BEHAVIOURAL, MORPHOLOGICAL AND CHEMICAL STRATEGIES IN SOCIAL INTEGRATIONS OF MYRMECOPHILIDAE CRICKETS TO HOST ANTS, *Paratrechina longicornis* AND *Anoplolepis gracilipes*

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by

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LIST OF ABBREVIATIONS

AI	Aggression index
ANOSIM	Analysis of similarities
CHC	Cuticular hydrocarbon
df	Degree of freedom
et al.	And others
NMDS	Non-metric multidimensional scaling
PERMANOVA	Permutational multivariate analysis of variance
PERMDISP	Multivariate homogeneity of multivariate variances
RH	Relative humidity
RI	Retention index
S.D.	Standard deviation
τ_b	Kendall's tau-b correlation
tr	trace amount
χ^2	Chi-square

PERILAKU, MORFOLOGI DAN STRATEGI KIMIA DALAM INTEGRASI SOSIAL CENGKERIK MYRMECOPHILIDAE TERHADAP SEMUT PERUMAH, *Paratrechina longicornis* DAN *Anoplolepis gracilipes*

ABSTRAK

Tesis ini bertumpu kepada strategi integrasi oleh cengkerik semut dari famili Myrmecophilidae dalam koloni-koloni semut invasif *Paratrechina longicornis* (Latreille, 1802) dan *Anoplolepis gracilipes* (Smith, 1857) (Hymenoptera: Formicidae) melalui perilaku, morfologi dan integrasi strategi kimia. Hasil kajian mendapati spesies cengkerik semut *Myrmecophilus albicinctus* Chopard, 1924 dan *Myrmecophilus americanus,* Saussure, 1877 kurang cenderung memakan anak semut seterusnya menyebabkan penurunan keagresifan semut perumah. *Myrmophilellus pilipes* (Chopard, 1928), *Myrmecophilus hebardi* Mann, 1920 dan *Myrmecophilus dubius* Saussure, 1877 lebih cenderung memakan anak semut dan menyebabkan semut perumah lebih agresif. Perilaku *Myrme. albicinctus* dan *Myrme. americanus* didapati lebih berintegrasi dalam koloni keasalannya. Mereka didapati lebih kerap merias semut perumah, aktiviti trofalaksis stomodeal, mendiami dalam sarang tiruan dengan semut perumah dan bergantung atas semut perumah untuk kemandirian. Sebaliknya, cengkerik semut yang tidak berintegrasi menggunakan strategi perilaku alternatif untuk penyesuaian diri melalui pelaburan dalam strategi pengelakkan yang lebih baik (melarikan diri lebih cepat atau perilaku yang tidak menimbulkan syak) dan mempunyai diet yang lebih pelbagai untuk berevolusi bersama semut perumah. *Myrmecophilus albicinctus* dan *Myrme. americanus* mempunyai nisbah panjang gigi/mandibel yang kecil dan hujung gigi tumpul yang menunjukkan pergantungan

kedua-dua cengkerik ini terhadap makanan berbentuk cecair daripada semut perumah. *Myrmecophilus dubius, Myrme. hebardi* dan *Myrmo. pilipes* menunjukkan nisbah panjang kaki/badan yang besar menunjukkan kedua-dua spesies cengkerik tersebut mempunyai kebolehan untuk melarikan diri dari semut perumah dengan tangkas. Dalam kajian ini, profil hidrokarbon kutikular (CHC) atas semut perumah (*A. gracilipes*), cengkerik yang berintegrasi (*Myrme. albicinctus*) dan cengkerik yang tidak berintegrasi (*Myrme. hebardi*) digunakan untuk menyiasat pengaruh keserasian kimia atas integrasi sosial antara cengkerik dan semut perumah. Secara kualitatif, tidak ada perbezaan yang telah dijumpa dalam mimikan kimia antara profil CHC kedua-dua cengkerik semut perumah dengan profil CHC semut perumah. Walaubagaimanapun, secara kuantitatif, apabila subset profil CHC tertentu dianalisasi (dimethylalkanes), menunjukkan cengkerik berintegrasi *Myrme. albicinctus* berkelompok dengan semut perumah asal. Dalam perbandingan dengan *Myrme. hebardi* (dengan dimethylalkanes: Kemiripan Bray-Curtis = 34 – 63), *Myrme. albicinctus* mempunyai keserasian kimia yang lebih tinggi (dengan dimethylalkanes: Kemiripan Bray-Curtis = 68 – 84) dengan profil CHC semut perumah. Ini mengurangkan perilaku agresif *A. gracilipes* terhadap *Myrme. albicinctus*. Kajian ini menunjukkan bahawa cengkerik menggunakan penggabungan strategi berbeza untuk berintegrasi dalam koloni semut perumah. Cengkerik yang berintegrasi menggunakan strategi yang lebih terperinci seperti penurunan virulensi dan mimikan kimia sementara cengkerik yang tidak berintegrasi lebih bergantung kepada perilaku dan morfologi yang umum untuk berintegrasi.

