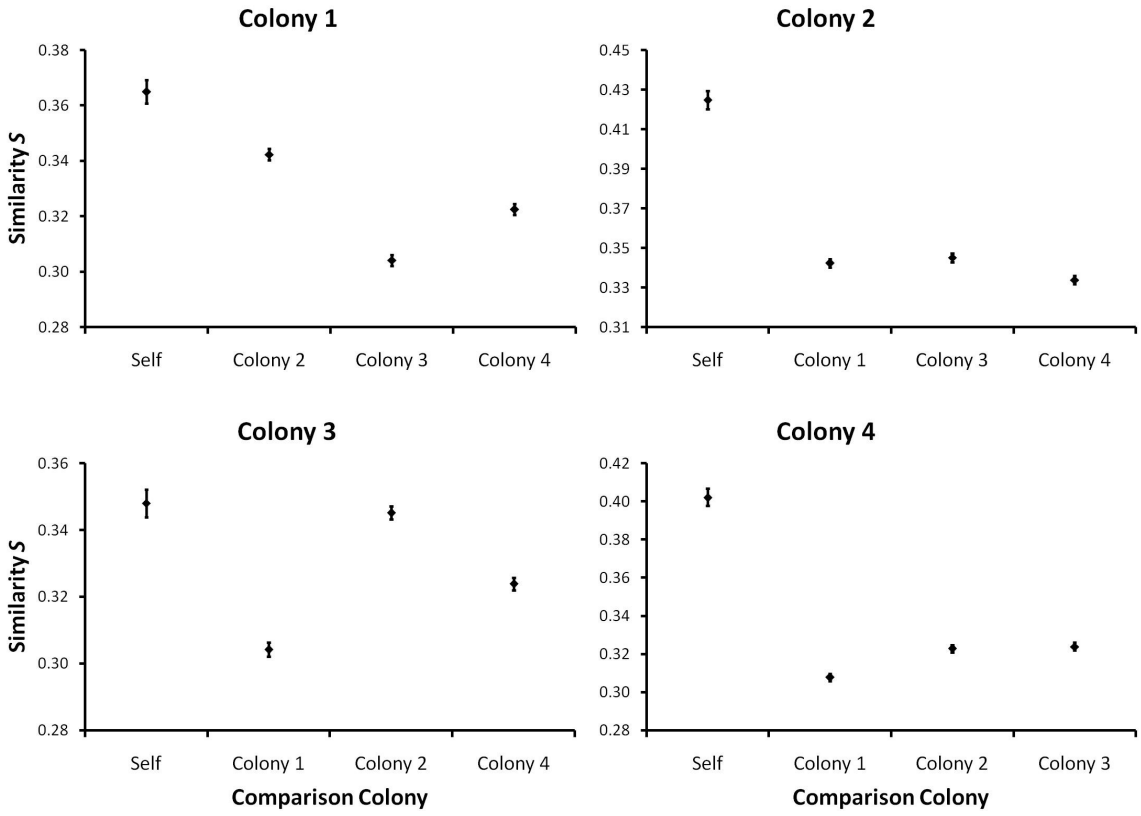

Figure 4-2**:** The mean similarity (with 95% confidence intervals) of weaver ants within a colony to each other (Self), and to ants from other colonies.

Individuals within colonies were, on average, significantly more similar to each other than to individuals from other colonies (Figure 4-2: $p < 0.0001$ in each case, except Colony 3 and Colony 2 [$p = 0.0048$]).

4.3.2 Nest allocation

When logistic regression was carried out on random pairs of groups, approximately 61.7% of individuals were correctly re-assigned to their group. In every case except Colony 1, the percentage of individuals correctly assigned to their nest of origin within a colony was significantly greater than this at the 0.01 level of significance (Table 4-2). In Colony 1, 75% of individuals were correctly assigned to nest *a*, which was significantly better than random ($p = 0.027$); however, the success rate for nest *b* (63.3%) was not better than random ($p = 0.461$); and the overall success rate for the nests in that colony (69.2%) was just shy of being significantly better than random ($p = 0.056$). Overall, 79.6% of individuals were assigned to the correct nest.

Except for Colony 1, individuals within nests were consistently more similar to each other than they were to colony mates in the other nest ($p < 0.0001$; Figure 4-3). In Colony 1, the

Table 4-2**:** Allocation of ants to nests within colonies using logistic regression. The shaded cells indicate the individuals assigned to the correct nest.

		Assigned Nest			
Colony/Nest of Origin					
Colony	**Nest**	**a**	**b**	**% Correct**	***p*[a]**
1	**a**	45	15	75.0	0.027
	b	22	38	63.3	0.461
	Total			69.2	0.056
2	**a**	46	14	76.7	0.003
	b	11	49	81.7	< 0.001
	Total			79.2	< 0.001
3	**a**	52	8	86.7	< 0.001
	b	9	51	85.0	< 0.001
	Total			85.8	< 0.001
4	**a**	50	10	83.3	< 0.001
	b	9	51	85.0	< 0.001
	Total			84.2	< 0.001

[a] The probability of achieving this result by chance

similarity of individuals to each other in nest *b* was not significantly different from their similarity to individuals from nest *a* ($p = 0.7182$). This would account for the low success rate at assigning ants to nest *b*: there was a high probability of being mistakenly assigned to nest *a*.

In most cases, individuals from one nest within a colony were significantly more similar to individuals from the other nest within the colony than they were to individuals from any other nest. The exceptions were as follows: In Colony 1, individuals from nest *a* were no more similar to individuals from nest *b* than they were to individuals from Colony 2, nest *a* ($p = 0.0858$). In Colony 4, individuals from nest *a* were no more similar to individuals from nest *b* than they were to individuals from Colony 2, nest *a* ($p = 0.0821$) or Colony 3, nest *a* ($p = 0.1074$). In Colony 3, individuals from both nests were actually more similar to individuals from Colony 2, nest *a* and *b*, and Colony 4, nest *a*, than to individuals from their sister nest ($p < 0.0001$ in each case). This is reflected in the fact that Colony 3 was more similar to Colony 2 than to any other colony (Figure 4-2), although the difference was still significant ($p = 0.0048$). Despite this, Colony 3 had the highest percentage overall of individuals correctly assigned to it (Table 4-1).

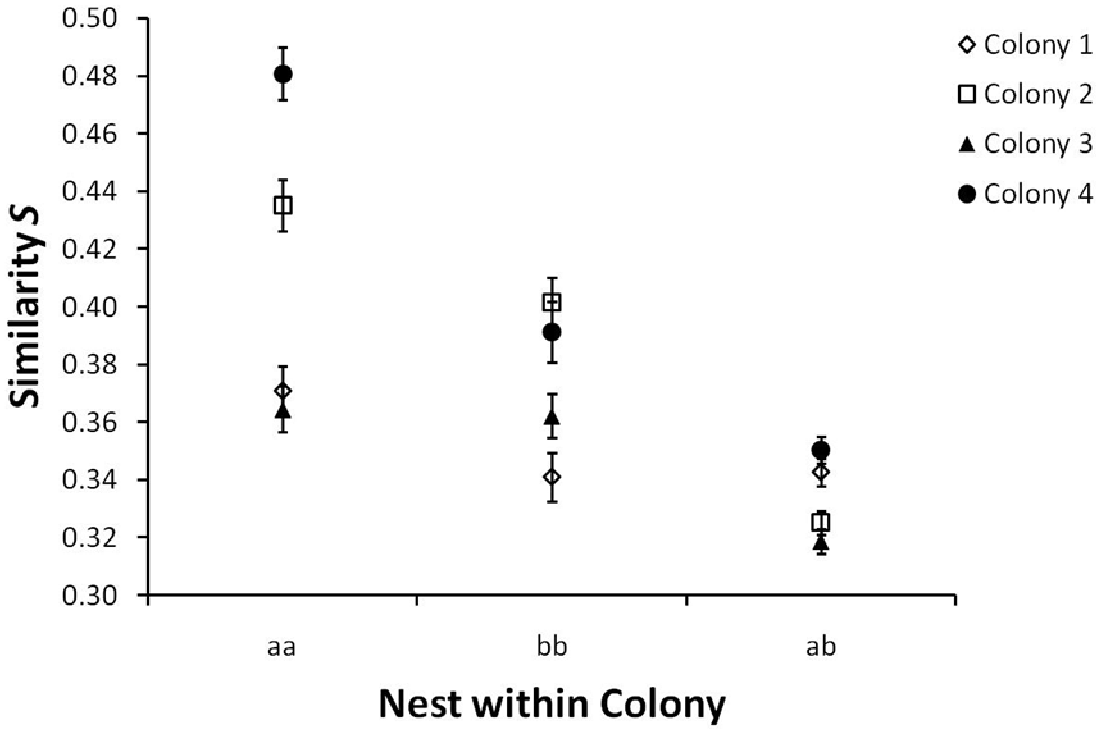

Figure 4-3**:** The mean similarity (with 95% confidence intervals) of weaver ants within each nest in a colony (*aa*, *bb*) compared with the similarity between the two nests (*ab*).

4.4 Discussion

The analysis shows that weaver ants can be correctly assigned to their colony of origin using spectra generated by scanning individuals in the near-infrared, with an overall success rate of 76.5%. This was more than twice what could be achieved by random allocation (37%). Furthermore, 74.6% of unknown individuals could be assigned to the correct colony.

The success rate was lower than the 86.4% attained by Heinze et al. (2002) for *Pachycondola cf. inversa* from four colonies, using GCMS to identify CHCs. However, these were small, single nest colonies maintained in the laboratory, and lower success rates might be expected using very large, wild polydomous colonies such as those of weaver ants. The success rate was also lower than the 83.9% attained by van Wilgenburg et al. (2006) with four colonies of polydomous Australian meat ants. However, the procedure for replacing missing values meant that the results were conservative, and when only individuals with all peaks present were used in the analysis, the results were closer to those of van Wilgenburg (80.8%). Using a similar cross-validation procedure to that used here, van Wilgenburg et al. (2006) achieved a success rate of 79.2%, compared with a success rate in this study of 74.6%. Furthermore, NIRS could assign, overall, 79.6% of individuals to the correct nest within a colony, while van Wilgenburg

et al. (2006) achieved a success rate of only 73.6%. That the inter-nest differentiation of *O. smaragdina* colonies is comparable to those in meat ant colonies is striking, given that weaver ant leaf nests are relatively evanescent, being abandoned when the leaves die, whereas meat ant nests can persist for decades (Greenslade 1974). The lower success rate attained when assigning individuals to colonies may result in part from broader variation within colonies of *O. smaragdina* compared to *I. purpureus*.

This study extends the findings of van Wilgenburg et al. (2006), and confirms that in large, polydomous ant colonies, individual nests vary in their chemical profile. Slight variations in microhabitat and diet, and imperfect exchange of chemical components between nests, probably account for these differences. However, the study also shows that this does not necessarily negate the existence of an overall colony gestalt (Crozier and Dix 1979), which may be able to accommodate these inter-nest differences, just as it can accommodate differences between individuals. In support of this, in most colonies, individuals from one nest within the colony were, on average, more similar to individuals from the other nest within the colony than they were to individuals from any nest from any other colony. The main exception to this was in Colony 3, where individuals from both nests were, on average, more similar to individuals from both nests of Colony 2, and one nest of Colony 4, than they were to individuals from their sister nest. Yet this did not result in confusion between these colonies when the logistic regression was performed. Indeed, Colony 3 had the highest proportion correctly assigned to it. It seems, therefore, that a distinctive colony identity can be maintained despite differences between nests within colonies, and similarities between individuals from nests in different colonies.

A novel feature of the present study was the use of NIRS, rather than analysis of extracted CHCs, to discriminate between colonies. While it may take up to 20 minutes to extract CHCs from a specimen, and a further 30 minutes to then process each sample, a specimen can be scanned in less than one minute using NIRS. I suggest that this technique offers considerable opportunities for the study of social insects and, indeed, other insect communities. While it is unlikely to replace GCMS when chemical precision is required, it may provide a useful alternative when such precision is not essential. Being able to assign unknown individuals to a colony may be valuable if colony boundaries are unclear, or foraging areas overlap. NIRS should be further explored to determine if it can be used to differentiate, for example, between types of larvae (male/female, gyne/worker) or even eggs. Dowell et al. (2005) demonstrated that Tsetse fly pupae could be sexed with accuracies ranging from 80 to 100% using NIRS. The ability to successfully define individual social insects with an efficient high-throughput technique such as NIRS presents significant opportunities for the evolutionary, physiological and behavioural study of social insects.

5 Aggression in weaver ants reflects differences in near-infrared spectra

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Abstract

CHCs play an important role in insect recognition systems. A growing body of evidence indicates that insects are able to act upon the information contained in CHCs. However, investigating the behavioural response of insects to cuticular compounds usually involves the extraction and analysis of CHCs using GCMS, which is a time consuming and expensive process. NIRS may provide a much faster and less expensive technique for studying the behavioural response of insects to cuticular compounds. Here I present a case study of inter-colonial aggression in the weaver ant. I show that the level of aggression expressed by colonies towards intruders increased as the spectral distance between colonies increased. The variability in the aggressive response also increased as the breadth of within-colony variation in spectra increased. This suggests that spectra generated using NIRS encode information to which weaver ants are able to respond. I discuss the implications of this for behavioural studies that have previously depended upon the extraction and analysis of CHCs.

5.1 Introduction

Although the primary function of insect CHCs is to prevent desiccation (Neville 1975), they also play a key role in insect recognition systems (reviewed by Howard and Blomquist 2005; Singer 1998). As previously discussed (Chapter 2) a growing body of evidence indicates that many insects are able to act upon the information contained in CHCs. However, investigating the behavioural response of insects to cuticular compounds usually involves the extraction and analysis of CHCs using GCMS. An alternative may be to use NIRS. Although this lacks the chemical specificity of GCMS, the spectra generated contain information that is related to the chemical content of the cuticle, including CHCs. In cases where it is not necessary to identify specific chemicals but simply to quantify the chemical difference between specimens, this method may provide sufficient information.

In the previous chapter I demonstrated that NIRS could be used to discriminate between individuals from four different colonies of weaver ants, with a success rate ranging from 76.5% to 80.3%, depending on the method used. If it can be shown that behaviour is correlated with the information contained in spectra, as it is correlated with the information contained in CHCs, this may facilitate a wide range of behavioural studies that seek to relate behaviour to the chemical profile of the individual or the colony.

Here I seek to determine whether the aggressive behaviour expressed by colonies of weaver ants towards conspecific intruders from other colonies is a function of the spectral distance between colonies, as measured using NIRS. Social insects generally react aggressively towards intruders, and in several species aggression has been shown to increase with the chemical distance between colonies (D'Ettorre et al. 2006; Foitzik et al. 2007; Kaib et al. 2004; Suarez et al. 2002). The level of aggression may also depend on the within-colony variation in the chemical profile, assuming that workers use this profile as a template against which to assess unknown individuals (Tsutsui 2004). Colonies with greater variation may be more tolerant towards intruders than colonies with less variation, because the acceptance threshold (Getz 1981; Reeve 1989) in these colonies is likely to be higher. Both polydomy (multiple nests per colony) (Van Wilgenburg et al. 2006) and greater genetic diversity, as a result of either multiple queens per colony (polygyny) or multiple mating of queens (polyandry) (Pirk et al. 2001; Starks et al. 1998), may increase the variation in the chemical profile, and reduce the levels of aggression shown towards intruders.

Colonies of weaver ants are aggressively territorial, with fierce battles often breaking out at colony boundaries (Hölldobler 1983b). Nevertheless, the level of aggression expressed between colonies appears to vary (personal observation). *Oecophylla smaragdina* is therefore a useful model for studying the behavioural response to the information contained in near-infrared spectra.

I sought to determine whether: (1) the level of aggression between weaver ant colonies decreased as the spectral distance between them decreased; (2) the level of aggression displayed by a colony towards intruders increased as the within-colony variation in spectra decreased, lowering the acceptance threshold; and (3) the variability in the aggressive response increased as the variability in the spectra within the recipient colony increased.

5.2 Methods

I collected 120 weaver ants from each of six colonies in the grounds of James Cook University, Cairns, North Queensland. None of the colonies were near neighbours. I stored samples at -4°C before thawing and scanning with a Bruker Optics Multi Purpose Analyzer ®.

5.2.1 Colony Spectra

The protocol for acquiring spectra is described in Chapter 4, and the same peak values were used for the present study.

I used principal components analysis to reduce the data to a set of six orthogonal factors (F1 to F6), accounting for 22.26, 17.14, 13.72, 9.46, 8.45 and 7.77% of the variance respectively, a total of 78.79%. These factors represent the position of each scanned individual in 6-dimensional Euclidean space. I calculated the mean Euclidean distance, that is, the straight-line distance in this 6-dimensional space, between an individual from one colony and every individual from another colony, repeating this for all 120 individuals in the first colony. The mean for all 120 individuals provided an estimate of the distance (D_o – distance to other) between the two groups. I also calculated the distance along each individual axis (F1 to F6), that is, in one dimension at a time. To estimate the breadth of each colony spectral profile I calculated the mean Euclidean distance between each individual from that colony and the 119 other individuals from that colony, using the mean for all individuals as an estimate of the internal similarity of colony members. This intra-colonial distance I designated D_s (distance to self). The smaller the distance, the greater the similarity and the narrower the colony spectral profile.

5.2.2 Aggression Bioassay

For the aggression bioassay, I collected a medium-size nest from five of the six colonies to serve as recipient colonies. While all six colonies provided intruders, I was unable to obtain a large enough nest from Colony 3 for it to serve as a recipient colony. Nests were maintained in small, ventilated plastic boxes for the duration of the trials and provided with diluted honey. For each trial, I introduced five individuals from the recipient colony into a small observation area. I applied a spot of water-based acrylic paint to the pronotum of an individual

from the intruder colony, and placed this individual into a small tube adjacent to the test area. An opening between the tube and the test area was initially sealed. I allowed several minutes for the recipient ants and intruder ant to acclimate, before removing the seal between the tube and the test area, permitting the intruder to enter the test area. I recorded the behaviour of the recipient ants towards the intruder at ten second intervals for a period of three minutes. Timing began from the moment of first contact, resulting in 19 observations per trial. The behaviours recorded were: aggressive posture (mandibles open towards the intruder, with or without raised gaster), pursuit and biting/grappling. I graded these in intensity from: 1 (aggressive posture); to 2 (pursuit); and 3 (biting/grappling). An index of aggression (A) was calculated:

$$A = \Sigma f_i i$$

where i is the intensity of response and f_i is the frequency of that response. A could assume a value ranging from 0 (no aggressive behaviour recorded) to 57 (biting recorded at every observation). I conducted 20 trials for each recipient-intruder combination, including a control in which intruders were derived from the recipient colony, with different recipient and intruder ants in each trial. On the rare occasions when the intruder seized and immobilised one of the recipient ants, the trial was repeated with different individuals. I was aware of the colony of origin of recipient and intruder ants, but because spectra were not analysed until the completion of the behavioural trials, I was blind to the spectral distance between recipient and intruder colonies.

Because the aggression index A was not normally distributed, I used a bootstrapping method to estimate the mean and 95% confidence intervals around the mean for each colony-colony interaction (See Appendix A.2). I randomly selected 20 values (with replacement) from among the original 20 values for each colony-colony interaction and re-calculated the mean. I repeated this 10,000 times and identified the 95% confidence intervals as the two values that encompassed the central 95% of the distribution (Efron 1979). Using the same methodology, I also generated an estimate for the standard deviation in aggression (A_{SD}) for each colony-colony interaction, as a measure of variability.

I analysed the relationship between aggression and distance using linear regression models, with mean D_o and mean D_s as the predictor variables in one model, and mean distance along each axis as predictor variables in the second model. I used a stepwise variable entry method, and tested for collinearity between predictor variables. All analyses and simulations were carried out using either SPSS 13.0 for MacIntosh or MS Excel 2007.

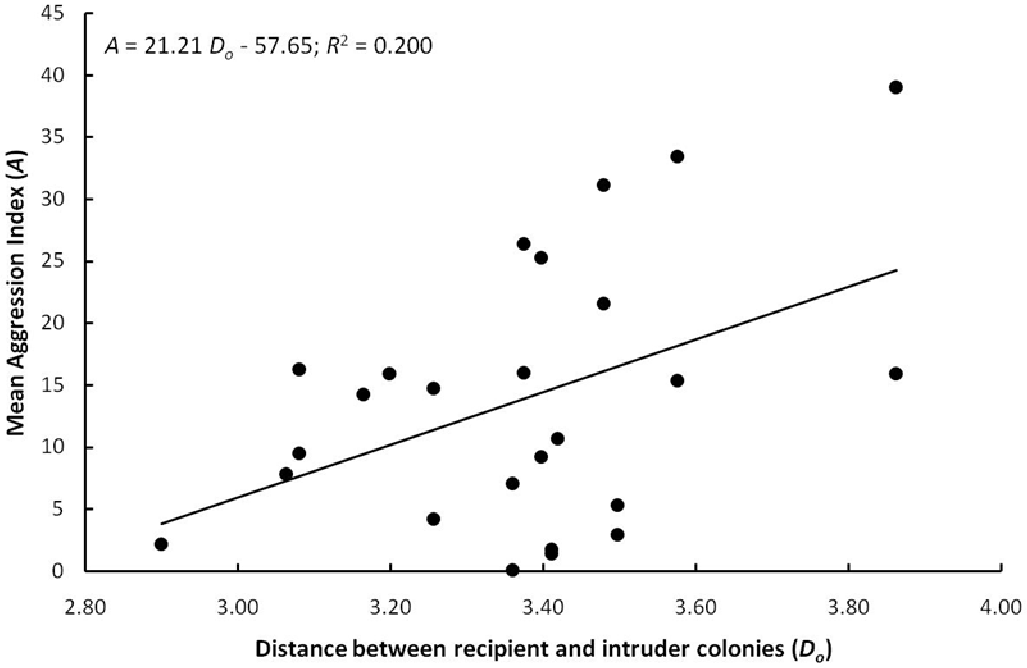

Figure 5-1**:** Relationship between the mean aggression index (*A*) of *O. smaragdina* colonies and the mean spectral distance (D_o) between individuals from the recipient colony and the intruder colony.

5.3 Results

The mean level of aggression displayed by recipient colonies towards colony-mates was very low (*A* = 0.07; 95% Confidence Interval [CI]: 0.01 – 0.15), and rarely consisted of more than occasional aggressive posturing. The level of aggression displayed towards individual intruders from other colonies was highly variable, resulting in large confidence intervals around the means. The aggression index *A* ranged from 4.80 (CI: 2.53 - 7.53) for Colony 2 to 18.10 (CI: 13.68 – 22.56) for Colony 4.

Aggression increased with the mean distance between individuals from the recipient and intruder colonies (D_o: $F_{1,23}$ = 5.767, R^2 = 0.200, p = 0.025; Figure 5-1), but the distance between individuals within the recipient colony had no significant effect on aggression (D_S: $F_{1,22}$ change = 1.524, R^2 change = 0.052, p = 0.230).

When the distances along the individual axes, F1 to F6, were entered into a multiple regression model as independent variables, with aggression as the dependent variable, only F4 was significant ($F_{1,23}$ = 5.767, R^2 = 0.300, p = 0.005; Figure 5-2). However, this result should be interpreted with caution, as the collinearity statistics indicated that some of these variables were

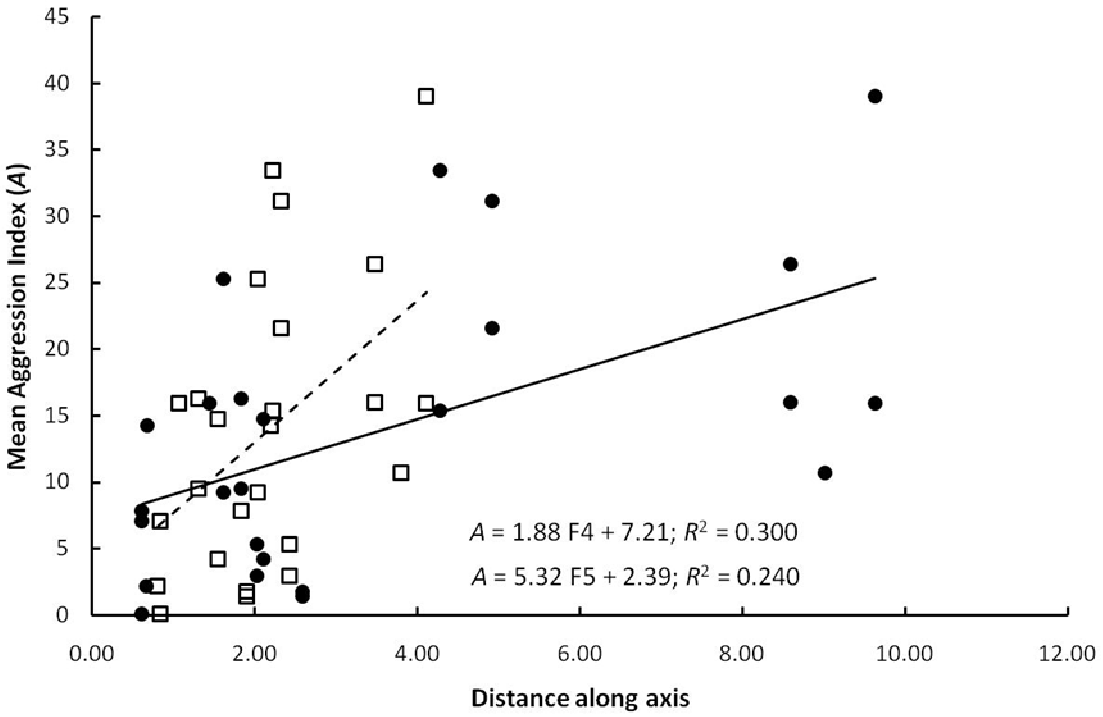

Figure 5-2**:** Relationship between the mean aggression index (*A*) of *O. smaragdina* colonies and the mean spectral distance between individuals from the recipient colony and the intruder colony along two axes, F4 (🡻) and F5 (□).

highly correlated. In particular, F4 and F5 were highly correlated ($r = 0.910$, $N = 25$, $p < 0.001$). With F4 omitted from the regression model, F5 contributed significantly ($F_{1,23} = 7.246$, $R^2 = 0.240$, $p = 0.013$; Figure 5-2). F4 and F5 were most closely connected with the parameters of P6 (4553 – 4651 per cm). F4 was highly correlated with the frequency of P6 ($r = 0.807$), while F5 was highly correlated with the intensity ($r = 0.836$) and width ($r = 0.791$) of P6.

