Mechanisms of Nestmate Recognition Cue Production in the European Honey Bee, Apis mellifera.

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Mechanisms of Nestmate Recognition Cue Production in the European Honey Bee, *Apis mellifera*.

by

Cassondra Vernier

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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Cassondra Vernier

Washington University in St. Louis

August 2019
Dedicated to my grandmothers, Benita “Oma” Klein and Rosemary “Uma” Vernier.
Abstract

ABSTRACT OF DISSERTATION


by

Cassondra Vernier

Doctor of Philosophy in Biology and Biomedical Sciences

Program in Evolution, Ecology, and Population Biology

Washington University in St. Louis, 2019

Professor Yehuda Ben-Shahar, Chair

Social insects are some of the world’s most ecologically successful animal groups, and their complex societies are considered one of the pinnacles of animal evolution. Since these organisms live in colonies composed of many individuals and stored resources, they are a target for intruders, such as parasites, predators and conspecific robbers. Therefore, many social insect species have evolved mechanisms for nest defense, including nestmate recognition, where guarding individuals at the entrance of the colony use cues on incoming individuals to determine whether they are nestmates or intruders. Although nestmate recognition is incredibly important for maintaining colony integrity and fitness, the behavioral and physiological mechanisms that underlie this behavior remain unknown for most species. My dissertation work focused on elucidating the mechanisms of colony-specific nestmate recognition cue production in the economically important honey bee. Previously, it was assumed that the colony-specific chemical signatures used for nestmate recognition are acquired by individual workers through the
homogenization of cuticular chemicals, called cuticular hydrocarbons (CHCs), via interactions with other colony members or hive materials. Although this mechanism seems to be used by few ant species, my dissertation work shows that in honey bee colonies, the CHC profiles of workers develop through a sequence of stereotypic qualitative and quantitative chemical transitions driven by environmentally-sensitive biosynthetic pathways, which result in the final mature colony-specific nestmate recognition cue in forager bees, independent of their genetic background (Chapter 2). These data suggest that, rather than acquiring cues via CHC homogenization mechanisms, honey bees intrinsically produce colony specific CHC profiles via environmentally-sensitive mechanisms. To further understand how hive-specific environmental factors might define the colony-level specificity of the nestmate recognition cue, independent of the bee’s genetics, I subsequently show that colony-specific gut microbial communities contribute to the development and perception of nestmate recognition cues in honey bee colonies (Chapter 3). My work highlights the link between gut microbial community and cue production in honey bees, but also remarkably suggests a link between gut microbial community and cue perception, implying a pleiotropic role of the gut microbiome in this recognition behavior. Overall, my dissertation work supports the model that colony-specific gut microbial communities drive the intrinsic production of colony-specific nestmate recognition cues across foragers of the same colony, as well as the ability to identify colony-specific cues in guard bees.
Chapter 1

Introduction

Social insects are some of the world’s most ecologically successful species (Holldobler and Wilson, 1990; Wilson, 1971). They form colonies composed of many individuals and are defined by reproductive division of labor, overlapping generations, and cooperative brood care; a social lifestyle that is considered one of the pinnacles of animal evolution (Maynard Smith and Szathmáry, 1995; Wilson, 1971). Due to this, nests contain many adult individuals, brood and stored food, making them a target to parasites, predators, and conspecific robbers (D’Ettorre et al., 2006a). Therefore, social insects have evolved sophisticated mechanisms of nest defense, including nestmate recognition. Conceptually, while identifying and deterring parasites and predators from entering a social insect colony seems trivial, identifying unrelated intruding conspecifics, especially in species with very large colonies, seems to be an impossible task. Yet, all social insects species seem to have evolved mechanisms that enable them to discriminate nestmates from conspecific intruders at the nest entrance, which is essential for colony defense and social cohesion (van Zweden and D’Ettorre, 2010; Vernier et al., 2019).

As a social-group form of “self” versus “non-self” behavioral discrimination, theoretical models predict that nestmate recognition involves the sensory matching of phenotypic “labels” carried by colony members to “templates” stored in the nervous systems of other colony
members. Thus, the degree of match (or mismatch) between the label and the template determines whether an individual will be accepted or rejected at the hive entrance (Buckle and Greenberg, 1981; Carlin and Hölldobler, 1986; Errard, 1994; Gamboa et al., 1986; Getz, 1982; Hölldobler and Michener, 1980; Lacy and Sherman, 1983; Reeve, 2002; Tsutsui, 2004; van Zweden and D’Ettorre, 2010). Most social insect species seem to have evolved the use of chemical cues in nestmate recognition, with a majority of evidence pointing to a blend of cuticular hydrocarbons (CHCs) that is colony specific (van Zweden and D’Ettorre, 2010).

1.1 CHCs and pheromonal communication in insects

CHCs are long chain, hydrophobic compounds found on the cuticle of insects and other organisms, including plants (Chung and Carroll, 2015; Jetter et al., 2006). Within arthropods, CHCs originally evolved as an anti-desiccant layer, but have subsequently been co-opted to function as pheromones in diverse insect communication systems (Chung and Carroll, 2015; Howard and Blomquist, 2005; McKinney et al., 2015). For example, CHCs are known to function as mating signals (Ferveur, 2005), aggregation pheromones (Saïd et al., 2005) and recognition cues (Howard and Blomquist, 2005), including playing an important role in nestmate recognition (van Zweden and D’Ettorre, 2010).

CHCs are synthesized in specialized cells called oenocytes, which are found beneath the cuticle in association with the fat body (Makki et al., 2014; Snodgrass, 1956), and are subsequently transported to the exterior of the cuticle (Chung and Carroll, 2015). Within the oenocytes, a biosynthetic pathway that relies on several classes of enzymes, including elongases, desaturases and reductases, converts Acetyl-CoA into long chain hydrocarbons (Chung and
Carroll, 2015). Typically, insect cuticles carry a blend of CHCs, composed of a variety of classes, including alkanes, methyl-branched alkanes, and alkenes, and this variation is thought to rely on the enzymatic properties of certain classes of CHC biosynthetic enzymes, namely elongases and desaturases (Chung and Carroll, 2015, Figure 1.1). Additionally, since all classes of CHCs are synthesized from a common pathway utilizing Acetyl-CoA, it is thought that changes in the production of one class could affect the production of another class, such that relative increases in the production of one class would yield decreases in another class (Chung and Carroll, 2015). In addition to direct changes in the biosynthetic pathway of CHCs, factors including climate and diet influence variations in the CHC profile. Due to the role of CHCs in desiccation resistance, climate is known to influence the evolution of CHC profiles such that animals living in drier climates have higher proportions of CHCs known to prevent desiccation (Chung et al., 2014; Gibbs and Pomonis, 1995; Rouault et al., 2004). In contrast, diet is thought to influence CHC profiles because different food sources provide different quantities of amino acids, of which certain kinds are required for the synthesis of certain classes of CHCs (Chung and Carroll, 2015). However, the relative contribution of these mechanisms to CHC profiles has evolved in conjunction with the roles that these compounds play in communication within species (Chung and Carroll, 2015; Menzel et al., 2017).

Within species, CHC profiles are typically qualitatively composed of the same compounds, and therefore, in most social insect species, nestmate recognition is thought to depend on quantitative variations in individual compounds across individuals from different colonies (Karl E Espelie et al., 1990; Martin et al., 2008; van Zweden and D’Ettorre, 2010; Vander Meer et al., 1989). A variety of studies support the role of CHCs in nestmate recognition, including work on social parasites, which overcome detection as intruders by adopting the host
colony’s CHC profile (A. Lenoir et al., 2001), and “perfuming” studies, which demonstrated that replacing an individual’s CHCs with those extracted from an unrelated conspecific would increase the likelihood of being rejected by nestmate guards, as well as specific topical application of different synthetic CHCs on individual nestmates which elicit negative behavioral responses from nestmate guards (van Zweden and D’Ettorre, 2010). However, despite many studies supporting the role of CHCs in nestmate recognition, the mechanisms mediating nestmate recognition cue development and colony-specificity remain unknown for most species.
Figure 1.1: The CHC biosynthesis pathway. Acetyl-CoA is converted into fatty acyl-CoA through fatty acid synthases (yellow). Desaturases (green) and elongases (blue) modify fatty acyl-CoA, converting it into aldehydes. Finally, aldehydes are converted into hydrocarbons via P450 decarboxylase (orange). Hydrocarbon class depends on the precursor, and the action of desaturases and elongases. Modified from Chung and Carroll 2015.


1.2 Study System

To better understand the genetic and physiological mechanisms that drive nestmate recognition systems in social insects, my dissertation aims to identify mechanisms of nestmate recognition cue production in the European honey bee, *Apis mellifera*. The European honey bee is one of the most well-studied social insect species, and is emerging as a model for understanding molecular and genetic mechanisms underlying social behavior. Honey bee colonies exhibit age-dependent division of labor amongst sterile workers: Typically, young adult bees take part in brood care and hive cleaning behaviors, while a small proportion of some older bees perform other specific in-hive tasks, such as undertaking, including removal of dead individuals, and guarding, including nestmate recognition behaviors. Eventually, all workers transition to foraging behavior at about three weeks of age (Robinson, 1992; Smith et al., 2008; Søvik et al., 2015a). While honey bee colonies are common targets for diverse vertebrate and invertebrate predators and parasites, some of the most harmful effects on the fitness of individual colonies come from members of unrelated hives. In times of dearth, when temperatures are warm but flowers are not in bloom, robbing between colonies becomes very prevalent, leading to the death of weak colonies due to starvation (Downs and Ratnieks, 2000). Therefore, in honey bees, nestmate recognition is extremely important to exclude robbers from the nest, and is likely employed mostly for this purpose, since guarding efforts change with nectar availability throughout the year (Downs and Ratnieks, 2000).

Evidence suggests that honey bees use both cuticular hydrocarbons (CHCs) (Arnold et al., 1996; Breed and Julian, 1992; Chaline, 2005; Dani, 2005; Page et al., 1991) and fatty acids (Breed, 1998; Breed et al., 2004) as recognition cues. These cues are colony-specific, and differ in the relative abundance of individual components between colonies (van Zweden and
D’Ettorre, 2010; Vernier et al., 2019), and their transfer between colony members has been hypothesized to occur via exposure to the comb wax (Breed et al., 1998; Margaret J. Couvillon et al., 2007; D’Ettorre et al., 2006b). Although the high genetic relatedness of nestmates would suggest that colony-specific chemical cues would be genetically determined (Arnold et al., 1996; Breed, 1983; Page et al., 1991), studies have shown that the effects of genetics are likely masked by environmental inputs (Downs and Ratnieks, 1999; Vernier et al., 2019). For example, studies have shown that although bees raised in a controlled lab environment are attacked more by non-relatives than by their sisters (Breed, 1983), bees raised, post-eclosion, in an unrelated colony are behaviorally accepted by that colony, but rejected by their natal colony (Downs and Ratnieks, 1999). These findings suggest that all individual workers have the capacity to develop and/or acquire the colony-specific nestmate recognition cue, independent of their genetic relatedness to other colony members. Additionally, work by me and others have indicated that the development of colony-specific chemical signatures seems to be mediated via an age-dependent process (Breed et al., 2004; Falcón et al., 2014; Vernier et al., 2019). Specifically, data indicate that newly eclosed bees have low levels of CHCs and fatty acids (Breed et al., 2004; Falcón et al., 2014; Vernier et al., 2019), young bees have a similar chemical signature across colonies (Breed et al., 2004; Vernier et al., 2019), and young bees are likely accepted at any colony (Breed et al., 2004; Vernier et al., 2019). However, once the nestmate recognition cue is mature in foragers, they are only accepted in their home hive (Vernier et al., 2019). While studies support the role of environment in defining nestmate recognition cues in the honey bee (Downs and Ratnieks, 1999; Vernier et al., 2019) and the dramatic transition from no-cue in day-old bees to a fully mature cue in foragers (Breed et al., 2004; Vernier et al., 2019), the genetic, physiological, and/or environmental factors that enable worker bees to develop colony-specific cues remain unknown.
1.3 Models of nestmate recognition cue production

Several models have been proposed for how members of individual social insect colonies acquire similar nestmate recognition cues. Since members of the same social insect colony are genetically related, it was originally thought that nestmate recognition cues were defined by genetic factors (R.H. Crozier and Dix, 1979; Getz, 1982, 1981). However, subsequent empirical studies indicated that environment plays a more important role than genetics in defining nestmate recognition cues across many social insect species, with studies implicating the role of diet (Buczkowski et al., 2005; Buczkowski and Silverman, 2006; Liang and Silverman, 2000; Richard et al., 2004, 2007), nesting materials (Breed et al., 1988; Margaret J Couvillon et al., 2007; D’Ettorre et al., 2006a; Karl E Espelie et al., 1990; Singer and Espelie, 1996), and the queen (Carlin and Hölldobler, 1988, 1987, 1986, 1983). Therefore, these data led to support for the alternative “Gestalt” model (R.H. Crozier and Dix, 1979).

Under the original inception of the “Gestalt” model, it was predicted that individuals produce CHCs via the biosynthetic pathway described above, but these CHCs are subsequently homogenized and redistributed between individuals, such that every individual carries a mean colony-specific CHC signature (R. H. Crozier and Dix, 1979). While CHC homogenization as a mechanism for generating colony-specific nestmate recognition cues is supported by work in a few ant species, it seems that in these specific species, transfer of CHCs between individuals depends on the action of the post-pharyngeal gland (PPG) (Boulay et al., 2000; Lenoir et al., 2001; Meskali et al., 1995; Soroker et al., 1994; Soroker et al., 1995; Van Zweden et al., 2010). However, most social insect species lack a PPG, and in many species, the regulation of CHC profiles is influenced by many factors, including genetic relatedness (Page et al., 1991; Teseo et al., 2014), age (Breed et al., 2004; Cuvillier-Hot et al., 2001; Falcón et al., 2014; Teseo et al.,
2014; Vernier et al., 2019) and task (Martin and Drijfhout, 2009; Sturgis and Gordon, 2013; Vernier et al., 2019; Wagner et al., 1999, 1998), rather than solely by contact with other colony members. Therefore, more recent models adopting “Gestalt”-like mechanisms include processes where multiple factors, including genetic, environmental and social factors, are combined to produce an individual’s chemical profile, but colony-specific cues are defined by the overlap of the chemical profile between individuals of the same colony (Esponda et al., 2015; Newey, 2011; Sturgis and Gordon, 2012).

Despite these revised parameters for what might constitute a “Gestalt”-like mechanism for the determination of nestmate recognition cues, the role of CHC homogenization remains the major explanation for how members of a colony acquire nestmate recognition cues, including in honey bees, which are thought to use comb wax as a homogenization medium (Breed, 2015). Likewise, more recently, the cephalic salivary gland (CSG) has been hypothesized to serve as the analogous gland to the ant PPG in honey bees (Martin et al., 2018). However, direct empirical evidence that indicates CHC homogenization is actually playing a role in the development of nestmate recognition cues in honey bee colonies is lacking.

