Contributions to the ecology of North American vespines: Rearing yellowjackets in nest boxes and unraveling pheromone-mediated nest defense in bald-faced hornets

by

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Abstract

To obtain an ample supply of study insects, we attempted to rear yellowjackets from spring-collected queens, achieving a high nest initiation rate for German yellowjackets, *Vespula germanica.* To study nest defense, we placed paired boxes near the nest entrance of bald-faced hornets, *Dolichovespula maculata*, and audio recorded sound impulses caused by nest mates striking the boxes. The number of strikes increased 27-fold when – compared to two control boxes – one of the two boxes was treated with venom sac extract (VSE), providing evidence for an alarm response. The VSE-treated box also induced a greater proportion of strikes than the corresponding control box, providing evidence for a target-oriented response. Analyzing VSEs by gas chromatographic-electroantennographic detection (GC-EAD) and GC-mass spectrometry, we identified seven candidate pheromone components which – based on molecular structure – triggered primarily alarm or target-oriented responses. VSE was more effective than synthetic pheromone in triggering alarm, indicating a missing alarm pheromone component.

Keywords: *Dolichovespula, Vespula*, alarm, pheromone, rearing

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List of Acronyms

MBA	*N*-3-Methylbutylacetamide
MBO	2-Methyl-3-buten-2-ol
MBP	*N*-3-methylbutylpropanamide

Glossary

Allomone	Semiochemical that mediates interspecific interactions benefiting the emitter but not the receiver.
Eusocial insects	Insects that display cooperative brood care, reproductive division of labour, and overlapping generations
Kairomone	Semiochemical that mediates interspecific interactions benefitting the receiver but not the emitter.
North American vespines	Invasive and endemic species of vespines in North America
Releaser pheromone	Semiochemical that mediates intraspecific interactions benefitting both the emitter and the receiver. The releaser pheromone triggers a behavioural response.
Primer pheromone	Intraspecific semiochemical that affects the physiological state of the receiver.

“Every [person] gets a narrower and narrower field of knowledge in which [they] must be an expert in order to compete with other people. The specialist knows more and more about less and less and finally knows everything about nothing.”
– Konrad Lorenz, 1973

Chapter 1.

Nest defense pheromones in vespids

1.1. Abstract

Like most insects, wasps use pheromones as their predominant means of communication. As eusocial insects, wasps need to protect their larvae and pupae from predation. They defend against vertebrate predators by cooperation and *en-masse* defense via alarm pheromones. The release of alarm pheromone by a wasp triggers alarm behaviour by nest mates that then counteract a threat. All alarm pheromones heretofore identified have been isolated from venom sacs. Alarm behaviour includes stinging and biting of the intruder by worker wasps. Pheromone-mediated alarm behaviour is not ubiquitous among vespids. Within the Vespinae subfamily, the phenomenon has been described for just nine species. However, even if pheromone-based defense behaviour has not yet been demonstrated in a particular species, this type of behaviour may still be revealed in the future with an improved bioassay design. While some components of the nest defense pheromone volatilize readily, prompting nest mates into defensive behaviour, other components mark perpetrators for directed and coordinated attacks. Further compounds isolated from venom sacs suppress aggressive behaviour towards nest mates. The study of nest defense signals will enhance our understanding of the evolution of eusociality and could result in better trap baits that might mitigate the ecological impact of invasive wasps.

Keywords: Vespidae, hornets, yellowjackets, nest defense pheromone, alarm pheromone, marker pheromone, nest defense

1.2. **From individuals to super-organism**

The nests of eusocial insects, such as honeybees and yellowjackets, are described as super-organisms with emergent behavioural traits (Smith et al. 2008, Gardner and Grafen 2009, Nowak et al. 2010). These traits include cooperative brood care, reproductive division of labor, and overlapping generations (Michener 1969, Smith et al. 2008). The increase in complexity of the patterns of behaviour displayed, from the individual to the entire colony, is proximately explained, in large, by a vast array of primer and releaser pheromones that serve various functions in separate contexts (Wilson 1965).