The mean breadth (± SD) of each colony spectral profile (D_s) was: 3.29 ± 0.47 for C1, 2.80 ± 0.48 for C2, 3.45 ± 0.73 for C4, 3.00 ± 0.64 for C5, and 3.00 ± 0.56 for C6. The standard deviation in the aggressive response (A_{SD}) increased significantly as D_s of the recipient colony increased ($F_{1,23} = 5.807$, $R^2 = 0.202$, $p = 0.024$). However, a pure error lack-of-fit test showed that the linear model provided only a marginally adequate fit to the data ($p = 0.051$). With the quadratic term added to the model, there was significant improvement ($F_{1,22} = 7.520$, R^2 change $= 0.203$, $p = 0.012$). A pure error lack-of-fit test showed that the new model, including both the linear and quadratic terms, was a good fit to the data ($p = 0.42$). This suggests that the increase began to fall away as D_s increased further (Figure 5-3). Colony C2, which had the narrowest spectral profile, clearly also had the most consistent aggressive response (Helmert Contrasts, *p*

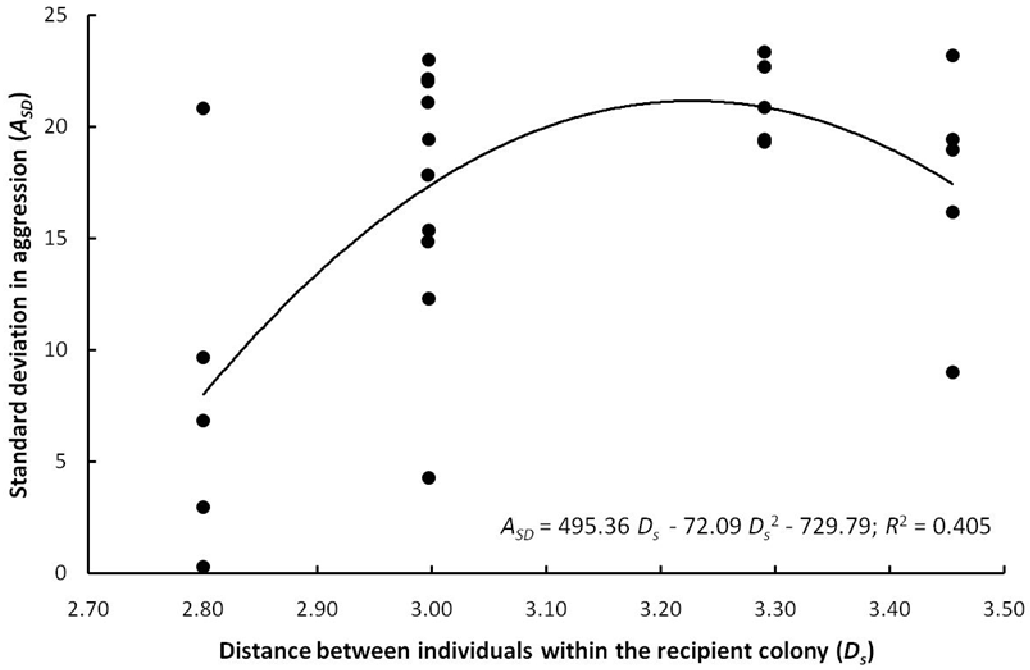

Figure 5-3**:** Relationship between the standard deviation in aggression (A_{SD}) shown by *O. smaragdina* colonies towards intruders and the mean spectral distance (D_s) between individuals from the recipient colony.

= 0.002), with a mean A_{SD} of 8.11. The total model with the linear and quadratic terms accounted for approximately 40% of the variation in A_{SD}.

5.4 Discussion

The CHCs of the insect cuticle contain a large quantity of information to which individual insects can potentially respond. That they do so has been demonstrated across a range of taxa in a variety of contexts. Among social insects, aggression between colonies has been found to increase with chemical distance in several taxa. In the present study, I demonstrated that spectra generated using NIRS also contain encode information to which weaver ants were able to respond. The level of aggression shown towards intruders increased as the mean spectral distance between individuals from the recipient and intruder colonies increased. Furthermore, two particular factors, correlated with the parameters of P6 (4553 – 4651 per cm), made the most significant contribution to the aggressive response. This suggests that information correlated with particular spectral features plays a role in determining the response of recipient colonies to intruders.

Contrary to expectations, the mean level of aggression did not increase as the within-colony spectral variation decreased. This contrasts with findings from other studies, in which either genetic relatedness or the level of polydomy is assumed to affect the breadth of the chemical profile. For example, Pirk et al. (2001) found that aggression was significantly correlated with the intra-nest relatedness of recipient colonies in *Formica pratensis*. Van Wilgenburg et al. (2006) demonstrated that ants from polydomous colonies of the Australian meat ant, *Iridomyrmex purpureus*, were less aggressive towards intruders than ants from oligodomous colonies. In contrast to this, Rosset et al. (2007) found that aggression did not vary significantly with queen number in colonies of the ant *Formica selysi*, and Pirk et al. (2001) detected no difference in the level of aggression expressed by polydomous and monodomous colonies of *F. pratensis*.

This negative result regarding within-colony variation is, therefore, well within the parameters of other studies. Although a broader chemical profile may increase the probability that an individual intruder falls below the acceptance threshold (Reeve 1989), this does not necessarily make colonies systematically less aggressive. Consider two colonies, *A* and *B*, where *A* has a narrow chemical profile and *B* a broad chemical profile, into which intruders from a third colony, *C*, are introduced. If all individuals from *C* are beyond the acceptance threshold of both *A* and *B* there is no reason to expect that *B* will be less aggressive towards *C* than *A*, despite *B*'s broader profile. A broader template does not decrease the level of aggression expressed towards intruders that fall beyond that threshold. This may account for the conflicting results obtained in other studies. I argue that the breadth of the colony spectral profile cannot be treated in isolation from the spectral distance between the recipient colony and the intruder. Furthermore, if individuals within the colony have divergent spectral profiles, as a result of environmental, dietary or genetic factors (Crozier and Pamilo 1996), then if chemicals are imperfectly shared throughout the colony (via trophallaxis and allogrooming), individuals may vary slightly in their recognition template; that is, a perfect gestalt model (Crozier and Dix 1979) might not apply. This would result in diverse responses to an intruder by different individuals within the recipient colony. Within a colony, some individuals are likely to be spectrally more distant from an intruder than others. The greater the diversity of spectra within the colony, the more pronounced this will be, and the more varied the aggressive response.

In support of this, I found that the aggressive response of the recipient colony became more variable as intra-colonial spectral distance increased. The general observation that the level of aggression directed towards intruders could range from zero (minimum) to 57 (maximum) in any colony-colony interaction, and that some recipient ants would ignore an intruder while others attacked it, supports the hypothesis that ants within the same colony may use different recognition templates, or may perceive themselves to be different distances from the intruder. The lack of aggression exhibited by some workers may also reflect the context in

which the aggression trials were conducted, if the test arena was not regarded by the ants themselves as defendable territory: the context in which encounters occur can influence acceptance thresholds (Liebert and Starks 2004). Nevertheless, individual reactions varied while the context remained constant. Furthermore, in aggression bioassays in which intruders were introduced directly into nest sites, the response to intruders could still vary considerably (Roulston et al. 2003). Future studies may wish to explore the relationship between spectra and aggression at the individual rather than the colony level.

When an individual is confronted with the task of classifying another individual as either self or non-self (Tsutsui 2004), two types of error can occur: inappropriately accepting non-self as self, analogous to a Type II statistical error (Reeve 1989), or inappropriately rejecting self as non-self, analogous to a Type I statistical error (Reeve 1989). Type II errors appear to be quite common among weaver ants, while Type I errors are rare. Both presumably incur fitness costs, but it appears that selection in weaver ants has favoured an acceptance threshold that permits high levels of Type II errors, but almost no Type I errors. A high threshold makes sense in large colonies, as encounters with self are much more likely than encounters with non-self (Reeve 1989).

Some studies have shown that aggressive behaviour is reduced between neighbour colonies (Heinze et al. 1996; Langen et al. 2000), due to a phenomenon known as the "dear enemy" effect. This avoids costly battles between neighbours. Frequently, however, the opposite effect is observed: aggression is greater towards neighbours than more distant conspecifics (Gordon 1989; Knaden and Wehner 2003; Sanada-Morimura et al. 2003; Thomas et al. 1999; Thomas et al. 2007). Neighbours may pose a more real and immediate threat than others, particularly if there is intense competition for resources. All the interactions in this study were between widely separated colonies. It is therefore possible that individuals did not act very aggressively towards intruders because they were unfamiliar with their odour, that is, they were not recognised as potentially dangerous neighbours. Both the dear enemy effect and its opposite presuppose that colony members can incorporate new information into their recognition template and modify their behaviour accordingly. Studying interactions between neighbouring colonies would help determine whether the dear enemy effect or its opposite occurs in weaver ants.

Finally, the failure of an individual to correctly identify an intruder does not necessarily compromise the integrity of the colony if another colony member is able to make a correct identification. Indeed, if all individuals possessed the same recognition template, some intruders would completely escape detection. There may, therefore, be a selective advantage in having a range of recognition templates operating within the colony. This is analogous to the body having a range of antibodies to deal with different types of intrusive pathogens.

I have shown that the behaviour of weaver ants is correlated with differences between colonies that are detected by NIRS. I confirmed that workers were more aggressive towards intruders that were spectrally more distant from their own colony, and that the degree of variability in the aggressive response increased as the within-colony variation in spectra increased. There remained a large proportion of unexplained variation in aggression, at the level of both the colony and the individual, and this requires further investigation. This unexplained variation means that spectral distance may be more suited to making qualitative rather than precise quantitative predictions concerning the level of aggression between colonies. Nevertheless, these are significant findings. The behavioural response to variations in CHCs is well-documented, but, as far as I am aware, this is the first time that a behavioural response to chemical variations encoded in spectra generated using NIRS has been demonstrated. This approach has the potential to save a great deal of time and money, and to facilitate a range of behavioural studies, where precise knowledge of the chemicals involved in recognition is not required. NIRS contains sufficient chemical information to make it possible to relate behaviour to chemical differences: indeed, it represents this information in a holistic form that may more realistically model what the individual encounters in the real world when confronted with important decisions. For example, studies of mate recognition and cooperative behaviour across populations, and its relationship to the chemical distance between populations, such as that recently undertaken for the scarab beetle, *Canthon cyanellus cyanellus* (Coleoptera : Scarabaeidae) (Ortiz-Dominguez et al. 2006a), could be carried out using NIRS, without the need to extract CHCs.

I recommend that future studies examine the impact of a range of other factors, such as diet, environment and genetic variation, on NIR spectra, so that the potential of this methodology might be fully realised.

6 Nest and colony specific spectra in the weaver ant

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Abstract

Animals in social groups need to differentiate between group members and others. In very large groups, such as those formed by many ant species, it is not possible to rely on individually specific cues to identify colony-mates. Instead, recognition must be based on colony-specific cues. Individual ant colonies tend to have a specific chemical gestalt that is maintained by the continual exchange of chemicals between workers. In very large polydomous colonies, the exchange of chemicals may be limited between nests within the colony, resulting in inter-nest variation in colony odour that might hinder identification of colony-mates or conspecific intruders. I used near-infrared spectroscopy to explore variation in the chemical profile between and within colonies of weaver ants. I found that differences between colonies were reflected in the position, amplitude and width of spectral peaks, while differences between nests within colonies were reflected mainly in amplitude. Furthermore, in the context of colony-mate recognition the behaviour of the ants themselves was positively correlated with colony-specific spectral characteristics, rather than with nest-specific characteristics. Thus colony spectra have features that are not obscured by intra-colonial variation and may potentially encode the chemical characteristics used by workers to identify colony-mates.

6.1 Introduction

In Chapter 4 I showed that NIRS could be used to assign individual weaver ant workers to their colony of origin, and also to their nest-within-colony of origin. This suggests that spectra contain information that differentiates both between colonies and between nests within colonies. In extensive polydomous colonies, with limited exchange of workers between nests, it may be difficult to maintain a single colony gestalt odour. However, there is no evidence for aggressive behaviour between weaver ant workers from different nests within the same colony (personal observation). This suggests that, in this species, the colony-specific chemical signature is not obscured by intra-colonial variation. In the present study I used five nests from each of five colonies to explore in more detail the variation in the spectral signature within and between colonies. I sought to differentiate between two hypotheses. The presence of both colony and nest-within-colony differences may be a question of magnitude: the differences between colonies may simply be greater than the differences between nests within colonies, although they involve the same parameters (quantitative hypothesis). Thus, for example, differences between both nests and colonies might be attributable to differences in the amplitudes (or positions or widths) of the same peaks, with differences between colonies simply being more pronounced. Alternatively, there may be qualitative differences: the differences between colonies may be characterised by some spectral components while the differences between nests may be characterised by others (qualitative hypothesis). For example, differences between colonies might be in the positions of peaks, while differences between nests might be in the amplitudes of peaks; or colonies may vary in the width of one peak, while nests vary in the width of another.

Finally, I explored the relationship between intra-colonial aggression and specific spectral characteristics. In Chapter 5 I demonstrated that aggression increased as the spectral distance between colonies increased. However, if the qualitative hypothesis is supported, I would expect the level of aggression between colonies to be related specifically to spectral features that varied between colonies, but not to features that varied between nests within colonies. On the basis of my findings here I reanalysed the data on aggression from Chapter 5 to test this hypothesis.

6.2 Methods

6.2.1 Colony and nest spectra

In September and October 2008, 20 workers were collected from each of five nests from five different colonies (C1 to C5) in the grounds of the Cairns campus of James Cook

Table 6-1**:** Main peaks identified using NIRS: mean location ± SD. The chemical bonds most likely associated with those peaks are listed

peak	location (wavenumber per cm)	chemical bonds*
P1	8659.70 ± 21.62	Second overtone: CH_2, CH_3
P2	7024.92 ± 23.12	First overtone of CH combinations; first overtone of H_2O, R-OH.
P3	5790.62 ± 5.41	First overtone: CH, CH_2, SH
P4	5640.78 ± 9.90	First overtone: CH
P5	5225.80 ± 9.44	R-$CONH_2$
P6	4599.62 ± 8.68	C-C combinations; R-CHO; R-NH_2
P7	4255.11 ± 23.04	CH, CH_2, CH_3 combinations

* Source: BrukerOptics (2008)

University and the surrounding suburbs. To ensure that nests were from the same colony, interconnecting trails were followed, and individuals from one nest were introduced into another nest to determine the response to the intruder: an aggressive response indicated that the intruder originated from a different colony. Colonies were separated by several hundred metres. Samples were stored at -4°C before thawing and scanning with a Bruker Optics Multi Purpose Analyzer ®. Samples were scanned within three days of collection.

Spectra were obtained following the protocol detailed in Chapters 4 and 5, with one modification: peak detection thresholds were lowered from 0.44% to 0.15% to reduce the proportion of undetected peaks. Scanning was conducted at room temperature (approximately 20 - 25°C). This resulted in the identification of seven spectral peaks, P1 to P7 (Table 6-1), to use in the analysis. I used the software package OPUS 6.0 (Bruker Optics Inc., Ettlingen, Germany; Build: 6,0,72, 1997e2006) to measure the position (wavenumber per cm), amplitude (absorbance units) and width at 50% amplitude (wavenumber per cm) of each peak.

I used a nested analysis of variance (ANOVA) design to determine whether there was a difference in peak position, amplitude and/or width for each peak (i) between nests within colonies and (ii) between colonies. With 21 variables and 25 nests in total, the normality of the data and equality of variances across all groups and subgroups could not be guaranteed. I therefore conducted a permutation version of the nested ANOVA (Anderson and Ter Braak 2003) in MS Excel 2007 (see Appendix A.3). To test for differences between nests within colonies, permutations were carried out on individual samples between nests within colonies; that is, the samples from all the nests in a colony were shuffled and randomly reassigned to one of five nests within that colony, but samples remained within their original colony. To test for

differences between colonies, permutations were carried out on nests between colonies; that is, nests were shuffled between colonies, with five nests per colony, but with each sample remaining in its original nest. In each case 10,000 permutations were performed. The test statistic used was the appropriate *F* statistic for each level of the analysis (Zar 1999):

$$F_{nest} = MS_{nest}/MS_{error}$$

$$F_{colony} = MS_{colony}/MS_{nest},$$

where MS is the mean sum of squares. If a difference was found at the colony level, nests within colonies were subsequently pooled and a (permutation) ANOVA conducted, followed by Tukey's HSD test to determine which colonies were different from each other (Appendix A.3).

6.2.2 Aggression and colony spectra

To determine the behavioural response of workers to different spectral components I re-analysed data from Chapter 5 in which the same spectral peaks (except P7) were used to determine the relationship between aggression and the spectral distance between colonies. Whereas in that study all peak parameters were used to calculate the distance between colonies, here I used (1) only those spectral characteristics that varied between colonies, but not between nests within colonies (D_1); and (2) all remaining spectral parameters (D_2). Details of the method used to calculate spectral distance can be found in Chapter 5. I then used linear regression models in SPSS 16.0 for Windows (copyright SPSS Inc. 1989-2007) to analyse the relationship between the aggression index *A* (see Chapter 5) and D_1 and D_2.

6.3 Results

6.3.1 Colony and nest spectra

The locations of P1, P2 and P5 differed significantly between colonies (Table 6-2). Post-hoc comparisons revealed that colonies could be differentiated into two distinct subgroups on the basis of P1, with C1 and C5 forming one group and C2, C3 and C4 forming the other group (Figure 6-1a). Colonies could be differentiated into three distinct subgroups on the basis of P2 (Figure 6-1b). The situation for P5 was more complicated, with C4 clearly differentiated from all colonies except C2, and C1 and C3 clearly differentiated from all colonies except C5; there was some overlap between other colony combinations (Figure 6-1c).

There were no significant differences in the positions of any peaks between nests within colonies (Table 6-2).

The amplitudes of P1, P2, P3 and P5 varied significantly between colonies (Table 6-3). However, there were also significant differences in the amplitudes of five of the seven peaks between nests within colonies (Table 6-3). Within C1 there were significant differences in the

Table 6-2**:** Variation in peak location (wavenumber per cm), obtained using NIRS, between colonies and between nests within colonies.

peak	between colonies				within colonies			
	F	***DF***	***R²***	***P***	***F***	***DF***	***R²***	***P***
P1	11.47	4/464	0.08	**	0.86	20/464	0.03	NS
P2	10.90	4/476	0.11	**	1.39	20/476	0.05	NS
P3	2.39	4/476	0.03	NS	1.36	20/476	0.05	NS
P4	2.52	4/476	0.02	NS	1.20	20/476	0.05	NS
P5	5.48	4/476	0.05	*	1.28	20/476	0.05	NS
P6	2.30	4/476	0.03	NS	1.53	20/476	0.06	NS
P7	2.84	4/453	0.02	NS	0.69	20/453	0.03	NS

There were five colonies (C1 to C5) and five nests within each colony. I performed a nested ANOVA for each peak location (nests within colony) using a permutation procedure (10 000 permutations). The appropriate *F*-statistics, degrees of freedom (*DF*), R^2 and significance levels (*P*) are reported.

* significant at the 0.01 level

** significant at the 0.001 level

NS not significant

amplitudes of P3 ($F_{4,95}$ = 3.46, P = 0.0088) and P5 ($F_{4,95}$ = 2.98, P = 0.0238); within C2 there were significant differences in P1 ($F_{4,95}$ = 2.0.0265), P2 (P2: $F_{4,95}$ = 2.89, P = 0.0265) and P4 ($F_{4,93}$ = 3.19, P = 0.0178); within C3 there were significant differences in P3 ($F_{4,95}$ = 6.07, P = 0.0003); and within C4 there were significant differences in P1 ($F_{4,95}$ = 2.89, P = 0.0265) and P5 ($F_{4,95}$ = 2.90, P = 0.027). There were no significant differences in the amplitudes of any peaks between nests within C5.

There were significant differences between colonies in the widths of all seven peaks (Table 6-4; post-hoc comparisons: Figure 6-2). There were also significant differences between the widths of two peaks (P3 and P7) between nests within colonies (Table 6-4). However, significant differences in P3 were restricted to C1 ($F_{4,95}$ = 3.98, P = 0.0048), and significant differences in P7 were restricted to C5 ($F_{4,95}$ = 3.98, P = 0.0048). Thus within C2, C3 and C4, there were no significant differences between the widths of any peaks, and there were no significant differences in the widths of P1, P2, P4, P5 or P6 within any colonies.

Although significant, the differences between colonies and nests within colonies in peak positions, amplitudes and widths accounted for only a small proportion of the total variation. For example, colony identity accounted for only 8% of the variation in the position of

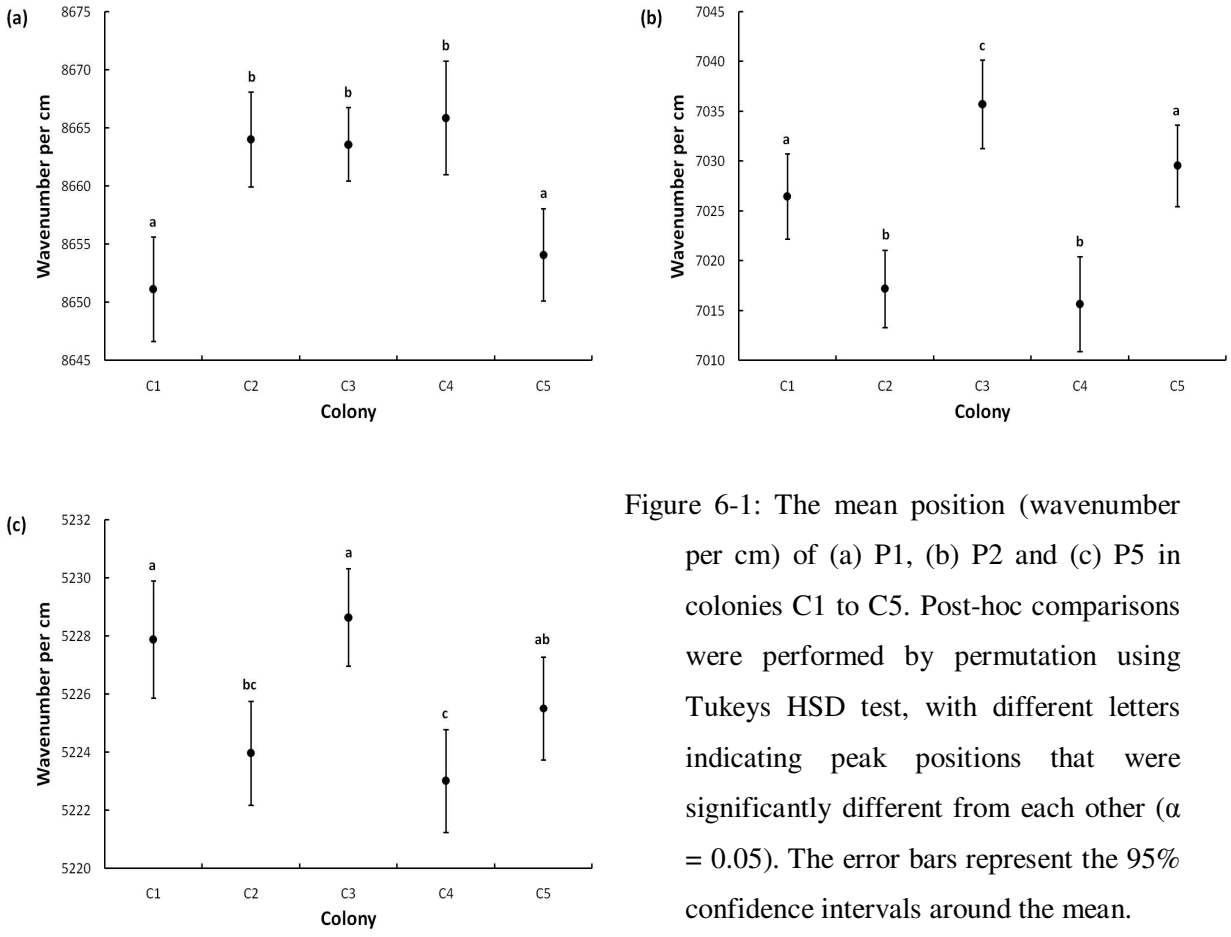

Figure 6-1: The mean position (wavenumber per cm) of (a) P1, (b) P2 and (c) P5 in colonies C1 to C5. Post-hoc comparisons were performed by permutation using Tukeys HSD test, with different letters indicating peak positions that were significantly different from each other ($\alpha = 0.05$). The error bars represent the 95% confidence intervals around the mean.

P1, 11% of the variation in the position of P2 and 5% of the variation in the position of P5 (Table 6-2). Similarly, colony identity accounted for only 8% of the variation in the amplitude of P2, 7% of the variation in the amplitude of P3 and 8% of the variation in the amplitude of P5; colony identity did, however, account for 21% of the variation in the amplitude of P1 (Table 6-3). There remained a large proportion of unexplained variation in spectral characteristics that may be attributed to differences between individuals.