Consequently, based on experimental data I present in Chapter 2 of my dissertation, I propose an alternative model for how individual honey bees acquire their colony-specific nestmate recognition cue. This model stipulates that worker honey bees develop their nestmate recognition cue via intrinsic mechanisms that are influenced by their environment and the physiological processes that drive their age-dependent division of labor, such that foragers of the same colony intrinsically produce a similar cue (Vernier et al., 2019). Predictions of this model and recent views on “Gestalt”-like mechanisms are not mutually exclusive since both models highlight the importance of the interaction between environmental and genetic factors in defining
cue specificity, and multiple environmental factors, including diet and social interactions, likely contribute to the influence of microbiome on CHC profiles (Chung and Carroll, 2015; Lee et al., 2018; Powell et al., 2014). However, my model is the first to suggest an indirect effect of the social environment on the cue by modulating the expression of CHC producing genes, highlighting the importance of environmentally-sensitive genetic factors over heritable genetic factors or directly-acquired environmental cues in defining cue specificity. Yet, which specific hive-environmental factor might shape the specificity of nestmate recognition cues in honey bee colonies, and how it does so, remains unknown (Vernier et al 2019).

1.4 The role of microbes in insect pheromone production

One possible hypothesis that could explain how hive-specific environmental factors might influence the developmental regulation of colony-specific chemical nestmate recognition cues is that colony specific gut microbial communities play a role in defining colony-specific nestmate recognition cues in foraging honey bees. The idea that gut microbes influence host digestion, metabolism, and immunity are not new, emerging data about the “gut-brain axis” indicate they also have a significant impact on the neural physiology and behavior of their hosts (Mayer et al., 2014). For example, studies in mammalian models have highlighted the role that microbes play in stress and anxiety (Clarke et al., 2013; Diaz Heijtz et al., 2011; Neufeld et al., 2011; Sudo et al., 2004), neurodevelopmental disorders, such as autism spectrum disorders (Desbonnet et al., 2014; Mayer et al., 2014), and mood (Benton et al., 2007; Schroeder et al., 2007). Likewise, symbiotic microbes have been implicated in vertebrate social interactions, including communication (Archie and Tung, 2015; Arentsen et al., 2015; Bharwani et al., 2017;
Buffington et al., 2016; Desbonnet et al., 2015; Ezenwa and Williams, 2014; Leclercq et al., 2017; Münger et al., 2018). Similarly, a growing body of literature indicates that microbes can also affect the production of insect pheromones, either via parasitic interactions, in which the microbe manipulates host pheromones to benefit their own transmission and dispersal at a cost to the host (Keesey et al., 2017), or via symbiotic interactions, in which microbial physiology and metabolism directly or indirectly contribute to pheromone syntheses in the host (Engl and Kaltenpoth, 2018). Examples include a role for microbe-influenced pheromones in mediating behaviors such as courtship and mating (Ami et al., 2010; Arbuthnott et al., 2016; Ben-Yosef et al., 2008; Damodaram et al., 2016; Engl and Kaltenpoth, 2018; Gavriel et al., 2011; Guo and Blomquist, 1991; Hoyt et al., 1971; Sharon et al., 2010), aggregation (R J Dillon et al., 2002; Wada-Katsumata et al., 2015; Zhao et al., 2015), anti-aggregation (repellants) (Brand et al., 1977; Hulcr et al., 2011; Kandasamy et al., 2016), and oviposition site location (Lam et al., 2007).

Microbes influence important host phenotypes in social insects as well, including the ability to identify individuals infected with pathogens as form of “social immunity”. In some cases, natural pathogen infections (McDonnell et al., 2013) or experimental immunostimulations (F. J. Richard et al., 2012, 2008) have been shown to lead to changes in the CHC profiles of the infected host, which presumably allows nestmates to identify and remove these individuals from the colony. Conversely, some microbes are known to disrupt social immunity behaviors, including the natural agricultural biocide fungus Beauveria bassiana, which has recently been shown to alter CHC profiles of honey bees such that they can more easily gain entrance into unrelated hives (Cappa et al., 2019). Similarly, symbiotic microbes have been implicated in mediating normal nestmate recognition behaviors in a few ant species (Dosmann et al., 2016;
Teseo et al., 2018). Together, these findings open the door for understanding the role of microbes in mediating nestmate recognition behaviors across social insect species.

In Chapter 3 of this thesis, I provide evidence that gut microbial communities play an important role in nestmate recognition in the honey bee. In honey bees, the gut microbial community consists of a stereotypic 8-10 phylotypes, is known to develop as a bee ages (Vincent G. Martinson et al., 2012b), and is acquired through interactions with older bees or nesting materials (J Elijah Powell et al., 2014). My work shows that honey bee colonies carry colony-specific gut microbial communities, which vary in the relative proportion of these common phylotypes between colonies, and that differences in gut microbial communities, both at the phylotype and strain-level, are sufficient to drive differences in CHC profile and recognition behaviors between genetically related bees. Remarkably, my work indicates that gut microbial community not only contributes to recognition cue production, but also its perception, highlighting a potential pleiotropic role that microbes play in mediating this host behavior.
Chapter 2

The cuticular hydrocarbon profiles of honey bee workers develop via a socially-modulated innate process

Note: This chapter is published as (Vernier et al., 2019).

2.1 Abstract

Large social insect colonies exhibit a remarkable ability for recognizing group members via colony-specific cuticular pheromonal signatures. Previous work suggested that in some ant species, colony-specific pheromonal profiles are generated through a mechanism involving the transfer and homogenization of cuticular hydrocarbons (CHCs) across members of the colony. However, how colony-specific chemical profiles are generated in other social insect clades remains mostly unknown. Here I show that in the honey bee (*Apis mellifera*), the colony-specific CHC profile completes its maturation in foragers via a sequence of stereotypic age-dependent quantitative and qualitative chemical transitions, which are driven by environmentally-sensitive intrinsic biosynthetic pathways. Therefore, the CHC profiles of individual honey bees are not likely produced through homogenization and transfer mechanisms, but instead mature in
association with age-dependent division of labor. Furthermore, non-nestmate rejection behaviors seem to be contextually restricted to behavioral interactions between entering foragers and guards at the hive entrance.

2.2 Introduction

The ability to recognize “self” plays an important role in regulating diverse processes across biological organizational levels (Tsutsui, 2004). Analogous to the acquired immunity system, which depends on self-recognition at the cellular and molecular levels (Boehm, 2006), adaptive organismal social interactions often depend on the recognition of kin and/or group-members to increase cooperation or to suppress inbreeding (W. D. Hamilton, 1964; W. D. D. Hamilton, 1964; Pusey and Wolf, 1996; Trivers, 1971; Stuart A West et al., 2007; Wilkinson, 1988). One remarkable example of organismal recognition of “self” comes from colonies of social insects, which depend on a robust non-nestmate discrimination system (more commonly called “nestmate recognition”) to prevent the loss of expensive resources to non-nestmates, and to maintain overall colony integrity (Hefetz, 2007; van Zweden and D’Ettorre, 2010).

As in other self-recognition systems, theoretical models suggest that nestmate recognition in social insect colonies depends on the ability of individual colony members to reliably match colony-specific phenotypic cues, or “labels”, carried by other colony members, to stored neural “templates” (Buckle and Greenberg, 1981; Errard, 1994; Gamboa et al., 1986; Getz, 1982; Hölldobler and Michener, 1980; Lacy and Sherman, 1983; Reeve, 2002; Tsutsui, 2004; van Zweden and D’Ettorre, 2010). In some social insect species, the cues used in recognizing individual members of the colony have been reported to be visual (Baracchi et al., 2015), but in most cases are thought to be chemical (van Zweden and D’Ettorre, 2010). Cuticular
hydrocarbons (CHCs), which evolved to function as hydrophobic, anti-desiccant barriers in terrestrial arthropods, have been co-opted to also function as pheromones in diverse insect communication systems, including nestmate recognition in social insect species (Chung and Carroll, 2015; van Zweden and D’Ettorre, 2010). Whether the overall profile, or more specific components of it, represent the actual nestmate recognition cue remains unknown. However, previous studies have indicated that variations in the relative amounts of each compound in the CHC profile across individuals from different colonies are likely sufficient for the chemical recognition of nest membership (van Zweden and D’Ettorre, 2010). Nevertheless, how large groups of hundreds to thousands of individuals coordinate the production and recognition of a robust colony-specific chemical cue remains unknown for most species.

Because members of social insect colonies are often genetically related, it was initially assumed that the production of similar colony-specific pheromones by individual colony members is intrinsically driven by shared allelic variants (R.H. Crozier and Dix, 1979; Getz, 1982, 1981). However, empirical studies revealed that, surprisingly, in many social insect species colony and social environmental factors play the most dominant role in defining colony-specific cues, and can often mask genetic relatedness (Breed et al., 1988; Downs and Ratnieks, 1999; Heinze et al., 1996; Lahav et al., 1999; Liang and Silverman, 2000; Singer and Espelie, 1996; Stuart, 1988). Although these colony “environmental” factors remain unknown for most social insect species, it has been suggested that contributions from nest building materials (Breed et al., 1988; Margaret J Couvillon et al., 2007; D’Ettorre et al., 2006a; Karl E. Espelie et al., 1990; Singer and Espelie, 1996), the queen (Carlin and Hölldobler, 1988, 1987, 1986, 1983), and diet (Buczkowski et al., 2005; Buczkowski and Silverman, 2006; Liang and Silverman, 2000; Richard et al., 2004, 2007) could, at least in part, provide unique chemical components to the
chemical signature shared by colony members. Consequently, empirical and theoretical studies suggested that individual colony members acquire their colony-specific chemical signature largely through a homogenization process involving the exchange of relevant chemicals, including CHCs, through interactions between colony members or contact with nest building materials, often referred to as the “Gestalt” model (R.H. Crozier and Dix, 1979). Empirical evidence in support of this model has been reported for a few ant species, which are known to transfer mixed blends of CHCs between individuals through trophallaxis and grooming via the action of the postpharyngeal gland (PPG) (Boulay et al., 2000; Lenoir et al., 2001; Meskali et al., 1995; Soroker et al., 1994; Soroker et al., 1995; Van Zweden et al., 2010). However, other studies suggest that such CHC homogenization processes might not fully represent how colony-specific chemical cues develop in all social insect species. For example, some ant species do not display robust trophallaxis behaviors, the main mode of chemical transfer across colony members (Soroker et al., 1994; Soroker et al., 1995), and in others, the CHC profiles of individual colony members are likely modulated by genetic relatedness (Teseo et al., 2014), age (Cuvillier-Hot et al., 2001; Teseo et al., 2014), and/or task (Martin and Drijfhout, 2009; Sturgis et al., 2012; Wagner et al., 1999, 1998). Together, these data suggest that the regulation of chemical cues in different species is more variable and complex than initially hypothesized (Esponda et al., 2015; Newey, 2011; Sturgis and Gordon, 2012), and remains unknown for most social insect species.

Consequently, here I investigated the development of CHC profiles and nestmate recognition cues in the European honey bee, *Apis mellifera*, a species of economic importance and one of the best studied social insect species. Numerous previous studies have demonstrated that honey bees exhibit a robust nestmate recognition system that is based on the chemical
recognition of pheromones (van Zweden and D’Ettorre, 2010). Analyses of CHC profiles showed that newly emerged honey bee workers express significantly lower amounts of total CHCs and lower overall CHC chemical diversity in comparison to older foragers, which are expected to elicit the strongest nestmate recognition response from guards at the entrance to the hive (Breed et al., 2004; Kather et al., 2011). Additionally, other studies have suggested that honey bee nestmate recognition cues might be derived from various environmental sources (Downs and Ratnieks, 1999), and hive building materials such as the honeycomb wax (Breed, 1998; Breed et al., 1988; Margaret J Couvillon et al., 2007; D’Ettorre et al., 2006a). Based on these studies, it has been hypothesized that, similar to some ant species, the CHC profile of newly eclosed workers represents a “blank slate” (Breed et al., 2004; Lenoir et al., 1999), and that nestmate recognition cues are subsequently acquired by individual workers primarily through the homogenization and transfer of chemicals via direct social interactions and intermediate environmental factors (Breed et al., 2015). Furthermore, it has recently been proposed that the cephalic salivary gland of honey bee workers is functionally analogous to the PPG in ants, and could be involved in the homogenization and transfer of the CHCs between colony members (Martin et al., 2018). However, when and how honey bee chemical nestmate recognition cues mature, and whether CHC homogenization mechanisms play a role in this process have not been directly investigated.

Here I provide empirical evidence that the maturation of the CHC profile of individual honey bee workers is primarily regulated by innate developmental processes associated with age-dependent behavioral tasks and modulated by the social colony environment, and that mature colony-specific recognition cues are primarily associated with the foraging task. Specifically, I find that individual workers exhibit stereotypic quantitative and qualitative changes in their CHC
profile as they transition from in-hive tasks to foraging outside, that these changes are associated with innate transcriptional changes in CHC biosynthetic pathway genes, and that only forager honey bees are behaviorally rejected from the entrance of an unrelated hive. Together, my findings suggest that not all members of honey bee colonies display a uniform cuticular chemical profile via the direct acquisition of CHC mixes. Instead, my data indicate that CHC profiles, and likely nestmate recognition cues, in honey bees are more likely a product of a genetically-determined developmental program that is modulated by colony-specific factors.