Releaser pheromones are chemical signals that are emitted by an individual and that when sensed by conspecifics change their behaviour (Karlson and Butenandt 1958). There would be no super-organism without pheromones, as they mediate social behaviour and link individuals to the super-organism. Key pheromone-mediated social behaviours include caste differentiation (i.e., division of labour between queens and workers) and alarm and defense (Wilson 1965, Smith et al. 2008). Prior to the study of excretory glands, their contents and their effects on organisms, proximate and ultimate questions on insect eusociality were practically impossible to answer (Wilson 1965).

Holarctic yellowjackets and hornets in the subfamily Vespinae display the most advanced level of social organization among wasps and are therefore potential model organisms for the study of eusociality (Akre et al. 1980, Landolt et al. 1998). Yellowjackets and hornets are part of the Vespidae family which - within the aculeata taxon - comprises ca. 15,000 species of true wasps worldwide (Hurd 1955). Most of these wasps utilize their sting as a predatory tool to subdue prey; vespine wasps, in contrast, utilize the sting mainly for defending the super-organism (Maschwitz 1964a, Jeanne 1981, Heath and Landolt 1988).

1.3. **Predatory pressures on eusocial wasps**

The high concentration of food in the nests of eusocial insects with cooperative brood care renders these nests attractive to vertebrate predators (Starr 1985). In temperate climates, mature vespine nests contain between 500 and 15,000 brood cells, with peak numbers of workers ranging between 75 and 5,000. In tropical climates, the number of workers per nest is even higher simply because there is no winter diapause, and nests continue to grow year round (Akre et al. 1980, Akre 1982).

Predators of social wasps that successfully harvest a nest consume large numbers of high-quality food items: larvae and pupae. For example, the larvae and pupae of *Vespula squamosa* (Drury) contain up to 72% of the niacin content (Vitamin B3) of that of cow liver (8.74 mg/100 g); thus, 100 g of *V. squamosa* brood are sufficient to satisfy the daily niacin requirements of a 1-year-old human infant (Ramos and Pino 2001). Larvae and pupae are also rich in minerals, containing up to 4.69 g/100 g of ash, 4.5 times higher than beef (Sales and Hayes 1996, Ramos and Pino 1998). Residents of the Mixteca region of Oaxaca, Mexico, consume *V. squamosa* larvae and pupae (pers. obs.) but we don't know the extent to which wasp brood contributes to their overall diet. We did notice, though, that 10 of 11 nests we had found in November 2014 had been harvested by humans. This leads us to hypothesize that humans affect *V. squamosa* populations in the Mixteca region and that the brood of eusocial wasps may be a crucial dietary component for humans.

The successful establishment of a nest, and ultimately the fitness of the super-organism, depends - among many factors - on the ability of nest mates to prevent nest predation and to allow gynes and males to reproduce (Sudd and Franks 1987, McCann et al. 2014a). Predatory pressures by non-human vertebrates on eusocial wasps are well documented. In North America, eastern yellowjackets, *V. maculifrons* (Buyson), suffer heavy predation from the stripped skunk, *Mephitis mephitis* Shreber (Preiss 1967). In the Pacific Northwest, bald-faced hornets, *Dolichovespula maculata* (L.), appear to suffer heavy predation from raccoons (per. obs.). All 15 nests that we had transplanted in the summer of 2014 and not protected by electric fence succumbed to repeated attacks by raccoons (pers. obs.). Raccoons are considered common predators of North

American vespids (Akre et al. 1980, MacDonald and Matthews 1981, Reed and Landolt 2000). Indeed, our personal observations and those of colleagues suggest that raccoons may rely on vespid brood as part of their diet, for at least part of the year, and that raccoons may play a role in determining wasp population densities.

Mammals are major predators of eusocial wasps in North America. Yet, birds like the honey buzzard, *Pernis apivorus* (L.), in Europe (Lack 1946) eat the brood of eusocial insects. Paper wasps in the Amazonian rain forest likely suffer a higher predation pressure from Red-throated Caracaras, *Ibycter americanus* (Boddaert), than from army ants (McCann et al. 2013, 2014a), which were thought to be the main predator of social wasps in the tropics. Much smaller birds, such as Black-throated Antshrikes, *Frederickena viridis* (Viellot), prey on *Polybia* paper wasps (McCann et al. 2014b). Furthermore, blue jays, *Cyanocitta cristata* (L.), and summer tanagers, *Piranga rubra* (L.), frequently consume brood of *Polistes* spp Latreille in North America (Starr 1985). Birds and mammals may have main impacts on population densities of eusocial wasps worldwide but there are very few studies that quantify the effect of predation on populations of eusocial wasps (Archer 1981,1985, Barlow 2002, Archer 2009, McCann 2014a).

