Sexual Communication in Yellowjackets (Hymenoptera: Vespidae)

by

Nathan Derstine

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Name:	**Nathan Derstine**
Degree:	**Master of Science**
Title:	***Sexual Communication in Yellowjackets (Hymenoptera: Vespidae)***
Examining Committee:	**Chair:** Harold Hutter Professor

Gerhard Gries
Senior Supervisor
Professor

Jenny Cory
Supervisor
Professor

Peter Landolt
Supervisor
Research Entomologist
US Department of Agriculture

Sheila Fitzpatrick
External Examiner
Research Entomologist
Agriculture and Agri-Food Canada

Date Defended/Approved: April 11, 2017

Abstract

To determine if and how pheromones mediate sexual communication of yellowjackets [*Dolichovespula arenaria, D. maculata, Vespula alascensis, V. pensylvanica, V. squamosa*], I took three approaches: (1) In field trapping experiments, I baited traps with a virgin queen (gyne) or a male and tested for their ability to attract prospective mates. I found that only gynes of *D. arenaria* attracted males. (2) In laboratory Y-tube olfactometer experiments with *D. arenaria, D. maculata and V. pensylvanica*, I used sibling or non-sibling gynes as a test stimulus, and found that only *D. maculata* gynes attracted conspecific males, provided they were non-siblings. These results imply an olfactory-based mechanism of nestmate recognition and inbreeding avoidance. (3) I tested the hypothesis that cuticular hydrocarbons (CHCs) differentiate sex, caste, and nest membership. I found that each caste had specific CHC profiles. My data demonstrate the diversity and complexity of sexual communication in yellowjacket wasps, and inspire follow-up studies to identify the sex pheromones.

Keywords: Vespidae; *Vespula*; *Dolichovespula*; sex pheromones; cuticular hydrocarbons

Dedication

This thesis is dedicated to my parents, Kenton and Rhoda Derstine, who have always nurtured my curiosity, even when it took me far from home. To my brother and sister, Adam and Christina, who have endured more conversations about insect sex than they ever imagined possible. To my friend and mentor Matt Siderhurst who set me on this path. To my partner Allison Cornell, whose love and intelligence have been a pillar of support. And to the much maligned yellowjackets, through which I've learned a great deal, and who thrive beautifully despite the ignorant hatred often directed their way.

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Chapter 1.

Introduction

The insect order Hymenoptera is incredibly speciose, comprising ants, bees, wasps, and sawflies that function as predators, pollinators, parasitoids and herbivores, and that represent almost every gradation from solitary to eusocial living. Sexual communication systems in the Hymenoptera seem to be as diverse as the insects, with single- or multi-component pheromones of many chemical classes, originating from 'myriad' glands in both sexes. This introductory chapter summarizes knowledge about the role of pheromones in the reproductive biology of Hymenoptera, and details advances in the knowledge of hymenopteran sex pheromones since the last major review in 2004.

1.1. The role of pheromones in the reproductive biology of Hymenoptera

The diversity of hymenopteran sex pheromones is best viewed in light of the variety of mating systems that have evolved. Sex pheromones function as long- and short-range attractants, substrate-borne arrestants, and contact-chemoreceptive elicitors of copulatory behavior. There are three types of rendezvous sites for prospective mates (Ayasse et al., 2001): (1) aggregations of females at sites of emergence, overwintering, sleeping, or oviposition; 2) resource-based sites such as flowers, and (3) landmarks (non-resource-based sites) (Ayasse et al., 2001). Rendezvous sites can then be divided between those that males do, or do not, occupy territorially (Ayasse et al., 2001). The distribution of females in space and time affects the mate-seeking tactic of males. When receptive females are aggregated, males tend to patrol these aggregations; when females are dispersed, rendezvous sites are resources visited by females, or are landmarks. Neither the degree of social organization nor the mating system of females (monandry/polyandry) seems to affect the type of rendezvous site or the presence of territorial behavior by males in the mating system (Ayasse et al., 2001). Moreover, tactics of males vary among conspecifics, and among

congeners (Leonard & Boake, 2006), especially when males differ in size (Alcock, 1997; Oliveira & Schlindwein, 2010).

Sex pheromones can be categorized by their chemical classes and by their specific functions in sexual communication systems. Often, a single pheromone component fulfills multiple behavioral functions. These can include mate attraction, recognition and assessment. Pheromones that attract both males and females to a resource which also serves as a mating site can be considered aggregation and sex pheromones. The multiple behavioral functions of certain pheromones are indications of the evolutionary path that led them from non-specific cues to pheromones (Weiss et al., 2013).

While there are many simple and familiar examples of female-produced sex pheromones that both attract males from a distance *and* release copulatory behavior, frequently, additional components are required to elicit the full suite of courtship behaviors that result in copulation. In an increasing number of species, cuticular hydrocarbons (CHCs) have been implicated as mediators of these behaviors.

1.2. Recognition and Assessment of Mates

The costs of copulation (expended energy, increased predation risk, loss of other mating opportunities) and the benefits of choosing a high-quality mate incentivize the ability to discriminate between individuals of varying fitness. In general, males seek virgin females as mates, thereby reducing sperm competition and ultimately increasing paternity (Johansson & Jones, 2007). Depending on their sex determination system, males also attempt to avoid females, and their siblings (Chuine et al., 2015; Heimpel & de Boer, 2008) that are non-fertile (but see Cowan & Stahlhut, 2004; Elias et al., 2009). Females, in turn, should choose males with good genes while avoiding males that are sperm-limited, if males mate multiply (Ruther et al., 2009).

A vast body of recent literature has implicated CHCs as a ubiquitous source of information on individual identity that is often linked to physiological state. Especially in social insects, CHCs are honest signals of fertility in both queens and workers (Bonckaert et al., 2012; Cuvillier-hot et al., 2001; de Biseau et al, 2004; Dietemann et al., 2003; Eliyahu et al., 2011; Foitzik et al., 2011; Liebig et al., 2000; Smith et al., 2013; Sramkova et al., 2008), and are reliable discriminators of nest-mates and non-nest-mates (Bonckaert et al., 2012; Cuvillier-Hot et al., 2001; Dani et al.,

2004; de Biseau et al., 2004; Eliyahu et al., 2011; Liebig et al., 2000; Ozaki et al., 2005; Ruther et al., 2009; Sharma et al., 2015; Smith et al., 2013). Interestingly, in the bumble bee *Bombus terrestris*, sterility rather than fertility is advertised by workers. Once workers develop ovaries, they lose characteristic octyl esters in Dufour gland secretions, thus rendering them queen-like (Amsalem et al., 2009). In the monandrous solitary bee *Amegilla dawsoni*, CHCs likely carry information that allows patrolling males to discriminate between virgin and mated females (Simmons et al., 2003).

CHCs of virgin and mated females can differ (Hora et al., 2008) and affect mate choice by male hymenopterans (Oppelt & Heinze, 2009; Simmons et al., 2003; Thomas, 2011) but whether CHCs convey relatedness or kin recognition cues that affect mate choice has yet to be demonstrated. Queens of *B. terrestris* do mate with siblings but only after significant lag time which could be sufficient to avoid inbreeding for a monandrous species in a competitive mating environment (Whitehorn et al., 2009). Whether and to what extent the CHCs of sibling and non-sibling *B. terrestris* males differ, and if females can read these differences, is not known. In the ant *Leptothorax gredleri,* there is no strong preference for non-nest mates as mates, suggesting that mechanisms other than nest specific CHCs, such as sequential production and dispersal of sexuals from the nest, contribute to inbreeding avoidance, and prevent sibling mating (Oppelt et al., 2008).

Mate quality can also be assessed indirectly through variation in the composition and quantity of sex pheromones (Johansson & Jones, 2007). Females of the parasitoid wasp *Nasonia vitripennis* prefer males with high pheromone titer, a phenotypic trait linked to highly functional fertility via sperm load (Ruther et al., 2009). In the European beewolf, *Philanthus triangulum*, ratios of the males' cephalic pheromone components vary according to family group, potentially providing females with a pre-copulatory mechanism to discriminate against siblings (Herzner et al., 2006).

In the next section, I will review recent pheromone identifications in various suborders of the Hymenoptera. For each suborder, the newly identified pheromone structures are depicted in a figure and summarized in a table (Tables and Figures 1-1, 1-2 and 1-3 for Symphyta, Aprocrita: Aculeata: Apiformes, and Apocrita: Parasitica, respectively). Bold numbers in the text refer to the corresponding compound in tables and figures.

1.3. Symphyta

Symphyta is an ancestral hymenopteran suborder of almost entirely phytophagous insects, commonly known as sawflies. Until recently, most knowledge of mating systems and sex pheromones in the Symphyta came from conifer sawflies (Diprionidae), and stem sawflies (Cephidae), both groups being important economic pests whose chemical ecology has previously been reviewed (Anderbrant, 1993, 1999; Ayasse et al., 2001; Keeling et al., 2004). Briefly, female conifer sawflies tend to produce single-component pheromones that are acetate or propanoate esters of methyl-branched 2-alkanols between 11 and 15 carbon atoms long, with up to four chiral centers. The presence of some non-natural enantiomers and diastereomers in synthetic pheromones inhibits attraction of males (Keeling et al., 2004). This general pattern is exemplified by females of *Gilpinia pallida* that produce acetate and propionate esters of (2*S*,3*R*,7*R*)3,7-dimethyl-2-tetradecanol **(8)** as the main pheromone component (Hedenström et al., 2006).

Since the most recent reviews (Ayasse et al., 2001; Keeling et al., 2004), sex pheromones have been identified in the Siricidae, Pergidae and Pamphiliidae, raising the number of sawfly families with known sex pheromones to six out of 14. Females of the pine false webworm, *Acantholyda erythrocephala* (Pamphiliidae), produce (6*Z*)-6,14-pentadecadienal **(1)**, possibly via abiotic oxidation of (Z,Z)-1,9,15-pentacosatriene. Synthetic (6*Z*)-6,14-pentadecadienal attracts males in sufficient numbers for potential monitoring of this pest (Staples et al., 2009). Virgin females of *Cephalcia tannourinensis* (Pamphiliidae), or hexane body extracts of virgin females, as trap baits attracted males to traps but the pheromone components were not identified (Nemer et al., 2007). Additional olfactometer tests determined the abdomen as the source of the female pheromone, and revealed that the response of males was dose dependent.

Males of woodwasp *Sirex noctilio* (Siricidae) produce an aggregation pheromone comprising (*Z*)-3-decen-1-ol **(2),** (*Z*)-4-decen-1-ol **(3)** and (*E*,*E*)-2-4-decadienal **(4)** (Cooperband et al., 2012). Moreover, specific CHC components of females [(*Z*)-7-heptacosene **(5)**, (*Z*)-7-nonacosene **(6)**, (*Z*)-9-nonacosene **(7)**] mediate attraction and copulation by males. Solvent-extracted dead females treated with these three CHCs induced copulatory attempts by males (Böröczky et al., 2009).

Field and laboratory experiments with the sawfly *Lophyrotoma analis* (Pergidae) provide strong evidence of a long-range, female-produced sex pheromone (Schmidt et al., 2006). Sticky

traps baited with virgin females captured males, and males could be prompted to copulate with freeze-killed females by surface-extracting their integument and then re-applying the extract.

1.4. Aprocrita: Aculeata: Apiformes

Since the last review (Keeling et al., 2004), numerous studies with aculeate Hymenoptera have implicated the presence of sex pheromones through behavioral experiments, but few pheromones have been identified. This is testament to the challenge posed by the complex behaviors and life cycles exhibited by social insects, and by their often unorthodox chemistry.

In the honey bee, *Apis mellifera*, (*E*)-9-oxodec-2-enoic acid (9-ODA) in mandibular gland secretions was long known to attract male drones outside the hive, and was later discovered to be a main component of the nine-component queen retinue pheromone (QRP) (Slessor et al., 2005). Recently, two QRP components [(*R*,*E*)-(−)-9-hydroxy-2-enoic acid (9-HDA; **9**), 10-hydroxy-2-decenoic acid (10-HDA; **10**) have been shown to increase contacts of drones with pheromone-baited dummies, but not to affect the number of drones attracted (Brockmann et al., 2006). All species of honey bees (*Apis* spp.) studied utilize similar components as part of their retinue pheromone, but achieve signal specificity by varying the relative amounts of pheromone components, as shown in *Apis florea* where 10-HDA, not 9-ODA as *A. mellifera*, is the most abundant component (Plettner et al., 1997). The same pattern may apply to their sex pheromones.

In a study with *B. terrestris*, freeze-killed queens elicited mounting and copulation attempts by males, whereas ether extracts of virgin queens re-applied to solvent-extracted "dummy" queens elicited only mounting attempts (Krieger et al., 2006). Coupled gas chromatographic-electroantennographic detection (GC-EAD) analyses of behaviorally-active queen body surface extracts using drone antennae as electroantennographic detectors led to the identification of 21 compounds that elicited antennal responses. A synthetic blend of these compounds formulated at ratios as found in queen body surface extracts elicited mounting but not copulatory attempts by drones.

Males of the bumblebee *B. muscorum* occasionally aggregate at mature conspecific nests, presumably to mate with emerging gynes (Darvill et al., 2007). Microsatellite data revealed that

these aggregating males were not related to workers of the nests, indicating that males had sensed mature nests and aggregated to find non-related mates.

(S)-(+)-Linalool is a female-produced sex attractant for patrolling males of the solitary bee *Colletes cunicularius* (Colletidae) but on its own does not elicit copulation by males (Borg-Karlson et al., 2003). Recent evidence implies that CHCs together with linalool serve as long-range attractants and that CHCs then mediate copulation (Mant et al., 2005). The synthetic blend of components in female cuticle extracts that elicited antennal responses from males increased "contacts" by males, but was not compared directly to a virgin female, making it difficult to assess if the synthetic pheromone components matched the attractiveness of the natural pheromone.

1.5. Aprocrita: Aculeata: Spheciformes

There continues to be fascinating progress on the mating system of the beewolf, *Philanthus triangulum* (Crabronidae), where territorial males deposit scent-marks that attract females for mating from nearby nests (Kroiss et al., 2006, 2010). The morphology of the males' pheromone gland has been meticulously studied (Goettler et al., 2007; Goettler & Strohm, 2008) but synthetic (candidate) pheromone components have never been tested. Lacking this step, it is not clear which components constitute the *P. triangulum* sex pheromone. It is thus surprising that many papers on the communication ecology of *P. triangulum* refer to a "pheromone" language, reporting chemical analyses, and even comparing quantities of "pheromone" components between species and populations (Borg-Karlson & Tengö, 1980; Kaltenpoth et al., 2007).

In a study with the parasitoid wasp *Cephalonomia tarsalis* (Bethylidae), dodecanal in female "footprints" has been identified as a male arrestant (Collatz et al., 2009). Dodecanal was found in extracts of filter paper exposed to young females and arrested males but not females in bioassays.

Blends of (*R*)-3-ethyl-4-methylpentan-1-ol **(11)** and methyl 6-methylsalicylate **(12)** are sex pheromones of two ant species (Castracani et al., 2008; Greenberg et al., 2004, 2007). Virgin alate (winged) queens of *Polyergus refuscens* and *P. breviceps* utilize these two components in ratios biased towards methyl salicylate to attract males. The queens produce almost exclusively the (*R*)-enantiomer, with the (*S*)-enantiomer being neither attractive nor inhibitory.

Four pyrazines are sex pheromone components of the wasp *Zaspilothynnus trilobatus* (Tiphiidae) (Bohman et al., 2014). 2-Ethyl-3,5-dimethylpyrazine **(13)**, 2-propyl-3,5-dimethylpyrazine **(14)**, 2-butyl-3,5-dimethylpyrazine **(15)**, and 2-hydroxymethyl-3,6-diethyl-5-methylpyrazine **(16)** were identified in extracts of both female wasps and the sexually deceptive orchid *Drakaea glyptodon* that relies on this wasp for pollination. “Dummy” wasps treated with blends of these four pyrazines elicited copulation attempts males.

In a study with the neotropical paper wasp *Chartergellus communis* (Vespidae), males were observed at a nest the day following experimental elimination of the foundress queen (Pizarro & Noll, 2014). No males had been seen in the preceding five months of observations, and the colony had not gone through a male production phase. Males were apparently able to sense the queenless state of the colony, and had arrived to mate. Whether this observation represents the norm for mate location in *C. communis,* and the mechanism(s) that mediated attraction of males, remain unknown.

Trapping and wind-tunnel-experiment data provide evidence that gynes of the common yellowjacket, *Vespula vulgaris* (Vespidae), produce a long-range male-attractant sex pheromone (Brown et al., 2013). In wind-tunnel bioassays, hexane extracts of gynes were more effective than controls at eliciting upwind flight of males. However, extracts were not as effective as live gynes and never induced contact by males.