6.3.2 Aggression and colony spectra

I identified 8 spectral parameters that varied between colonies, but not between nests within colonies, and used these (the position of P1, P2 and P5 and the width of P1, P2, P4, P5 and P6) to calculate the spectral distance (D_1) between colonies and analyse the relationship between spectral distance and aggression. The aggression index A increased significantly as D_1 increased (Figure 6-3a; $F_{1,23} = 9.00$, $R^2 = 0.281$, $P = 0.006$). I then used the remaining parameters (excluding P7, which was not used in the earlier study) to calculate D_2 and regressed A against this: there was no significant relationship between D_2 and A (Figure 6-3b; $F_{1,23} = 0.74$, $R^2 = 0.031$, $P = 0.398$). The relationship between D_1 and A accounted for more variance

Table 6-3: Variation in peak amplitude (absorbance units), obtained using NIRS, between colonies and between nests within colonies.

peak	between colonies				within colonies			
	F	***DF***	R^2	***P***	***F***	***DF***	R^2	***P***
P1	16.79	4/464	0.21	***	2.03	20/464	0.06	**
P2	4.76	4/476	0.08	**	1.59	20/476	0.06	*
P3	4.95	4/476	0.07	*	2.36	20/476	0.09	***
P4	1.29	4/476	0.03	NS	2.74	20/476	0.09	***
P5	8.74	4/476	0.08	**	1.51	20/476	0.06	*
P6	2.23	4/476	0.02	NS	1.36	20/476	0.05	NS
P7	1.99	4/453	0.01	NS	2.43	20/453	0.06	NS

For details of analysis see Table 6-2.

* significant at the 0.05 level

** significant at the 0.01 level

** * significant at the 0.001 level

NS not significant

(28.1%) than the original model which used all spectral parameters to calculate distance (20.0%; Chapter 5), and had a lower *P*-value (0.006 compared to 0.025).

6.4 Discussion

The analysis of the near-infrared spectra of weaver ant workers from each of five nests within five colonies revealed significant differences both between colonies, and between nests within colonies, supporting the earlier finding that spectra could be used to assign individual workers to colonies and to nests within colonies (Chapter 4). Consistent patterns emerged supporting the qualitative rather than the quantitative hypothesis. Specifically, differences between colony spectra were generated by differences in the positions, amplitudes and widths of peaks, whereas differences between nests within colonies were generated only by differences in peak amplitudes and the width of two of the seven peaks. This strongly suggests that variations in peak position and width are the main spectral components in the colony-specific signal, and that this signal is not masked by variations between nests within colonies.

Previous studies of the response of ants (Akino et al. 2004; Martin et al. 2008) and other social insects (reviewed by Dani 2006) to cuticular hydrocarbons (CHCs) demonstrate

Table 6-4**:** Variation in peak width, obtained using NIRS, between colonies and between nests within colonies.

peak	between colonies				within colonies			
	F	***DF***	R^2	***P***	***F***	***DF***	R^2	***P***
P1	14.02	4/464	0.08	***	0.75	20/464	0.03	NS
P2	5.50	4/476	0.06	**	1.38	20/476	0.05	NS
P3	6.69	4/476	0.08	**	1.74	20/476	0.06	*
P4	20.76	4/476	0.13	***	0.85	20/476	0.03	NS
P5	10.73	4/476	0.09	***	1.17	20/476	0.04	NS
P6	3.91	4/476	0.05	*	1.58	20/476	0.06	NS
P7	3.68	4/453	0.06	*	2.19	20/453	0.08	**

(For details of analysis see Table 6-2.

* significant at the 0.05 level

** significant at the 0.01 level

** * significant at the 0.001 level

NS not significant)

that they respond to complex mixtures in various proportions. Furthermore, chemicals other than CHCs may also sometimes be involved in the nestmate recognition process (Hernandez et al. 2006). NIRS provides a means of representing this chemical complexity by means of a single spectrum, admittedly at the cost of greater precision. There is, however, sufficient precision to differentiate between colonies and between nests within colonies. In the present analysis, variations in the positions of P1, P2 and P5 and the widths of P1, P2, P4, P5 and P6 contain information that is not obscured by intra-colonial and potentially encode chemical characteristics that may facilitate colony-mate recognition. I have previously demonstrated that the level of aggression expressed by weaver ant workers is positively correlated with the spectral distance between colonies (Chapter 5). Here I demonstrated further that aggression increased specifically in response to the distance between spectra derived from spectral characteristics that varied between colonies but not between nests within colonies. Furthermore this model accounted for a greater proportion of the variance in aggression than a model in which distance was derived from all spectral characteristics (cf. Chapter 5). There was no significant increase in aggression when distance was derived from spectral characteristics that varied between nests within colonies. This suggests that NIR spectra do to some extent encode the chemical characteristics that weaver ants use as colony-mate recognition cues.

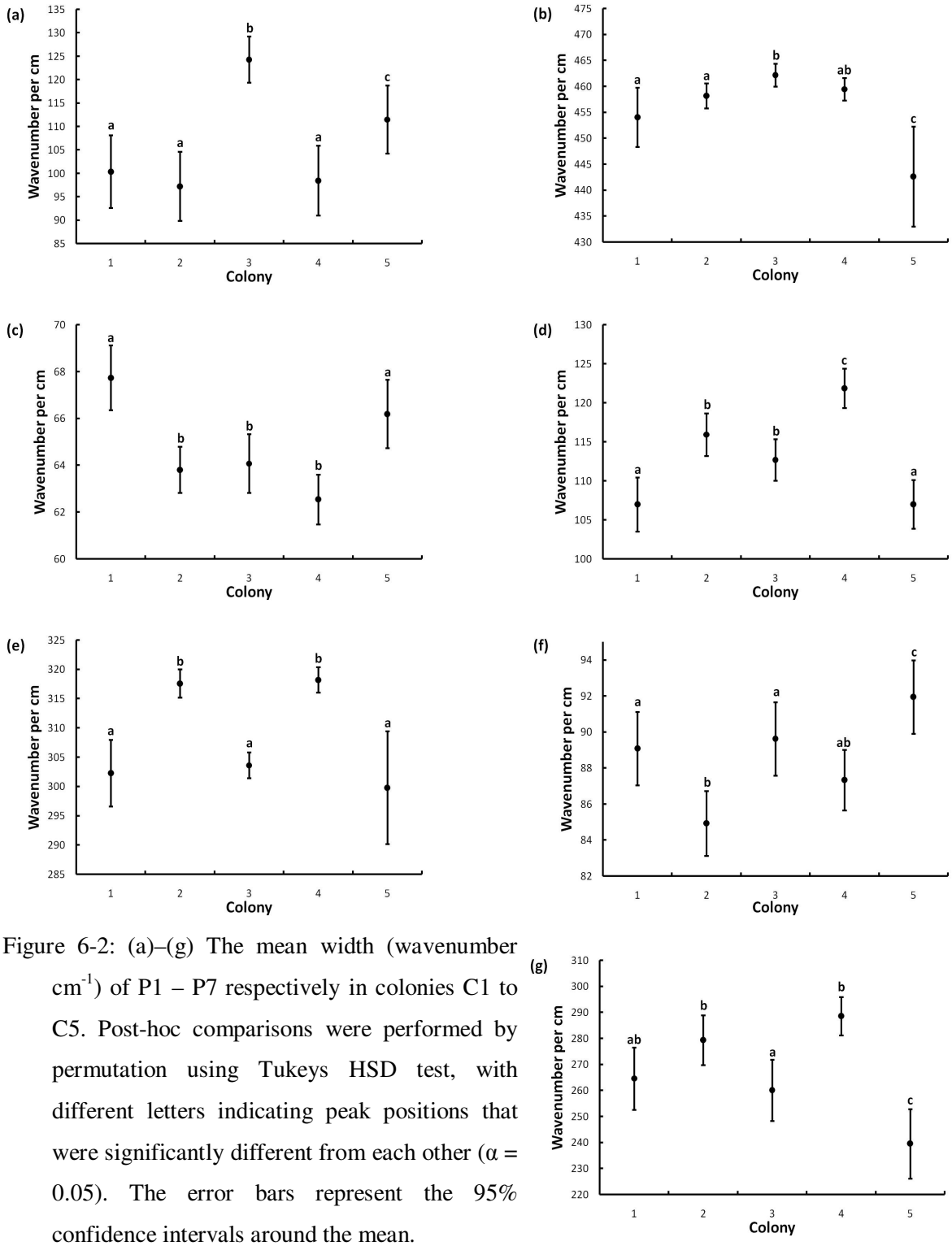

Figure 6-2: (a)–(g) The mean width (wavenumber cm^{-1}) of P1 – P7 respectively in colonies C1 to C5. Post-hoc comparisons were performed by permutation using Tukeys HSD test, with different letters indicating peak positions that were significantly different from each other (α = 0.05). The error bars represent the 95% confidence intervals around the mean.

There was also considerable overlap in the spectral characteristics of colonies, and it is likely that spectra reflect other chemical differences between colonies that play no role in recognition, and which obscure the characteristics that may be related to recognition. However, it is also possible that recognition cues do themselves overlap between colonies. The scope for

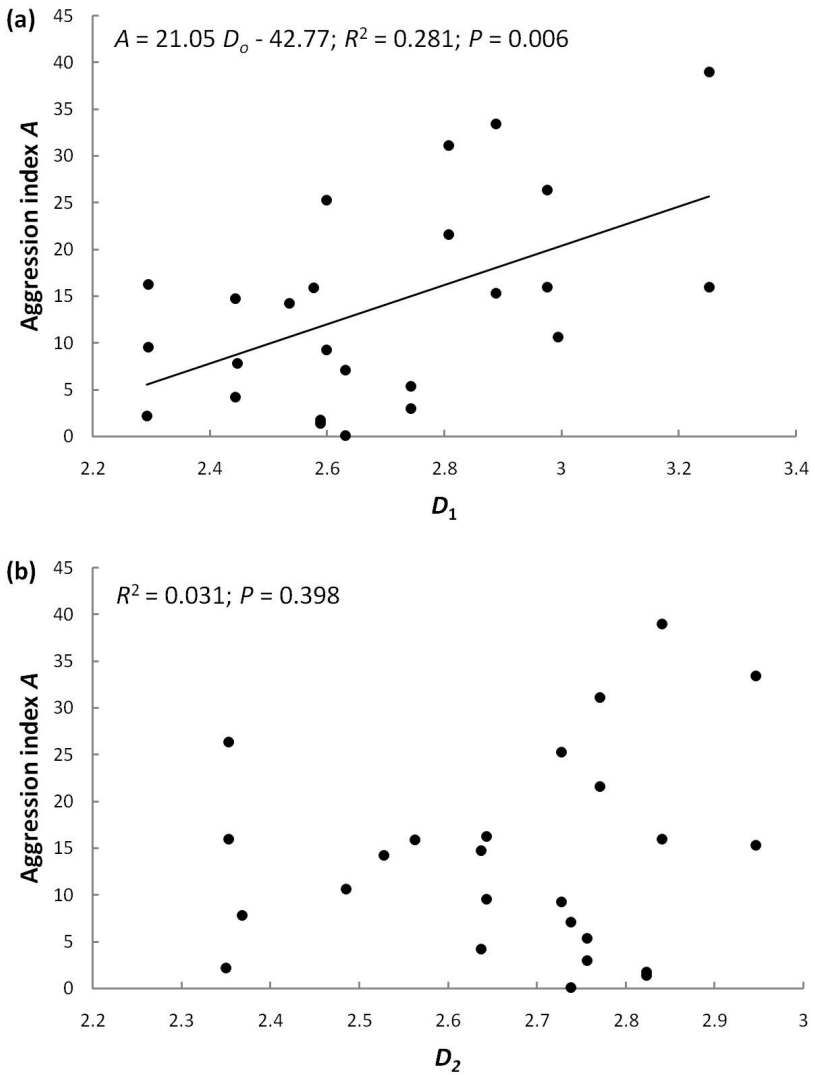

Figure 6-3: (a) The relationship between the mean aggression index A and the distance D_1 between colonies, calculated using only those spectral parameters that differed between colonies but not between nests within colonies; (b) the relationship between the mean aggression index A and the distance D_2 between colonies, calculated using the remaining spectral parameters.

variation within the population may be limited. If this is the case, workers are often likely to misidentify workers from some colonies as colony-mates. This does, in fact, appear to be the case (Chapter 5). Additional research is required to determine which particular chemical characteristics are involved in the recognition system of weaver ants, and how these characteristics are encoded in NIR spectra.

The different levels of aggression between colonies may also be affected by other factors. For example, not all conspecific colonies are likely to represent the same level of threat. Neighbouring colonies, for example, may present a more immediate threat than more distant

colonies (Gordon 1989), the opposite of the dear enemy effect (Fisher 1954). It is possible that workers may learn to discriminate more accurately the odour of a neighbouring colony, whereas it is less important to be able to recognise an occasional stray worker from a more distant colony. Whether this is the case for weaver ants remains to be determined.

Using NIRS, the proportion of variation in peak positions, amplitudes and widths that could be attributed to colony or nest of origin was small. This suggests that there is considerable variation at the level of the individual. Comparisons with studies of CHCs are difficult. Most of these involve data reduction using factorial analysis, followed by discriminant analysis. While this provides information about how much variation in odour may be explained by particular factors, it does not reveal anything about how that variation is distributed between and within colonies, or between individuals. In the case of weaver ants, considerable variation between the spectra of individuals is compatible with the existence of both colony- and nest-specific characteristics. Furthermore, such individual spectral variation is consistent with the variation that is often observed in the level of aggression shown towards intruders by different individuals, in both this and other species (Chapter 5; Roulston et al. 2003). This variation at the level of the individual warrants further investigation. In general, the amount of individual variation and colony overlap among potential cues, while undoubtedly resulting in recognition errors, does not appear to undermine colony defences. The sheer weight of numbers is probably sufficient to guarantee that a potentially hostile intruder will be recognised as such by at least some colony members. For this reason there may be little selective pressure for recognition cues to become more finely differentiated.

NIRS does not generate the type of specific chemical information that is provided by the analysis of CHCs. Nevertheless, I have presented evidence that NIRS can be used to differentiate between weaver ant colonies and nests within colonies. I have demonstrated that colony-specific characteristics and nest-specific characteristics are related to different components of the spectra. Furthermore, in the context of colony-mate recognition the behaviour of the ants themselves was positively correlated with colony-specific spectral characteristics, rather than with nest-specific characteristics. This is further evidence that NIRS is a valuable tool in the study of social insects.

7 Temporal variation in recognition cues: implications for the social life of weaver ants.

[The content of this chapter has been published as:

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Abstract

Recognition systems are involved in a range of evolutionary and biological processes, and animals use a variety of visual, auditory, tactile and olfactory cues to determine the status of others. The use of chemical cues is widespread, occurring among mammals, fish, reptiles and birds, but particularly among insects. Yet chemical cues may be influenced by diet and environment, and can change over time, potentially limiting their reliability as recognition cues. Nevertheless, they play a key role in colony-mate recognition among social insects. I created colony isolates from large multi-nest colonies of weaver ants and asked (1) whether colony spectral profiles (determined using NIRS) changed over time; (2) whether spectral profiles of colony isolates diverged from those of original colonies; and (3) whether the behaviour of original colonies towards colony isolates changed over time. Although I detected changes in the spectral profile of original colonies and colony isolates, they did not diverge significantly from each other. As far as I am aware, this is the first time that such parallel changes in colony odour have been observed. Aggression towards individuals from colony isolates did not increase, although levels of trophallaxis did. This demonstrates that colony-mate recognition among weaver ants was unimpaired by temporal variation in recognition cues. I discuss the characteristics of weaver ants that might give rise to this system. I also discuss the implications for other organisms when, for example, ontogenetic changes in the expression of individual cues may hamper their usefulness as kin recognition cues.

7.1 Introduction

In Chapters 4 and 6 I showed that the spectral characteristics of weaver ants varied significantly between colonies and between nests within colonies. I also demonstrated in Chapters 5 and 6 that workers respond to the chemical information encoded in spectra. However, in several ant species, colony odour is also known to change over time (Dahbi and Lenoir 1998; Lenoir et al. 2001; Liu et al. 1998; Nielsen et al. 1999; Provost et al. 1993; Suarez et al. 2002; Vander Meer et al. 1989), giving rise to the question of how this might affect the recognition system. In small colonies this is unlikely to be an issue, as the colony gestalt may be continually updated and maintained by the exchange of chemicals between workers (Boulay et al. 2000). However, maintaining a single colony odour may be difficult in large, polydomous colonies, where exchange of workers between some nests may be limited. If the chemical profiles of nests were to diverge from each other, this could have serious consequences for the integrity and survival of the colony.

I have demonstrated that weaver ant colonies have nest specific spectral profiles, and I assume that these differences result from an incomplete exchange of chemicals between individuals from different nests. If partial isolation between nests results in chemical divergence between nests, complete isolation could result in a more dramatic divergence, as no chemical exchange with the rest of the colony would be possible. This is even more likely if there is variation in the composition of chemical cues over time, or if there is an environmental or dietary component to the colony gestalt. Eventually, this would result in the breakdown of the colony gestalt and increased aggression between workers from different parts of the colony. The purpose of the present study was to determine whether the chemical profiles of weaver ant colonies provide reliable recognition cues for workers. Most studies of recognition systems among ants have focused on the ability to identify alien conspecifics, in the context of colony defence. Here I focus on whether colony members retain the ability to identify colony-mates, despite periods of separation. The integrity and viability of a colony may be threatened as much by a breakdown of colony-mate recognition as by a failure to identify conspecific intruders. Using NIRS, I sought to determine: (1) whether *O. smaragdina* colony spectral profiles changed over time; (2) whether the spectral profiles of isolated fragments diverged over time from those of the original colonies; and (3) whether the behaviour of individuals from the original colony towards individuals from isolated fragments changed over time.

7.2 Methods

Beginning in July 2007, I collected medium-sized nests (>2000 workers, plus brood) from six colonies of weaver ants in the grounds of James Cook University, Cairns, Queensland, one each week over a period of six weeks. I designated the time at which each colony was first collected as T1. These nests (referred to as colony isolates) were maintained in clear plastic boxes (two or more food containers, 18 cm x 12 cm x 7 cm, connected by 12 mm clear, flexible vinyl tubing), inside a shadehouse with unregulated temperature and humidity. They were sustained on 2 – 3 mealworms (*Tenebrio* spp. larvae) and dilute honey, provided 2 – 3 times per week. It was not possible to collect all nests and conduct all tests at the same time, so time periods are measured relative to the time when each nest was first collected.

7.2.1 Colony Spectra

At T1, 80 individuals were collected from each colony and stored at -4°C. Spectra were obtained using the Bruker Optics Multi Purpose Analyzer®, following the protocol detailed in Chapter 6. Twenty individuals from each colony were randomly selected to represent the original colony at T1 and 20 were selected to represent the colony isolate. At T2, after approximately six weeks (mean ± SD: 41.08 ± 2.57 days), 20 additional individuals from each original colony and each colony isolate were scanned. This was repeated at T3, after approximately 12 weeks of isolation (87.30 ± 1.89 days; except for one colony, the colony isolate of which did not survive to T3), and at T4, after approximately 18 weeks of isolation (126.90 ± 3.48 days).

I identified seven peaks (P1 to P7) for analysis, located respectively at the following wavenumber per cm: 8668.63 ± 20.58, 7026.61 ± 23.17, 5791.41 ± 3.48, 5647.50 ± 12.76, 5228.38 ± 10.78, 4615.53 ± 19.45 and 4212.68 ± 25.69. The location, intensity, and width (at 50% intensity) of each peak were recorded for each individual, resulting in 21 parameters for a total of 1120 samples. Some peaks were undetected in a small percentage of samples: 2.23% for P1, 0.09% for P3, 6.96% for P6 and 0.36% for P7. I assumed that these peaks were not absent, but were below the threshold of detectability. To avoid losing the information represented by other peaks detected in these individuals, missing values were replaced with the overall mean for that peak, in the case of location and width, and zero in the case of intensity. I acknowledge that replacing missing values with sample wide means may reduce the power to detect differences over time within or between original colonies and colony isolates.

I used principal components analysis to reduce the spectral data to six orthogonal factors: F1, F2, F3, F4, F5, and F6. These accounted for 17.71, 17.10, 15.02, 7.52, 6.75, and 5.45% of the variance respectively: a total of 69.55%. These factors represent the position of

each scanned individual in 6-dimensional Euclidean space, and I used this to determine the mean spectral distance between samples. I first calculated the mean Euclidean distance between an individual from one sample and every individual from another sample, repeating this for every individual in the first sample. The mean of these values was then used to determine the distance between the two samples. Using this method, I estimated the distance between the two random samples of 20 individuals selected to represent each original colony and its corresponding isolate at T1. This established a baseline measurement for the distance between any two groups of 20 individuals from the same colony, with which subsequent distances were compared. I then estimated the distance at T2, T3 and T4 between the original colony and the corresponding colony isolate, to determine whether the isolated fragments diverged from the original colony over time. I also determined how far the spectra of each original colony and each colony isolate diverged over time from their spectra at T1 by estimating the distance between the 20 individuals at T2 and those from the same colony or colony isolate at T1, the 20 individuals at T3 and those from the same colony or colony isolate at T1, and so on. I used the same procedure to measure divergence in a particular dimension, that is, along the axes represented by F1 to F6, to determine the direction of movement.

7.2.2 Behavioural Bioassays

Immediately upon collection at T1, I conducted a series of behavioural bioassays, with one nest of the original colony as the recipient colony and 20 individuals from that nest and 20 from the corresponding colony isolate as intruders. These trials were used to establish a baseline for the behaviour expressed by the original colony towards colony mates and towards individuals from the isolated fragment.

The protocol for the behavioural bioassays was the same as for Chapter 5, except that I also recorded the number of times (during the 19 observations) that the intruder was ignored (no interaction), was antennated, or was engaged in trophallaxis or grooming. Antennation was interpreted as exploratory behaviour. Both trophallaxis and grooming were assumed to have (among other functions) a role in redistributing chemical cues. No attempt was made to distinguish between occasions when the intruder adopted a passive or active role in these behaviours: in either case the behaviour indicated acceptance of the intruder as a colony-mate. The index of aggression (A) was calculated as before:

$$A = \Sigma f_i i$$

where i is the intensity of response and f_i is the frequency of that response. Mean values from each series of trials were used in the subsequent analysis.

To determine whether the behaviour of workers from the original colonies towards workers from colony isolates changed over time, at T2 I collected another small nest from each original colony and used ants from this as recipients in another series of aggression bioassays,

following the same protocol. Ants from that nest and from the corresponding colony isolate were introduced as intruders. I repeated this procedure at T3 and T4.

7.2.3 Statistics

I used repeated measures analysis of variance (ANOVA) to detect significant divergence over time from the original colony spectra at T1, and between the spectra of the original colonies and their colony isolates. All variables were normally distributed and, in general, met the assumption of sphericity. In a few cases, where this was not so, I used the Greenhouse-Geisser correction and report corrected degrees of freedom and significance values. I tested for changes in both the mean Euclidean distance and in the distance along each dimension (F1 to F6).

I used the same procedure to test for changes in behaviour over time. In the case of aggression, some heteroscedasticity was apparent, so I complemented the parametric tests with non-parametric tests as a precaution (see Results for details).

The relationships between behaviour and spectral characteristics were analysed using linear regression models. Each behavioural characteristic was regressed against the spectral distance between the original colony and the colony isolate, and against the distance along each axis. In the latter case I used stepwise variable entry.

Additional statistical procedures are described with the results. All statistical tests were carried out using SPSS® Release 13.0.0 (SPSS for Mac OSX, Chicago, IL, USA), with significance levels set at 0.05. I report the mean ± SD, and partial η^2 values as a measure of effect size.

7.3 Results

7.3.1 Colony Spectra

7.3.1.1 *Change over Time*

The mean baseline spectral distance between two randomly selected groups of individuals from each colony at T1 was 2.74 ± 0.38. The mean distance between colonies at T2 and their earlier counterparts at T1 was 3.17 ± 0.26, representing a significant increase over a period of six weeks ($F_{1,8}$ = 6.486, partial η^2 = 0.448, p = 0.034; Figure 7-1). The mean distances at T3 and T4 were also greater than at T1 (T3: $F_{1,8}$ = 33.530, partial η^2 = 0.807, $p < 0.001$; T4: $F_{1,8}$ = 17.285, partial η^2 = 0.684, p = 0.003), but did not differ significantly from the distance at T2, or from each other. There was no difference between the response over time of the original colonies and the colony isolates ($F_{1,8}$ = 0.719, partial η^2 = 0.082, p = 0.421), and no interaction

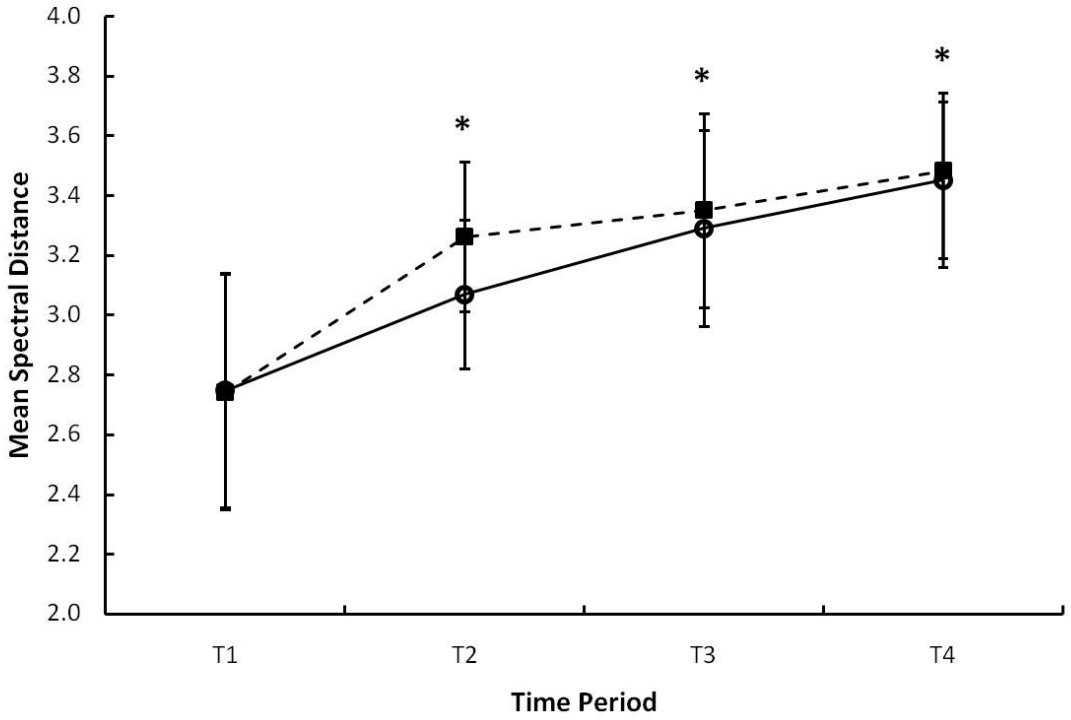

Figure 7-1: The mean spectral distance between the original colonies (↓, —) and the colony isolates (■, ----) and the original colony spectra at T1, measured over four time intervals, T1 to T4. The distance at T1 is the mean distance between two randomly selected samples from each colony. The error bars represent the standard deviation around the mean. *Indicates that the distance at that time was greater than the distance at T1.

between them ($F_{1.396,11.164} = 0.325$, partial $\eta^2 = 0.039$, $p = 0.653$). Although the distances at T3 and T4 from the original colony spectra were not significantly greater than the distance at T2, this did not mean that colony spectra remained static after T2. To determine whether colony spectra continued to change, I calculated the mean distance between individuals within colonies at T2, and then calculated the mean distance between these individuals and individuals from the same colonies at T3. The mean distance between individuals at T2 (2.72 ± 0.43) was significantly less than the mean distance between individuals at T2 and T3 (3.05 ± 0.32; paired *t*-test: $t_9 = -3.997$, $p = 0.003$). This indicates that colonies continued to move through this 6-dimensional spectral space, although a movement from their position at T2 did not necessarily increase the distance from their position at T1.