BEHAVIOURAL, MORPHOLOGICAL AND CHEMICAL STRATEGIES IN SOCIAL INTEGRATIONS OF MYRMECOPHILIDAE CRICKETS TO HOST ANTS, *Paratrechina longicornis* AND *Anoplolepis gracilipes*

ABSTRACT

This thesis focusses on the integration strategies of ant crickets from the family Myrmecophilidae in colonies of invasive ants, *Paratrechina longicornis* (Latreille, 1802) and *Anoplolepis gracilipes* (Smith, 1857) (Hymenoptera: Formicidae) through behavioural, morphological, and chemical integration strategies. Results obtained demonstrated that ant cricket species of *Myrmecophilus albicinctus* Chopard, 1924 and *Myrmecophilus americanus,* Saussure, 1877 showed low behavioural tendency in the consumption of ant broods and thus resulting in lower aggression exerted by their host ants. *Myrmophilellus pilipes* (Chopard, 1928), *Myrmecophilus hebardi* Mann, 1920 and *Myrmecophilus dubius* Saussure, 1877 consumed a higher percentage of ant brood and elicited greater aggression levels from host ants. *Myrmecophilus albicinctus* and *Myrme. americanus* were more behaviourally integrated in their colony of origin by frequently grooming host ants, frequently engaging in stomodeal trophallaxis, inhabiting inside the artificial nest with their host ants and depended on their host ants for survival. On the other hand, non-integrated ant crickets utilized alternative behavioural strategies for adaptation by investing in better avoidance strategies (swift escapes or a prudent behaviour) and having a broader diet to co-evolved with their host ants. *Myrmecophilus albicinctus* and *Myrme. americanus* showed smaller relative tooth/mandible length and a reduced cutting edge in the incisor, reflecting on their heavy reliance on liquid food from ants. *Myrmecophilus dubius, Myrme. hebardi* and

Myrmo. pilipes showed a longer relative hind leg/body lengths, reflecting on their reliance of frequent swift escape movements and avoidance from worker ant contact. The cuticular hydrocarbon (CHC) profiles of host ants (*A. gracilipes*), integrated ant cricket (*Myrme. albicinctus*) and non-integrated ant cricket (*Myrme. hebardi*) were investigated to determine if chemical accuracy to host CHC profiles influences social integration. Qualitatively, no differences were found in similarity patterns between both the ant cricket CHC profiles to host CHC profiles. However, quantitatively, integrated ant crickets of *Myrme. albicinctus* were found to cluster together with host worker ants from their colony of origin when specific subsets of CHC profiles (dimethylalkanes) was analyzed. *Myrmecophilus albicinctus* showed higher chemical accuracy (with dimethylalkanes: Bray-Curtis similarities = 68 – 84) to host CHC profile compared to *Myrme. hebardi* (with dimethylalkanes: Bray-Curtis similarities = 34 – 63), reflecting on the lowered aggression exhibited by *A. gracilipes* towards *Myrme. albicinctus*. This study has shown that ant crickets utilized a combination of differential strategies to integrate in host ant colonies. Integrated ant crickets utilized more elaborate strategies such as lowered virulence and chemical mimicry whereas non-integrated ant crickets relied more on their generalized behavior and morphology for integration.

CHAPTER 1

GENERAL INTRODUCTION

Parasitism is regarded as one of the most successful ways of life among living organisms due to the rate it has evolved and the diversity of parasites (Foitzik et al., 2001; de Meeûs and Renaud, 2002; Combes, 2005) thus, making it a model system for co-evolutionary studies. Parasites are often involved in a co-evolutionary arms race with their host since both groups have differential interest in maximizing fitness. Parasites will continuously evolve adaptations to overcome host defenses and utilized host resources, whereas host responds by evolving behavioral, chemical, or immunological defenses against parasites (Dawkins and Kreb, 1979; Schmid-Hempel, 2011; Poulin, 2011) until a dynamic equilibrium is achieved. Also, host-parasite interactions often result in peculiar distortions and deformations of certain characters in host or parasite (e.g., behavior or morphological) due to intense selection pressures driving the changes (Parker, 2016).

One particular system to investigate host-parasite co-evolutionary interactions is between ants and their symbionts. Although ant colonies are well-defended environments against intruders, a large variety of parasites from different taxa of vertebrates and invertebrates, have successfully exploited and deceived ants (Kistner, 1979; Hölldobler and Wilson, 1990; Schmid-Hempel, 1998; Rettenmeyer et al., 2011). The abundant resources available (e.g., immobile brood, harvested food sources, refuse) and "predator free-space" have made ant nests a desirable microhabitat for various organisms. These ant-associated organisms or symbionts are known as 'myrmecophiles' (Kronauer and Pierce, 2011). In other words, an organism that relies

on ants for at least a fraction of their lifecycle is considered a myrmecophile (Kronauer and Pierce, 2011; Parker, 2016) includes plants, bacteria, fungi, and various mutualistic trophobionts. However, this thesis will only focus on insects since most organisms that evolved myrmecophily are parasitic insects (Kistner, 1979).

Myrmecophiles may exhibit fitness costs towards ants by consuming their resources, ant brood, or even bringing about mortality on the queen or workers (Hölldobler and Wilson, 1990; Buschinger, 2009; von Beeren et al., 2011a). In response, host ants defend their nest by aggression (e.g., biting, stinging, or spraying defensive chemicals) (Hölldobler and Wilson, 1990; Ayasse and Paxton, 2002) and evolved a highly complex recognition system based on cuticular hydrocarbons (CHC), to discriminate between nestmate and non-nestmate (Blomquist and Bagnères, 2010). However, several myrmecophiles are ignored, fed, groomed, or transported by ants while also behaving as if they are assimilated into the colony (Refer to 2.3 for examples) (Hölldobler and Wilson, 1990; Witte et al., 2008). They evolved specialized adaptations to fit their ant-myrmecophile association and cope with ant aggression by mimicking ant CHC (e.g., chemical mimicry or chemical camouflage), advanced behavioral adaptations, acoustic mimicy, or a lowered selection pressure (e.g., lowering virulence) (Barbero et al., 2009; Akino, 2008; Stöffler et al., 2011; von Beeren et al., 2011a; Pérez-Lauchad et al., 2015; Parmentier et al., 2016). These myrmecophiles are an "integrated species". They are regularly found at all areas in the nest, including the brood chambers (Kistner, 1979).

