Larval Recognition in Two Host Species (*Temnothorax longispinosus* Roger and *Temnothorax ambiguus* Emery) of the Slave-Making Ant *Protomognathus americanus* Emery (Hymenoptera: Formicidae).

by

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A thesis submitted to the Department of Biological Sciences,
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in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

Slave-making ants exploit the labour of their own or another species. These social parasites raid the nests of host species, kill or displace the resident adults, and abscond with the host brood. Following eclosion the captured workers accept and tend to the slave-maker brood and other captured host-species brood. *Temnothorax ambiguus* and *T. longispinosus* are both host species to the obligatory slave-making ant *Protomognathus americanus* and are facultative slave-makers themselves. I offered laboratory colonies of *T. ambiguus* and *T. longispinosus* a series of choices among different larval types to better understand their brood discrimination abilities. Workers of both species preferentially accepted nestmate over non-nestmate larvae. There was also some evidence that *T. ambiguus,* but not *T. longispinosus,* preferred non-nestmate conspecifics originating from the same geographical location to non-nestmates from other sites. Unrelated conspecific larvae were not preferentially retrieved over allospecific larvae for either species; however, *T. ambiguus* workers consumed more allospecifics than conspecifics. When presented with conspecific versus *P. americanus* larvae, both species manifested a clear bias towards conspecific larvae in terms of earlier retrieval and preferential acceptance. Moreover, reciprocal contact between *P. americanus* and conspecific larvae increased *T. longispinosus* acceptance of the slave-maker larvae, but did not appear to lessen the acceptability of conspecific larvae. Results from experiments using larval-sized pieces of silicone ("baits") that had been left in contact with larvae demonstrated that discriminator substances present on the larval cuticle transfer through direct physical contact. These discriminator substances were not sufficient on their own, however, to allow discrimination within or between species. The biases in retrieval order, acceptance and antennation indicate these species ability to discriminate among larvae.

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"Throughout the evolutionary history of the ants and other social insects there has been an intense selective pressure to sharpen recognition ability, because favors bestowed on an unrelated individual are wasted in the remorseless crucible of natural selection."

— Bert Hölldobler and E.O. Wilson 1990

GENERAL INTRODUCTION

Ants (Hymenoptera: Formicidae) are important pollinators, movers of soil, foragers, decomposers, and predators of other insects (Hölldobler and Wilson 1990). They epitomize how variation in social organization can overcome a plethora of ecological challenges. These 'eusocial' insects have been subjected to intensive scientific scrutiny aimed at understanding the intricacies of their advanced and complex societies. Within ant colonies, non-reproductive workers sacrifice personal reproductive output in favour of investment in reproductive kin, which maximizes their own inclusive fitness (Hamilton 1964; Wilson 1975a).

The presence of a worker caste in eusocial insects necessitates a more open recognition system than that of lower units of biological organization such as individual organisms (Wilson 1971). Although such social organization requires robust recognition mechanisms, there are several instances where the lines of communication and recognition within ant societies have been extended to incorporate other species (Hölldobler and Wilson 1990; Howard et al. 2001; Orivel et al. 2004). For example, several arthropod species (e.g., staphylinid beetles) follow the odour trails of foraging ants, preying on the spoils of the ant workers (Quinet and Pasteels 1996). Some arthropods adopt the chemical signature of a colony to utilize the nest infrastructure (e.g., *Formicoxenus nitidulus*, Buschinger 1976a,b) or to better prey on the ants themselves (e.g., *Cosmophasis bitaeniata*: Elgar and Allan 2004, 2006). Certain ants have entered into mutulistic relationships with honeydew-producing homopterans (Way 1963; Buckley 1987a,b), whereas others cultivate fungal gardens (e.g., *Atta spp.*: Chapela et al. 1994). Remarkably, some socially parasitic ants specialize in exploiting the labour of other

'host' species (Buschinger 1986). In this thesis, I examine aspects of brood recognition and explore the nature of the chemical cues underlying that recognition in two host species of the socially parasitic ant *Protomognathus americanus* — *Temnothorax longispinosus* and *T. ambiguus.*

Social Parasitism

While ant societies are typified by cooperation, socially parasitic ants exploit the labour of their own or another species (Buschinger 1986). Although social parasites do not take advantage of individual host physiology, they are rightfully called parasites in that they capitalize on the social structure of their hosts, exerting an appreciable fitness cost on their hosts while benefiting themselves (Hasegawa and Yamaguchi 1994; Savolainen and Deslippe 1996; Mori et al. 2000; Foitzik et al. 2001; Foitzik and Herbers 2001b; Herbers and Foitzik 2002; Fischer-Blass et al. 2006). By redirecting host labour they divert resources to themselves, presumably reducing their host's reproductive output if not eliminating reproduction all together (but see Hare and Alloway 2001).

The phenomenon of social parasitism occurs within and between species and has been documented in a wide range of taxa. Lace bugs (*Gargaphia spp.*: Tallamy 1985) and many waterfowl species (Rohwer and Freeman 1989) commonly lay their own clutch of eggs among those of an unrelated conspecific. If the foster parent is unable to differentiate between the 'dumped' eggs and her own, she will incur the costs of caring for extra young (Petrie and Møller 1991). Avian social parasites such as brown-headed cowbirds (*Molothrus ater*) and common cuckoos (*Cuculus canorus*) lay their eggs in the nests of passerine hosts (Brooke and Davis 1988; Rothstein 1990; Woolfenden et al.

2003). Within the nest, these parasitic chicks are fierce competitors, often killing host chicks outright (Davies et al. 1998) or causing their death indirectly (Payne 1977; Lorenzana and Sealy 1999). Social parasitism has also been documented in fish (Wisenden 1999), amphibians (Harris et al. 1995) and in a wide range of arthropod species (Thomas et al. 1989; Müller et al. 1990; Akino et al. 1999; Allan and Elgar 2001; Cervo 2006). Humans, too, are arguably social parasites whenever we exploit the labour of members of our own species or domesticate individuals of other species.

To successfully infiltrate the social order of others, a social parasite must utilize inherent sensory preferences of their host. This can be accomplished if the parasite can adequately mimic their host's sensory cues necessary for recognition (Howard et al. 2001). The termitophile *Trichopsenius frosti*, for example, biosynthesizes the same chemical signature as its termite host *Reticulitermes flavipes* (Howard et al. 1980). Alternatively, supernormal stimuli may release exaggerated levels of host response. The relatively large size and vocal mimicry of cuckoo chicks persuade their warbler host to deliver increased quantities of food (Davies et al. 1998). Similarly, larvae of the vespid social parasite *Polistes sulcifer* are able to solicit more food from workers of their host *P. dominulus* than host larvae can (Cervo et al. 2004).

Several theories have emerged to explain why host species have not ultimately evolved means of rejecting their social parasites. Social parasites may represent a relatively new selection pressure and hosts have not had the time necessary to evolve an appropriate defense (evolutionary lag). This may happen when a social parasite is introduced into a new geographical location (Brooke et al. 1998; D'Ettorre et al. 2004) or where parasites switch host species (Fischer-Blass et al. 2006). Parasite-host relationships

may then be locked in an evolutionary arms race (Dawkins and Krebs 1979) or a 'Red Queen' scenario (Van Valen 1973) in which the parasite is currently ahead (Foitzik et al. 2003; Fischer and Foitzik 2004). Some have proposed that parasites simply have too little impact for their hosts to evolve means of recognizing them as enemies (Gladstone 1981). Like physiological parasites, social parasites may have evolved to have less impact on their hosts (Brooker and Brooker 1996; Hare and Alloway 2001). More likely, however, host species have reached an evolutionary equilibrium in the stringency of their recognition mechanisms (Maynard Smith 1982). That is, the costs of accepting a social parasite are on average outweighed by the possibility of rejecting one's relatives.

Social Parasitism Among Ants

Within the Formicidae social parasitism is wide-spread, encompassing approximately 200 of the 9000 known species of ants (Buschinger 1986; Hölldobler and Wilson 1990). Social parasitism in ants can be grouped into four general categories — xenobiosis, temporary parasitism, inquilism, and dulosis (reviewed in Hölldobler and Wilson 1990). For the purpose of convenience and to adhere to convention, I have adopted this classification system, however, these groupings are somewhat ill-defined. Within each category, individual species exhibit much variation in the specifics of their parasitic adaptations. Also, there is considerable overlap in behaviours among categories.

Xenobiotic or 'guest' ants live within the colony of another species and freely intermingle with host workers. Commonly, xenobiotic ants obtain solid food from their hosts or, in more socially advanced species (e.g. *Formicoxenus provancheri*: Wheeler 1903, *F. nitidulus*: Buschinger 1976a, 1976b), solicit liquid food by trophalaxis from

their hosts. Most often, the brood is kept separate from that of the host (Hölldobler and Wilson 1990).

Temporary parasites are dependant on a host species for colony foundation but can otherwise care for themselves. In this system a newly mated queen gains access to a host colony either by force (Buschinger and Winter 1983; Buschinger et al. 1990) or by otherwise placating (e.g. *Polyergus breviceps*: Topoff and Zimmerli 1993) the host workers. The invader makes her way to the nest chamber, kills the resident queen and usurps her as the primary reproductive in the colony. She may kill the host queen directly (e.g. *Myrmicoxenus corsica*: Buschinger and Winter 1985) or persuade the resident workers to assassinate their own queen (e.g. *Temnothorax kutteri*: Allies et al. 1986; Franks et al. 1990). The host workers then care for the parasite's first brood. Once the brood eclose, a fully mixed colony exists. In time, however, with no reproductive of their own, the host population dwindles, leaving a pure colony of the temporary parasite (Hölldobler and Wilson 1990).

Inquiline ants are also reliant on other species for colony foundation and are permanent parasites having lost the ability to care for themselves (Hölldobler and Wilson 1990). Often these species consist exclusively of reproductives or the superfluous worker caste, if it still exists, has become degenerate (Hölldobler and Wilson 1990; Heinze and Alloway 1991). Inquiline ants are therefore completely reliant on their host species workers and spend their entire lifecycle in a host colony. Some inquiline queens murder host queens and produce reproductives only as long as the original host surplus lives (Queen Intolerant Inquilines: *Leptothorax goesswaldi,* Buschinger 1981; *Leptothorax wilsoni,* Heinze 1989b). In other species, inquilines do not eliminate the host queen but

live within the nest chamber with her and thus enjoy a constant renewal of host workers (Queen Tolerant Inquilines: e.g. *Pogonomyrmex colei*, Rissing 1983; *Plagiolepis xene*, Passera et 2001; *Temnothorax minutissimus*, Smith 1942; Buschinger and Linksvayer 2004). The inquiline *Teleutomyrmex schneideri* is so well adapted to its parasitic lifestyle that it has lost the worker caste and developed morphological, physiological and behavioural adaptations that promote complete dependence on their hosts (Gosswald 1953; Wilson 1971).

Within the Formicidae, the most extensively studied social parasites are the dulotic or 'slave-making' ants (Hölldobler and Wilson 1990). To varying degrees these ants have become dependant on host workers (Mori and Moli 1988; Stuart and Alloway 1985), which they keep as slaves. Obligatory slave-makers practice non-independent colony foundation similar to that observed in temporary parasites (Buschinger 1986; Stuart 2002). To supplement their host worker force, however, these ants raid the nests of other species, kill or displace adults, and abscond with worker brood (Alloway 1979). Upon eclosion, host workers augment the parasite's slave work force, performing usual worker functions including foraging, nest maintenance, colony defense, care for their captor's queen and brood and even accompany them on subsequent raids (Huber 1810; Wilson 1975 b; Alloway 1979; 1980; 1982; Buschinger et al. 1980). Several dulotic ants have become so specialized that they are completely reliant on their hosts for feeding and brood care (Wilson 1975b; Stuart and Alloway 1985).

Dulosis in ants can be classified further into three subcategories. Alloway (1980) postulated that these subcategories represent an ordered series of evolutionary stages towards advanced and obligatory social parasitism in some myrmicine slave-making

systems; however, this gradient towards a parasitic lifestyle is not necessarily representative of all slave-making ants. In its most rudimentary form, ant slavery occurs facultatively within a species. Following territorial conflicts between colonies, the victorious workers invade the defeated colony's nest-chamber and seize the brood (Alloway 1980). Some of the captured brood is allowed to eclose and is incorporated into their captor's work force. Because these ants (e.g. the honeypot ant *Myrmecocystus mimicus*: Hölldobler 1976; 1981) enslave members of their own species, there is little room for adaptations directed towards a slave-making lifestyle.

Facultative interspecific slavery is the next subcategory of dulotic ants. These ants can found and maintain colonies, forage, and rear young independent of a slave work force, but will occasionally capture brood of other species (e.g. *Temnothorax ambiguus*, *T. curvispinosus, T. longispinosus*: Wilson 1975; Alloway 1980). Their habit of enslaving other species has promoted the evolution of adaptations for a parasitic lifestyle. Across taxa facultative slave-makers exhibit varying degrees of specialization towards a parasitic lifestyle. The range of specialization is seen best within the sanguinary ants (*Formica sanguinea* group). This group of ants demonstrates the full range of parasitic adaptations from the accidental capture of brood following a territorial skirmish to highly organized raiding behaviour (Huber 1810; Hölldobler and Wilson 1990; Mori et al. 2001).

With the advancement of a slave-making lifestyle, some species have become specialized to the point of complete dependency on other species for colony function. The degree of specialization within obligatory slave-makers appears to be correlated with the degeneration of domestic abilities (Wesson 1939; Stuart and Alloway 1985; Mori and Moli 1988). While species such as *Harpagoxenus canadensis* and *H. sublaevis* can

expand their behavioural repertoire when deprived of slaves, other species including several species within the genera *Polyergus* and *Myrmoxenus,* and *Protomognathus americanus* have become so specialized for slave-raids that their 'normal' worker ant functions have atrophied (Creighton 1950; Marikovsky 1963; Wilson 1975; Buschinger and Winter 1983; Stuart and Alloway 1985). Obligatory slave-makers are the archetype of social parasitism, relying on host workers for colony foundation, brood care, foraging, and nest maintenance.

Evolution of Ant Slavery

Establishing a general theory for the evolution of slavery in ants is difficult because of the diversity of behavioural, morphological and physiological adaptations for slave-making among dulotic species. Convergent evolution of obligate dulosis has been documented in two phylogenetically distinct lineages of ants — Subfamilies Formicinae and Myrmicinae (Buschinger 1986; Hölldobler and Wilson 1990; Stuart 2002). It is believed that slave-making has evolved independently at least nine times, but recent molecular evidence suggests that evolution of a slave-making lifestyle may be much more frequent (Buschinger 1986; Hölldobler and Wilson 1990; Stuart 2002; Beibl et al. 2005). Slave-making has been observed in three genera in the Formicinae (*Formica spp.*; *Polyergus spp.*; and *Rossomyrmex spp.*) and eight genera in the Myrmicinae (*Chalepoxenus spp.*; *Myrmoxenus spp.* — formerly *Epimyrma,* Schulz and Sanetra 2002; *Harpagoxenus spp.; Protomognathus sp.* — formerly *Harpagoxenus; Stronylognathus* and *Temnothorax* — formerly *Leptothorax;* Huber 1810; Kutter 1969; Buschinger 1986; Wilson 1971; 1975b; Alloway 1980; Buschinger et al. 1980; 1981; 1983; Hölldobler and

Wilson 1990; Bolton 2003). Species within the myrmicine tribe Formicoxenini (formerly Leptothoracini, Bolton 2003) seem to be particularly pre-adapted for a socially parasitic lifestyle (Buschinger 1990; Heinze 1989a; Heinze and Alloway 1991).

A common theme observed in nearly all dulotic systems is that slave-makers hold a close phylogenetic relationship with their hosts ("Emery's rule": Emery 1909; Buschinger 1990; Heinze 1991; Baur et al. 1996). This trend is likely due to a parasite's need for hosts that can fulfill their nutritional and behavioural requirements. Although there has been convergence in many of the traits necessary for a parasitic lifestyle, the pathway by which ants came to it are not necessarily the same. Hypotheses addressing the evolution of obligatory slavery in ants must account for two aspects of their life history — slave-raiding and non-independent colony foundation. Three hypotheses have been advanced to explain slavery in ants but these hypotheses are by no means mutually exclusive.

Darwin (1859) was the first to develop a hypothesis to explain ant slavery. He proposed that slavery developed from species predisposed to looting neighbouring colonies nest chambers for food. Brood would be carried back to the raider's own nest and cannibalized. Where some brood eclosed before they could be eaten, the new adults then started working for their captors. Where this extra work proved more beneficial than the food from cannibalizing the larvae, the raiders evolved to allow more brood to reach adulthood. This hypothesis is unsatisfying in that it only seems to apply to species that are already predisposed for brood predation (Topoff and Zimmerli 1991; Mori et al. 2000). Further, this hypothesis does not address the origin of non-independent colony foundation.

The territoriality hypothesis states that slavery evolved as a byproduct of territorial interactions (Wilson 1971, 1975). Following conflict between neighbouring colonies, the victors apprehend the defeated colony's brood and carry it to their nest. If some of this captured brood remains uneaten long enough, they will mature in the alien nest. If these workers form a social attachment to their captors, selection might sanction continued maturation of captured brood (Alloway 1980; Stuart and Alloway 1982, 1983). In the genus *Myrmecocystus* territorial conflict leads to intraspecific slavery (Hölldobler 1976; Kronauer et al. 2003) and has been documented in laboratory studies of some *Temnothorax* species (Alloway 1980; Stuart and Alloway 1982; Alloway et al. 1991). Similar to Darwin's (1859) hypothesis, however, the territoriality hypothesis does not account for non-independent colony foundation and therefore cannot fully explain the evolution of slavery in ants.

It is currently held that the evolution of dulosis has its inception in polygynous (containing more than one queen) and polydomous (occupying more than one nest) species. The polygyny/polydomy hypothesis (Buschinger 1986) posits that non-independent colony foundation originated from the regular exchange of queens among multiple nests (Alloway et al. 1982) or from the return of recently mated gynes to their maternal colony (Heinze and Keller 2000). If a queen cannot find her maternal nest, she may attempt to access nests of unrelated conspecifics or closely related species. With time, selection would act to increase the queen's chances of establishing in the alien nest. Once non-independent colony foundation is established, behaviours involved in the exchange of brood among nests and raiding following territorial conflicts could be

refined to resemble slave-raiding behaviour (Elmes 1973; Buschinger 1990; Bourke and Franks 1991; D'Ettorre and Heinze 2001; Stuart 2002).

Recognition Among Ants

For cooperation and altruism of the type regularly observed in ant societies to evolve via natural selection, individuals must be able to discriminate nestmates from non-nestmates. Unity within an ant society depends heavily on kin selection, whereby altruistic acts are directed towards a relative, thus increasing the actor's own inclusive fitness (Hamilton 1964; Wilson 1975a). Lax recognition mechanisms leave species susceptible to exploitation by social parasites. For organisms generally (Holmes and Sherman 1983; Sherman and Holmes 1985) and social insects specifically (Hölldobler and Michener 1980), four mechanisms for discriminating kin from non-kin have been put forward —recognition alleles, spatial location, familiarity, and phenotype matching.

Hamilton (1964) originally suggested allelic recognition as a theoretical strategy for kin identification. Under this strategy, recognition of kin is innate and inherited rather than learned. Termed the "green beard effect" by Dawkins (1976; see also Crozier 1987a;), such a system would require genes that code for a recognition cue (label) along with the sensory network to perceive that cue in others and predispose bearers to behave altruistically towards other green beards in a true green beard system. Specific gene complexes have been shown to regulate queen recognition and social organization in *Solenopsis invicta* (Keller and Ross 1998; Krieger and Ross 2002; Ross and Keller 2002); however, this strategy is not likely realized in other species (e.g. *Temnothorax* spp.), as it would be inefficient in polygynous species and in species whose queens mate multiple

times. Also, most species exhibit plasticity in their recognition process to accommodate variation in the recognition signatures of colony members. Newly eclosed workers tend to be accepting of and are accepted by whatever adults they are exposed to early in life (Carlin and Hölldobler 1983; Alloway and Ryckman 1991; Stuart 1992).

Where kin are reliably found in certain locations, some species rely on spatial segregation for discrimination of relatives. Adult bank swallows (*Riparia riparia*) appear to identify newly hatched chicks as kin based solely on chicks being situated within their nest (Sargent 1962). Although nest sites may be recognized by geographical cues (Tinbergen and Kruyt 1938) and environmental cues may refine an individual's chemical signature (Singer and Espelie 1992), I know of no examples in ants where kin recognition depends on spatial segregation.

If relatives are predictably associated with a location over time, kin recognition may be accomplished through social familiarity (Waldman et al. 1988). Cross-fostering experiments demonstrate that previous association may be necessary (Holmes 1984) or sufficient to allow kin recognition (Holmes and Sherman 1982). Recognition by direct association is an unlikely strategy employed by ants. Results from previous studies that seem to demonstrate recognition by social familiarity (e.g. *Formica rufa*: Le Moli and Passetti 1977; 1978) are likely the consequence of another learning mechanism (see phenotype matching below). Indeed, recognition by direct familiarization would be especially limiting in species with heavily populated colonies. Further, ants are often amicable towards colony members that they have not previously encountered (Hölldobler and Wilson 1990). When species that are formerly aggressive towards conspecifics

experience severe genetic bottlenecks, colonies commonly intermingle and coalesce (Tsutsui et al. 2000; 2003).

The most likely mechanism underlying colony recognition in social insects involves phenotype matching (Holmes and Sherman 1982). Under a phenotype-matching mechanism individuals form a template of what phenotypic cues represent kin based on a reliable referent. This recognition template is then compared to the phenotype of encountered individuals. During this recognition process, ants likely focus on differences between the encountered phenotype and the template over similarities between the two (Lahav et al. 1999). In ant societies, queens, other workers, brood and environmental cues may contribute labels that contribute to a recognition template (Fielde 1905; Kalmus and Ribbands 1952; Blomquist and Jackson 1973; Crozier and Dix 1979; Jutsum et al. 1979; Hölldobler and Michener 1980; Buckle and Greenburg 1981; Carlin and Hölldobler 1983; 1986; 1987; Jaffe and Villegas 1985; Mintzer and Vinson 1985; Gamboa et al. 1986; Stuart 1987a; Bourke 1988; Obin and Vander Meer 1989; Provost 1989; 1991; Vander Meer et al. 1989; Alloway and Hodgson 1990; Howard et al. 1990; Singer and Espelie 1992; Heinze et al. 1996; Liang and Silverman 2000). Alternatively, individuals may use themselves as a referent ("self-referent phenotype matching"; Hauber et al. 2000; Mateo and Johnston 2000). While theoretically appealing, the limited empirical evidence for self-referent phenotype matching remains the subject of contention (Hare et al. 2003; Hauber and Sherman 2003). An early learning period during which individuals form a template of what constitutes kin may be necessary for discrimination (Jaisson 1975; Le Moli and Passetti 1977; Le Moli 1980; Alloway and Ryckman 1991). Recognition templates should be less exacting as heterogeneity of genetic and

environmentally derived discriminator substances increases (Reeve 1989). In colonies that occupy multiple nests (polydomy), with multiple queens (polygyny) that mate multiply (polyandry), selection would favour a broader acceptance of what constitutes a colony member (Hölldobler and Michener 1980; Brandt et al. 2005).