2.3 Results

2.3.1 CHC profiles of individual honey bee workers exhibit qualitative and quantitative age-dependent changes.

Given that newly emerged honey bees have lower amounts of total CHCs, and exhibit less chemical diversity compared to older bees (Breed et al., 2004), I initially sought to determine the age at which the CHC profile of individual honey bee workers matures. To achieve this goal, I analyzed the CHC profiles of individual workers from a single age-cohort that was reintroduced back into its source colony and then collected at different ages. This analysis revealed that the total amount of CHCs increases between one-day post-reintroduction and 14-days post-reintroduction and then remains stable (Figure 2.1A, Kruskal-Wallis, H = 9.21, df = 3, p = 0.026, FDR pairwise contrasts: Day 1 vs. Day 7 p = 0.11, Day 1 vs. Day 14 p = 0.036, Day 1 vs. Day 21 p = 0.04, Day 7 vs. Day 14 = 0.613, Day 7 vs. Day 21 p = 0.691, Day 14 vs. Day 21 p = 0.79). Additionally, individual compounds vary in total amount across bees of different ages (Figure 2.1C, D, Table 2.1). Independently of the age-related quantitative changes, I also found that the CHC profiles of workers exhibit age-related qualitative changes in the overall CHC chemical
composition (Figure 2.1B, Permutation MANOVA, F(1,31) = 22.86, R² = 0.43, p < 0.001, FDR pairwise contrasts: Day 1 vs. Day 7 p = 0.002, Day 1 vs. Day 14 p = 0.002, Day 1 vs. Day 21 p = 0.002, Day 7 vs. Day 14 = 0.017, Day 7 vs. Day 21 p = 0.002, Day 14 vs. Day 21 p = 0.31), as well as in the relative amounts of individual CHCs (Figure 2.1E, F, Table 2.2). These data confirm that not all members of a honey bee colony share a common CHC profile (Kather et al., 2011), and suggest that age-dependent processes might be playing an important role in the regulation of both the quantitative and qualitative dimensions of the cuticular chemical profiles of individual honey bee workers.
Figure 2.1: CHC profiles of bees exhibit quantitative and qualitative changes in association with age. (A) Total CHC amounts (μg) extracted from sister bees of different ages. (B) CHC profiles of sister bees of different ages. (C) Statistically significantly changing amounts (μg) of individual CHCs across sister bees of different ages. (D) A subset of C with low amounts. (E) Statistically significantly changing proportions of individual CHCs across sister bees of different ages. (F) A subset of C with low proportions. Statistics in A using ANOVA followed by Tukey’s HSD post-hoc. Statistics in B using Permutation MANOVA followed by FDR pairwise contrasts shown as a non-metric multidimensional scaling plot depicting Bray-Curtis dissimilarity between samples. Statistics for C & D are listed in Table 1, statistics for E & F are listed in Table 2. Lowercase letters above bars in A and legend in B denote posthoc significance (p < 0.05). Sample size per group, N = 8.
Table 2.1: Individual CHCs vary in total amount (ng) across different aged sister bees of a single colony. Numbers represent mean amount (ng) of compound across bees of that age ± standard error. All p-values are from parametric ANOVA or nonparametric Kruskal Wallis ANOVA (denoted by “KW”). Letters denote statistically significant age groups across individual compounds via Tukey’s HSD (ANOVA post-hoc) or Dunn’s Test with FDR adjustment (KW post-hoc) (p < 0.05).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Name</th>
<th>Retention Time</th>
<th>Day 1</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Foraging</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>C18:1</td>
<td>( z(-9) )-Octadecenoic acid</td>
<td>20.23</td>
<td>146.91 ± 52.98 (A)</td>
<td>1213.9 ± 250.45 (AB)</td>
<td>2193.35 ± 360.14 (B)</td>
<td>2465.05 ± 779.73 (B)</td>
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<tr>
<td>C23:1</td>
<td>Tricosene</td>
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<td>121.69 ± 7.05 (A)</td>
<td>86.74 ± 9.13 (B)</td>
<td>68.64 ± 9.57 (B)</td>
<td>61.79 ± 9.87 (B)</td>
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</tr>
<tr>
<td>C23</td>
<td>Tricosane</td>
<td>21.21</td>
<td>1372.77 ± 115.62 (A)</td>
<td>811.16 ± 97.45 (B)</td>
<td>688.47 ± 50.32 (B)</td>
<td>662.47 ± 70.72 (B)</td>
<td>0.001 (KW)</td>
</tr>
<tr>
<td>C24</td>
<td>Tetracosane</td>
<td>22.79</td>
<td>43.54 ± 2.33</td>
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<td>Pentacosene</td>
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<td>167.38 ± 15.77</td>
<td>131.94 ± 10.87</td>
<td>122.74 ± 13.46</td>
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<td>Pentacosane</td>
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<td>1280.62 ± 78.61</td>
<td>1253.94 ± 145.03</td>
<td>1364.18 ± 51.8</td>
<td>1465.39 ± 195.17</td>
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<tr>
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<td>Heptacosene</td>
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<td>359.94 ± 109.71 (A)</td>
<td>67.55 ± 5.22 (B)</td>
<td>299.98 ± 33.28 (A)</td>
<td>340.49 ± 29.27</td>
<td>0.006</td>
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<td>C27</td>
<td>Heptacosane</td>
<td>26.87</td>
<td>1372.77 ± 115.62 (A)</td>
<td>811.16 ± 97.45 (B)</td>
<td>688.47 ± 50.32 (B)</td>
<td>662.47 ± 70.72</td>
<td>0.001 (KW)</td>
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<td>11,13 methyl C27</td>
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<tr>
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<td>484.12 ± 21.65 (B)</td>
<td>515.79 ± 37.33</td>
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<tr>
<td>C29</td>
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<td>3891.37 ± 203.93</td>
<td>3361.26 ± 373.3</td>
<td>3535.4 ± 161.9</td>
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<td>1888.38 ± 76.35 (A)</td>
<td>621.6 ± 51.82 (B)</td>
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<td>482.86 ± 24.65</td>
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<td>3030.3 ± 152.07 (B)</td>
<td>3336.99 ± 139.27 (B)</td>
<td>3437.57 ± 146.12</td>
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<td>2623.84 ± 145.02 (B)</td>
<td>2756.09 ± 101.15 (B)</td>
<td>2748.6 ± 137.39</td>
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<td>Foraging</td>
<td>p-value</td>
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<td>6.68 ± 1.83 (B)</td>
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<td>0.25 ± 0.02 (B)</td>
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<td>0.13 ± 0.01 (B)</td>
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<td>11.06 ± 1.29 (B)</td>
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<td>1.95 ± 0.09 (B)</td>
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<td>Octacosane</td>
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<td>1.21 ± 0.04 (AB)</td>
<td>1.35 ± 0.06 (B)</td>
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<td>6.62 ± 0.24 (A)</td>
<td>1.79 ± 0.08 (B)</td>
<td>1.37 ± 0.08 (B)</td>
<td>1.37 ± 0.08 (B)</td>
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<tr>
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<td>1.35 ± 0.08 (AB)</td>
<td>1.19 ± 0.13 (AB)</td>
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Table 2.2: Individual CHCs vary in proportion across different aged sister bees of a single colony. Numbers represent mean percentage of compound across bees of that age ± standard error. All p-values are from parametric ANOVA or nonparametric Kruskal Wallis ANOVA (denoted by “KW”). Letters denote statistically significant age groups across individual compounds via Tukey’s HSD (ANOVA post-hoc) or Dunn’s Test with FDR adjustment (KW post-hoc) (p < 0.05).
2.3.2 The CHC profiles of individual workers are task-related.

Honey bee workers exhibit age-related division of labor, which is characterized by a stereotypic sequence of in-hive behavioral tasks such as nursing and food handling, followed by the final transition to foraging outside the colony at about three weeks of age (Robinson, 1992; Smith et al., 2008; Søvik et al., 2015a). Consequently, under natural colony settings, it is impossible to separate the possible independent impacts of ‘age’ and ‘task’ on the expression of forager-specific CHC profiles. Therefore, I next analyzed the CHC profiles of individual nurse and forager bees from single-cohort-colonies (SCC), a well-established experimental approach to uncouple behavioral maturation from chronological age (Ben-Shahar, 2002; Ben-Shahar et al., 2004; Greenberg et al., 2012; Robinson et al., 1989; Whitfield et al., 2003). Because these artificial colonies are initially comprised of a single age-cohort of day-old bees, a small proportion of these young workers will accelerate their behavioral maturation to become precocious foragers that are the same age as typical nurses (~7 days old) (Ben-Shahar, 2002; Greenberg et al., 2012; Huang and Robinson, 1992). The comparison of the CHC profiles of typical young nurses and precocious foragers of identical age revealed a significant effect of task on the CHC profile of individual workers (Figure 2.2A, Permutation MANOVA, F(1,15) = 13.79, R² = 0.50, p < 0.001). Similarly, I observed a significant effect of task on the CHC profiles of individual “over-aged” nurses and typical-aged foragers at three weeks of age (Figure 2.2B, Permutation MANOVA, F(1,15) = 45.41, R² = 0.76, p < 0.001). In contrast, task and age had no effect on total CHC amount (Figure 2.2C, Two-way ANOVA, age: F(1,28) = 0.55, p = 0.46, task: F(1,28) = 0.37, p = 0.55, age*task: F(1,28) = 5.37, p = 0.03). Together, these data suggest that processes associated with the behavioral maturation of honey bee workers, not
chronological age, are primarily responsible for the observed forager versus nurse CHC profiles of individual honey bee workers.

Previous studies in Harvester ants suggested that exposure to the environment outside the nest is sufficient to induce stereotypical changes in the CHC profiles of individual social insects (Wagner et al., 1999). Therefore, I next asked whether spending time outside the hive is sufficient to induce the observed forager-specific CHC profile by comparing the CHC profiles between “undertakers”, nurses, and foragers from typical colonies. “Undertakers” are a small group of highly specialized older pre-foraging workers (2-3 weeks of age), which are responsible for removing dead bees by carrying them outside and away from the colony (Robinson, 1992; Smith et al., 2008; Søvik et al., 2015a; Trumbo et al., 1997). Therefore, because undertakers and foragers perform their respective tasks outside the hive, while nurses and other younger, pre-foraging bees rarely do, I reasoned that if outdoor exposure defines the distinct forager-specific CHC profile then the CHC profiles of undertakers should be more similar to foragers than to nurses. However, I found that the CHC profiles of undertakers are markedly different from those of foragers, and are more similar to those of nurses (Figure 2.2D, Permutation MANOVA, F(2,23)=12.60, R^2 = 0.55, p < 0.001, FDR pairwise contrasts: undertaker vs. forager p = 0.003, undertaker vs. nurse p = 0.176, forager vs. nurse p = 0.003). These data suggest that some outdoor exposure is not sufficient to drive forager-specific CHC profiles.

I next asked whether the CHC profiles of foragers are a direct consequence of their behavioral state by using “big back colonies” (Ben-Shahar et al., 2000; Withers et al., 1995), which allowed us to compare active foragers to bees of a similar age and behavioral state that are unable to forage outside (see methods). I found that the overall CHC profiles of “big-back” bees were different from those of their actively foraging sisters (Figure 2.2E, Permutation MANOVA,
F(1,14)=5.91, R² = 0.313, p < 0.001). These data suggest that the physiological transition to foraging behaviors is not the sole factor that defines forager-specific CHC profiles, and that it could be modulated by additional factors associated with the act of foraging itself and/or extended exposure to various outdoor environmental factors. However, the fact that foraging nestmates express very similar CHC profiles, which are markedly different from those of non-nestmate foragers sharing a similar foraging environment (Figure 2.3, Permutation MANOVA, F(1,15) = 12.5, R² = 0.47, p < 0.001) suggests that forager-specific CHC profiles are not simply defined by the foraging environment. Additionally, to test whether extended exposure to outdoor environmental factors induces predictable changes in CHCs, I compared the relative amounts of individual compounds between forager bees and in-hive bees across my various experiments. I did not find a single compound that varied between foragers and in-hive bees in a consistent manner across my experiments (e.g. always increases or always decreases in association with foraging activity) (Table 2.3), indicating that CHCs do not change in a stereotypic manner in association with extended outdoor exposure, as they do in Harvester ants (Wagner et al., 1999). Nevertheless, to further examine whether forager-specific CHC profiles were solely environmentally determined, I also analyzed the CHC profiles of typical-age foragers that were forced to revert to a nursing state (Robinson et al., 1992). However, I did not find any differences between the CHC profiles of reverted nurses and active foragers (Figure 2.2F). These data suggest that once foragers acquire their signature CHC profile, it remains stable independent of the task they perform and despite the typical short CHC half-life in insects (Kent et al., 2007). Together, these data suggest that forager-specific CHC profiles are derived from a combination of factors associated with an innate behavioral maturation process, as well as being physically engaged in foraging activity.
Figure 2.2: Effect of task on the CHC profile of bees is independent of age. Single cohort colony bees differ in CHC profile by behavioral task at one week of age (typical nurse age, (A)) and three weeks of age (typical forager age, (B)). (C) SCC bees do not differ in total CHC amount due to age and/or task. (D) Undertakers and nurses differ from foragers in CHC profile. (E) “Big-back” bees differ from same-aged actively foraging sisters in CHC profile. Total CHC statistics (C) using ANOVA followed by Tukey’s HSD with FDR correction. CHC profile statistics (A, B, D, E) using Permutation MANOVA followed by FDR pairwise contrasts, shown as non-metric multidimensional scaling plots depicting Bray-Curtis dissimilarity between samples. Letters in graphs and legends denote posthoc statistical significance (p < 0.05). Sample size per group, N = 8.
Figure 2.3: CHC profiles differ between unknown-aged foragers from two different colonies at a single location. Statistics using Permutation MANOVA followed by FDR pairwise contrasts shown as a non-metric multidimensional scaling plot depicting Bray-Curtis dissimilarity between samples. Lowercase letters in legend denote posthoc significance (p < 0.05). Sample size per group, N = 8.
<table>
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<tr>
<th>Compound</th>
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<th>SCC 3 weeks</th>
<th>Undertaker</th>
<th>“Big-back”</th>
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<tr>
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<td>0.51 (+)</td>
<td>1.56 (+)</td>
<td>0.78 (+)</td>
<td>ns</td>
</tr>
<tr>
<td>C_{23}</td>
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<td>3.48 (+)</td>
<td>11.45 (*)</td>
<td>10.8 (+)</td>
<td>-0.85 (+)</td>
</tr>
<tr>
<td>C_{24}</td>
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<td>0.54 (+)</td>
<td>0.52 (*)</td>
<td>-0.09 (+)</td>
</tr>
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<td>4.15 (+)</td>
<td>1.66 (*)</td>
<td>ns</td>
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<td>24.22 (+)</td>
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<td>ns</td>
<td>-0.29 (+)</td>
</tr>
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<td>-2.58 (*)</td>
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<td>-1.56 (+)</td>
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**Table 2.3:** CHCs vary in relative proportion between foragers and in-hive bees across studies. Numbers represent difference in mean percentage of each compound in forager bees relative to in-hive bees. “Hive 1” denotes forager bees minus Day 14 bees corresponding to Figure 2.1; “SCC week 1” and “SCC week 3” denote forager bees minus nurse bees corresponding to Figure 2.2A and 2.2B, respectively; “Undertaker” denotes forager bees minus undertaker bees corresponding to Figure 2.2D; “Big-back” denotes forager bees minus big-back bees corresponding to Figure 2.2E. Statistics using Student’s t-test or Mann-Whitney U between the forager and in-hive bee group. Asterisks (*) or plus sign (+) denote statistical significance for t-test or Mann-Whitney U, respectively. “ns” denotes non-significant differences.
2.3.3 The development of individual CHC profiles is a regulated process modulated by the colony environment.