On the other hand, some myrmecophiles are identified as intruders and provoked aggression in the host nest (Parmentier et al., 2016). They lack the specialized adaptations, but they are still able to adapt to their host by evolving simpler adaptations such as avoiding contacts, hiding (von Beeren et al., 2011b; Parmentier et

al., 2018), possessing anatomical modifications for swift escapes, shielding from attacks or usage of defensive glands (Komatsu et al., 2009; Stöeffler et al., 2011; Brückner and Parker 2020). They are known as "non-integrated species" and occupy by the periphery, outside of the nest, and/or at refuse deposits of ant nest as niches (Kisner, 1979; von Beeren et al., 2011a; Parmentier et al., 2016).

Myrmecophile adaptations may vary among different taxa and even closely related species, and some may require a combination of more than one adaptation to inhabit with ants (Witte et al., 2009; von Beeren et al., 2012; Pérez-Lauchad et al., 2015; Parmentier et al., 2017; von Beeren et al., 2021). Most studies on the underlying mechanisms of integration strategies or adaptations have been biasedly focused on either specialized myrmecophiles or taxa with the majority of myrmecophiles from orders of Coleoptera, Hymenoptera or Diptera (Parker, 2016). Outside these orders, cases of myrmecophily are infrequent; however, the question still arises whether if specialized or unspecialized strategies are universal among these sporadic instances of myrmecophily.

The yellow crazy ants (*Anoplolepis gracilipes* (Smith, 1857)) and the longhorn crazy ants (*Paratrechina longicornis* (Latreille, 1802)) are ant species that are both highly invasive and damaging in their introduced ranges (Holway et al., 2002; O'Dowd et al., 2003; Wetterer, 2008). Despite forming large and polygynous supercolonies, they are not known to host a diverse community of myrmecophiles (Wetterer, 2008; Hoffman, 2015). However, a variety of different species of tiny, wingless ant crickets from the family Myrmecophilidae, have been found in nests of these two species of ants (Hsu et al., 2020). Recent studies have shown two species of ant crickets, from the genus *Myrmecophilus* Berthold, 1827, receiving differential treatment from host ants and displaying dissimilar intimacy towards ants (Komatsu et al., 2008; 2010); One

species (*Myrmecophilus albicinctus* Chopard, 1924) were constantly in contact with ants and were tolerated, whereas, another species (*Myrmecophilus formasanus* Shiraki, 1930) were recognized as foreign entities and provoked aggression in ants (Komatsu et al., 2009), suggesting two different forms of ant-myrmecophile association.

The underlying mechanisms of myrmecophile social integration remain mostly unknown for the family Myrmecophilidae and quantitative comparison studies accessing the different adaptations as a predictor variable of host ant aggression between different ant cricket species remains largely missing. Hence, this study was initiated (1) to determine and compare the behavioral strategies of integrated ant crickets and non-integrated ant crickets in their host ant colony of origin, (2) to examine and compare morphological adaptations of integrated ant crickets and non-integrated ant crickets that fit their ant-myrmecophile association and lastly, (3) to determine the role of chemical mimicry as an integration strategy for *Myrme. albicinctus* (integrated ant cricket) and *Myrmecophilus hebardi* (non-integrated ant cricket) in their respective *A. gracilipes* colony.

CHAPTER 2

LITERATURE REVIEW

2.1 Invasive Ants

Several species of ants, known as "tramp" ants, have established populations in their introduced range on oceanic islands or urban cities (Hölldobler and Wilson, 1990; McGlynn, 1999). However, a small group of them are categorized as invasive due to their spread into native territories and disrupting the natural community assembly of native ants, invertebrates and vertebrate, through competition or predation (McGlynn, 1999). These species of invasive ants include the yellow crazy ants, *Anoplolepis gracilipes* (Smith, 1857), the Argentine ant, *Linepithema humile* (Mayr, 1868), the crazy ant, *Paratrechina longicornis* (Latreille, 1802), the red imported fire ant, *Solenopsis invicta* Buren, 1972, and the little fire ant, *Wasmannia auropunctata* (Roger, 1863) (Hoffman et al., 1999; Tsutsui et al., 2000; O'Dowd et al., 2003; Espadaler et al., 2004; Errard et al., 2005).

A study by Sanders et al., (2003), found a drastic change in native ant community organization within a year at a biological preserve of northern California, which shifted from a structured and segregated pattern to random occurrences patterns of native ant species due to interference in food-chains by the Argentine ants, *L. humile*. In other studies, the introduction of an alien invasive species indirectly affects essential processors of symbiosis and functions of organisms in natural communities. For instance, mutualistic relationships between *A. gracilipes* and honeydew-secreting Homopteran have led to positive feedback in densities of scale insects and the presence

of sooty molds (due to excess honeydew deposited on leaves) which may impair photosynthesis functions of the host plant (O'Dowd et al., 2003).

Aside from disrupting local communities, invasive ants brings about considerable amount of economic damage, with $211 million/year loss in damages and foregone opportunities in critical economic sectors reported at Hawaii (Gutrich et al., 2007), while a total of $581 million were reportedly needed to control, repair or replace damaged items in urban and agricultural areas of Texas (Lard et al., 2002). Despite the aggression and damage shown, there are still other invertebrates that persist and co-inhabit with invasive ants, such as the Cucurbit Shield Bug (*Megymenum affine* Boisduval, 1835), spider species of *Cryptothele* L. Koch, 1872, woodlice (Hoffman, 2015) and *Myrmecophilus* Berthold, 1827 crickets (e.g. *Myrmecophilus albicinctus* Chopard, 1924 and *Myrmecophilus formasanus* Shiraki, 1930) (Komatsu et al., 2009) in *A. gracilipes* colonies, and *Myrmecophilus americanus* Saussure, 1877 in *P. longicornis* colonies (Wetterer and Hugel, 2008).