In insects, chemoreception is the primary sensory channel mediating kin recognition (Breed and Bennett 1987; Howard and Blomquist 2005). Within Hymenoptera, it is widely accepted that hydrocarbons located on the cuticle are important discriminator substances (Wilson 1971; Carlin and Hölldobler 1986; Breed and Bennett 1987; Howard 1993; Espelie et al. 1994; Lorenzi et al. 1996; Lahav et al. 1999) and that these chemicals are detected by direct contact (Wilson 1965; Walsh and Tschinkel 1974; Hölldobler and Michener 1980). Hare (1996) summarized that studies of recognition in Hymenoptera reveal that: 1) variation in the hydrocarbon profiles among nests is greater than that within nests (Vander Meer et al. 1989; Page et al. 1991); 2) social discrimination among individuals correlates with differences in their cuticular hydrocarbon profiles (Obin 1986; Bonavita-Cougourdan et al. 1987; Morel and Vander Meer 1987; Nowbahari et al. 1990; Espelie et al. 1994; Foitzik et al. 2007); and 3) social parasites have similar hydrocarbon profiles as their hosts (Howard et al. 1980; Vander Meer and Wojcik 1982; Vander Meer et al. 1989; Franks et al. 1990; Howard et al. 1990; Yamaoka 1990; Bagnères et al. 1991; Kaib et al. 1993; Lenoir et al. 2001; Brandt et al. 2005). Removal of hydrocarbons precludes nestmate recognition, whereas addition of cuticular hydrocarbons reinstates discrimination (Bonavita-Cougourdan et al. 1987; Clément et al. 1987; Signer and Espelie 1992; Hare 1996; Lahav et al. 1999).

Ant societies with an open social structure provide an opportunity to explore the evolution of the mechanisms underlying chemical communication. Several studies have focused on recognition among adults, pupae or eggs (Greenberg 1979; Breed 1981; Alloway 1982; Getz and Smith 1983; Pfenning et al. 1983; Stuart 1987a; 1987c, Carlin 1988; Gamboa 2004; Johnson et al 2005). Of particular interest, however, is the ability of workers to discriminate among larvae (Hare and Alloway 1987; Carlin 1988; Alloway and Hare 1989; Hare 1996). Larvae represent a critical resource to a colony in future workers and reproductives and require more care and attention than pupae and eggs (Brian 1975). Sophisticated levels of brood care have only been realized in advanced insect societies and may represent an essential element in the evolution of their social organization (Wilson 1971). Larvae evoke a more pronounced reaction in *Myrmica rubra* workers than pupae (Brian 1975), perhaps owing to their direct behavioural responses to workers given their feeding, and are more commonly accepted in allospecific colonies than adult workers (Bagnères et al. 1991). Larvae serve important trophic functions by contributing to the 'collective gut' of a colony and are major contributors to colony recognition signatures (Hölldobler and Wilson 1990). Larvae are also the life-stage that workers are most reliably exposed to early in life (Wilson 1971). Finally, knowledge of larval recognition is critical for understanding of social parasitism. The mechanisms by which immature stages of parasites deceive their hosts remain a poorly understood phenomenon.

Hare and Alloway (1987) discovered that *T. ambiguus* and *T. longispinosus* workers reared in the presence of conspecific larvae preferentially accept conspecific larvae over larvae of the other species. In a similar bioassay, Alloway (1982) found that

both species preferentially accept conspecific over allospecific pupae. In both studies, workers adopted congeneric brood but to a lesser degree, suggesting preferential rather than exclusive brood acceptance (Alloway 1982; Hare and Alloway 1987). Also, *T. longispinosus* workers tended to be more accepting of *T. ambiguus* brood than visa versa, possibly indicating that *T. ambiguus* have more exacting recognition mechanisms (Alloway 1982; Hare and Alloway 1987). While exposure to conspecific brood early in life seemingly activates a later preference for conspecifics, exposure to allospecific brood or social isolation results in non-preferential brood acceptance (Hare and Alloway 1987). Hare and Alloway (1987) hypothesized that this early learning mechanism could cause these species to be especially susceptible to social parasitism. In a later study Alloway and Hare (1989) found that *T. longispinosus* workers preferentially adopt larvae of the slave-making ant *P. americanus* regardless their early exposure regime (i.e. exposure to conspecific larvae, *P. americanus* larvae or social isolation). These results suggest that *P. americanus* larvae posses some element (likely a brood pheromone) that is highly attractive to *T. longispinosus* workers (Alloway and Hare 1989).

Prelude to Experiments

In this thesis I report results of experiments investigating larval recognition in *Temnothorax* (formerly *Leptothorax*, Bolton 2003) *longispinosus* Roger and *T.* (formerly *Leptothorax*, Bolton 2003) *ambiguus* Emery. I also explore the chemical mechanisms underlying larval recognition in these species. The polygynous/polydomous lifestyle of *T. longispinosus* and *T. ambiguus* makes these species interesting subjects for nestmate recognition studies, owing to the presumably increased variation in genetic and

environmentally derived discriminator substances (Alloway et al. 1982; Del Rio Pesado and Alloway 1983; Stuart 1985; Herbers 1986). Further, both species along with the closely related *T. curvispinosus* are hosts to the slave-making ants *Protomognathus americanus* (Creighton 1927, 1929, 1950; Sturtevant 1927; Buschinger and Alloway 1977; Alloway 1979; Alloway 1980; Alloway et al. 1982; Alloway and Del Rio Pesado 1983; Foitzik and Herbers 2001b), *Temnothorax duloticus* Wesson (Wesson 1937; 1940; Talbot 1957; Wilson 1975; Alloway 1979; 1980) and the currently undescribed *T. pillagens* (Alloway 1997; Herbers and Foitzik 2002; Alloway personal communication) and are facultative slave-makers themselves (Stuart and Alloway 1982). The experiments described in Chapter 1 examine the ability of *T. longispinosus* and *T. ambiguus* workers to differentiate nestmate from non-nestmate larvae and explores the nature of the cues underlying larval recognition. In Chapter 2 I test whether workers discriminate larvae at the species level. Finally, in Chapter 3, I describe the results of experiments designed to explore the ability of *T. longispinosus* and *T. ambiguus* to recognize slave-maker larvae and test whether chemical cues underlying brood recognition transfer among larvae.

T. longispinosus are black ants that are found throughout the Eastern United States and Southern Ontario (Wesson 1940; Headley 1943; Creighton 1950; Talbot 1957; Alloway 1980; Herbers and Foitzik 2002). *T. ambiguus*, are light-orange and occupy areas surrounding and North of the Great Lakes and have been reported as far west as St. Malo, Manitoba (Wesson 1940; Headley 1943; Creighton 1950; Talbot 1957; Alloway 1980; Wheeler et al. 1989). Adults of both species are small (2-3 mm long) and commonly nest in hollow twigs, under tree bark, in preformed rock cavities, and in old *Carya ovata* and *C. cordiformes* hickory nuts and *Quercus alba* and *Q. rubra* acorns that

have previously been hollowed out by Coleoptera or Lepidoptera larvae (Creighton 1927; 1950; Sturtevant 1927; Buschinger and Alloway 1977; Alloway et al. 1982). Typically, individual nests contain between 10 and 100 workers and zero and four queens (Alloway et al. 1982; Del Rio Pesado and Alloway 1983). *T. longispinosus* queens may establish colonies individually (haplometrosis) or with other queens (pleometrosis), while *T. ambiguus* queens found colonies only pleometrotically (Alloway et al. 1982; Foitzik et al. 2004).

GENERAL METHODS

Collection and Culturing

A total of 825 acorn (*Quercus alba*) and hickory nut (*Carya ovata*) ant nests was collected from various sites in Peel and Halton Counties of southern Ontario during 6-10 October 2006 and 2-7 May 2007. GPS coordinates of the 19 collection sites were recorded using a Garmin GPS72 (Garmin Ltd. Kansas City, KS, U.S.A.). In the field, nuts were partially opened to confirm the presence of the desired species, and then sealed in individual plastic bags until cultures were established in the lab. The precise species composition of the nest was not known, however, until the nuts were reopened in the lab. The collection included 250 nests comprised solely of *T. longispinosus*, 484 purely *T. ambiguus* nests, 18 nests with a mix of *T. longispinosus* and *T. ambiguus,* 51 nests with at least one *P. americanus* worker present, and 22 other species nests. Of the 51 *P. americanus* nests, 12 had only *T. longispinosus* slaves, 9 had only *T. ambiguus* slaves and 30 had a mix of both slave species. The ants collected in October were left in their natural nests and overwintered in a cooler located in an insulated garage from 13 January to 27 March 2008. Temperature, measured every five days, within the cooler ranged from -8 to 5 °C (mean ± SE: -1.85 ± 3.30 °C). Nests collected in May did not require vernalization but were refrigerated at approximately 4 °C for transportation to the University of Manitoba prior to experimentation. Because *T. longispinosus* and *T. ambiguus* are facultatively polydomous and *P. americanus* is likely polydomous (Alloway et al. 1982; Del Rio Pesado and Alloway 1983), individual nests cannot be deemed separate colonies. Consequentially, I refer to ants collected from an individual nest as a culture. For

experimental purposes I assumed that cultures were unrelated only when collected from different sites (Hare 1996).

Ants were removed from their acorn/hickory nuts by gently tapping on the outside of opened nuts with forceps over large plastic culture dishes (150 mm diameter, 25 mm depth). Along with providing an area in which the ants could forage, these culture dishes served as experimental arenas. Inside each culture dish, I placed an artificial nest (see Alloway 1979) consisting of an opaque green plastic dish (32 mm diameter, 8 mm depth) with a transparent orange Plexiglas^{TM} lid. The lid had a grove along the periphery allowing the lid to snap firmly into place. Because insect photoreceptors are relatively insensitive to longer wavelengths in the visible spectrum (Rossel 1989) the transparent orange lid allowed ready viewing of cultures leaving the ants undisturbed. A small hole (approximately 1 mm diameter) bored into the side of the base served as the only access point into the nest. Colonies were fed a diet consisting of water, agar, honey, hen egg, and a multivitamin (Bhatkar and Whitcomb 1970) three times a week. Because it is unresolved whether this diet is sufficient for normal brood production (Buschinger and Pfeifer 1988; Alloway et al. 1991), I supplemented each experimental culture's diet with five frozen *Drosophila melanogaster* (variety Canton S) each week. Within each culture dish, ants had *ad libitum* access to water via a cotton-stoppered bottle (15 mm diameter, 50 mm height). Cultures were maintained at approximately 23°C on a 14-hour light : 10-hour dark cycle (light from 07:00 to 21:00 CDT).

General Experimental Protocol

I used clean camel-hair brushes to handle larvae and larval baits during experimentation. A camel-hair brush is ideal for transporting ant brood because the bristles are stiff enough to easily pick up larvae but pliable enough to not puncture the cuticle. Different brushes were used for larvae from separate cultures and following each use, brushes were soaked in 85% ethanol followed by distilled water and thoroughly dried. For each experiment I employed unique cultures of ants so that cultures weren't made familiar with larvae or larval baits in the context of earlier testing. Only larvae originating from queenright nests were used in experimentation.

Statistical Analyses

All statistical analyses were done on a Macintosh™ computer using Statview 5.01 (SAS Institute; Cary, NC, U.S.A.). For all data sets, I performed Kolmogorov-Smirnov tests to determine whether data were drawn from a normally distributed population of differences and equality of variances *F*-tests (F-max) to establish whether error variances were homogeneous across treatments. Whenever these parametric assumptions were not met and a subject culture received all possible treatments, I used Wilcoxon signed-ranks tests when evaluating the effects of the main independent variables. When comparisons called for non-parametric unpaired tests, I used Mann-Whitney *U* tests. If parametric assumptions of normality and homogeneity of variance were met, I used analyses of variance (ANOVA) for comparisons with greater than one factor or where independent variables had more than one group. Single factor binary comparisons were carried out

using paired or unpaired t-tests. Except where stated differently, differences were considered significant where $P \leq 0.05$.

Chapter 1: Nestmate recognition in the ants *Temnothorax longispinosus* and *T. ambiguus*

INTRODUCTION

Social insects take altruism to extremes where members of a non-reproductive worker caste sacrifice personal reproductive output to promote the reproductive effort of a few reproductive individuals (Hölldobler and Wilson 1990). The cooperation commonly observed in insect societies can be explained by inclusive fitness theory (Hamilton 1964; Wilson 1975a), but could also be maintained by factors independent of kin selection (Jaisson 1991; Wilson and Hölldobler 2005). Apparent altruism may develop as a byproduct of mutualism (Eberhard 1975), if altruistic acts enhance social status (Zahavi 1995), or from benefits that accrue via reciprocity (Trivers 1971). Regardless, where the coefficient of relatedness within a colony is greater than zero (e.g. *Temnothorax ambiguus, T. longispinosus* — Herbers and Grieco 1994), cooperation among nestmates constitutes a form of nepotism. Preferentially directing care towards nestmates increases a worker's inclusive fitness but requires robust recognition mechanisms whereby individuals competently differentiate nestmate from non-nestmate conspecifics. Discrimination of nestmates from non-nestmates would also prove adaptive from a colony fellowship perspective (Jaisson 1991; Wilson and Hölldobler 2005). Determining the mechanisms of nestmate recognition and discrimination remains a central issue in the study of eusocial insect behaviour (reviewed in Hölldobler and Michener 1980; Carlin 1988; Gamboa 2004).

Nestmate discrimination has been documented in several Hymenopteran species (Greenberg 1979; Breed 1981; Getz and Smith 1983; Pfenning et al. 1983; Stuart 1987a, 1987c; Carlin 1988; Gamboa 2004). *T. curvispinosus* workers isolated as pupae are accepted by their maternal colony but are commonly attacked and killed when introduced into a non-related conspecific nest (Stuart 1987b). *T. ambiguus* discriminate between nestmate queens and non-nestmate queens (Alloway and Ryckman 1991). *T. longispinosus* workers, meanwhile, discriminate among conspecific brood and prefer nestmate over non-nestmate larvae (Hare 1996). Typically, nestmate recognition is indicated by aggression towards foreign intruders or by the preferential transport of nestmate brood (Lenoir 1981; Carlin 1988; Hare 1996; Lenoir et al. 1999). Because brood encountered outside the nest tends to be carried into the nest by workers fairly quickly (reviewed in Carlin 1988), brood recognition can easily be demonstrated using an assay of this type. Alloway and Hare (1989) even report that the order in which *T. longispinosus* workers retrieve larvae into their nest correlates with the relative long-term survival of larvae.

Levels of discrimination may not end at the colony-member/ non-colony-member level. Individuals may discriminate among workers based on relatedness even within the colony, preferentially directing altruistic behaviour towards closer relatives (Wilson 1974; Getz and Smith 1983; Visscher 1986; 1987). Tenable examples of discrimination based purely on relatedness in ants, however, are uncommon and recognition systems founded on kinship have likely expanded to include all nestmates ("fellowship hypothesis"; Jaisson 1991). In general, the ability to recognize brood would facilitate efficient transport, feeding and grooming and therefore prove adaptive (Carlin 1988).

Ants tend to act aggressively towards unfamiliar adults, but are more accepting of unfamiliar brood (Bagnères et al. 1991). Foreign larvae and pupae are often accepted in conspecific nests and occasionally received in allospecific nests (Elmes and Wardlaw 1982; Alloway and Hare 1987; Carlin et al. 1987). In nature, unfamiliar brood may be incorporated into a colony following a slave-raid or territorial conflict (Wilson 1975b; Alloway 1980; Buschinger 1986). Captured brood provides obvious benefits to a colony as an immediate food resource or as a future supplement to the work force. We might then expect ants to have evolved sophisticated brood-recognition abilities that allow workers to bias care towards original nestmates.

The experiments described in this chapter were designed to further our understanding of brood-recognition in the ants *T. longispinosus* and *T. ambiguus*. In particular, I tested the hypothesis that these ants can differentiate nestmate from non-nestmate larvae. I endeavored to corroborate Hare's (1996) finding that *T. longispinosus* discriminate nestmate from non-nestmate larvae and to test for the first time *T. ambiguus* worker's ability to discriminate among conspecific larvae. Previous studies concerning the larval discrimination abilities of *T. longispinosus* and *T. ambiguus* workers have shown that they prefer conspecific to allospecific larvae (Hare and Alloway 1987).

Beyond overall larval preference, it is possible to identify the importance of individual sensory channels in larval recognition. An organism's perception of the world is limited to the senses it possesses and it only attends to signals that are biologically relevant (Uexküll 1909). Because the inside of a *T. longispinosus* and *T. ambiguus* nest is devoid of light (Creighton 1927; Sturtevant 1927; Buschinger and Alloway 1977), larval

recognition cannot be achieved through visual stimuli. Auditory stimuli are also unlikely to contribute to larval recognition because Formicidae larvae lack the necessary 'drumming' or stridulatory sound-producing organs sometimes found in adults (Markl 1965; Markl et al. 1977; Stuart and Bell 1980). Similarly, I doubt that behavioural cues add much to larval recognition (but see Le Masne 1953). While some social insects utilize certain actions to solicit trophalaxis from colony members (O'Neal and Markin 1973; Lenoir 1982; Bonavita-Cougourdan 1983) and for recruitment ('tandem running': Wilson 1959; Hölldobler 1971; Hölldobler et al. 1974; Stuart 1986), the importance of behavioural cues to over-all recognition appears limited (Brian 1975; Hölldobler and Wilson 1990). When presented with a dead or anesthetized alien individual, workers often violently attack the alien, but will accept the corpse of a former nestmate (Walsh and Tschinkel 1974; Bonavita-Cougourdan et al. 1987). Discrimination of larvae by *T. longispinosus* in particular appears to be independent of any behavioural cues in that *T. longispinosus* workers do not preferentially accept unanaesthetized over anaesthetized larvae (Hare 1996). Recognition must therefore be achieved through surface chemicals detected by direct contact (Hölldobler and Michener 1980), pheromones released by glands (Visicchio et al. 2001), or by tactile cues such as larval shape, or surface texture (Brian 1968, 1975).

Most social insects appear to discriminate colony members from non-colony members based on chemicals borne on the cuticle (Carlin and Hölldobler 1986; Breed and Bennett 1987; Howard 1993; Espelie et al. 1994; Lorenzi et al. 1996; Lahav et al. 1999; Howard and Blomquist 2005). Structural features including hairiness, turgidity and overall shape likely contribute only to subtly refine the discrimination process (Brian

1975). Therefore, along with testing the degree to which *T. longispinosus* and *T. ambiguus* can differentiate among whole larvae, I used larval-sized pieces of silicone ('baits') left in contact with larvae as models to test the degree to which they can discriminate nestmate larvae from unrelated conspecific larvae based on contact pheromones alone. The use of baits in this way does not in and of itself eliminate the possibility that volatiles contribute to larval recognition. That said, the similar treatment of anesthetized and non-anesthetized larvae (Hare 1996) and the considerable amount of antennal contact workers direct towards baits treated with cuticular extract (Bigley and Vinson 1975), certainly suggests the predominance of contact pheromones in the recognition process. Silicone baits have previously been shown to absorb attractive chemical substances borne on the cuticle of *T. longispinosus* larvae (Hare 1996). Other studies have employed filter paper left in contact with individuals or imbibed with cuticular extract in similar behavioural assays (Bigley and Vinson 1975; Watkins and Cole 1966; Glancey 1970). By presenting larval models instead of actual larvae, differences in ant behaviour towards baits can be attributed to differences in chemical substances alone and avoid potential influences of other variables.

Hare (1996) demonstrated that *T. longispinosus* workers spent more time in antennal and oral contact with larval-sized pieces of silicone that had previously contacted nestmate larvae than untreated silicone baits. These results show that chemical cues borne on the larval cuticle are sufficient to promote inspection by nestmate workers and are likely sufficient to allow larval discrimination, but several unanswered questions remain. It is unknown how long these baits must be in contact with larvae before adults preferentially attend and retrieve them over untreated baits. Homogeneity of a brood

odour may be maintained at least in part through direct physical contact; however, Hare (1996) suggested that transfer of discriminator substances through direct contact may be insufficient to completely overwhelm a larva's own recognition signature. Given that initial acceptance of slave-maker queens by host colonies and acceptance of unrelated larvae/ pupae by slaves in an established slave-maker colony may be facilitated by contact with brood (Wheeler 1903; Viehmeyer 1908; Wasmann 1908; Hölldobler and Wilson 1990; Crozier and Dix 1979; Franks et al. 1990; Hare 1996; Elgar and Allan 2004), it is also important to understand the time necessary for the transfer of chemical discriminator substances to take place. Understanding the time taken for the attractive material to be transferred provides a preliminary understanding of the nature of the chemicals involved. To determine the time necessary for transfer of any recognition chemical, I measured the response of workers from *T. longispinosus* and *T. ambiguus* cultures presented with silicone baits left in contact with larvae for different periods of time to untreated baits. Understanding the time necessary for chemical transference also sets a practical precedent for future assays. I also presented cultures of *T. longispinosus* and *T. ambiguus* with larval models that had been left in contact with nestmate larvae and models left in contact with unrelated conspecific larvae to explore the importance of contact pheromones in nestmate brood-recognition.

METHODS

Nestmate Recognition

To determine whether *T. ambiguus* and *T. longispinosus* discriminate among conspecific larvae, I performed a behavioural assay similar to that reported in Hare (1996). I presented *T. longispinosus* and *T. ambiguus* cultures with a coverslip containing larvae extracted from their own nest (nestmate: NM), a coverslip containing conspecific larvae from a nest collected at the same site as the subject culture (non-nestmate: NNM) and a coverslip with conspecific larvae derived from a nest collected from a different site of origin (other site: OS). Approximately one hour prior to experimentation the food slide and water bottle were removed from a subject culture dish. Removal of these visual obstructions allowed tracking of larvae during experiments. 10 NM, 10 NNM and 10 OS larvae were then removed from queenright nest chambers. All 10 larvae of each type were removed from the same nest. Separate camel-hair brushes were used to extract larvae from each nest. The 10 larvae of each type were transferred to separate clean glass coverslips (18 mm x 18 mm) and arranged so that larvae were proximate to one another but not touching. For the hour preceding experimentation, each larvae-containing coverslip was stored in an isolated artificial nest along with a moist piece of cotton to prevent brood desiccation. Within each replicate, the 10 larvae of each type were matched as closely as possible by size to avoid biased retrieval order based on size or life stage (Lenoir 1981).