Dichloromethane (DCM) whole-body extracts of gynes of the European hornet, *Vespa crabro* (Vespidae), applied to “dummy” workers did elicit attraction of males and copulatory attempts (Spiewok et al., 2006), whereas dummy workers treated with a solvent control did not. Chemical analyses of DCM extracts revealed almost exclusively CHCs of 21 to 33 carbon atoms. No synthetic compounds were tested for attraction of males.

1.6. Apocrita: Parasitica

Mate-seeking males of the parasitoid wasp *Aphytis melinus* (Aphelinidae) respond to substrate borne, but not airborne, pheromone components of virgin females (Bernal & Luck, 2007). This applies also to males of the parasitoid wasp *Metaphycus luteolus* (Encyrtidae) (Kapranas et al., 2013), where males and females disperse from their natal patch, with males searching for and being arrested by the “chemical footprints” of females. Y-tube olfactometer

bioassays with the parasitoid *Phasgonophora sulcata* (Chalcididae) indicate a female-produced volatile sex pheromone (Roscoe et al., 2015). Virgin, 1- to 15-day-old females attracted males, whereas males did not elicit significant responses from females.

Males of the parasitoid wasp *Dibrachys cavus* (Pteromalidae) are attracted to CHC extracts of 1- to 2-day-old females but not to CHC extracts of newly eclosed females (Ruther et al., 2011). However, bioactive extracts do not elicit the complete range of courtship behavior, including copulation. Principal component analysis (PCA) of extracts revealed a subset of primarily methyl-branched alkanes that distinguish the 1- to 2-day-old females from newly eclosed females.

The sexual communication system of the parasitoid wasp *Lariophagus distinguendus* (Pteromalidae) exemplifies the complexity possible in a hymenopteran mating system. Immature, developing males distract their adult male competitors by producing the female sex pheromone, which degrades 32 hours after eclosion, likely to prevent molestation by mate-seeking males (Steiner et al., 2005). Further analytical and behavioral studies of sex pheromone components associated with developmental stages and sexes of these wasps implicated CHCs as the pheromone source (Steiner et al., 2007), and identified 3-methylheptacosane (3me-27) **(17)** as the primary sex pheromone component (Kühbandner et al. 2012). The absolute configuration of 3me-27 is not critical for the response of males (Kühbandner et al., 2013) but 3me-27 elicits characteristic mating behavior in males only when it is presented together with cuticular lipids, suggesting that female *L. distinguendus* produce a sex pheromone comprising CHCs and triacylglycerides (TAGs) (Kühbandner et al., 2012).

Both males and females of the jewel wasp *Nasonia vitripennis* (Pteromalidae) produce pheromones that affect sexual behavior. Protandrous males emerge and await the emergence of females, attracting females with a blend of (4*R*,5*R*)-5-hydroxy-4-decanolide (4*R*,5*R*-HDL) **(18)**, (4*R*,5*S*)-5-hydroxy-4-decanolide (4*S*,5*S*-HDL) **(19)**, and 4-methylquinazoline **(20)** (Ruther et al., 2007, 2008). The females' responses to pheromone are not enantioselective, as they also respond to (4*S*,5*S*)-HDL which the males do not produce enantiospecifically. In addition, the females' responses to the pheromone are dependent on their mating status. Immediately after copulation, females are either indifferent or avoidant of HDL. This represents both the first identification of a male-produced sex pheromone in parasitic Hymenoptera, and the first time HDL has been identified in insects. Males, in turn, are attracted to the CHCs of females but the most attractive subset of CHCs are not yet known (Steiner et al., 2006).

In *Spalangia endius* (Pteromalidae), a parasitoid wasp of house fly, *Musca domestica* pupae, males are arrested by two female-specific pheromone components: methyl-6-salicylate and an unknown (Nichols et al., 2010).

The parasitoid wasp *Leptopilina heterotoma* (Figitidae) has been used as a model system to show how iridoids that were initially produced as non-specific defensive compounds evolved as competition avoidance semiochemicals and subsequently became sex pheromones (Stökl et al., 2012; Weiss et al., 2013). The sex pheromone of *L. heterotoma* comprises a blend of five iridoids: (–)-iridomyrmecin **(21)**, (+)-isoiridomyrmecin **(22)**, a 3rd iridomyrmecin with unknown stereochemistry, and two iridodials. The major component of the blend is (–)-iridomyrmecin, accounting for ~80% of the pheromone. The pheromone blend is inactive if any one of the components is missing, or if the (+)-isomer of iridomyrmecin is substituted. Two other *Leptopilina* species (*L. boulardi, L. victoriae*) have iridoid sex pheromones that differ from that of *L. heterotoma* in the ratio of iridoid components and in the contributing role of CHCs (Weiss et al., 2015). In *L. boulardi,* iridoids and female CHCs in combination, but not alone, elicit wing-fanning by males. In *L. victoriae*, iridoids and female CHCs in combination, as well as female CHCs on their own, elicit wing-fanning by males. The iridoids on their own elicit stronger wing-fanning than controls, but not as strong as the female CHCs, or the iridoids and female CHCs combined.

Females of *Asobara tabida* (Braconidae), parasitoid wasps of *Drosophila* vinegar flies, produce a remarkably complex substrate-borne pheromone comprising three chemical classes: CHCs, fatty acid acetates (FAAs), and an ester (methyl-6-methylsalicylate) (Stökl et al., 2014). Behavioral assays measuring wing-fanning by males in response to extracts of females or to synthetic compounds show that all three chemical classes carry important information, but are not as active as female extracts. This pheromone is secreted by females as they are walking.

Females of the parasitoid wasp *Glyptapanteles flavicoxis* (Braconidae) produce a 4-component, substrate-borne pheromone (Danci et al., 2006). The specific compounds have yet to be identified but their biological activity has been demonstrated through extensive bioassays. A study of the parasitoid wasp *Lysiphlebus testaceipes* (Braconidae) revealed evidence for a female-produced sex pheromone (Pinto et al., 2013). Males wing-fanned when presented with whole-body extracts of females, but not of males, or solvent controls.

Males of the parasitoid wasp *Spathius agrili* (Braconidae) produce an aggregation pheromone with at least three components: dodecanal, (4*R*,11*E*)-tetradecen-4-olide **(23)**, and (*Z*)-

10-heptadecen-2-one **(24)** (Cossé et al., 2012). In wind tunnel bioassays, a blend of these components elicited upwind flight and contact with the pheromone source in both males and females. Synthetic pheromone may prove useful for monitoring the establishment of *S. agrili* populations as biocontrol measures for the emerald ash borer.

Females of the parasitoid wasp *Trissolcus brochymenae* (Platygastridae), produce two pheromone components [tetradecyl acetate **(25)**, (Z)-11-hexadecen-1-yl acetate **(26)**] that elicit electrophysiological and behavioral responses from males (Salerno et al., 2012). This is the first sex pheromone identified in the platygastrid family.

1.7. Future Directions

The advice given by authors of previous reviews on how to advance the field of Hymenopteran chemical ecology are still valid today. Bioassay-driven experimentation and testing of synthetic candidate pheromones are key, despite possible obstacles. The complexity of pheromones in terms of number of components, their absolute configuration, and potential synergism all can make the development of appropriate bioassays daunting (Ayasse et al., 2001). The difficulty in rearing or field-collecting study organisms and their scarcity and short-term availability all hinder the development of suitable bioassays. Still, too often potential pheromone components are painstakingly identified without ever testing synthetic components for behavioral activity (Keeling et al., 2004).

Social wasps (Vespidae) are near-ubiquitous across the globe and serve as model organisms for studying the evolution of social behavior. Yet, not a single vespid sex pheromone has been identified to date (Claudia et al., 2010), presenting a large gap in our understanding of sexual communication in the Hymenoptera. Understanding the role of pheromones in the sexual communication of vespids would allow for interesting comparisons with other social aculeate hymenopterans, namely the bees and ants. These comparisons would help clarify if and how life-history traits shape the evolution of pheromones and mating systems.

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1.9. Tables and Figures

Table 1-1. Summary of sex pheromones recently identified in the Symphyta. Bold-face numbers refer to chemicals shown in Fig. 1-1.

Taxon	Source	Behavior	Sex pheromones	References
MEGALODONTOIDEA				
Pamphilidae				
Acantholyda erythrocephala	female	male attractant	(6*Z*)-6,14-pentadecadienal **(1)**[1]	Staples et al. 2009
Cephalcia tannourinensis	female	male attractant	?	Nemer et al. 2007
SIRICOIDEA				
Siricidae			(3*Z*)-3-decen-1-ol **(2)**	
Sirex noctilio	male	male aggregation & female sex attractant	(4*Z*)-4-decen-1-ol **(3)** (2*E*,4*E*)-2,4-decadienal **(4)**	Cooperband et al. 2012
	female	male attractant	(7*Z*)-7-heptacosene **(5)** (7*Z*)-7-nonacosene **(6)** (9*Z*)-9-nonacosene **(7)**	Böröczky et al. 2009
TENTHREDINOIDEA			acetate & propionate esters of (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tetradecanol **(8)**	Hedenström et al. 2006
Diprionidae				
Gilpinia pallida	female	male attractant		
Pergidae			?	Schmidt et al. 2006
Lophyrotoma analis	virgin females	male attractant		

[1] possible abiotic oxidation from (9*Z*,15*Z*)-1,9,15-pentacosatriene

Table 1-2. Summary of sex pheromones recently identified in aculeate bees, wasps and ants. Bold-face numbers refer to chemical shown in Fig. 1-2.

Taxon	Source	Behavior	Sex pheromones	References
APOIDEA (Apiformes)				
Apidae			(2*E*)-9-hydroxydecenoic acid **(9)**	Brockmann et al. 2006
Apis mellifera	queen	male attractant	(2*E*)-10-hydroxydecenoic acid **(10)**	
Bombus terrestris	female	male mounting	21 compounds	Krieger et al. 2006
Bombus muscorum	mature nest	male aggregation at nest	?	Darvill et al. 2007
Colletidae		male attractant & contact sex pheromone	linalool + (7*Z*)-C21 + (7*Z*)-C23 + (7*Z*)-C25	Mant et al. 2005
Colletes cunicularius	female			
Crabronidae		female attractant	?	Kroiss et al. 2010
Philanthus triangulum	male			
CHRYSIDOIDEA		male attractant	dodecanal	Collatz et al. 2009
Bethylidae	female cocoons			
Cephalonomia tarsalis				
VESPOIDEA		male attractant	(3*R*)-3-ethyl-4-methylpentan-1-ol **(11)** & methyl 6-methylsalicylate (12) at 1:6 ratio	Greenberg et al. 2007
Formicidae				
Polyergus breviceps	gyne (MG?)			
Polyergus refuscens	gyne (MG?)	male attractant	(3*R*)-3-ethyl-4-methylpentan-1-ol & methyl 6-methylsalicylate at 3:1 to 50:1 ratio	Castracani et al. 2008
Tiphiidae			2-ethyl-3,5-dimethylpyrazine **(13)**	Bohman et al. 2014
Zaspilothynnus trilobatus	female	male attractant	2-propyl-3,5-dimethylpyrazine **(14)** 2-butyl-3,5-dimethylpyrazine **(15)** 2-hydroxymethyl-3,6-diethyl-5-methylpyrazine **(16)**	
Vespidae	queenless nest	male attractant	?	Pizarro & Noll 2014
Chartergellus communis				
Vespula vulgaris	gyne	male attractant	?	Brown et al. 2013

Table 1-3. Summary of sex pheromones recently identified in the Parasitica. Bold-face numbers refer to chemicals in Fig. 1-3.

Taxon	Source	Behavior	Sex pheromones	References
CHALCIDOIDEA	female "footprint"	male attractant	?	Bernal & Luck 2007
Aphelinidae				
Aphytis melinus				
Chalcididae				
Phasgonophora sulcata	female	male attractant	?	Roscoe et al. 2015
Encyrtidae				
Metaphycus luteolus	female "footprint"	male attractant	?	Kapranas et al., 2013
Pteromalidae		contact sex pheromone/ male arrestant	CHCs; 3-methylnonacosane & 3-methylhentriacontane implicated	Ruther et al. 2011
Dibrachys cavus	female			
Lariophagus distinguendus	female & young male	contact	3me-27 (17), triacylglycerides	Kühbandner et al. 2012; Steiner et al. 2005, 2007
Nasonia vitripennis	male	female attractant	(4*R*,5*R*)-5-hydroxy-4-decanolide **(18)** (4*R*,5*S*)-5-hydroxy-4-decanolide **(19)** 4-methylquinozoline **(20)**	Ruther et al. 2007, 2008, 2011
	female	substrate-borne sex pheromone	CHCs 25-37 carbon atoms long	Steiner et al. 2006
Spalangia endius	female	male attractant	methyl 6-methylsalicylate	Nichols et al. 2010
CYNIPOIDEA			(-)-iridomyrmecin **(21)**	Weiss et al. 2015
Figitidae			(+)-isoiridomyrmecin **(22)**	
Leptopilina heterotoma	female	male attractant	& analogs & minor role of CHCs	

Table 1-3 continued

Taxon	Source	Behavior	Sex pheromones	References
Leptopilina boulardi	female	male attractant	iridoids or CHCs induce wing fanning but less than female extract	Weiss et al. 2015
Leptopilina victoriae	female	male attractant	CHCs elicit full response behavior	Weiss et al. 2015
ICHNEUMONOIDEA				
Braconidae			CHCs, fatty acid acetates &	Stökl et al. 2014
Asobara tabida	female	male attractant	methyl-6-methylsalicylate	
Glyptapanteles flavicoxis	female	male attractant	4 components, unidentified	Danci et al. 2006
Lysiphlebus testaceipes	female	male attractant	unidentified, CHCs implicated	Pinto et al. 2013
Spathius agrili	female	male and female attractant	dodecanal, (4*R*,11*E*)-tetradecen-4-olide **(23)**, (*Z*)-10-heptadecen-2-one **(24)**	Cossé et al. 2012
PLATYGASTROIDEA				
Platygastridae			tetradecyl acetate **(25)**	Salerno et al. 2012
Trissolcus brochymenae	female	male attractant	(11*Z*)-11-hexadecen-1-yl acetate **(26)**	

Figure 1-1. Sex pheromone components recently identified in the Symphyta.

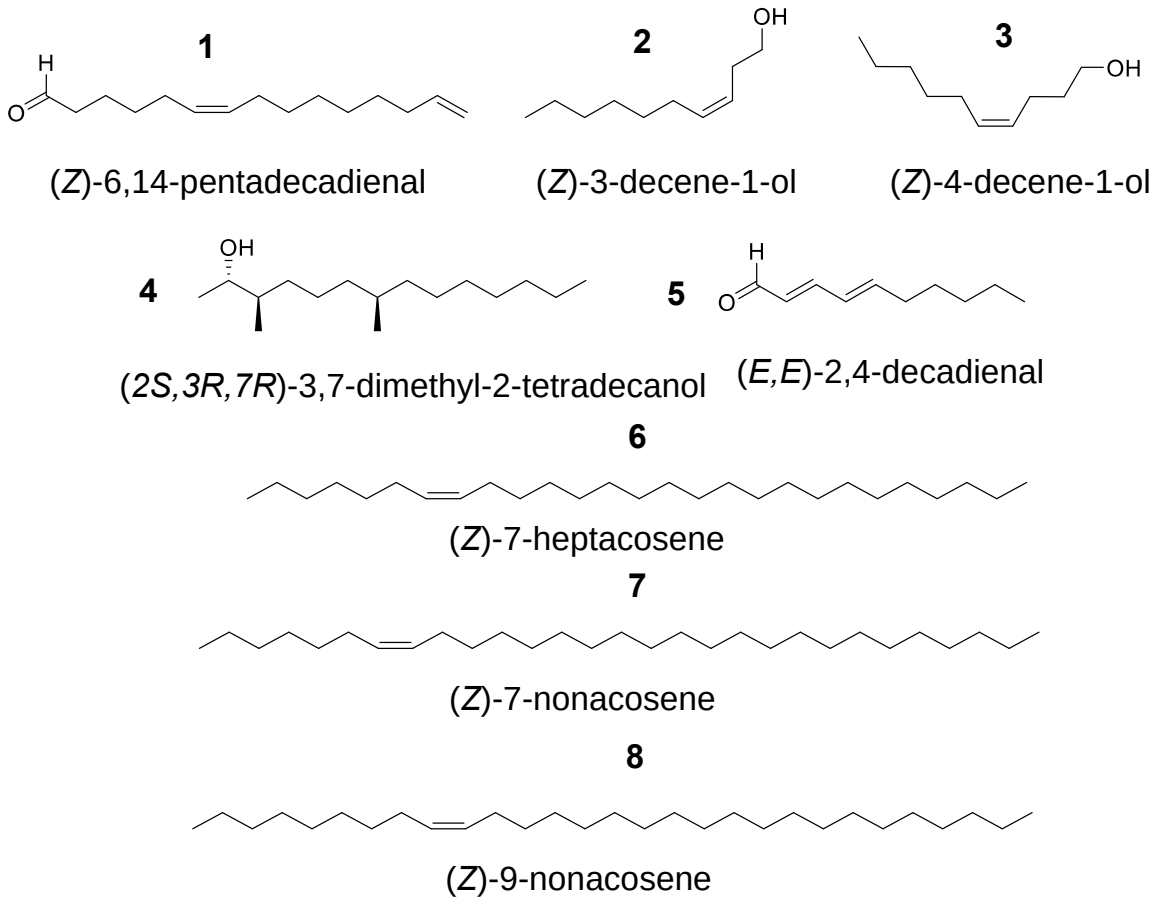


Figure 1-2. Sex pheromone components recently identified in aculeate bees, wasps, and ants.