2.1.1 Characteristics of Invasive Ants

Most invasive ants that establish and dominate in new environments share common traits among them such as multiple queens in the nest (e.g., polygyny), low intraspecific aggression, colony fission via budding, generalized diet and simple nesting habits (Holway et al., 2002; O'Dowd et al., 2003; Tsutsui and Suarez, 2003; Errard et al., 2005; Abbott, 2006; Wetterer, 2008). While these traits also have been found to exist in other non-invasive ants, only unicoloniality commonly exist within invasive ants but seldom occur among non-invasive ants (Passera, 1994; Tsutsui and Suarez, 2003).

Unicoloniality or supercolony is defined as the formation of numerous interconnected nests separated over a considerable distance containing multiple queens, with workers from each satellite nest lacking any aggression or behavioral differences among each other. The low aggression between workers from distinct nest benefits the supercolony as it reduces the cost of territoriality and directs resources to develop foraging ability, colony growth and interspecific defense and competition through high worker densities in its introduced range (Holway et al., 1998; Holway et al., 2002; Tsutsui and Suarez, 2003; Thomas et al., 2010).

A few hypotheses have been proposed to elucidate the evolution of unicoloniality such as i) a supercolony is founded based on a genetic bottleneck event, where a fragment of a native population is established in an alien territory, and due to low genetic variation, low phenotypic variability is found in the nestmate recognition of workers (Tsutsui and Suarez, 2003; Payne et al., 2004; Errard et al., 2005), ii) supercolonies are formed through 'genetic cleansing', whereby colonies with more common recognition alleles would have reduced cost of territoriality and would gradually fuse to become a supercolony, whereas colonies with rare recognition alleles would be subjected to aggression and progressively be eliminated (Giraud et al., 2002), and lastly iii) a system which is unique only among *S. invicta* colonies, where the differences between the formation of monogyny or polygyny colony is determined by genotypes located at gene *Gp-9* (*B* or *b* allelic variant), and the ability of the workers to distinguished colony queen number between monogyny or polygyny form (Krieger, 2005).

2.2 Communication in Ants

Communication is essential for both solitary and social insects. In solitary insects, communication is mostly limited to sexual signals and information on kinship (Lihoreau et al., 2007; Wyatt, 2014). As the social organization becomes more complex (e.g., solitary to eusocial), information sharing is diversified to sustain the hundreds to millions of individuals in a social insect colony. This is particularly true for ants, as messages transferred between individuals include labor division, alarm or defensive actions, queen signaling, and resource utilization, which could ultimately enhance task efficiency of the colony (Leonhardt et al., 2016). However, communication does more than any task or alarm related scenarios, as it also maintains group cohesion and plays a role in the recognition behavior of ants.

The continuous persistence and abundance of ants in environments have constrained them to regular intra- and interspecific competition or exploitation from various alien species. To compete and defend resources and territory, ants would need to recognize their nestmates from con- and heterospecifics (Sturgis and Gordon, 2012). Therefore, ants have evolved a complex nestmate recognition system that could distinguish alien individuals among their large number of nestmates (van Zweden and d'Ettorre, 2010). Also, this characteristic may have contributed to the dominance found in unicolonial ants, as all conspecific individuals treat each other peacefully despite satellite nest being separated over large distance areas (e.g., 4,000 km) (Tsutsui et al., 2000; Tsutsui and Case, 2001; Holway et al., 2002; Tsutsui and Suarez, 2003; Torres et al., 2007).

Colony-specific cues and mechanisms to detect and respond to these cues are needed to discriminate nestmates from non-nestmates (d'Ettorre and Lenoir, 2010). In

general, (1) cues are expressed by the emitter, followed by (2) detection of the cues by the receiver, (3) matchmaking the cues to a template cue stored in the memory and finally (4) an acceptance or rejection response by the receiver (Vander Meer and Morel, 1998; Newey et al., 2010). Information transfer of cues between ants are mostly chemical (e.g., queen signaling, alarm signals, recruitment, and nestmate recognition) (Wyatt, 2014; Leonhard et al., 2016), although other forms of cues such as tactile, vibrational/acoustic and visual can still occur (Hölldobler and Wilson, 1990). In this study, only tactile and chemical cues in ants will be discussed as these two information transfer modes are reported to be widely used among ants (Hölldobler and Wilson, 1990; Jackson et al., 2006; Martin et al., 2008a).

2.2.1 Tactile Cues

Tactile cues form a vital part of ants' communication, even though chemical cues are more persistent among insects. The role of touch in recognition is best illustrated through the grooming of nestmates among ants. Grooming is known as a coordinated hygienic defense by ants to remove ectoparasites such as mites, nematodes, fleas, and various fungi infections or dust particles, and are performed either through self-grooming or between individual workers (allogrooming) (Schmid-Hempel, 1998; Hughes and Boomsma, 2004; Walker and Hughes, 2009). Individual workers clean themselves or their nestmates using their tongues or forelegs, either by licking or rubbing together their legs, while also spreading antibiotic secretions secreted from their exocrine glands (Farish, 1972; Hölldobler and Wilson, 1990).

Grooming is likely to be an essential part of recognition as it is usually carried out by a nestmate. However, it has been reported that other arthropods have taken

advantage of this prophylactic behavior, as seen in the case of a staphylinid from the genus *Diploeciton* Wasmann, 1923, a guest of the ant *Neivamyrmex* Borgmeier, 1940, and a silverfish, whereby the grooming process by the ant guest calms the ants and paralyzes them partially, allowing acquiring the colony odor or lowering ant aggression before a swift escape (Akre and Torgerson, 1968; Hölldobler and Wilson, 1990).

One of the most fundamental activities carried out among social insects is the capability to share food. Although solid food is consumed freely among individual ants, however, liquid food is shared among individual ants through regurgitation via orally (e.g., stomodeal trophallaxis) or anally (e.g., abdominal trophallaxis). Nevertheless, stomodeal trophallaxis is the most common form of liquid sharing in ants. An ant obtains the liquid carbohydrate by stimulating the forager using tactile signals by tapping the labium of the latter lightly and repeatedly with either the foreleg or antennae, to trigger a regurgitator reflex and regurgitate the liquid food from the crop. During the flow of the liquid food, both the worker and the forager continue with their rapid antennae play, most likely to maintain contact while liquid food is being regurgitated (Hölldobler and Wilson, 1990).