Previous work indicates that guard bees will accept foraging-age nestmates and reject foraging-age non-nestmates, independent of genetic relatedness (Downs and Ratnieks, 1999). This suggests that factors associated with the hive environment play a dominant role in specifying the colony-specific chemical signatures used for nestmate recognition. Yet, my data also indicate that CHC profile development in individual workers is a developmentally-regulated process that is closely associated with the age-dependent division of labor among workers. To address this potential conundrum, I next asked whether the effects of task and colony environment on the development of CHC profiles of individual workers are independent by using a reciprocal cross-fostering strategy. To achieve my goal, I introduced cohorts of newly eclosed bees from two different typical colonies back into their source colony, as well as a reciprocal foster colony, and then recollected marked workers from both cohorts in each reciprocal colony at different ages. CHC analyses revealed that through Day 14, the CHC profiles of bees were more similar to the profiles of their same-aged non-nestmate sisters than those of unrelated nestmates of similar age (Figure 2.4A, Two-way Permutation MANOVA, foster colony (environment): F(1,31) = 2.19, R² = 0.06, p = 0.06, source colony (genetics): F(1,31) = 5.94, R² = 0.16, p < 0.001, foster colony*source colony: F(1,31)=0.46, R² = 0.01, p = 0.82; Figure 2.4B, Two-way Permutation MANOVA, foster colony: F(1,31) = 1.13, R² = 0.03, p = 0.33, source colony: F(1,31) = 3.18, R² = 0.09, p = 0.02, foster colony*source colony: F(1,31) = 1.78, R² = 0.05, p = 0.15; sample size assessment depicted in Figure 2.5 indicates sample size is adequate). In contrast, once workers shift to foraging activity, I found that the CHC profiles of fostered bees are different from the profiles of both foraging sisters raised in the source colony and unrelated host foragers of similar age (Figure 2.4C, Two-way Permutation MANOVA, foster colony:
F(1,31) = 4.04, R² = 0.10, p = 0.02, source colony: F(1,31) = 7.65, R² = 0.19, p = 0.001, foster colony*source colony: F(1,31)=0.48, R² = 0.01, p = 0.67). Together, these data suggest that genetic variations, or other long-term effects associated with the source colony, play an important role in defining the CHC profiles of individuals during the early phases of the age-dependent behavioral development of worker honey bees. However, by the time bees start foraging, the mature CHC profile of individual workers is defined by an interaction between factors associated with both the source and foster colonies (Figure 2.4C).
Figure 2.4: Cross-fostering indicates colony environment drives the signature CHC profiles of foragers. Age-matched cross-fostered bees differ in CHC profile by source colony at Day 7 (A) and Day 14 (B), and by both source colony and foster colony when they are foragers (C). Number to left of arrow in legend represents the bee’s source colony, and the number to the right represents the bee’s foster colony. All statistics using Permutation MANOVA followed by FDR pairwise contrasts, shown as non-metric multidimensional scaling plots depicting Bray-Curtis dissimilarity between samples. Letters in legends denote posthoc statistical significance (p < 0.05). Sample size per group, N = 8.
Figure 2.5: Sample size assessment of cross-fostered bees indicates sample size of 8 is adequate. Pseudo multivariate dissimilarity-based standard error “levels off” around a sample size of 7 for all cross-fostered groups at Day 7 (A), Day 14 (B), and when they are foragers (C).
2.3.4 The development of CHC profiles of individual workers is associated with the regulation of CHC biosynthesis genes.

Homogenization models for the development of colony-specific nestmate recognition cues predict that cue specificity is acquired by individuals via physical contact with other colony members and/or environmental sources of hydrocarbons (Breed, 2015; R.H. Crozier and Dix, 1979; a. Lenoir et al., 2001; Meskali et al., 1995; Soroker et al., 1994; Victoria Soroker et al., 1995; Van Zweden et al., 2010). However, because my data indicate that the maturation of the CHC profile of individual honey bees is actually regulated in association with the stereotypic age-dependent division of labor in this species, I next hypothesized that the CHC profiles of worker honey bees develop, at least in part, via an intrinsic age-dependent regulation of the CHC biosynthetic pathways in the pheromone producing oenocytes (Chung and Carroll, 2015; Falcón et al., 2014; Makki et al., 2014; Yew and Chung, 2015). Thus, I next examined whether age and/or task are associated with the mRNA expression levels of genes that encode elongases and desaturases, the primary CHC diversity producing classes of enzymes in the CHC biosynthesis pathway (Chung and Carroll, 2015). To identify candidate genes for my analyses, I first used a bioinformatic approach to identify all putative members of both protein families in the honey bee genome (Table 2.4). Subsequently, I used real-time quantitative RT-PCR to compare mRNA levels of each candidate gene in dissected abdominal cuticles from bees of different ages raised in their source colony, as well as foraging sister bees raised in either their source colony or an unrelated foster-colony. My analyses revealed that the expression levels of at least one elongase and two desaturase genes are associated with either age or colony environment (Figure 2.6 and Table 2.5). Thus, my data suggest that individual worker honey bees regulate CHC expression through an innate age-dependent developmental process that is further modulated by other factors such as task and the social environment.
Figure 2.6: Age and social environment affect the expression level of CHC biosynthesis genes. (A) Elongase gene. (B-C) Desaturase genes. Only genes with different expression levels between at least two groups are shown (See Table 7 for results for all studied genes). Black bars represent bees raised in their own colony. Grey bars represent sister forager bees that were raised in an unrelated colony (“Fostered”). (D) Heat map of relative expression levels of all genes tested. Aging bee statistics using ANOVA followed by Tukey HSD post-hoc, or Kruskal-Wallis followed by Dunn’s Test with FDR adjustment post-hoc, with letters denoting posthoc statistical significance (p < 0.05). Between colony statistics using Mann-Whitney U test, with asterisks above grey bars denoting statistical significance from foraging bees raised in their own colony (*, p < 0.05). Sample size per group, N = 4.
<table>
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<tr>
<th>Gene</th>
<th>Function</th>
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<th>Forward primer</th>
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<th>Previously Published</th>
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Table 2.4: CHC biosynthesis genes, BLAST E-values, and quantitative real-time PCR primers used in this study. BLAST E-values listed with known *Drosophila melanogaster* enzyme gene compared to.
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<td>0.71 ± 0.10 (AB)</td>
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<td>1.95 ± 0.09</td>
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Table 2.5: Genes differ in relative mRNA expression level between bees of different ages (Age), and foraging sister bees raised in two different colonies (Hive). Numbers represent mean relative mRNA expression level ± standard error across four biological replicates. All p-values are from parametric ANOVA or nonparametric Kruskal Wallis ANOVA (denoted by “KW”). Letters denote statistically significant age groups across individual compounds via Tukey’s HSD (ANOVA post-hoc) or Dunn’s Test with FDR adjustment (KW post-hoc) (p < 0.05).
2.3.5 Age and task play a role in defining nestmate recognition cues in honey bee colonies.

Because previous studies have indicated that nestmate recognition in honey bee colonies is likely driven by components of the CHCs profile (van Zweden and D’Ettorre, 2010), and my discovery that the CHC profiles of individual workers seem to mature in association with the well-described age-dependent division of labor in this species (Robinson, 1992; Smith et al., 2008; Søvik et al., 2015a), I next hypothesized that, in honey bees, nestmate recognition cues themselves mature in association with age-dependent division of labor, and reach maturation during foraging. To test this hypothesis, I investigated the behavioral responses of guard bees to related and unrelated focal bees of different ages (Day 1, Day 7, Day 14, and foragers on Day 21). At each test colony, the behavioral responses of guards to random related and unrelated returning foragers of unknown age were used as the benchmark for the baseline level of nestmate recognition behavior. Behavioral observations revealed that bees are accepted at the entrance of their own colony, regardless of age (Figure 2.7A, Pearson’s Chi-Squared, Day 1: $\chi^2 = 49.05$, df = 2, $p < 0.001$, Day 7: $\chi^2 = 19.07$, df = 2, $p < 0.001$, Day 14: $\chi^2 = 44.89$, df = 2, $p < 0.001$, Day 21: $\chi^2 = 28.32$, df = 2, $p < 0.001$). In contrast, at the entrance to an unrelated colony, bees were accepted on Days 7 and 14, but rejected as foragers (Day 21) (Figure 2.7B, Day 1: $\chi^2 = 11.61$, df = 2, $p = 0.003$, Day 7: $\chi^2 = 15.51$, df = 2, $p < 0.001$, Day 14: $\chi^2 = 11.91$, df = 2, $p = 0.002$, Day 21: $\chi^2 = 7.35$, df = 2, $p = 0.04$). These data support the hypothesis that nestmate recognition cues in honey bee colonies mature in association with age-dependent division of labor, and suggest that nestmate recognition is specific to behavioral interactions between guards and foragers at the entrance to the hive.
Surprisingly, I also observed that while young Day 1 bees are accepted by related guards, they are often rejected by unrelated guards (Figure 2.7B). This finding contradicts the broadly accepted “blank slate” hypothesis, which predicts that because day-old bees are devoid of any defining chemical signatures, they should be always accepted by guards independent of relatedness (Breed et al., 2004). While I do not yet know which specific components of the CHC profile of young bees, if any, might have triggered a rejection by unrelated guards in my colonies, one plausible interpretation of these data is that the observed response of guards to unrelated Day 1 bees is an artifactual experimental outcome of a forced behavioral interaction between two bee groups, which in colonies with a typical demography, do not normally interact in the context of the hive entrance.
Figure 2.7: Forager bees have mature colony-specific recognition cues. (A) Bees are accepted at a similar rate as Colony 1 foragers at the entrance to their source colony (Colony 1) at all ages. (B) Bees are rejected at a similar rate as Colony 1 foragers at an unrelated colony (Colony 2) on Day 1 and Day 21. However, bees are accepted at a similar rate as Colony 2 foragers at an unrelated colony (Colony 2) on Day 7 and Day 14. All statistics using Pearson’s Chi-Square. Asterisks or letters denote posthoc statistical significance (p < 0.05), ns denotes non-significant comparisons. Sample size per group, N = 18-29.
2.4 Discussion

The ability of colonies of social insects to reliably recognize group membership is one of the remarkable adaptations that enabled their immense ecological success. Yet, the molecular and physiological mechanisms that underlie this complex trait remain unknown for most species. In the well-studied honey bee, previous studies suggested that the chemical cues that drive nestmate recognition are absent in newly eclosed bees, and subsequently develop primarily through the homogenization and transfer of chemicals between colony members via direct interactions such as allogrooming and trophallaxis, and indirect interactions such as physical contact with wax and other nest materials (Breed, 2015; Breed et al., 2004). However, the data I present here suggest that the overall development of individual CHC profiles of honey bee workers primarily depends on an innate developmental process that is associated with the stereotypic age-dependent division of labor in this species, and that colony-specific cues are likely only carried by foragers. Therefore, I posit that it is unlikely that CHC profiles in honey bees develop through homogenization and transfer mechanisms between nestmates and hive materials. Furthermore, given the established implicated role of CHCs in nestmate recognition (van Zweden and D’Ettorre, 2010), I additionally posit that CHC homogenization mechanisms are unlikely to play a key role in the production of colony-specific cues in honey bees.

A major line of investigation in understanding nestmate recognition of social insects has been to determine how colony-specific cues are determined. Cue specificity has historically been proposed to be determined by mechanisms under genetic control or acquired from the environment (Crozier and Dix, 1979). Although my studies do not directly address the mechanism by which cue specificity is determined in honey bees, data from cross-fostering experiments suggest that cue development and specificity are defined by interactions between
factors derived from the colony-of-origin of individual workers and the actual hive environment they develop in. Therefore, my data suggest that CHC profiles of honey bee workers develop via a biphasic process that is governed, at least in part, by the intrinsic physiology of individual workers, the specific behavioral tasks they are engaged in, and the hive environment they age in. In phase one, similar to other social insect species (Soroker et al., 1995), the total CHC amount builds up, possibly to increase the resistance of workers to desiccation while still inside the protective hive environment (Chung and Carroll, 2015). In phase two, the total amount of CHCs remains constant but the relative abundances of individual components shift in association with the age-dependent behavioral maturation of workers, at least in part, via the transcriptional regulation of CHC biosynthetic enzymes.

Which specific components of the honey bee CHC profile represent the nestmate recognition cue remains unknown. Although it has been shown that CHCs are likely used for nestmate recognition in honey bees (van Zweden and D’Ettorre, 2010), it is unlikely that all components of the CHC profile contribute to this process (Akino et al., 2004; Dani, 2005; Dani et al., 2001; Martin et al., 2008; Ruther et al., 2002). In fact, it has previously been shown that alkenes seem to play a more prominent role in nestmate recognition in the honey bee than alkanes (Dani, 2005). My data also indicate that although unrelated foragers raised in the same colony are equally accepted, their overall CHC profiles remain somewhat qualitatively different (Figure 2.4C). These data provide two important insights. First, guards are not likely using the full CHC profile of individuals to determine group membership. Second, differences in the CHC profiles of co-fostered nestmate foragers of similar age that originated from different source colonies indicate that the chemical profiles of individual workers are not likely to be the product of a stochastic CHC homogenization and transfer between colony members.
The observation that the mRNA expression levels of genes that encode CHC-biosynthesis enzymes vary in association with age and/or task further indicate that the primary mechanism for the dynamic regulation of the CHC profile of individual honey bee workers is directly associated with the well-established age-dependent division of labor in the honey bee (Robinson, 1992; Smith et al., 2008; Søvik et al., 2015a). Although these data do not directly exclude the possibility that some particular CHCs are transferred across colony members, they do indicate that the overall observed qualitative age- and task-dependent changes in the CHC profiles of individual workers are affected by intrinsic molecular dynamics of the CHC synthesis pathway. However, my studies also importantly show that genetically-related bees that age in different colonies exhibit qualitatively different CHC profiles and CHC biosynthesis gene expression levels, which suggests that the CHC synthesis process is also plastic and could be modulated by factors associated with the hive/social environment.

I were initially surprised by my observation that Day 1 bees are accepted at the entrance to their source colony but rejected by guards at the entrance of an unrelated colony since previous studies hypothesized that the lower amounts of total CHCs in young bees represent a “blank slate” in terms of the nestmate recognition cue because these bees are readily “accepted” when introduced into unrelated colonies (Breed et al., 2004). In fact, this phenomenon was exploited here to introduce cohorts of bees to foster colonies, typically by placing the new bees on the top frames of experimental hives. This apparent conundrum highlights an important, yet often underappreciated, aspect of the nestmate recognition system in honey bees and other social insect species, which is that the “rejection” behavior by guards is highly contextual. Conceptually analogous to other biological systems responsible for the detection of “self” versus “non-self” (e.g., the acquired immunity system in vertebrates), behaviors associated with
nestmate recognition are restricted to interactions between guards and incoming bees at the entrance to the hive (Couvillon et al., 2013). Therefore, I speculate that because nestmate recognition is spatially restricted to specific behavioral interactions between entering bees and guards at the entrance, the commonly observed “acceptance” of day old bees outside the specific context of the hive entrance actually represents the lack of behavioral “rejection” rather than a true self-recognition-dependent “acceptance”. Consequently, the observation that Day 1 bees are rejected at the entrance of an unrelated colony suggests that nestmate recognition of young bees either depends on components of the CHC profile that are already present in Day 1 bees, non-CHC chemical cues, or an altogether different sensory modality. Alternatively, because newly eclosed bees usually perform cell cleaning behaviors at the interior of the hive, and therefore do not typically interact with guards at the hive entrance (Robinson, 1992; Smith et al., 2008; Søvik et al., 2015a), differences in rejection of Day 1 bees between these two colonies might represent an experimental artifact resulting from differences in tolerance to the forced behavioral interaction between two bee groups that normally do not interact. Additionally, it has previously been shown that observed levels of guarding behaviors in honey bees are plastic, and could fluctuate in response to various environmental factors such as seasonal weather patterns, overall colony size, food availability, and “robbing” pressures from other colonies or predators (Downs and Ratnieks, 2000). Likewise, more extreme forms of plasticity in nestmate recognition systems have been reported in other social species. For example, some social insects can switch between using visual or chemosensory modalities for nestmate recognition under different circumstances (Baracchi et al., 2015). Together, it seems that instead of being driven by simple binary decisions, nestmate recognition systems in the honey bee and other social insect species depend
on a plastic recognition of “friends” versus “foes” as part of a broader group-level optimization of colony fitness.