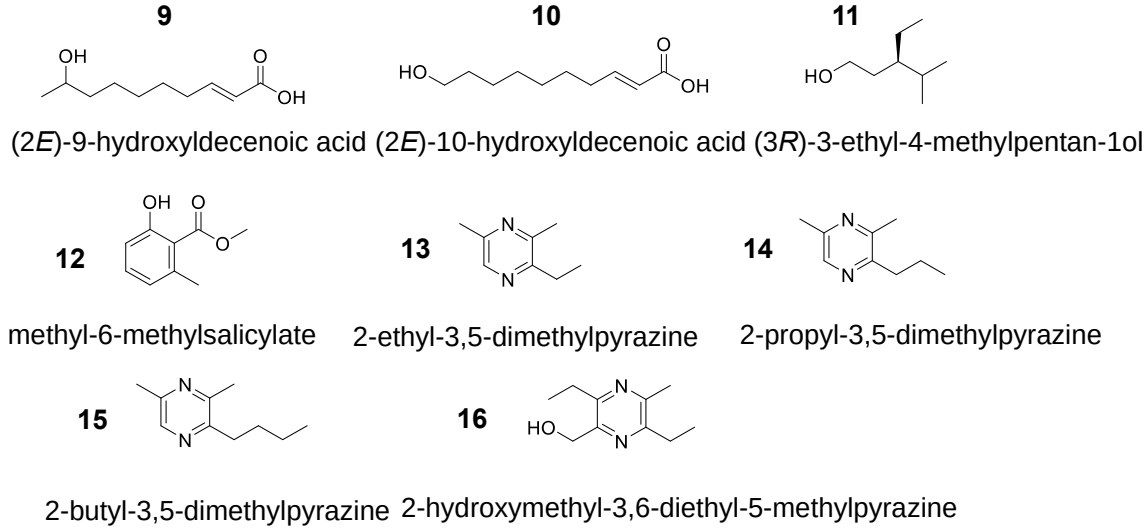
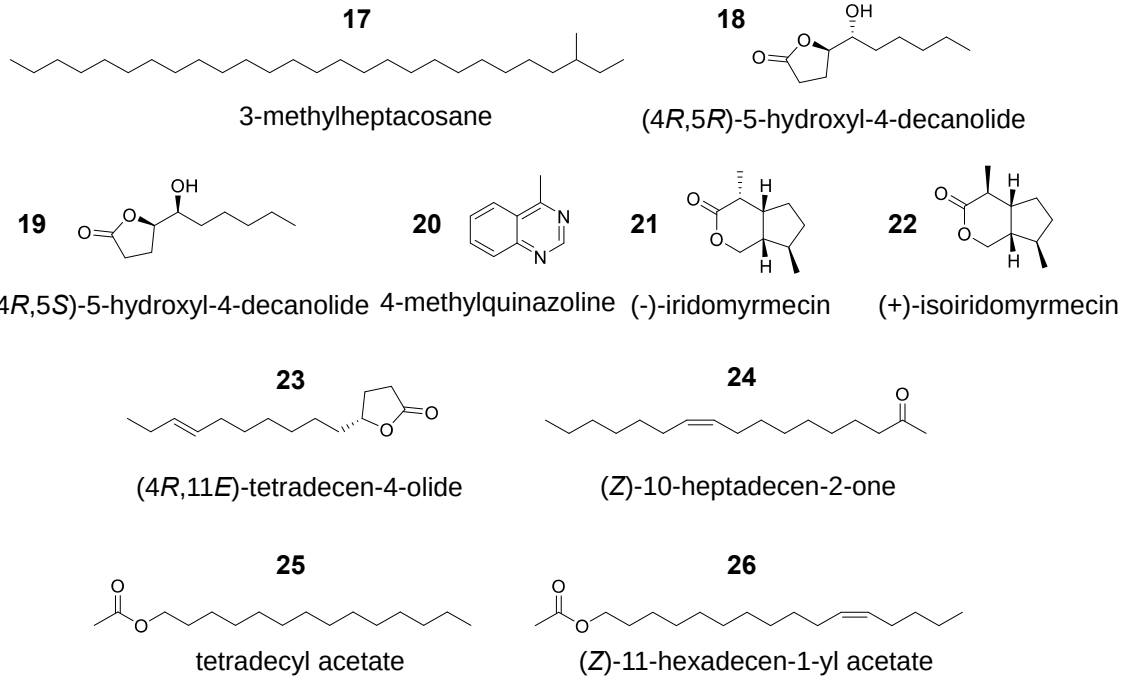


Figure 1-3. Sex pheromone components recently identified in the Parasitica.



Chapter 2. Evidence for sex pheromones and inbreeding avoidance in select North American yellowjacket species (Hymenoptera: Vespidae)

2.1. Abstract

Little is known about the roles of sex pheromones in mate-finding behavior of social wasps (Vespidae). Working with the aerial yellowjacket, *Dolichovespula arenaria* (Fabricius), baldfaced hornet, *D. maculata* (L.), western yellowjacket, *Vespula pensylvanica* (Saussure), southern yellowjacket, *V. squamosa* (Drury), and *V. alascensis* Packard, we tested the hypotheses (1) that gynes produce an airborne sex pheromone attractive to males, and (2) that males are more strongly attracted to non-sibling gynes based on olfactory cues. A field experiment provided the first definitive evidence that *D. arenaria* gynes attract males. Surprisingly, we did not find such evidence in similar field experiments for sexual attractiveness of gynes of *V. squamosa*, *V. pensylvanica*, *V. alascensis*, or *D. maculata*. In Y-tube olfactometer experiments with three of these species (*D. arenaria, D. maculata, V. pensylvanica*), only *D. maculata* gynes attracted males, provided they were non-siblings, implying an olfactory-based mechanism of nestmate recognition and inbreeding avoidance. Lack of sex attraction responses for *V. pensylvanica*, *V. alascensis*, and *V. squamosa* in this study does not rule out pheromone-mediated sexual communication. Instead, it highlights the possibility that pheromonal signaling may be dependent upon the presence of appropriate contextual cues.

2.2. Introduction

Many taxa in the Hymenoptera are known to use sex pheromones for mate location (Ayasse et al., 2001; Keeling et al., 2004) but in yellowjackets (Vespidae) the role and molecular structure of sex pheromones have not been elucidated. To help close this knowledge gap, we studied mate location behavior in five yellowjacket species: the aerial yellowjacket, *Dolichovespula arenaria* (Fabricius), baldfaced hornet, *D. maculata* (L.), western yellowjacket, *Vespula pensylvanica* (Saussure), southern yellowjacket, *V. squamosa* (Drury), and *V. alascensis* Packard. We selected these species because they represent both genera and four of the six species-groups (*maculata*, *norwegica*, *vulgaris*, *squamosa*) that comprise yellowjackets (Lopez-Osorio et al., 2017), thus portraying a broad cross section of this group of social insects. Studying multiple species provided a larger time-frame to gather data, as the seasonal phenology of these five species does not entirely overlap.

For these five vespids, there is limited information on the mechanisms of mate location (Landolt et al., 1998; Bruschini et al., 2010), or the importance of barriers that prevent inbreeding (Heimpel & de Boer, 2008). Many *Vespula spp.* display similar courtship behavior (MacDonald et al., 1974; Greene et al., 1978; Edwards et al., 1980; Post, 1980; Akre et al., 1982; Ross, 1983; Reed & Landolt, 1990a; Kovacs et al., 2008) but the mechanisms that bring males and virgin reproductive females (gynes) together prior to courtship may vary drastically between species, as in bumblebees (Paxton, 2005; Darvill et al., 2007) and paper wasps (Beani et al., 1992).

Field studies of vespid mating behavior describe a variety of strategies, even within a single species (Alcock et al., 1978). In many *Polistes* paper wasps, males may lek, swarm, or patrol (Mathis-Sears & Alcock, 1986; Beani & Turillazzi, 1990; Polak, 1993a,b), often according to dominance interactions regulated by body size (Turillazzi & Rita, 1982; Post & Jeanne, 1983a; Beani & Turillazzi, 1988; Beani et al., 1992) and male ornamentation (Izzo & Tibbetts, 2012). In contrast, males of the Asian giant hornet, *Vespa mandarinia* Smith, seek conspecific nests and intercept gynes as they leave (Matsuura &

Yamane, 1984), and males of the European hornet, *Vespa crabro* L., patrol for gynes without exhibiting territorial behavior (Spiewok et al., 2006).

The involvement of sex pheromones in the communication ecology of vespids has been demonstrated in a variety of laboratory and field studies. These studies provide evidence for pheromone-mediated mate-location and copulatory behavior in *Polistes spp.* (Post & Jeanne, 1983b, 1984; Reed & Landolt, 1990b; MacKenzie et al., 2008), *Vespa spp.* (Ono & Sasaki, 1987), the eusocial wasp *Belonogaster petiolata* (De Geer) (Keeping et al., 1986), and the Southern yellowjacket, *Vespula squamosa* (Drury) (Reed & Landolt, 1990a). Recent laboratory and field studies in New Zealand demonstrated that gynes of the common yellowjacket, *Vespula vulgaris* (L.), produce an airborne sex pheromone that attracts males (Brown et al., 2013). This finding supports previous observations and hypotheses of sex attractant pheromones produced by females of *Dolichovespula sylvestris* (Scopoli) (Sandeman, 1938), the western yellowjacket*, V. pensylvanica* (Saussure) (MacDonald et al., 1974)*,* the Eastern yellowjacket, *V. maculifrons* (Buysson) (Post, 1980; Ross, 1983), and *V. squamosa* (Reed & Landolt, 1990a). Despite mounting behavioral evidence for sex pheromones, including sex attractant pheromones, in the Vespidae, none has yet been identified (Landolt et al., 1998; Ayasse et al., 2001; Keeling et al., 2004; Bruschini et al., 2010).

The sex-determination systems of most hymenopterans favor outbreeding, thus avoiding the fitness costs associated with the production of inviable or sterile diploid males (Zayed & Packer, 2005; Heimpel & de Boer, 2008). Sibling mating in yellowjackets does occur in the laboratory (Ross, 1983; Kovacs et al., 2008), but not in natural populations, as genetic analyses of German yellowjackets, *V. germanica* (Fabricius) (Goodisman et al., 2002*), V. squamosa* and *V. maculifrons* (Hoffman et al., 2008) have shown. These data point to the presence of pre- or post-copulatory inbreeding avoidance mechanisms. Compared to the (typically) polyandrous *Vespula spp.* (Loope et al., 2014), queens of the (typically) monandrous *Dolichovespula spp.* (Foster et al., 2001) have one sperm source and thus are expected to incur higher costs from sib-mating, and be more likely to exhibit inbreeding avoidance behaviors (van Wilgenburg et al., 2006).

Working with *Dolichovespula arenaria*, *D. maculata*, *Vespula pensylvanica*, *V. squamosa* and *V. alascensis* in field and laboratory experiments, we tested the hypotheses: (1) that gynes produce an airborne sex pheromone attractive to males, and (2) that males prefer non-sibling gynes based on olfactory cues.

2.3. Methods and Materials

2.3.1. Experimental Insects

Five species of yellowjackets were studied in laboratory and field trapping experiments to test for the presence of sex attractant pheromones. *Dolichovespula arenaria*, *D. maculata*, *V. pensylvanica*, and *V. alascensis* are native to British Columbia (BC, Canada), with both *Dolichovespula spp.* being widely distributed in North America (Miller 1961; Akre et al., 1981). *Vespula squamosa* is present from Central America to the Great Lakes region of the United States (Akre et al., 1981; Landolt et al., 2009; Kimsey & Carpenter, 2012) and was tested in this study near Santiago Apoala, Oaxaca, Mexico.

Nests of yellowjackets from BC were collected between May and September in 2013, 2014 and 2015, and transported to a fenced grass lot surrounded by trees on three sides, on the Burnaby campus of Simon Fraser University, as previously described (Jimenez et al., 2016). Nests were located through advertisements in social media, contacts with bee-keepers and local parks and recreation agencies, and through searches of potential habitats. To collect wasps, entire colonies were anesthetized by plugging the entrance hole of nests with an ether-soaked cotton ball. Underground nests were then dug up and aerial nests were detached by severing the branches or the nest paper that served as attachment points. Harvested nests were suspended with metal wire or hot melt glue within acrylic nest boxes (for aerial nests) or plywood nest boxes (for ground nests) that were placed on tables surrounded by electric fence to deter raccoon predation. Acrylic boxes (30 × 30 × 46 cm) were modified insect cages with the door removed to allow wasps to enter and exit. Plywood boxes (15 × 15 × 30 cm) had a 2.5-cm entrance hole, and a hinged door allowing observations, as described previously (Jimenez et al., 2015). Fiberglass trays placed on top of nest boxes provided shade and protection from rain. Nests that were gathered before the onset of reproductive caste production were left to develop until gynes and males could be collected, as indicated by the presence of capped queen cells or reproductive

adults. Queen cells, housing either gynes or males, were recognized by their larger diameter compared to worker cells.

Wasps tested in experiments in BC were either taken as adults from harvested nests or reared from brood combs in the laboratory. For rearing, combs with capped queen cells were housed inside clear acrylic cages in an environmental chamber (BioChambers Inc., model ER-75, Winnipeg, MB, Canada) kept at 28 °C, 60% RH, and a photoperiod of 14L:10D. Combs were checked daily for newly eclosed wasps, which were separated by caste and age and provided with water and honey *ad libitum*. This procedure provided a source of virgin gynes and males for experiments.

In Washington (WA, USA), nests of both aerial and western yellowjackets were collected in late season (August) into plastic bags that were placed for transport into a cooler with ice. At the laboratory, wasps were separated from nest combs, and combs with capped queen cells were placed in screened cages in a greenhouse. Daily, newly eclosed wasps were removed, separated by caste and age, and placed in cages with water and sugar water on cotton balls. The gynes reared from these nest combs were used in trapping experiments.

Nests of *V. squamosa* were collected in Mexico and immediately dissected to harvest wasps for a field trapping experiment. Harvested wasps were separated by caste (gyne, worker, male) and held overnight in sturdy Ziploc containers (~15 × 15 × 5 cm) modified with screen mesh lids for ventilation. Gynes of *V. squamosa* deployed as trap baits in the field trapping experiment were dissected after the trapping period and examined for the presence of eggs, as a proxy for mating status. In perennial, polygynous colonies, visually distinguishing between virgin gynes and mated and/or functionally reproductive queens (which are likely not attractive to males) can be difficult as the functionally reproductive queens are not always physiogastric and physically worn (Ross and Matthews, 1982; Landolt et al., 2009).

2.3.2. Field trapping experiments

Field experiments were designed to determine whether males would be captured in traps baited with live gynes, as evidence for mate attraction. These tests were carried out in BC, WA and Oaxaca (Mexico) (Table 2-1), using a randomized complete block design, with 12 or 15 blocks (replicates) in each experiment. In each block, custom-built, adhesive-coated Delta traps (Fig. 2-1, a), or Pherocon VI white Delta traps with adhesive-coated insert (Fig. 2-1, b) (Trécé Inc., West

Adair, OK 74330, USA), were suspended ~1.5 m (BC) or 4 m (WA) above ground with inter-trap spacing within and between blocks of 5-10 m. Within each block, traps were baited with a caged gyne or a caged male that was provisioned with a sugar water soaked wick (Fig. 2-1, a), or a sugar water-containing vial (Fig. 2-1, b), whereas the unbaited control trap was fitted with a cage containing only the sugar water soaked wick or the sugar water-containing vial. Every day for 2-3 weeks, deceased gynes or males were replaced and captured male wasps were recorded and removed from traps. All field sites had known populations of the study species. Traps in the BC field site were also in proximity to transplanted nests of study species.