Despite functioning to distribute food evenly throughout the colony, trophallaxis also serves as a recognition behavior to reduce hostile encounters. Interactions between competing species or colonies among ants usually consist of threats, fighting, and escape. However, some ants have been reported to regurgitate liquid food to the competitor, similar to the behavior displayed between two ant nestmates, to reduce or delay attacks. For example, laboratory experiments of encounters between workers of *Pheidole* Westwood, 1839 ants with fire ants, *Solenopsis geminata* (Fabricius, 1804) or *S. invicta*, were found to be initially

aggressive. However, the former species of ants were found to appease the latter species through trophallaxis and, ultimately, lowering the frequency of aggression (Bhatkar, 1979a, b).

2.2.2 Chemical Cues

Information transfer by the chemical is the most dominant method of communication in insects and some vertebrate animals (Wyatt, 2014). In ants, they are known as 'walking chemical factories' (Hölldobler and Wilson, 1990), and over half of the 100 exocrine glands described in social insects belong to them (Billen, 2004). Therefore, it is highly likely that the recognition system in ants will be mainly based on chemical cues or profile. Nestmate recognition relies on matching a particular cue or profile (e.g., the chemical odor) to a template (e.g., the neural portrayal of the colony odor stored in the memory of the ant), and finally, acceptance or rejection response from the recipient depending on the similarity between the profile/cue and template (Vander Meer and Morel, 1998; Newey et al., 2010).

Ants can detect volatile, non-volatile cues or profile through their antennae. When sweeping over the donor's cuticle, cue signals are transmitted and interpreted by the central nervous system, by comparing the cues with the colony's template stored in the recipient's memory (e.g., higher-order brain centers) and subsequently initiate the appropriate behavioral response (Vander Meer and Morel, 1998). However, in some cases, recognition could be achieved without the antennae's contact on the cuticle, over a distance of 1-2 cm (Vander Meer and Morel, 1998).

A template is defined as a depiction of nestmate recognition cues of the colony (Lacy and Sherman, 1983) stored within the memory of the recipient worker ant. Both

Jaisson (1991) and Errard (1994) observed that callow workers displayed little aggression towards non-nestmates and could mix with other heterospecific callows. In contrast, such behavior was absent using adult workers, suggesting that early encounters within the nest are correlated with the early development of template cues (Lorenzi et al., 1999; Errard et al., 2008). Besides, templates may not remain permanent and require workers to continually update it with the dynamic odor of nestmates (e.g., Gestalt odor) (Errard and Hefetz, 1997; Lenoir et al., 2001; Leonhardt et al., 2007). The recipient's response (peaceful or aggressive) towards the cue donor after matchmaking is expressed based on a graded response, from exploring to probing to threat or hostile behavior (Obin and Vander Meer, 1988; Errard and Hefetz, 1997).

2.2.2(a) Cuticular Hydrocarbons (CHC) in Ants

In general, hydrocarbons are abundantly found in ants and are present on cuticles, in hemolymph, glands, and crop of the insect (Attygalle and Morgan, 1984). Cuticular hydrocarbons (CHC) predominantly functions as a protection against desiccation (Ramsay, 1935) and as communication (Blomquist and Bagnères, 2010), but have also been found to function as lubrication on the cuticles (Cooper et al., 2009), as footprints left by insects (Wüst and Menzel, 2017), and even a protection layer against microbes (Wurdack et al., 2017).

Biosynthesis of hydrocarbons occurs within oenocytes found in fat bodies or epidermal layers. They convert fatty acyl-CoAs to long-chain fatty acids and form hydrocarbons when the carboxyl group is removed (Nelson and Blomquist, 1995; Howard and Blomquist, 2005; Blomquist, 2010). Cuticle hydrocarbons have chain lengths between 21 to >40 carbons. They are made up of five types of structural classes: *n*-alkanes, methyl-branched hydrocarbons, mono-, di- and tri-alkenes (Martin

and Drijfhout, 2009a). The presence of differential chain lengths and multiple structural class allows for the formation of combinations and complex hydrocarbon structural compounds on the cuticle of an insect, which is essential for an intricate recognition system found in ants (Sturgis and Gordon, 2012).

2.2.2(b) Cuticular Hydrocarbons (CHC) as Nestmate Recognition Signals

Cuticular hydrocarbons (CHC) of ants have been implicated in containing the necessary recognition cues to discriminate between nestmates and non-nestmates (Howard and Blomquist, 2005). The role of CHC's in the importance of nestmate recognition in earlier publications began with correlative evidences in studies of CHC patterns between host and myrmecophiles or termitophiles: through experiments of removal and substitution studies between ants (Vander Meer and Morel, 1998).

Vander Meer and Wojcik (1982) found that when an isolated myrmecophile beetle of *Myrmecaphodius excavaticollis* (Blanchard, 1843) was placed in a new *Solenopsis* colony, the beetle was initially attacked but aggression decreased as beetles survived longer in the nest. As the beetles are exposed longer in the colony, colony specific CHCs from host ants were found to accumulate on the beetle's cuticle correlated with the reduction in aggression, suggesting that CHC may influence the recognition behavior in ants. Consequently, another study done by Nowbahari et al., (1990) found that the ant, *Cataglyphis cursor* (Fonscolombe, 1846), adopted alien conspecifics when both share similarity in CHC profiles according to Nei's distances, whereas conspecifics were rejected when CHC profiles diverged, concluding that colony recognition is correlated to similarity in CHC composition.