At the onset of experimentation, the larvae containing coverslips were simultaneously placed in a subject culture dish adjacent to one another and five cm from the nest entrance. The relative position of the three coverslips was randomized among

subject cultures (using a random numbers table). The culture dish was then placed under a dissecting microscope (Wild model M3, Wild Leitz Ltd. Ottawa, ON, Canada) at 64x magnification, so that worker behaviour could be easily observed. So as not to disorient subject workers, I did not use an additional light source while using the microscope throughout this or any other experiment described in this thesis. I recorded the order in which each larva was retrieved into the nest entrance along with the time from presentation to retrieval. I also noted any occurrences of larval cannibalism and instances of larvae being abandoned in a refuse pile outside the nest (midden). Observations ceased after all offered larvae were taken into the nest chamber, abandoned in a midden, or cannibalized. Because an assistant was not available, observations were not blind due to difficultly in establishing a self-blind protocol. Larvae were then assigned an ordinal number between 1 and 30 depending on their retrieval order, where 1 denotes the first and 30 the last larva retrieved (as in Hare 1996). Abandoned or cannibalized larvae were given an ordinal value equal to the mean ordinal value of all unretrieved larvae. For example, if two larvae were cannibalized, they were both assigned ordinal values of 29.5 [(29+30)/2]. The mean ordinal values were then calculated for each larval type. This process was repeated from 26 October to 3 November 2007 on 16 *T. ambiguus* cultures from 9 collection sites containing between 35 and 168 workers (mean ± SE: 86.31 ± 11.527) and from 27 to 30 November 2007 on 16 *T. longispinosus* cultures from 8 collection sites containing between 25 and 196 workers (mean ± SE: 71.94 ± 11.722). A careful census of subject cultures was performed on the same day of experimentation. I recorded the number of eggs, larvae, pharate pupae, worker pupae, male pupae, adult workers, adult males, alate gynes, dealate gynes, gynadromorphs (individuals with both

male and female characteristics) and ergatogynes (individuals with intermediate morphology between a queen and a worker) in each culture. Table 1-1 summarizes colony demographics of both species.

I examined differences among mean retrieval scores of the three larval types using non-parametric tests because for some comparisons between treatment types, my data were not drawn from a normally distributed population of differences (Kolmogorov-Smirnov tests) and error variances were not homogeneous across treatments (equality of variances *F*-tests). Table 1-2 summarizes the results of Kolmogorov-Smirnov and equality of variances *F*-tests among the three larval types for both species. I tested for differences among mean retrieval scores of NM, NNM and OS larvae using Wilcoxon signed-ranks tests. I also tested for differences in retrieval scores among the three larval types for each replicate using Wilcoxon signed-ranks tests. The probability values associated with contrasts among the mean ranks of the different larval types were adjusted using the sequential Bonferoni technique to avoid inflation of type I error (Rice 1989). For each species, I tested whether the number of larvae consumed or abandoned differed across the three larval types using a chi-square test on a 3 X 2 contingency table. Mann-Whitney U tests were used to determine whether the number of eggs, larvae, pharate pupae, worker pupae, male pupae, adult workers, adult males, alate gynes, dealate gynes, gynadromorphs and ergatogynes differed between species.

Control Assay

A potential problem concerning the methods described above is that chemical marking or trail laying could affect the retrieval behaviour of workers and therefore

retrievals within each replicate may not be independent of one another. While *T. longispinosus* and *T. ambiguus* recruit nestmates to foraging sites and territorial interactions by tandem running and not by laying chemical trails (Möglich 1978), I cannot offhandedly discount the possibility of chemical recruitment and the bias that such a mechanism would impose. Because of the chance that the order in which larvae were retrieved could have been due to the segregation of larvae among coverslips and not due to the differences between larval types, I performed a control assay using only unrelated (collected at least 0.5 km away from subject culture) conspecific larvae. Except where noted, methods mirrored those described above. Four coverslips containing five conspecific larvae each were presented simultaneously to a subject nest. Within each replicate, all larvae were extracted from the same nest. I then recorded the order in which larvae from each coverslip were retrieved into the subject nest and assigned each larva an ordinal rank depending on order of retrieval. This procedure was repeated using 10 *T. ambiguus* cultures collected from seven sites, containing between 24 and 89 workers (mean ± SE: 45.00 ± 6.862) and 10 *T. longispinosus* cultures collected from six sites, containing between 19 and 49 workers (mean ± SE: 31.40 ± 3.40) from 3 to 10 December 2007. Because the parametric assumptions of normality (Kolmogorov-Smirnov tests; Table 1-6) and homogeneity of variance (equality of variances *F*-tests; Table 1-6) were met, I used single factor analyses of variance (ANOVA) to test for differences in the mean retrieval scores of larvae among the four coverslips for each species.

Transfer of Conspecific Recognition Cues

To determine whether workers were attracted to chemical cues present on the larval cuticle and the time necessary for transfer of these cues by direct contact, I presented *T. longispinosus* and *T. ambiguus* cultures with larval-sized pieces of silicone ('baits') that had been left in contact with conspecific larvae for different periods of time. Ten *T. longispinosus* subject cultures collected from 10 sites and containing between 46 and 211 workers (mean ± SE: 110.60 ± 15.77) and 10 *T. ambiguus* subject cultures collected from 10 sites and containing between 32 and 227 workers (mean ± SE: 76.50 ± 17.508) were employed in this experiment. Treatment of baits involved positioning five late-instar larvae, extracted from the same nest, against the bait so that one larva contacted each exposed side of the bait. Larvae were removed from their maternal nests with a clean fine-tipped camel-hair brush and left in contact with a bait in an isolated nest. Care was taken to ensure that the brush used to handle the larvae did not directly contact the bait. To avoid brood desiccation, a moist piece of cotton was placed inside the nest. Silicone baits were exposed to either *T. longispinosus* or *T. ambiguus* larvae for 10 min, 1 hr, 4 hrs, 8 hrs or 16 hrs. Hare (1996) previously demonstrated that 16 hrs is sufficient for transfer of attractive chemical cues for *T. longispinosus* under this protocol.

At the onset of experimentation, I placed a clean glass coverslip (18 mm X 18 mm) containing a silicone bait that had been left in contact with conspecific larvae and an untreated silicone bait two cm outside the nest entrance. I visually inspected baits under a microscope and removed any particulate matter using a clean camel-hair brush. The relative positions of the two baits on the coverslip were randomized and each was placed approximately 0.5 cm apart from the other using separate camel-hair brushes. The

subject culture dish was then placed on the stage of a dissecting microscope (Wild model M3, Wild Leitz Ltd. Ottawa, ON, Canada) at 64x magnification for observation. Observations lasted five min, during which time I recorded the time that workers contacted each bait with their maxilla, maxillary palps, mandibles or antennae (see Hare 1996). I am basing this assay on the assumption that workers recognize chemical cues by taste (mouth parts) or smell (antennae) (Wilson 1958; Hölldobler and Wilson 1990). I also recorded any instances where a bait was retrieved into a nest. Following five minutes, the coverslip was removed and two similarly treated baits were introduced on a new coverslip. Five such presentations were given to each culture. The mean time of oral or antennal contact and the total number of retrievals were used in analyses. Each subject culture received a set of presentations like this using baits that had contacted conspecific larvae for 10 min, 1 hr, 4 hrs, 8 hrs and 16 hrs (total of 25 presentations per culture). The order that each of these contact times were tested was randomized (using as random numbers table) and no culture received more than one set of presentations within 24 hours. Presentations took place from 13 to 29 July 2007. To avoid bias due to relatedness or differential familiarity, baits were treated with larvae derived from queenright nests collected at sites other than that from which the subject culture was derived. I censused all subject cultures on 1 and 2 August 2007 upon completion of the trials (see Table 1-7).

For both *T. longispinosus* and *T. ambiguus* I tested for differences in the time workers antennated or orally contacted treatment and control baits using Wilcoxon signed-ranks tests. This non-parametric test was used because the parametric assumptions of normality of differences and homogeneity of variance were not met. Kolmogorov-Smirnov tests revealed that the data for *T. longispinosus* ($\chi^2 = 21.16$;

$P < 0.001$; $N = 50$) and *T. ambiguus* ($\chi^2 = 23.04$; $P < 0.001$; $N = 50$) were not drawn from a normally distributed population of differences. Equality of variances *F*-tests showed that error variances were not homogeneous for *T. longispinosus* ($F = 0.117$; $P < 0.001$; $N = 50$) and *T. ambiguus* ($F = 0.015$; $P < 0.001$; $N = 50$) data. For both species, I tested whether the number of baits carried into nests differed with treatment using chi-square tests on a 2 X 2 contingency, with the Yates correction for continuity.

I tested for differences in the amount of time each species contacted treated baits that had been left in contact with larvae for 10 min, 1 hr, 4 hrs, 8 hrs and 16 hrs using a single factor ANOVA where the amount of time that larvae were left in contact with larvae was the independent variable. For these tests the parametric assumptions of normality (Kolmogorov-Smirnov tests; Table 1-8) and homogeneity of variance (equality of variances F-tests; Table 1-8) were met. I justify omitting the contact times of control baits from analyses because the same control was presented in all time periods and these data may increase the variance of contact time to the point that differences among treatments were no longer detectable. Lastly, I tested for differences in the amount of time *T. longispinosus* and *T. ambiguus* contacted treated baits using a non-parametric Mann-Whitney *U* test. The data for this comparison were not drawn from a normally distributed population of differences ($\chi^2 = 7.840$; $P = 0.040$; $N = 50$) and variances were heterogeneous between species ($F = 5.173$; $P < 0.001$; $N = 50$). The number of eggs, larvae, pharate pupae, worker pupae, male pupae, adult workers, adult males, alate gynes, dealate gynes, gynadromorphs and ergatogynes of both species were compared using Mann-Whitney U tests.

Chemical Influence on Nestmate Recognition

To determine whether chemical cues present on the larval cuticle are sufficient to promote nestmate recognition, I presented cultures of *T. longispinosus* and *T. ambiguus* with silicone baits treated with nestmate larvae and baits treated with unrelated conspecific larvae. Except where noted, methods were similar to those reported above. Baits were left in contact with larvae for 16 hours, as time of exposure to conspecific larvae did not affect contact time and this proved to be the most convenient time for experimental purposes, since baits could be established with treatment larvae in the afternoon, and then be ready for testing the next morning. Further, only one pair of baits was presented to each subject culture.

16 hours prior to experimentation, five larvae were removed from the subject nest and were placed in direct contact with a larval-sized piece of silicone. At this time, five conspecific larvae were also removed from a nest collected from a different site from that of the subject culture and were placed in contact with another silicone bait. Following 16 hours, the larvae were removed from the pieces of silicone and both baits were randomly arranged (using a random numbers table) in the middle of a clean glass coverslip. I placed the coverslip in the subject culture dish two cm from the nest entrance. Over the next five minutes I recorded the total time that workers spent contacting each bait with their antennae, mandibles, maxilla and maxillary palps along with any cases where a bait was carried into a nest. This process was repeated using 15 *T. longispinosus* cultures collected from six sites and 15 cultures of *T. ambiguus* collected from 11 sites. The *T. longispinosus* cultures contained between 29 and 143 workers (mean ± SE: 65.07 ± 9.15) and the *T. ambiguus* cultures contained between 30 and 154 workers (mean ± SE: 83 ±

11.78). This experiment took place from 2 to 6 October 2007 and a census of each subject culture was conducted the same day as experimentation (Table 1-9).

I compared the time that each species spent in contact with nestmate baits and non-nestmate baits using Wilcoxon signed-ranks tests. The data from *T. ambiguus* tests met the assumptions of normality (Kolmogorov-Smirnov: $\chi^2 = 1.200$; $P > 0.999$; $N = 15$) and homogeneity of variance (equality of variances *F*: $F = 1.255$; $P = 0.676$; $N = 15$). The data from *T. ambiguus* tests met the assumption of normality (Kolmogorov-Smirnov: $\chi^2 = 1.286$; $P > 0.999$; $N = 15$), but not homogeneity of variance (equality of variances *F*: $F = 4.448$; $P = 0.011$; $N = 15$). I also compared the time that *T. longispinosus* contacted baits to the time *T. ambiguus* spent contacting baits using a Mann-Whitney *U* test, because not all of the parametric assumptions were met (Kolmogorov-Smirnov: $\chi^2 = 13.936$; $P = 0.002$; $N = 30$, equality of variances *F:* $F = 0.511$; $P = 0.081$; $N = 30$). I used a chi-square test on a 2 x 2 contingency table, with the Yates correction for continuity, to determine whether the total amount of baits carried into nests differed across nestmate and non-nestmate treatments. Mann-Whitney U tests were used to determine whether colony demographics differed between species.

RESULTS

Recognition of Nestmate Larvae

Figure 1-1 summarizes the mean retrieval ranks of nestmate larvae (NM), non-nestmate larvae (NNM) and larvae collected from another site (other site: OS) for *T. ambiguus* and *T. longispinosus*. After adjusting probability values for inflation of type I error, differences among the three larval types are considered significant where

$P \leq 0.017$. For trials involving *T. ambiguus*, there were no significant differences in the mean retrieval ranks of NM versus NNM ($Z = -1.862$, $P = 0.0627$, $N = 16$) or between NNM and OS ($Z = -1.591$; $P = 0.1118$; $N = 16$); however, nestmate larvae (NM) were retrieved significantly earlier than larvae from another site (OS: $Z = -2.637$, $P = 0.0084$, $N = 16$). Among the 16 *T. ambiguus* replicates, nine cultures retrieved NM larvae earlier than NNM larvae (Table 1-3) and 12 cultures retrieved NM larvae before OS larvae (Table 1-4). Retrieval scores of OS and NNM larvae were significantly different for 10 *T. ambiguus* cultures, with seven and three cultures preferring NNM and OS larvae respectively (Table 1-5). Overall, *T. ambiguus* workers consumed or abandoned 9 NM, 18 NNM and 28 OS larvae. A chi-square test on a 2 X 3 contingency table revealed that this difference was significant ($\chi^2 = 11.130$, $P = 0.0038$).

For *T. longispinosus* trials, there were significant differences in the mean retrieval ranks of NM and NNM ($Z = -2.637$, $P = 0.0084$, $N = 16$) and NM and OS ($Z = -2.534$, $P = 0.0113$, $N = 16$), with nestmate larvae tending to be retrieved earlier. There was no significant difference in the mean retrieval ranks of NNM and OS ($Z = -0.377$, $P = 0.7064$, $N = 16$). Eight *T. longispinosus* cultures retrieved NM larvae before NNM larvae (Table 1-3) and seven cultures retrieved NM larvae earlier than OS larvae (Table 1-4). The retrieval scores of NNM and OS larvae were significantly different in only one *T. longispinosus* culture, where NNM larvae were retrieved earlier (Table 1-5). There was a significant difference in the number of each type of larvae consumed or abandoned in *T. longispinosus* trials ($\chi^2 = 16.125$; $P < 0.0001$). In total, *T. longispinosus* workers cannibalized or abandoned 3 NM, 16 NNM and 23 OS larvae.

Table 1-1 summarizes the culture demographics of both species. There were no significant differences between species in any of the demographic categories measured.

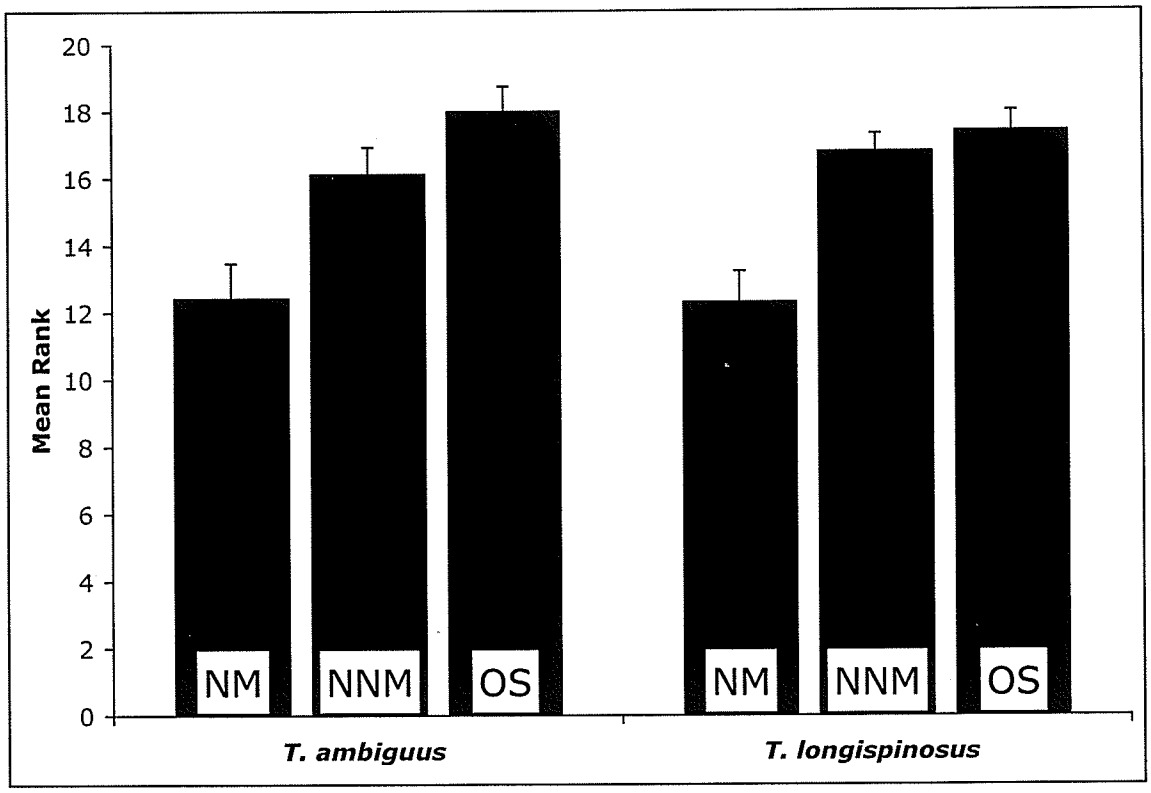

Figure 1-1: Mean retrieval rank (+ SE) of *T. ambiguus* and *T. longispinosus* larvae taken from the same nest (nestmate: NM), a nest from the same site of origin (non-nestmate: NNM), and a nest from a different site of origin (other site: OS).

Table 1-1: Results from Mann-Whitney *U*-tests comparing colony demographics of *T. ambiguus* and *T. longispinosus* cultures used in experiment testing these species ability to recognize nestmate larvae.

# of	*T. ambiguus* Mean ± SE	*T. longispinosus* Mean ± SE	Mann-Whitney *U* *Z*	*P*	*n*
Eggs	0.000 ± 0.000	0.000 ± 0.000	0.000	>0.999	16
Larvae	44.750 ± 6.897	67.313 ± 10.370	-1.771	0.077	16
Pupae	3.250 ± 1.293	2.313 ± 0.700	-0.075	0.940	16
Male Pupae	0.250 ± 0.250	0.500 ± 0.224	-1.112	0.266	16
Pharate pupae	0.625 ± 0.352	0.250 ± 0.250	-0.584	0.559	16
Males	2.875 ± 1.268	1.250 ± 0.629	-0.923	0.356	16
Workers	86.313 ± 11.527	71.938 ± 11.722	-0.999	0.318	16
Gynadromorphs	0.000 ± 0.000	0.000 ± 0.000	0.000	>0.999	16
Ergatogynes	0.000 ± 0.000	0.000 ± 0.000	0.000	>0.999	16
Alate Gynes	0.000 ± 0.000	0.000 ± 0.000	0.000	>0.999	16
Dealate Gynes	1.375 ± 0.202	1.625 ± 0.239	-0.678	0.498	16

Table 1-2: Results of Kolmogorov-Smirnov (K-S) tests for normality and equality of variances *F*-tests (*F*-max) among nestmate (NM), non-nestmate (NNM), and other site (OS) larval retrieval data for *T. ambiguus* and *T. longispinosus*.

		K-S		F-Max		
		χ^2	*P*	*F*	*P*	*n*
T. ambiguus	NM, NNM	10.125	0.013	1.677	0.327	16
	NM, OS	15.125	0.001	1.939	0.211	16
	NNM, OS	6.125	0.094	1.156	0.782	16
T. longispinosus	NM, NNM	12.500	0.004	3.000	0.041	16
	NM, OS	10.125	0.013	2.241	0.129	16
	NNM, OS	2.000	0.736	0.747	0.579	16

Table 1-3: Results of Wilcoxon signed-ranks tests comparing mean (± SE) retrieval ranks of larvae taken from the same nest (nestmate: NM) and a nest from the same site of origin (non-nestmate: NNM) for *T. ambiguus* and *T. longispinosus* individual trials. Significant differences ($\alpha \leq 0.0167$) are in bold.

Species	Culture	NM Mean± SE		NNM Mean± SE		*Z*	*P*	*n*
	T.a. 1-1	13.20±	3.189	12.50±	2.921	-0.070	0.944	10
	7-46	13.60±	2.262	13.10±	3.142	-0.866	0.386	10
	13-13	**11.95±**	**2.771**	**16.85±**	**2.364**	**-2.666**	**0.008**	**10**
	13-5	19.10±	2.413	11.90±	2.477	-2.380	0.017	10
	T.a. 5-13	**9.50±**	**1.167**	**22.00±**	**2.422**	**-2.803**	**0.005**	**10**
	11-53	**10.90±**	**2.900**	**14.20±**	**2.351**	**-2.429**	**0.015**	**10**
T. ambiguus	17-1	**10.60±**	**1.933**	**16.30±**	**3.246**	**-2.599**	**0.009**	**10**
	15-36	**11.30±**	**2.499**	**14.20±**	**2.736**	**-2.803**	**0.005**	**10**
	T.l. 6-84	11.50±	2.473	15.80±	2.662	-1.955	0.051	10
	T.a. 6-13	**9.50±**	**1.962**	**16.80±**	**2.538**	**-2.803**	**0.005**	**10**
	T.a. 6-3	**9.20±**	**2.318**	**16.10±**	**2.041**	**-2.803**	**0.005**	**10**
	9-26	14.90±	2.734	16.15±	3.044	-1.478	0.139	10
	T.a. 6-14	17.95±	2.620	16.50±	2.654	-1.890	0.059	10
	9-59	**7.60±**	**1.579**	**22.55±**	**1.926**	**-2.803**	**0.005**	**10**
	T.a. 1-2	**14.90±**	**2.558**	**12.30±**	**2.495**	**-2.395**	**0.017**	**10**
	7-63	**5.50±**	**0.957**	**20.50±**	**1.916**	**-2.803**	**0.005**	**10**
	19-3	**10.50±**	**2.083**	**18.15±**	**2.591**	**-2.803**	**0.005**	**10**
	1-14	15.40±	2.956	16.70±	2.852	-0.102	0.919	10
	T.l. 6-43	**18.70±**	**2.624**	**12.75±**	**3.054**	**-2.666**	**0.008**	**10**
	19-17	**10.00±**	**2.160**	**19.00±**	**2.565**	**-2.803**	**0.005**	**10**
	11-57	**8.80±**	**2.430**	**19.50±**	**2.295**	**-2.803**	**0.005**	**10**
	T.l. 1-11	15.00±	3.246	15.70±	2.733	-0.561	0.575	10
T. longispinosus	T.l. 5-6	**14.60±**	**2.713**	**16.80±**	**2.931**	**-2.395**	**0.017**	**10**
	T.l. 1-6	15.80±	2.444	15.75±	2.999	-1.019	0.308	10
	T.l. 6-72	**8.40±**	**1.827**	**18.00±**	**2.422**	**-2.803**	**0.005**	**10**
	T.l. 6-8	10.90±	2.340	12.70±	2.432	-1.733	0.083	10
	T.l. 5-12	**10.60±**	**2.166**	**17.95±**	**2.781**	**-2.803**	**0.005**	**10**
	14-3	15.10±	2.558	15.60±	2.922	-0.510	0.610	10
	T.l. 5-31	**7.90±**	**2.068**	**18.10±**	**2.751**	**-2.803**	**0.005**	**10**
	16-5	11.90±	1.215	17.80±	3.363	-1.937	0.053	10
	T.l. 6-56	**6.30±**	**1.248**	**19.40±**	**2.222**	**-2.803**	**0.005**	**10**
	7-51	**17.10±**	**2.946**	**14.70±**	**2.914**	**-2.650**	**0.008**	**10**

Table 1-4: Results of Wilcoxon signed-ranks tests comparing mean (± SE) retrieval ranks of larvae taken from the same nest (nestmate: NM) and a nest from a different site of origin (other site: OS) for *T. ambiguus* and *T. longispinosus* individual trials. Significant differences ($\alpha \leq 0.0167$) are in bold.