2.3.3. Y-tube olfactometer experiments

Anemotactic responses of *D. arenaria, D. maculata,* and *V. pensylvanica* males toward live conspecific gynes were bioassayed between mid-August and mid-October 2014-2015. All bioassays were run at 24-27 °C in Y-shaped Pyrex® glass olfactometers (stem 23 cm long × 23 mm ID; side arms at 120º; 18 cm long) angled at a 45º inclination (Fig. 2-1, c) and illuminated from above by two horizontal fluorescent lights (one light: Sylvania T12 daylight deluxe 40W, 6500K; the other: Phillips F40T12 Plant and Aquarium 40W, 6500K) suspended 66 cm above the bioassay table. For each replicate, the treatment and the control stimuli were randomly assigned to, and placed at, the opening of the side arms. A live gyne as the treatment stimulus was held in a glass tube (60 × 22 mm OD) with stainless steel mesh covering both openings. An empty glass tube served as the control stimulus. A vacuum pump drew air at 0.5 L min^{-1} through the Y-tube, carrying volatiles of the live gyne towards the male that exited a glass holding tube (26 cm × 22 mm OD) attached to the Y-tube stem via a ground glass joint (Fig. 2-1, c). For each replicate, a clean Y-tube and a new male wasp were employed. Prior to bioassays, each male was confined in his respective holding tube for 10 min so that he could acclimate to the experimental setting. Holding tubes were labeled with the male's nest of origin, so that responding males could be recorded as sibling or non-sibling to the stimulus gyne. The order of sibling and non-sibling wasps tested within an experiment was randomized. A male was scored as a non-responder if after 5 min he did not approach within 3 cm of either the treatment or control stimulus. All males bioassayed in experiments were capable of flight. As some bioassay males were harvested directly from collected nests, their age was not known. Males reared from combs and bioassayed were > 6 days old. Non-responding male wasps are reported but were excluded from statistical analyses. Olfactometers were washed in warm water with Sparklene™ detergent, rinsed with cold tap water followed by distilled water, and oven-dried at 120 °C for at least 2 hours. An experiment

with an empty control tube in both side arms was run to test for potential experimental design asymmetries that could have biased data (see Results section).

2.3.4. Statistics

Data obtained in field trapping experiments were analyzed by a t-test or by ANOVA followed by Tukey's HSD (honest significant difference) test for multiple comparisons of means, depending on the number of treatments (two or three) tested in each experiment. To determine whether males in Y-tube olfactometer experiments selected gynes more often than could be expected by chance (50%), data were analyzed using a binominal (χ^2) test. To further test for a difference in the proportion of sibling and non-sibling males responding to caged gynes, data were analyzed by a Pearson's χ^2 test with Yates' correction for continuity. All data were analyzed with the statistical software R (version 3.3.0), in RStudio (version 0.99.902).

2.4. Results

2.4.1. Field trapping experiments

Gynes of *D. arenaria* attracted males to traps (Fig. 2-2). In BC, 12 traps baited with a *D. arenaria* gyne captured a total of 44 males during 10 days (mean $\pm$ SE per trap: 3.67 $\pm$ 1.19; $F_{2,33}$ = 9.5, P<0.001; Fig. 2-2, a), whereas neither male-baited traps nor control traps captured any males. In WA, *D. arenaria* gyne-baited traps captured a total of 130 males during 18 days of trapping (13.0 $\pm$ 4.47; t_9 = 2.9, P = 0.002; Fig. 2-2, b), whereas control traps captured no males. The number of *D. arenaria* males captured varied widely between blocks, and between days (BC: range 0-13; WA: range 0-48). Male wasps were not captured in traps baited with gynes of *D. maculata*, *V. pensylvanica, V. squamosa, or V. alascensis*. In Oaxaca, all *V. squamosa* colonies found were polygynous. No eggs were found in post-experiment dissections of gynes used in field trapping experiments.

2.4.2. Y-tube olfactometer experiments

Gynes of *D. arenaria* (n = 8) had no effect on the orientation of conspecific males (χ^2 = 0.17, P = 0.68, n = 52; Fig. 2-3, a). The hypothesis that gyne-male relatedness affects attraction of males could not be tested for *D. arenaria* due to a paucity of nests collected. Moreover, two of

the three nests we collected had almost exclusively males, whereas the third nest had mostly gynes.

Gynes of *D. maculata* (*n* = 9) attracted non-sibling males (χ^2 = 4.2, P = 0.04, *n* = 95) but caused avoidance by sibling males (χ^2 = 4.7, P = 0.03, *n* = 69) (Fig. 2-3, b). There was a significant difference in the proportion of sibling and non-sibling *D. maculata* males responding to gynes (χ^2 = 8.9, P = 0.003, *n* = 164).

Gynes of *V. pensylvanica* (*n* = 7) did not have a statistically significant effect on the orientation behavior of sibling males (χ^2 = 4.2, P = 0.066, *n* = 24) or non-sibling males (χ^2 = 4.1, P = 0.054, *n* = 78) (Fig. 2-3, c). There was no significant difference in the proportion of sibling and non-sibling *V. pensylvanica* males responding to gynes (χ^2 = 0.35, P = 0.558, *n* = 24).

When (empty) control stimuli were presented in each of the two side arms of the olfactometer, eight *D. maculata* males responded to the left side arm and seven to right side arm (χ^2 = 0, P = 1), indicating no side bias in the experimental design.

2.5. Discussion

Our field tests with five yellowjacket species sought evidence for gyne-produced sex attractant pheromones that mediate attraction of males. The resulting field data provide the first experimental evidence that *D. arenaria* gynes produce a long-range sexual communication signal that attracts males, as evidenced by their capture in gyne-baited traps (Fig. 2-2). This signal is most likely a sex attractant pheromone but further studies are needed to rule out any roles of non-chemical signals involved in the attraction of males and their entry into traps. Surprisingly, we did not find equivalent evidence for gynes of *V. squamosa*, *V. pensylvanica*, *V. alascensis*, or *D. maculata*.

Y-tube olfactometer experiments yielded results that differed from those of field experiments. In olfactometers, there was no evidence of *D. arenaria* males orienting towards gynes. Conversely, *D. maculata* gynes as test stimuli in olfactometers attracted males provided they were non-siblings, implying an olfactory based mechanism of nestmate recognition and inbreeding avoidance. Olfactometer results were consistent with field results for *V. germanica*,

V. pensylvanica, *V. alascensis*, with no evidence of male orientation to gynes in either species. Below, we elaborate on these findings with reference to the two hypotheses we tested.

2.5.1. Hypothesis 1: Gynes produce an airborne sex pheromone attractive to males

Gynes of *D. arenaria* attracted numerous males in the field in both British Columbia and Washington State. The attractiveness of these gynes to males is likely to be due to a sex attractant pheromone because visual cues associated with caged gynes were obscured by both the screened holding cage and the trap accommodating it. Attraction of males simply to sugar water wicks can be ruled out because males were not captured in control traps containing sugar water wicks. Although long-range acoustic signaling by gynes is conceivable, there is no prior evidence for it in vespids (Hunt & Richard, 2013). Vespids simply lack the (stridulatory) organs for sound production found in many other insects with acoustic sexual communication systems. Even though honey bees, *Apis mellifera* L. and certain ants (Hickling & Brown, 2000) are known to communicate also by sound (Michelson et al., 1986, 1987) they do so in a non-sexual intra-colony context. There is, however, evidence for courtship sound signals in the solitary parasitoid wasp *Glyptapanteles flavicoxis* (Marsh) (Danci et al., 2010).

If the attractiveness of *D. arenaria* gynes was indeed due to a pheromone, it functioned as a sex attractant pheromone rather than as an aggregation pheromone because it attracted only males (not gynes). Because traps baited with a *D. arenaria* male failed to capture any gynes, or other males, it appears that *D. arenaria* males do not signal for mate attraction, but instead seek signaling (sex pheromone emitting) gynes. Captures of males in gyne-baited traps in early July in BC, and in mid-September in WA, suggest that the mating season varies across geographic locations and might be strongly affected by local environmental factors such as temperature and precipitation. That certain gynes attracted many more males than others (e.g., range of 0-48 in WA, and 0-13 in BC) could have been due to the superior attractiveness of individual gynes or their placement in a particularly suitable micro-location for attracting males.

Gynes of *V. alascensis*, *V. pensylvanica, V. squamosa,* and *D. maculata* all failed to attract conspecific males in field experiments, not supporting the hypothesis that males locate females by a long-distance pheromone. It is conceivable, however, that males are attracted to a gyne-produced pheromone in a different context. The latter interpretation is supported by wind-tunnel experiments, showing that *V. squamosa* gynes do attract males (Reed & Landolt, 1990a). It is not

likely that our trapping periods were out of sync with the mating season of target species. We initiated trapping based on (*i*) reported time frames of wasp mating seasons (Spradbery, 1973; Post, 1980) and (*ii*) the presence of gynes and males in multiple local nests. Only for the field experiment with *V. squamosa* gynes was the trapping period and site based on the presence of a single nest full of gynes and males, implying that local populations were in mating mode. Gynes of *V. squamosa* deployed as trap baits in this field experiment were likely all virgin, because their dissections after the trapping study revealed no evidence of eggs.

The natural rates of successful mate attraction and mating (Rhainds, 2010) in female yellowjackets, are not well studied but they are likely quite low. In a study by Stein and Fell (1994), 18-100% of spring queens sampled were not inseminated. Thus, it is possible that we did not detect attraction of males to gynes in most of our field studies because attracting or locating a mate in yellowjackets may be difficult and relatively rare.

With evidence that confined *D. arenaria* gynes emitted sex pheromone (this study), and previous evidence that confined *V. vulgaris* gynes do the same (Brown et al., 2013), it is not likely that trap confinement of *V. alascensis*, *V. pensylvanica, V. squamosa* and *D. maculata* gynes in our study inhibited their pheromone emission. Rather, it seems more likely that some species may rely on social cues such as semiochemicals or behavior of nestmates, or on environmental cues such as temperature, precipitation and photoperiod, to initiate pheromone production or emission. Such cues have been intensely studied in other social hymenopterans like ants (Dunn et al., 2007; Noordijk et al., 2008) that engage in synchronized nuptial flights triggered by environmental cues (Boomsma & Leusink, 1981), pheromones (Alonso & Vander Meer, 1997) or acoustic signals (Markl et al., 1977). Such cues may also affect the communication and mating behavior of yellowjackets but have yet to be studied.

Data from field trapping experiments, but not Y-tube olfactometer experiments, suggest that *D. arenaria* gynes produce an airborne sex pheromone attractive to males (Figs. 2,3). Conversely, data from Y-tube olfactometer experiments (Fig. 2-3, b), but not field experiments, suggest that *D. maculata* gynes emit an airborne sex pheromone. These seemingly contradictory results highlight the importance of studying insect behavior both in the laboratory and the field. Both *D. maculata* and *D. arenaria* gynes seem to produce airborne sex pheromones, but the combination of laboratory and field data shows that the functional context, or the effective distance, of pheromonal signaling and responses to these sex pheromones vary among species. The specific phenology, the mating sites, and the mechanisms that trigger mate-seeking in males,

or mating receptivity in gynes, need to be considered for each species. The age of both gynes and males is likely also a factor, although males initiate copulation over several weeks of their life (N. Derstine, unpubl. data).

The contrasting data obtained with *D. arenaria* and *D. maculata* in laboratory and field experiments support previous evidence that vespids differ in their mating strategies (Matsuura & Yamane, 1984; Beani et al., 1992). These differences may have evolved due to specific life-history traits. For example, nests of *D. arenaria,* but not *D. maculata,* have a strongly biased sex ratio. Greene et al. (1976) found that 50% and 31% of the *D. arenaria* nests they collected over two years had a 3:1 and 1:3 ratio of male to queen cells, respectively.

2.5.2. Hypothesis 2: Males prefer non-sibling gynes based on olfactory cues

In the Hymenoptera, many pre-copulatory mechanisms exist to limit inbreeding, including dispersal from the natal nest (Tabadkani et al., 2012), cuticular hydrocarbon profiles delineating nestmates from non-nestmates (Oppelt et al., 2008), and hereditary blends of pheromone components indicative of family groups (Herzner et al., 2006). The avoidance of sibling gynes by *D. maculata* males (Fig. 2-4, b) indicates that males sense gyne relatedness through olfactory cues. This ability then provides a barrier to inbreeding that otherwise would result in a higher rate of sterile or inviable diploid male offspring, as shown in most Hymenopterans with single-locus complementary sex determination systems (Zayed & Packer, 2005). Sterile or inviable diploid males are particularly detrimental for monandrous species that cannot rely upon post-copulatory mechanisms like selective fertilization to avoid fitness costs (van Wilgenburg et al., 2006). Males of the paper wasp *Polistes dominula* (Christ) can discriminate nestmate females through unknown mechanisms (Liebert et al., 2010) but whether this ability affects mate choices in natural settings is not clear, given that diploid males and their triploid offspring have been found in multiple *Polistes spp.* (Liebert et al., 2004, 2005). Males of the halictid bee *Lasioglossum zephurum* (Smith) use semiochemical cues to recognize the relatedness of potential mates, showing less interest in mating as the relatedness of females increases (Smith, 1983). That *V. pensylvanica* males did not discriminate against sibling gynes (Fig. 2-4 c) seems to suggest that either alternative inbreeding avoidance mechanisms (i.e., sequential dispersal of reproductives from natal nests which makes kin recognition in a mating context unnecessary) are in effect, or that inbreeding avoidance behavior is evident only in the specific context of sexual attraction, which was not provided in our study.

In conclusion, we provide evidence for pheromone-mediated mate attraction in *D. arenaria* and *D. maculata*, and for the ability of *D. maculata* males to gauge the relatedness of prospective mates based on olfactory cues. Lack of similar results for *V. pensylvanica, V. alascensis,* and *V. squamosa* does not rule out olfactory sexual communication in these vespids. Rather, we must consider that certain social or environmental cues conducive to mate-finding behavior in these species may not have been appropriate. Studying these cues may be essential to determine the sexual communication systems and mating strategies of these and other vespid wasps.

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2.7. Tables and Figures

Table 2-1. Summary of experiments that tested for evidence of pheromonal communication in *Dolichovespula* (*D*) and *Vespula* (*V*) yellowjackets, the trapping locations and dates, and the number of replicates (*n*) deployed in each experiment.

Species	Location	Trapping period	*n*	Males captured
D. arenaria	SFU, Burnaby, BC, Canada (Latitude: 49°16'31.02"N, Longitude: 122°54'59.45"W)	6 –16 July 2013	12	Yes
D. arenaria	Union Gap, WA, U.S.A (Latitude: 46°29'38.753"N, Longitude: 120°56'54.459"W)	12 Sept. – 01 Oct. 2008	10	Yes
D. maculata	SFU, Burnaby, BC, Canada	28 Aug. – 13 Sept. 2013	15	No
D. maculata	SFU, Burnaby, BC, Canada	14 – 25 Sept. 2015	12	No
V. pensylvanica	SFU, Burnaby, BC, Canada	6 – 23 Oct. 2013	15	No
V. pensylvanica	Moxee, WA, U.S.A. (Latitude: 46°30'19.335"N, Longitude: 120°10'5.991"W)	06 – 20 Oct. 2008	10	No
V. alascensis	SFU, Burnaby, BC, Canada	14 – 25 Sept. 2015	12	No
V. squamosa	Santiago Apoala, Oaxaca, Mexico (Latitude: 17°39'0.26"N, Longitude: 97° 8'22.96"W)	26 Nov. – 09 Dec. 2014	15	No

Figure 2-1. **Graphical illustrations of experimental designs. In field trapping experiments (Table 2-1) (a, b), Delta traps were baited with a live gyne that was confined within a 50-ml plastic Falcon tube (110 mm long × 30 mm wide) whose open faces were covered with stainless steel mesh (a) or within a welded hardware cloth mesh cage (65 mm long × 50 mm wide × 25 mm tall) (b) while being provisioned with a sugar water-soaked cotton wick (a) or a sugar water-filled vial (b). In laboratory experiments (c), the Y-tube olfactometer (stem 23 cm × 23 mm ID; side arms at 120º; stem: 18 cm long) was kept at a 45º inclination and via a ground glass joint connected to holding tube (26 cm × 22 mm OD) from which the bioassay male entered the Y-tube in response to a conspecific gyne that was held in an open glass tube (60 × 22 mm OD) with stainless steel mesh covers. The gyne-containing tube and the empty control tube were inserted into the side arms of the olfactometer.**