Akino et al., (2004) found that the mixture of hydrocarbons present on cuticle was responsible for nestmate recognition and that the combination of both *n*-alkanes and (Z)-9-alkenes classes functions as signals to discriminate nestmate or non-nestmate. CHC extracted from non-nestmates and applied on glass dummies produce aggressive responses from ants whereas less attention was given to CHC extracts from nestmates. Furthermore, the mixture of both *n*-alkanes and (Z)-9-alkenes using synthetic hydrocarbons, produced almost identical reactions as the test with live workers (e.g. aggressive reactions towards non-nestmates and peaceful reactions to nestmate). However, when only either *n*-alkanes or (Z)-9-alkenes extracts were used on the glass dummies, no significant aggressive reactions were seen but only peaceful reactions using extracts from non-nestmate or nestmates.

In contrast, Martin et al., (2008b) found that only the (Z)-9-alkene portion is subject to nestmate discrimination rather than the combination of different class mixture of hydrocarbons or from the alkane component, in the ant *Formica exsecta* Nylander, 1846. When profiles of nestmates were altered by adding single component classes (a Z9 alkene or an alkane), only by the addition of a Z9-alkene increased aggression of nestmates towards the focal worker whereas addition of an alkane component had no effect on the worker, similar to the addition of the control hexane component, suggesting that ants only respond to changes in the Z9-alkene component (Martin et al., 2008b).

2.2.2(c) Olfactory Perception and Discrimination of CHC

CHC odors are identified in the antenna, where it contains a variety of odorant receptors. They are interpreted through an olfactory sensillae in the antenna (Nakanishi

et al., 2009). The CHC odor then diffuses into the sensillum lymph and is transported via an odorant-binding protein to the odorant receptors (Fleischer et al., 2018; Fleischer and Krieger, 2018). Processing and interpretation of CHCs then occur in the glomeruli located in antennal lobes (Trible et al., 2017). Consequently, the ants' ability to detect the various CHCs depends on the different genetic expressions of the odorant receptor families (Zhou et al., 2012; McKenzie et al., 2016).

It was long suggested that discrimination between nestmates and non-nestmates is based on the similarity between all CHC compounds and the formation of a "gestalt" colony odor (Crozier and Dix, 1979; Boomsma and Franks, 2006). However, recent studies have found that only a few compounds within the CHC profile, rather than all compounds, are recognizable by ants as nestmate recognition signals. For example, the cuticle of the ant *F. exsecta* is dominated mainly by *n*-alkanes and a homologous series of Z9-alkenes, but only the altered proportions of Z9-alkenes elicited aggression between ants (Martin et al., 2008b). In addition, a study conducted by Martin et al., (2008a) reported that the genus of ants, *Formica* Linnaeus, 1758, had elevated productions of either Z9-alkenes or dimethyl alkanes, and interestingly, their production of CHCs were found to be species-specific. Removal of the major groups (Z9-alkenes or dimethyl alkanes) diminished the species relationships, suggesting that these compounds contain information.

On the other hand, between populations or conspecifics, similar CHCs (dominant Z9-alkenes or dimethyl alkanes) were found to vary in proportions or quantitatively whereas individual ants within colonies show similarity in proportions of CHC (Martin et al., 2008a; Martin et al., 2008c; Guillem et al., 2016). Aside from proportions, ants were also found to be able to discriminate between conspecifics based on the compound structure, such as the differential position of the methyl-group

or double bonds (van Wilgenburg et al., 2010). Although studies found that discrimination occurred based on a single structural group, combination of two or more structural classes contributing to nestmate recognition could occur, as demonstrated in the combination of *n*-alkanes and Z9-alkenes in the ant *Formica japonica* Motschoulsky, 1866 (Akino et al., 2004) and the combination of three structural class (*n*-alkane, alkenes and methyl alkanes) in the ant *L. humile* (Greene and Gordon, 2007).

These differences are mostly dictated by genetic distances, as the more genetically diverse a colony, the more diverse the compounds found on the CHC profile (Brandt et al., 2009; Teseo et al., 2014). Within a colony, CHC profiles between individuals show less variation compared to between species or between conspecifics, however, the differences found is usually either queen-worker differences or between behavioral castes (Greene and Gordon, 2003; van Oystaeyen et al., 2014). On top of the findings mentioned above, it was also found that each odorant receptors found in the antenna of ants only respond to particular or a narrow spectrum of CHCs and that the different positions of the methyl chains also affected the odorant receptor activities (Pask et al., 2017). This suggests that ants do not rely on all CHC compounds to form a colony odor, but instead rely on selected integrated compounds (Sprenger and Menzel, 2020). In the same study, aside from the selected response, odorant receptor activities were also found to be dose-dependent (Pask et al., 2017).

2.2.2(d) Factors Influencing CHC Composition

2.2.2(d)(i) Abiotic Factors

CHC has been found to be influenced by environmental factors, such as temperatures and humidity, where warmer and drier conditions will lead to an increase in abundances of linear *n*-alkanes and a decrease in the more fluid unsaturated or methyl CHCs (Sprenger et al., 2018). Consequently, it was also found that ant CHC profiles were able to acclimatize to the nest materials found around it, although it does not necessary change the entire profile, but it does add a few non-CHC compounds into the profile (Pickett et al., 2000; Katzav-Gozansky et al., 2004).

2.2.2(d)(ii) Biotic Factors

The type of diet too can affect CHC profiles in two ways: firstly, the type of food consumed would have different ratios of fatty acids or amino acids, which are essential building blocks in CHC synthesis via malonyl-CoA or methylmalonyl-CoA (Kleeberg et al., 2017; Schultzhaus et al., 2018). Secondly, CHCs found on prey could be directly incorporated on the cuticles of ants (Liang and Silverman, 2000; Silverman and Liang, 2001), as CHC could be transported to the haemolymph from the digestive tract, and then transported to the epicuticle (Fan et al., 2004).