Species	Culture	NM Mean± SE		OS Mean± SE		*Z*	*P*	*n*
T. ambiguus	T.a. 1-1	**13.20±**	**3.189**	**20.80±**	**1.227**	**-2.497**	**0.013**	**10**
	7-46	**13.60±**	**2.262**	**19.80±**	**2.614**	**-2.803**	**0.005**	**10**
	13-13	**11.95±**	**2.771**	**17.70±**	**3.083**	**-2.666**	**0.008**	**10**
	13-5	19.10±	2.413	15.50±	3.036	-1.992	0.046	10
	T.a. 5-13	9.50±	1.167	15.00±	3.000	-2.191	0.028	10
	11-53	**10.90±**	**2.900**	**21.50±**	**2.155**	**-2.803**	**0.005**	**10**
	17-1	**10.60±**	**1.933**	**19.60±**	**2.468**	**-2.803**	**0.005**	**10**
	15-36	**11.30±**	**2.499**	**21.50±**	**2.522**	**-2.803**	**0.005**	**10**
	T.l. 6-84	**11.50±**	**2.473**	**18.80±**	**2.711**	**-2.488**	**0.013**	**10**
	T.a. 6-13	**9.50±**	**1.962**	**20.20±**	**2.832**	**-2.803**	**0.005**	**10**
	T.a. 6-3	**9.20±**	**2.318**	**21.20±**	**2.715**	**-2.803**	**0.005**	**10**
	9-26	14.90±	2.734	15.45±	2.852	-0.815	0.415	10
	T.a. 6-14	**17.95±**	**2.620**	**12.05±**	**2.961**	**-2.666**	**0.008**	**10**
	9-59	**7.60±**	**1.579**	**16.35±**	**2.486**	**-2.803**	**0.005**	**10**
	T.a. 1-2	**14.90±**	**2.558**	**19.30±**	**3.070**	**-2.701**	**0.007**	**10**
	7-63	**5.50±**	**0.957**	**20.50±**	**1.928**	**-2.803**	**0.005**	**10**
T. longispinosus	19-3	**10.50±**	**2.083**	**17.85±**	**3.122**	**-2.803**	**0.005**	**10**
	1-14	15.40±	2.956	14.40±	2.790	-0.764	0.445	10
	T.l. 6-43	**18.70±**	**2.624**	**15.05±**	**2.589**	**-2.666**	**0.008**	**10**
	19-17	**10.00±**	**2.160**	**17.50±**	**2.922**	**-2.803**	**0.005**	**10**
	11-57	**8.80±**	**2.430**	**18.20±**	**2.484**	**-2.803**	**0.005**	**10**
	T.l. 1-11	15.00±	3.246	15.80±	2.632	-0.510	0.610	10
	T.l. 5-6	14.60±	2.713	15.10±	2.945	-0.459	0.647	10
	T.l. 1-6	15.80±	2.444	15.35±	3.131	-1.376	0.169	10
	T.l. 6-72	**8.40±**	**1.827**	**20.10±**	**2.669**	**-2.803**	**0.005**	**10**
	T.l. 6-8	**10.90±**	**2.340**	**22.30±**	**1.906**	**-2.803**	**0.005**	**10**
	T.l. 5-12	**10.60±**	**2.166**	**17.95±**	**2.922**	**-2.803**	**0.005**	**10**
	14-3	15.10±	2.558	15.80±	3.123	-0.408	0.684	10
	T.l. 5-31	**7.90±**	**2.068**	**20.50±**	**1.778**	**-2.803**	**0.005**	**10**
	16-5	11.90±	1.215	16.80±	3.158	-2.191	0.028	10
	T.l. 6-56	**6.30±**	**1.248**	**20.80±**	**2.037**	**-2.803**	**0.005**	**10**
	7-51	17.10±	2.946	14.70±	2.716	-2.039	0.042	10

Table 1-5: Results of Wilcoxon signed-ranks tests comparing mean (± SE) retrieval ranks of larvae taken from the same (non-nestmate: NNM) and from a different site of origin (other site: OS) for *T. ambiguus* and *T. longispinosus* individual trials. Significant differences ($\alpha < 0.0167$) are in bold.

Species	Culture	NNM Mean± SE		OS Mean± SE		*Z*	*P*	*n*
	T.a. 1-1	**12.50±**	**2.921**	**20.80±**	**1.227**	**-2.497**	**0.013**	**10**
	7-46	13.10±	3.142	19.80±	2.614	**-2.521**	**0.012**	10
	13-13	16.85±	2.364	17.70±	3.083	-1.125	0.260	10
	13-5	11.90±	2.477	15.50±	3.036	-1.820	0.069	10
	T.a. 5-13	**22.00±**	**2.422**	**15.00±**	**3.000**	**-2.701**	**0.007**	**10**
	11-53	**14.20±**	**2.351**	**21.50±**	**2.155**	**-2.803**	**0.005**	**10**
T. ambiguus	17-1	16.30±	3.246	19.60±	2.468	-2.293	0.022	10
	15-36	**14.20±**	**2.736**	**21.50±**	**2.522**	**-2.666**	**0.008**	**10**
	T.l. 6-84	15.80±	2.662	18.80±	2.711	-1.330	0.183	10
	T.a. 6-13	**16.80±**	**2.538**	**20.20±**	**2.832**	**-2.488**	**0.013**	**10**
	T.a. 6-3	**16.10±**	**2.041**	**21.20±**	**2.715**	**-2.650**	**0.008**	**10**
	9-26	16.15±	3.044	15.45±	2.852	-1.125	0.260	10
	T.a. 6-14	**16.50±**	**2.654**	**12.05±**	**2.961**	**-2.666**	**0.008**	**10**
	9-59	**22.55±**	**1.926**	**16.35±**	**2.486**	**-2.666**	**0.008**	**10**
	T.a. 1-2	**12.30±**	**2.495**	**19.30±**	**3.070**	**-2.803**	**0.005**	**10**
	7-63	20.50±	1.916	20.50±	1.928	0.000	>0.999	10
	19-3	18.15±	2.591	17.85±	3.122	-0.059	0.953	10
	1-14	16.70±	2.852	14.40±	2.790	-1.733	0.083	10
	T.l. 6-43	12.75±	3.054	15.05±	2.589	-2.310	0.021	10
	19-17	19.00±	2.565	17.50±	2.922	-0.770	0.441	10
	11-57	19.50±	2.295	18.20±	2.484	-1.125	0.260	10
	T.l. 1-11	15.70±	2.733	15.80±	2.632	-0.102	0.919	10
T. longispinosus	T.l. 5-6	16.80±	2.931	15.10±	2.945	-1.185	0.236	10
	T.l. 1-6	15.75±	2.999	15.35±	3.131	-0.357	0.721	10
	T.l. 6-72	18.00±	2.422	20.10±	2.669	-2.090	0.037	10
	T.l. 6-8	**12.70±**	**2.432**	**22.30±**	**1.906**	**-2.803**	**0.005**	**10**
	T.l. 5-12	17.95±	2.781	17.95±	2.922	-0.237	0.813	10
	14-3	15.60±	2.922	15.80±	3.123	-0.560	0.575	10
	T.l. 5-31	18.10±	2.751	20.50±	1.778	-1.599	0.110	10
	16-5	17.80±	3.363	16.80±	3.158	-1.478	0.139	10
	T.l. 6-56	19.40±	2.222	20.80±	2.037	-1.680	0.093	10
	7-51	14.70±	2.914	14.70±	2.716	-0.204	0.839	10

Table 1-6: Results from Kolmogorov-Smirnov (K-S) tests for normality and equality of variances *F*-tests (*F*-max) among conspecific larvae in relative coverslip positions 1, 2, 3 and 4 for *T. ambiguus* and *T. longispinosus*.

		K-S		F-max		
		χ^2	*P*	*F*	*P*	*n*
T. ambiguus	1,2	3.200	0.404	0.870	0.839	10
	1,3	5.000	0.164	1.134	0.855	10
	1,4	3.200	0.404	0.483	0.293	10
	2,3	0.800	>0.999	1.303	0.700	10
	2,4	5.000	0.164	0.555	0.393	10
	3,4	5.000	0.164	0.426	0.219	10
T. longispinosus	1,2	3.200	0.404	2.114	0.280	10
	1,3	1.800	0.812	2.177	0.262	10
	1,4	1.800	0.812	0.776	0.712	10
	2,3	0.800	>0.999	1.029	0.966	10
	2,4	3.200	0.404	0.367	0.152	10
	3,4	1.800	0.812	0.357	0.141	10

Transfer of Conspecific Recognition Cues

Figure 1-2 shows the total time *T. ambiguus* and *T. longispinosus* workers spent in oral or antennal contact with untreated baits and baits left in contact with conspecific larvae for 10 min, 1 hr, 4 hrs, 8 hrs and 16 hrs. Overall *T. longispinosus* workers spent more time than *T. ambiguus* antennating or orally contacting baits that had been left in contact with conspecific larvae (Mann-Whitney *U*: $Z = -2.027$; $P = 0.043$; $N = 50$). There were no differences in colony demographics between species (Table 1-7).

A Wilcoxon signed-ranks test revealed that *T. ambiguus* spent significantly more time contacting baits that had been left in contact with conspecific larvae than untreated pieces of silicone ($Z = -4.600$; $P < 0.0001$; $N = 50$). The time that baits were left in contact with larvae did not affect the time *T. ambiguus* spent in contact with them (ANOVA: $F = 1.640$; $P = 0.181$; df = 4). There were significantly more treated baits than untreated baits carried into *T. ambiguus* nests ($\chi^2 = 4.217$; $P = 0.040$). Six of the 250 treated baits that had been left in contact with *T. ambiguus* larvae were carried into the nest while none of the untreated baits were retrieved.

T. longispinosus spent significantly more time contacting baits left in contact with conspecific larvae than untreated baits (Wilcoxon signed-ranks: $Z = -4.914$; $P < 0.0001$; N = 50), but the time that baits were left in contact with larvae did not affect contact time (ANOVA: $F = 0.891$; $P = 0.477$; df = 4). Of the 500 silicone baits presented to *T. longispinosus* cultures, 21 treated baits and zero untreated pieces of silicone were carried into their nest. This difference in the number of each type of bait retrieved was significant ($\chi^2 = 19.883$; $P < 0.001$).

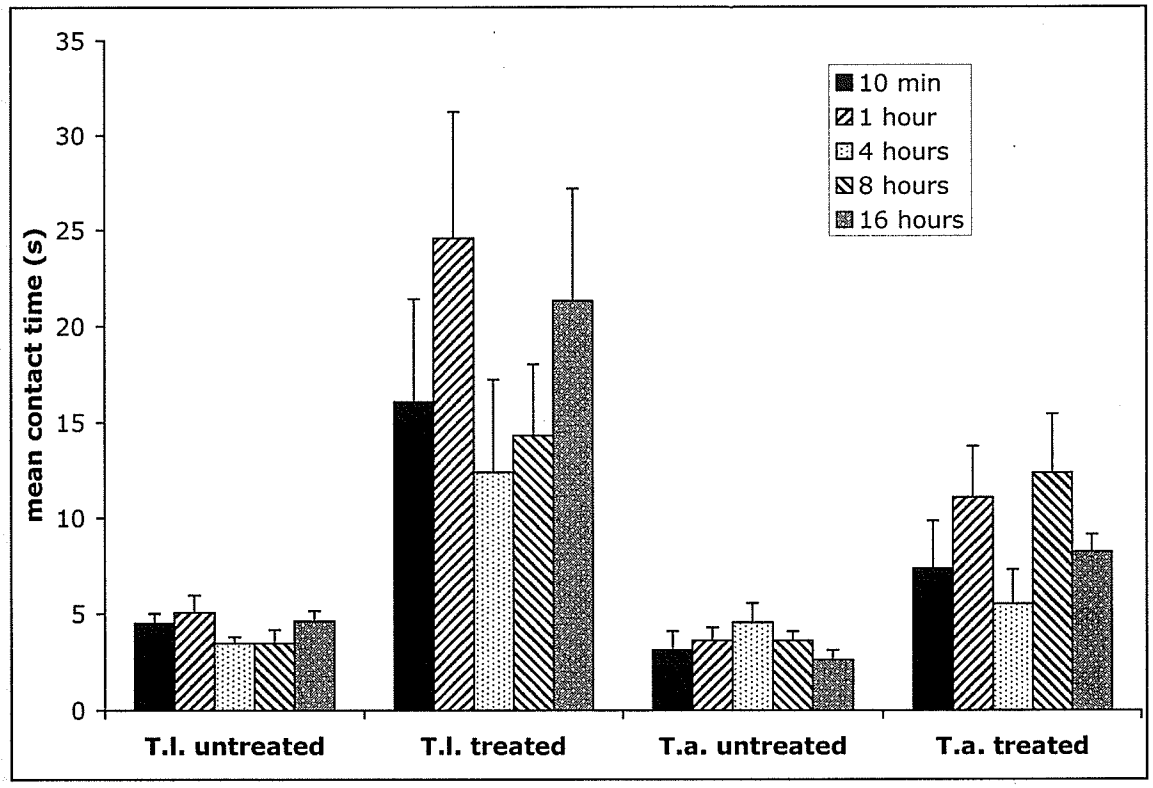

Figure 1-2: Mean time (+ SE) *T. longispinosus* and *T. ambiguus* workers spent in oral or antennal contact with untreated silicone baits and baits left in contact with conspecific larvae for 10 minutes, 1 hour, 4 hours, 8 hours and 16 hours.

Table 1-7: Results from Mann-Whitney *U* tests comparing colony demographics of *T. ambiguus* and *T. longispinosus* cultures used in experiment testing transfer of conspecific recognition cues.

	T. ambiguus	*T. longispinosus*	Mann-Whitney *U*		
	Mean ± SE	Mean ± SE	*Z*	*P*	*n*
Eggs	1.50 ± 1.500	0.00 ± 0.000	-0.348	0.706	*10*
Larvae	72.30 ± 12.575	113.40 ± 27.179	-1.209	0.227	10
Pupae	3.80 ± 2.154	1.00 ± 0.632	-0.983	0.326	10
Male Pupae	1.10 ± 0.674	0.00 ± 0.000	-1.134	0.267	10
Pharate pupae	0.30 ± 0.300	0.70 ± 0.423	-0.718	0.473	10
Males	2.50 ± 1.655	2.60 ± 1.558	-0.038	0.970	10
Workers	76.50 ± 17.508	110.60 ± 15.774	-1.776	0.078	10
Gynadromorphs	0.10 ± 0.100	0.00 ± 0.000	-0.378	0.706	10
Ergatogynes	0.00 ± 0.000	0.00 ± 0.000	0.000	>0.999	10
Alate Gynes	0.00 ± 0.000	0.00 ± 0.000	0.000	>0.999	10
Dealate Gynes	1.70 ± 0.335	1.30 ± 0.213	-0.794	0.427	10

Table 1-8: Results from Kolmogorov-Smirnov (K-S) tests for normality and equality of variances *F*-tests (*F*-max) among baits left in contact with conspecific larvae for 10 minutes, 1 hour, 4 hours, 8 hours and 16 hours for *T. ambiguus* and *T. longispinosus*.

		K-S		F-max		
		χ^2	*P*	*F*	*P*	*N*
	10 min, 1 hr	1.800	0.813	0.866	0.834	10
	10 min, 4 hrs	1.800	0.813	1.936	0.339	10
	10 min, 8 hrs	7.200	0.055	1.291	0.710	10
	10 min, 16 hrs	5.000	0.164	2.609	0.169	10
T. ambiguus	1 hr, 4 hrs	3.200	0.404	2.235	0.247	10
	1 hr, 8 hrs	1.800	0.813	1.490	0.562	10
	1 hr, 16 hrs	3.200	0.404	3.012	0.116	10
	4 hrs, 8 hrs	7.200	0.055	0.667	0.556	10
	4 hrs, 16 hrs	7.200	0.055	1.348	0.664	10
	8 hrs, 16 hrs	3.200	0.404	2.022	0.309	10
	10 min, 1 hr	1.800	0.813	0.649	0.530	10
	10 min, 4 hrs	0.800	>0.999	1.219	0.773	10
	10 min, 8 hrs	0.800	>0.999	2.074	0.292	10
	10 min, 16 hrs	1.800	0.813	0.831	0.787	10
T. longispinosus	1 hr, 4 hrs	3.200	0.404	1.879	0.361	10
	1 hr, 8 hrs	3.200	0.404	3.196	0.099	10
	1 hr, 16 hrs	1.800	0.813	1.280	0.719	10
	4 hrs, 8 hrs	1.800	0.813	1.701	0.441	10
	4 hrs, 16 hrs	3.200	0.404	0.681	0.577	10
	8 hrs, 16 hrs	3.200	0.404	0.401	0.189	10

Chemical Influence on Nestmate Recognition

The mean time that *T. longispinosus* and *T. ambiguus* spent in oral or antennal contact with baits treated with nestmate and non-nestmate larvae is summarized in Figure 1-3. There was no significant difference in the time *T. longispinosus* (Paired t-test: $t = 0.534$; $P = 0.5936$; $N = 15$) or *T. ambiguus* workers ($t = 0.408$; $P = 0.6832$; $N = 15$) contacted the different bait types. Overall, *T. longispinosus* workers contacted baits more than *T. ambiguus* workers ($Z = -2.817$; $P = 0.0049$; $N = 30$). There were no significant differences in the number of nestmate and non-nestmate baits retrieved into the nest for either species. *T. longispinosus* workers retrieved 6 and 2 nestmate and non-nestmate baits respectively ($\chi^2 = 1.534$; $P = 0.215$), while *T. ambiguus* retrieved 2 nestmate baits and 2 non-nestmate baits ($\chi^2 = 0.000$; $P > 0.999$). I detected no differences in the number of eggs, larvae, pharate pupae, worker pupae, male pupae, adult workers, adult males, alate gynes, dealate gynes, gynadromorphs and ergatogynes between species (Table 1-9).

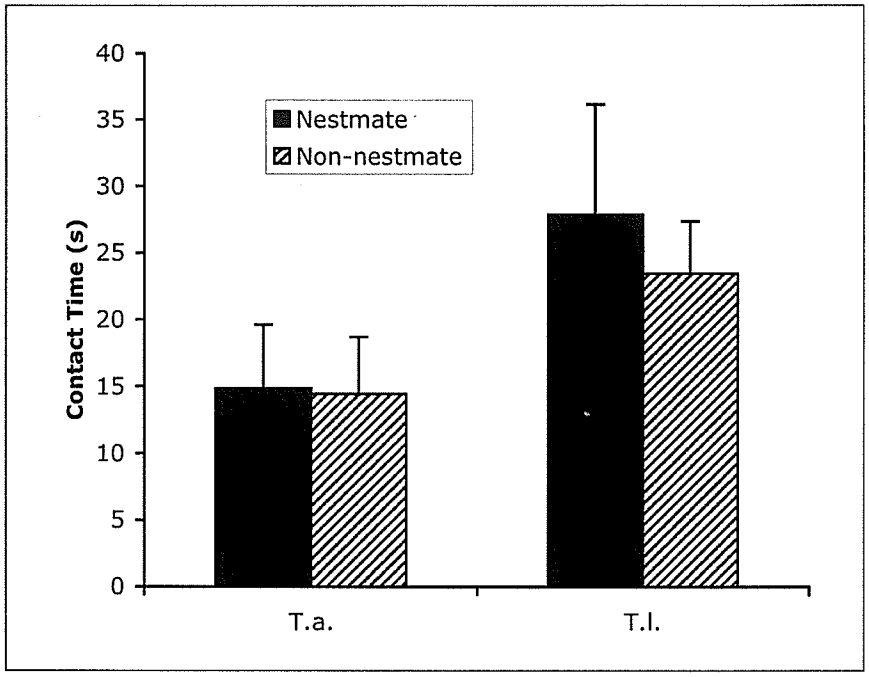

Figure 1-3: Mean time (+ SE) *T. longispinosus* and *T. ambiguus* workers spent in oral or antennal contact with silicone baits left in contact with nestmate larvae and baits left in contact with unrelated conspecific larvae for 16 hours.

Table 1-9: Results from Mann-Whitney *U* tests comparing colony demographics of *T. ambiguus* and *T. longispinosus* cultures used in experiment testing chemical influence on nestmate recognition.

	T. ambiguus Mean ± SE		*T. longispinosus* Mean ± SE		Mann-Whitney *U* *Z*	*P*	*n*
Eggs	0.00 ±	0.000	0.00 ±	0.000	0.000	>0.999	15
Larvae	58.73 ±	6.388	65.73 ±	10.932	-0.041	0.967	15
Pupae	3.07 ±	1.364	1.07 ±	0.511	-1.016	0.310	15
Male Pupae	0.47 ±	0.322	0.33 ±	0.232	-0.062	0.950	15
Pharate pupae	0.53 ±	0.322	0.53 ±	0.307	0.000	>0.999	15
Males	2.73 ±	1.201	2.47 ±	1.264	-0.104	0.917	15
Workers	83.00 ±	11.781	65.07 ±	9.153	-1.058	0.290	15
Gynadromorphs	0.00 ±	0.000	0.00 ±	0.000	0.000	>0.999	15
Ergatogynes	0.07 ±	0.067	0.00 ±	0.000	-0.311	0.756	15
Alate Gynes	0.00 ±	0.000	0.00 ±	0.000	0.000	>0.999	15
Dealate Gynes	1.67 ±	0.287	1.33 ±	0.126	-0.311	0.756	15

DISCUSSION

These experiments demonstrate unequivocally that *T. ambiguus* and *T. longispinosus* workers discriminate among conspecific larvae. What's more, if we assume that order of retrieval correlates with larval preference (Alloway and Hare 1989; Hare 1996), both species appear to favour nestmate larvae over conspecific larvae from other nests. Workers of both species retrieved nestmate larvae earlier than conspecific larvae from different nests and abandoned or cannibalized less nestmate larvae than larvae from different nests. These findings are consistent with those of Hare (1996) who first demonstrated that *T. longispinosus* workers prefer nestmate larvae over non-nestmates from the same site and those from a different site, and add *T. ambiguus* to the catalog of species in the Hymenoptera that discriminate among conspecific brood (e.g., *Lasius niger*: Lenoir 1981; *Cataglyphis cursor*: Lenoir 1984; *T. longispinosus*: Hare 1996; *Polistes spp.*: Gamboa 2004; *Polyergus breviceps*: Johnson et al. 2005). Improved inclusive fitness (Hamilton 1964; Wilson 1975a), benefits resulting from cooperation (Jaisson 1991), or both would promote a preference for nestmate brood. My results alone, however, do not indicate that *T. longispinosus* and *T. ambiguus* can recognize larvae on the basis of kinship alone. Future studies must correlate the relatedness of individuals within nests with larval retrieval order to better understand kin recognition ability in these ants.