I next considered the distance in each dimension individually, to determine the direction of the movement. Along F1, the distance was significantly greater at T3 ($F_{1,8} = 5.481$, partial $\eta^2 = 0.407$, $p = 0.047$), but not at T2 ($F_{1,8} = 1.370$, partial $\eta^2 = 0.146$, $p = 0.275$) or T4

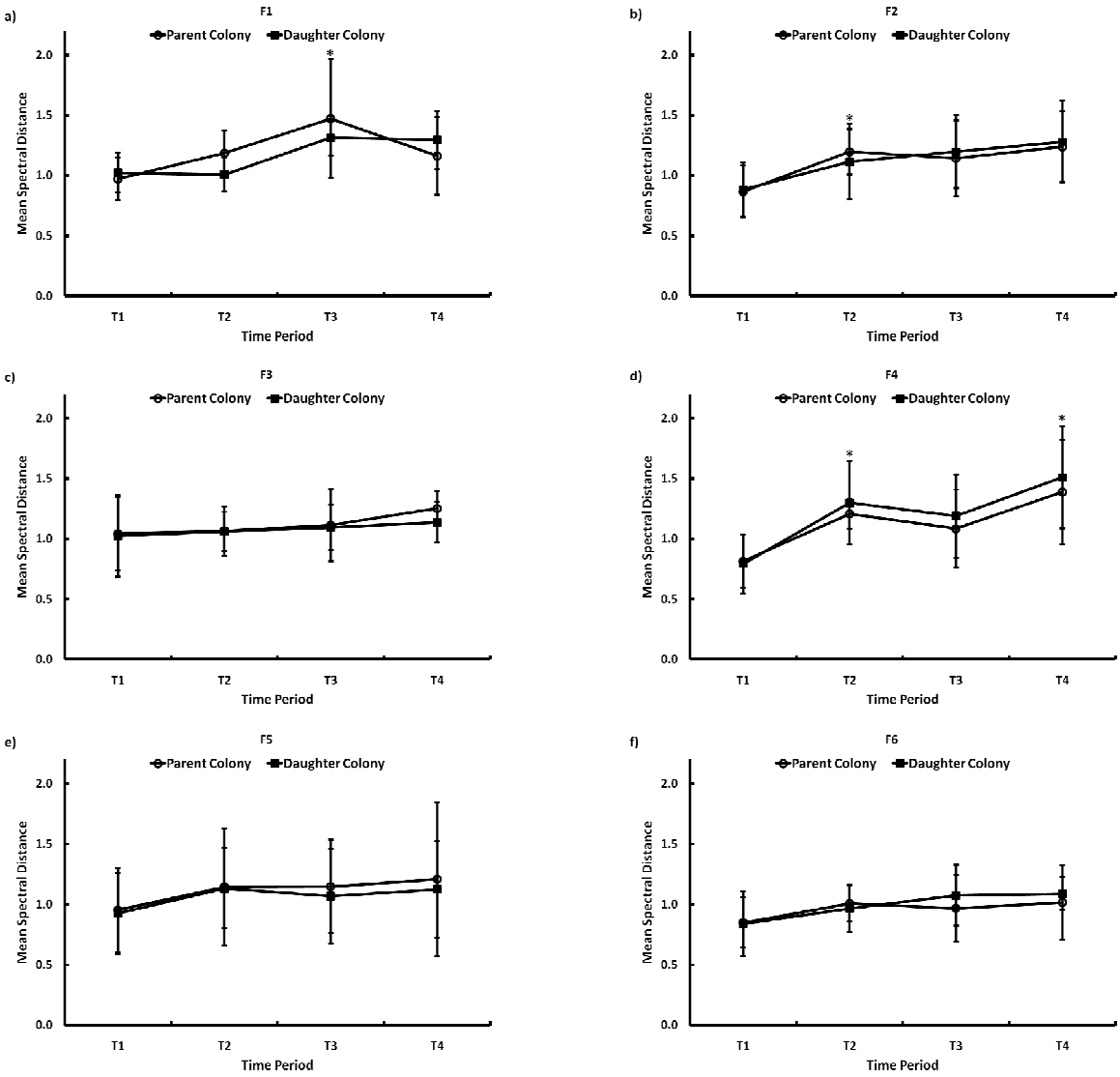

Figure 7-2: The mean spectral distance between the original colonies (↓, —) and colony isolates (■, ----) and the original colony spectra at T1, measured along each axis (F1 to F6), at four time intervals, T1 to T4. The distance at T1 is the mean distance between two randomly selected samples from each colony. The error bars represent the standard deviation around the mean. *Indicates that the distance at that time was greater than the distance at T1.

($F_{1,8}$ = 2.552, partial η^2 = 0.242, p = 0.149; Figure 7-2a). Along F2, the distance was significantly greater at T2 ($F_{1,8}$ = 22.629, partial η^2 = 0.739, p = 0.001), T3 ($F_{1,8}$ = 24.400, partial η^2 = 0.753, p = 0.001), and T4 ($F_{1,8}$ = 12.279, partial η^2 = 0.605, p = 0.008; Figure 7-2b). Along F3, the distance only became significantly greater at T4 ($F_{1,8}$ = 9.215, partial η^2 = 0.535, p = 0.016; Figure 7-2c). This was also the case along F5 ($F_{1,8}$ = 7.754, partial η^2 = 0.492, p = 0.024; Figure 7-2e). Distance did not increase along F4 ($F_{3,24}$ = 2.776, partial η^2 = 0.258, p =

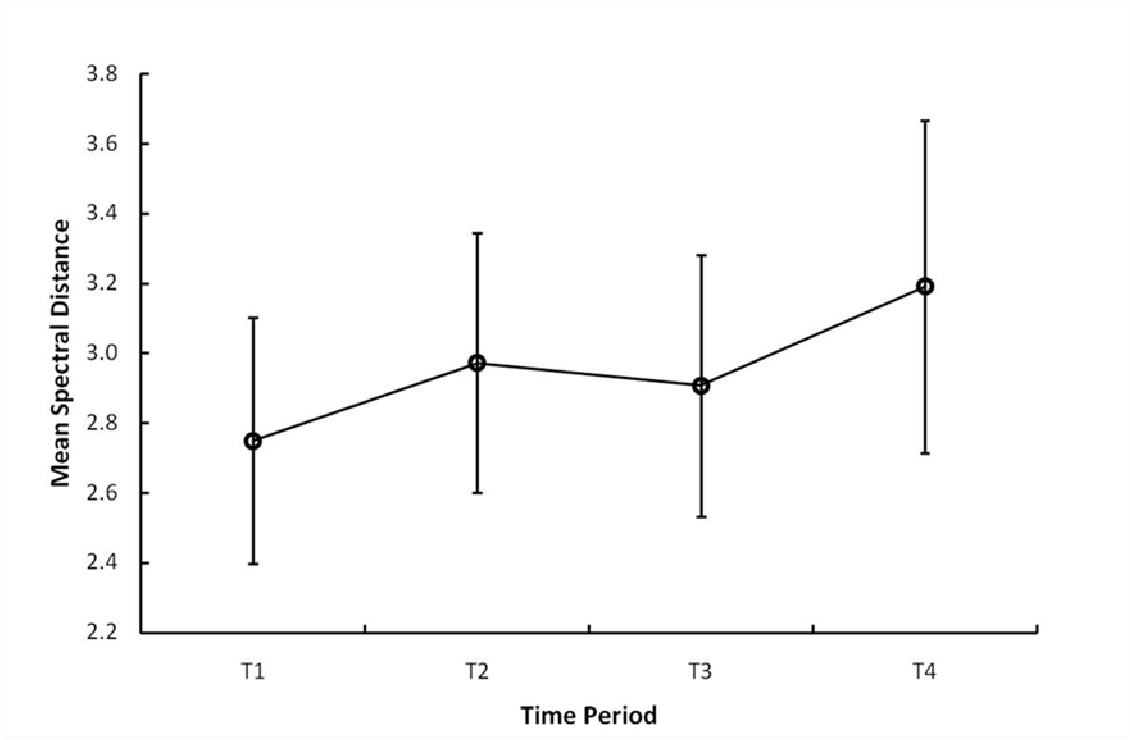

Figure 7-3: The mean spectral distance between the original colonies and colony isolates at four time intervals, T1 to T4. The error bars represent the standard deviation around the mean.

0.063) or F6 ($F_{3,24}$ = 2.220, partial η^2 = 0.217, p = 0.112; Figure 7-2d,f). The degree and timing of movement varied along the different axes, but there was significant movement in four out of six dimensions (F1, F2, F3 and F5), while colonies remained relatively stationary in the remaining two dimensions (F4 and F6). There were no significant interactions or differences between colony types, suggesting that the movement of original colonies and colony isolates through the 6-dimensional space was parallel.

7.3.1.2 *Divergence between Original Colonies and Colony Isolates*

There was no significant increase in the mean distance between the original colonies and the corresponding colony isolates over time ($F_{3,12}$ = 0.693, partial η^2 = 0.148, p = 0.573; Figure 7-3). Nor was there any significant increase in distance along F1 ($F_{3,12}$ = 3.365, partial η^2 = 0.457, p = 0.055), F2 ($F_{3,12}$ = 0.272, partial η^2 = 0.064, p = 0.844), F3 ($F_{3,12}$ = 2.678, partial η^2 = 0.401, p = 0.094), F4 ($F_{3,12}$ = 0.136, partial η^2 = 0.033, p = 0.937), F5 ($F_{3,12}$ = 1.198, partial η^2 = 0.231, p = 0.352) or F6 ($F_{3,12}$ = 0.065, partial η^2 = 0.016, p = 0.977). This confirms that, insofar as colony spectra tended to change along a particular axis (as indicated by the increased

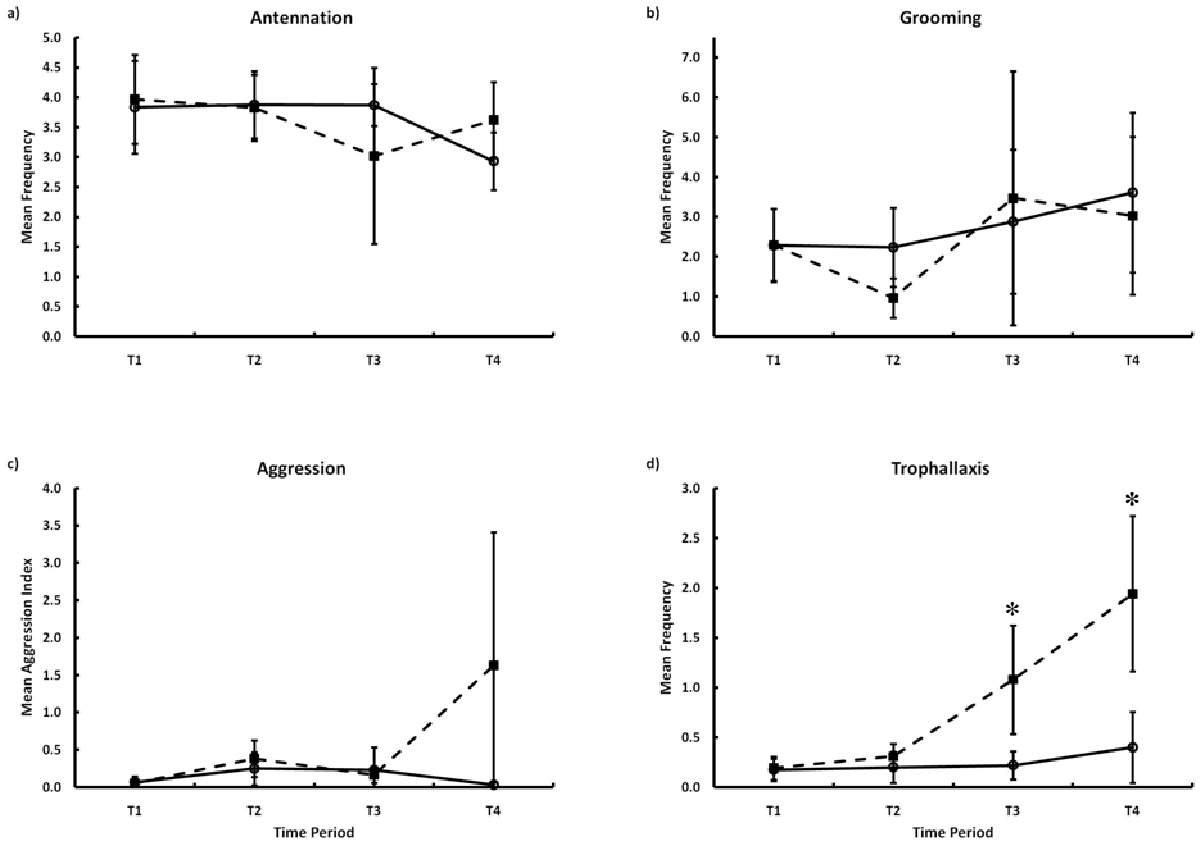

Figure 7-4**:** The mean frequency of antennation (a), grooming (b) and trophallaxis (d), and the mean level of aggression (c), between recipients from the original colonies and intruders from the original colonies (↓**,** —) and colony isolates (■, ----), at four time intervals, T1 to T4. The error bars represent the standard deviation around the mean. * indicates a significant difference at that time.

distance from the original spectra), movements in the original colonies were matched by parallel movements in the colony isolates.

7.3.2 Behaviour

There was no significant change over time, towards intruders from either the original colonies or colony isolates, in the frequency of antennation ($F_{3,24}$ = 1.120, partial η^2 = 0.123, p = 0.361; Figure 7-4a), grooming ($F_{3,12}$ = 1.826, partial η^2 = 0.186, p = 0.169; Figure 7-4b), or aggression ($F_{3,12}$ = 1.703, partial η^2 = 0.176, p = 0.193; Figure 7-4c). As the variance in aggression was heteroscedastic, I checked this result using the non-parametric Friedman test for related samples. This confirmed that aggression did not increase significantly towards intruders from parent colonies (χ^2_3 = 1.786, p = 0.618). While there was a non-significant trend for

aggression to increase towards intruders from colony isolates ($\chi^2_3 = 7.667$, $p = 0.053$), a Mann-Whitney *U*-test detected no significant difference between colony types at T4 ($U = 7.000$, $N = 12$, $p = 0.220$).

There was no significant increase in the level of trophallaxis between recipients and intruders from the original colonies ($F_{3,12} = 0.916$, partial $\eta^2 = 0.186$, $p = 0.462$); but there was a significant increase between recipients and intruders from colony isolates ($F_{3,12} = 13.057$, partial $\eta^2 = 0.765$, $p < 0.001$); the interaction between colony types was significant ($F_{3,12} = 7.669$, partial $\eta^2 = 0.489$, $p = 0.007$; Figure 7-4d).

7.3.3 Spectra and Behaviour

The frequency of trophallaxis increased significantly as the distance between the original colonies and colony isolates increased ($F_{1,20} = 7.485$, $R^2 = 0.236$, $p = 0.013$; Figure 7-5a). Among the individual factors, a significant increase in trophallaxis was evident with increasing distance along F3 ($F_{1,20} = 10.943$, $R^2 = 0.354$, $p = 0.004$; Figure 7-5b). There were no other significant relationships between spectra and behaviour.

7.4 Discussion

The analysis demonstrated that the near-infrared spectral profiles of weaver ant colonies changed over time, corresponding, I suggest, to changes in the colony gestalt odour. Similar changes have been documented in a number of ant species, based on analysis of cuticular hydrocarbons. Lahav et al. (2001) detected significant changes in colonies of *Cataglyphis niger* after one month, and Provost et al. (1993) detected significant changes in colonies of *Temnothorax lichtensteini* (formerly *Leptothorax lichtensteini*) after only 15 days. By T2, after approximately six weeks, weaver ant colonies had already diverged from their original spectra at T1. It is possible that significant shifts in colony spectra had already occurred prior to this.

Despite the temporal shift in colony spectra, the spectra of colony isolates did not diverge significantly from those of the original colonies measured at the same time. This was true of total distance and along each dimension individually. While the use of sample-wide means to replace missing values may have reduced the probability of detecting a difference, it is reasonable to claim that any divergence between original colonies and colony isolates over time was less pronounced than changes over time within both colony types. This is only the second time, as far as I am aware, that the effects of segregation on the colony odour of a polydomous, monogynous ant species have been tested experimentally. In the only other species to be studied (Cataglyphis iberica; Dahbi and Lenoir 1998) colonies consisted of only two or three nests, with several hundred workers in each. The colonies of *O. smaragdina* were much larger, with many

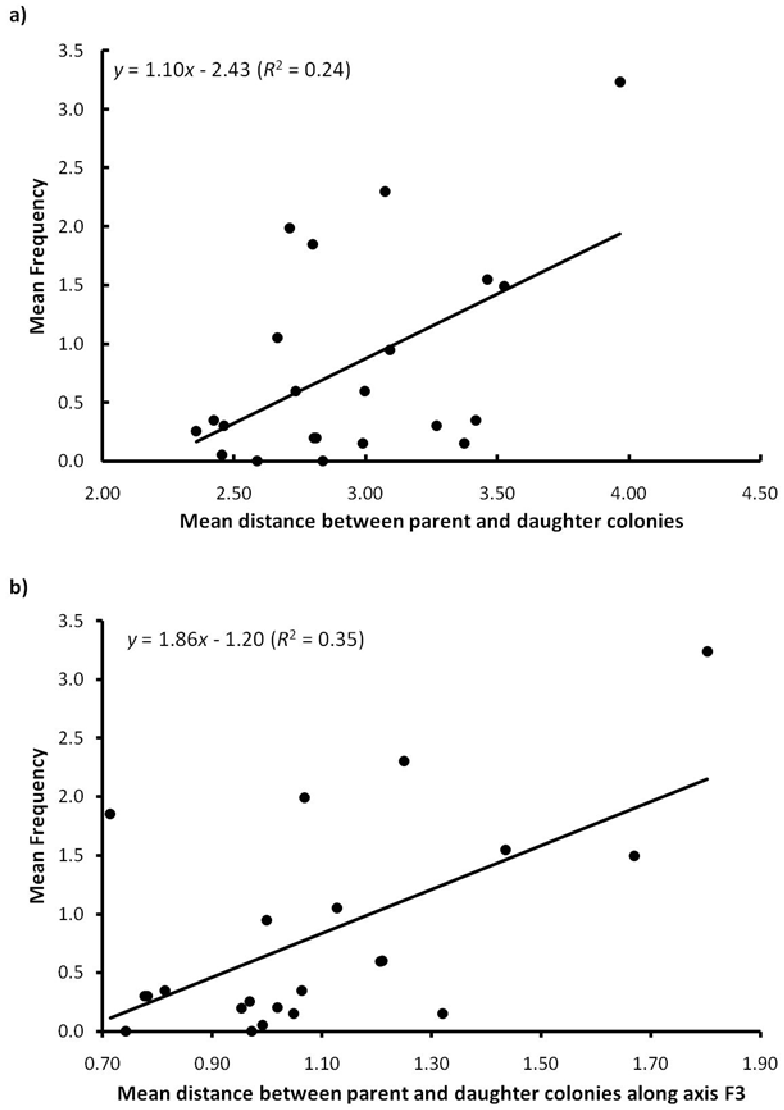

Figure 7-5**:** The mean frequency of trophallaxis plotted against (a) the mean Euclidean distance and (b) the distance along axis F3, between the original colony (recipient) and the colony isolate (intruder). The regression line and equation are shown.

more nests, and several thousand workers per nest. Dahbi and Lenoir (1998) found that separated sections of colonies diverged chemically after 12 months, but divergence was more pronounced after a five month period between nests in which hibernation was induced than nests in which it was not (Dahbi and Lenoir 1998). It is possible that the spectra of the weaver ant colony isolates might have diverged from those of the original colonies after a longer period of separation; however, the colony isolates were unlikely to have survived much beyond the four month period of this study.

As far as I am aware, this is the first report of parallel changes over time in the odour of a colony and an isolated fragment of that colony. Although Lahav et al. (2001) found that time had a greater effect than separation on colony odour in mother and daughter colonies of the polygynous desert ant *C. niger*, they nevertheless detected significant divergence between mother and daughter colonies after three months (Lahav et al. 2001). The absence of any significant divergence in *O. smaragdina* suggests that there may be a strong genetic component to the expression of colony odour. The unusual social structure of weaver ant colonies may lie at the heart of this: species with very large polydomous colonies and a single queen are rare. It would be interesting to see whether the same phenomenon is observed in other populations of *O. smaragdina*, in which polygyny is reported to occur (Peng et al. 1998).

Aggression has been shown to increase over time between isolated segments of some ant colonies. For example, nests of the polydomous mound-building ant *Formica exsecta* undergo a natural period of isolation during winter hibernation, and levels of aggression between nests of the same colony are correspondingly higher during spring than during summer or autumn (Katzerke et al. 2006). When colonies of *Formica aquilonia* were subdivided and maintained on different diets, levels of aggression between separated segments increased significantly over a period of four weeks (Sorvari et al. 2008). Even between segments maintained on the same diet there was a significant increase in aggression after two weeks compared to a control treatment involving no separation, although the difference was no longer evident after four weeks (Sorvari et al. 2008). Stuart and Herbers (2000) found that formerly friendly neighbouring nests in a New York population of *Temnothorax longispinosus* (formerly *Leptothorax longispinosus*) became aggressive towards each other following three months of separation in the laboratory. I found that the level of aggression expressed by weaver ants towards intruders from colony isolates did not increase significantly after a separation of four months; nor did it increase with the spectral distance between the original colonies and colony isolates. There was, however, a significant increase in the level of trophallaxis shown towards intruders from colony isolates (but not the original colonies), both over time and with increasing spectral distance between the original colonies and colony isolates. Trophallaxis also increased significantly with distance along F3. Dahbi and Lenoir (1998) reported an increase in the level of antennation over time between separated sections of *C. iberica* colonies. In *Camponotus fellah*, individuals isolated from their colony for one or three days provoked significantly greater levels of trophallaxis after re-introduction into their colony than those introduced into the colony as controls without any period of isolation (Boulay et al. 2000). Given that the original colonies and colony isolates of *O. smaragdina* did not diverge significantly from each other along F3, individuals appear particularly sensitive to changes in this part of the spectrum, and increased trophallaxis may be a way of correcting a perceived discrepancy. Even small

changes were sufficient to trigger a significant alteration in behaviour. Nevertheless, the capacity for colony-mate recognition was unimpaired.

There are several reasons why the chemical profile of a colony might change over time. Dietary and environmental factors could play a role. Changes in chemical profiles have been associated with changes in diet in the Argentine ant, *Linepithema humile* (Buczkowski et al. 2005; Liang and Silverman 2000; Suarez et al. 2002), and the fungus growing ant *Acromyrmex subterraneus subterraneus* (Richard et al. 2004). Changes in colony odour may also reflect changes in the genetic composition of a colony, if the relative contribution of different queens in a polygynous colony, or the differential use of male sperm in a colony with a multiply-mated queen, changes over time. Changes in colony odour may also reflect genetically programmed seasonal changes. As I found that the original colonies and colony isolates changed in parallel, the first two explanations are unlikely for *O. smaragdina*. I therefore suggest that changes were the result of genetically programmed seasonal changes. Colonies were initially sampled from the end of July to mid-August, the height of the dry season in the study area. This probably represents the period of lowest activity within weaver ant colonies. The second period of sampling took place from the end of August to mid-October. During this time, temperature and humidity levels were rising, and this may have triggered changes within the colony. It is at about this time that colonies begin to produce larvae destined to become gynes (reproductive females), which generally mature from November through to January (Lokkers 1990). Although the colony isolates could not produce gynes, genetically programmed chemical changes may take place regardless, preparing workers for this task. This hypothesis might be tested by following spectral changes over a yearly cycle, and relating these directly to the appearance of larval gynes and/or males.