In addition, CHC profiles of most insects have been found to be affected by pathogens and parasites. In ants, it was found that when the pupae of the ant *Lasius neglectus* Van Loon et al., 1990, were infected with a fungus, *Metarhizium brunneum,* CHC profiles of pupae diverged and it triggered the hygienic behavior of the worker ants (Pull et al., 2018). Another study found that when the ant *Temnothorax nylanderi*

(Foerster, 1850) workers were infected with tapeworms, *Anomotaenia brevis* (Clerc, 1902), CHC profiles of workers were found to be different from healthy workers, and those infected were found to stay within the nest, regardless of age and were cared by nurses (Beros et al., 2017).

However, although environmental factors influence CHC profiles, the quantitative and qualitative variation between species and within species is still primarily affected by genetically determined cues, as demonstrated by van Zweden et al., (2009)'s study. In each species, CHC's are genetically heritable (Martin et al., 2008a; Guillem et al., 2016) and may differ qualitatively between species due to genetic drift or an accumulation of supercolony mutations (Drescher et al., 2010). Hydrocarbons are shared among nestmates through genetic relatedness (Drescher et al., 2010; Nehring et al., 2011), learned or adsorb when as callows (Nowbahari et al., 1990; Lenoir et al., 2001), grooming and trophallaxis (Soroker et al., 1995; Foitzik et al., 2007). Consequently, continuous CHC transfer (e.g. gestalt odor via grooming, trophallaxis, mixing of hydrocarbons through PPG) homogenizes/mixes the colony odor to form an intermediate odor. Any isolation of an individual worker or a satellite nest may result in a cue divergence from the central nest and be recognized as an intruder (Soroker et al., 1994; Meskali et al., 1995; Dahbi and Lenoir, 1998).

However, a recent study has shown that individual ant adjusts their own biochemical process to match the dominant profile, rather than mixing to form an intermediate profile like a gestalt odor. For example, Martin et al., (2019) showed that when alien workers or callows of the ant *F. exsecta* was placed in a host conspecific fragment colony for a period of 20 days, alien workers were found to adjust their dominant (Z)-9-alkene chain lengths (from C23 to C27 or vice versa) to match chain lengths of their host rather than forming an intermediate profile. This may further

implicate those ants are aware of their own and their nestmates profile and will continually adjust their profiles to match the dominant odor.

2.2.2(e) Differential Importance in CHC Structural Classes

Ants possessed a total of ten distinctive CHC groups such as, *n*-alkanes, monomethyl alkanes, dimethyl alkanes, trimethyl alkanes, tetramethyl alkanes, alkenes, dienes, methyl alkenes, methyl alkadienes, and trienes. Despite the many different structural groups found in the cuticles of ants, *n*-alkanes, monomethyl alkanes, dimethyl alkanes and alkenes dominate the profiles of most ant species. Structural groups becomes more complex, fewer species produces them (Martin and Drijfhout, 2009a). The different structural groups exist on a similar alkane backbone, with the addition of double bonds (e.g. single or multiple double bonds), methyl groups (e.g. single or multiple methyl groups; differential positional isomers) or a combination of double bonds and methyl groups (Martin and Drijfhout, 2009a).

The number of hydrocarbon structural class and the possible positional variations of each structural class has made CHC an ideal candidate as a complex nestmate recognition system. However, in most studies, it has been found that each peak or structural class may not function as colony or nestmate signals but are independently encoded as either fertility or task cues within the same CHC profile, whereas nestmate discrimination are based on only a specific group compounds or structural class.

2.2.2(e)(i) Alkanes

Alkanes are commonly found in most species of ants and often appear as odd-numbered C_{25} to C_{35} *n*-alkanes, covering approximately more than 50% of the CHC profile (Martin and Drijfhout, 2009a). In all insects, alkanes functions as part of the major compounds responsible to prevent desiccation and waterproofing of cuticles (Edney, 1977). Although some studies have shown that alkanes do not illustrate any form of species or colony level recognition (see Châline et al., 2005; Martin et al., 2008c; Guerrieri et al., 2009; Martin and Drijfhout, 2009b), research has found that alkanes instead encodes simple messages related to task (Wagner et al., 1998), with ant foragers showing greater amounts (total ng) of alkanes compared to non-foragers (Martin and Drijfhout, 2009b).

This is because foragers are frequently exposed to varying temperatures and relative humidity (RH) when foraging compared to non-foragers and they would require greater amounts of alkanes for desiccation resistance. For example, exposure of non-foragers of the desert harvester ant, *Pogonomyrmex barbatus* (Smith, 1858), to high temperatures and low humidity for a short term, were found to trigger an increase in the proportion of alkanes in the CHC profiles (Wagner et al., 2001). This also suggest that alkanes are unstable within the colony and ineligible for nestmate recognition (Boomsma et al., 2003; Martin and Drijfhout, 2009b). In addition, alkanes act as cues to regulate the number of foragers, as it was found that a brief encounter with a worker with higher alkanes (e.g. patroller worker ant) influenced the number of foragers leaving the nest (Greene and Gordon, 2003).

2.2.2(e)(ii) Monomethylalkanes

Monomethylalkanes are saturated hydrocarbons that possessed a methyl group attached to the alkane backbone and appear to arise at a variety of homologous series. With an addition of a methyl group, melting points of the saturated hydrocarbons are lowered (Gibbs and Pomonis, 1995). Therefore, the cuticle of the insects exists as a solid-liquid phase over a wide range of temperatures with the presence of both alkanes and monomethylalkanes (Gibbs, 1995).