In conclusion, I propose that nestmate recognition cue production and acquisition in honey bees are not likely to be primarily driven by CHC homogenization and transfer mechanisms as previously described in some ant species (Boulay et al., 2000; a. Lenoir et al., 2001; Meskali et al., 1995; Soroker et al., 1994; Victoria Soroker et al., 1995; Van Zweden et al., 2010). Instead, I propose a new model for the regulation of nestmate recognition in honey bee colonies, which stipulates that unknown factors associated with the hive environment play a direct or indirect role in defining the developmental kinetics and specificity of nestmate recognition cues by modulating the cellular and molecular processes that are responsible for pheromone synthesis. Thus, it is plausible that the colony/social environment drives the intrinsic development of similar pheromone profiles by individual colony members, which in typical honey bee hives, is associated with the physiological processes that drive age-dependent division of labor. If true, this model could resolve previous seemingly contradictory data which suggested that honey bee CHC profiles are defined by genetic (Page et al., 1991) versus environmental (Downs and Ratnieks, 1999) factors, as well as open the door for comparative mechanistic studies of how complex social traits evolve and function in different social insect clades.

2.5 Methods

2.5.1 Animal Husbandry and Bee Collections.

Honey bee (*Apis mellifera*) colonies were reared and managed using standard beekeeping techniques across two locations near St. Louis, MO: Tyson Research center (38° 31’N, 90°
33°W) and a residential home. For all experiments that included collections of bees at specific ages, capped brood frames were taken from a colony and placed in a humidified 32°C incubator. Once eclosed, about 1000 bees (<24 hours old) were marked with a spot of paint (Testors, Vernon Hills, IL, USA) on their thorax, and then reintroduced into either their source or a foster colony, depending upon the experiment. For collections of bees at specific ages, marked bees were collected from internal frames of the colony one day post reintroduction (Day 1), seven days post reintroduction (Day 7), 14 days (Day 14) post reintroduction, and as returning foragers, identified by pollen loads on their hind legs or having a distended abdomen due to nectar loads, between 18 and 21 days post reintroduction. Bees used for chemical and molecular analyses were placed in individual 1.7 mL microtubes and immediately placed on dry ice. All samples were kept at -80°C until further analysis.

2.5.2 Single-cohort colonies.

Single-cohort colonies (SCC) were established as previously reported (Ben-Shahar, 2002; Ben-Shahar et al., 2004; Greenberg et al., 2012; Robinson et al., 1989; Whitfield et al., 2003). In short, about 1000 newly eclosed bees (<24 hours old) were placed in a small wooden nucleus hive-box with a young, unrelated mated queen, one honey frame from their source colony, an empty comb frame, and three new frames with wax covered plastic foundation. Bees were collected as typical-aged nurses and precocious foragers one week after introduction, and as over-aged nurses and typical-aged foragers at three weeks after introduction. Bee samples were collected and stored as above.

2.5.3 Undertaker collection.
To induce “undertaking” behavior, about 1000 dead bees were placed into the top of two different colonies, and the first 20 bees that were observed removing dead bees from the colony were collected from the entrance. Returning foragers and in-hive nurses of unknown ages were also collected from each colony at the same time. Samples were stored and processed as described above.

2.5.4 Big-back colony.

Big-back colonies were established as previously described (Ben-Shahar et al., 2000; Withers et al., 1995). In short, bees were introduced in two cohorts to a 5-frame hive box containing 3 empty comb frames, 2 brood frames, and a new queen. In the first cohort, 200 day-old bees were collected as described above and marked on the thorax with paint. Half of these bees were marked with a plastic tag attached to the thorax (~3 mm diameter, ~1 mm thick; “big-back” bees). Day-old bees in the other cohort were collected and introduced 4 days later as described above to increase the proportion of precocious foragers in the first group. The entrance to the colony was blocked by a piece of Plexiglas with holes in it that prevented “big-back” bees from leaving the hive, but allowed paint marked bees to leave. Bees were collected at 7 days of age: returning foragers were collected as described above, and “big-back” bees were collected as they were attempting to leave the hive via the holes in the plastic.

2.5.5 Reversion colony.

Reversion colonies were made by collecting ~1000 foragers from a single source colony by vacuuming them directly into a sealed 5-frame hive box containing two brood frames, one honey frame, and two empty comb frames. The hive was sealed and moved to a new location ~30 miles
away from the source colony, and a new queen was added that night. The hive was sealed for 3 days, and then was opened to allow normal foraging activity to resume. During this time, in the absence of nurses, some foragers reverted back to nursing behaviors (Robinson et al., 1992). Actively foraging bees were collected at the hive entrance as described above and reverted nurses were collected from internal frames as described above.

2.5.6 Cross-fostering experiment.

1000 day-old bees from two independent source colonies were collected and marked as above. Half of the bees in each marked cohort were randomly reintroduced to both their own source colony and the reciprocal foster colony. Subsequently, marked bees of defined age were recollected from internal frames of each colony as described above.

2.5.7 Nestmate recognition assay.

Every day over a three-week period, newly eclosed bees (<24 hours old) from a single source colony were collected as described above, uniquely color-marked, and then reintroduced into their source colony. Subsequently, on each experimental day, bees from the following groups were collected, placed in individual 15 mL plastic tubes (Corning, Corning, NY, USA), and chilled on wet ice in an ice cooler up to 10 minutes before the assay in order to limit heat related stress: bees of the focal age (identified by color of mark), returning nectar foragers (denoted by distended abdomen and lack of pollen) of unknown age from the source colony, and returning nectar foragers of unknown age from an unrelated colony. All foragers, which served as behavioral controls, were painted the same color as the experimental bees just after collection.
Tubes were numbered in a randomized order and blinded to the experimenter conducting the behavioral assays. Fifteen bees per group were prepared for each colony each experimental day.

Behavioral assays were conducted simultaneously at two colonies (source and unrelated) by two researchers, as well as recorded using digital video cameras. As described previously (D’Ettorre et al., 2006a; Downs and Ratnieks, 2000), acceptance at the colony entrance was used as a proxy for nestmate recognition by placing individual bees on a modified entrance platform and recording the behavioral reactions of guard bees for ~5 min. Bees were considered ‘Rejected’ if they were bit, stung and/or dragged by at least one guard bee. Bees were considered ‘Accepted’ if they were approached by guards, antennated and/or licked and then left alone (not bit), if they immediately entered the colony and were not removed by other bees, or if they remained on the platform and did not receive aggression. After 5 min, focal bees that remained on the platform outside the colony were removed before the next assay. All behaviors were scored in real time, and videos were retained as back-up. All behavioral assays were conducted during a period of 10 days, between 12 and 4pm, with two days focusing on each age of experimental bee (N = 20-30 bees per group).

2.5.8 Cuticular Hydrocarbon Extractions and GC analysis.

CHCs were extracted from whole bees by placing individual bees into 6 mL glass vials fitted with 16mm PTFE/silica septa screw caps (Agilent Crosslab, Santa Clara, CA, USA). Bee CHCs were extracted in 500 uL hexane containing 10 ng/μl of octadecane (C\textsubscript{18}) and 10 ng/μl of hexacosane (C\textsubscript{26}) (Millipore Sigma, St. Louis, MO), which served as injection standards. To achieve efficient extraction, each vial was gently agitated by vortexing (Fisher Scientific, Waltham, MA, USA) for 2 min at minimum speed. Extracts were immediately transferred to new
2 mL glass vials fitted with 9mm PTFE lined caps (Agilent Crosslab, Santa Clara, CA, USA). In cases where experiments involved forager honey bees, all bees (including non-foragers) had their hind legs removed prior to extraction, in order to ensure removal of pollen. 100 ul of each extract was transferred to a new 2 mL glass vial and stored at -20°C for further analysis; the remaining 400 uL was stored at -80°C as back-up.

Representative pooled samples of foragers and nurses of known age were first analyzed by combined gas chromatography/mass spectrometry (GC/MS) for compound identification. Samples were run from 150⁰ (3 min hold) to 300⁰ at 5⁰/ min. Compounds were identified by their fragmentation pattern as compared to synthetic compounds. For profile characterizations of individual bees, samples were analyzed using an Agilent 7890A gas chromatograph system with a flame ionization detector (GC/FID) and PTV injector (cool-on-column mode), and outfitted with a DB-1 20 m x 0.18 mm Agilent 121-1022 fused silica capillary column (Agilent Technologies, Inc. Santa Clara, CA, USA). Sample volumes of 1.0 μl were injected onto the column. Helium was the carrier gas and applied at a constant flow rate of 1 ml/min. Analysis of the extract was carried out with a column temperature profile that began at 50C (held for 1 min) and was ramped at 36.6⁰C/min to 150C and then at 5C/min to 280C, where it was held for 10 min. The injector and FID temperatures were programmed to 280C and 300C, respectively. Agilent OpenLAB CDS (EZChrom Edition) software was used to calculate the retention time and total area of each peak. Data was normalized to known quantity (ng) of internal standard hexacosane.

2.5.9 CHC Biosynthesis Gene Identification, RNA Isolation and Quantitative Real-Time PCR.
Members of the highly conserved desaturase and elongase gene families were identified in the honey bee genome by using the protein BLAST search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with annotated *Drosophila melanogaster* amino acid sequences (https://flybase.org) of elongase and desaturase genes known to play a role in CHC biosynthesis (Chung and Carroll, 2015). Initial homologs in the honey bee genome were chosen by picking the top match (highest total score and query cover, lowest E value) for each *D. melanogaster* gene, and possible paralogs of these putative genes were identified by subsequently using the NCBI protein BLAST tool (RRID:SCR_004870) with these genes’ amino acid sequences. Many of these putative elongase and desaturase genes have previously been identified as possible CHC biosynthesis pathway genes in the honey bee (Falcón et al., 2014). E-values from the BLAST scans of the honey bee genome by using three canonical *Drosophila melanogaster* CHC biosynthesis genes, *EloF* (elongase subfamily), *Elo68α* (elongase subfamily), and *desat1* (fatty acid desaturase subfamily), are listed in Table 2.6.

To measure mRNA levels of individual genes, the cuticles from the abdomens of four bees per group were dissected out, and total RNA was extracted using the Trizol Reagent (Life Technologies, Grand Island, NY, USA). SuperScript II (Life Technologies, Grand Island, NY, USA) reverse transcriptase was used to generate cDNA templates from 500ng of total RNA per sample by using random hexamers. A Bio-Rad (Hercules, CA, USA) CFX Connect Real-Time PCR Detection System and Bio-Rad iTaq Universal SYBR Green Supermix were subsequently used for estimating relative differences in mRNA levels across samples (N=4 per group, run in triplicate technical replications). Expression levels of the *EIF3*-S8, a housekeeping gene that has previously been used as a reference gene in honey bee studies of gene expression by us and others (Alaux et al., 2009; Fischer and Grozinger, 2008; Greenberg et al., 2012; Mao et al., 2015;
F.-J. Richard et al., 2008), was used as a loading control. To further ensure that the reported expression data for the experimental genes are robust, I first confirmed that the raw $\text{EIF3-S8 C}_t$ values per total RNA used in the individual RT reactions were not affected by any of the studied groups included in my current study (Kruskal-Wallis, $H = 3.299, df = 4, p = 0.5091$). The specific RT-PCR primers for each gene-specific assay are listed in Table 2.6.

### 2.5.10 Statistical Analysis.

All CHC analyses included a set of 19 peaks that represent well-established honey bee CHCs, identified by comparing GC traces to published data (Kather et al., 2011). For the comparisons of total CHCs across groups (as in Figure 2.1A), total ng of all identified CHCs in each bee were analyzed using ANOVA followed by Tukey’s HSD in R 3.3.2 (R Core Team, 2016). For the remainder of the datasets, the relative proportion of each compound in each sample was calculated and then used in further statistical analysis. For each dataset, a permutation MANOVA was run using the ADONIS function in the vegan package of R (RRID:SCR_011950) with Bray-Curtis dissimilarity measures (Oksanen et al., 2017). Pairwise comparisons with FDR p-value correction were subsequently run on experiments where more than two groups were compared. Data were visualized using non-metric multidimensional scaling (metaMDS function in the vegan package of R (RRID:SCR_011950) (Oksanen et al., 2017)) using Bray-Curtis dissimilarity, and either 2 or 3 dimensions in order to minimize stress to $< 0.1$. For Table 2.1, Table 2.2, Table 2.3, and Table 2.4 an ANOVA followed by Tukey’s HSD post-hoc comparison, or Kruskal-Wallis followed by Dunn’s Test with FDR adjustment was performed using total ng (Table 2.1 and Table 2.2) or proportions (Table 2.3 and Table 2.4) of each compound across bees of the four time point collections. For cross-fostering studies,
power was assessed by performing pseudo multivariate dissimilarity-based standard error, a method for assessing sample-size adequacy in multivariate data, as described in and using code from Anderson and Santana-Garcon (2015). For behavioral data, the proportion of bees accepted by guard honey bees was calculated for each experimental group at each colony at each day of age. A Pearson’s chi-square was run for each day of age at each colony with subsequent pairwise comparisons. For qPCR data, relative expression levels were calculated as previously described (Greenberg et al., 2012; Hill et al., 2017; Zheng et al., 2014), using eIF3-S8 as a loading control. Fold-expression data were generated by using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and designating a single individual from the “Day 1” group (Figure 2.6) as a calibrator. For statistical analyses, the $2^{-\Delta\Delta CT}$ scores were compared within each gene across bees of different groups using an ANOVA followed by Tukey’s HSD post-hoc comparison, or Kruskal-Wallis followed by Dunn’s Test with FDR adjustment. Overall test p-values were then adjusted using FDR correction to account for 16 independent comparisons (Benjamini and Hochberg, 2018).

2.6 Acknowledgements

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Chapter 3:
Gut microbiome factors define group membership in honey bee colonies

3.1 Abstract

Social insect colonies rely on colony-specific chemical signatures in order to recognize group members and prohibit intruders from entering the colony. In previous work I have shown that in the honey bee (*Apis mellifera*), nestmate recognition cues are intrinsically produced by individual workers in association with their age-dependent division of labor, and that cue specificity is determined by colony-specific environmental factors. However, the identity of these factors was unknown. Here I show that one important colony-specific factor in defining colony-specific chemical nestmate recognition cues is the gut microbial community. Specifically, my data indicate that nestmate workers share colony-specific gut microbial communities, and that qualitative and quantitative differences in gut microbial community at the phylotype and strain levels are sufficient to drive differences in the CHC profile of genetically related bees, and surprisingly, their ability to behaviorally perceive these CHC profile differences. Together, this work indicates that the gut microbial community may play a pleiotropic role in nestmate recognition cue production and perception, and more broadly, highlights the importance of
symbiotic bacterial communities in complex animal social traits, including driving inter-individual behavioral variations of their hosts.