Maintaining the effectiveness of a recognition system, despite temporal variation in recognition cues, may require the continuous updating of the recognition template (Liu et al. 1998; Mateo 2004; Provost 1989; Vander Meer et al. 1989), or the continuous exchange of chemical cues (Boulay et al. 2000), and extended periods of separation could result in costly recognition errors. This is a challenge faced by taxa other than the social insects. Among mammals and other vertebrates, chemosignals arising from expression of the genes of the major histocompatibility complex (MHC) are involved in conveying reliable genotypic information (Brennan and Kendrick 2006; mammals: Brown and Eklund 1994; fish: Rajakaruna et al. 2006; amphibians: Villinger and Waldman 2008). However, these signals are not necessarily invariable. In male ring-tailed lemurs (*Lemur catta*) chemical cues provide reliable information about the genetic distance between individuals, but only during the mating season (Charpentier et al. 2008). Cues of genetic origin can, therefore, exhibit temporal variability as long as they are reliable at the appropriate time and in the right context. The ontogenetic development of odour may also threaten the reliability of recognition cues. In Belding's ground squirrel,

Spermophilus beldingi, individual odour changes over time, at least up until the age of 28 days (Mateo 2006), making it necessary for mothers to constantly update their recognition template. Odour may continue to change after 28 days, as squirrels fail to recognize previously familiar individuals after a prolonged period of hibernation, although they continue to discriminate between littermates (kin) and non-littermates (non-kin; Mateo and Johnston 2000). It may be that previously familiar conspecifics were no longer remembered, or that their odour had changed (Mateo 2006). The continued recognition of kin could be based on a self-referential mechanism which would not depend on memory, but which would also imply that changes in odour followed a parallel path in close kin; whereas the recognition of non-kin would depend on the continuous updating of recognition templates (either because odours changed or because memories faded). Such a parallel development of odour among close kin would be analogous to the parallel change in colony odour in segregated sections of *O. smaragdina* colonies.

Among social insects chemical cues sometimes have a genetic origin and are sometimes influenced by environmental and dietary factors. The latter are not necessarily unreliable cues of kinship if diet and environment are relatively heterogeneous (Tsutsui 2004). Conversely, cues of genetic origin are not necessarily reliable cues of group membership if there are low levels of genetic diversity between groups. The reliability of kinship cues presumably reflects the relative importance of kin recognition in the life history of the organism. For example, increased aggression in spring between workers from different nests of *F. exsecta* colonies could result in a split in the colony, with workers rejecting closely related conspecifics from nearby nests. However, this may not have high fitness costs, as many colonies are polygynous, and individual nests may possess or adopt a new queen. Indeed, this could be a mechanism that encourages colony budding if colonies pass a critical size threshold. In contrast, workers of the polydomous and facultatively polygynous ant *Ectatomma tuberculatum* display only low levels of aggression towards workers from nearby colonies (Zinck et al. 2008). New colonies form by budding, and because of the close relatedness of neighbouring colonies, genetic cues are unreliable indicators of the colony of origin of individual workers. However, for the same reason, the fitness costs of accepting an alien conspecific as a colony-mate are low. These examples suggest that high reliability of recognition cues may be under little selective pressure if the costs of mistakenly rejecting a colony-mate (*F. exsecta*) or mistakenly accepting a non-colony-mate (*E. tuberculatum*) are low.

In the present study population, weaver ant colonies have only a single queen, and colonies are established by new queens following a nuptial flight during which they mate with one or more males. A queenless colony does not adopt a new queen, and no isolated fragment can become established as a separate colony. In these circumstances, a loss of colony integrity may have high fitness costs, and although trophallaxis contributes to maintaining a single colony odour, it may not be entirely effective in such extensive colonies. In weaver ants,

therefore, selection for reliable recognition cues is likely to be strong. I identified aspects of the colony spectra (axes F4 and F6) that diverged little over time, and these may be closely linked with colony identity. However, at present I cannot exclude the possibility that these are species specific. Nevertheless, even spectral characteristics that changed over time changed in a similar manner in isolated parts of the colony, and could encode chemical characteristics that serve as reliable recognition cues.

Temporal variation in recognition cues presents a challenge to organisms seeking to maximize inclusive fitness by showing preferential behaviour towards kin, or seeking to avoid inbreeding. The present study shows that the reliability of kin recognition cues is not necessarily undermined by their variability. In the case of weaver ants, reliability is maintained by synchronous changes throughout the colony. Among other animals, ontogenetic changes in the expression of individual cues may hamper their usefulness as kin recognition cues, particularly if individuals experience extended periods of separation (for example, because of hibernation), unless changes follow a similar path within kin groups. In the context of mate selection, seasonal variability in recognition cues will not affect their reliability if they convey accurate information at the appropriate point in the life cycle of the organism or group. In the absence of these mechanisms, maintaining the reliability of variable cues may require the perceiver to keep track of changes as they occur, and update its recognition template accordingly, which may not always be possible. Further study of a broad range of taxa may shed light on the life history characteristics and ecological conditions under which these various recognition systems operate.

8 Weaver ants Oecophylla smaragdina encounter nasty neighbours rather than dear enemies

Abstract.

The evolution of territorial behaviour requires that the benefits of territoriality outweigh the costs. The costs are primarily those of territorial defence against encroaching neighbours or against floaters seeking to establish their own territory. One way to reduce the cost of defence might be to restrict serious conflict to encounters with those posing the greatest threat. Studies of many animals have found that less aggression is shown towards neighbours than towards strangers, a phenomenon known as the "dear enemy effect". However, the opposite can also be true, namely that more aggression is shown towards neighbours than strangers: the "nasty neighbour effect". This may be particularly true of group-living species that defend a resource-based territory. Here I sought to determine whether colonies of weaver ants responded more aggressively to intruders from neighbouring colonies than from more distant colonies, or vice versa. I found that 1) workers were able to recognize a greater proportion of workers from neighbouring colonies as non-colony members and 2) that, when recognized as non-colony members, more aggression was exhibited towards neighbours than non-neighbours. I found no evidence that weaver ant workers were better able to recognize workers from previously unknown colonies, or responded more aggressively to them, even after a 10-day period of contact. This contrasts with other ant species in which rapid learning of the identity of new potential enemies has been demonstrated. I suggest that such a response is unnecessary for weaver ants, as encounters with intruders from non-neighbouring colonies are probably rare and of little consequence. This study adds to the growing body of evidence that the nasty neighbour effect may be much more common than the dear enemy effect among group living species.

8.1 Introduction

In previous chapters I have considered recognition in the context of colony odour, as measured using NIRS. However, the spectral distance between colonies may not be the only factor determining the level of aggression shown towards intruders. Some colonies may represent a greater threat than others. Here I explore the aggressive behaviour of weaver ants in the context of the spatial relationships between colonies.

The evolution of territorial behaviour requires that the benefits of territoriality outweigh the costs. Benefits include access to a reliable pool of resources, such as food, nesting sites, potential mates, and refuge from predators. Costs are primarily those of territorial defence against encroaching neighbours or against floaters seeking to establish a territory. One way to reduce costs is to restrict serious conflict to encounters with those posing the greatest threat. Among territorial animals, the greatest threat often arises from floaters, who might seek to establish territory at the expense of existing territory holders. Neighbours already in possession of a territory pose less threat, because their status is generally known and they have less to gain from a conflict. The expression of less aggression towards a neighbour than a stranger is known as the dear enemy effect (Fisher 1954), and has been widely observed among individuals or breeding pairs defending a territory (reviewed in Temeles 1994). However, if there is intense competition for resources the opposite effect may also occur (Temeles 1994): more aggression may be shown towards neighbours than towards strangers. This has been termed the "nasty neighbour" effect (Muller and Manser 2007).

Temeles' (1994) review of the dear enemy effect included only two group-living species, both ants. The dear enemy effect was evident in one and the nasty neighbour effect in the other (Temeles 1994). In subsequent studies of group-living species, evidence for the dear enemy effect was found for the green woodhoopoe, *Phoeniculus purpureus* (Radford 2005), but most studies suggest that the dear enemy effect might occur less frequently than the nasty neighbour effect in a range of taxa including mammals (Herbinger 2004; Muller and Manser 2007), birds (Botero et al. 2007; Koetz et al. 2007) and social insects (Dunn and Messier 1999; Sanada-Morimura et al. 2003). This makes intuitive sense, as groups may compete more intensely than individuals for resources, and may fluctuate in size, increasing the demand for resources as they grow. Furthermore, an individual from a neighbouring group may present a greater threat than a lone wandering individual from further away, as the former is more likely to represent the vanguard of a potential invasion force, and can recruit reinforcements if necessary.

Social insects provide a useful model for exploring these issues. Many ant species control and defend large territories to provide sufficient resources for rearing large numbers of reproductive males and females. Furthermore, large colonies may be unable to relocate if their

territory is threatened by conspecifics. There is evidence for the nasty neighbour effect in several ant species (Gordon 1989; Knaden and Wehner 2003; Thomas et al. 2005; Van Wilgenburg 2007) and in some termites (Dunn and Messier 1999). Some studies report evidence of the dear enemy effect, but there are often confounding factors. Low levels of aggression towards neighbours may occur because neighbours share common foraging materials (Jutsum et al. 1979), live in a homogeneous habitat (Heinze et al. 1996), or are closely related (Zinck et al. 2008). Observations of an apparent dear enemy effect need to be corroborated experimentally by determining whether aggression towards unknown intruders decreases after repeated exposure. This appears to be the case for *Pheidole tucsonica* and *P. gilvescens*, and Langen et al. (2000) hypothesise that this is because colonies occasionally relocate, and strangers may represent scouts searching for new nesting sites.

Both the dear enemy and nasty neighbour effects have implications for recognition systems. The dear enemy effect indicates a capacity to differentiate between different types of non-self, as well as between self and non-self, and to modify behaviour accordingly. This constitutes a shift in the response component of the recognition process. The nasty neighbour effect, on the other hand, is open to two interpretations. Failure to react aggressively to a stranger may mean that types of non-self can be differentiated, but that behaviour is adjusted only when the unknown other becomes a greater threat, as signalled by increased contact. This constitutes a shift in the response component. Alternatively, failure to react aggressively may arise from recognition errors: unknown strangers are recognized as other only after a period of familiarisation. This implies a shift in the perception component of recognition: all conspecifics recognised as non-self are responded to aggressively, but they are only recognised as non-self with experience.

I sought to shed light on these issues by exploring the response of weaver ant colonies to intruders from neighbouring and distant colonies. Where colonies meet, extensive battles can sometimes be observed (Hölldobler 1983b). This suggests that the dear enemy effect might be absent in this species. However, colonies also exhibit some aggression towards individuals from distant colonies (Chapter 5), and it has been unclear whether they respond differently to neighbours than to strangers. I observed the behaviour of weaver ant workers towards intruders from both neighbouring and distant colonies. I also sought to determine if and how the behaviour of a colony changed after extended contact with a previously unknown colony. My predictions for this species were that (1) the nasty neighbour effect would be present and (2) colonies would become more aggressive towards previously unknown colonies after a period of exposure. I also explored whether differential treatment of neighbours and strangers reflected a difference in behaviour, perception, or both. In this way I sought to increase our understanding of the mechanisms determining self/non-self recognition systems, particularly among species living in social groups.

8.2 Methods

8.2.1 Experiment 1: field nests

Throughout April and May 2008 I selected 10 colonies of weaver ants from the campus of James Cook University, Cairns, Queensland, to serve as recipient colonies (R_1 to R_{10}), collecting a small nest from each, as well as a nest from a neighbouring colony (N_1 to N_{10}), the foraging area of which was seen to overlap that of the R colony, and from a more distant colony (D_1 to D_{10}) with which the R colony could have had no previous interaction. Each group of three nests was taken to the laboratory, where a series of aggression bioassays were conducted between workers from R colonies and workers from N and D colonies over a period of two or three days.

The protocol for the behavioural bioassay was similar to that used in previous chapters but was modified to increase its sensitivity and resolution. For each trial, five individuals were introduced from the R colony into a small observation area. A spot of water-based acrylic paint was applied to the rear of the head capsule of an individual from the N or D colony, which was then placed into a small tube adjacent to the test area. N and D workers were used alternately to eliminate any bias introduced by changes in levels of aggression over time. An opening between the tube and the test area was initially sealed. The recipient ants and intruder ant were given several minutes to acclimate, before the seal between the tube and the test area was removed and the intruder was permitted to enter the test area. The behaviour of the recipient ants towards the intruder was recorded over a period of five minutes, timed from the moment of first contact between the recipients and intruder. When recording behaviour, if any observed behaviour was maintained for a period of five seconds it was scored as another instance of that behaviour, and so on for each subsequent period of five seconds. The first five recipient ants were left overnight in the test arena to increase the likelihood that they would identify the test arena as defensible territory. Twenty encounters were staged for each colony pair, with different recipient and intruder ants for each trial. On the rare occasions when the intruder seized and immobilized one of the recipient ants, the trial was repeated with different individuals.

I recorded the frequency with which recipients antennated the intruder (however briefly), the frequency with which a recipient and the intruder engaged in trophallaxis and the frequency with which one or more recipients groomed the intruder. Antennation was interpreted as exploratory behaviour. Both trophallaxis and grooming were interpreted as acceptance of the intruder as a colony-mate. I also recorded the frequency with which one or more recipients visibly avoided the intruder. While avoidance is sometimes interpreted as a low level aggressive response (Roulston et al. 2003), I believe it is more appropriately interpreted as an alternative strategy: in some circumstances it may be more cost effective to avoid an intruder than to

engage in dangerous conflict. I also recorded the following overtly aggressive behaviours: threatening posture (mandibles open towards the intruder, with or without raised gaster), pursuit, biting (some part of the intruder's body held in the mandibles of one or more recipient) and grappling (one or more recipient ant with its body curled around the intruder while biting – once this occurs the position is held until both ants are dead). These were graded in intensity as follows: (1) threatening posture, (2) pursuit, (3) biting and (4) grappling. An index of aggression (*A*) was calculated:

$$A = \frac{\sum f_i i}{T}$$

where i is the intensity of response, f_i is the frequency of that response and T is the total number of interactions observed. I calculated the mean and maximum aggression score for each colony pair. I also determined the proportion of individuals that elicited either an aggressive or aversive response as a measure of the extent to which intruders were correctly identified as alien conspecifics. I re-calculated the mean aggression score, using only these individuals, to determine whether the level of aggression expressed towards those correctly identified as aliens differed between intruder types. In this way I sought to determine whether differences in aggression could be attributed to differences in perception or differences in behaviour. I calculated the mean proportion of avoidance behaviour for each colony pair to determine if different strategies were adopted towards intruders from familiar and unfamiliar colonies. Finally, I calculated the mean proportion of grooming and trophallaxis, as an indication of the extent to which intruders were accepted as colony-mates.

I demonstrated in Chapter 5 that weaver ants responded more aggressively to intruders from other colonies as the spectral distance, measured using NIRS, increased. I therefore determined the mean spectral distance between colonies to control for any confounding effect on the level of aggression between colonies. Spectra of 20 individuals from each colony were obtained following the protocol used in Chapter 6. I identified seven key peaks, located respectively at the following wavenumber per cm: 8668.63 ± 20.58, 7026.61 ± 23.17, 5791.41 ± 3.48, 5647.50 ± 12.76, 5228.38 ± 10.78, 4615.53 ± 19.45 and 4212.68 ± 25.69. The location, intensity, and width (at 50% intensity) of each peak were recorded for each individual, resulting in 21 parameters. I used principal components analysis to reduce the spectral data to six orthogonal factors, and calculated the mean value of each factor to locate the centroid of each colony in 6-dimensional Euclidean space. I then calculated the Euclidean distance between these centroids as an estimate of the spectral distance between colonies.

8.2.2 Experiment 2: manipulated nests

Between August 2008 and February 2009, I collected nests from 10 additional colonies in the university grounds and surrounding suburbs. Each nest was divided into two

approximately equal sections, an "a" section and a "b" section, designated Ra_1 to Ra_{10} and Rb_1 to Rb_{10} respectively. At the same time I collected nests from 10 other colonies that were distant from each of the first colonies. These were similarly divided into two sections, designated Ia_1 to Ia_{10} and Ib_1 to Ib_{10}. Nest sections were maintained in clear plastic boxes (food containers, 18 cm x 12 cm x 7 cm) inside a shadehouse with unregulated temperature and humidity and provided with mealworms (*Tenebrio* spp. larvae) and dilute honey as required. I conducted an initial behavioural bioassay, as previously described, between each Ra_i/Ia_i and Rb_i/Ib_i pair shortly after collection (t_1). Ra_i and Ia_i nests (treatment) were then connected by clear polyvinyl tubing to a shared third plastic box that was divided in two by a double layer of 1.6 mm aluminium mesh. Workers were able to contact each other through the mesh, and were even able to seize legs and antennae across the mesh, but were unable to gain access to the other side. These were now considered to be neighbours. Rb_i and Ib_i nests continued to be maintained separately as a control. After 9 or 10 days (t_2) the behavioural bioassays were repeated between each Ra_i/Ia_i and Rb_i/Ib_i pair to determine whether prolonged contact with previously unfamiliar conspecifics resulted in behavioural changes. I again calculated for each colony pair: the mean and maximum aggression score, the proportion of individuals that elicited either an aggressive or aversive response, and the mean proportion of grooming and trophallaxis.

8.2.3 Statistical analysis

Prior to statistical analysis, proportional data were transformed using the arcsine-square root transformation. All variables were normally distributed and I used regression and ANOVA models to analyse the results using S-PLUS® 8.0 for Windows (Insightful Corp., 2007, Seattle, Washington). I report the mean ± 95% confidence intervals in the results.

8.3 Results

8.3.1 Experiment 1: field nests

A simple linear regression of aggression against spectral distance revealed, as expected, that the mean level of aggression increased as the spectral distance between colonies increased ($F_{1,18} = 4.967$, $R^2 = 0.216$, $p = 0.039$; Figure 8-1). A one-way ANOVA with intruder type (N or D) as the predictor variable and spectral distance as a covariate showed that colonies were twice as aggressive towards N colonies than towards D colonies (N = 1.45 ± 0.53, D = 0.72 ± 0.38; $F_{1,8} = 17.299$, $p = 0.003$; Figure 8-2a). Aggression towards N colonies remained higher even when only those correctly identified as alien conspecifics were included in calculating the aggression index (N = 1.70 ± 0.53, D = 1.10 ± 0.39; $F_{1,8} = 14.792$, $p = 0.005$; Figure 8-2b). The maximum level of aggression was also higher between R and N colonies than between R and D

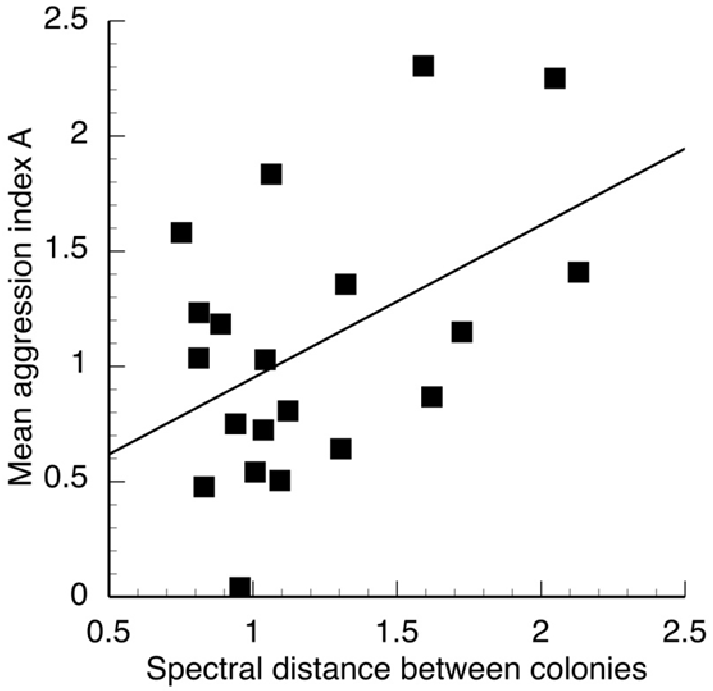

Figure 8-1**:** The mean aggression index *A* plotted against the spectral distance between colonies of weaver ants.

colonies (N = 3.49 ± 0.25, D = 2.94 ± 0.50; $F_{1,9}$ = 5.442, p = 0.045; Figure 8-3). The proportion of intruders correctly identified as alien conspecifics by R colonies was significantly greater when intruders were from N colonies than when they were from D colonies (N = 1.21 ± 0.13, D = 0.93 ± 0.17; $F_{1,9}$ = 17.314, p = 0.002; Figure 8-4).

The mean proportion of avoidance behaviour did not differ between intruder types (N = 0.12 ± 0.06, D = 0.12 ± 0.08; $F_{1,9}$ = 0.052, p = 0.825). The mean proportion of grooming and trophallaxis was significantly greater in R – D encounters than in R – N encounters (N = 0.08 ± 0.07, D = 0.31 ± 0.17; $F_{1,9}$ = 13.218, p = 0.005; Figure 8-5).

8.3.2 Experiment 2: manipulated nests

Being in contact with the intruder nest for a period of 9-10 days had no effect on the behaviour of the ants in the recipient nest, compared to the control. Using a repeated measures ANOVA model I detected a non-significant trend for aggression to increase over time ($F_{1,18}$ = 4.041, p = 0.059), but there was no significant interaction between time and treatment ($F_{1,18}$ = 1.147, p = 0.298). Thus, if aggression increased, it increased in a similar fashion for recipient nests that were connected to the intruder colony and those that were not. The maximum aggression score did not increase over time ($F_{1,18}$ = 0.036, p = 0.852) and there was no interaction between time and treatment ($F_{1,18}$ = 0.546, p = 0.469). The proportion of intruders

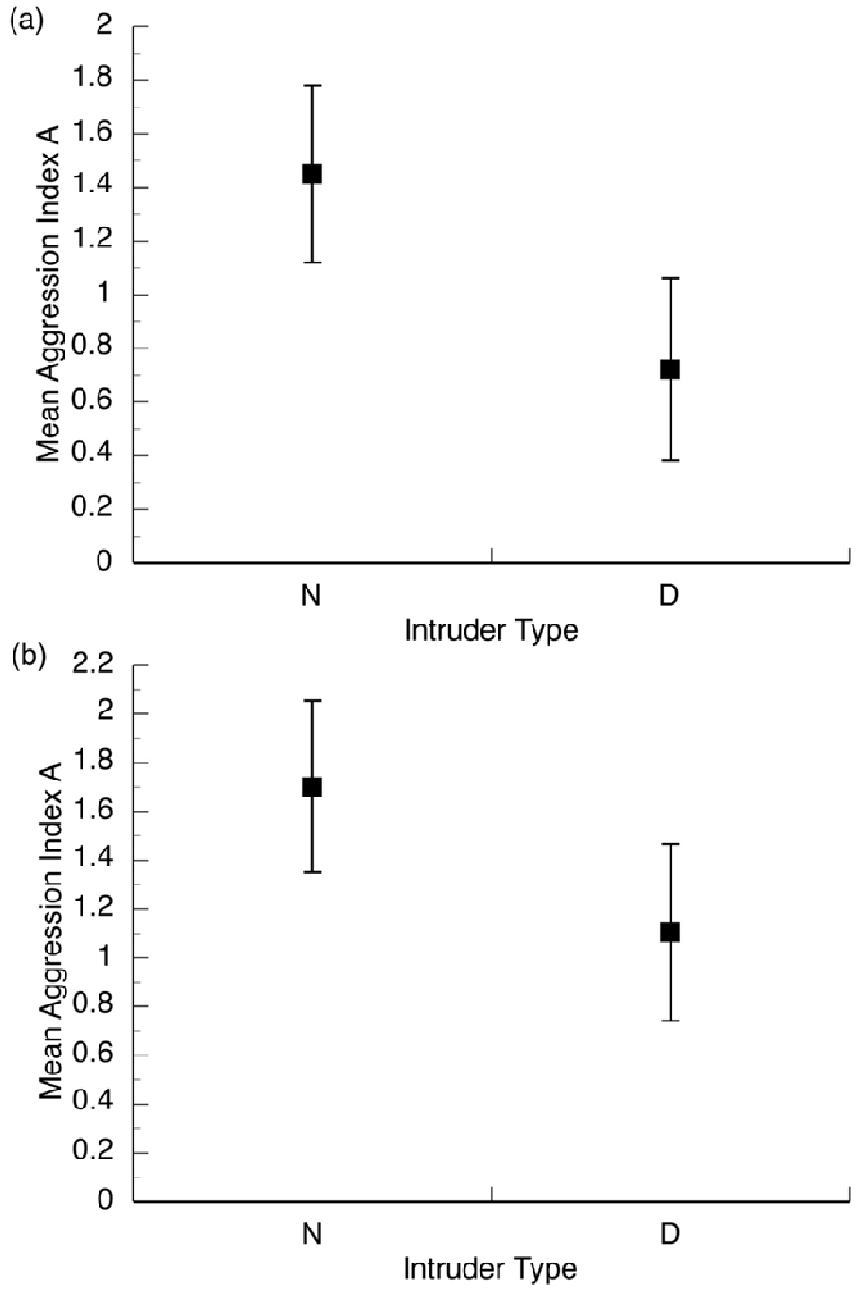

Figure 8-2: The mean aggression index *A* towards intruders from neighbouring colonies (N) and distant colonies (D), (a) with all intruders included and (b) with only those included that were correctly identified as alien conspecifics. The error bars indicate the 95% CI around the mean.

identified correctly also remained unchanged (time: $F_{1,18} = 1.648$, $p = 0.059$; interaction: $F_{1,18} = 0.321$, $p = 0.578$). The proportion of grooming and trophallaxis increased significantly from t_1 to t_2 ($F_{1,18} = 4.536$, $p = 0.047$), but with no interaction between time and treatment ($F_{1,18} = 1.109$, $p = 0.306$).