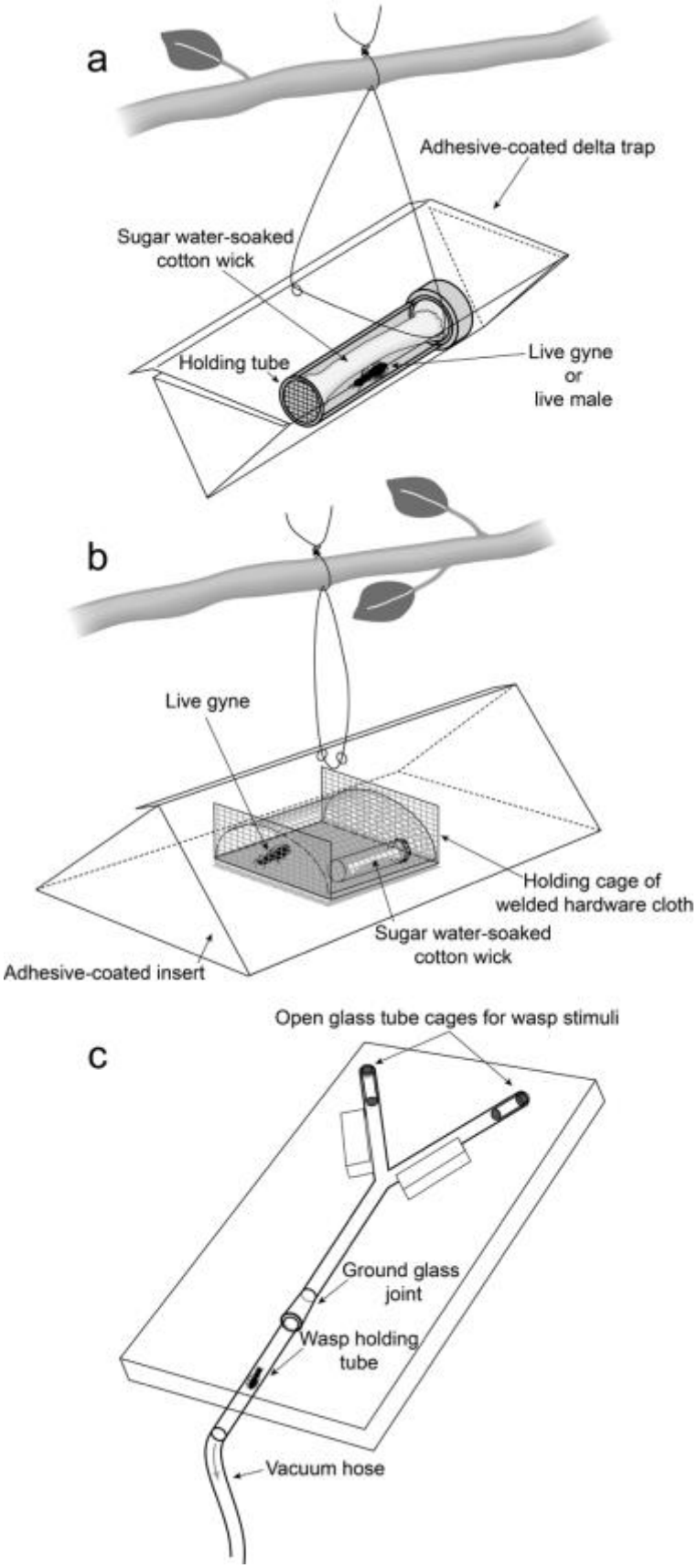

a
Adhesive-coated delta trap
Sugar water-soaked
cotton wick
Holding tube
Live gyne
or
live male
b
Live gyne
Holding cage of
welded hardware cloth
Sugar water-soaked
cotton wick
Adhesive-coated insert
c
Open glass tube cages for wasp stimuli
Ground glass
joint
Wasp holding
tube
Vacuum hose

Figure 2-2. **Captures of *D. arenaria* males in traps that were baited with a live conspecific gyne (Figure 1, a, b) or a live conspecific male, or that were left unbaited (controls) in field experiments ran in British Columbia (a) and in Washington State (b) (see Table 2-1 for details). Boxplots show the mean, median lower and upper quartiles, and ± whiskers (minimum/maximum data points) of male captures. In each experiment, different letters (a,b,c) above test stimuli indicate significant differences in captures of males; ANOVA followed Tukey's HSD test (P<0.05).**

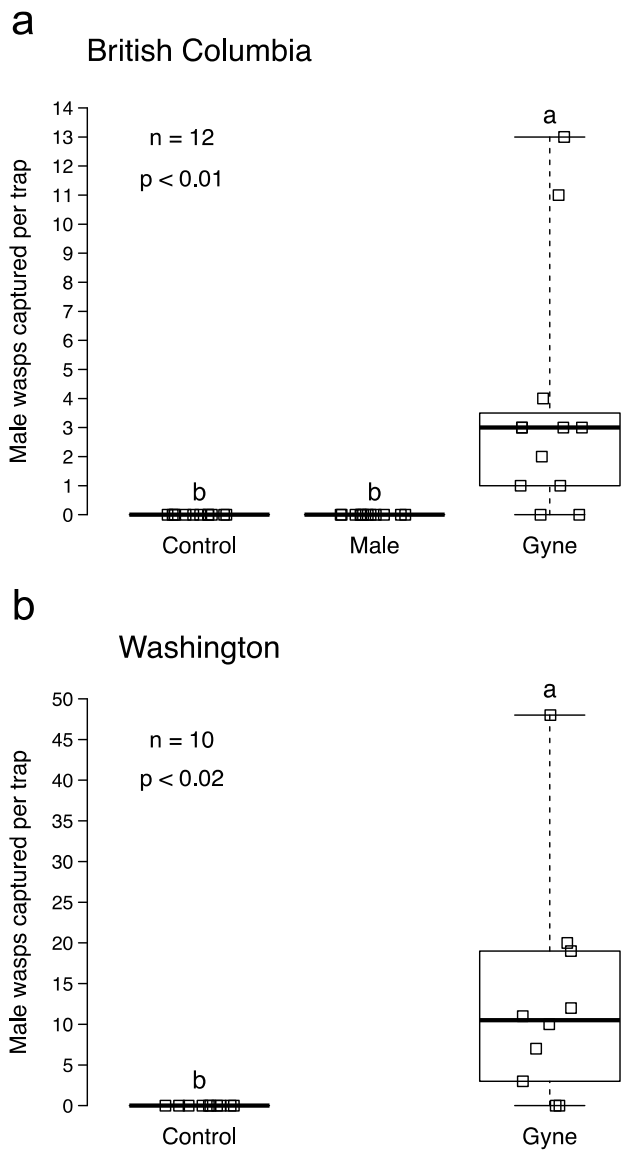
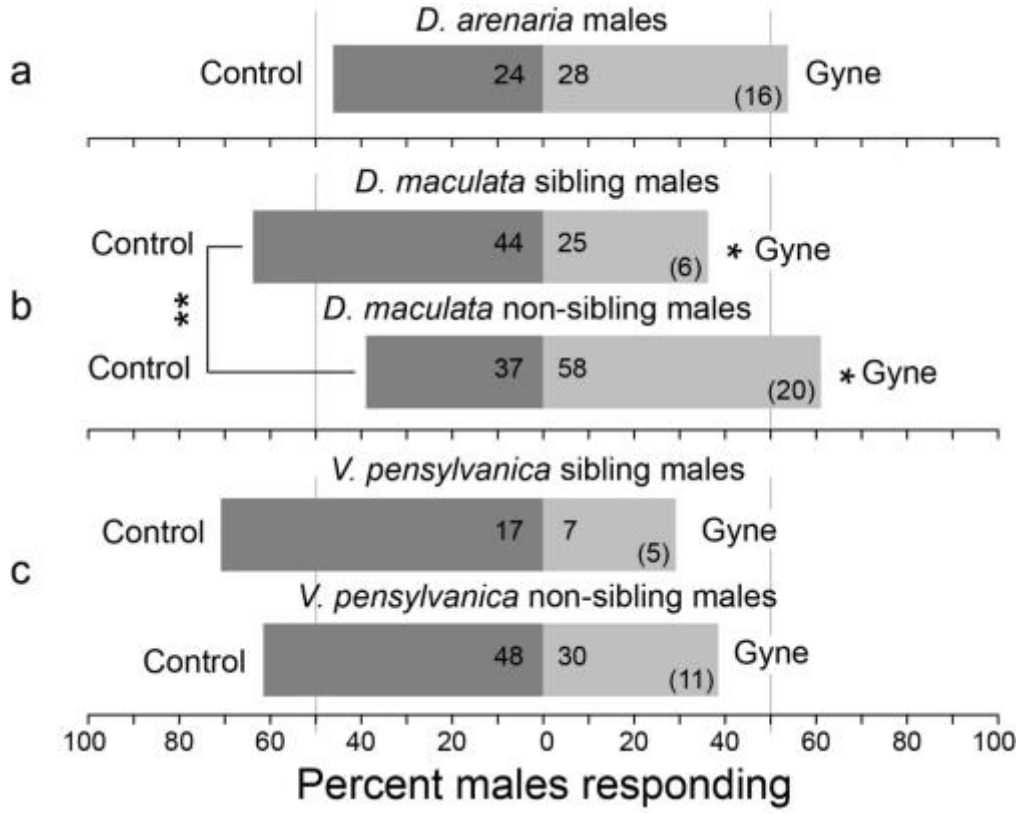


Figure 2-3. **Responses of sibling or non-sibling males of *Dolichovespula arenaria* (a), *D. maculata* (b) and *Vespula pensylvanica* (c) to conspecific gynes in Y-tube olfactometer experiments (Figure 1, c). In each experiment, the asterisk (*) indicates a significant preference for a specific test stimulus; χ^2-test, P<0.05; in b, the proportion of sibling and non-sibling *D. maculata* males responding to gynes differed significantly, as indicated by the double asterisk (**); Pearson's χ^2 test with Yates' correction for continuity (P<0.05); numbers in parentheses indicate the number of non-responding males.**



Chapter 3. Cuticular hydrocarbons discriminate sex, caste, and nest membership in each of four species of yellowjackets (Hymenoptera: Vespidae)

3.1. Abstract

Cuticular hydrocarbons (CHCs) of social insects have typically been studied for their roles in reproductive signaling (i.e., fertility) rather than sexual signaling (i.e., interest in mating), resulting in little information about CHCs of males and virgin females and their contributions to sexual signaling. This dearth of information applies particularly to social wasps. We tested the hypothesis that CHCs differentiate sex, caste, and nest membership in each of four yellowjacket species (baldfaced hornets, *Dolichovespula maculata;* southern yellowjackets, *Vespula squamosa;* western yellowjackets, *V. pensylvanica; V. alascensis*). Cold-euthanized queens (21), gynes (81), workers (125), and males (77) from 35 nests were extracted with pentane, and each of the resulting 304 extracts was analyzed by gas chromatography (GC) and GC-mass spectrometry to identify and quantify CHC constituents (aliphatic alkanes and alkenes; mono-, di- and tri-methylbranched alkanes). To determine whether caste and sex differ in CHC profiles of wasps, linear discriminant analyses were performed, using Z-transformed relative CHC peak areas as predictor variables and sex and caste, or nest, as grouping variables. When caste and sex were used as a grouping variable, plots of the first two discriminant functions revealed that wasps from each of the four species clustered into their respective groups (queens, gynes, workers, males), with significant differences in group centroids, as measured by Wilks' lambda. When nest was used as a grouping variable, plots of the first two discriminant functions revealed that workers from each of the four species, and males from each of three species (insufficient sample size for *V. pensylvanica*) clustered according to nest. Diagnostic power calculations show greater inter-caste than inter-nest variation. Our data support the above hypothesis and inspire future studies to determine the definitive role(s) that gyne- and male-specific CHCs play in the context of sexual communication, from the perspective of both males and females.

3.2. Introduction

Cuticular hydrocarbons (CHCs) provide critical information about the physiological state and identity of insects (Blomquist and Bagnéres 2010). Important across diverse taxa, CHCs drive

sexual selection [e.g., *Timema* stick insects (Schwander et al. 2013; Riesch et al. 2017); sagebrush crickets, *Cyphoderris strepitans* (Steiger et al. 2013)] and indicate breeding status [e.g., burying beetles, *Nicrophorus vespilloides* (Steiger et al. 2007)]. In many social insects, CHCs honestly signal fertility (Dietemann et al. 2003; de Biseau et al. 2004; Sramkova et al. 2008; Liebig et al. 2009; Liebig 2010; Bonckaert et al. 2012; Smith et al. 2013), reliably reveal nest membership (Wagner et al. 2000; Ruther et al. 2002; Ozaki et al. 2005; Cournault and de Biseau 2009; van Zweden and Ettorre 2010), and function as queen pheromones that regulate worker sterility (van Zweden et al. 2009; Holman et al. 2010; Van Oystaeyen et al. 2014; Oi et al. 2015, 2016; Oliveira et al. 2015). CHCs in social insects have often been studied for their roles in reproductive signaling (i.e., fertility) rather than sexual signaling (i.e., interest in mating), resulting in little information about CHCs of males and virgin females and their contributions to sexual signaling. This dearth of information applies particularly to social wasps (Butts et al. 2001).

In vespine wasps, CHCs play a functional role in regulating the reproduction of worker wasps (Bonckaert et al. 2012; Oi et al. 2016). If CHCs in vespid wasps were to differ between virgin queens (gynes), workers and males, and if they were to define nest membership, then CHCs could conceivably function in sexual signaling, mediating attraction or recognition of mates and rejection of nest mates as potential mates (Ingleby 2015). Caste-specific CHCs would raise questions about nestmate recognition cues, such as how these signals or cues are produced and maintained simultaneously. Inbreeding avoidance is particularly important for insects with single-locus complementary sex determination systems, like most hymenopterans, where inbreeding may result in sterile or inviable diploid males in lieu of females (Zayed and Packer 2005; Heimpel and de Boer 2008). Surprisingly, CHC profiles of male yellowjacket wasps are still not known.

Our objectives were to (1) describe the CHCs associated with gynes, workers and males of four yellowjacket species (baldfaced hornets, *Dolichovespula maculata*, southern yellowjackets, *Vespula squamosa*, western yellowjackets, *V. pensylvanica*, and *V. alascensis*) and (2) test the hypothesis that CHCs differentiate the sex, caste, and nest membership of nest mates in each of the four study species. These four species represent two genera and three of the six species-groups (*vulgaris*, *maculata*, and *squamosa*) that comprise yellowjackets (Lopez-Osorio et al., 2017), thus allowing us to gain a rather broad understanding of sex-, caste-, and nest-based CHC variation in yellowjackets.

3.3. Methods and Materials

3.3.1. Experimental Insects

Specimens from four species of yellowjackets were used for CHC analyses. Three species (*D. maculata*, *V. pensylvanica*, *V. alascensis*) are native in British Columbia (BC) (Akre et al. 1981) and were collected in various sites in BC. The fourth species (*Vespula squamosa*) is present from Central America to the Great Lakes region of the United States (Akre et al. 1981; Landolt et al. 2009; Kimsey and Carpenter 2012), and was collected near Santiago Apoala, Oaxaca, Mexico

Nests of yellowjackets in B.C. were collected between May to September in 2013, 2014 and 2015, and transported to an enclosed "wasp garden" on the Burnaby campus of Simon Fraser University, as previously described (Ibarra Jimenez et al., 2016). Nests of *V. squamosa* were collected in November 2014 and immediately dissected to harvest specimens for CHC extractions.

3.3.2. Body surface extracts

Cold-euthanized wasps were placed with clean stainless steel forceps into separate 4-ml vials and covered with HPLC-grade pentane (1.5 ml for a queen or gyne; 1 ml for a worker or a male). After 10 min of extraction, the supernatant was withdrawn into a 2-ml vial, evaporated to dryness in a fume hood, and reconstituted with redistilled hexane (300 µl) containing (*E*9)-octadecen-1-yl acetate (10 ng/µl) as an internal standard (IS) for quantification of CHC constituents.

3.3.3. Analyses of CHCs

Aliquots (1-2 µL) of CHC extracts were analyzed by GC-flame ionization detection (FID) and GC-mass spectrometry (MS). Here, a Hewlett Packard 6890 gas chromatograph was fitted with a DB-5 column [50 m × 0.32 mm inner diameter (i.d.); J&W Scientific, Folsom, CA, USA)]. Helium was used as the carrier gas (35 cm s^{-1}) with the following temperature program: 100 °C for 1 min, 20 °C min^{-1} to 300 °C (50 min). The injector port and FID were set at 300 °C.

To identify CHC constituents, extracts were analyzed in electron impact (EI) and chemical ionization (CI) modes by a Saturn 2000 Ion Trap GC-MS or by an Agilent 5977A MSD fitted with a DB-5 MS column (30 m × 0.25 mm i.d.). Reagent gases for CI analyses were acetonitrile (Saturn Ion Trap) or methane (Agilent 5977 MSD). Helium was used as the carrier gas (35 cm s^{-1}) with the following temperature program: 100 °C for 1 min, 20 °C min^{-1} until 300 °C (10 min). The injector port and ion trap were set to 280 °C and 260 °C, respectively.