When presented with four groups of conspecific larvae drawn from the same unrelated nest, neither *T. ambiguus* nor *T. longispinosus* showed differential larval retrieval among the groups. These results suggest that these species do not lay chemical

trails during larval transport and that the retrieval assay employed in the present experiment was unbiased.

I found evidence that the treatment of non-nestmate larvae by *T. ambiguus* differed with location of collection site. *T. ambiguus* workers differentially accepted the three larval types, accepting nestmate larvae (NM) first, non-nestmate larvae from the same collection site (NNM) secondly, and larvae from another site (OS) last. The number of larvae derived from different sources that were consumed or abandoned by *T. ambiguus* also followed this sequential pattern. I speculate that any or all of the following hypotheses might explain the earlier retrieval of NNM over OS larvae. NNM larvae may have originated from nests within the same polydomous colony as the subject culture to which they were offered (Alloway et al. 1982; Del Rio Pesado and Alloway 1983; Stuart 1985; Herbers 1986; Hare 1996). This hypothesis could be tested by fine-scale mapping of nests within colonies upon collection (e.g. Del Rio Pesado and Alloway 1983; Stuart 1987c) followed by a similar retrieval assay where nestmate, non-nestmate colony-member and non-colony-member larvae are presented (as suggested by Hare 1996). Other studies estimate conspecific nests collected within 96 cm to 3 m of each other to be part of the same polydomous colony (Alloway et al. 1982; Foitzik and Herbers 2001a). Where larvae were not drawn from the same polydomous colony as a subject culture, intraspecific slavery could have familiarized subject workers with former nestmates of the presented NNM larvae (as suggested by Hare 1996). Alternatively, individuals from sympatric nests may share more genetically derived chemical discriminators, owing to the philopatric nature of newly mated *Temnothorax* queens

(Stuart 1987c). Additionally, there may be greater variation of environmentally derived discriminator substances between than within sites.

Unlike their congener, *T. longispinosus* showed no differential treatment of NNM versus OS larvae. Overall there were no differences in the retrieval order or the frequency of cannibalism for NNM and OS larvae. The differences in retrieval data for *T. longispinosus* and *T. ambiguus* may simply reflect less non-nestmate colony-member larvae offered in *T. longispinosus* trials. The relative timing of both experiments may also have affected retrieval results. *T. longispinosus* trials were conducted approximately one month after *T. ambiguus* trials. Later in the year, these ants may be more prone to accept nestmate over non-nestmate larvae in preparation for hibernation. Alternatively, differences in life history between species may have been responsible for the differences observed. It is possible that *T. ambiguus* have more nests per polydomous colony, increasing the likelihood that non-nestmate colony-member larvae would be included as NNM, although I know of no studies documenting such a difference. These species also tend to demonstrate fluidity in their colony structure throughout the year, expanding to more nests in the spring and coalescing to fewer nests in the fall (Herbers and Tucker 1986). Because I collected ants shortly before and shortly after hibernation, differences in the relative timing of colony expansion/ consolidation may contribute to species differences.

T. ambiguus and *T. longispinosus* workers spent significantly more time contacting silicone baits that had been left in contact with conspecific larvae than untreated baits, revealing that chemicals present on the cuticle of conspecific larvae are sufficient to attract attention from workers. Both species also carried more treated baits

into their nests than untreated baits. Similar findings have been shown in a study involving only *T. longispinosus* (Hare 1996). My results suggest that in the absence of tactile and behavioural stimuli, chemical cues are, at least in part, involved in larval recognition and that these pheromones may transfer among nestmates.

Interestingly, the amount of time that baits were exposed to larvae did not affect the amount of time they were contacted by workers. In as little as 10 minutes chemical cues transferred from larvae and could be detected by conspecific workers. Within a brood pile, such rapid transference of recognition cues by direct physical contact could serve to quickly make a homogenous brood odor ('Gestalt': Crozier and Dix 1979). This may be especially beneficial to polygynous/polydomous species or those with polyandrous queens because of the increased variability in genetically and environmentally derived discriminator substances. Transfer of discriminator substances by direct contact may also, however, render a species more susceptible to social parasitism. Transfer of pheromones from host larvae to parasitic queens or among larvae within a mixed species brood pile could enhance the acceptance of socially parasitic queens and brood by their hosts (Franks et al. 1990; Elgar and Allan 2002).

I found no evidence that the chemicals that transferred from larvae to silicone baits were sufficient to allow nestmate discrimination. Both species antennated and orally contacted silicone baits treated with nestmate and non-nestmate larvae for similar periods of time. So while these ants clearly manifest a refined capacity to discriminate nestmate from non-nestmate larvae, the mechanisms involved in this process remain obscure. Larval recognition in these species is likely achieved through contact pheromones (Hare 1996); however, it is possible that the elements necessary for discrimination of nestmates

do not transfer through direct cuticle-cuticle contact. Further examination into the chemistry of *T. ambiguus* and *T. longispinosus* larval discriminator substances is clearly warranted. I suggest soaking larvae in a non-polar solvent (e.g., Hexane: Johnson et al. 2001, pentane: Vienne et al. 1995; Brandt et al. 2005) to draw an array of hydrocarbons from the cuticle. These hydrocarbons could then be separated and individually identified via gas-chromatography and mass-spectrometry (Bergström and Löfqvist 1968; Franks et al. 1990; Habersetzer and Bonavita-Cougourdan 1993; Kaib et al. 1993). To determine the biological relevance of the extract, baits treated with the solution could then be presented to workers in a similar assay to that described in this chapter.

T. longispinosus workers spent more time contacting treated baits with their antennae and mouth parts than *T. ambiguus*. I cannot conclude outright, however, that the cuticular chemicals of *T. longispinosus* larvae are generally more attractive than those of *T. ambiguus* larvae. Under these experimental conditions *T. longispinosus* workers may be more active or more prone to inspect baits than *T. ambiguus* workers. I can, however, disregard the possible influence of a culture's demographics on their proclivity to inspect baits as none of these variables differed between species. In Chapter 2, I separate the influence of larval attractiveness and workers' proclivity to inspect baits along with both species' abilities to discriminate conspecific from allospecific larvae.

Chapter 2: Interspecific brood recognition of the ants *Temnothorax longispinosus* and *T. ambiguus*

INTRODUCTION

The experiments described in this chapter explored the ability of *T. longispinosus* and *T. ambiguus* workers to discriminate conspecific from allospecific larvae, and endeavored to provide preliminary insight into the nature of the chemicals involved in larval discrimination at the species level. *T. ambiguus* and *T. longispinosus* capacity to recognize and bias care toward nestmate brood (see Chapter 1) is advantageous from an inclusive fitness perspective and/ or from benefits resulting from cooperation (Hamilton 1964; Wilson 1975a; Jaisson 1991). Where workers discriminate among conspecific larvae, it follows that they should be able to discriminate at a species level. Ants form a recognition template based on cues synthesized internally and/or acquired from external sources (Hölldobler and Michener 1980; Stuart 1987b) and variation in recognition cues is typically greater among than within species (Brant et al. 2005). When encountering an unfamiliar organism, an individual likely compares the cues emanating from that individual to their underlying recognition template, determining familiarity based on differences from its recognition template more so than similarities (Lahav et al. 1999). The accuracy of this recognition process depends greatly on the ecology and life history of a species. Species that occupy more than one nest (polydomous) and inhabit colonies that contain more than one queen (polygynous), for example, are faced with a difficult task when evaluating relatedness owing to the increased diversity of environmental and genetically derived recognition cues (Alloway et al. 1982; Del Rio Pesado and Alloway

1983). We might expect that such species would be obliged to lower their nestmate recognition threshold to include variant individuals (Reeve 1989; Brandt et al. 2005).

A number of researchers have demonstrated that interspecific mixed colonies of ants can form following experimental introduction of brood (reviewed in Carlin 1988; Hölldobler and Wilson 1990; Lenoir et al. 2001). In nature, mixed colonies may come about if allospecific brood, captured during slave-raids or following territorial interactions, are allowed to eclose and integrate into their captors' colony (Wilson 1975b; Alloway 1980; Buschinger 1986). Typically, interspecific introductions are more successful when taxa are phylogenetically close (Carlin 1988). At the proximate level, this trend likely stems from similar recognition cues shared by closely related species. Ultimately, species should evolve to adopt more closely related workers that are better able to meet their behavioural needs. Although acceptance of allospecific brood is common in socially parasitic species and in experimental situations, discrimination among species still may occur (Alloway 1982; Hare and Alloway 1987; Carlin et al. 1987; Gamboa et al. 1987; Alloway and Hare 1989; Carlin 1988). Indeed, *T. longispinosus* and *T. ambiguus* show preferential rather than exclusive acceptance of different species of larvae and pupae (Alloway 1982; Alloway and Hare 1989; Hare and Alloway 1987; Carlin 1988; Hare 1996).

Early learning appears to play a critical role in the recognition abilities of most Hymenopteran species studied. Moreover, the presence of brood seems to be an important factor involved in the formation of an individual's recognition template (Pfenning et al. 1983). *Formica polyctena*, *F. lugubris* and *F. rufa* all appear to 'imprint' to whatever species of brood they are exposed to early in life, later accepting that species

nearly exclusively (Jaisson 1975; Jaisson and Fresneau 1978; Le Moli and Passetti 1977; 1978; Le Moli and Mori 1982; but see Hare and Alloway 1987 and Carlin 1988). While early learning may contribute to an ant's recognition template (Le Moli and Passetti 1977; Alloway and Ryckman 1991) it is not necessarily imprinting per se. *T. longispinosus* and *T. ambiguus* workers prefer conspecific larvae if exposed to conspecific brood early in life, but exhibit non-preferential brood acceptance if exposed to larvae of a congener or if socially isolated (Hare and Alloway 1987). Moreover, *T. longispinosus* appear to prefer larvae of the slave-maker *Protomognathus americanus* independent of their early experience with ant larvae (Alloway and Hare 1989).

Although unmanipulated workers from whole cultures of *T. longispinosus* favour conspecific over allospecific larvae (Hare 1996), such a preference in *T. ambiguus* has not previously been documented. Other studies that have shown both species prefer conspecific larvae or pupae have specifically addressed the ontogeny of recognition and effects of enslavement (Alloway 1982; Hare and Alloway 1987; Alloway and Hare 1989). Here I test directly the larval preference of *T. longispinosus* and *T. ambiguus* under identical experimental conditions documenting not only the ability of workers developing in natural colonies to discriminate congeneric larvae at the species level, but a comparison of the strength of discrimination behaviour between species.

Discriminator substances that underlie nestmate larval recognition in *T. longispinosus* are present on the larval cuticle and can be transferred to larval models through physical contact (Hare 1996; but see Chapter 1). In a mixed species colony, transfer of recognition pheromones might also occur among larvae in a common brood pile (Hare 1996). It remains unclear whether these pheromones are sufficient for

species-level discrimination. To determine whether pheromones present on the cuticle of *T. longispinosus* and *T. ambiguus* larvae are adequate to promote inspection by workers of the other species, I offered cultures of these species baits left in contact with congeneric larvae and untreated baits. Further, by simultaneously presenting cultures with baits left in contact with conspecific larvae and baits left in contact with allospecific larvae I tested whether these species discriminate between larval species based solely on contact-transferable attractant chemicals borne on the cuticle (see Chapter I for rationale).

METHODS

Larval Species Recognition

To determine whether *T. ambiguus* and *T. longispinosus* discriminate conspecific from allospecific larvae, I simultaneously offered *T. longispinosus* and *T. ambiguus* larvae to cultures of both species. Presentations took place from 14 to 23 November 2007 on 15 *T. ambiguus* cultures from 7 collection sites containing between 26 and 154 workers (mean ± SE: 62.33 ± 9.58) and 15 *T. longispinosus* cultures from 8 collection sites containing between 29 and 163 workers (mean ± SE: 79.73 ± 10.89). Except where noted, presentations followed the methods reported in Chapter 1. I placed two clean glass coverslips (18 mm x 18 mm) each containing 10 *T. longispinosus* larvae and 10 *T. ambiguus* larvae, respectively, five cm from the nest entrance (See Alloway and Hare 1989 and Hare 1996 for similar assays). Then, after placing the culture dish on the stage of a dissecting microscope (Wild model M3, Wild Leitz Ltd. Ottawa, ON, Canada) at 64x magnification, I observed the order in which each larva was retrieved into the nest

entrance and any instances of larval cannibalism or abandonment in a midden. After all larvae were carried into the nest, eaten or abandoned, I assigned each larva an ordinal value between 1 and 20 depending on the order in which they were carried into the nest. The ordinal value of abandoned or cannibalized larvae was equal to the average rank of all larvae left unretrieved (see Chapter 1).

For each species, I tested for differences between mean retrieval scores for *T. longispinosus* and *T. ambiguus* using Wilcoxon signed-ranks tests because of the paired nature of the presentations and the failure of the data to meet the parametric assumptions of normality. Kolmogorov-Smirnov tests showed that the data for both species were not drawn from normally distributed populations of differences (*T. longispinosus*: $\chi^2 = 10.800$; $P = 0.009$; $N = 15$, *T. ambiguus*: $\chi^2 = 10.800$; $P = 0.009$; $N = 15$). Equality of variances F-tests revealed that variances were homogeneous between treatments for *T. longispinosus* ($F = 1.000$; $P > 0.999$; $N = 15$) and *T. ambiguus* ($F = 1.000$; $P > 0.999$; $N = 15$). I also tested for differences in the retrieval scores of conspecific and allospecific larvae for each replicate using Wilcoxon signed-ranks tests. To determine whether the total number of larvae consumed versus accepted differed with species of larvae I performed a chi-square test on a 2 X 2 contingency table, with the Yates correction for continuity, for *T. ambiguus* and *T. longispinosus*.

All larvae offered in this experiment were late-instar isolated in separate artificial nest chambers for one hour prior to experimentation. Within each replicate the relative position of the two coverslips was randomized (using a random numbers table) and the 10 larvae of each type were matched as closely as possible for size. Observations were not blind because an assistant was not available at this time. I censused each subject culture

on the same day as experimentation, recording the number of eggs, larvae, pharate pupae, worker pupae, male pupae, adult workers, adult males, alate gynes, dealate gynes, gynadromorphs and ergatogynes (See Table 2-1 for a summary of colony demographics). Mann-Whitney U tests were then used to determine whether any of these demographic categories differed between species.

Transfer of Allospecific Recognition Cues

To test whether chemical cues present on the cuticle of allospecific larvae were sufficient to promote attraction of workers, I presented cultures of *T. longispinosus* and *T. ambiguus* with untreated baits and baits left in contact with larvae of the other species. Treatment of baits and experimental methods generally followed those described in Chapter 1. Observations lasted for five minutes following the introduction of baits, during which time I recorded the total time workers spent antennating or contacting baits with their mandibles, maxilla or maxillary palps and any occurrence of baits being carried into the nest. The relative positions of the baits on the coverslip were randomized using a random numbers table. Presentations took place from 3 to 14 August 2007. This procedure was repeated using 15 cultures of *T. longispinosus* containing between 37 and 211 workers (mean ± SE: 98.93 ± 14.09) that were collected from 10 sites and 15 cultures of *T. ambiguus* containing between 32 and 227 workers (mean ± SE: 85.2 ± 15.12) collected from 9 sites. To avoid bias resulting from previous familiarity, baits were left in contact with larvae collected from sites other than those of the subject culture. Cultures were censused on the same day as experimentation (see Table 2-3).

For both species, I tested for normality of differences between the bait types using Kolmogorov-Smirnov tests and homogeneity of variance using equality of variances *F*-tests. The data for *T. longispinosus* was not drawn form a normally distributed population of differences ($\chi^2 = 8.533$; $P = 0.028$; $N = 15$) and variances were not homogeneous between bait types ($F = 5.803$; $P = 0.002$; $N = 15$). The data for *T. ambiguus* met the parametric assumption of normality ($\chi^2 = 1.200$; $P > 0.999$; $N = 15$), but not the assumption of homogeneity of variance ($F = 34.255$; $P < 0.001$; $N = 15$). I therefore compared the average time workers spent in oral or antennal contact with each type of bait using Wilcoxon signed-ranks tests. I compared the time that *T. longispinosus* workers versus *T. ambiguus* workers spent in contact with treated baits using a Mann-Whitney *U* test as these data failed to meet the parametric assumptions of normality ($\chi^2 = 13.333$; $P = 0.003$; $N = 15$), however, the variances were homogeneous between species ($F = 1.287$; $P = 0.502$; $N = 15$). I also tested whether culture demographics differed between species using Mann-Whitney *U* tests.

Chemical Influence on Species Recognition

To test whether a chemical attractant present on the cuticle of *T. longispinosus* and *T. ambiguus* larvae is sufficient for species level discrimination, I presented cultures of *T. longispinosus* and *T. ambiguus* with baits left in contact with either species of larvae. One presentation was given to each subject culture where one bait left in contact with five *T. longispinosus* larvae for 16 hrs and one bait left in contact with *T. ambiguus* larvae for that same 16 h period were simultaneously presented on a clean coverslip. Over the five min after presentation I recorded the time that each bait was antennated or

contacted orally by workers along with any instances of bait retrieval. Presentations of this sort were given to 15 cultures of *T. longispinosus* collected from eight sites and 15 cultures of *T. ambiguus* collected from nine sites on 6, 7, and 16 October 2007. The *T. longispinosus* cultures contained between 29 and 205 workers (mean ± SE: 86.40 ± 14.60) and the *T. ambiguus* cultures contained between 28 and 169 workers (mean ± SE: 75.8 ± 11.67). Larvae used to treat baits were taken form nests collected from sites other than that of the subject colony, which received the bait.

The data for *T. ambiguus* were drawn from a normally distributed population of differences ($\chi^2 = 1.200$; $P > 0.999$; $N = 15$) and variances were homogeneous between treatments ($F = 1.255$; $P = 0.6763$; $N = 15$). The data for *T. longispinosus* also met the parametric assumption of normality ($\chi^2 = 1.200$; $P > 0.999$; $N = 15$) but failed to meet the assumption of homogeneity of variance ($F = 4.438$; $P = 0.009$; $N = 15$). I therefore compared the time that workers contacted either bait type using a non-parametric Wilcoxon signed-ranks test for both species. To test whether the number of baits carried into nests differed depending on treatment, I performed a chi-square test on a 2 X 2 contingency table, with the Yates correction for continuity, for both species. I compared the time that each species spent in contact with baits using a non-parametric Mann-Whitney *U* test. The population of differences in contact time between species was not normally distributed ($\chi^2 = 15.000$; $P = 0.001$; $N = 30$), but variances were homogeneous between species ($F = 0.547$; $P = 0.110$; $N = 30$). Mann-Whitney *U* tests were also used to ascertain whether colony demographics differed between species.

RESULTS

Larval Species Recognition

The mean retrieval scores of *T. longispinosus* larvae were lower than those of *T. ambiguus* larvae for *T. longispinosus* cultures; however, this difference fell short of significance ($Z = -1.791$; $P = 0.0736$; $N = 15$; Figure 2-1). Among the 15 *T. longispinosus* replicates eight cultures retrieved conspecific larvae significantly earlier and two cultures retrieved allospecific larvae earlier (Table 2-2). Either because of cannibalism or abandonment, *T. longispinosus* workers did not retrieve 21 conspecific larvae and 26 allospecific larvae. A 2 X 2 contingency analysis revealed that this difference was not significant ($\chi^2 = 0.404$; $P = 0.5252$).

T. ambiguus also showed a trend towards earlier retrieval of conspecific over allospecific larvae, but this difference was not significant ($Z = -1.732$; $P = 0.083$; $N = 15$; Figure 2-1). Six *T. ambiguus* cultures carried conspecific larvae into the nest significantly earlier while one culture retrieved allospecific larvae earlier (Table 2-2). *T. ambiguus* workers either cannibalized or abandoned *T. longispinosus* larvae more frequently than conspecific larvae ($\chi^2 = 6.098$; $P = 0.0132$). In the *T. ambiguus* trials a total of 25 *T. ambiguus* and 44 *T. longispinosus* larvae were consumed or left in a midden. The number of eggs, larvae, pupae, pharate pupae, male pupae, workers, males, alate gynes, dealate gynes, gynadromorphs and ergatogynes were not significantly different between species (Table 2-1).

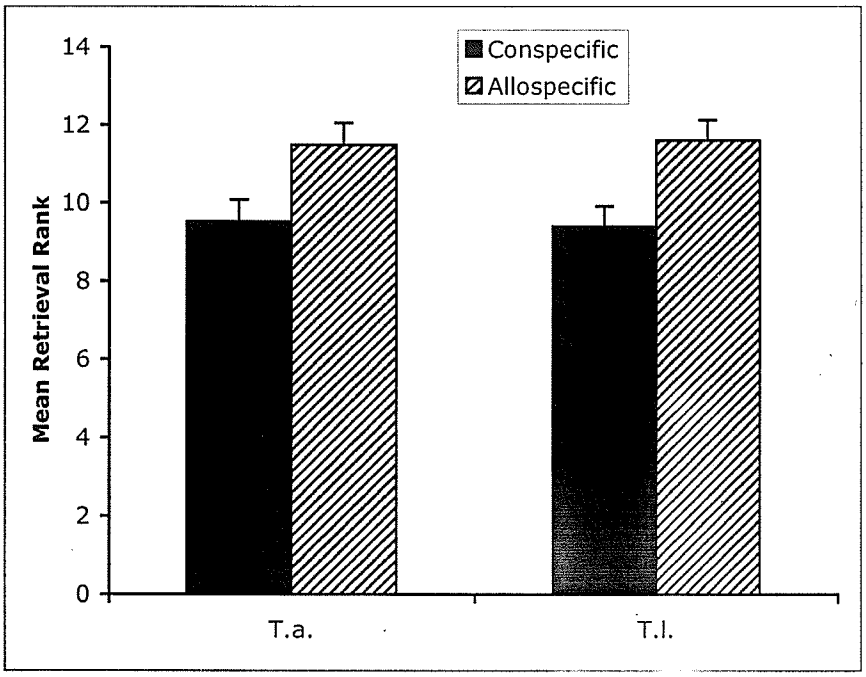

Figure 2-1: Mean retrieval rank (+ SE) of conspecific and allospecific larvae taken by *T. ambiguus* and *T. longispinosus* workers.

Table 2-1: Results from Mann-Whitney *U* tests comparing colony demographics of *T. ambiguus* and *T. longispinosus* cultures used in the experiment testing these species' ability to differentiate conspecific from allospecific larvae.