3.2 Introduction

My previous work has indicated that in honey bees, nestmate recognition cues are defined by social environmental factors, which drive the intrinsic physiological processes that define the shared colony-specific chemical profiles of foragers from the same colony (Vernier et al., 2019). These findings indicate that although the development of nestmate recognition cues seems to be genetically determined, cue specificity is in fact determined by an unknown social/hive environmental factor. To resolve this apparent conundrum, I tested the hypothesis that colony-specific nestmate recognition cues are defined via an interaction between the host bees and symbiotic microbial communities shared by members of the same colony. If true, this model would provide a relatively simple solution for how an acquired environmental factor could drive an innate genetic program that defines social group membership.

Previous studies have indicated that symbiotic microbes play an important role in driving and modulating diverse biological aspects of their animal hosts, from nutrition and health to behavior (Cryan and Dinan, 2012; Engel and Moran, 2013; Ezenwa et al., 2012; Ezenwa and Williams, 2014; Flint et al., 2012; Hooper et al., 2012; Münger et al., 2018). Recent studies suggest that the gut-brain axis is a vital driving force of complex host behavior, with studies supporting the role of gut microbes in anxiety-like behaviors (Clarke et al., 2013; Degroote et al., 2016; Diaz Heijtz et al., 2011; Kim et al., 2018; Neufeld et al., 2011) and social interactions (Arentsen et al., 2015; Bharwani et al., 2017; Buffington et al., 2016; Desbonnet et al., 2015;
Leclercq et al., 2017), including communication (Archie and Tung, 2015; Ezenwa and Williams, 2014; Münger et al., 2018), across multiple host species. Microbes have been implicated as sources of olfactory cues used in conspecific recognition (Albone et al., 1974; E S Albone and Perry, 1976; Gorman, 1976), including familiarity with specific individuals (Gorman, 1976), mate preferences (Arbuthnott et al., 2016; Lizé et al., 2014; Morimoto et al., 2017; Ringo et al., 2011b; Sharon et al., 2010), and the recognition of group membership (K R Theis et al., 2012) and kin (Kirchner and Minkley, 2003; Lizé et al., 2014; Minkley et al., 2006). Although the precise mechanisms by which bacteria affect the chemical signatures of their hosts are unknown, both direct (Albone et al., 1974; E S Albone and Perry, 1976; R. J. Dillon et al., 2002; Gorman, 1976; K R Theis et al., 2012) and indirect effects on the chemical signatures of individual hosts have been reported (Engl and Kaltenpoth, 2018; Sharon et al., 2010; Vernier et al., 2019). Specifically in insect hosts, it has been hypothesized that symbiotic bacteria could affect social interactions via their effects on the production of cuticular hydrocarbons, which are commonly used as pheromones (Engl and Kaltenpoth, 2018) including several social insect species (de Souza et al., 2011; Kenji Matsuura, 2001; Teseo et al., 2018).

Although honey bees have a relatively simple gut microbial community, mostly composed of eight to ten phylotypes, recent studies suggest that it plays an important role in regulating various aspects of the honey bee host biology (Kwong and Moran, 2016). Although the possible effect of the gut microbiota on the CHC profiles of individual bees has not been investigated, several lines of evidence suggest that such a link might exist. First, both the CHC profiles (Breed et al., 2004; Falcón et al., 2014; Vernier et al., 2019) and gut microbial communities (Martinson et al., 2012) of individual worker bees change as a function of age. Second, both nestmate recognition cues (Breed et al., 1998, 1988; Margaret J Couvillon et al.,
and the gut microbial communities (Martinson et al., 2012) are influenced by the social environment. Third, gut bacterial communities (Moran et al., 2012) and CHC profiles (Vernier et al., 2019) are similar across individual members of a colony but vary across hives.

Here, I provide empirical evidence that the gut microbiome plays an important role in nestmate recognition in honey bees. Specifically, I show that honey bee colonies have colony-specific microbial communities, measured at both the phylotype and the strain level, which are associated with differences in CHC profile. Specifically, I demonstrate that experimentally induced differences in gut microbial communities between genetically related bees is sufficient to induce differences in CHC profile, and that differences in gut microbiome across individuals are sufficient to induce differential acceptance behaviors by groups of bees inoculated with a similar or different microbiome. Remarkably, I also demonstrate that bees inoculated with different microbiomes show differences in recognition abilities, suggesting that in addition to mediating cue production, gut microbiome likely plays a role in mediating nestmate recognition cue perception. These data suggest that gut-microbiome-derived factors are important for nestmate recognition via the pleiotropic coupling of nestmate recognition cue production and perception.

3.3 Results

3.3.1 Gut microbial communities differ between worker bees from different colonies.

To first address the possible contribution of the honey bee gut microbiota to nestmate recognition, I investigated whether variations in the gut microbiome are associated with
variations in the CHC pheromonal profile of individual bees. To achieve this goal, I compared the CHC profiles and gut microbial communities between forager bees across three different colonies by using gas chromatography and 16S rRNA sequencing, respectively. This analysis revealed that foragers from different honey bee colonies indeed differ in their CHC profiles (Figure 3.1A, Permutation MANOVA, F(2,23) = 8.54, R² = 0.45, p < 0.001) and their gut microbiome beta diversity (Figure 3.1B, Permutation MANOVA, F(2,29) = 2.00, R² = 0.13, p = 0.014), but not alpha diversity (Figure 3.1C, ANOVA, F(2,27) = 0.19, p = 0.83). These data indicate that although the phylogenetic diversity of honey bee gut microbial communities across colonies are similar at the phylotype-level, individuals from different colonies exhibit varying relative abundances of specific phylotypes, which are associated with differences in their CHC profiles.
Figure 3.1: Gut microbial communities differ between foragers from different colonies. Forager honey bees across different hives differ in CHC profile (A) and gut microbial community beta diversity (B), but not in alpha diversity (C). Forager bee gut microbial community is determined by colony environment (D-E). Gut microbiome data via 16S rRNA sequencing. Statistics in (A), (B) and (D) using Permutation MANOVA followed by FDR pairwise contrasts, shown as non-metric multidimensional scaling plots depicting Bray-Curtis dissimilarity between samples. Statistics in (C) and (E) using ANOVA with Shannon Index alpha diversity metric. Letters in legends denote posthoc statistical significance (p < 0.05). Sample size per group, N = 8 and 10, for CHC and microbiome data, respectively.
3.3.2 The honey bee gut microbial community is influenced by colony environment.

Because previous studies have suggested that honey bees acquire their microbiome through interactions with older bees and nest materials (Martinson et al., 2012; Powell et al., 2014), I next tested the hypothesis that the colony-specific composition of the gut microbiome in individual bees depends on interactions with their hive environment. To achieve this goal, I cross-fostered cohorts of newly eclosed bees from two different typical colonies by either placing them back into their source hive or a reciprocal unrelated foster hive, and analyzed their gut microbiomes with 16S rRNA sequencing. This analysis revealed that bees differed in their gut microbial community beta diversity as a function of their environment (Figure 3.1D, Permutation MANOVA, $F(3,33) = 2.44, R^2 = 0.20, p = 0.001$), but not alpha diversity (Figure 3.1E, ANOVA, $F(3,30) = 0.606 , p = 0.616$). These data suggest that, similar to the CHC profiles of individual foragers (Vernier et al 2019), qualitative measures of the gut microbial communities of individual workers are shaped, at least in part, by social environment, further supporting a relationship between gut microbial community and CHC pheromone profiles of individual foragers.

3.3.3 Gut microbial communities can mask the effect of host genotype on the development of individual bees’ CHC profiles.

In order to directly test the link between gut microbial community and CHC profile, I assessed whether experimentally manipulating gut microbiome between sister honey bees would be sufficient to drive differences in their CHC profiles. To accomplish this, I first examined the effect of antibiotics on the CHC profiles of treated versus untreated sister bees, which revealed a strong effect of treatment on the CHC profiles of these individuals (Figure 3.2, Permutation
MANOVA, $F(1,15) = 3.66, R^2 = 0.21, p = 0.005$). Similarly, when sister bees were inoculated with live inoculum or heat-killed inoculum, they developed different CHC profiles (Figure 3.2D, Permutation MANOVA, $F(1,31) = 6.17, R^2 = 0.17, p = 0.006$) and gut microbial communities (Figure 3.2E, Permutation MANOVA, $F(1,39) = 6.93, R^2 = 0.15, p = 0.001$) depending on inoculation treatment.

Because previous research has shown that young bees acquire their microbiome via interactions with older bees or nest materials (Martinson et al., 2012), I reasoned that exposure of newly eclosed bees to older bees from different colonies would result in the development of markedly different microbiomes and CHC profiles. Indeed, I found that when raised with older bees from two different colonies, sister bees developed different CHC profiles (Figure 3.2F, Permutation MANOVA, $F(1,15) = 5.44, R^2 = 0.28, p = 0.01$) and gut microbial communities (Figure 3.2G, Permutation MANOVA, $F(1,19) = 8.60, R^2 = 0.32, p = 0.001$), but not when these older bees were first treated with antibiotics (Figure 3.2H, Permutation MANOVA, $F(1,13) = 1.55, R^2 = 0.11, p = 0.194$). Additionally, I was not able to amplify the V4 region of the 16S rRNA gene from bees that were raised with older bees first treated with antibiotics, which indicated very low bacterial load in these focal bees. The lack of any microbiome signatures in the focal bees confirmed that the treated older bees had very low bacterial loads, as well as the likely transfer of antibiotics between the older and experimental bees. Regardless, these findings indicate that young bees acquire microbial communities from their older sisters, and that this microbiome transfer helps define their CHC profiles.
Figure 3.2: Sister bees inoculated with different honey bee gut microbial communities develop different CHC profiles. Sister bees differ in CHC profile when they are treated with antibiotics (A). Antibiotic treated bee guts (B) show less growth when plated than those treated a control sugar solution (C). Sister bees differ in CHC profile (B) and gut microbial community (C) when inoculated with live inoculum vs. heat-killed inoculum. Sister bees differ in CHC profile (F) and gut microbiome (G) when they are inoculated by older bees from two different colonies (F), but not when the older bees are first treated with antibiotics (H-I). Gut microbiome data via 16S rRNA sequencing. All statistics using Permutation MANOVA followed by FDR pairwise contrasts, shown as non-metric multidimensional scaling plots depicting Bray-Curtis dissimilarity (A,D,F,H) or Weighted UniFrac (E,G) between samples. Letters in legends denote posthoc statistical significance (p < 0.05). Sample size per group, N = 8 and 10, for CHC and microbiome data, respectively.
3.3.4 The gut microbiome contributes to group recognition behaviors.

To test whether variations in the gut microbiome can directly influence the chemical cues used by bees for nestmate recognition, I manipulated the gut microbiomes of bees by inoculating them with specific phylotypes and then assessed the behavioral responses of groups of inoculated bees to unfamiliar individuals that were inoculated with the same or different phylotype. In the Ben-Shahar laboratory, I can reliably culture two common honey bee phylotypes on standard LB plates in the lab (Figure 3.3A), which I have identified as *Gilliamella apicola* and *Lactobacillus Firm-4* by sequencing the V4 region of the 16S rRNA gene, and then inoculate groups of bees with these phylotypes. Initially I verified that sister bees inoculated with either *G. apicola* or *Lactobacillus Firm-4* developed different CHC profiles (Figure 3.3B, Permutation MANOVA, F(1,15) = 7.33, R² = 0.34, p = 0.004) and gut microbial communities (Figure 3.3C, Permutation MANOVA, F(1,18) = 3.09, R² = 0.15, p = 0.004). I then used in-lab behavioral assays to measure the impact of inoculations on acceptance versus rejection behaviors. I found that bees inoculated with *G. apicola* were able to distinguish sister bees inoculated with the same phylotype versus those inoculated with a *Lactobacillus Firm-4* (Figure 3.3D, Pearson’s chi squared, $\chi^2 = 7.59, p = 0.01$). In contrast, bees inoculated with *Lactobacillus Firm-4* were not able to discriminate between sister bees inoculated with the same phylotype versus those inoculated with *G. apicola* (Figure 3.3E, Pearson’s Chi Squared, $\chi^2 = 0.02, p = 1$). Together, these data suggest that some members of the honey bee gut microbial community are sufficient to drive nestmate recognition cues in honey bees. Even more remarkable, my observations suggest that the gut microbiome not only plays a role in chemically defining recognition cues, but also plays a role in their perception via mechanisms that remain unknown.
**Figure 3.3: Gut microbiome plays a role in recognition in honey bees.** Two honey bee phylotypes are easily culturable on LB plates in lab (A): *Gilliamella apicola* (black arrow) and *Lactobacillus Firm-4* (green arrow). Bees inoculated with *Gilliamella apicola* differ in CHC profile from their sister bees inoculated with *Lactobacillus* (B). Bees inoculated with *Gilliamella apicola* differ in gut microbiome from their sister bees inoculated with *Lactobacillus* (C). Bees inoculated with *Gilliamella apicola* are able to distinguish sister bees inoculated with the same phylotype vs. those inoculated with *Lactobacillus* Firm-4 (D), while those inoculated with *Lactobacillus* cannot distinguish between those inoculated with the same phylotype vs. those inoculated with *Gilliamella apicola* (E). Statistics in (B-C) using Permutation MANOVA followed by FDR pairwise contrasts, shown as non-metric multidimensional scaling plots depicting Bray-Curtis dissimilarity between samples; statistics in (D-E) using Pearson’s Chi Squared followed by FDR pairwise contrasts. Asterisks and “ns” in graphs denote statistical significance and insignificance, respectively. Sample size per group, $N = 8$, 10, and 15 for CHC, microbiome and behavioral data, respectively.
3.3.5 *Gilliamella apicola* strain-level genetic diversity is associated with differences in honey bee CHC profile.

My current data suggest that bees with different microbial communities measured at the phylotype level have different CHC profiles and acceptance rates. However, since recent reports have indicated that there is substantial genetic diversity in the honey bee gut microbiome at the sub-phylotype level (Ellegaard and Engel, 2019; Kwong et al., 2017), I next wished to assess whether within-phylotype strain-level diversity further specifies the CHC profiles of individuals. To do this, I first characterized the population level genetic diversity of the culturable *Gilliamella apicola* bacterial clones from the mid- and hind-guts of foragers from four different colonies, and sequenced the coding gene *elongation factor Tu* (*tuf*), which has previously been used to assess honey bee microbiome strain diversity across multiple common honey bee gut phylotypes (Kwong et al., 2017). Based on the number of SNPs identified in the *tuf* ORF from individual clones of *G. apicola*, I found that specific strains of this phylotype were more distantly related to each other when collected from forager bees from different colonies than between those from the same colony (Figure 3.4A, Kruskall-Wallis, H = 72.802, p < 0.001).