Aliphatic alkanes and mono-methyl CHCs were identified based on their retention indices (RI) (van Den Dool and Kratz 1963) and mass spectra (Howard and Blomquist 1982), and where possible, by comparing their RIs and mass spectra with those of authentic standards. To determine the double bond position(s) in unsaturated CHCs, aliquots of extracts were treated with dimethyl disulfide (Dunkelblum et al. 1985) and analyzed by GC-MS. Unsaturated hydrocarbons and select mono- and di-methylated CHCs were synthesized to confirm methyl branch positions that had been tentatively assigned based on mass spectral information (Howard and Blomquist 1982).

3.3.4. Synthesis of CHCs

General Experimental Procedures

All syntheses were performed with distilled solvents under argon atmosphere, using flame-dried glassware with standard vacuum line techniques. Flash chromatography was performed on silica gel (230–400 mesh) with eluents listed. Unless otherwise stated, solutions of crude products were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure by rotary evaporation.

Syntheses of methylalkanes

All methylalkanes were synthesized by Wittig reaction coupling phosphorus ylides and methylketones or isovaleraldehyde (for 2Me-C26), producing a mixture of *E*- and *Z*-isomers of methylalkenes. These mixtures were quickly purified by flash chromatography (silica, hexane as eluent) and subjected to hydrogenation in ethyl acetate with 10 % Pd/C as a catalyst. Final purification of the hydrogenated material afforded the methylalkanes. The general reaction scheme is given in Fig. 3-8.

A solution of the phosphonium salt (0.4 mmol, 1.0 eq) in anhydrous THF (15 ml) was cooled to −78 °C, and *n*-BuLi (1.6 M in hexanes, 0.3 ml, 0.48 mmol, 1.2 eq) was added dropwise. After stirring the reaction mixture at −78 °C for 1 h, the methylketone (0.4 mmol, 1.0 eq) was added, the mixture was stirred again at −78 °C for 1 h, and then slowly warmed to ambient temperature and stirred for an additional 6 h before it was treated with saturated aqueous NH_4Cl (15 ml). The aqueous layer was separated and extracted with EtOAc (15 ml). The combined organic layers were washed sequentially with H_2O and brine, then dried over Na_2SO_4 and concentrated. The residue was quickly purified by flash chromatography (hexane) to provide an olefin mixture of *E*- and *Z*-isomers. To this mixture were added 10 mL of ethyl acetate and 10% Pd on carbon (0.1 eq). After stirring the suspension under hydrogen for 14 h, the mixture was filtered through a plug of Celite (rinsing with hexane) and concentrated *in vacuo* to give the methylalkane as a colorless oil.

Synthesis of 2-methylhexacosane (2Me-C26)

A solution of the phosphonium salt (0.3 mmol, 1.0 eq) in anhydrous THF (15 ml) was cooled to −78 °C, and *n*-BuLi (1.6 M in hexanes, 0.23 ml, 0.36 mmol, 1.2 eq) was added dropwise. The reaction mixture was stirred at −78 °C for 1 h, then isovaleraldehyde (0.3 mmol, 1.0 eq) in THF (1 mL) was added. The reaction mixture was stirred at −78 °C for 1 h, then slowly warmed to ambient temperature, and stirred again for 6 h before it was treated with saturated aqueous NH_4Cl (15 ml). The aqueous layer was separated and extracted with EtOAc (15 ml). The combined organic layers were washed sequentially with H_2O and brine, then dried over Na_2SO_4 and concentrated. The residue was quickly purified by flash chromatography (hexane) to provide an olefin mixture of *Z*- and *E*-isomers. To this mixture were added 10 mL of ethyl acetate and 10% Pd on carbon (0.1 eq). The suspension was stirred under hydrogen for 14 h. The mixture was then filtered through a plug of Celite (rinsing with hexane) and concentrated *in vacuo* to give 2Me-C26 as a colorless oil. The reaction scheme is given in Fig. 3-9.

3.3.5. Statistical analyses

To determine whether caste and sex differed in CHC profiles, linear discriminant analyses (DA) were performed, using Z-transformed relative CHC peak areas as predictor variables and sex and caste or nest as grouping variables. For each analysis, only those compounds were included which were >1% of the total GC peak area, and present in all groups. These peaks were then re-standardized to 100% and transformed according to the Aitchinson's formula [Z_{ij} =

$\ln[Y_{ij}/g(Y_j)]$ (Aitchison 1986), as used previously in multivariate statistical analyses of CHCs (Dietemann et al. 2003; Ferreira-Caliman et al. 2010; van Zweden et al. 2014a). For discriminant analyses, variables that were highly correlated ($r > 0.8$ or < -0.8) were removed to improve the interpretability of standardized scoring coefficients. Wilks' lambda, which tests against the null hypothesis of no difference between group centroids, was used to measure the significance of group discrimination. We then compared the mean fold difference in relative abundance of individual CHCs to assess their specificity to a given caste or sex. In addition, we calculated the diagnostic power (DP) for each compound that had been selected for multivariate analyses. Traditionally, DP has been used as a measure of between-nest variation to within-nest variation to determine those highly nest-specific compounds that are likely nestmate recognition cues (van Zweden et al. 2010, 2014b; van Zweden and Ettorre 2010). Here, we used the same formula (van Zweden and Ettorre 2010) to calculate the degree of between-caste variation to within-caste variation, seeking highly caste-specific compounds. For DP calculations with *V. squamosa*, caste groups were queen, gyne, worker, and male. In all other species, only gyne, worker, and male groups were used due to insufficient numbers of queens.

3.4. Results

3.4.1. Chemical Analysis of CHC Extracts

GC-FID analyses of CHC extracts revealed 10-24 CHCs each with relative abundance of >1% of the total GC peak area (Figs. 3-1:4). GC-MS analyses of CHC extracts indicated that these CHCs were aliphatic alkanes and alkenes, as well as mono-, di- and tri-methyl branched alkanes (Table 3-2). Considering only CHCs quantified for discriminant analyses, we summarize below the major findings for *D. maculata, V. alascensis, V. pensylvanica* and *V. squamosa,* reporting in parentheses the relative difference in abundance of select CHCs in body surface extracts of gynes and workers, and of males and workers. Sample sizes for specific species and castes are given in Table 3-1.

Gynes of *D. maculata* had larger relative amounts of 9-$C_{29:1}$ (6.7-fold), 13me-C_{29} (3.1), 7-$C_{29:1}$ (2.6) and 13me-C_{27} (2.1) than workers, whereas males had larger relative amounts of 13me-C_{29} (6.0), 13me-C_{27} (3.4) , 9-$C_{29:1}$ (2.9) and x,x-diMe-C_{25} (2.4) than workers.

Gynes of *V. alascensis* had larger relative amounts of C_{30} (8.6-fold), C_{29} (7.6), 12me-C_{28} (2.4), and C_{27} (2.2) than workers, whereas males had larger relative amounts of 3me-C_{29} (2.4), C_{30} (2.4), C_{28} (2.2) and C_{29} (2.0) than males.

Gynes of *V. pensylvanica* had larger relative amounts of 3me-C_{29} (6.0-fold), C_{29} (3.8), x,x-diMe-C_{30} (3.5) and 11me-C_{27} (2.2) than workers, whereas males had larger relative amounts of 11me-C_{29} (2.8) and x,x-diMe-C_{30} (2.1) than workers.

Gynes of *V. squamosa* had 2.1-fold more x,x-diMe-C_{28} than workers, whereas males had no CHC more than twice as abundant as workers.

3.4.2. Caste and Sex Discrimination

When using caste and sex as a grouping variable in linear discriminant analyses, plots of the first two discriminant functions reveal that wasps from all four yellowjacket species cluster into their respective groups (Fig. 3-5). In each of the four species, these clusters represent significant differences in group centroids, as measured by Wilks' lambda (Table 3-3).

In *D. maculata* (Fig. 3-5, a), seven CHCs (x,x-dimethylpentacosane, 13-methylheptacosane, 3-methylheptacosane, (Z7)-nonacosene, nonacosane, 13-methylnonacosane, and x,x-dimethylnonacosane) were included in discriminant analyses. The first two discriminant functions represented 68% and 23.7% of the variation, respectively.

In *V. alascensis* (Fig. 3-5, b), eight CHCs (pentacosane, hexacosane, x,x-dimethylhexacosane, 3-methylheptacosane, nonacosane, 3-methylnonacosane, x,x-octacosane, and 3-methylhentriacontane) were included in discriminant analyses. The first two discriminant functions represent 54.4% and 32.1% of the variation, respectively.

In *V. pensylvanica* (Fig. 3-5, c), 16 CHCs (tricosane, pentacosane, 13-methylpentacosane, 3-methylpentacosane, (Z9)-heptacosene, heptacosane, 13-methylheptacosane, 3-methylheptacosane + x,x-dimethyl-heptacosane, 12-methyloctacosane, (Z9)-nonacosene, nonacosane, 11-methylnonacosane, 3-methylnonacosane, 11/13/15-methyltriacontane, (Z9)-hentriacontene) were included in discriminant analyses. The first two discriminant functions represented 61.2% and 27.2% of the variation, respectively.

In *V. squamosa* (Fig. 3-5, d), 17 CHCs (pentacosane, 11-methylpentacosane, x,x-dimethylpentacosane, 3-methylpentacosane, hexacosane, 13-methylhexacosane, 2-methylhexacosane, 13-methylheptacosane, x,x-dimethylheptacosane (RI: 2763), 2-methylheptacosane, x,x-dimethyloctacosane (RI: 2873), nonacosane, 13-methylnonacosane, x,x-dimethyl-nonacosane (RI: 2955), x,x-dimethyl-nonacosane (RI: 3008), 3-methylnonacosane, and x,x-dimethyltriacontane) were included in discriminant analyses. The first two discriminant functions represented 55.4% and 41.1% of the variation, respectively.

3.4.3. Nest Discrimination in Males

When using nest as a grouping variable and male CHC area counts as predictors, plots of the first two discriminant functions show that male wasps from each of *D. maculata*, *V. alascensis* and *V. squamosa* cluster according to nest (Fig. 3-6, a-c; Table 3-4). In *V. pensylvanica*, males from only two nests were obtained, so the analysis (producing one discriminant function) could not be graphed in the same manner as for the other species.

3.4.4. Nest Discrimination in Workers

When using nest as a grouping variable and worker CHC area counts as predictors, plots of the first two discriminant functions show that worker wasps from each of *D. maculata*, *V. alascensis*, and *V. squamosa* cluster according to nest (Fig. 3-7, a-d; Table 3-5). In *V. pensylvanica*, workers from two of the seven nests plotted overlap.

3.4.5. Diagnostic Power (DP) Calculations

DP calculations show that CHCs of *D. maculata*, *V. alascensis*, *V. pensylvanica,* and *V. squamosa* vary more between caste than they do between nest (Table 3-6). The compounds with the highest DP values were as follows: (*i*) *D. maculata*: heptacosane (2.92), x,x-dimethyl-heptacosane (2.34), and (Z9)-nonacosene (2.1); (*ii*) *V. alascensis*: triacontane (3.33), 13-methylheptacosane (3.19), and 3-methylheptacosane (2.54); (*iii*) *V. pensylvanica*: 3-methylnonacosane (1.85), 11/13/15-methyltriacontane (1.57), and x,x-dimethylnonacosane (1.3); and (*iv*) *V. squamosa*: heptacosane (3.1), 6-methylhexacosane (2.71), and nonacosane (2.57).

3.5. Discussion

In each of the four species of yellowjackets that we studied, CHC profiles differentiate caste, sex, and nest membership. CHC profiles of gynes and of males differ from those of workers and reproductive queens (Table 3-2; Fig. 3-1:4). The data for *D. maculata* are particularly obvious in that both gynes and males have strongly elevated relative amounts of specific CHCs compared to workers (Fig. 3-1). This caste- and sex-specific differential abundance of CHCs is as striking as that found for compounds considered queen pheromones in odor analyses of queens and workers of vespine wasps (Oi et al. 2016), implicating CHCs as potential sexual communication signals in yellowjackets. Alternatively, gyne-specific CHCs could reflect adaptations to specific life-history constraints such as the need to over-winter.

Amazingly, discriminant analyses also revealed that yellowjacket males and workers each have nest-specific CHC profiles (Figs. 3-6, 3-7) that could provide cues by which gynes avoid sibling mating and workers discriminate non-nestmates. Chemical cues are thought to inform avoidance of sib-mating in other hymenopterans, such as the ichneumonid parasitoid *Venturia canescens* (Metzger et al. 2010; Chuine et al. 2015).

Multiple lines of evidence suggest that CHCs mediate nestmate recognition in social insects (van Zweden and Ettorre 2010). This recognition informs altruistic or antagonistic behaviors between individuals based on nest membership. The most popular model for how nest-specific odor is generated describes the transfer of nest-specific odors through physical interactions between individuals (e.g., trophallaxis, allogrooming) or through contact with nest paper to form a “Gestalt” nest odor (Soroker et al. 1994, 1995, 1998; Vienne et al. 1995; Lahav et al. 1998). Acceptance and rejection error rates of discriminating nestmates are reduced by homogenizing specific odor cues around a mean value (van Zweden and Ettorre 2010). However, as social wasps vary not only in the relative (and absolute) abundance of CHCs between nests, but also between castes and sexes, and as CHCs are sensed as blends, it is difficult to conceive a mechanism for transferring only nest- but not caste-specific odors. If physical transfer and homogenization are the mechanisms producing nest-specific odor, then caste- and sex-specific odors would be muddled in the homogenization. This is obviously not the case in yellowjackets: our discriminant analyses show distinct caste-specific CHC profiles (Fig. 3-5), and diagnostic power calculations show that CHC variation between castes is greater than CHC variation between nests, for almost all CHCs (Table 3-6). Dietemann et al. (2003) argue against the Gestalt nest odor model based on their findings that both fertile queens and workers of *Myrmecia gulosa*

red bull ants have specific CHC profiles within the same nest. Similarly, male and female reproductives of the termite *Zootermopsis nevadensis* each have specific CHC profiles that differ greatly from those of workers (Liebig et al. 2009). Nonetheless, data from a cross-fostering experiment with *Formica rufibarbis* ants support the Gestalt nest odor model, demonstrating that a subset of CHCs is heritable and has different transfer rates among workers than the non-heritable CHCs that are not means of nestmate recognition (Van Zweden et al. 2010). A mechanism for this differential transfer is not proposed.

One potential (albeit speculative) mechanism is the transfer of all CHCs and selective degradation of some. As experiments with radiolabeled CHC precursors in ants have shown, CHCs shared through trophallaxis are mixed in the postpharyngeal gland (PPG) and then re-applied to the cuticle through grooming (Soroker et al. 1994; Meskali et al. 1995). Perhaps enzymes in the PPG degrade “ambiguous” CHCs before the others are re-applied onto the cuticle or transferred through trophallaxis. If instead of being transferred between individuals, nest and caste specific CHC profiles are primarily the result of heritable genetic factors, then these cues would be true kin recognition cues, and should show higher within nest variability among species with higher effective paternity (Foster and Ratnieks 2001).

Gynes of some yellowjacket species are known to produce airborne long-range sex pheromones that attract conspecific males (Reed and Landolt 1990; Brown et al. 2013; Derstine et al. 2017). In addition to these sex attractant pheromones, non-volatile CHCs could play a role in some other context of sexual communication. For example, following mate-location, CHCs could affect mate-choice and help avoid inbreeding which is detrimental in hymenopterans with single-locus complementary sex determinations systems (Zayed and Packer 2005; van Wilgenburg et al. 2006).

Our findings that CHC profiles differentiate caste, sex, and nest membership in yellowjackets are consistent with previous reports that aliphatic alkanes and methyl-branched hydrocarbons signal fertility and act as queen pheromones in vespines (Oi et al. 2016; van Zweden et al. 2014). Future studies with yellowjackets should manipulate CHCs in perfuming experiments (e.g., Riesch et al. 2017) to determine the role(s) that gyne- and male-specific CHCs play in the context of sexual communication and mating behavior, from the perspective of both males and females. By manipulating the relative amounts of specific CHCs, their effect on mate attractiveness, mate choice and mate acceptance may become apparent. Studies with radio-labelled CHCs may offer insight into CHC transfers between nestmates and their relative

contributions to the Gestalt nest odor. Tracking the presence of labelled CHCs belonging to specific chemical groups may verify the variability that seems to exist in the transfer of nestmate recognition CHCs and other CHC constituents.

3.6. References

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3.7. Tables and Figures

Table 3-1. Number of yellowjacket nests and individuals sampled for cuticular hydrocarbon (CHC) analyses per species.

Species	Queens	Gynes	Workers	Males	Nests
Dolichovespula maculata	1	47	29	21	10
Vespula pensylvanica	3	11	39	10	7
Vespula alascencis	2	13	27	15	4
Vespula squamosa	19*	10**	30	31	4

* Nests studied here were polygynous, allowing more queens than nests to be sampled.