# of	*T. ambiguus* Mean ± SE	*T. longispinosus* Mean ± SE	Mann-Whitney *U* *Z*	*P*	*n*
Eggs	0.00 ± 0.000	0.00 ± 0.000	0.000	>0.999	15
Larvae	39.43 ± 5.637	53.47 ± 6.631	-1.555	0.120	15
Pupae	4.07 ± 1.577	1.47 ± 0.533	-0.456	0.648	15
Male Pupae	0.00 ± 0.000	0.40 ± 0.235	-0.933	0.351	15
Pharate pupae	0.27 ± 0.182	0.00 ± 0.000	-0.622	0.534	15
Males	0.60 ± 0.434	1.73 ± 0.589	-1.472	0.141	15
Workers	62.33 ± 9.583	53.80 ± 8.451	-0.705	0.481	15
Gynadromorphs	0.00 ± 0.000	0.00 ± 0.000	0.000	>0.999	15
Ergatogynes	0.00 ± 0.000	0.00 ± 0.000	0.000	>0.999	15
Alate Gynes	0.00 ± 0.000	0.00 ± 0.000	0.000	>0.999	15
Dealate Gynes	1.60 ± 0.254	1.53 ± 0.236	-0.083	0.934	15

Table 2-2: Results of Wilcoxon signed-ranks tests comparing mean retrieval ranks (± SE) of conspecific and allospecific larvae for *T. ambiguus* and *T. longispinosus* individual trials. Significant differences ($\alpha \leq 0.05$) are in bold.

		Conspecific Mean± SE		Allospecific Mean± SE		*Z*	*P*	*n*
	15-79	10.300±	1.844	10.700±	1.915	-0.592	0.554	10
	T.a. 5-5	**13.400±**	**1.698**	**7.600±**	**1.607**	**-2.803**	**0.005**	**10**
	7-75	**6.600±**	**1.376**	**14.400±**	**1.416**	**-2.803**	**0.005**	**10**
	15-88	10.400±	1.836	10.600±	1.884	-0.169	0.866	10
	T.a. 5-59	**5.500±**	**0.957**	**15.500±**	**0.850**	**-2.803**	**0.005**	**10**
	15-17	9,700±	2.086	11.300±	1.685	-1.599	0.110	10
T. ambiguus	19-27	10.250±	1.965	10.750±	1.859	-0.178	0.859	10
	13-2	10.300±	2.000	10.700±	1.838	-0.663	0.508	10
	11-8	**8.100±**	**1.810**	**12.900±**	**1.588**	**-2.521**	**0.012**	**10**
	11-61	**9.700±**	**1.674**	**11.300±**	**2.082**	**-1.955**	**0.051**	**10**
	16-19	**6.400±**	**1.352**	**14.600±**	**1.310**	**-2.803**	**0.005**	**10**
	15-49	9.800±	1.853	11.200±	1.947	-1.422	0.155	10
	16-24	12.400±	1.962	8.600±	1.621	-1.303	0.193	10
	11-71	**8.600±**	**1.893**	**12.400±**	**1.727**	**-2.090**	**0.037**	**10**
	7-54	11.350±	2.028	9.650±	1.764	-1.631	0.103	10
	T.l. 6-35	9.100±	2.001	11.900±	1.701	-1.478	0.139	10
	T.l. 6-75	10.200±	1.812	10.800±	1.993	-1.120	0.263	10
	16-2	**7.250±**	**1.834**	**13.750±**	**1.274**	**-2.599**	**0.009**	**10**
	7-58	**8.900±**	**1.494**	**12.100±**	**2.142**	**-2.650**	**0.008**	**10**
	15-21	**12.600±**	**1.607**	**8.400±**	**1.875**	**-2.521**	**0.012**	**10**
	15_7	10.200±	1.723	10.800±	2.051	-0.652	0.515	10
T. longispinosus	16-3	**9.000±**	**1.155**	**12.000±**	**2.352**	**-2.039**	**0.042**	**10**
	15-85	**8.200±**	**1.873**	**12.800±**	**1.611**	**-2.521**	**0.012**	**10**
	11-31	9.100±	2.068	11.900±	1.636	-1.784	0.075	10
	T.l. 1-16	**8.500±**	**1.727**	**12.500±**	**1.869**	**-2.497**	**0.013**	**10**
	T.l. 6-28	**9.000±**	**1.931**	**12.000±**	**1.722**	**-2.521**	**0.012**	**10**
	T.l. 6-4	**5.900±**	**1.159**	**15.100±**	**1.135**	**-2.803**	**0.005**	**10**
	14-1	**12.900±**	**1.956**	**8.100±**	**1.509**	**-2.803**	**0.005**	**10**
	T.l. 5-7	**6.900±**	**1.386**	**14.100±**	**1.609**	**-2.803**	**0.005**	**10**
	T.l. 5-8	10.200±	1.794	10.800±	2.037	-2.019	0.208	10

Transfer of Allospecific Chemicals

T. longispinosus contacted baits left in contact with allospecific larvae significantly longer than untreated baits (Z = -2.613; P = 0.009; N = 15; Figure 2-2); however, there was no significant difference in the time *T. ambiguus* workers spent in contact with either type of bait (Z = -0.534; P = 0.5936; N = 15; Figure 2-2). *T. longispinosus* workers retrieved two and zero allospecific and untreated baits respectively but this difference was not significant (χ^2 = 0.536; P = 0.4642). There was also no difference in the amount of either type of bait *T. ambiguus* workers retrieved (χ^2 = 0.000; P > 0.9999). *T. ambiguus* workers carried one allospecific bait and zero untreated baits into their nests. Overall, *T. longispinosus* workers contacted treated baits more than *T. ambiguus* workers (Z = -2.344; P = 0.019; N = 15). There were no differences in colony demographics between species (Table 2-3).

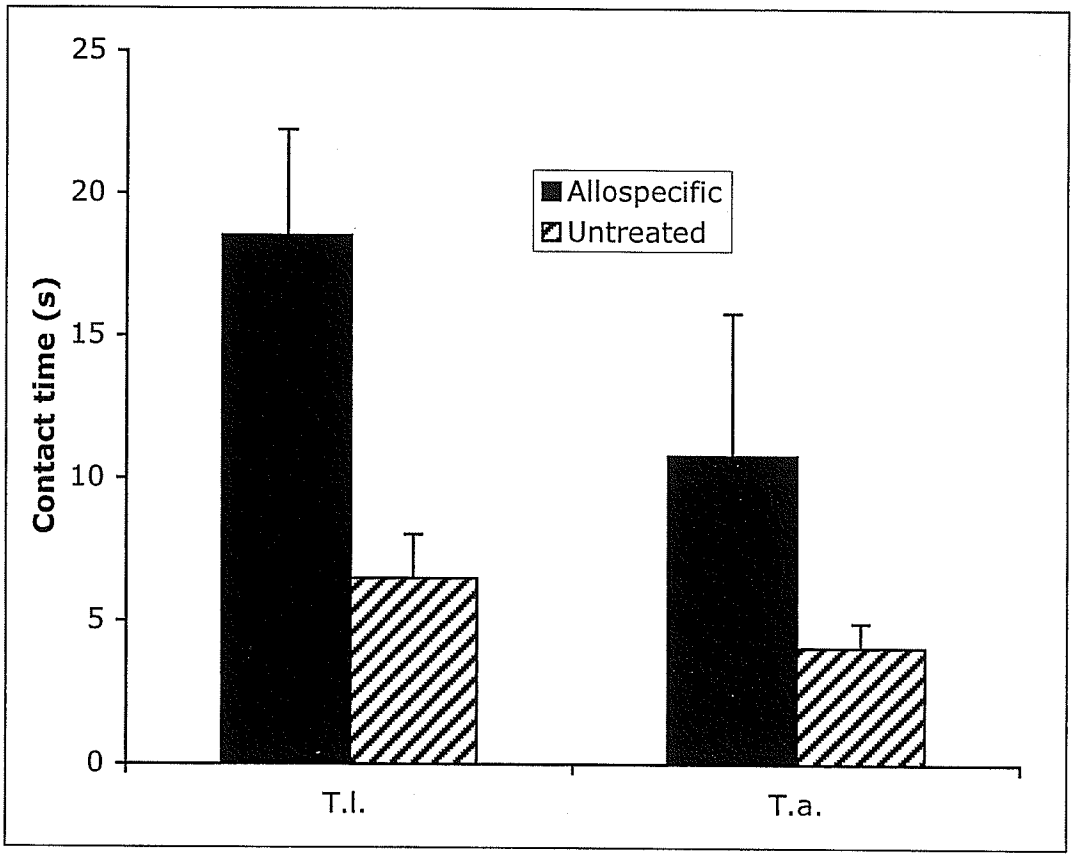

Figure 2-2: Mean time (+ SE) *T. longispinosus* and *T. ambiguus* workers spent in oral or antennal contact with untreated silicone baits and baits left in contact with allospecific larvae for 16 hours.

Table 2-3: Results from Mann-Whitney *U* tests comparing colony demographics of *T. ambiguus* and *T. longispinosus* cultures used in the experiment testing transfer of allospecific recognition cues.

	T. ambiguus Mean±SE		*T. longispinosus* Mean±SE		Mann-Whitney *U* *Z*	*P*	*n*
Eggs	1.13±	0.999	0.60±	0.412	0.000	>0.999	15
Larvae	84.47±	15.350	92.93±	19.844	-0.166	0.868	15
Pupae	3.60±	1.505	1.40±	0.592	-1.016	0.310	15
Male Pupae	1.13±	0.576	0.27±	0.182	-0.705	0.481	15
Pharate pupae	0.33±	0.270	0.47±	0.291	-0.311	0.756	15
Males	3.67±	1.632	3.33±	1.472	-0.062	0.950	15
Workers	85.20±	15.121	98.93±	14.090	-0.664	0.507	15
Gynadromorphs	0.00±	0.000	0.00±	0.000	0.000	>0.999	15
Ergatogynes	0.00±	0.000	0.07±	0.067	-0.311	0.756	15
Alate Gynes	0.00±	0.000	0.00±	0.000	0.000	>0.999	15
Dealate Gynes	1.60±	0.235	1.33±	0.211	-0.767	0.443	15

Chemical Influence on Species Recognition

There was no difference in the time *T. longispinosus* workers antennated or orally contacted baits treated with conspecific larvae and baits left in contact with allospecific larvae ($Z = -0.057$; $P = 0.9547$; $n = 15$; Figure 2-3). Similarly, the time *T. ambiguus* workers contacted conspecific and allospecific baits did not differ significantly ($Z = -0.408$; $P = 0.6832$; $n = 15$; Figure 2-3). Overall, *T. longispinosus* workers contacted both types of bait longer than *T.* ambiguus workers ($Z = -2.942$; $P = 0.0033$; $n = 30$; Figure 2-3). There were no significant differences in the amount of each type of bait that *T. longispinosus* ($\chi^2 = 0.000$; $P > 0.9999$) or *T. ambiguus* ($\chi^2 = 0.288$; $P = 0.5912$) retrieved. *T. longispinosus* workers retrieved two conspecific baits and three allospecific baits, while *T. ambiguus* workers retrieved three conspecific baits and one allospecific bait. Colony demographics did not differ significantly between species (Table 2-4).

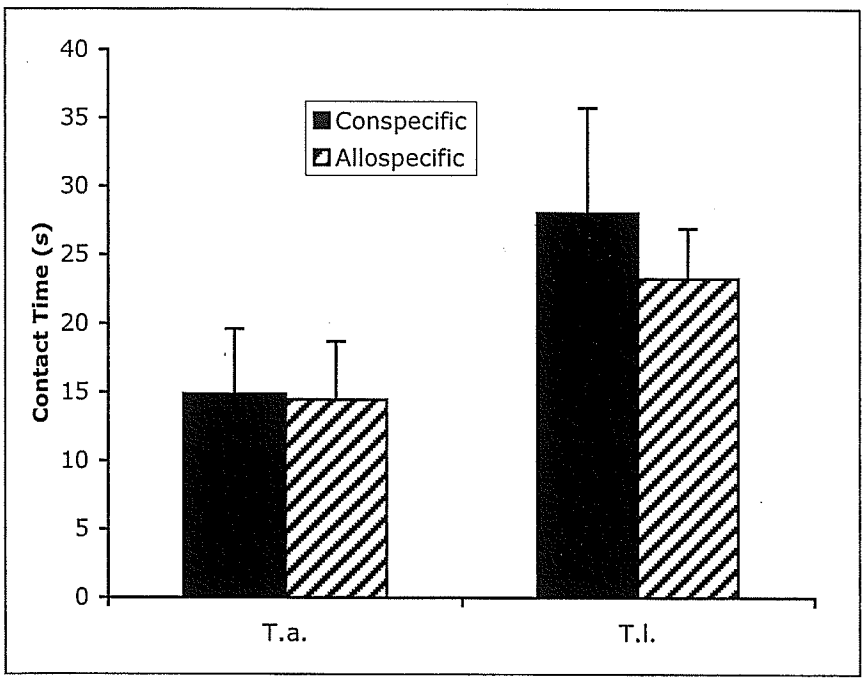

Figure 2-3: Mean time (+ SE) *T. longispinosus* and *T. ambiguus* workers spent in oral or antennal contact with silicone baits left in contact with conspecific larvae versus baits left in contact with allospecific larvae.

Table 2-4: Results from Mann-Whitney *U*-tests comparing colony demographics of *T. ambiguus* and *T. longispinosus* cultures used in experiment testing chemical influence on species recognition.

	T. ambiguus Mean±SE		*T. longispinosus* Mean±SE		Mann-Whitney *U* *Z*	*P*	*n*
Eggs	0.00±	0.000	0.00±	0.000	0.000	>0.999	15
Larvae	47.73±	6.827	83.20±	19.896	-1.286	0.199	15
Pupae	1.93±	0.658	1.47±	0.584	-0.436	0.663	15
Male Pupae	0.73±	0.330	0.40±	0.289	-0.850	0.395	15
Pharate pupae	0.87±	0.496	0.40±	0.214	-0.166	0.868	15
Males	2.73±	1.221	3.20±	1.461	-0.062	0.950	15
Workers	75.80±	11.669	86.40±	14.603	-0.353	0.724	15
Gynadromorphs	0.00±	0.000	0.00±	0.000	0.000	>0.999	15
Ergatogynes	0.00±	0.000	0.07±	0.067	-0.311	0.756	15
Alate Gynes	0.00±	0.000	0.00±	0.000	0.000	>0.999	15
Dealate Gynes	1.40±	0.235	1.33±	0.187	-0.041	0.967	15

DISCUSSION

T. longispinosus and *T. ambiguus* workers tended to retrieve conspecific larvae earlier than the larvae of a congener, but this trend was not significant for either species. The lack of preference for larvae of the same species is surprising in light of other studies that demonstrate a clear preference for conspecific brood using similar assays (Hare 1996; Hare and Alloway 1987; Alloway 1982). *T. ambiguus* workers did consume or abandon significantly more allospecific than conspecific larvae, suggesting that they do discriminate among larvae based on their species. I found little indication, however, that *T. longispinosus* exhibit such favoritism for their own species' larvae. Previous work has also shown that *T. ambiguus* workers are more adept at discriminating con- from allospecific larvae than *T. longispinosus* (Hare and Alloway 1987). The greater tolerance of *T. longispinosus* towards allospecific larvae could render this species more susceptible to social parasitism and may partially explain why *T. longispinosus* is the preferred host species of *P. americanus* (Blatrix and Herbers 2003).

Where *T. ambiguus* workers preferentially accept conspecific over allospecific larvae it is likely representative of the close odour similarity between conspecific larvae and nestmate brood based on their closer phylogenetic relationship. No differential treatment between conspecific and allospecific larvae suggests that *T. longispinosus* accept conspecific and allospecific larvae non-preferentially. If the extent of inter-colony competition within a species is similar to that between species (Herbers 1986), we shouldn't expect preferential treatment of unrelated conspecifics. What's more, accepting both species of larvae equally suggest that they provide similar benefit when incorporated into a *T. longispinosus* colony.

My results do not suggest that discrimination between adults of either species is not possible. Generally, larvae are accepted more readily than adults (Carlin 1988; Bagnères et al. 1991). In the lab some species (e.g. *Camponotus* spp.) initially adopt allospecific brood but kill the adoptees as they eclose (Plateaux 1960; Wilson 1975b; Carlin 1988). While *Temnothorax* spp. tend to be more hostile to invading allospecific than unrelated conspecific workers (Stuart 1991; Heinze et al. 1996), they commonly allow congeneric larvae to integrate into the colony upon maturation (Alloway 1979; 1980).

Likely, recognition on a species level, where it exists, is based upon chemical cues borne on the larval cuticle and is detected by direct contact (Hölldobler and Michener 1980; Carlin and Hölldobler 1986; Breed and Bennett 1987; Howard 1993; Espelie et al. 1994; Lorenzi et al. 1996; Lahav et al. 1999). *T. longispinosus* workers spent more time contacting silicone baits treated with allospecific larvae than untreated baits. This suggests that attraction to *T. ambiguus* larvae by *T. longispinosus* workers depends at least in part on chemical cues located on the larval cuticle and that these chemicals can transfer from the larvae to silicone baits and possibly to other larvae. Hare (1996) reported similar findings using baits treated with *T. longispinosus* larvae. *T. ambiguus* workers did not contact allospecific treated baits significantly more than control baits. This indicates that chemicals present on the cuticle of *T. longispinosus* larvae are not sufficient in the absence of other sensory stimuli to promote attraction of *T. ambiguus* workers or, perhaps is an artifact of the generally low responsiveness of *T. ambiguus* workers to treated silicone baits that I observed. Alternatively chemical cues involved in recognition of *T. longispinosus* larvae by *T. ambiguus* do not transfer to baits.

In Chapter 1, I established that *T. longispinosus* workers spent more time contacting silicone baits left in contact with conspecific larvae than *T. ambiguus* larvae. Because *T. longispinosus* also showed a more pronounced interest in baits treated with allospecific larvae I can conclude that *T. longispinosus* workers generally inspect treated baits more than their congener. These findings may reflect the tendency of *T. longispinosus* to accept a greater proportion of allospecific brood than *T. ambiguus* (Alloway 1982; Hare and Alloway 1987) or overall differences in the species' activity budgets (Trampus 1997).

Neither species showed a difference in the amount of time they antennated or orally contacted conspecific or allospecific baits. Chemical cues that transfer from the larval cuticle may then not be sufficient on their own to facilitate larval discrimination between species. These species are facultative slave-makers and commonly place captured larvae and pupae in a common brood pile with nestmates (Stuart and Alloway 1982). Lack of chemical transfer through contact might facilitate preferential treatment of closely related nestmate brood even within a common brood pile. Indeed, direct contact with *T. ambiguus* larvae does not seem to affect the acceptance of conspecific larvae by *T. longispinosus* (Hare 1996). This does not preclude the possibility that discriminator substances transfer by other means. For example, trophalaxis between colony members is suspected to be important in establishing homogeneity of odour within a colony (Lenoir et al. 1999). Trophalaxis is the mouth-to-mouth (stomodeal) or abdominal tip-to-mouth (proctodeal) exchange of alimentary fluids between individuals. In studies that have determined the cuticular hydrocarbon profiles of ants in single species and in mixed species cultures, the species-specific recognition odours are commonly modified in the

mixed species cultures (Bagnères et al. 1991). For social parasites in particular, matching their hosts' chemical signature is likely an important strategy when invading host nests (Akino et al. 1999; Bergström and Löfqvist 1968; Brandt et al. 2005). Even species that occupy host nests by force (e.g. *Protomognathus americanus* — Herbers and Foitzik 2002) would benefit from reduced aggression brought about by host chemical acquisition. Once established in *Temnothorax* nests, the slave-making ant *P. americanus* adjusts its chemical signature according to the host species composition of its nests (Brant et al. 2005). Moreover, slave-maker presence triggers a reciprocal adjustment in cuticular chemical profiles of its hosts *T. ambiguus* and *T. longispinosus* (Brant et al. 2005). In Chapter 3 I test whether culticular chemicals transfer between *P. americanus* and *Temnothorax* larvae by direct contact and examine *T. ambiguus* and *T. longispinosus* workers' ability to differentiate between conspecific and slave-maker larvae.

Chapter 3: The ants *Temnothorax longispinosus* and *T. ambiguus* discriminate conspecific from slave-maker (*Protomognathus americanus*) larvae

INTRODUCTION

The unity of ant societies depends on the capacity of workers to recognize colony members and reject foreign individuals. Colony unity is essential if altruistic behaviour is to be directed towards kin (Hamilton 1964; Wilson 1975a) or even unrelated cooperating colony members (Jaisson 1991), and is maintained through a recognition system predicated upon lipid cues borne on the cuticle (Howard 1993; Lorenzi et al 1996). Cuticular hydrocarbons in particular are considered to be critical in the recognition process (Lahav et al. 1999; Howard and Blomquist 2005). Typically, colony members share a common chemical signature or "gestalt odour" (Crozier and Dix 1979). Shortly after eclosion, workers form a recognition template based on this colony odour and subsequently spurn adult individuals whose chemical signature does not match that template (Gamboa 1996; Crozier and Dix 1979; Lenoir et al. 1999). Unfamiliar larvae and pupae, however, tend to be accepted more readily than adults (Bagnères et al. 1991). Even so, species level discrimination among brood is common (Carlin 1988).

Hydrocarbons that make up a distinctive colony odour may be acquired through outside environmental sources including nesting material and food (Singer and Espelie 1992; Stuart 1987a; Heinze et al. 1996) or colony members themselves may synthesize elements that contribute to the colony odour (Buckle and Greenburg 1981; Mintzer 1982; Mintzer and Vinson 1985; Vander Meer et al. 1989). Queens in particular may produce cues that proliferate through a colony, greatly influencing the recognition signature of

their workers (Carlin and Hölldobler 1986; 1987; Provost 1989; 1991). Homogeneity of odour among colony members is accomplished through direct contact, allo-grooming and trophalaxis (Meskali et al. 1995; Soroker et al. 1995; Vienne et al. 1995; Dahbi et al. 1997; Franks et al. 1990). It is thought that recognition cues are stored in the post-pharyngeal gland and transferred to the cuticle via self- or allo-grooming (Soroker et al. 1995; Lenoir et al. 1999). In many species, individuals undergo a period of chemical insignificance before they obtain their colony's smell (Lenoir et al. 2001; Cervo et al. 2008). An individual's cuticle tends to be especially permeable to discriminator substances during the larval and callow stages (Hölldobler and Wilson 1990).

The integrity of ant colonies is compromised when social parasites subvert the recognition systems of host species and benefit from the host's labour and physical resources. Integration of a social parasite into a host colony may be accomplished if the parasite can adequately mimic their host's chemical signature (Dettner and Liepert 1994). This may involve the active biosynthesis of host recognition cues by the parasite itself, as is the case with at least one slave-making ant and certain myrmecophiles (Kaib et al. 1993; Howard et al. 1990; Howard 1993) or acquisition of cues from host individuals as has been demonstrated in predatory spiders (Elgar and Allan 2004). Prior to gaining cues from host individuals, social parasites may gain initial access into a colony through force (Herbers and Foitzik 2002), by masking their own chemical signature or by being chemically inert ("odourless hypothesis" — Lenoir et al. 2001) or chemically neutral ("chemical blend that is not meaningful to the host" — Cervo et al. 2008). Alternatively, social parasites may achieve acceptance by their hosts by manipulating the recognition processes of host individuals (Jaisson 1975; Alloway 1982; Mori et al. 2000).