Therefore, I next hypothesized that genetically distinct strains of *G. apicola* could drive the development of different CHC profiles in sister bees. To test this hypothesis, I inoculated newly eclosed bees with *G. apicola* strains that were the most distantly related based on the number of *tuf* gene SNPs between them. I found that sister bees inoculated with different strains of *G. apicola* developed significantly different CHC profiles (Figure 3.4B, Permutation MANOVA, F(3,28) = 7.74, R² = 0.48, p < 0.001). Together, these data indicate that strain-level genetic diversity of a single phylotype in the honey bee gut is sufficient to induce differences in the CHC profiles of their honey bee host. Whether these CHC differences could also elicit nestmate-recognition behaviors will be explored in the near future.
Figure 3.4: Strain level diversity is associated with differences in CHC profile and recognition in honey bees. *Gilliamella apicola* coding gene *tuf* has more SNPs between forager bees from different colonies than between those from the same colony (A). Sister bees inoculated with different strains of *G. apicola* develop different CHC profiles (B). Statistics in (A) using Kruskal-Wallis followed by Dunn’s test pairwise contrasts, shown as violin plots with points representing median and lines represent inter-quartile region. Statistics in (B) using Permutation MANOVA followed by FDR pairwise contrasts, shown as non-metric multidimensional scaling plots depicting Bray-Curtis dissimilarity between samples. Letters in graph (A) and legends (B) denote *posthoc* statistical significance (p < 0.05). Sample size per group for CHC data, N = 8.
3.4 Discussion

Previous studies have suggested that colony-specific chemical nestmate recognition cues in honey bees are acquired by individual honey bee workers through “Gestalt” mechanisms via CHC homogenization (Breed, 2015). Nonetheless, I have recently shown that, in contrast to the original predictions of the "Gestalt” model (R. H. Crozier and Dix, 1979), honey bee workers acquire their colony-specific nestmate recognition cues via an intrinsic, developmentally regulated process, and that cue specificity depends on environmental hive-specific factors (Vernier et al., 2019). Yet, the identity of these hive factors, and the mechanisms by which they help define chemical cue specificity remained unknown. Here, I show that in the honey bee, nestmate recognition cue specificity is driven, at least in part, by the symbiotic gut microbiome. Specifically, my data show that differences in colony-specific gut microbial communities, measured at both the phylotype and strain levels, are sufficient to drive stable differences in CHC profiles across colonies, independent of the host genetics. Furthermore, although the precise mechanism remains unknown, my data suggest that the gut microbiome may not only define colony-specific cues, but may also play a role in their perception. Therefore, although the idea that the microbiome plays a key role in modulating host behavior is not new (Cryan and Dinan, 2012; Ezenwa et al., 2012; Ezenwa and Williams, 2014), the data presented here represents one of the first demonstrations of the direct impact that the genetic diversity of symbiotic bacteria can have on the fitness of their animal hosts via the modulation of complex social interactions.

My findings could also have important implications for understanding the maintenance of insect eusociality. It is commonly assumed that in social insects, nestmate recognition systems have evolved to enable groups of genetically-related individuals to protect stored resources, which are essential for maintaining optimal group fitness (van Zweden and D’Ettorre, 2010).
Yet, previous research highlights the importance of environment in defining nestmate recognition cues across social insect species (Breed et al., 1988; Downs and Ratnieks, 1999; Heinze et al., 1996; Lahav et al., 1999; Liang and Silverman, 2000; Singer and Espelie, 1996; Stuart, 1988), and the data I present here suggest that, in natural colony settings, colony members are mostly blind to their actual genetic relatedness to other nestmates. Instead, honey bees seem to, either directly or indirectly, rely on chemical cues from symbiotic bacteria to define group membership. Although the precise selective pressure that led to the emergence of this mechanism for defining group membership is not known, one possible explanation is that my observations represent a highly derived trait, which can still reliably serve as a proxy for genetic relatedness because in natural colonies, members are typically genetically related (Strassmann and Queller, 2013; Stuart A. West et al., 2007). However, establishing a role for the gut microbiome in defining group membership in other closely related insect lineages, such as stingless bees (Meliponini), another eusocial group in the Corbiculate clade (Kocher and Paxton, 2014), may, instead, suggest that the role of symbiotic bacteria in defining group membership may have emerged early as a basal trait that facilitated the evolution of eusociality.

Finding that symbiotic gut microbes contribute to the development of colony-specific chemical signatures is also puzzling from a neuroethological perspective. If group membership is indeed defined by stochastic environmentally-acquired factors, such as the gut microbiome, then it is hard to explain how guards at the entrance would “know” which specific chemical signatures represent group membership for their colony, and how their chemosensory system could be specifically tuned to these cues in the context of nestmate recognition. Accordingly, this has been under debate for quite some time (Dalton, 2000; Esponda et al., 2015; Lacy and Sherman, 1983; Ozaki, 2005; Ozaki and Hefetz, 2014; Sherman et al., 1997; van Zweden and
D’Ettorre, 2010). Although the studies I present here were not designed to specifically address this question, I was surprised to find that inoculating genetically related bees with different phylotypes impacted their ability to discriminate between bees that were inoculated with the same phylotype versus those that were not (Figure 3.3D-E). One simple interpretation of these findings is that the inoculation of bees with G. apicola had a coordinated pleiotropic effect on both the production and perception of specific CHCs associated with nestmate recognition.

Empirical and theoretical work from the Ben-Shahar lab (Zelle et al., 2019) and others (Arnold et al., 2006; Boake, 1991; Bousquet et al., 2011; Butlin and Ritchie, 1989; Fukamachi et al., 2009; Hoy et al., 1977; Kronforst et al., 2006; Lande, 2006; O’Donald, 1962a; Shaw et al., 2011; Singh and Shaw, 2012; Wiley et al., 2012) have indicated that genetic and hereditary factors play an important role in maintaining a robust coupling between the production and release of mating signals, and the receptor cells and neural circuits that perceive and process these signals. While these studies support the functional coupling of pheromone production and perception at the evolutionary timescale, my data suggest that coupling could also occur at the developmental and/or physiological timescales via, at least in part, the action of the symbiotic gut microbiome.

Although my findings suggest that gut microbiomes in different honey bee colonies are often comprised of different relative abundances of the common eight to ten phylotypes (Kwong and Moran, 2016), my data point to the intriguing hypothesis that strain-level genetic diversity of some of the phylotypes might be important for the development of colony-specific nestmate recognition cues. Although the majority of the known microbial phylotypes found in the bee gut are not culturable under standard lab conditions, I was able to establish that strain level genetic diversity of the common honey bee phylotype, G. apicola, is high across colonies (Figure 3.4A), and was sufficient to drive differences in the CHC profiles of genetically related hosts (Figure
3.4B). This finding raises the intriguing hypothesis that strain-level microbial communities may gain fitness benefits from living in a social insect colony, since a colony of individuals serves as a larger somatic mass as a host than individual insects, and therefore, could support larger mass of individual microbial strains. Additionally, my finding that a bee’s microbiome is a product of their social environment (Figure 3.1D) suggests that strain-level microbial communities may also gain fitness benefits from nestmate recognition behaviors by their hosts because it limits competition with unrelated strains of the same phylotype that are carried by bees from other hives. Although highly speculative, these hypotheses suggest that microbes may have facilitated in the evolution of nestmate recognition behaviors, and possibly even eusociality itself. The phenomenon of microbiomes influencing host pheromones in a manner that is beneficial to themselves, as well as the host, is not likely to be unique to social insects, and might be more common than is currently appreciated (Engl and Kaltenpoth, 2018).

In conclusion, my data indicate that the microbiome plays an important role in defining the chemical cues used for discriminating self from non-self in honey bee colonies. The gut-brain axis is emerging as major field of study in biology. While variations in the composition of the gut microbiome have historically been implicated in influencing host physiology, including aiding in digestion and contributing to diseases such as obesity, a growing body of literature highlights the connection between the gut microbiome and brain function (Cryan and Dinan, 2012; Sharon et al., 2016). In the last decade or so, the microbiome has been linked to anxiety and stress-related behaviors (Foster and McVey Neufeld, 2013) and even neurodevelopmental disorders, such as autism (Buie, 2015). Additionally, the microbiome has been implicated in communication behaviors across a variety of organisms, including invertebrates (Archie and Tung, 2015; Ezenwa and Williams, 2014; Münger et al., 2018). Specifically, variations in
microbial community composition have been linked to differences in pheromonal cues in several mammal and insect species (Albone et al., 1974; E S Albone and Perry, 1976; Arbuthnott et al., 2016; R J Dillon et al., 2002; Engl and Kaltenpoth, 2018; Gorman, 1976; Kirchner and Minkley, 2003; Lizé et al., 2014; Minkley et al., 2006; Morimoto et al., 2017; Ringo et al., 2011a; Sharon et al., 2010; K R Theis et al., 2012). The work presented here provides evidence that in the honey bee, variations in the relative abundance of gut microbial phylotypes are sufficient to drive differences in group recognition cues, but also highlights the potential role that strain-level diversity has on host cues and behavior, as well as the role that the microbiome may play in the pleiotropic coupling of recognition cues and receptors.

3.5 Methods

3.5.1 Animal Husbandry and Bee Collections.

Honey bee (*Apis mellifera*) colonies were reared and managed using standard beekeeping techniques across two locations near St. Louis, MO: Tyson Research center (38° 31’N, 90° 33’W) and a residential home. For all experiments that included in-lab treatments or cross-fostering, capped brood frames were taken from a colony and placed in a humidified 32°C incubator. For in-lab treatments, newly eclosed bees (<24 hours old) were marked with a spot of paint (Testors, Vernon Hills, IL, USA) on their thorax and placed in groups of 50 in Plexiglas treatment boxes in a humidified 32°C incubator. For antibiotic treatments (as in Figure 3.3A), newly eclosed bees were placed back into their source colony for three days and then recollected before they were placed in treatment boxes. For cross-fostering experiments, 1000 newly eclosed bees from two independent source colonies were marked with a spot of paint on their thorax, and
then half of each group were randomly reintroduced into both their source and a foster colony. Marked bees were then collected from internal frames of the colony one day post reintroduction (Day 1), seven days post reintroduction (Day 7), 14 days (Day 14) post reintroduction, and as returning foragers, identified by pollen loads on their hind legs or having a distended abdomen due to nectar loads, at 18 days post reintroduction. All bees used for chemical and molecular analyses were placed in individual 1.7 mL microtubes and immediately placed on dry ice. All samples were kept at -80°C until further analysis.

3.5.2 In lab treatments.

In-lab treated bees were kept in a humidified 32°C incubator in groups of 50 in Plexiglas boxes with a sterilized pollen patty and a hanging, inverted, sterile 1.7 mL microtube whose bottom 100 uL was removed with a razor blade. Each microtube contained sterilized 25% sugar water with the specific treatment and was replaced daily. For antibiotic treatments, a mixture of three antibiotics known to perturb insect microbiomes was used (Sharon et al., 2010). The following 1000x antibiotic solutions were made: 50 mg/mL tetracycline in water, 200 mg/mL rifampicin in DMSO, and 100 mg/mL streptomycin in water; 50 ul of each stock solution was added to 50 mL 25% sugar water, and 1.5 mL of this working solution was added to a new inverted microtube for each treatment box every treatment day. For Figure 3.3A, newly emerged bees were placed in their source colony for 3 days and then recollected and placed in treatment boxes where they received either 25% sugar water or antibiotic treatment for 15 days. For Figure 3.3D, forager bees were collected from their source colony and were placed in treatment boxes, where they received either 25% sugar water or antibiotic treatment for 3 days. Groups of 10 of these bees were then transferred to a new treatment boxes with 40 newly eclosed bees and fed 25% sugar
water for 16 days. For treatments with live inoculum, 6 forager honey bee mid- and hind-guts from a single colony were dissected under sterile conditions (Engel et al., 2013), homogenized in 1 mL sterile 25% sugar water, centrifuged at 2700 rpm for 1 min, and 250 uL of the supernatant was added to 1.3 mL 25% sugar water in a new inverted microtube for each treatment box. For heat-killed treatments, the remainder of the supernatant was heated at 95°C for 20 minutes, chilled on ice, and 250 uL was added to 1.3 mL 25% sugar water in a new inverted microtube for each treatment box. This was repeated every treatment day (17 days). For treatments with specific cultures, single colonies of bacteria were cultured in standard LB overnight to an OD of ~1 and placed in the refrigerator. Every day for the treatment period (15 days), 50 uL of this culture was added to 1.5 mL 25% sugar water in a new inverted microtube for each treatment box. In all cases, dead bees were removed daily from the treatment boxes. Bees were kept in treatment boxes until 14-18 days old, depending on the experiment and survival rate, when they were flash frozen and kept at -80°C until further analysis, or used for behavioral assays.

### 3.5.3 In lab recognition assay.

To assess recognition behaviors, I performed a modified version of the intruder assay (Li-byarlay et al., 2014; F. Richard et al., 2012). In short, four groups of bees were inoculated bees with either *Gilliamella apicola* or *Lactobacillus* (two groups each) for 16 days following the protocol described above. After the inoculation period, bees from one of each type of treatment group were placed in groups of three in a petri dish in a humidified 32°C incubator overnight. On the day of the trial, these petri dishes were moved to a humidified 25°C 1 hour before behavioral assays. During this time, bees from the other two treatment groups were marked with a spot of paint and placed in individual 15 mL tubes until the behavioral assay. These bees served as
“intruders”, and each one was introduced into one petri dish with bees inoculated with either the same phylotype or a different phylotype. Behavioral interactions were video-taped for 20 minutes and videos were subsequently scored by a blinded researcher. Intruder bees were scored as ‘Rejected’ or ‘Accepted’, where they were considered ‘Rejected’ if they were bit, stung and/or dragged by at least one bee, and ‘Accepted’ if they never received aggression from the other bees.

### 3.5.4 Cuticular Hydrocarbon Extractions and GC analysis.

CHCs were extracted from whole bees by placing individual bees into 6 mL glass vials fitted with 16mm PTFE/silica septa screw caps (Agilent Crosslab, Santa Clara, CA, USA). Bee CHCs were extracted in 500 uL hexane containing 10 ng/μl of octadecane (C_{18}) and 10 ng/μl of hexacosane (C_{26}) (Millipore Sigma, St. Louis, MO), which served as injection standards. For extraction, each vial was gently vortexed (Fisher Scientific, Waltham, MA, USA) for 2 min at minimum speed. Extracts were immediately transferred to new 2 mL glass vials fitted with 9mm PTFE lined caps (Agilent Crosslab, Santa Clara, CA, USA). In cases where experiments involved forager honey bees, all bees (including non-foragers) had their hind legs removed prior to extraction to ensure removal of pollen. 100 ul of each extract was transferred to a new 2 mL glass vial and stored at -20°C for further analysis; the remaining 400 uL was stored at -80°C as back-up.