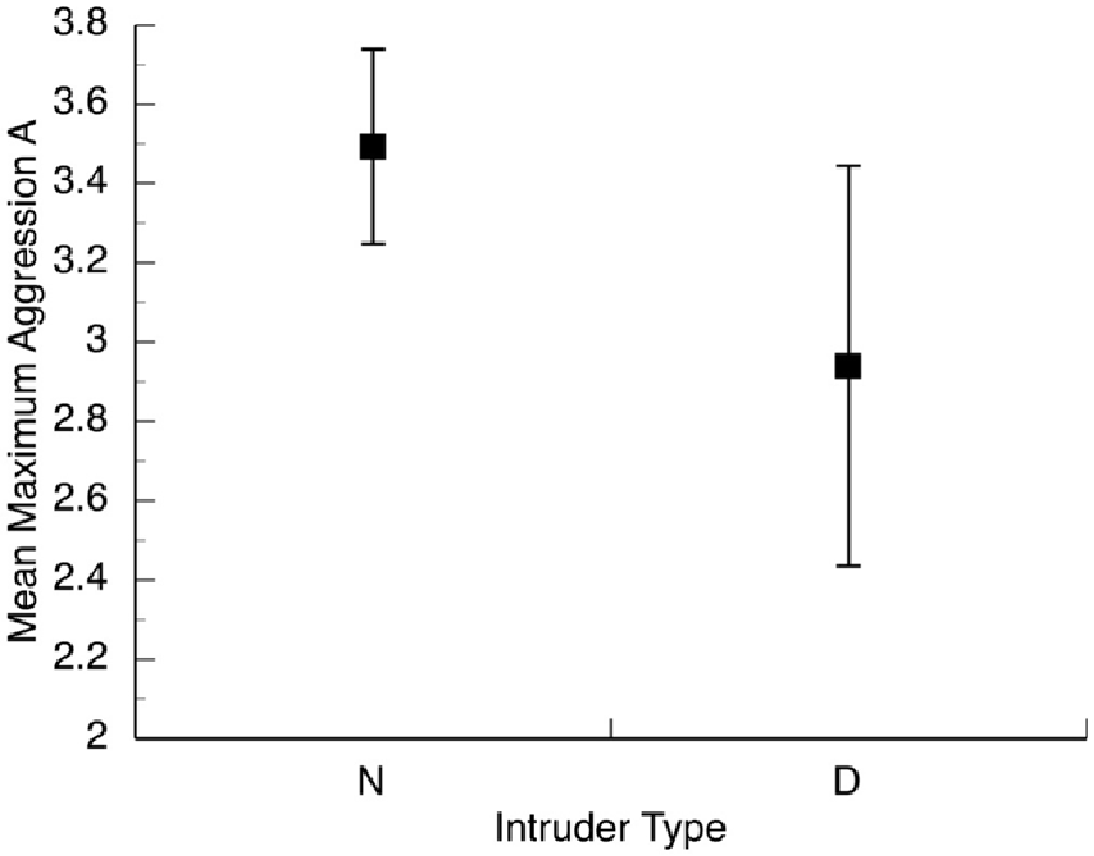

Figure 8-3: The mean maximum level of aggression shown towards intruders from neighbouring colonies (N) and distant colonies (D). The error bars indicate the 95% CI around the mean.

8.4 Discussion

These results revealed that workers from colonies of weaver ants were more aggressive towards intruders originating from neighbouring colonies than towards intruders from more distant colonies. Both the mean level of aggression and the maximum level of aggression were greater towards neighbours than strangers. This strongly suggests that the nasty neighbour effect occurs in this species rather than the dear enemy effect. This adds to the growing number of group living organisms in which this is the case.

Colonies were both more aggressive towards individuals, and aggressive towards a higher proportion of individuals, from neighbouring colonies than from distant colonies. This suggests that there is both a behavioural and a perceptual component to this effect. The behavioural component is evident from the fact that among individuals correctly identified as alien conspecifics, the aggressive response was greater towards intruders from neighbouring

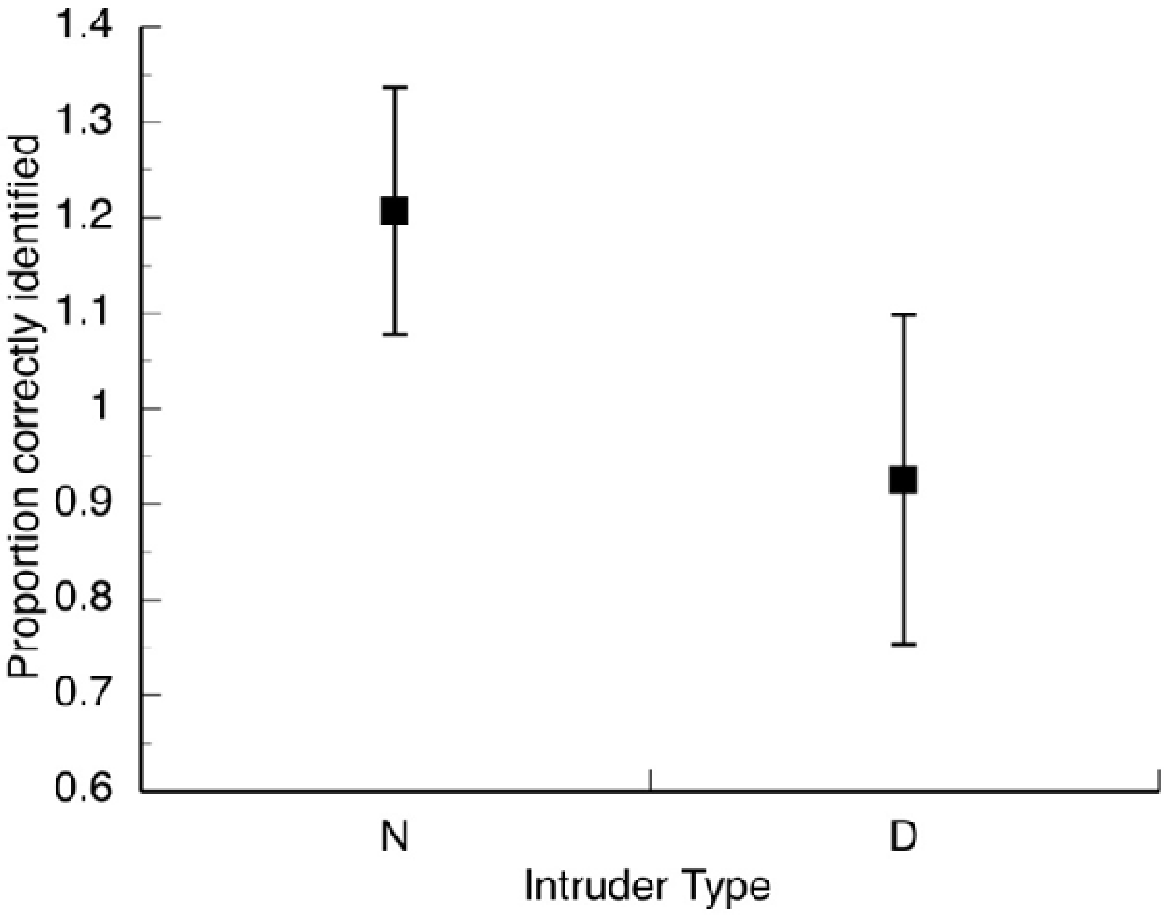

Figure 8-4: The mean proportion (arcsine-square root transformed) of intruders from neighbouring colonies (N) and distant colonies (D) correctly identified by recipient colonies as alien conspecifics. The error bars indicate the 95% CI around the mean.

colonies. The perceptual component is indicated by the fact that a smaller proportion of workers from distant colonies than from neighbouring colonies elicited any aggressive or aversive response. While I cannot completely rule out the possibility that recipients made a behavioural decision to treat some workers from a colony aggressively and some non-aggressively, it seems more likely that the difference was the result of misidentification. There was no evidence that intruders from distant colonies were avoided rather than engaged in conflict, as might have been expected if they were recognized as alien conspecifics. Furthermore, workers from recipient colonies were more likely to engage in grooming or trophallaxis with workers from unfamiliar than from familiar colonies. I therefore conclude that intruders from distant colonies were more likely to be misidentified as colony mates than workers from neighbouring colonies; in addition, when intruders were correctly identified as alien conspecifics, they were greeted with a more aggressive response when they originated from a neighbouring colony than when they originated from a more distant colony. It seems clear from this that experience plays an

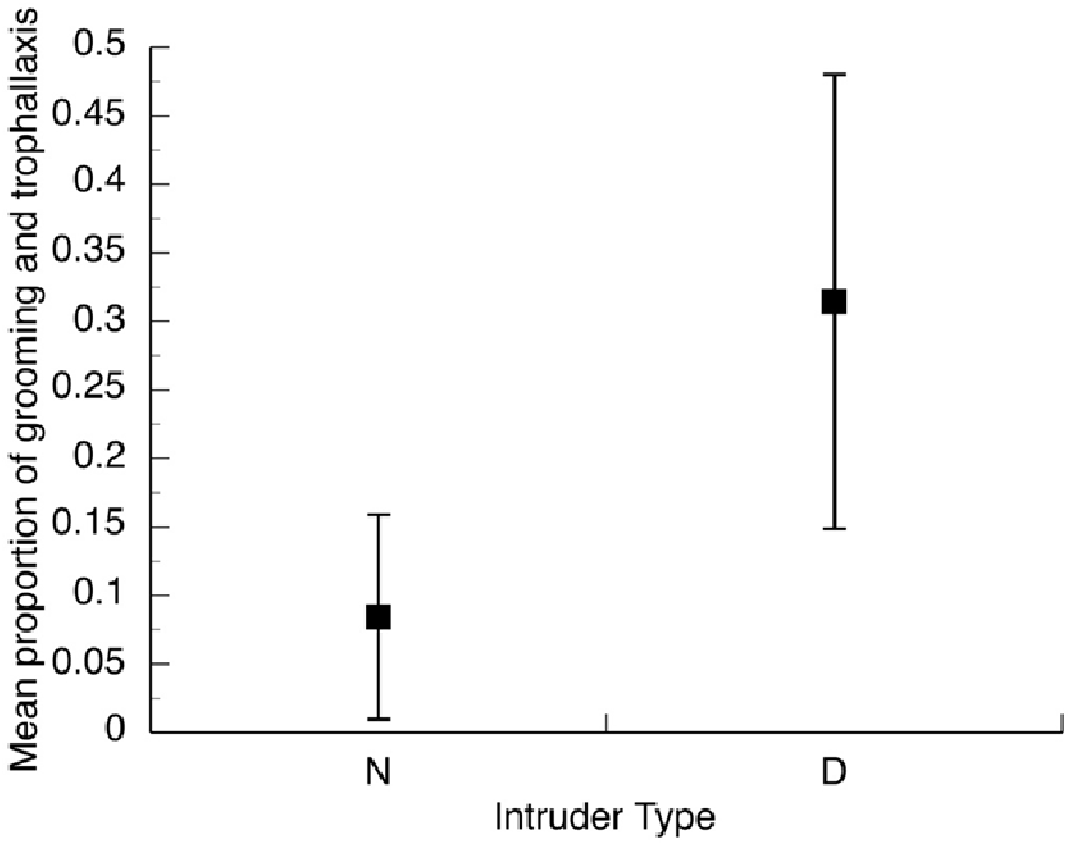

Figure 8-5: The mean proportion (arcsine-square root transformed) of grooming and trophallaxis shown towards intruders from neighbouring colonies (N) and distant colonies (D). The error bars indicate the 95% CI around the mean.

important role in both the ability of weaver ants to differentiate between self and non-self, and in determining the level of aggression directed towards those identified as non-self.

In this study I was unable to detect any significant change in the behaviour of workers in nests that were forced to spend time as neighbours, compared to workers in control nests that remained apart. This contrasts with the results of Sanada-Morimura et al.'s (2003) study of *Pristomyrmex punctatus* (previously *P. pungens*) which found that colonies exhibited increased hostility towards new neighbours within a day of first contact. However, unlike weaver ants, *P. punctatus* colonies relocate approximately once every two weeks during the warmer months, so workers are likely to encounter new neighbours quite frequently (Sanada-Morimura et al. 2003). New neighbours represent either a rival whose territory is to be taken, or a rival threatening to take over the colony's territory. In these circumstances, neighbours and strangers are ephemeral phenomena, and a capacity to continually re-evaluate the threat is essential.

Knaden and Wehner (2003) also found, in contrast to this study, that the aggression shown towards intruders by *Cataglyphis fortis* workers was greater if there had been a prior encounter only 18 hours earlier. However, these ants form only small colonies of about 50

workers, and are spaced sufficiently widely apart that encounters with workers from other colonies are rare (Knaden and Wehner 2003). In this context, there are no real neighbours, only strangers.

For most weaver ant colonies, neighbours are an ever-present and fairly stable reality. While the boundaries of colonies fluctuate, and colonies expand and contract seasonally (Lokkers 1990), entire colonies do not relocate, and the identity of neighbours remains fairly constant over time. New neighbours are therefore likely to be encountered only rarely, and only repeated encounters over an extended period of time is likely to result in a modified response. The 9 or 10 days during which the experimental nests lived as neighbours may not have been long enough to generate any behavioural change. Colonies in the field may have spent years as neighbours, perhaps from shortly after their establishment.

It is also possible that individual weaver ants are unable to learn the identity of a new potential enemy after an older enemy has been identified: the template for "enemy" may become fixed after an encounter with an alien conspecific. This does not preclude the possibility that aggression might increase after repeated or prolonged exposure to the known enemy, or that more individuals would recognize neighbours than non-neighbours as enemies, both of which are consistent with the results of the first experiment. However, it could mean that workers identify as an enemy the first non-colony-mate that they encounter, which is much more likely to be a neighbour than a non-neighbour, and do not have the capacity to add other conspecifics to their list of enemies. In this case, only subsequent workers would be able to learn the identity of a new enemy, a system that would work quite effectively if neighbours changed only rarely.

It is clear that weaver ants learn at some stage in colony development that neighbours represent a serious threat. Non-neighbours are frequently not identified as non-self, and even if they are recognized, they are treated less aggressively than neighbours. The ability to recognise new potential threats may exist, but if colonies, once established, are fairly stable, there may be little benefit in being able to recognise and respond aggressively to non-neighbouring alien conspecifics if they represent only the occasional stray worker. Enough workers do respond aggressively to suggest that few strays would ultimately survive. However, even if they did survive, they represent little threat to the colony: they are simply additional "free" workers in which the colony has made no investment. The only real threat would arise from neighbouring colonies that can invade in large numbers and steal valuable territory and resources.

I suggest that there is now sufficient evidence, from a wide range of taxa, to argue that the nasty neighbour effect may be a general phenomenon among group living animals that defend a territory and compete for limited resources. With weaver ants the challenge remains to determine the extent to which, and mechanism by which, the identity of new enemies can be learnt.

9 Know thine enemy: Why some weaver ants do but others don't

Abstract

Social insects generally defend their colony against intruding conspecifics. It is usually assumed that they use the shared colony odour as a template against which to assess strangers. However, individual behaviour varies considerably, and this is inconsistent with a model in which the odour and template are fully shared. The sources of this variation have remained largely unexplored, because research on social insects often focuses on the colony rather than the individual. Here I show that variation in the behaviour of individual weaver ants arises more from the identity of the recipient than the intruder and, contrary to previous findings, that this often results from perceptual and not only from motivational differences. These findings indicate that recognition in weaver ants may involve a template based on the individual's odour prior to intermingling with other odours, rather than a common odour. This also suggests that a common odour might be more important for the survival of the colony than a shared template. Conversely, possessing a range of templates may provide a colony with additional fitness benefits. By focussing on the differences among individual workers within colonies, this study reveals complexities in nestmate recognition that might otherwise have gone unnoticed.

9.1 Introduction

As previously discussed (Chapter 2), recognition systems involve three components: an expression component, consisting of a phenotypic cue borne by the target of the recognition process; a perception component, consisting of the template against which the cue borne by the target is assessed, together with the referent on which this template is based; and an action component, which is the response to the cue bearer (Tsutsui 2004). Disentangling the perception component from the action component can be difficult (Gamboa et al. 1991; Liebert and Starks 2004). Positive evidence that recognition has occurred is only obtained when it is accompanied by a differential response towards bearers of particular cues. The lack of any such differential response may result from either a recognition error or from a lack of motivation to behave differently towards individuals bearing different phenotypic cues. A lack of recognition, therefore, cannot be inferred from the lack of such a differential response.

Among eusocial insects it is generally acknowledged that the expression component consists of an odour that is particular to each colony (Crozier and Pamilo 1996; Hölldobler and Wilson 1990; Wilson 1971) and that cuticular hydrocarbons constitute a key component of this (Akino et al. 2004; Dani 2006; Howard and Blomquist 2005; Lahav et al. 1999). Researchers have long been aware of inter-colonial variation in the response towards conspecific intruders, and have generally attributed this to differences in the breadth of the recognition template. It is argued that colonies with greater genetic diversity will possess a broader colony odour, and a correspondingly broader recognition template (Breed and Bennett 1987), which in turn will make them more tolerant towards (response) or less able to recognise (perception) alien conspecifics. The U-present (undesirable-present) model for phenotype matching, recently proposed by Gerrieri et al. (2009), provides a mechanism for understanding this. According to this model, undesirable components present in the intruder's, but not the recipient's, phenotype elicit an aggressive response. Thus, in the carpenter ant *Camponotus herculeanus*, workers responded aggressively only to intruders bearing a cuticular chemical component absent from their own profile (Guerrieri et al. 2009). A colony with greater genetic diversity will presumably possess a greater range of cuticular chemical components and workers will therefore be less likely to encounter intruders possessing chemicals absent from their own profile.

While this inter-colonial variation has been the subject of considerable theoretical discussion and empirical research, intra-colonial variation has received much less attention. While it is evident from most behavioural bioassays that there is much individual variation within colonies in the response to intruders (Chapter 5; Roulston et al. 2003), this variation remains largely unexplored. One exception is the study by Crosland (1990) of *Rhytidoponera confusa* in which he explored variations in the behaviour of individual workers towards non-

nestmates. He identified a few individual ants that were highly aggressive in each colony, whereas others were relatively non-aggressive, and attributed this to differences in the aggressor rather than the victim (Crosland 1990). However, he did not determine whether this difference in behaviour could be attributed to a difference in the response or perception component of the recognition system. Individual variation in response has generally been discussed in the context of the division of labour within the colony. The tasks of foraging and defence, the more dangerous tasks within the colony, are often thought to be taken up by older workers (Hölldobler and Wilson 2009). Thus, particular individuals are likely to demonstrate consistently a more aggressive response than others. This probably represents a difference in response rather than perception, although the latter cannot be excluded if workers become more proficient at identifying intruders as they age.

Using weaver ants I explored in greater detail the variation between individuals within the same colony to conspecific intruders. By employing a novel experimental design, I first sought to determine whether variation in the response to intruders could be attributed principally to the recipient or the intruder during one-to-one encounters. I then sought to determine whether variation in the behaviour of the recipient was the result of a difference in the perception or response component of recognition. This could have important implications for understanding how recognition systems operate, and particularly for the issue of template formation.

9.2 Methods

9.2.1 Experiment 1

From November 2008 to March 2009, I collected medium-sized nests from 10 weaver ant colonies in the grounds of James Cook University, Cairns, Queensland. Nests from five colonies were used as recipient nests and nests from the other five colonies were used as intruder nests. I conducted two series of behavioural bioassays between workers from these colonies, Treatment 1 and Treatment 2. In both treatments, I placed a recipient worker in a small observation arena and allowed it to acclimate for 5 minutes. I then introduced an intruder into the arena. The antennae of the intruder were clipped to prevent an aggressive response towards the recipient: without active antennae a worker is unable to determine the status of another ant and tends to behave passively (personal observation). I then observed the behaviour of the recipient towards the intruder for a period of 5 minutes from the time that the former first made antennal contact with the latter. I recorded the frequency of the following behaviours of the recipient towards the intruder: antennation, grooming, trophallaxis, avoidance, recoil,

Table 9-1: Observed behaviours and associated score (*s*)

Behaviour	**Description**	***s***
Antennation	Recipient's antennae make contact with any part of intruder's body	0
Grooming	Recipient "licks" any part of intruder's body	-1
Trophallaxis	Recipient and intruder exchange fluids orally	-2
Avoidance[1]	Recipient abruptly changes direction on contact with intruder	1
Recoil[1]	Recipient violently recoils from the intruder after contact	2
Aggressive posture	Recipient opens mandibles towards intruder	1
Bite	Recipient seizes some part of intruder's body with mandibles	2
Grapple	Recipient wraps body around intruder while biting	3

[1] Avoidance and Aggressive posture, are regarded as alternative behaviours of roughly equivalent intensity rather than behaviours of different intensity on the same scale. The same is true of Recoil and Bite.

aggressive posture, biting and grappling (see Table 9-1). If any observed behaviour was maintained for a period of 5 seconds it was scored as another instance of that behaviour, and so on for each subsequent period of 5 seconds. I assigned these behaviours a value from -2 to +3 (Table 9-1). Avoidance was given the same score as an aggressive stance, and recoil was given the same score as biting. I believe this is justified because it is more appropriate to regard aversive behaviour as an alternative to aggressive behaviour, rather than as a less intense aggressive response.

In Treatment 1, 10 individual recipients were confronted consecutively with the same intruder. This was repeated 5 times (5 trials for each colony pair), with a new intruder and 10 new recipients each time. This involved a total of 50 recipients and 5 intruders. In Treatment 2, a single recipient was confronted consecutively with 10 different intruders. This was also repeated 5 times (5 trials for each colony pair), involving 5 recipients and 50 intruders.

Using the frequencies of each type of behaviour, I first measured the similarity between each pair of encounters in each trial using the Bray-Curtis Index (BCI):

$$\mathrm{BCI} = \frac{\sum |f_{i,1} - f_{i,2}|}{\sum (f_{i,1} + f_{i,2})}$$

where $f_{i,1}$ is the frequency of a particular behaviour in the first encounter of the pair and $f_{i,2}$ is the frequency of the same behaviour in the second encounter of the pair. I determined the mean similarity for each trial for each colony pair, in each treatment.

I also calculated a response index RI:

$$\mathrm{RI} = \frac{\sum f_i s_i}{T} + 2$$

where f_i is the frequency of a behaviour, s_i is the score for that behaviour, and T is the total number of observed interactions. I added 2 to the right hand term to avoid negative values so that I could calculate the coefficient of variation (CV) for this variable in each trial. I determined the mean CV for each colony in each treatment. Because, in Treatment 2, individual recipients were subjected to repeated consecutive trials, I tested for the possibility that this might have a systematic effect on their response using regression models with trial number (1 to 10) as the independent variable. There was no significant linear or quadratic relationship between trial number and RI (Linear: $F_{1,248} = 0.009$, $R^2 = 0.000$, $p = 0.926$; Quadratic: $F_{2,247} = 0.508$, $R^2 = 0.004$, $p = 0.603$).

I used a repeated measures ANOVA to compare the effects of Treatment 1 and Treatment 2 on both BCI and the CV of RI. These variables were normally distributed and met the assumptions for this test. Analyses were performed in SPSS 16.0 for Windows.

9.2.2 Experiment 2

In March and April 2009 I collected a nest from each of 18 additional colonies in the grounds of James Cook University. Six of these were used as recipient colonies and 12 as intruder colonies. Individual encounters were staged as described above between two workers from each of the recipient colonies and five workers from each of two intruder colonies. Different intruders were used for each of the recipients. Thus each recipient encountered five intruders from one colony and five from another. This was repeated for each of the six recipient colonies. I determined the mean response index RI for each series of encounters.

To determine whether one recipient worker from a colony was consistently more aggressive than the other, I conducted a two-way ANOVA for each recipient colony with RI as the response variable, and recipient and intruder colony as fixed effects. Because sample sizes were small, I used a permutation method (Appendix A.4), testing for an interaction by permuting the residuals, after accounting for main effects, following Anderson and Ter Braak (2003). A significant interaction between the responses of the two workers to intruders from the different colonies would indicate a difference in the perceptual component of the recognition system, whereas a consistent difference between them would indicate a difference in the response component.

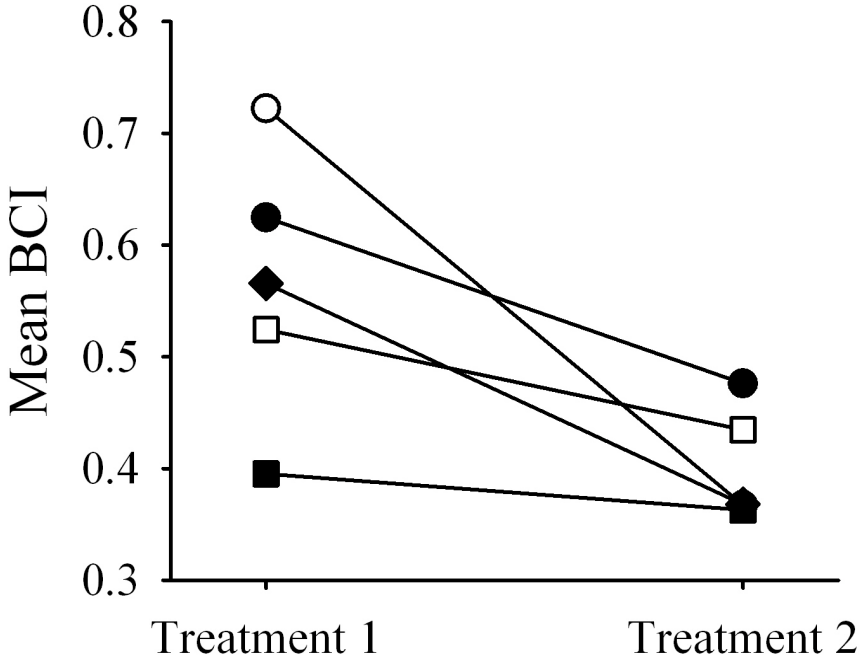

Figure 9-1: The mean Bray-Curtis Index (BCI) for each colony for one intruder facing several recipients (Treatment 1) and one recipient facing several intruders (Treatment 2).