The obligatory slave-making ant *Protomognathus americanus* exploits the labour of its host species *Temnothorax longispinosus*, *T. ambiguus*, and *T. curvispinosus* (Creighton 1927; Sturtevant 1927; Alloway 1979). *P. americanus* exemplifies specialization towards a dulotic lifestyle and is, perhaps, the most studied of all socially parasitic ants. Following her mating flight a *P. americanus* queen infiltrates a host nest, kills or displaces the resident queen and usurps her role as the primary reproductive in that colony (Herbers and Foitzik 2002; Blatrix and Herbers 2003). *P. americanus* queens possess many of the morphological, ecological and behavioural traits that suggest they initially rid a host colony of adult workers; however, I do not know of any direct observations of this behaviour. These traits include: large size relative to their hosts, a thick cuticle, broad post-petiole, antennal scrobes (grooves in the head into which antennae can be placed for protection), and the tendency to invade small host colonies (Wesson 1939; Buschinger 1974; Wilson 1975; Herbers and Foitzik; Brandt et al. 2005). Established *P. americanus* colonies obtain additional slaves by pillaging local host nests of their pupae and late-instar larvae (Alloway 1979). Upon eclosion, host workers perform the menial colony tasks for their captors including foraging, nest maintenance and brood care (Buschinger 1986; Stuart and Alloway 1985). Typical *P. americanus* colonies contain a single queen, 2-12 workers, 20-50 slaves and occupy more than one nest (Wesson 1939; Buschinger and Alloway 1979; Del Rio Pesado and Alloway 1983; Foitzik and Herbers 2001a; Brandt et al. 2005). They conduct between 2 and 11 raids a year (Foitzik and Herbers 2001; Foitzik et al. 2001; Blatrix and Herbers 2003).

While *P. americanus* capitalizes on the labour of its hosts, the exploitation of brood care behaviour is of particular interest. When host workers tend to their captor's

brood, they presumably increase the parasite's fitness at their own expense. Other species of ants including *Formica cunicularia* and *F. rufa* appear to 'imprint' to whatever species they are exposed to early in life subsequently directing care preferentially to that species (Le Moli and Passetti 1977; Le Moli and Mori 1987). *T. longispinosus*, *T. ambiguus* and *T. curvispinosus* all demonstrate heightened acceptance of alien pupae when enslaved by *P. americanus* (Alloway 1982). Although enhancing pupae-acceptance behaviour in slaves is clearly adaptive, the mechanism by which *P. americanus* encourages such behaviour remains unknown. While early learning may contribute to an ant's recognition template (Jaisson 1975; Le Moli and Passetti 1977; Le Moli 1980; Alloway and Ryckman 1991) it is not necessarily imprinting per se. Instead, *T. longispinosus* and *T. ambiguus* seemingly require the presence of conspecific larvae early in life to activate a later preference for conspecific larvae (Hare and Alloway 1987). Following eclosion in the presence of conspecific brood, workers accept conspecific larvae preferentially, but exhibit non-preferential larval acceptance if exposed to allospecifics or if socially isolated prior to eclosion to adulthood (Hare and Alloway 1987). Alloway and Hare (1989) reported that isolated *T. longispinosus* workers preferentially retrieve and tend *P. americanus* larvae regardless of early experience. What's more, the long-term survival of larvae within a nest correlates with the order of larval retrieval (Alloway and Hare 1989). These results suggest that *P. americanus* larvae do not simply mimic species-specific odours or rely on early learning mechanisms, but produce some element that exploits inherent sensory preferences of *T. longispinosus* workers (Alloway and Hare 1996). Preliminary results comparing gas-chromatography (GC) profiles of solids sampled whole larvae of *P. americanus*, *T. longispinosus,* and *T. ambiguus* reveal that *P.*

americanus larvae have increased concentrations of three of the 25 hydrocarbon chains shared by all three species (J.F. Hare, A.B. Attygalle, and T. Eisner unpublished data: referred to in Hare 1996). Such exaggerated concentrations in the slave-maker GC spectrum may serve as a supernormal stimulus, releasing preferential acceptance and tending of slave-maker larvae by host species workers. We cannot be sure, however, that these patterns are based exclusively on cuticular chemicals that serve as discriminator substances and therefore cannot attribute differences in GC profiles to recognition signals in that Hare, Attygalle and Eisner crushed whole larvae into the column in their unpublished study. Adult *P. americanus* workers certainly share a common hydrocarbon profile with their hosts (Brandt et al. 2005), but do not appear to exhibit the enhanced concentrations of certain elements indicative of a supernormal attractant.

The experiments described in this chapter were designed to further our understanding of the seemingly preferential treatment of *P. americanus* larvae by their hosts. I tested whether the preferential treatment of slave-maker larvae by isolated groups of *T. longispinosus* workers (Alloway and Hare 1989) extends to workers within queenright, unenslaved colonies and whether *T. ambiguus* shares this preference. If hosts adopt the parasite larvae preferentially, it is likely due to the larvae having an attractive pheromone similar to that proposed by Alloway and Hare (1989). Alternatively, if host species workers accept conspecific over slave-maker larvae it may be the result of the close odour similarity of conspecific larvae with colony members (Brandt et al. 2005) or may be because the host workers from otherwise intact *Temnothorax* maternal colonies recognize the slave-makers as enemies (Alloway 1989). This experiment also tested whether reciprocal contact between host and *P. americanus* larvae affects retrieval by *T.*

longispinosus and *T. ambiguus*. Hare (1996) showed that *T. longispinosus* preferentially retrieve conspecific larvae over those of *T. ambiguus* but found no evidence that transferable cuticular cues underlying larval recognition for these species transfer among larvae. In the context of social parasitism, transfer of recognition cues among larvae may increase slave-maker acceptance by their slaves (Vander Meer and Wojcik 1982; Dettner and Liepert 1994; Johnson et al. 2001; Lenoir et al. 2001) thereby ensuring that all captured broods are cared for by enslaved workers or could serve as a mechanism by which slave-makers "brand" their slaves, reducing the opportunity for slaves to return to their maternal nest (Alloway 1982). Additionally, I used baits to test whether contact pheromones are sufficient for recognition of slave-maker larvae (see Chapter 1 for rationale).

METHODS

Chemical Influence on Slave-Maker Recognition

To evaluate the ability of *T. longispinosus* and *T. ambiguus* to differentiate slave-maker from conspecific brood, I simultaneously offered four types of larvae to cultures of both host species. The four treatment groups were as follows: Five host species larvae that were each left in contact with five *P. americanus* larvae for 16 hours (conspecific manipulated: CM); b) five *P. americanus* larvae that were each left in contact with five host species larvae for 16 hours (*Protomognathus* manipulated: PM); c) five unmanipulated host species larvae isolated for 16 hours (conspecific unmanipulated: CU); and d) five unmanipulated *P. americanus* larvae isolated for 16 hours (*Protomognathus* unmanipulated: PU).

Sixteen hours prior to experimentation, 10 larvae were extracted from nests of *P. americanus*, and 10 larvae were taken from host species nests. The 10 host species larvae were of the same species as the subject culture being tested in each replicate and all larvae used in this experiment were from nests collected at least 0.5 km from the subject culture. All larvae used in this experiment were late-instar and derived from queenright nests. The larvae were divided into the four treatment groups and isolated in separate nest chambers. A moist piece of cotton was present in the nest chambers to prevent brood desiccation. Handling of larvae and contact manipulation followed the same protocol described in Chapter 1 for treatment of silicone baits.

Larval presentation generally followed the methods reported in studies by Hare and Alloway (1987) and Hare (1996). Just prior to presentation, larvae were transferred to separate clean glass coverslips according to treatment type. The four coverslips were

then juxtaposed inside a subject culture dish, five cm from the nest entrance. The relative position of each coverslip was randomized for each replicate using a random numbers table. I then placed the culture dish on the stage of a dissecting microscope (Wild model M3, Wild Leitz Ltd. Ottawa, ON, Canada) at 64x magnification for easy observation. I recorded the order in which each larva was retrieved into the nest entrance along with the time of retrieval. I also recorded any instances where larvae were cannibalized or abandoned in a midden. Observations ceased once all larvae were retrieved, eaten or abandoned. Following experimentation, I assigned each larva an ordinal number between 1 and 20 based on the order in which it was taken into the nest and the mean retrieval ranks for all treatment types were calculated. Cannibalized or abandoned larvae were given an ordinal number equal to the mean of all larvae not retrieved (see Chapter 1). This process was repeated using 12 *T. longispinosus* cultures containing between 36 and 107 workers (mean ± SE: 63.417 ± 6.583) that were collected from 8 sites and 12 *T. ambiguus* cultures collected from 10 sites containing between 34 and 159 workers (mean ± SE: 72 ± 10.269). The limited number of *P. americanus* larvae available in my collection restricted my sample size to 12. Experimentation took place from 7 to 24 October 2007 and the number of eggs, larvae, pupae, pharate pupae, male pupae, workers, males, alate gynes, dealate gynes, gynadromorphs, and ergatogynes of each species was determined on the same day as experimentation (Table 3-1).

Although the data for both species met the parametric assumption of homogeneity of variance, they were not drawn from a normally distributed population of differences (see Table 3-2 for results of Kolmogorov-Smirnov and equality of variances *F*-tests). Therefore, for both species, differences in mean retrieval ranks among the four larval

types were evaluated using non-parametric Wilcoxon signed-rank tests. To avoid inflation of type I error, I corrected the probability values for focused contrasts among the four possible larval types using the sequential Bonferroni technique (Rice 1989). Because only five of each type of larvae were presented, statistical comparisons among the four larval types for each replicate were not possible. I therefore combined data into strictly conspecific and *P. americanus* categories and compared the retrieval ranks of these categories for each replicate using Wilcoxon signed-ranks tests. Chi-square tests on a 2 x 2 contingency table, with the Yates correction for continuity, tested whether the number of larvae consumed or abandoned differed between species and whether contact with other species affected the consumption of either species of larvae. A series of Mann-Whitney U tests were used to determine whether colony demographics differed between *T. ambiguus* and *T. longispinosus* subject cultures.

Transfer of Slave-Maker Recognition Cues

To test whether host cultures could discriminate between conspecific and slave-maker larvae based on chemicals borne on the larval cuticle, cultures of *T. longispinosus* and *T. ambiguus* were presented with silicone baits left in contact with conspecific larvae and baits left in contact with *P. americanus* larvae. One hour prior to experimentation, the food tray and water bottle of a *T. longispinosus* or *T. ambiguus* culture was removed. A clean glass coverslip (18 mm x 18 mm) containing a silicone bait that had been left in contact with five *P. americanus* larvae and a bait that had left in contact with five conspecific larvae for 16 hours was then introduced into the subject culture two cm from the nest entrance. Within each replicate, the relative size of the two

baits was visually matched as closely as possible and the relative position of each bait on the coverslip was randomized using a random numbers table. Observations lasted five minutes following presentation, during which time I recorded the amount of time workers contacted each bait with their antennae, mandibles, maxilla or maxillary palps. Additionally, I recorded any incidences where a bait was carried into the nest chamber. This process was repeated using 15 *T. longispinosus* cultures collected from 8 sites containing between 29 and 205 workers (mean ± SE: 86.400 ± 14.603) and 15 *T. ambiguus* cultures collected from 9 sites containing between 28 and 169 workers (mean ± SE: 75.800 ± 11.669).

Treatment of baits followed the same experimental protocol as that described in Chapter 1. The larvae used to treat baits were derived from queenright cultures collected at different sites from the site where the subject culture had been collected. Presentations took place from 2 to 6 October 2007 and a census of subject cultures was performed on the same day as experimentation (Table 3-4). As an assistant was available on these dates, I set up a single blind protocol where, the assistant randomized the relative positions of the two bait types on the coverslip and I was unaware of the relative position of each type of bait until after experiments were complete. For *T. longispinosus* and *T. ambiguus* trials, the data met the parametric assumptions of normality (Kolmogorov-Smirnov tests: *T. ambiguus* — $\chi^2 = 2.133$, $P = 0.688$, $N = 15$; *T. longispinosus* — $\chi^2 = 2.133$, $P = 0.688$, $N = 15$) and homogeneity of variance (equality of variances F-tests: *T. ambiguus* — $F = 0.335$, $P = 0.059$, $N = 15$; *T. longispinosus* — $F = 0.555$, $P = 0.283$, $N = 15$). I therefore tested for differences in the time spent in contact with conspecific and *P. americanus* baits for both species using paired t-tests. I

compared the time that *T. ambiguus* and *T. longispinosus* spent in contact with baits using non-parametric Mann-Whitney *U* tests, because the parametric assumptions were not met (Kolmogorov-Smirnov: $\chi^2 = 8.067$, $P = 0.035$, $N = 30$; equality of variances *F*-tests — $F = 0.251$, $P < 0.001$, $N = 30$). Mann-Whitney *U* tests were also used to test whether colony demographics differed between species.

RESULTS

Transfer of Slave-Maker Recognition Cues

There were no differences in colony demographics between *T. ambiguus* and *T. longispinosus* subject cultures (Table 3-1). Figure 3-1 depicts the mean retrieval ranks while Table 3-3 summarizes the results of Wilcoxon signed-rank tests comparing the mean retrieval ranks of manipulated *P. americanus* larvae (PM), manipulated conspecific larvae (CM), unmanipulated *P. americanus* larvae (PU), and unmanipulated conspecific larvae (CU) for *T. ambiguus* and *T. longispinosus* cultures. After adjusting the probability value of focused contrasts among larval types using the Bonferroni technique, differences are only considered significant where $P \leq 0.008$. Except where stated differently there were no significant differences in retrieval order among PM, CM, PU and CU.

T. ambiguus workers retrieved CU significantly earlier than PU ($Z = -2.668$, $P = 0.0076$, $n = 12$). Manipulated conspecific larvae were also retrieved significantly earlier than unmanipulated *P. americanus* larvae ($Z = -2.981$, $P = 0.0029$, $n = 12$) and CM significantly earlier than PM ($Z = -2.944$, $P = 0.0032$, $N = 12$). In three of the 12 *T. ambiguus* replicates conspecific larvae were retrieved significantly earlier than *P.*

americanus larvae (Table 3-3). *T. ambiguus* workers consumed or abandoned 22 PM, 25 PU, 14 CM and 10 CU larvae. While there were significantly more *P. americanus* larvae consumed or abandoned than conspecific larvae ($\chi^2 = 9.681$, $P = 0.002$), this trend was not affected by larval manipulation ($\chi^2 = 0.451$, $P = 0.502$).

T. longispinosus workers also preferentially retrieved unmanipulated conspecific over unmanipulated slave-maker larvae ($Z = -2.707$ $P = 0.0068$, $N = 12$). The mean retrieval rank of PU was also greater than that of PM, although this difference only approaches significance ($Z = -5.514$, $P = 0.0119$, $N = 12$). CM was also retrieved earlier than PU but this difference was also not quite significant ($Z = -2.436$, $P = 0.0149$, $N = 12$). Conspecific larvae were retrieved significantly earlier than *P. americanus* larvae by seven of the 12 *T. longispinosus* cultures. *T. longispinosus* workers also consumed or abandoned significantly more *P. americanus* than conspecific larvae ($\chi^2 = 8.309$, $P = 0.004$), but this trend was not affected by larval manipulation ($\chi^2 = 2.352$, $P = 0.125$). In total *T. longispinosus* workers consumed or abandoned 13 PM, 21 PU, 10 CM and 5 PU larvae.

Table 3-1: Results from Mann-Whitney *U* tests comparing colony demographics of *T. ambiguus* and *T. longispinosus* cultures used in experiment testing transfer of slave-maker recognition cues.

	T. ambiguus	*T. longispinosus*	Mann-Whitney *U*		
	Mean ± SE	Mean ± SE	*Z*	*P*	*n*
Eggs	0.00 ± 0.000	0.00 ± 0.000	0.000	>0.999	12
Larvae	52.25 ± 7.888	60.67 ± 6.655	-0.953	0.341	12
Pupae	2.27 ± 1.175	1.83 ± 0.644	0.462	0.644	12
Male Pupae	0.27 ± 0.249	0.00 ± 0.000	-0.346	0.729	12
Pharate pupae	0.17 ± 0.160	0.00 ± 0.000	-0.346	0.729	12
Males	1.67 ± 1.108	1.42 ± 0.595	-0.577	0.564	12
Workers	72.00 ± 9.832	55.42 ± 6.737	-1.155	0.248	12
Gynadromorphs	0.00 ± 0.000	0.00 ± 0.000	0.000	>0.999	12
Ergatogynes	0.00 ± 0.000	0.00 ± 0.000	0.000	>0.999	12
Alate Gynes	0.00 ± 0.000	0.00 ± 0.000	0.000	>0.999	12
Dealate Gynes	1.75 ± 0.292	1.25 ± 0.125	-0.953	0.341	12

Table 3-2: Results of Kolmogorov-Smirnov (K-S) tests for normality, equality of variances *F*-tests (*F*-max) and Wilcoxon signed-ranks tests among conspecific manipulated (CM), *P. americanus* (PM), conspecific unmanipulated (CU), and *P. americanus* unmanipulated (PU) larval retrieval data for *T. ambiguus* and *T. longispinosus*.

		K-S		F-max		Wilcoxon		
		χ^2	*P*	*F*	*P*	*Z*	*P*	*N*
T. ambiguus	PM, CM	6.000	0.100	2.939	0.088	-2.944	0.003	12
	PM, PU	4.167	0.249	1.562	0.472	-0.942	0.346	12
	PM, CU	4.167	0.249	0.950	0.933	-1.334	0.182	12
	CM, PU	13.500	0.002	0.531	0.309	-2.981	0.003	12
	CM, CU	4.167	0.249	0.323	0.074	-0.628	0.530	12
	PU, CU	13.500	0.002	0.608	0.422	-2.668	0.008	12
T. longispinosus	PM, CM	4.167	0.249	0.831	0.765	-1.580	0.114	12
	PM, PU	10.667	0.010	1.016	0.980	-2.514	0.012	12
	PM, CU	6.000	0.100	0.622	0.444	-1.726	0.084	12
	CM, PU	13.500	0.002	1.222	0.746	-2.436	0.015	12
	CM, CU	1.500	0.945	0.749	0.639	-0.889	0.374	12
	PU, CU	13.500	0.002	0.613	0.430	-2.707	0.007	12

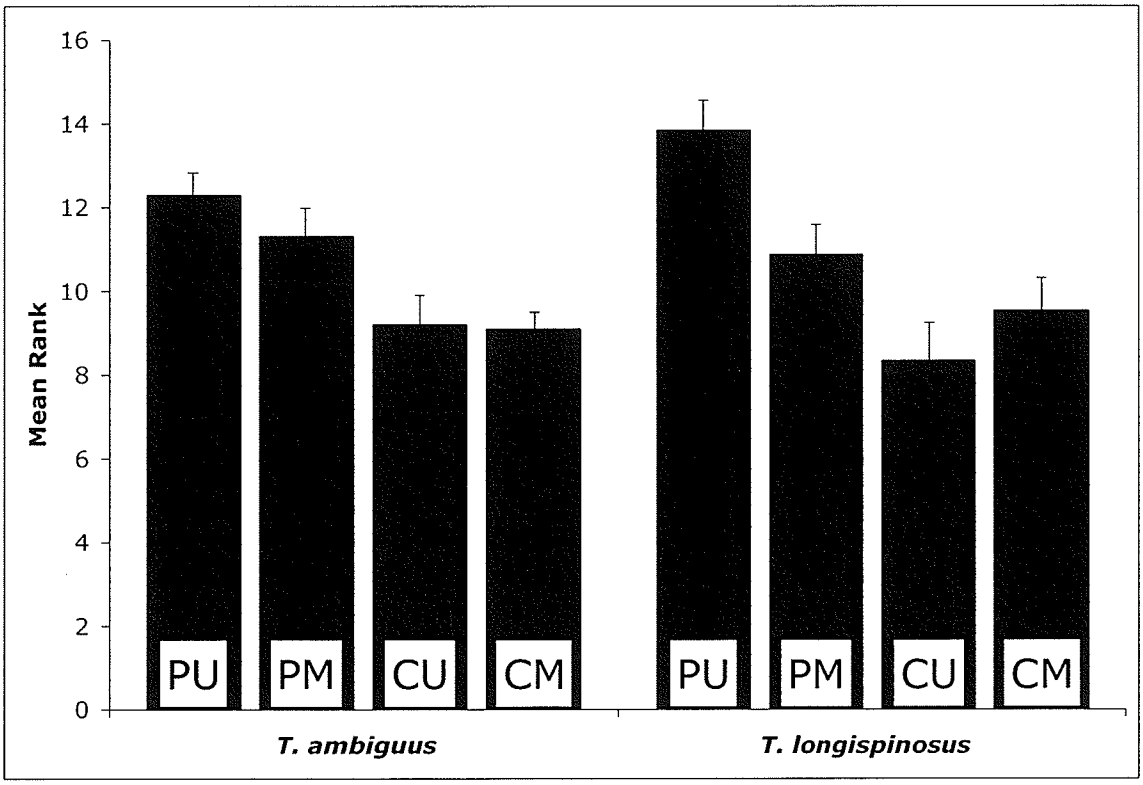

Figure 3-1: Mean retrieval rank (+ SE) by *T. ambiguus* and *T. longispinosus* of conspecific larvae that were left in contact with 5 *P. americanus* larvae for 16 hours (conspecific manipulated: CM), *P. americanus* larvae that were left in contact with 5 conspecific larvae for 16 hours (*P. americanus* manipulated: PM), unmanipulated conspecific larvae isolated for 16 hours (conspecific unmanipulated: CU), and 5 unmanipulated *P. americanus* larvae isolated for 16 hours (*P. americanus* unmanipulated: PU).

Table 3-3: Results of Wilcoxon signed-ranks tests comparing mean retrieval ranks (± SE) of conspecific and *P. americanus* larvae for *T. ambiguus* and *T. longispinosus* individual trials. Significant differences ($\alpha \leq 0.008$) are in bold.