Representative pooled samples of foragers and nurses of known age were first analyzed by combined gas chromatography/mass spectrometry (GC/MS) for compound identification. Samples were run from 150^0 (3 min hold) to 300^0 at 5^0/min. Compounds were identified by their fragmentation pattern as compared to synthetic compounds. For profile characterizations of
individual bees, samples were analyzed using an Agilent 7890A gas chromatograph system with a flame ionization detector (GC/FID) and PTV injector (cool-on-column mode), and outfitted with a DB-1 20 m x 0.18 mm Agilent 121-1022 fused silica capillary column (Agilent Technologies, Inc. Santa Clara, CA, USA). Sample volumes of 1.0 μl were injected onto the column. Helium was the carrier gas and applied at a constant flow rate of 1 ml/min. Analysis of the extract was carried out with a column temperature profile that began at 50C (held for 1 min) and was ramped at 36.6 °C/min to 150C and then at 5C/min to 280C, where it was held for 10 min. The injector and FID temperatures were programmed to 280C and 300C, respectively. Agilent OpenLAB CDS (EZChrom Edition) software was used to calculate the retention time and total area of each peak. Data was normalized to known quantity (ng) of internal standard hexacosane.

3.5.5 Gut microbiome DNA extraction, 16S rRNA sequencing and analysis.

Individual honey bee guts were homogenized by maceration with a disposable mixer sterile pestle (VWR Products). The homogenate was added to a PowerSoil Bead Solution tube (Mo Bio), and DNA was extracted using a DNeasy PowerSoil DNA isolation kit (Mo Bio, Carlsbad, CA), following the manufacturer’s instructions. The hypervariable V4 region of the 16S rRNA gene was amplified by PCR in triplicates using primers and barcodes designed in (Caporaso et al., 2012). Before sequencing, the PCR products were visualized on 1.5% agarose gels, and samples that did not have a band were removed from further analysis. All samples were pooled based upon concentrations and were sequenced on an Illumina MiSeq with 2x250bp paired-end reads. The samples for this paper were split among three sequencing pools: #1: Figure 3.1B-C, #2: Figure 3.1D-E and Figure 3.2E, #3: Figure 3.2H-I and Figure 3.3C.
After obtaining sample sequences, sequences were demultiplexed using QIIME2, and paired end reads were truncated at the first base with a quality score of <Q3 using DADA2. Paired-end reads were then merged and “amplicon sequence variants” (ASV) were identified using DADA2. Chimeric ASVs were removed and the remaining ASVs were taxonomically classified using the Greengenes database. Sample data was subsequently rarified to the lowest reasonable sample read count. Samples with a read count lower than the rarified amount were removed from the data.

For sequencing pool #1 (corresponding to Figure 3.1B-C), 1,723,236 (861,618 pairs) total sequence reads were obtained, 798,864 pairs were filtered and denoised, 710,716 pairs were merged and identified as non-chimeric (82.4%), 233 ASVs were identified, and data was rarified to 11,675 reads per sample. For sequencing pool #2 (corresponding to Figure 3.1D-E and Figure 3.2E), 1,429,358 (714,679 pairs) total sequence reads were obtained, 692,814 pairs were filtered and denoised, 672,361 pairs were merged and identified as non-chimeric (94.1%), 633 ASVs were identified, and data was rarified to 1794 reads per sample. For sequencing pool #3 (corresponding to Figure 3.2H-I and Figure 3.3C), 1,280,076 (640,038 pairs) total sequence reads were obtained, 606,799 pairs were filtered and denoised, 582,712 pairs were merged and identified as non-chimeric (91%), 183 ASVs were identified, and data was rarified to 1126 reads per sample.

3.5.6 Gilliamella apicola strain diversity measurement.

Gilliamella apicola colonies were cultured as described above, with four colonies per individual honey bee from four bees across four colonies (a total of 64 cultures), being analyzed. DNA was extracted from these cultures using a Qiagen DNeasy Blood and Tissue kit (Mo Bio, Carlsbad,
CA), and a PCR was performed targeting the *Elongation factor Tu (tuf)* gene using primers CGATACACCAACTCGTCACT and AACAACACCAGCACCAACAG. PCR products were subsequently run on a 1.5% agarose gel, the amplicon band was excised and DNA was extracted from the gel piece using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI), following the manufacturer’s directions with an extended (3 minute) elution step using 60°C heated nuclease free water, which was repeated by re-using the first elution flow-through. The extracted DNA was then sent for Sanger sequencing by Genewiz (https://www.genewiz.com), using the forward and reverse *tuf* primers separately in order to obtain 2x coverage. Sequencing was repeated for samples that had low quality scores (<30), and if the repeated sequence still had low quality scores the sample was removed from the data set. The resulting sequences were merged using USEARCHv10.0. Individual sample sequences were globally aligned in a pairwise manner across all samples using USEARCHv10.0 and the proportion of sequence overlap for each comparison was calculated and used in downstream analysis.

### 3.5.7 Statistical Analysis.

All CHC analyses included a set of 19 peaks that represent well-established honey bee CHCs, identified by comparing GC traces to published data (Kather et al., 2011). For the comparisons of CHC profiles, the relative proportion of each compound in each sample was calculated. This data was subsequently rescaled within each CHC compound to a value between 0 and 1 using the calculation $z_i = (x_i - \min(x)) / (\max(x) - \min(x))$, where $x = (x_1, ..., x_n)$ and $z_i$ is the rescaled value, in order to limit the influence of highly expressed CHCs to the overall CHC profile. These rescaled values were used in further statistical analysis. For comparisons of gut microbiome data, ASV counts were used. For each dataset, a permutation MANOVA was run using the ADONIS
function in the vegan package of R with Bray-Curtis dissimilarity measures (Oksanen et al., 2017). Pairwise comparisons with FDR p-value correction were subsequently run on experiments where more than two groups were compared. Data were visualized using non-metric multidimensional scaling (metaMDS function in the vegan package of R (Oksanen et al., 2017)) using Bray-Curtis dissimilarity for CHC data, or Bray-Curtis dissimilarity or Weighted UniFrac (Lozupone et al., 2011; Lozupone and Knight, 2005), depending on which visualization method best depicts the statistical comparisons, for microbiome data. For behavioral data, the proportion of intruder bees accepted by the group of bees was calculated and a Pearson’s chi-square was run with subsequent pairwise comparisons. For Gilliamella apicola alignment data, comparisons using an ANOVA were made using the proportion of overlap between samples from the same bee, between samples from the same honey bee colony, and between samples from different honey bee colonies. These data were visualized using violin plots (Hintze and Nelson, 1998).

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Chapter 4: Discussion

4.1 Introduction

In this dissertation, I used the European honey bee as a model for studying the mechanisms of nestmate recognition cue production in social insects. I show that in contrast to the assumptions of prior models, which suggested that honey bees acquire their colony-specific chemical signatures via inter-individual transfer and homogenization of CHCs, honey bees do not rely on CHC homogenization mechanisms to define nestmate recognition cues. Instead, individual honey bee workers develop colony-specific nestmate recognition cues via intrinsic biosynthetic processes that are influenced by hive-environmental factors and are driven by the physiological processes that define age-dependent division of labor in this species, such that only forager bees carry a cue specific to the colony they were raised in (Chapter 2). Furthermore, I show that the colony-specific nestmate recognition cue is defined, at least in part, by distinct genetic variations in gut microbial communities across colonies, which drive differences in nestmate recognition cues between bees from different colonies, possibly by modulating the intrinsic synthesis of cuticular hydrocarbons (CHCs) (Chapter 3).
4.2 The “symbiont genotype – host phenotype” interaction

An organism’s phenotype is its observable characteristics and traits, including morphology, physiology, and behavior, and is the unit on which natural selection acts (Lewontin, 1970). Historically, an organism’s phenotype has been defined by the interaction of the organism’s genotype and its environment (Gupta and Lewontin, 1982). My work highlights the role of an environmental factor, the microbiome, in mediating a honey bee phenotype: nestmate recognition. However, a closer look puts an interesting, more complex spin, on the relationship between the genotype, the environment, and the impact of their interaction on the phenotype. My finding that *G. apicola* strain level diversity influences CHC profiles of their hosts (Figure 3.4) indicates that variations in this symbiont’s genotype not only influences its own phenotype, but also the phenotype of its host. Certainly, such a “symbiont genotype – host phenotype” interaction is not restricted to this specific host-symbiont interaction, especially given the importance of symbionts in influencing host biology across taxonomic groups (Cryan and Dinan, 2012; Engel and Moran, 2013; Ezenwa et al., 2012; Ezenwa and Williams, 2014; Flint et al., 2012; Hooper et al., 2012; Münger et al., 2018), and, in fact, symbiont genotype has been known to influence host phenotype in other cases (Dunbar et al., 2007; Tsuchida et al., 2014). Therefore, these studies and my dissertation work together highlight the importance of considering variations in symbiont genotype when considering the factors that might influence interindividual variations in complex host phenotypes.
4.3 The gut – brain axis: is this concept to narrow?

The gut-brain axis is defined as the communication between microbes in the gut and the central nervous system, and is emerging as an important aspect of animal biology (John F Cryan and Dinan, 2012). While many studies support the direct cross-talk between gut microbiome and the central nervous system (Benton et al., 2007; Clarke et al., 2013; Degroote et al., 2016; Desbonnet et al., 2015, 2014; Diaz Heijtz et al., 2011; Kim et al., 2018; Neufeld et al., 2011; Schroeder et al., 2007), many studies also support influences of the gut microbiome on animal behavior that do not necessarily act directly through the central nervous system. For example, data from this dissertation, as well as other studies across a variety of animal clades, support the role of the microbiome in influencing host pheromones (Albone et al., 1974; E. S. Albone and Perry, 1976; Engl and Kaltenpoth, 2018; Ezenwa and Williams, 2014; Gorman, 1976; Ringo et al., 2011b; Sharon et al., 2010; Kevin R. Theis et al., 2012). While the microbiome influences host behavior in these cases, it does not directly “talk to” the central nervous system. Instead, the microbiome affects pheromone synthesis in the host, which affects central nervous system functioning in a more indirect way. Therefore, my work highlights the role that gut microbes play in mediating behavior without necessarily requiring a direct gut-brain cross-talk, indicating that the impact that gut microbiomes play in host behavior are much more complex than initially thought.

4.4 Gut microbial communities may have played a role in the evolution of insect communication and sociality.

Microbes have previously been hypothesized to play a role in mediating nestmate recognition in social insects. Previous studies have suggested that gut microbes can play a role in nestmate
recognition behaviors in an ant species (Teso et al., 2018), and termites (Kirchner and Minkley, 2003; K. Matsuura, 2001). In termites, microbial communities are well-established as an essential component of the host biology, which rely on them to digest their nutrient-poor diets and to mediate other important biological processes (Brune, 2014; Ohkuma, 2008). Termites acquire their colony-specific gut microbial communities through physical contact with nestmates and/or their feces (Nalupa, 2015; Ohkuma et al., 2009) (K. Matsuura, 2001), which they utilize for nestmate recognition cues (Kirchner and Minkley, 2003; K. Matsuura, 2001). In this dissertation, I have shown that honey bees also have colony-specific gut microbial communities, which they rely on for nestmate recognition cues. Although speculative, support for the role of microbes in defining nestmate recognition cues in two independent eusocial clades indicates that interactions with gut microbes may represent a convergent trait that facilitated the evolution of nestmate recognition behaviors across eusocial groups. Furthermore, many other studies support the role of microbial symbionts in defining pheromones across a variety of insect species (Englund and Kaltenpoth, 2018), suggesting a more general role of microbe-host interactions in the evolution of communication and recognition systems across Insecta.

The data presented here also raise the intriguing hypothesis that components of the gut microbiome may have co-evolved with their hosts to support the behavioral and physiological innovations associated with eusociality. For example, it is possible that the somatic host expansion associated with the evolutionary transition from the ancestral solitary- to the derived eusocial-lifestyle, which includes colonies of related individuals living together in a common space, has had a major positive impact on bacterial fitness. During this transition, in return for having an increased habitat mass and access to resources, symbiotic bacteria provided fitness benefits to their hosts by supplying a mechanism for the development of a colony-specific
nestmate recognition system. While highly speculative, this model provides testable hypotheses, which could be studied in additional social species. Of particular interest are facultatively eusocial insect species, including some species of Halictid sweat bees (Davison and Field, 2016; Eickwort et al., 1996; Field et al., 2010; Packer, 1990), in which different individuals or populations exhibit different levels of sociality. One possible hypothesis is that the association with specific bacteria could be driving individual bees to choose either a solitary or eusocial lifestyle.

4.5 Environmental stressors, the microbiome and implications in honey bee health

The European honey bee, Apis mellifera, is the main pollinator of many major crop species, making it an important economic species (Klein et al., 2007). Recent declines in honey bee populations are therefore a threat for food sources around the world, not to mention economic stability. This has led to an increase in funding for studies on the health of honey bees, and recently the microbiome has emerged as a very important aspect of honey bee biology (Bonilla-Rosso and Engel, 2018; Hamdi et al., 2011; Waldan K Kwong and Moran, 2016; Raymann and Moran, 2018). While several studies highlight the negative impacts of human-mediated environmental stressors in honey bee biology (Balbuena et al., 2015; Søvik et al., 2015b), recent studies have implicated pesticides, including glyphosate, the main component of Roundup weed killer, as a disruptor of honey bee microbiomes (Blot et al., 2019; Dai et al., 2018; Motta et al., 2018). As of yet, the main negative impact of these stressors on honey bee health has been through sensitizing them to pathogens (Blot et al., 2019; Motta et al., 2018), since their
microbiome stabilizes them against pathogen attacks (Hamdi et al., 2011; Raymann and Moran, 2018). However, the work presented here indicate that the effects of microbiome disturbance could also have negative effects on honey bee health by disrupting important adaptive behaviors. For example, the recent finding that the insecticide glyphosate, and its main metabolite amino-methylphosphonic acid, reduce the growth of the honey bee symbiotic bacterium *G. apicola* in *vivo* and *in vitro*, respectively (Blot et al., 2019), suggest that nestmate recognition behaviors could be affected by this herbicide, as well as other environmental stressors that perturb the honey bee microbiome (Raymann et al., 2017). Although my studies do not directly address this aspect of the honey bee microbiome, my work highlights new directions for understanding honey bee health via complex interactions between human mediated environmental stressors, the microbiome and behavior.

### 4.6 Conclusions

In conclusion, my dissertation work supports a model where colony-specific gut microbial communities drive the intrinsic production of colony-specific nestmate recognition cues across foragers of the same colony, as well as the ability to identify colony-specific cues in guard bees. My data have broader implications in honey bee health, understanding symbiont-host-behavior interactions and causes of variation in host phenotype, and highlights the potential role that microbes play in the evolution of insect communication and sociality.
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