Invertebrate and vertebrate predators exert selective pressures on wasps that, over time, have resulted in predator-specific defense tactics (McCann et al. 2014a). Chemical defense tactics against invertebrate predators are well documented among paper wasps (Jeanne 1996). The paper wasp *Polistes dominulus* (Christ) and its social parasite *P. sulcifer* (Zimmerman) exemplify a chemical defense tactic. Both insects produce a mixture of unsaturated fatty acids which they place on nest petioles to repel predatory ants of at least three species (Dani et al. 1996). Vertebrate predation on wasps, on the other hand, has resulted in defense strategies that range from nesting in inaccessible cavities (Edmunds 1974) to mass stinging and biting of a nest perpetrator by nest mates (Post et al. 1984, Heath and Landolt 1988, Landolt et al. 1998).

1.4. **Defense of the super-organism**

The sting and venom of eusocial aculeate hymenopterans epitomize effective defense. The sting with its venom induces pain and immediately informs the perpetrator that it has been injured. This ultimately forces the perpetrator to abandon its pursuit of prey larvae and pupae (Schmidt 1986a, Sudd and Franks 1987). Stinging is a common response of nest mates to vertebrate intruders but not all eusocial wasp species may respond this way (Landolt et al. 1998, Fortunato et al. 2004, Bruschini et al. 2006).

Prolonged pain in the area of the sting coupled with local edema and erythema are immunoglobulin E mediated responses to the venom, achieved by an increased permeability of blood vessels (Nakajima 1986, Klotz et al. 2009). The pain of a sting may persist for several hours, and the itchiness of the stung body part may last for several days. In extremely rare cases, anaphylactic reactions have led to death in humans (Nakajima 1986, Sampson et al. 2005, Klotz et al. 2009). The main pharmacological components in vespid venoms are amines, peptides and proteins, as well as allergens and neurotoxins (Nakajima 1986).

The constituents of a venom sac not only serve to inform the perpetrator that it is under attack, they have also been selected over evolutionary time to inform nest mates about an incipient or ongoing nest attack (Schmidt 1986a, Landolt et al. 1998). As shown in many hymenopterans, defensive compounds co-function as alarm signals (Saslavaski et al. 1973, Hölldobler and Wilson 1990, Landolt et al. 1998).

Single- or multiple-component alarm pheromones are released in the presence of danger (Wilson 1971, Ono et al. 2003, Wyatt 2003). The capacity to release and sense these pheromones is a major factor contributing to the success of eusocial wasps (Wilson 1965). Through time, some defense compounds have been selected for high volatility and thus disperse quickly through space, allowing nest mates to engage in defense with a minimal lag period (Wilson 1971). The volatility of alarm pheromones also facilitates their rapid waning, allowing nest mates to resume normal activities as soon as the danger has passed (Wilson 1971, Ono et al. 2003). Alarm pheromones act by either reducing the threshold required to attack or by marking potential intruders as a source of danger (Jeanne 1982, Landolt et al. 1998, Reed and Landolt 2000).

1.5. **Pheromone-mediate defense behaviour in vespids**

The term “alarm behaviour” has been used to describe many responses to potential danger, ranging from running to or from the source of danger. As such, there is no one specific response type that solely qualifies as alarm behaviour; however, species-specific experiments to test alarm behaviour have been developed (Hölldobler and Wilson 1990). These experiments have resulted in the observation and documentation of pheromone-mediated alarm behaviour among many Hymenoptera, particularly ants, bees, and eusocial wasps (Hölldobler and Wilson 1990, Landolt et al. 1998, Bruschini et al. 2010).