9.3 Results

9.3.1 Experiment 1

Overall, the behavioural response of a single recipient towards several intruders was significantly more consistent than that of several recipients towards the same intruder (Figure 9-1; $F_{1,20}$ = 36.862, partial η^2 = 0.662, p < 0.001). However, there was also a significant difference between colonies tested ($F_{4,20}$ = 4.082, partial η^2 = 0.449, p = 0.014), and a significant interaction between treatment and colony ($F_{4,20}$ = 3.682, partial η^2 = 0.424, p = 0.021). The interaction was driven by colonies differing significantly with regard to the effect of Treatment 1 ($F_{4,20}$ = 6.564, partial η^2 = 0.568, p = 0.002), but not Treatment 2 ($F_{4,20}$ = 1.186, partial η^2 = 0.192, p = 0.347). Thus colonies varied in the extent to which the same intruder

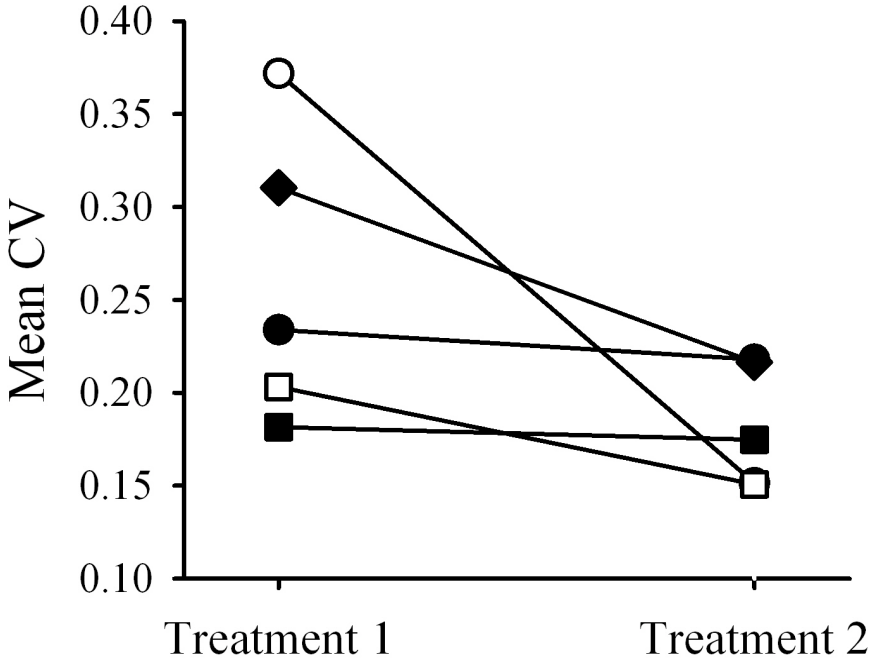

Figure 9-2: The mean coefficient of variation (CV) of the response index (RI) for each colony for one intruder facing several recipients (Treatment 1) and one recipient facing several intruders (Treatment 2).

elicited a different response from different recipients, but not in the extent to which the same recipient responded differently to different intruders.

The CV of the response index revealed a similar pattern. It was also significantly lower in Treatment 2 than in Treatment 1 (Figure 9-2; $F_{1,20} = 12.604$, partial $\eta^2 = 0.387$, $p = 0.002$), there was a significant difference between colonies ($F_{4,20} = 4.682$, partial $\eta^2 = 0.484$, $p = 0.008$), and a significant interaction between treatment and colony ($F_{4,20} = 3.310$, partial $\eta^2 = 0.385$, $p = 0.038$). The interaction was again driven by colonies differing significantly with regard to the effect of Treatment 1 ($F_{4,20} = 6.489$, partial $\eta^2 = 0.565$, $p = 0.002$), but not Treatment 2 ($F_{4,20} = 1.100$, partial $\eta^2 = 0.180$, $p = 0.384$).

9.3.2 Experiment 2

I detected a significant interaction in three of the six colonies tested (Figure 9-3). This strongly suggests that in these colonies individual workers perceive intruders differently, rather than just responding differently to them. In colony 1, the RI of the first recipient was higher towards intruders from the first colony than that of the second recipient ($F_{1,8}$ = 6.488, p = 0.040), but their response towards intruders from the second colony did not differ significantly ($F_{1,8}$ = 2.700, p = 0.166). In colony 5, there was no significant difference in the response to intruders from the first colony ($F_{1,8}$ = 1.366, p = 0.271), but the RI of the first recipient towards the second intruder colony was lower than that of the second recipient ($F_{1,8}$ = 6.160, p = 0.047). In colony 6, the response of the first recipient was similar towards both intruders ($F_{1,8}$ = 14.098, p = 0.022), while that of the second recipient varied significantly between intruder colonies ($F_{1,8}$ = 1.378, p = 0.301).

In two colonies the RI of one recipient was significantly higher than that of the other (colony 4: $F_{1,16}$ = 41.746, $p < 0.001$; colony 6: $F_{1,16}$ = 50.593, $p < 0.001$), suggesting that some individuals consistently had a stronger response towards intruders than others.

Finally, in colony 2 the RI of both recipients towards intruders from both colonies was similar; while in colony 3, the RI of both recipients to intruders from the first colony was greater than their response to intruders from the second colony (first recipient: $F_{1,16}$ = 11.341, p = 0.032; second recipient: $F_{1,16}$ = 8.402, p = 0.031).

9.4 Discussion

The results of the first experiment demonstrate that there is greater variation in the response of recipients to a common intruder than in the response of a single recipient to several intruders. This suggests that the response of the recipient towards an intruder is determined more by characteristics of the recipient than of the intruder. This is consistent with Mintzer's (1982) finding at the colony level in the acacia ant *Pseudomyrmex ferruginea*: variation in aggression was greater when colonies were considered as recipients in aggressive encounters than when they were considered as intruders. At the level of the individual, this finding also supports that of Crosland (1990), but provides even stronger evidence, as I used the same recipient with several intruders, on the one hand, and the same intruder with several recipients, on the other. This could suggest that some *O. smaragdina* workers play a more aggressive role in the defence of the colony than others, in accordance with the division of labour within the colony, as Crosland concluded with respect to *Rhytidoponera confusa.*

However, the second experiment does not support this conclusion. While a recipient's response is consistent towards intruders from any given colony, it is not always consistent

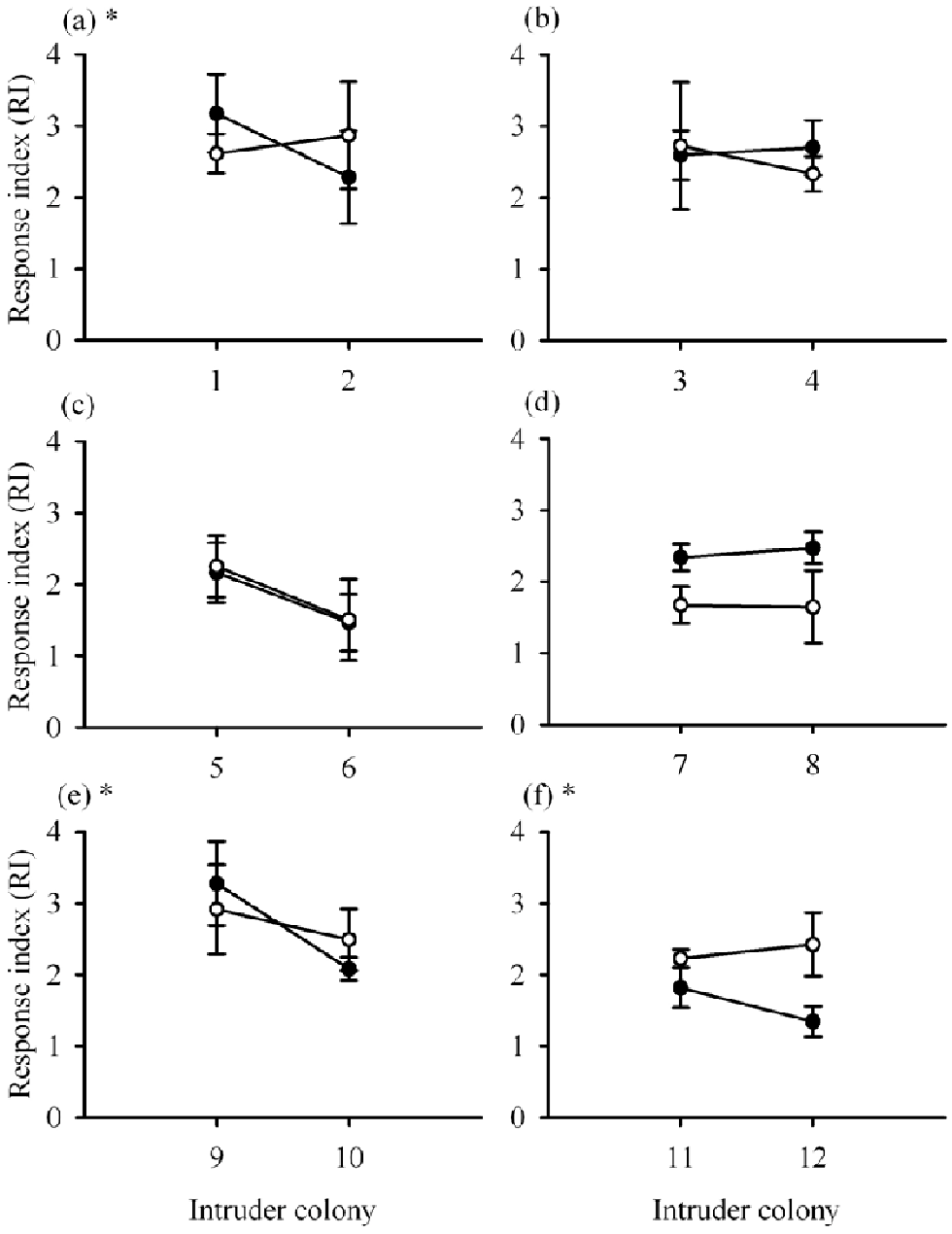

Figure 9-3: The mean response index (RI) of recipients facing intruders from two different colonies. Parts (a) – (f) show the results for recipient colonies 1 – 6 respectively. Hollow circles and solid circles represent the two individuals from each recipient colony, and * indicates a significant interaction between the responses of the two recipients to intruders from different colonies. The error bars represent the 95% confidence intervals around the mean.

towards intruders from different colonies. While some recipients appear to be consistently more aggressive than others, regardless of the colony of origin of the intruder, the significant interaction term in some colonies indicates that an individual worker from a recipient colony

can be aggressive towards intruders from one colony but not towards intruders from another colony; whereas the reaction of another worker from the same recipient colony may be the opposite of this. Hölldobler (1983b) attributed the role of colony defence primarily to older weaver ant workers. However, it is clear from the present study that other workers are also capable of undertaking a defensive role. Furthermore, if age alone were the determining factor, we might expect to see an individual behaving consistently towards intruders, whatever their colony of origin. The present results suggest, rather, that different individuals undertake the defence of the colony, depending on the colony of origin of the intruder. This provides the first evidence that the variation in response arises from a difference in perception rather than just a difference in behaviour: conspecific intruders from different colonies are recognised as such and responded to aggressively by different workers within the recipient colony.

These findings are consistent with a model in which workers use different templates for assessing intruders, and in which the common colony odour is not the referent for this template. One possible alternative for this referent is the individual's own odour, prior to any mixing. Both genetic variation (Dronnet et al. 2006; Foitzik et al. 2007; Lahav et al. 2001; Stuart 1988) and differences in microclimate and diet (Buczkowski et al. 2005; Buczkowski and Silverman 2006; Liang and Silverman 2000; Richard et al. 2004; Richard et al. 2007) during rearing might contribute to this individual variation in odour. According this model, different workers confronted with the same alien intruder will respond differently, as the distance between each worker's template and the odour of the intruder will vary. The chemical distance between an individual recipient's current odour and that of the intruder is unlikely to be correlated with this behavioural response, as the original odour of the recipient no longer exists except as a template. Nevertheless, there is likely to be a correlation between mean colony distances and mean colony responses, as the colony gestalt odour is effectively the mean of the individual odours which form the referent for the individual templates. This is consistent with observed results (Chapter 5; Suarez et al. 2002).

Although I did not detect a significant interaction in all colonies this does not invalidate this interpretation. Some individuals within any colony are always likely to have a similar template. If individual variation in odour has a genetic basis, this is more likely to occur in colonies with lower genetic variation. Because odour is shared to some extent, the current odour of individuals within the colony does not necessarily reflect the genetic diversity within the colony. However, if individuals use their original odour as a template, I predict that an interaction such as that described in the second experiment above will be more likely to occur in colonies with high genetic diversity than in colonies with low genetic diversity. The interaction detected in the first experiment, in which colonies varied in the extent to which the same intruder elicited a different response from different recipients, but not in the extent to which the same recipient responded differently to different intruders, is also consistent with this model.

The greater variation among recipients in some colonies is not mirrored by a greater variation among intruders in some colonies, as might be expected if it had its basis in existing differences in the odours of individuals. Thus whatever causes the variability between the responses of different recipients is not detectable among the intruders. It is most likely masked by the sharing of odour cues throughout the colony. If the proposed model is correct, those colonies with the greatest behavioural variability between recipients will also have the greatest genetic variability.

Having a range of templates rather than a single template may result in some fitness benefits for the colony. Starks et al. (1998) found that greater genetic diversity resulted in a higher number of recognition errors in polygynous colonies of *Pseudomyrmex pallidus* compared to monogynous colonies. However, this may not always be the case. A colony with low genetic diversity, in which all workers share a common template, may consistently misidentify intruders from another colony if that colony has a similar odour, whereas in a colony with a variety of templates, some workers may identify the intruder and raise the alarm. In this case selection may favour a variety of templates rather than a shared template, although it may still favour a shared colony odour if mistaking a colony-mate for an intruder is more costly than mistaking an intruder for a colony-mate. In the case of weaver ants, the first type of error could result in "civil war" and the collapse of the colony; whereas the second type of error might result only in the occasional adoption of an alien worker, who represents a "free" worker in which the colony has invested no resources.

By focussing on the differences among individual workers within colonies, this study reveals complexities in nestmate recognition that might otherwise have gone unnoticed. This individuality may itself contribute to the fitness of the colony. The evolution of polyandry (multiple queen mating) among social insects has been hypothesised to arise (among other things) from the benefits gained by having a genetically diverse workforce capable of performing the range of tasks necessary for the functioning of the colony, and from the benefits gained in having greater resistance to pathogens (Crozier and Pamilo 1996). Rather than regarding the failure to recognise an intruder from a particular colony as an "error", this variability in the capacity of individual workers to recognise intruders from different colonies may represent another factor favouring selection for greater intra-colonial genetic diversity.

10 General discussion

Distinguishing self from non-self (Tsutsui 2004) is at the heart of a range of evolutionary, biological and social processes, including the immune response, reproductive barriers, mate choice, kin selection and the evolution of parasitism (reviewed in Mateo 2004). In the context of immunity, inappropriate rejection of self can give rise to auto-immune diseases, whereas the failure to recognise an invading cancer or pathogen can result in serious illness or death. In an analogous fashion, in the context of co-operative behaviour, kin selection and inclusive fitness (Hamilton 1963, 1964; Trivers and Hare 1976), inappropriate rejection of close kin may result in the loss of inclusive fitness or even injury, if rejection is a dangerous activity, whereas accepting non-kin as kin may result in valuable resources being diverted from true kin.

Because of their complex social systems eusocial insects, and ants in particular, provide an excellent model for studying recognition systems. The colony constitutes a new level of "self". Weaver ants, with their large colonies, in terms of both population size and physical extent, their habit of constructing a large number of ephemeral, discrete nests in trees, and the prevalence of monogyny, provide an opportunity to study recognition processes within a context that generates particular challenges for both the colony and the individual. Weaver ants constitute an extreme example of the difficulties faced by a social organism in differentiating self from non-self, and, with the exception of the study by van Wilgenburg et al. (2006) of *Iridomyrmex purpureus*, no other studies of the recognition systems of ants with large, polydomous colonies have been conducted.

My overall aim in this project was to advance our understanding of self/non-self recognition systems by studying weaver ants, with a particular focus on the variability in time and space of potential recognition cues, and the variability in response to those cues among individuals. The former is concerned with the expression component of the recognition system, while the latter is concerned with the perception and response components of the recognition system. Each of the preceding chapters has been written in the form of a stand-alone publication, and the significance of the results has already been discussed in a broad context. In this chapter I will present a more general review of the thesis as a whole, and suggest some directions for future study. First, however, I will comment on the application of near-infrared spectroscopy to the study of social insects.

10.1 Near-infrared spectroscopy

Although the main aim of this project was to study colony-mate recognition, this involved the application of a novel methodology, the development of which became a

secondary objective of the project. Prior to this study, NIRS had been applied to the study of insects mainly within the context of pest control. For the most part, this was restricted to the detection and identification of pest species in stored grain products (reviewed by Liu et al. 2009). The only previous application of NIRS to the study of social insects was by Aldrich et al. (2007), who used it to differentiate between four species/subspecies of the termite genus, *Zootermopsis*. The lack of application to insect studies is surprising, given the importance of chemical cues in the lives of insects, and the ability of NIRS to detect organic chemicals.

This project advances the application of NIRS in three ways:

1. This is the first application of NIRS to the study of any ant species, indeed, to the study of any of the Hymenoptera. This group of insects is important not only in the context of evolutionary biology, but also for economic reasons. It is hoped that the present project, together with the publications that have emerged from it, will encourage other ecologists and evolutionary biologists to explore ways of applying NIRS to the study of insect species.
2. While NIRS has been used to differentiate between between insect species, this is the first study to show that it can also be used to differentiate between groups within species. The ability to differentiate between weaver ants from different colonies, and even between ants from different nests within the same colony, demonstrates that NIRS is very sensitive to changes in cuticular compounds. Although not included within this study, I also found some evidence that NIRS could be used to differentiate between nurse and forager honeybees (*Apis mellifera*). The capacity to differentiate groups within a species, and even within a single colony of a species, has the potential to facilitate a range of ecological and evolutionary studies.
3. This is the first study to correlate the behavioural response of any insect to the information contained in NIR spectra. I clearly demonstrated that the level of aggression exhibited by weaver ant workers towards intruders from conspecific colonies increased as the spectral distance between colonies increased. This may be particularly important in the field of behavioural ecology, in which wet chemical analyses can consume much of the researcher's available time and resources.

The methods of acquiring and analysing spectra used in this project could almost certainly be refined to increase the sensitivity and resolution of the technique. However, there is no suggestion that NIRS can completely replace the need to extract and analyse particular chemical compounds. The analysis of CHCs is an important area of social insect research that has provided many significant insights. It is now important that the relationship between NIR spectra and the cuticular chemical profile of weaver ant colonies, and other insect species, be investigated directly. Determining the relationship between CHCs and NIR spectra will not only serve to further validate research using NIRS, but may also facilitate CHC research if relationships between spectra and CHCs can be established.

10.2 Spatial and tempoaral variation in potential recognition cues

For any recognition system to be effective, there need to be reliable recognition cues. We have seen that insect cuticular chemicals may derive from dietary components (Buczkowski et al. 2005; Buczkowski and Silverman 2006; Liang and Silverman 2000; Richard et al. 2004; Richard et al. 2007) or environmental sources such as nesting materials (Couvillon et al. 2007; D'Ettorre et al. 2006; Singer and Espelie 1996); or that they may have a genetic origin (Dronnet et al. 2006; Foitzik et al. 2007; Lahav et al. 2001; Stuart 1988). Cues derived from diet or environment are not necessarily unreliable indicators of kinship if these are relatively heterogeneous among colonies (Tsutsui 2004). However, weaver ant colonies often extend across many trees consisting of a variety of species. It is highly likely that diet and nesting materials are quite heterogenous both between and within colonies. In this context, the threat to the colony arises not from incorrectly accepting an alien conspecific as a colony-mate, but from rejecting a colony-mate as an alien intruder. Hostilities between nests could result in the collapse of the colony.

NIRS revealed that there were clear differences in spectra both between colonies and between nests within colonies. However, close analysis of the spectra revealed that the differences between colonies varied qualitatively from the differences between nests within colonies. The differences between nests within colonies were restricted mainly to the amplitudes of peaks, whereas differences between colonies were also found in the positions and widths of peaks. This means that there is not simply a gradient from colony-mate to non-colony-mate, but, rather, a clear discontinuity between them. There are some spectral features that remain relatively constant across nests within colonies, while others vary. This suggests two things:

1. Chemical exchange between nests within colonies is restricted. Weaver ant workers engage extensively in trophollaxis and allogrooming, suggesting that chemicals are, indeed, regularly exchanged. However, differences in spectra between nests indicate that exchanges within nests are more frequent than exchanges between nests. It is unlikely that exchanges could occur in which some spectral features were transferred between nests, but not others. Hölldobler (1983a) found that workers in the "barracks" nests at the edges of colonies demonstrated a high degree of site fidelity. Evidence from spectra suggests that this may be true of other nests within colonies. This possibility needs to be confirmed by means of direct behavioural observations.
2. Spectral features that remain relatively invariable across the breadth of the colony are likely to be under genetic control. This also requires confirmation by direct investigation.

NIRS also revealed that the odour of weaver ant colonies changed over time. However, the odour of an isolated nest did not diverge significantly from that of the colony from which it

was taken, despite changes in the odour of that colony. This suggests, not only that colony odour is under genetic control, but that temporal changes in colony odour may also be under genetic control. As far as I am aware, this is the first study to detect such a phenomenon in any social insect.

Behavioural bioassays clearly demonstrated that aggression between colonies increased as the spectral distance between them increased. Most importantly, however, the response was stronger when distance was calculated using only those spectral parameters that differentiated between colonies, and was completely absent when distance was calculated using spectral parameters that differentiated between nests within colonies. Furthermore, aggression did not increase significantly between the original colony and nests that had been isolated from that colony for a period of up to four months, suggesting that even complete isolation did not impair the ability of workers to recognise colony-mates.

All of this suggests that maintaining colony identity is particularly important for weaver ants. In the present study population, colonies have only a single queen, and are established by new queens following a nuptial flight during which they mate with one or more males. A queenless colony does not adopt a new queen, and no isolated fragment can become established as a separate colony. In these circumstances, a loss of colony integrity will have high fitness costs. This may not be the case for polygynous species in which individual nests may possess or adopt a new queen (eg. Formica exsecta; Katzerke et al. 2006). Comparative studies between closely related species with different colony structures would shed further light on this, as would similar studies on populations of *O. smaragdina* in which polygyny has been reported (Peng et al. 1998).

10.3 Individual variation in recognition skills

As far as I am aware, only one other study has explored individual variation in the response of ants to intruders (Crosland 1990), although such variation is everywhere apparent. Here I determined that variation in the individuals making the assessment, rather than in the individual being assessed, was the principle source of variation in encounters between weaver ants. This is consistent with Mintzer's (1982) finding at the colony level in the acacia ant *Pseudomyrmex ferruginea* that variation in aggression was greater when colonies were considered as recipients in aggressive encounters than when they were considered as intruders. At the level of the individual, this finding also supports that of Crosland (1990) with respect to *Rhytidoponera confusa*, but provides even stronger evidence. Unlike Crosland, I found only limited evidence that some individuals were consistently more aggressive than others, and that this, therefore, constitutes a division of labour within the colony. Instead, I was able to locate this variation in the perception component rather than the response component of the

recognition system, at least in some cases. This result suggests that different individuals undertake the defence of the colony, depending on the colony of origin of the intruder, which may provide the colony with defence against a broader range of intruders than might otherwise be the case. Further research into the relationship between the age and experience of workers and their response to intruders would also be valuable.

I suggest that the results of this particular study require us to re-examine our understanding of recognition templates and their formation within ant colonies. At the very least, examining recognition at the level of the individual rather than the colony reveals a complexity that might otherwise be overlooked. I have proposed two hypothesies that would be fairly easy to test, and which I restate here:

1. If individuals use their original odour as a template, an interaction such as that described in the second experiment of Chapter 9 will be more likely to occur in colonies with high genetic diversity than in colonies with low genetic diversity.
2. If the proposed model is correct, those colonies with the greatest behavioural variability between recipients will also have the greatest genetic variability.

I have argued that having a range of templates rather than a single template may result in some fitness benefits for the colony, if it enables the colony to identify a broader range of intruders. Selection may favour a variety of templates rather than a shared template, although it may still favour a shared colony odour, if mistaking a colony-mate for an intruder is more costly than mistaking an intruder for a colony-mate. In the case of weaver ants, the first type of error could result in "civil war" and the collapse of the colony; whereas the second type of error might result only in the occasional adoption of an alien worker, who represents a "free" worker in which the colony has invested no resources.

The evolution of polyandry among social insects has been hypothesised to arise (among other things) from the benefits gained by having a genetically diverse workforce capable of performing the range of tasks necessary for the functioning of the colony, and from the benefits gained in having greater resistance to pathogens (Crozier and Pamilo 1996). Rather than regarding the failure to recognise an intruder from a particular colony as an "error", I have argued that this variability in the capacity of individual workers to recognise intruders from different colonies may represent another factor favouring selection for greater intra-colonial genetic diversity.

10.4 Other significant findings: Know thine enemy

I was able to demonstrate decisively that weaver ant colonies were more aggressive towards intruders from neighbouring colonies than towards strangers from more distant colonies. Most importantly, it is clear that the difference in the response to neighbours

compared to strangers can be partly attributed to the perception component of recognition, not just the response component. Workers were less successful at identifying strangers than neighbours as intruders, suggesting that the identity of the enemy has to be learnt, at least to some extent. Such learning has been demonstrated previously in other ant species (Knaden and Wehner 2003; Sanada-Morimura et al. 2003). Unfortunately I was unable to demonstrate experimentally that learning took place in weaver ants, and this requires further investigation.