** Five gynes emerged after collection, five had no eggs.

Table 3-2. Summary of cuticular hydrocarbons (CHCs) identified (ID) in body surface extracts of queens (♕), gynes (☿), workers (♀), and males (♂) of *Vespula squamosa*, *V. pensylvanica*, *Dolichovespula maculata*, and *V. alascensis*, the retention indices (RI) of these CHCs, and their mean relative abundances in extracts. Sample sizes are given in Table 1. Cells with a "+" symbol indicate that the CHC was present but was not quantified.

Peak	ID	RI	MW	*V. squamosa*				*V. pensylvanica*				*D. maculata*				*V. alascensis*			
				♕	☿	♀	♂	♕	☿	♀	♂	♕	☿	♀	♂	♕	☿	♀	♂
1	C_{23}	2300	324	+	+	+	+	2.8	1.5	11.2	5.2					+	+	+	+
2	11me-C_{23}	2338	338	+	+	+	+												
3	3me-C_{23}	2375	338	+	+	+	+	+	+	+	+								
4	C_{24}	2400	338	+	+	+	+	+	+	+	+					+	+	+	+
5	11me-C_{24}	2436	352	+	+	+	+												
6	2me-C_{24}	2459	352	+	+	+	+												
7	C_{25}	2500	352	8.3	9.6	11.4	10.4	9.2	5.7	17.0	19.6	+	+	+	+	18.7	4.8	17.0	20.1
8	11/13me-C_{25}	2536	366	3.3	4.2	4.0	5.1	1.7	1.3	4.6	4.3					+	+	+	+
9	5me-C_{25}	2550	366																
10	x,x-C_{25}	2564	380	1.7	1.6	1.8	2.4												
11	3me-C_{25}	2574	366	1.1	2.5	3.7	4.0	4.0	1.7	6.4	7.2					+	+	+	+
12	x,x-C_{25}	2583	380									+	+	+	+				
13	C_{26}	2600	366	5.3	3.1	5.0	5.1					+	+	+	+	4.8	2.0	3.5	3.0
14	x,x-C_{25}	2601	380									2.1	1.8	1.6	3.8				
15	x,x-C_{25}	2608	380																
16	13/12-meC_{26}	2632	380	1.5	2.2	2.4	2.6												
17	6me-C_{26}	2644	380	0.6	0.8	2.3	2.1												

18	2me-C_{26}	2658	380	1.9	2.6	6.1	5.3												
19	9-$C_{27:1}$	2679	378					3.2	5.5	6.5	3.5	+	+	+	+				
20	7-$C_{27:1}$	2687	378									+	+	+	+				
21	C_{27}	2700	380	40.1	13.0	9.5	8.3	19.6	13.8	10.1	13.8	47.0	16.4	37.1	14.7	36.7	28.2	12.9	17.6
22	13me-C_{27}	2733	394	11.7	21.1	15.9	17.2	3.0	4.2	6.2	9.8	1.6	6.4	3.1	10.4	4.0	1.7	17.3	6.5
23	x,x-C_{27}	2760	408									+	+	+	+	+	+	+	+
24	x,x-C_{27}	2763	408	5.0	6.6	6.6	6.4												
25	3me-C_{27}	2773	394					10.2	5.6	5.5	6.1	12.0	12.0	13.1	19.4	0.8	0.8	9.8	3.7
26	2me-C_{27}	2773	394	3.0	7.3	8.0	8.6												
27	x,x-C_{27}	2775	408									+	+	+	+				
28	5,x-C_{27}	2781	408	+	+	+	+												
29	C_{28}	2800	394	+	+	+	+	+	+	+	+					3.6	4.5	4.0	8.6
30	x,x-C_{27}	2808	408	4.2	4.1	4.2	4.0	5.0	4.6	8.0	4.2	15.0	12.0	24.4	13.3				
31	14/13/12-C_{28}	2832	408	1.3	2.8	2.9	2.7	1.1	2.7	2.5	2.1					3.2	3.6	1.5	1.9
32	x,x,x-C_{27}	2838	422									+	+	+	+				
33	6me-C_{28}	2840	408	0.3	0.6	1.0	1.0												
34	2me-C_{28}	2858	408	0.8	1.3	2.0	1.7	+	+	+	+								
35	x,x,x-C_{27}	2858	422									+	+	+	+				
36	x,x-C_{28}	2873	422	0.3	2.0	0.9	0.8												
37	Z9-$C_{29:1}$	2880	406					2.0	3.6	6.0	2.5	2.7	22.8	3.4	10.0				
38	Z7-$C_{29:1}$	2890	406									1.0	7.1	2.7	2.3	+	+	+	+
39	x,x-C_{28}	2890	422	0.5	0.9	1.6	1.1												
40	C_{29}	2900	408	3.4	1.5	0.8	0.8	21.8	15.7	4.1	5.3	11.7	9.5	5.8	7.6	15.3	22.9	3.0	6.0
41	15/13/11me-C_{29}	2931	422	2.1	6.6	4.0	4.4	3.5	7.7	3.5	9.8	6.8	6.8	2.2	13.3	3.9	5.0	14.5	10.5
42	x,x-C_{29}	2955	436	1.0	1.3	1.3	1.3									+	+	+	+

43	x,x-C_{29}	2958	436									3.48	5.2	6.5	5.2				
44	x,x-C_{29}	2963	436	0.6	1.7	2.0	2.0												
45	3me-C_{29}	2968	422	0.5	1.1	1.0	1.0	7.0	13.5	2.2	1.6	+	+	+	+	1.4	2.8	3.5	8.3
46	C_{30}	3000	422	+	+	+	+	+	+	+	+					2.4	12.7	1.5	3.5
47	x,x-C_{29}	3008	436	0.6	1.4	1.6	1.5	2.7	4.9	2.8	1.5								
48	11/13/15-C_{30},	3032	436					1.5	4.0	1.1	2.4					+	+	+	+
49	2me-C_{30}/dm-C_{30}?	3058	436/450					+	+	+	+								
50	$C_{31:1}$	3081	434					1.72	3.8	2.1	1.0								
51	C_{31}	3100	436					+	+	+	+					+	+	+	+
52	x,x-C_{30}	3130	450													2.6	8.3	7.9	7.2
53	11/13/15-C_{31}	3130	450					+	+	+	+								
54	x,x-C_{30}	3155	464													+	+	+	+
55	x,x-C_{31}	3160	464					+	+	+	+					+	+	+	+
56	3me-C_{31}	3175	450					+	+	+	+					+	+	+	+
57	C_{32}	3200	450					+	+	+	+					+	+	+	+
58	C_{33}	3300	464													+	+	+	+
59	x,x-C_{32}	3329	478													1.2	3.7	3.8	3.0
60	x,x-C_{33}	3358	492													+	+	+	+
62	C_{34}	3400	478													+	+	+	+

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Table 3-3. Summary of statistics from linear discriminant analyses of yellowjacket species using caste and sex (queen, gyne, worker, male) as grouping variables, and Z-transformed cuticular hydrocarbon (CHC) peak areas as predictor variables.

Total model comparisons between sex and caste (linear discriminant analyses)			
Species	Wilks' lambda	Approximate F value	P-value
Dolichovespula maculata	0.0098	77.06	< 0.0001
Vespula alascensis	0.0003	28.01	< 0.0001
Vespula pensylvanica	0.0116	19.95	< 0.0001
Vespula squamosa	0.0058	13.99	< 0.0001

Table 3-4. Summary of statistics from linear discriminant analyses of male yellowjackets using nest membership as a grouping variable, and Z-transformed cuticular hydrocarbon (CHC) peak areas as predictor variables.

Comparing nest variation in males (linear discriminant analyses)			
Species	Wilks' lambda	Approximate F value	P-value
Dolichovespula maculata	< 0.0001	7.49	< 0.0001
Vespula alascensis	< 0.0001	5.82	0.0006
Vespula pensylvanica	0.0117	10.55	0.0229
Vespula squamosa	< 0.0001	8.33	< 0.0001

Table 3-5. Summary of statistics from linear discriminant analyses of worker yellowjackets using nest membership as a grouping variable, and Z-transformed cuticular hydrocarbon (CHC) peak areas as predictor variables.

Comparing nest variation in workers (linear discriminant analyses)			
Species	Wilks' lambda	Approximate F value	P-value
D. maculata	0.0002	12.71	< 0.0001
V. alascensis	< 0.0001	9.94	0.0002
V. pensylvanica	0.0001	3.67	< 0.0001
V. squamosa	0.0065	10.49	< 0.0001

Table 3-6. Diagnostic power (DP) values on Z-transformed relative peak areas for all cuticular hydrocarbons (CHCs) selected for multivariate analyses. Values are ranked largest to smallest based on the DP values for caste variation. For example, a value of 3 for a specific compound would mean that for this compound there is 3 times more variation among all castes than within castes; id = compound identity.

D. maculate			**V. alascensis**			**V. pensylvanica**			**V. squamosa**		
DP values			DP values			DP values			DP values		
id	Caste	Nest	id	Caste	Nest	id	Caste	Nest	id	Caste	Nest
C_{27}	2.92	1.51	C_{30}	3.33	1.25	3me-C_{29}	1.85	1.11	C_{27}	3.10	1.29
x,x-C_{27}	2.34	1.73	13me-C_{27}	3.19	1.30	11/13/15me-C_{30}	1.57	1.08	6me-C_{26}	2.71	1.23
Z9-$C_{29:1}$	2.10	1.55	3me-C_{27}	2.54	1.21	x,x-C_{29}	1.30	1.11	C_{29}	2.57	1.27
Z7-$C_{29:1}$	1.87	1.76	C_{27}	2.43	1.21	3me-C_{25}	1.26	0.99	2me-C_{26}	2.32	1.18
13me-C_{27}	1.78	1.25	C_{25}	2.28	1.03	C_{23}	1.24	1.00	x,x-$C_{27.2}$	2.00	1.28
13me-C_{29}	1.72	1.41	13me-C_{29}	2.18	1.38	C_{29}	1.20	1.08	6me-C_{28}	1.88	1.38
x,x-C_{29}	1.68	1.61	12me-C_{28}	2.16	1.09	11me-C_{29}	1.18	0.96	2me-C_{27}	1.84	1.24
3me-C_{27}	1.67	1.36	3me-C_{29}	2.09	1.00	13me-C_{25}	1.13	1.04	x,x-$C_{28.1}$	1.77	1.40
x,x-C_{25}	1.21	1.28	C_{29}	1.95	1.09	Z9-$C_{31:1}$	1.11	1.05	13me-C_{29}	1.75	1.22
C_{29}	1.05	1.12	C_{28}	1.47	1.43	13me-C_{27}	1.10	1.12	x,x-$C_{29.2}$	1.73	1.22
			x,x-C_{30}	1.30	1.08	C_{27}	1.08	1.06	14me-C_{28}	1.70	1.28
			x,x-C_{32}	1.20	1.12	Z9-$C_{27:1}$	1.08	0.98	C_{26}	1.64	1.30
			C_{26}	1.16	1.01	C_{25}	1.06	1.05	x,x-$C_{28.2}$	1.57	1.17
						Z9-$C_{29:1}$	1.05	1.02	3me-C_{25}	1.40	1.41
						x,x-C_{27}	1.05	1.10	x,x-C_{30}	1.35	1.20
						12me-C_{28}	0.99	1.06	2me-C_{28}	1.33	1.34

D. maculata			V. alascensis			V. pensylvanica			V. squamosa		
	DP values			DP values			DP values			DP values	
id	Caste	Nest	id	Caste	Nest	id	Caste	Nest	id	Caste	Nest
						3me-C_{27}	0.98	1.05	13me-C_{27}	1.31	1.15
									3me-C_{29}	1.18	1.41
									x,x-$C_{27.1}$	1.16	1.09
									11me-C_{25}	1.11	1.29
									x,x-$C_{29.1}$	1.09	1.04
									x,x-C_{25}	1.08	1.29
									C_{25}	1.06	1.18
									13me-C_{26}	0.99	1.01

Table 3-7. Summary of cuticular hydrocarbons identified (ID) in body surface extracts of *Vespula squamosa*, *V. pensylvanica*, and *V. alascensis* and referred to in Figures 3-2:4; RI = retention index; MW = molecular weight

Peak #	ID	RI	MW	Peak #	ID	RI	MW
1	C_{23}	2300	324	18	14/13/12-C_{28}	2832	408
2	C_{25}	2500	352	19	6me-C_{28}	2840	408
3	11/13me-C_{25}	2536	366	20	2me-C_{28}	2858	408
4	x,x-C_{25}	2564	380	21	x,x-C_{28}	2873	422
5	3me-C_{25}	2574	366	22	Z9-$C_{29:1}$	2880	406
6	C_{26}	2600	366	23	x,x-C_{28}	2890	422
7	13/12-meC_{26}	2632	380	24	C_{29}	2900	408
8	6me-C_{26}	2644	380	25	15/13/11me-C_{29}	2931	422
9	2me-C_{26}	2658	380	26	x,x-C_{29}	2955	436
10	9-$C_{27:1}$	2679	378	27	x,x-C_{29}	2963	436
11	C_{27}	2700	380	28	3me-C_{29}	2968	422
12	13me-C_{27}	2733	394	29	C_{30}	3000	422
13	x,x-C_{27}	2763	408	30	x,x-C_{29}	3008	436
14	3me-C_{27}	2773	394	31	11/13/15-C_{30}	3032	436
15	2me-C_{27}	2773	394	32	$C_{31:1}$	3081	434
16	C_{28}	2800	394	33	x,x-C_{30}	3130	450
17	x,x-C_{27}	2808	408	34	x,x-C_{32}	3329	478

Figure 3-1. Representative chromatograms (FID) of body surface extracts of a *Dolichovespula maculata* queen, gyne, worker and male. Note the profound differences in cuticular hydrocarbon profiles between sexes and castes.

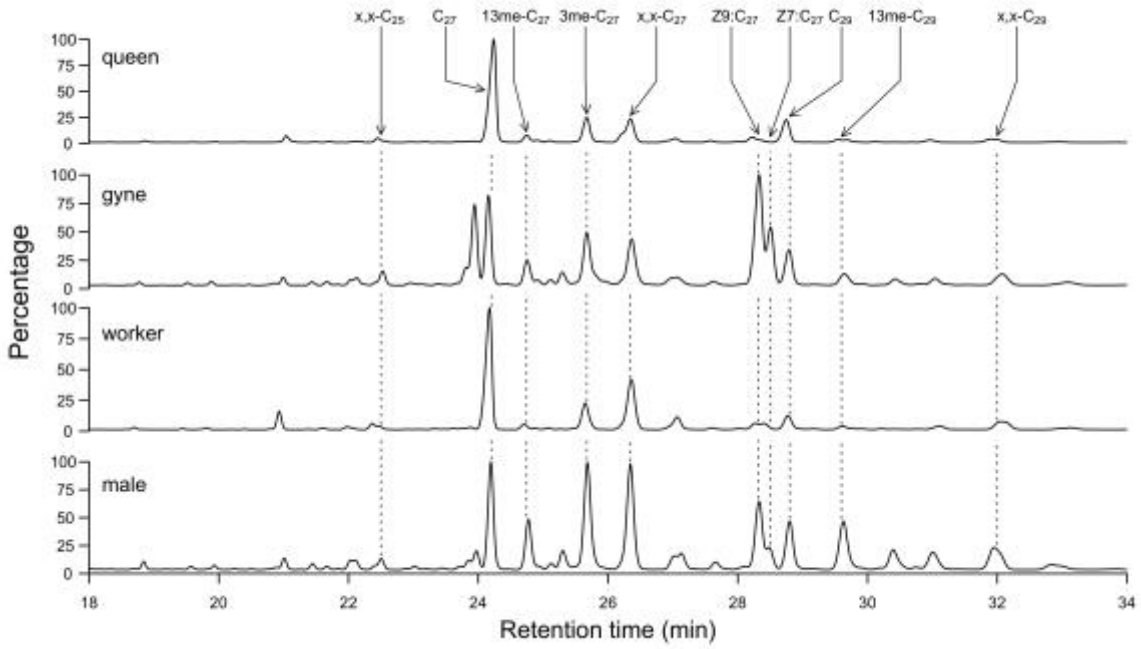


Figure 3-2. Representative chromatograms (FID) of body surface extracts of a *Vespula alascensis* queen, gyne, worker and male. Note the profound differences in cuticular hydrocarbon profiles between sexes and castes. Compounds numbers are identified in Table 3-7.