Species	Culture	Conspecific mean± SE	*P. americanus* mean± SE	*Z*	*P*	*N*
	18-1	9.100± 1.785	11.900± 1.912	-1.718	0.086	10
	15-86	9.500± 2.098	11.800± 1.744	-1.580	0.114	10
	11-43	9.750± 1.618	11.250± 1.748	-0.676	0.499	10
	T.a. 2-17	**7.200± 1.705**	**13.400± 1.166**	**-2.369**	**0.018**	**10**
	T.a. 5-33	9.900± 1.822	11.100± 1.969	-2.820	0.069	10
T. ambiguus	7-13	**7.650± 1.836**	**13.350± 1.138**	**-2.366**	**0.018**	**10**
	T.a. 6-19	8.700± 1.265	12.200± 2.257	-1.682	0.093	10
	18-12	9.350± 1.821	11.300± 1.748	-1.066	0.286	10
	16-20	**8.800± 1.541**	**12.200± 2.065**	**-2.192**	**0.028**	**10**
	14-4	10.000± 1.890	11.000± 1.925	-1.007	0.314	10
	18-14	9.800± 1.289	11.200± 2.347	-0.866	0.386	10
	T.a. 2-30	10.100± 2.014	10.900± 1.785	-0.829	0.407	10
	16-7	9.600± 1.851	11.400± 1.863	-1.470	0.142	10
	T.l. 6-31	**6.600± 1.360**	**14.400± 1.462**	**-2.803**	**0.005**	**10**
	1-15	11.400± 2.029	9.600± 1.727	-1.244	0.214	10
	19-9	12.650± 1.806	8.350± 1.745	-1.784	0.075	10
	T.l. 5-3	**7.250± 1.611**	**13.750± 1.500**	**-2.497**	**0.013**	**10**
T. longispinosus	7-26	7.950± 1.749	12.350± 1.908	-1.886	0.059	10
	7-47	**7.300± 1.932**	**13.700± 1.126**	**-2.039**	**0.042**	**10**
	T.l. 6-42	**8.400± 1.536**	**12.600± 1.998**	**-2.803**	**0.005**	**10**
	7-32	**7.100± 1.464**	**13.900± 1.629**	**-2.701**	**0.007**	**10**
	13-7	**11.600± 1.579**	**14.300± 1.126**	**-2.023**	**0.043**	**10**
	7-71	8.300± 1.764	12.100± 1.815	-1.753	0.080	10
	18-6	**9.100± 1.441**	**11.900± 2.203**	**-1.937**	**0.053**	**10**

Chemical influence on slave-maker recognition

There was no difference in the time *T. longispinosus* ($t = 0.041$; $P = 0.968$; $N = 15$; Figure 3-2) and *T. ambiguus* ($t = 0.448$; $P = 0.661$; $N = 15$; Figure 3-2) workers antennated or orally contacted baits treated with conspecific larvae and baits left in contact with *P. americanus* larvae. Overall, there was no significant difference in the time that *T. longispinosus* and *T. ambiguus* workers contacted both types of baits ($Z = -1.759$; $P = 0.0785$; $N = 30$; Figure 3-2). *T. longispinosus* workers retrieved two conspecific baits and one *P. americanus* bait, while *T. ambiguus* workers retrieved zero conspecific baits and one *P. americanus* bait. Neither of these differences was significant (*T. longispinosus*: $\chi^2 = 0.000$, $P > 0.999$; *T. ambiguus*: $\chi^2 = 0.000$, $P > 0.999$). Colony demographics did not significantly differ between species (Table 3-4).

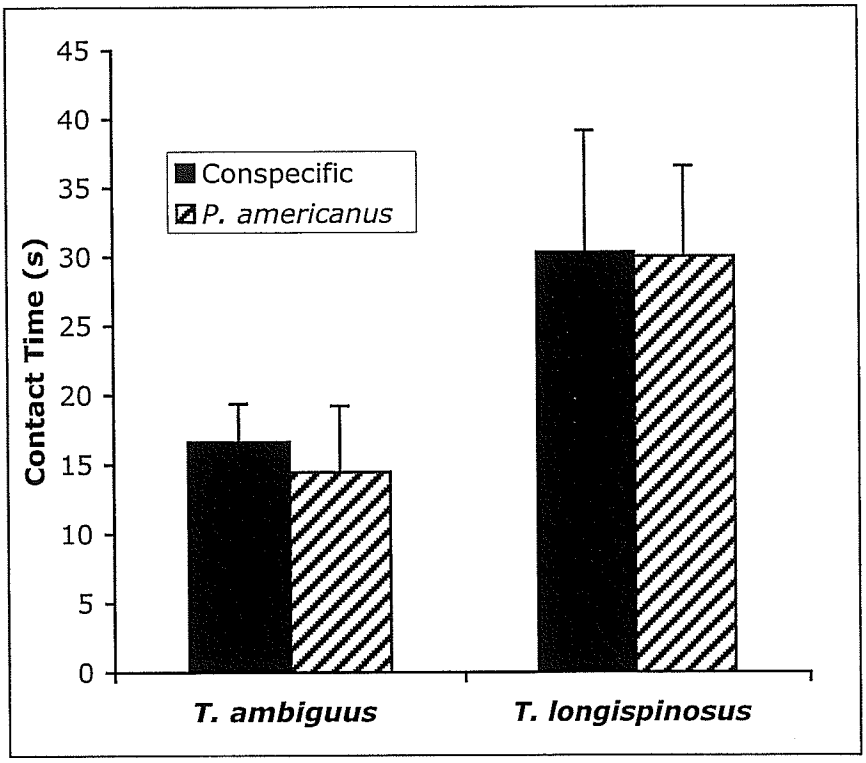

Figure 3-2: Mean time (+ SE) *T. longispinosus* and *T. ambiguus* workers spent in oral or antennal contact with silicone baits left in contact with conspecific larvae and baits left in contact with *P. americanus* larvae for 16 hours.

Table 3-4: Results from Mann-Whitney *U* tests comparing colony demographics of *T. ambiguus* and *T. longispinosus* cultures used in experiment testing chemical influence on slave-maker recognition

	T. ambiguus	*T. longispinosus*	Mann-Whitney *U*		
	Mean ± SE	Mean ± SE	*Z*	*P*	*n*
Eggs	0.00 ± 0.000	0.00 ± 0.000	0.000	>0.999	15
Larvae	62.27 ± 5.910	73.80 ± 12.218	-0.207	0.836	15
Pupae	3.07 ± 1.205	0.73 ± 0.316	-1.493	0.135	15
Male Pupae	0.27 ± 0.153	0.00 ± 0.000	-0.933	0.351	15
Pharate pupae	0.40 ± 0.289	0.47 ± 0.236	-0.518	0.604	15
Males	2.27 ± 1.161	1.87 ± 1.158	-0.767	-0.443	15
Workers	77.47 ± 9.932	82.73 ± 11.222	-0.166	0.868	15
Gynadromorphs	0.07 ± 0.067	0.00 ± 0.000	-0.311	0.756	15
Ergatogynes	0.00 ± 0.000	0.00 ± 0.000	0.000	>0.999	15
Alate Gynes	0.00 ± 0.000	0.00 ± 0.000	0.000	>0.999	15
Dealate Gynes	1.47 ± 0.236	1.40 ± 0.190	0.041	0.967	15

DISCUSSION

Throughout the evolution of communication and recognition, different intentions of signalers and receivers may result in an evolutionary arms race where the signaler benefits by deceiving the receiver (Dawkins and Krebs 1978). In this scenario, with time, the receiver should become less likely to accept these unreliable signals (Dawkins and Krebs 1978; Foitzik et al. 2001, 2003). With this in mind, we would expect that *T. ambiguus* and *T. longispinosus* should have evolved means to differentiate *P. americanus* from conspecific individuals. Previous studies have shown that *P. americanus* can have a severe impact on host fitness, resulting in a coevolutionary arms race between host and parasite (Foitzik et al. 2001; Foitzik and Herbers 2001; Blatrix and Herbers 2003; Brandt and Foitzik 2004; but see Hare and Alloway 2001). My results revealed that *T. longispinosus* and *T. ambiguus* workers both retrieved unmanipulated *P. americanus* larvae later than unmanipulated conspecific larvae. In seven of the 12 *T. longispinosus* replicates and three of the *T. ambiguus* replicates, conspecific larvae were carried into the nest earlier than *P. americanus* larvae, whereas there were no cases where *P. americanus* larvae were retrieved earlier. Further, both species consumed or abandoned more slave-maker than conspecific larvae. These findings suggest that workers of both host species are able to recognize slave-maker larvae and deem them less appealing than larvae of their own species. Unenslaved *T. longispinosus* and *T. ambiguus* workers also recognize *P. americanus* workers as well as enslaved conspecifics as enemies (Alloway 1989; Alloway and Keough 1990). These results do not indicate that the hosts have won the arms race, as both species are still commonly parasitized. It merely appears that *Temnothorax* workers currently residing in intact host cultures do not perceive *P.*

americanus larvae as inherently attractive. Because all *P. americanus* larvae were eventually carried into a nest, a refuse pile or cannibalized, I can also rule out the possibility that larvae are chemically inert or neutral (Lenoir et al. 2001; Cervo et al. 2008).

Several authors advocate manipulation of host recognition processes as the primary means in which slave-makers gain acceptance by their slaves (Jaisson 1975; Le Moli 1980; Lenoir et al. 1987; Mori et al. 2000). Early learning certainly seems to play an important role in the brood discrimination abilities of *T. longispinosus* and *T. ambiguus*. Exposure to conspecific larvae early in life activates a later preference for conspecific larvae, whereas exposure to allospecific larvae or social isolation fosters non-preferential larval acceptance (Hare and Alloway 1987). Interestingly, Alloway and Hare (1989) showed that *T. longispinosus* prefer *P. americanus* larvae independent of early experience with larvae (i.e., exposure to conspecific larvae, slave-maker larvae, or social isolation), suggesting that *P. americanus* larvae bear a potent attractant. Nevertheless, my results reveal a clear preference for conspecific over slave-maker larvae. I can only speculate as to the reasons for the apparent contradictory results between Alloway and Hare's (1989) experiment and my own. In their study, Alloway and Hare (1989) employed groups of *T. longispinosus* workers that had been isolated from their maternal nests while pupae. These pupae were allowed to eclose in the presence of five conspecific larvae, five *P. americanus* larvae or in social isolation. If we assume that individuals learn cues characteristic of their colony to form a template that is later compared to the phenotype of encountered individuals (Holmes and Sherman 1982), then the workers in Alloway and Hare's (1989) study were deprived of several possible referents that might

contribute to a recognition template ensuring preferential acceptance of conspecific nestmates. Moreover, long-term learning processes may shape brood-acceptance behaviour. Queens in particular can influence the recognition ability of workers. For example, *Camponotus* spp. workers raised in queenright cultures are less tolerant of unfamiliar individuals than workers from queen-less cultures (Carlin and Hölldobler 1986). *T. ambiguus* workers surely form a social attachment to whatever species of queen is present early in life (Alloway and Ryckman 1991). Also, colony takeover by queens of the dulotic *Polyergus breviceps* cannot be achieved in *Formica gnava* nests that lack a queen (Topoff et al 1988; Topoff and Zimmerli 1993). Social interactions among workers could also modify brood acceptance behaviour (Le Moli and Mori 1982). The transmission of pheromones among workers (likely mediated by trophalaxis) is also important for the development of social behaviour (Stuart 1987a, b, c, 1988).

Despite my findings, I cannot disregard Alloway and Hare's (1989) discovery of a chemical substance found on *P. americanus* larvae that attracts *T. longispinosus* workers. While not enough to override the recognition mechanisms established in workers in my study, I suspect that the true significance of such an attractant is realized within an established slave-making nest. I hypothesize that Alloway and Hare's (1989) attractant could stimulate host individuals raised in a slave-maker nest (slaves) to preferentially care for slave-maker larvae over captured brood while simultaneously ensuring tending of the next generation of slaves. This hypothesis could be tested in a 2 x 2 x 2 factorial experiment in which the preference for conspecific and *P. americanus* larvae is contrasted for free-living and enslaved *T. longispinosus* and *T. ambiguus* workers. I predict that free-living workers prefer conspecific larvae while enslaved individuals

prefer *P. americanus* larvae. It may be unnecessary for *P. americanus* larvae to appeal to unenslaved host workers as free-living hosts would only encounter slave-maker brood in the rare occurrence of a reverse slave-raid (Alloway 1980). I must be cautious comparing my findings to those reported by Alloway and Hare (1989), as differences in experimental protocol may be responsible for the apparently contradictory results. Specifically, the timing of experimentation and the duration of culturing under uniform conditions could influence larval preference.

Beyond the overall preference for conspecific larvae, I found some evidence that physical contact with conspecific larvae made slave-maker larvae more attractive to *T. longispinosus*. The only significantly different retrieval ranks among the four larval types (unmanipulated *P. americanus* — PU; manipulated *P. americanus* —PM; unmanipulated conspecific — CU; manipulated conspecific — CM) in *T. longispinosus* trials was between CU and PU, where PU larvae were retrieved later than CU larvae. While just shy of significance, *T. longispinosus* workers also tended to retrieve PM larvae before PU and CM before PU. Taken collectively, these results suggest that contact with *T. longispinosus* larvae increases the acceptance of *P. americanus* larvae, but contact with *P. americanus* larvae does not affect the acceptance of conspecific larvae in *T. longispinosus* cultures. Therefore, when discriminating between conspecific and *P. americanus* larvae, *T. longispinosus* appear to rely on similarities rather than differences between their recognition template and the encountered larval chemical signature. The opposite trend appears in the nestmate recognition mechanisms of *Cataglyphis niger* (Lahav et al. 1999).

Invading queens of the socially parasitic *Temnothorax kutteri* and the myrmecophilous spider *Cosmophasis bitaeniata* may acquire brood pheromones by immersing themselves in a brood pile (Franks et al. 1990; Elgar and Allan 2002). *P. americanus* queens may use a similar strategy when invading *T. longispinosus* nests. Combined with the effect of propaganda substances, such chemical disguise may facilitate the integration of a slave-maker queen (Allies et al. 1986; Topoff et al. 1988). Even after a slave-making queen has overtaken a host colony her brood are still confronted by the possibility of being recognized by the slave-workers. Alternatively or perhaps additionally, the transfer of brood pheromones that may occur over the longer term in a mixed-species brood pile may increase the acceptance of *P. americanus* brood by host workers. While it is pleasing to muse over such possibilities, further study is clearly necessary to ascertain the function and chemistry of brood pheromones. Specifically, a detailed description of cuticular hydrocarbon profiles, using gas chromatography—mass spectrometry, of *P. americanus* larvae relative to its hosts is required.

Contact between larvae did not appear to affect *T. ambiguus* preference for conspecific over *P. americanus* larvae. This suggests that transfer of cues through contact is not enough to significantly affect a *T. ambiguus* larva's own recognition signature or that *T. ambiguus* workers are impervious to such alterations. If, as I proposed, acceptance of *P. americanus* queens and/or larvae by *T. longispinosus* is improved by acquiring pheromones from larvae, the lack of transferability from *T. ambiguus* larvae suggests that *P. americanus* cannot use this technique when enslaving *T. ambiguus*. Such a scenario

may further explain why *T. longispinosus* is the preferred host species of *P. americanus* (Blatrix and Herbers 2003).

Regardless of the preference for conspecific over *P. americanus* larvae, both species showed no difference in the amount of time they contacted baits treated with either species of larvae. Any discriminator substances that transferred to baits by direct physical contact were not sufficient on their own for the species level discrimination seen with whole larvae. As I suggested in Chapter 1, other methods of treating baits, such as soaking them in larval extract, may better reveal the importance of cuticular chemicals in larval recognition.

GENERAL DISCUSSION

Preferential Versus Exclusive Brood Acceptance

Throughout my experiments, several larvae were either consumed or abandoned; however, most were ultimately carried into the nest chamber. So even where *T. ambiguus* and *T. longispinosus* workers can discriminate among larvae, they appear to practice preferential rather than exclusive larval acceptance. Other studies testing the brood-discrimination behaviour of these species report similar findings (Alloway 1982; Hare and Alloway 1987; Alloway and Hare 1989; Hare 1996). While nonexclusive brood acceptance is common in eusocial species (Carlin 1988; Gamboa et al. 1987), it is by no means universal (Jaisson 1975; Jaisson and Fresneau 1978; Le Moli and Passetti 1977; 1978). For example, when *Formica lugubris* workers are offered conspecific pupae and pupae of their congener *F. rufa,* only the former are accepted (Le Moli and Mori 1982). Other ants such as those in the genus *Camponotus* accept alien brood into their nests but kill those individuals after maturation (Carlin 1988). Because I did not follow the long-term survival of larvae offered in my study, I cannot be certain of their fate; however, both species regularly form mixed-species colonies and typically prey on brood only after territorial conflicts (Alloway 1980; Stuart and Alloway 1983). Moreover, retrieval preference is correlated with survival of larvae for *T. longispinosus* (Alloway and Hare 1989). Because *T. ambiguus* and *T. longispinosus* workers contribute to whatever nest in which they mature (Trampus 1997), accepting foreign brood provides obvious benefits by increasing the work force. This may be especially important in *Temnothorax* species whose colonies rarely contain more than 100 workers (Alloway 1980; Alloway et al. 1982; Stuart and Alloway 1983) and less so in species with more

populous colonies. Ants of the genus *Formica* can have upwards of 2000 workers per colony and all tend to exclusively accept familiar to unfamiliar larvae (Jaisson 1975; Le Moli and Passetti 1977; 1978). Colony size may be an important influence regulating brood acceptance behaviour.

Culturing Effects and Consequences

The minute size and cryptic nesting habits of *Temnothorax* ants necessitates a laboratory setting when directly observing their behaviour. Performing behavioural experiments under uniform laboratory conditions eliminates many effects brought on by unforeseen environmental variables. In uniform conditions, however, we must be wary when drawing conclusions concerning the differences between species. Indeed, behaviour displayed in the lab is out of the natural context and certain actions may be modified. For example, artificial nests larger than those found in nature can cause polydomous *Temnothorax* colonies to amalgamate into one nest (Herbers and Tucker 1986). Artificial diet that is lacking in certain elements may also cause species to alter their behaviour (Buschinger and Pfeifer 1988). Laboratory settings such as temperature, humidity and light intensity may be better suited for one species over the other. Headley (1943), Creighton (1950), Talbot (1957), Alloway and Hare (personal communication) all suspect that *T. ambiguus* are more commonly found in warm sunny areas, whereas *T. longispinosus* prefers cooler, shady habitats. Based on personal observations I doubt that such wide-ranging separation of gross habitats exists between these species; however, interspecific competition has likely partitioned each species into a different ecological niche and therefore different abiotic conditions between the species may exist on average.

Observed differences such as the greater propensity of *T. longispinosus* to contact larval baits may simply be an artifact of the lab. Alternatively, these findings may reflect general differences in activity patterns between the species (Trampus 1997).

Colony odour can be influenced by the environment and food (Liang and Silverman 2000). With time, discriminators that had been derived from the environment could diminish or be lost altogether. Where ants assess their relatedness to encountered individuals based on differences from their recognition template (Holmes and Sherman 1982), recognition systems may become less exacting with homogeneity of environmental cues. Eventually, under standardized culturing regimes, individuals may become less hostile towards each other (Stuart 1987; Heinze et al. 1996; Liang and Silverman 2000; Florane et al. 2004). *Solenopsis invicta* workers, for example, react less aggressively to non-colony members reared in uniform laboratory conditions than to individuals originating from natural conditions (Obin 1986). The ants in my experiments may have been made artificially amenable to each other with culturing and may have accepted more unpreferred larvae than they naturally would. In this case the behavioural patterns I observed would be more pronounced in the field. It would be useful to replicate my experiments shortly after nest collection or perform a separate controlled recognition study to determine whether culturing time affects larval acceptance.

Contact Chemistry

Considered collectively, my experiments involving silicone baits show that chemical cues present on the larval cuticle are sufficient in the absence of tactile and behavioural stimuli to attract attention from workers. Further, these chemicals transfer

through direct contact. The cues that transferred to silicone baits, however, were insufficient to allow discrimination among bait types. *T. longispinosus* and *T. ambiguus* workers did not spend significantly more time contacting baits treated with nestmate versus non-nestmate, conspecific versus allospecific, or conspecific versus slave-maker larvae, nor did they demonstrate a proclivity to retrieve any one bait type over the other. My findings do not necessarily indicate that cuticular pheromones are not the primary means of discriminating among larvae, but do suggest that contact alone is inadequate to acquire a complete chemical signature. It is possible that many of the surface chemicals necessary for refined discrimination did not transfer to the pieces of silicone.

Although I have found various differences in behaviour towards different types of larvae, I cannot say with certainty which chemical elements contribute to larval recognition in *T. ambiguus* and *T. longispinosus*. Future studies must employ gas-liquid chromatography (GC) techniques to compare the cuticular hydrocarbon profiles of *P. americanus*, *T. longispinosus* and *T. ambiguus* larvae. By coupling GC with mass spectrometry (MS), one could hypothesize which constituents of the GC profile contribute to species recognition. Finally, by combining GC-MS with behavioural assays, accurate conclusions could be made regarding the chemical mechanisms controlling larval recognition in *T. ambiguus* and *T. longispinosus*. While it has been established that adult *P. americanus* (Brant et al. 2005), other socially parasitic ants (Bergström and Löfqvist 1968; Franks et al. 1990; Habersetzer and Bonavita-Cougourdan 1993; Kaib et al. 1993) and myrmecophilous beetles (Vander Meer and Wojcik 1982) have similar cuticular spectra as their host(s), such a study would provide the first comprehensive data on larvae within a dulotic system.

Summary and conclusions

In the preceding chapters I described experiments in which cultures of *T. ambiguus* and *T. longispinosus* were simultaneously offered different types of larvae. I attempted to elucidate the degree that these species can discriminate nestmate from non-nestmate, conspecific from allospecific, and conspecific from the slave-maker *P. americanus* larvae. Further, by offering cultures silicone baits that had been left in contact with different larval types, I explored transference of larval contact pheromones along with their importance in recognition.

I found that both species preferentially retrieved nestmate larvae over non-nestmate conspecific larvae and consumed less nestmate than non-nestmate larvae. Generally, the ability to discriminate nestmates from foreign individuals is vital to the social organization of ant colonies and is advantageous in the contexts of inclusive fitness, cooperation or both (Hamilton 1964; Wilson 1975a; Jaisson 1991). I also found evidence that *T. ambiguus* workers retrieve non-nestmate larvae from their own collection site earlier than non-nestmates from other collection sites, likely reflecting the polydomous nature of this species (Alloway et al. 1982; Del Rio Pesado and Alloway 1983; Stuart 1985; Herbers 1986); however, no such trend was evident in *T. longispinosus* trials. *T. ambiguus* discriminated conspecific from allospecific larvae while *T. longispinosus* did not, suggesting a greater proficiency or perhaps persistence of species-level recognition in *T. ambiguus*. Both species exhibited a clear preference for conspecific larvae over the larvae of the slave-making ant *P. americanus*, but reciprocal contact between *P. americanus* and conspecific larvae only affected the response of *T. longispinosus*. Recognizing *P. americanus* larvae as enemies (Alloway 1989) could serve

as a counter-adaptation of *T. ambiguus* and *T. longispinosus* to resist social parasitism (Foitzik et al. 2001). Because a number of slave-maker larvae were still accepted into host nests, however, such a defense mechanism would be far from perfect. My results on the surface appear to contradict those of Alloway and Hare (1989) who showed that manipulated *T. longispinosus* workers prefer *P. americanus* larvae to conspecific larvae. Differences in the potential referents available to workers in Alloway and Hare's (1989) study and my own likely account for these different findings, and as such, offer insight into as yet unexplored mechanisms contributing to the normal ontogeny of larval recognition and acceptance in these species. A future study must test whether enslaved and free-living *Temnothorax* workers differentially accept conspecific and *P. americanus* larvae. Further studies are also needed to identify the exact components that contribute to a developing worker's recognition template.

T. ambiguus and *T. longispinosus* workers spent significantly more time inspecting larval-sized pieces of silicone with their antennae and/ or mouth parts when the baits had been left in contact with whole larvae. Further, the time of contact did not seem to affect the amount of attention baits received. Although these results suggest that some discriminators transfer by direct physical contact from the larval cuticle to the baits, these discriminators were not enough on their own to enable discrimination within or between species.

Rigorous examination of the intricate communication and recognition systems within the ants has generated an abundance of knowledge that contributes to our understanding of their social organization. The experiments described in this thesis

provide insight into the brood-discrimination abilities of two *Temnothorax* species and offer a foundation for future behavioural, chemical and ecological studies.

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