The fact that the identity of enemies has to be learned argues against any simple mechanistic understanding of the recognition process in ants. Two main models have been proposed for the way in which social insects evaluate the phenotype of an unknown individual with respect to their recognition template. The D-present model suggests that an unknown individual is accepted as a colony-mate when she possesses desirable cues that are usually associated with colony-mates (Sherman et al. 1997). According to the U-absent model an unknown individual is rejected if she possesses undesirable cues that are absent on colony-mates (Sherman et al. 1997). I would argue that the U-present model, recently proposed by Gerrieri et al. (2009) as an alternative, is really identical to the U-absent model: undesirable components present in the intruder's, but not the recipient's, phenotype elicit an aggressive response. All these models presuppose a fairly simple mechanism according to which intruders are either recognised or not recognised as such. Once learning and experience come into play, such mechanisms are not sufficient for explaining the recognition process. For example, a novel cue not previously encountered, or the absence of a cue that is ordinarily present, might not elicit an immediate aggressive response, since there is a possibility that this might be a new element present within the colony, and a prematurely aggressive response may be counter-productive. Only with ongoing experience might it be possible to determine whether this new element constitutes a potential threat. Given the high cost associated with rejecting colony-mates, it might be better to err on the side of caution. This proposal clearly requires further investigation, both of weaver ants and other ant species.

10.5 General conclusion

Colony-mate recognition among weaver ants and other social insects is a very complex process and many questions remain unanswered. Two issues in particular stand out, both of which may require us to rethink our approach to the topic.

First, recognition is usually examined in the context of the ability of workers to identify potential conspecific intruders. However, I would suggest that, except when there is potential for serious conflict over resources, or when there is the potential for conspecific parasitism to occur, the cost to the colony of inappropriately accepting a conspecific alien as a colony-mate has been exaggerated. In most cases, adoption of a stray worker from another colony represents

little, if any, cost to the adoptive colony. Only the “nasty neighbour” constitutes a real threat. However, when a lone, alien worker is adopted, the cost to that worker is very high indeed, as she is working to raise unrelated offspring. This may be the best option, if she has completely lost contact with her own colony. However, it is expected that there will be very strong selective pressure against a worker mistaking another colony for her own. I would suggest that further advances in understanding the recognition systems of ants may be made by shifing attention away from the recipient towards the intruder. This could include both theoretical modelling and behavioural studies.

Second, because colonies of social insects are often regarded as “superorganisms” (Hölldobler and Wilson 2009) little attention has been paid to variation among individuals within colonies. The present study has shown that this individual variation may be a source of valuable information, revealing aspects of recognition (and probably other aspects of colonial living) that would otherwise go unnoticed.

While there are obviously enormous advances yet to be made by employing genetic and chemical tools in the study of social insects, even relatively simple and inexpensive behavioural studies may prove enormously valuable if these two focal shifts are adopted by the community of scientists studying these fascinating and important organisms.

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Appendix A

Here I include the Visual Basic scripts (Visual Basic 6.5, © 1987 – 2006 Microsoft Corp.) that were written for use in Excel 2007 to conduct the various randomisation and bootstrapping procedures described in the text.

A.1 Comparison of means

```
Sub multimean()
Dim A(100, 1000), index(100, 1000), A1(100, 1000), index1(100, 1000), N(1000)
Dim sum_samples(1000), mean(1000), harmean(1000), sum_samples_sq(1000), Asq(100, 1000)
Dim temp(100, 1000), key(1000), temp1(2, 1000), pq(1000, 1000)
Workbooks("multi-mean").Activate
Worksheets("multi-mean").Activate
Nsamples = Cells(2, 1)
iterate = Cells(4, 1)
        'Read in the data
            For k = 1 To Nsamples
              i = 1
              While Cells(i + 1, k + 2) <> "eof"
                A(k, i) = Cells(i + 1, k + 2)
        'Calculate sums and sums of squares
                Asq(k, i) = A(k, i) ^ 2
                sum_all_sq = sum_all_sq + Asq(k, i)
                sum_samples(k) = sum_samples(k) + A(k, i)
                i = i + 1
                N(k) = i - 1
              Wend
              mean(k) = sum_samples(k) / N(k)
              Ntotal = Ntotal + N(k)
              sum_all = sum_all + sum_samples(k)
            Next k
          'Calculate the harmonic mean of sample sizes
            For k = 1 To Nsamples
              harmN = harmN + 1 / N(k)
            Next k
            harmN = 1 / (harmN / Nsamples)
          'Calculate degrees of freedom
            C = sum_all ^ 2 / Ntotal
            TotalSS = sum_all_sq - C
            TotalDF = Ntotal - 1
            ErrorDF = Ntotal - Nsamples
            GroupsDF = Nsamples - 1
```

```
                    'Calculate F-value
                        Call ANOVA(Nsamples, A, N, C, TotalSS, TotalDF, GroupsDF, ErrorDF, F,
                    real_variance)
                        Worksheets("output").Activate
                        Cells(2, 2) = F
'Repeat the section below "iterate" times
For q = 1 To iterate
Cells(1, 1) = q
'Prepare to permute the data
            'Make an index array for the data
                    For i = 1 To Nsamples
                        For j = 1 To N(i)
                            index(i, j) = True
                        Next j
                    Next i
            'Randomly assign within groups
                    Randomize
                    For i = 1 To Nsamples - 1
                        For j = 1 To N(i)
0:                          x = Int((Nsamples) * Rnd + 1)
                            y = Int((N(i)) * Rnd + 1)
                                If index(x, y) = False Then GoTo 0 Else index(x, y) = False
                                A1(i, j) = A(x, y)
                        Next j
                        z = 1
                        For j = 1 To Nsamples
                            For k = 1 To N(j)
                                If index(j, k) = True Then
                                    A1(Nsamples, z) = A(j, k)
                                    z = z + 1
                                End If
                            Next k
                        Next j
                    Next i
                'Calculate the F-value for the permuted samples
                    Call ANOVA(Nsamples, A1, N, C, TotalSS, TotalDF, GroupsDF, ErrorDF, F1, v)
                    If F1 >= F Then t = t + 1
                    p = t / iterate
                    Cells(2, 3) = p
Next q
                    If p > 0.05 Or Nsamples = 2 Then GoTo 1
'If there is a significant difference, run Tukey
                'First,rank the means in order of magnitude
Cells(3, 10).Font.Color = RGB(255, 0, 0)
```

```
Cells(3, 10) = "Please wait. Running post-hoc comparisons."
                    For i = 1 To Nsamples
                       Cells(i + 1, 6) = i
                       Cells(i + 1, 7) = N(i)
                       Cells(i + 1, 8) = mean(i)
                    Next i
                    Columns("F:H").Select
                    ActiveWorkbook.Worksheets("output").Sort.SortFields.Clear
                    ActiveWorkbook.Worksheets("output").Sort.SortFields.Add
                    key:=Range("H2:H545") _
                    , SortOn:=xlSortOnValues, order:=xlDescending, DataOption:=xlSortNormal
                    With ActiveWorkbook.Worksheets("output").Sort
                       .SetRange Range("F1:H545")
                       .Header = xlYes
                       .MatchCase = False
                       .Orientation = xlTopToBottom
                       .SortMethod = xlPinYin
                       .Apply
                    End With
      'Compare samples
                 For i = 1 To Nsamples - 1
                    For j = i + 1 To Nsamples
                       SE = Sqr(real_variance / 2 * (1 / N(i) + 1 / N(j)))
                       q = Abs(mean(i) - mean(j)) / SE
               'Permute these two samples
                    For z = 1 To iterate
                       sum_temp1 = 0
                       sum_temp2 = 0
                       For k = 1 To N(i)
                          index(i, k) = True
                       Next k
                       For k = 1 To N(j)
                          index(j, k) = True
                       Next k
                       Randomize
                       For k = 1 To N(i)
2:
                          x = Int((2) * Rnd + 1)
                          If x = 1 Then x = i Else x = j
                          y = Int(N(x) * Rnd + 1)
                          If index(x, y) = False Then GoTo 2 Else index(x, y) = False
                          temp1(1, k) = A(x, y)
                          sum_temp1 = sum_temp1 + temp1(1, k)
                       Next k
```

```
                    mean_temp1 = sum_temp1 / N(i)
                    For k = 1 To N(j)
3:
                      x = Int((2) * Rnd + 1)
                      If x = 1 Then x = i Else x = j
                      y = Int(N(x) * Rnd + 1)
                      If index(x, y) = False Then GoTo 3 Else index(x, y) = False
                      temp1(2, k) = A(x, y)
                      sum_temp2 = sum_temp2 + temp1(2, k)
                    Next k
                    mean_temp2 = sum_temp2 / N(j)
                    qtest = Abs(mean_temp1 - mean_temp2) / SE
                    If qtest >= q Then pq(i, j) = pq(i, j) + 1
                  Next z
                  pq(i, j) = pq(i, j) / iterate
                  Next j
                Next i
Cells(3, 10) = ""
Cells(3, 10).Font.Color = RGB(0, 0, 0)
                For i = 1 To Nsamples
                  Cells(1, 10 + i) = i
                  Cells(i + 1, 10) = i
                Next i
                For i = 1 To Nsamples - 1
                  For j = i + 1 To Nsamples
                    Cells(i + 1, 10 + j) = pq(i, j)
                    If pq(i, j) < 0.05 Then Cells(i + 1, 10 + j).Font.Color = RGB(255, 0, 0)
                  Next j
                Next i
1: End Sub

Sub ANOVA(Nsamples, A, N, C, TotalSS, TotalDF, GroupsDF, ErrorDF, F, v)
Dim sum_samples(1000), sum_samples_sq(1000)
                    For k = 1 To Nsamples
                      For i = 1 To N(k)
                        sum_samples(k) = sum_samples(k) + A(k, i)
                      Next i
                      sum_samples_sq(k) = sum_samples(k) ^ 2 / N(k)
                      sum_sum_samples_sq = sum_sum_samples_sq + sum_samples_sq(k)
                    Next k
                    GroupsSS = sum_sum_samples_sq - C
                    ErrorSS = TotalSS - GroupsSS
                    GroupsMS = GroupsSS / GroupsDF
                    ErrorMS = ErrorSS / ErrorDF
```

```
                v = ErrorMS
                F = GroupsMS / ErrorMS
End Sub
```

A.2 Bootstrapped confidence intervals

```
Sub ci()
' Calculate mean and confidence intervals
   n = Application.InputBox("Enter sample size: ", Type:=1)
   For j = 1 To 10000
         Randomize
         sumup = 0
         For i = 1 To n
            a = Int((n - 1 + 1) * Rnd + 1)
            choice = Cells(a+1, 1)
            sumup = sumup + choice
         Next i
         mean = sumup / n
         Cells(j, 7) = mean
         Cells(1, 8) = j
   Next j
   Columns("G:G").Select
   Selection.Sort Key1:=Range("G1"), Order1:=xlAscending, Header:=xlGuess,
      OrderCustom:=1, MatchCase:=False, Orientation:=xlTopToBottom
End Sub
```

A.3 Nested ANOVA

```
Sub nested_mean()
Dim A(10, 1000, 1000), index(10, 1000, 1000), A1(10, 1000, 1000), N(10, 1000), index1(10,
                    1000)
Workbooks("multi-mean").Activate
Worksheets("nested").Activate
Aa = Cells(2, 1)
Bb = Cells(4, 1)
maxS = Cells(6, 1)
iterate = Cells(8, 1)
Btot = Aa * Bb
'Read in the data
                    For k = 1 To Aa
                       For j = 1 To Bb
                          i = 1
                          While Cells(i + 1, ((2 + j) + (k - 1) * Bb)) <> "eof"
                             A(k, j, i) = Cells(i + 1, (2 + j) + (k - 1) * Bb)
                             SS = SS + A(k, j, i) ^ 2
                             i = i + 1
                             N(k, j) = i - 1
                          Wend
                       Next j
                    Next k
'Calculate the F-values and R-sqr values
                    Call Fvalues(SS, Aa, Bb, A, N, Fsubgroups, Fgroups, Rsubgroups, Rgroups)
                    Worksheets("output_nested").Activate
                    Cells(2, 2) = Fsubgroups
                    Cells(3, 2) = Fgroups
                    Cells(2, 8) = Rsubgroups
                    Cells(3, 8) = Rgroups
'Now perform within subgroups permutations to test for subgroup effect
For q = 1 To iterate
          Cells(1, 1) = q
          'Make an index array for the data
                    For i = 1 To Aa
                       For j = 1 To Bb
                          For k = 1 To N(i, j)
                             index(i, j, k) = True
                          Next k
                       Next j
                    Next i
          'Randomly assign within groups
                    Randomize
```

```
                For i = 1 To Aa
                  For j = 1 To Bb - 1
                    For k = 1 To N(i, j)
0:                    x = Int((Bb) * Rnd + 1)
                    y = Int((N(i, x)) * Rnd + 1)
                    If index(i, x, y) = False Then GoTo 0 Else index(i, x, y) = False
                    A1(i, j, k) = A(i, x, y)
                  Next k
                Next j
                z = 1
                For j = 1 To Bb
                  For k = 1 To N(i, j)
                    If index(i, j, k) = True Then
                      A1(i, Bb, z) = A(i, j, k)
                      z = z + 1
                    End If
                  Next k
                Next j
              Next i
'Recalculate F values on the basis of the new permutation
              Call Fvalues(SS, Aa, Bb, A1, N, F1subgroups, F1groups, Rsubgroups,
              Rgroups)
              If F1subgroups >= Fsubgroups Then Nsubgroups = Nsubgroups + 1
              Psubgroups = Nsubgroups / iterate
              Cells(2, 5) = Psubgroups
2: 'Now perform between groups permutations to test for group effect
        'Make an index array for the subgroups
              For i = 1 To Aa
                For j = 1 To Bb
                  index1(i, j) = True
                Next j
              Next i
        'Randomly assign subgroups to groups
              Randomize
              For i = 1 To Aa
                For j = 1 To Bb
1:                x = Int(Aa * Rnd + 1)
                y = Int(Bb * Rnd + 1)
                If index1(x, y) = False Then GoTo 1 Else index1(x, y) = False
                For k = 1 To N(i, j)
                  A1(i, j, k) = A(x, y, k)
                Next k
                Next j
              Next i
```

```
                Call Fvalues(SS, Aa, Bb, A1, N, F1subgroups, F1groups, Rsubgroups,
                Rgroups)
                If F1groups >= Fgroups Then Ngroups = Ngroups + 1
                Pgroups = Ngroups / iterate
                Cells(3, 5) = Pgroups
Next q
End Sub

Sub Fvalues(SS, Aa, Bb, A, N, F1, F2, R1, R2)
Dim sumBinA(10, 1000), SSBinA(10, 1000), sumA(10), SSA(10)
Dim NA(10), meanA(10), Asq(10)
'Calculate the sums of squares and mean squares
  'Sum of B nested in A
                For k = 1 To Aa
                  For i = 1 To Bb
                    For j = 1 To N(k, i)
                      sumBinA(k, i) = sumBinA(k, i) + A(k, i, j)
                    Next j
                   'Sum of squares of B nested in A
                    SSBinA(k, i) = sumBinA(k, i) ^ 2 / N(k, i)
                    sumSSBinA = sumSSBinA + SSBinA(k, i)
                  Next i
                Next k
  'Sum of A
                For i = 1 To Aa
                  For j = 1 To Bb
                    sumA(i) = sumA(i) + sumBinA(i, j)
                    Asq(i) = Asq(i) + sumA(i) ^ 2
                    NA(i) = NA(i) + N(i, j)
                  Next j
                'Sum of squares of A, means of A, total sum
                  meanA(i) = sumA(i) / NA(i)
                  SSA(i) = sumA(i) ^ 2 / NA(i)
                  sumSSA = sumSSA + SSA(i)
                  sumall = sumall + sumA(i)
                  sumAsq = Asq(i) + sumAsq
                  TotalN = TotalN + NA(i)
                Next i
  'C - "correction term"
                C = sumall ^ 2 / TotalN
                TotalSS = SS - C
  'Other SS
                subSS = sumSSBinA - C
                ErrorSS = TotalSS - subSS
```

```
                GroupsSS = sumSSA - C
                subgroupsSS = subSS - GroupsSS
    'Degrees of freedom
                TotalDF = TotalN - 1
                GroupsDF = Aa - 1
                subDF = Aa * Bb - 1
                subgroupsDF = subDF - GroupsDF
                ErrorDF = TotalDF - subDF
    'MS values
                subMS = subSS / subDF
                ErrorMS = ErrorSS / ErrorDF
                GroupsMS = GroupsSS / GroupsDF
                subgroupsMS = subgroupsSS / subgroupsDF
    'F values
                F1 = subgroupsMS / ErrorMS
                R1 = subgroupsSS / TotalSS
                F2 = GroupsMS / subgroupsMS
                R2 = GroupsSS / TotalSS
End Sub
```

A.4 Two-way ANOVA with interaction

```
Sub interaction()
Dim levels(2), matrix(10, 10, 10000), sum_group(10, 10), mean_factor(10, 10), error_matrix(10,
                10, 10000), sum_error(10, 10)
Dim mean_error(10, 10), index(10, 10, 10000), rand_matrix(10, 10, 10000), sum_factor(2, 10),
                f1_matrix(10, 10, 10000)
Dim f2_matrix(10, 10, 10000)
Worksheets("input_interaction").Activate
levels(1) = Cells(3, 2)
levels(2) = Cells(4, 2)
sample_size = Cells(7, 2)
N = levels(1) * levels(2) * sample_size
'Input data into matrix
   For i = 1 To levels(1)
      For j = 1 To levels(2)
         For k = 1 To sample_size
            matrix(i, j, k) = Cells((j - 1) * sample_size + 2 + k, i + 3)
            'Cells((j - 1) * sample_size + 2 + k + 20, i + 3) = matrix(i, j, k)
         Next k
      Next j
   Next i
'Calculate all means
Call mean_calc(matrix, sample_size, N, levels, mean_factor, total_mean, sum_group, sum_factor,
                Grand_Total)
'Calculate residuals
   For i = 1 To levels(1)
      For j = 1 To levels(2)
         For k = 1 To sample_size
            error_matrix(i, j, k) = matrix(i, j, k) - mean_factor(1, i) - mean_factor(2, j) + total_mean
            'Cells((j - 1) * sample_size + 2 + k + 20, i + 3) = error_matrix(i, j, k)
         Next k
      Next j
   Next i
'Calculate SS etc for error_matrix
Call mean_calc(error_matrix, sample_size, N, levels, mean_factor, total_mean, sum_group,
                sum_factor, Grand_Total)
Call SS(error_matrix, sample_size, N, levels, F1, F2, F12, sum_group, sum_factor, Grand_Total)
Cells(10, 2) = F12
'Run permutations
sum_count = 0
For q = 1 To 10000
```

```
'Permutation of residuals (error_matrix)
'Set index matrix values to True
   For i = 1 To levels(1)
      For j = 1 To levels(2)
         For k = 1 To sample_size
            index(i, j, k) = True
         Next k
      Next j
   Next i
'Choose random numbers x,y,z
Randomize
   For i = 1 To levels(1)
      For j = 1 To levels(2)
         For k = 1 To sample_size
0:
            x = Int(levels(1) * Rnd + 1)
            y = Int(levels(2) * Rnd + 1)
            z = Int(sample_size * Rnd + 1)
            If index(x, y, z) = True Then
               rand_matrix(i, j, k) = error_matrix(x, y, z)
               index(x, y, z) = False
               Else: GoTo 0
            End If
         Next k
      Next j
   Next i
Call mean_calc(rand_matrix, sample_size, N, levels, mean_factor, total_mean, sum_group,
                     sum_factor, Grand_Total)
Call SS(rand_matrix, sample_size, N, levels, F1, F2, F_rand, sum_group, sum_factor,
                     Grand_Total)
If F_rand >= F12 Then sum_count = sum_count + 1
p = sum_count / q
Cells(11, 2) = p
Next q
'If p < 0.05 Then GoTo 1
Cells(12, 1) = "Please wait: Testing for main effects."
'Calculate means etc for original data
Call mean_calc(matrix, sample_size, N, levels, mean_factor, total_mean, sum_group, sum_factor,
                     Grand_Total)
Call SS(matrix, sample_size, N, levels, F1, F2, F12, sum_group, sum_factor, Grand_Total)
Cells(13, 2) = F1
Cells(15, 2) = F2
sum_count1 = 0
sum_count2 = 0
```

```
For q = 1 To 10000
'Choose random numbers x,y,z: randomizing for Factor 1 within each level of Factor 2
'Set index matrix values to True
   For i = 1 To levels(1)
      For j = 1 To levels(2)
         For k = 1 To sample_size
            index(i, j, k) = True
         Next k
      Next j
   Next i
Randomize
   For i = 1 To levels(1)
      For j = 1 To levels(2)
         For k = 1 To sample_size
2:
            x = Int(levels(1) * Rnd + 1)
            y = j
            z = Int(sample_size * Rnd + 1)
            If index(x, y, z) = True Then
               f1_matrix(i, j, k) = matrix(x, y, z)
               index(x, y, z) = False
               Else: GoTo 2
            End If
         Next k
      Next j
   Next i
'Choose random numbers x,y,z: randomizing for Factor 2 within each level of Factor 1
'Set index matrix values to True
   For i = 1 To levels(1)
      For j = 1 To levels(2)
         For k = 1 To sample_size
            index(i, j, k) = True
         Next k
      Next j
   Next i
   For j = 1 To levels(2)
      For i = 1 To levels(1)
         For k = 1 To sample_size
3:
            x = i
            y = Int(levels(2) * Rnd + 1)
            z = Int(sample_size * Rnd + 1)
            If index(x, y, z) = True Then
               f2_matrix(i, j, k) = matrix(x, y, z)
```

```
                index(x, y, z) = False
                Else: GoTo 3
            End If
        Next k
    Next i
  Next j
'Calculate means, SS for f1_matrix
Call mean_calc(f1_matrix, sample_size, N, levels, mean_factor, total_mean, sum_group,
                sum_factor, Grand_Total)
Call SS(f1_matrix, sample_size, N, levels, F1_rand, F2_rand, F12, sum_group, sum_factor,
                Grand_Total)
If F1_rand >= F1 Then sum_count1 = sum_count1 + 1
p1 = sum_count1 / q
Cells(12, 2) = ""
Cells(14, 2) = p1
'Calculate means, SS for f2_matrix
Call mean_calc(f2_matrix, sample_size, N, levels, mean_factor, total_mean, sum_group,
                sum_factor, Grand_Total)
Call SS(f2_matrix, sample_size, N, levels, F1_rand, F2_rand, F12, sum_group, sum_factor,
                Grand_Total)
If F2_rand >= F2 Then sum_count2 = sum_count2 + 1
p2 = sum_count2 / q
Cells(16, 2) = p2
Next q
Cells(12, 1) = ""
1:
End Sub

Sub mean_calc(data, sample_size, N, levels, mean_factor, Grand_Mean, sum_group,
                sum_factor, Grand_Total)
'Calculate means
  For i = 1 To 10
    For j = 1 To 10
      sum_group(i, j) = 0
      sum_factor(1, j) = 0
      sum_factor(2, j) = 0
    Next j
  Next i
  For i = 1 To levels(1)
    For j = 1 To levels(2)
      For k = 1 To sample_size
        sum_group(i, j) = sum_group(i, j) + data(i, j, k)
      Next k
    Next j
```

```
    Next i
'Calculate factor means: Factor 1 for each level of Factor 2, ie. levels(1) means
    For i = 1 To levels(1)
        For j = 1 To levels(2)
            sum_factor(1, i) = sum_factor(1, i) + sum_group(i, j)
        Next j
    Next i
    For i = 1 To levels(1)
        mean_factor(1, i) = sum_factor(1, i) / (levels(2) * sample_size)
    Next i
'                   Factor 2 for each level of Factor 1, ie. levels(2) means
    For j = 1 To levels(2)
        For i = 1 To levels(1)
            sum_factor(2, j) = sum_factor(2, j) + sum_group(i, j)
        Next i
    Next j
    For i = 1 To levels(2)
        mean_factor(2, i) = sum_factor(2, i) / (levels(1) * sample_size)
    Next i
'Calculate Grand Mean
Grand_Total = 0
    For i = 1 To levels(1)
        For j = 1 To levels(2)
            Grand_Total = Grand_Total + sum_group(i, j)
        Next j
    Next i
Grand_Mean = Grand_Total / N
End Sub

Sub SS(data, sample_size, N, levels, F1, F2, F12, sum_group, sum_factor, Grand_Total)
'Sum of all squared
all_squared = 0
    For i = 1 To levels(1)
        For j = 1 To levels(2)
            For k = 1 To sample_size
                all_squared = all_squared + data(i, j, k) ^ 2
            Next k
        Next j
    Next i
C = Grand_Total ^ 2 / N
Total_SS = all_squared - C
total_df = N - 1
cells_SS = 0
    For i = 1 To levels(1)
```

```
        For j = 1 To levels(2)
            cells_SS = cells_SS + sum_group(i, j) ^ 2
        Next j
    Next i
cells_SS = cells_SS / sample_size - C
cells_df = levels(1) * levels(2) - 1
Error_SS = Total_SS - cells_SS
error_df = levels(1) * levels(2) * (sample_size - 1)
F2_SS = 0
    For i = 1 To levels(2)
        F2_SS = F2_SS + sum_factor(2, i) ^ 2
    Next i
F2_SS = F2_SS / (levels(2) * sample_size) - C
F2_df = levels(2) - 1
F1_SS = 0
    For i = 1 To levels(1)
        F1_SS = F1_SS + sum_factor(1, i) ^ 2
    Next i
F1_SS = F1_SS / (levels(1) * sample_size) - C
F1_df = levels(1) - 1
Interaction_SS = cells_SS - F1_SS - F2_SS
interaction_df = F1_df * F2_df
F1 = (F1_SS / F1_df) / (Error_SS / error_df)
F2 = (F2_SS / F2_df) / (Error_SS / error_df)
F12 = (Interaction_SS / interaction_df) / (Error_SS / error_df)
End Sub
```