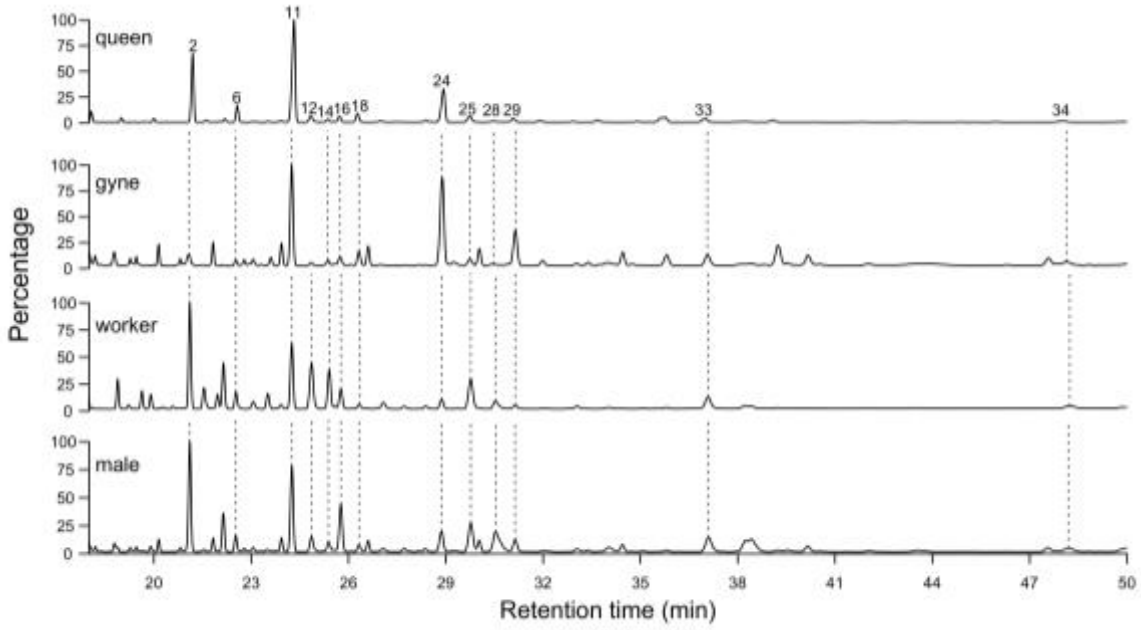


Figure 3-3. Representative chromatograms (FID) of body surface extracts of a *Vespula pensylvanica* queen, gyne, worker and male. Note profound differences in cuticular hydrocarbon profiles between sexes and castes. Compounds numbers are identified in Table 3-1.

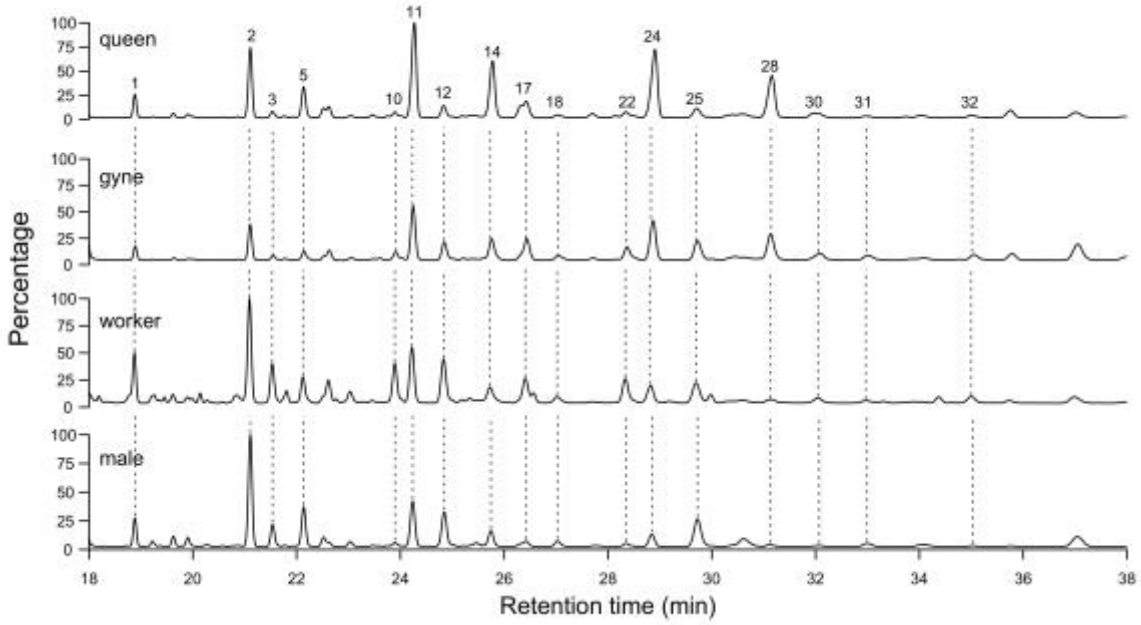


Figure 3-4. Representative chromatograms (FID) of body surface extracts of a *Vespula squamosa* queen, gyne, worker and male. Note profound differences in cuticular hydrocarbon profiles between sexes and castes. Compounds numbers are identified in Table 3-7.

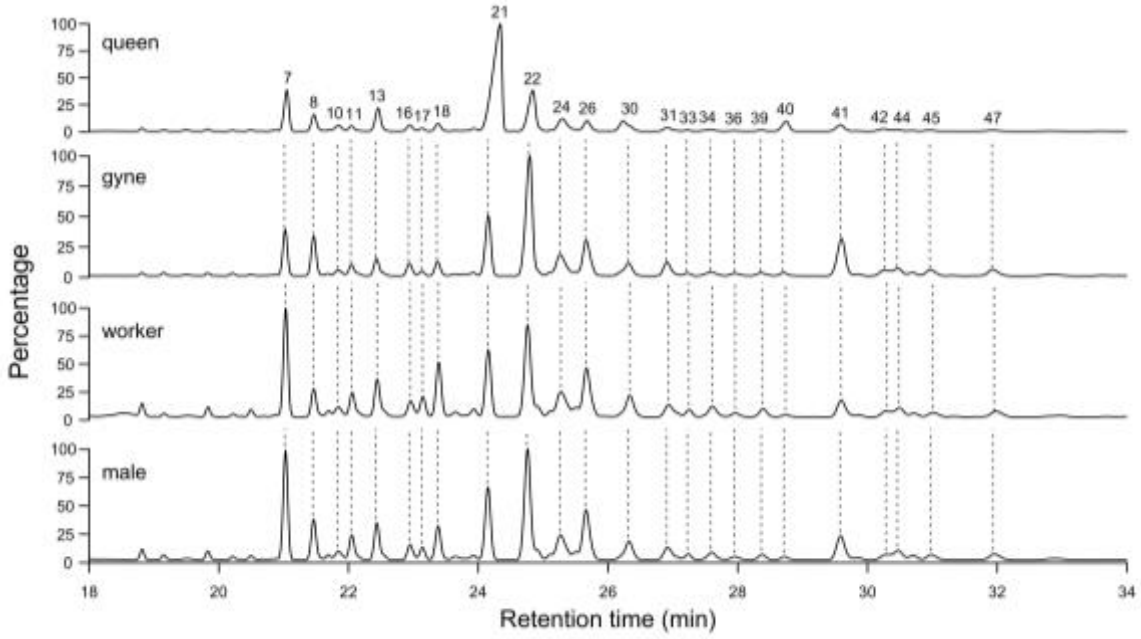


Figure 3-5. **Plots of the first two linear discriminant analysis (LDA) functions depicting the clustering of sex and caste groups (queen, gyne, male, worker) based on differences in cuticular hydrocarbon (CHC) profiles for four yellowjacket species (*Dolichovespula maculata, Vespula alascensis, V. pensylvanica, V. squamosa*). The percent of total variation in the sample described by the discriminant functions is given in parentheses on each axis.**

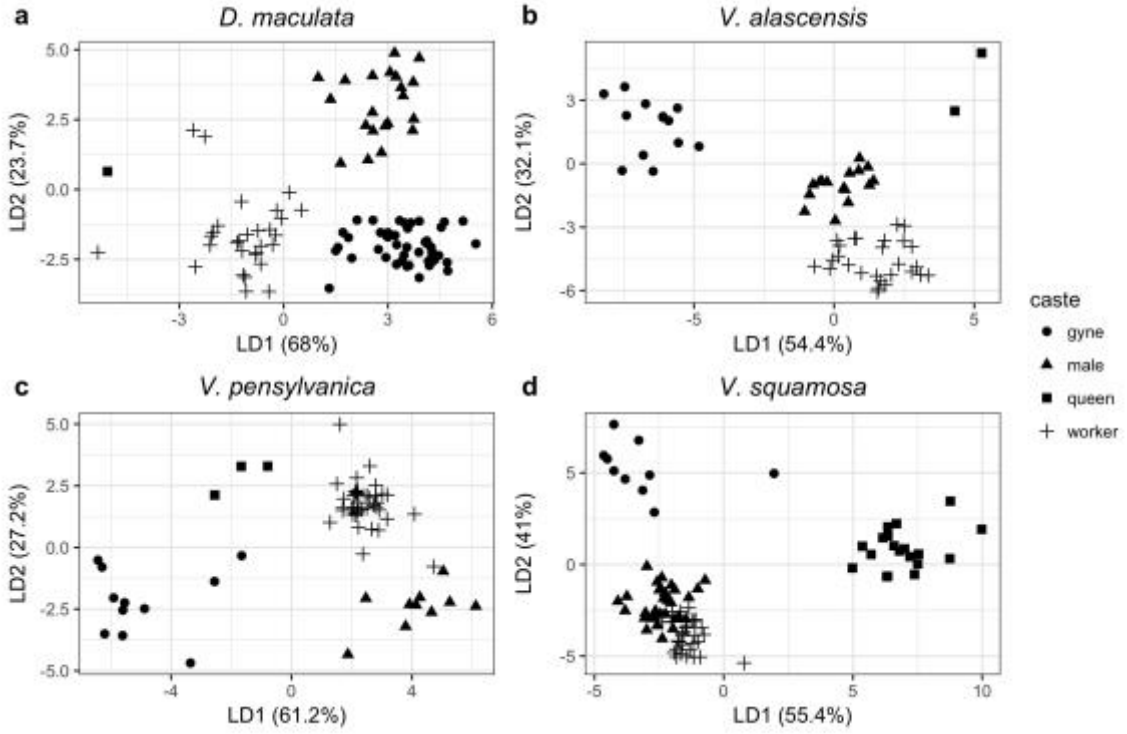


Figure 3-6. **Plots of the first two linear discriminant analysis (LDA) functions depicting the clustering of male wasps (a-c) into nest groups based on differences in cuticular hydrocarbon (CHC) profiles for four yellowjacket species (*Dolichovespula maculata, Vespula alascensis,* and *V. squamosa*). The percent of total variation in the sample described by the discriminant functions is given in parentheses on each axis.**

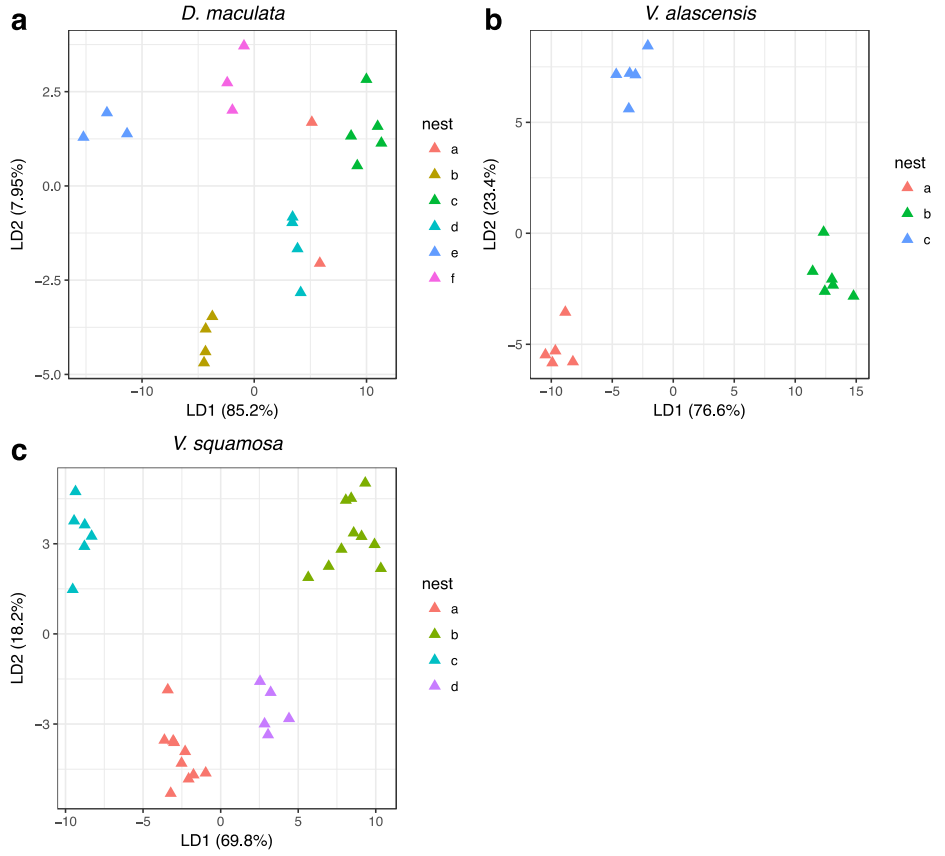


Figure 3-7. **Plots of the first two linear discriminant analysis (LDA) functions depicting the clustering of worker wasps (a-d) into nest groups based on differences in cuticular hydrocarbon (CHC) profiles for four yellowjacket species (*Dolichovespula maculata, Vespula alascensis, V. pensylvanica,* and *V. squamosa*). The percent of total variation in the sample described by the discriminant functions is given in parentheses on each axis.**

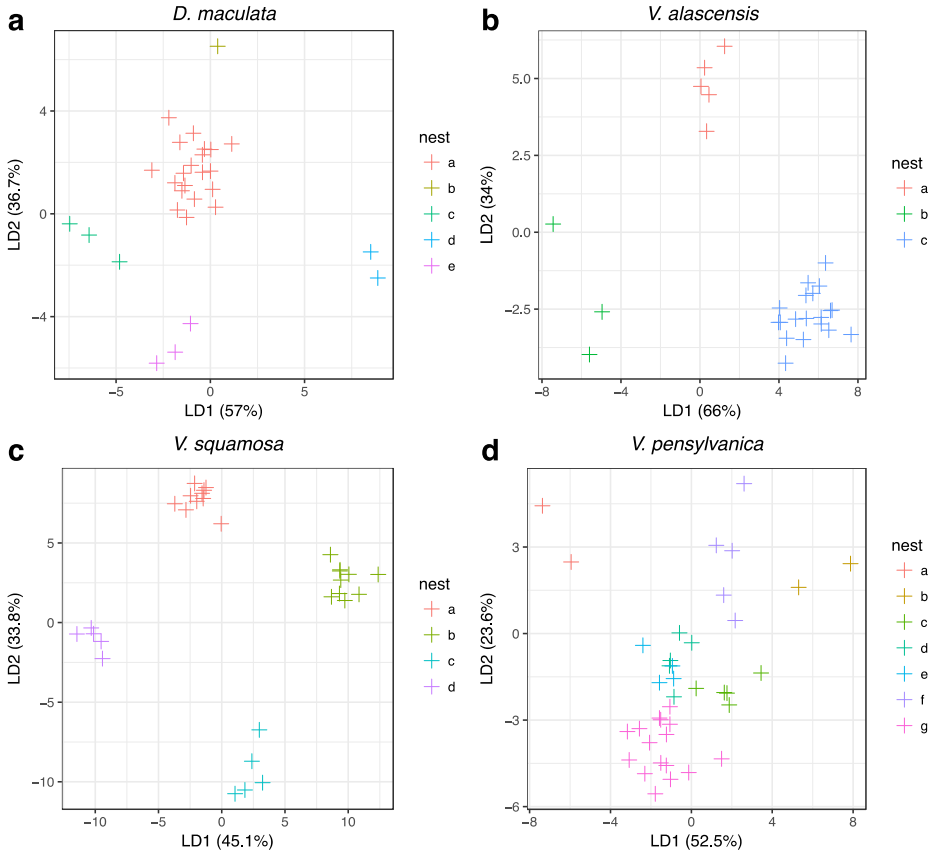
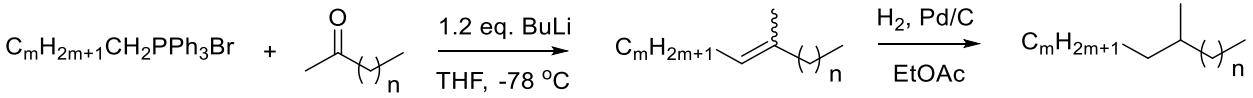


Figure 3-8. Scheme of methylalkane synthesis.



(a): m = 12, n = 9 product: 11Me-C24
(b): m = 19, n = 4 product: 6Me-C26
(c): m = 13, n = 10 product: 12Me-C26
(d): m = 12, n = 11 product: 13Me-C26
(e): m = 13, n = 12 product: 14Me-C28
(f): m = 15, n = 11 product: 13Me-C29

Figure 3-9. Scheme of 2-methylhexacosane (2Me-C26) synthesis.