It has been hypothesized that monomethylalkanes could be involved in chemical communication (Nelson, 1993), however, their pervasiveness like alkanes suggests that they are only common signals (Martin and Drijfhout, 2009a). Despite of that, studies have shown that ants do respond to changes in monomethylalkanes ratios and different positional isomers (van Wilgenburg et al., 2010; Krasnec and Breed, 2013), whereas a combination of monomethylalkanes and other compounds of a different structural class have been found to elicit a strong aggressive response from non-nestmates (Greene and Gordon, 2007).

2.2.2(e)(iii) Dimethylalkanes

Among the structural classes of CHC, dimethylalkanes has by far the most number of compounds identified and the highest number of homologous series (Martin and Drijfhout, 2009a). In addition, the large number of possible isomer positions has made dimethylalkanes by far the most diverse group of structural class and potentially suited as species-specific and colony-specific recognition cues (Martin et al., 2008a; Guillem et al., 2016). For example, studies on the relationship of CHCs have shown that dimethylalkanes exhibit both species-specific and colony-specific information

among ants. Removal of these group of compounds diminished the species and colony relationships, whereas, within species, similar dimethylalkanes were found but most exist in different quantitative variations (e.g. relative proportions) and with different isomers (Martin et al., 2008a; Martin et al., 2008b; Martin and Drijfhout, 2009a; Krasnec and Breed, 2013; Guillem et al., 2016).

Empirically, only a single study performed by Guerrieri et al., (2009) using synthetic hydrocarbons showed dimethylalkanes exhibit nestmate recognition properties. The study demonstrated that ants only reacted aggressively to stimuli and live ants supplemented with dimethylalkanes and not to other CHC structural classes (e.g. alkanes and monomethylalkanes).

2.2.2(e)(iv) Olefins

Olefins are unsaturated hydrocarbons that consist of one or more double bonds in the carbon chain. The addition of a double bond in a carbon chain effectively lowers the melting temperatures and the combination with alkanes and monomethylalkanes produces a solid-liquid phase cuticle over a wide range of temperatures (Gibbs 1998).

Like dimethylalkanes, some ants specialized in olefin productions. Consequently, double bonds of these compounds can occur at a wide variety of isometric positions and consists of a number of homologous series, making it suitable to encode recognition cues (Martin et al., 2008a; Martin and Drijfhout, 2009a; Kather and Martin, 2015). However, the above situation is limited to only compounds with a single double bond (e.g. alkenes), as dienes and trienes rarely occur and may be due a restricted biosynthesis which involves a double desaturation steps (Wicker-Thomas and Jallon, 2001).

In addition, combination of a methyl group and a double bond (e.g. methylalkene) in a single compound rarely occurs, and although it was found in some species of wasps and bees (Kather and Martin, 2015), it seldom occurs in ants but in two species (Lucas et al., 2004; D'Ettorre et al., 2004). Martin and Drijfhout (2009a) revealed that, for the species that do not specialize in the production of methyl groups, were found to instead produce an array of alkene compounds. Species of ants were differentiated based on presence or absence of alkene compounds or bearing different isomers, whereas, within species, alkenes were found to differ in relative proportions (Martin et al., 2008a; Martin et al., 2008c; Guillem et al., 2016). Moreover, it has been shown in several experiments that proportions of alkenes were relatively stable within colonies and that ants responded from tolerance to aggression to changing alkene concentration (Martin et al., 2008b; Martin et al., 2008c).

2.3 Myrmecophiles

Social insect symbionts of ants are called myrmecophiles, which means 'ant lover' in Greek, 'myrmex' for ants whereas 'philos' for loving (Kistner, 1982, Kronauer and Pierce, 2011). Organisms are associated with myrmecophily if their whole lifecycle or a part of their lifecycle relied on ants. The earliest record of myrmecophile diversity was done by Erich Wasmann, an Austrian Entomologist and Jesuit priest, where he documented at least 1,000 species of animals associated with ants (Wasmann, 1894). At present, there are an estimated of 10,000 to 100,000 of myrmecophiles from 100 families of mainly arthropods (Thomas et al., 2005; Hughes et al., 2008; Parker and Grimaldi, 2014). A large diversity of this number consists of beetles, flies, butterflies, mites, crickets, bristletails, millipedes, isopods, snails, aphids, wasps and scale insects' groups that have evolved to depend on ants at one

way or the other (Hölldobler and Wilson, 1990), with both staphylinid beetles and mites the most prevalent (Kronauer and Pierce, 2011).

Living inside an ant nest has its selected advantages. Firstly, ant colonies are described as factories within fortresses (Wilson, 1968), in which ant nest are well-defended by the numerous sister workers and forming a predator free-space for both ants and their symbionts (Keller and Genoud, 1997; Hughes et al., 2008). Secondly, a large ant nest can provide a buffered and homeostatic microhabitat (Odling-Smee et al., 2003; Hughes et al., 2008; Kronauer and Pierce, 2011) where temperature and humidity are maintained at stable conditions for survival. Thirdly, ants are known to be one of the most ecologically dominant species on earth, competing with other organisms for resources (e.g. as predators, scavengers, herbivores and mutualist) (Hölldobler and Wilson, 1990; Brady et al., 2006). Therefore, thriving colony of ants may possess abundant food resources which may benefit other organisms (Kronauer and Pierce, 2011; Parmentier et al., 2017).

In general, larger ant colonies are found able to shelter a higher number and diversity of myrmecophiles whereas smaller colonies may have smaller number or no myrmecophiles present (Hölldobler and Wilson, 1990; Rettenmeyer et al., 2011; Pérez-Lachaud and Lachaud, 2014). For example, a study on the symbiont communities of the army ant *Eciton burchellii* (Westwood, 1842), an ant species that has an approximately 500,000 workers in a single colony, recorded a staggering 557 symbionts, with an estimated of 300 different species of symbionts dependent on ants. Those dependent on ants consist of different species of mites, beetles, millipedes, springtails, flies, wasps and bristletails (Rettenmeyer et al., 2011).