In vespids, pheromone-mediated alarm behaviour has been well described, although the occurrence of the phenomenon may be patchy (Akre 1982, Landolt et al. 1998, Fortunato et al. 2004). Within the Polistinae subfamily, venom sac content of the following species has been shown to elicit defense behaviour: *Polistes canadensis* (L.) (Jeanne, 1982), *P. exclamans* Viereck and *P. fuscatus* (F.) (Post et al. 1984), *P. nimphus* (Chirst), *P. dominulus*, and *P. gallicus* L. (Bruschini et al. 2006, 2008), *Polybia occidentalis* Olivier (Jeanne 1981), *P. rejecta* (F.) (Overal et al. 1981), *Ropalidia romandi* (Le Guillou) (Kojima 1994), *R. opifex* Vecht, three species in the *R. flavopicta* (Smith) group (Fortunato et al. 2004), and *Polybioides raphigastra* (de Saussure) (Sledge et al. 1999).

The spiroacetal (*E,E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane has been isolated from venom sacs of several *Polybia* species but it has not been tested consistently for bioactivity. The spiroacetal makes workers of *Polybia occidentalis* (Olivier) gather outside the nest, which is deemed evidence for pheromonal activity of this compound (Dani et al. 2000). The same compound occurs in venom sacs of the European paper wasp *Polistes dominulus* and the hover wasps *Parischnogaster mellyi* (de Saussure) and *P. jacobsoni* (du Buysson) (Dani et al. 1998, Bruschini et al. 2006) but pheromonal activity has not yet been demonstrated in these species.

Other spiroacetals have been isolated from species across all sub-families of the Vespidae. (*E*,*E*)-2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane, (*E*,*E*)-7-ethyl-2-methyl-1,6-dioxaspiro[4.5]decane, (*Z*,*E*)-2-ethyl-7-methyl-1,6-dioxapsiro[4.5]decane, and (*E*,*Z*)-7-

ethyl-2-methyl-1,6-dioxaspiro[4.5]decane have been reported in venom sacs of the hover wasps *Parischnogaster mellyi* and *P. jacobsoni* (Dani et al. 1998). In *Paravesulpa vulgaris* (=*Vespula vulgaris* (L.)), *P. germanica* (=*Vespula germanica* (F.)), and *Dolichovespula saxonica* (F.) (Francke et al. 1978,1979), these spiroacetals, along with other alkyl-1-6-dioxaspiro[4.5]decanes, have been shown not to trigger alarm behaviour, as observed in polistine wasps, but to have repellent or aggression-inhibiting effects.

Within eusocial wasps, pheromone-mediated defense behaviour was first reported in vespines (Maschwitz 1964a,b), where venom sacs are the main source of pheromone. Since then, pheromone-mediated defense behaviour has been described for nine species in four genera of vespines. In three of these species, single- or multiple-component blends elicit alarm behaviour (Table 1).

N-3-Methylbutylacetamide (MBA) is a major alarm pheromone component of at least two species of yellowjackets (Table 1; Landolt et al. 1995). It has been isolated from venom sacs of all polistine and most vespine wasps studied thus far, except for any of the V*espa spp.* L. (Saslavaski et al. 1973, Veith et al. 1984, Heath and Landolt 1988, Landolt et al. 1995, Dani et al. 2000, Bruschini et al. 2006).

The relative proportion of both MBA and (*E,E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane is cast-specific in *P. dominulus.* Foundresses have more MBA than workers, and workers - in turn- have more (*E,E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane than foundresses (Bruschini et al. 2008). Moreover, nests of *P. dominulus* are most likely to display alarm behaviour when presented with venom sac extract of workers, which contains more of the spiroacetal than venom sac extract of foundresses (Bruschini et al. 2008). This phenomenon exemplifies the complexity of the super-organism, reflected in queen-worker phenotypic differences such as division of labour mediated by pheromones.

Spiroacetals seem to trigger alarm behaviour in some polistines but to suppress it in some vespines (Francke et al. 1979). Similarly, MBA triggers alarm behaviour in vespines, but in polistines it appears to play a role in intra- and interspecific interactions (Bruschini et al. 2008, Landolt et al. 1995).There are other volatile components in venom sacs of social wasps that may interact with alarm pheromones, creating a complex

matrix of messages that are context-dependent, as shown for *P. fuscatus* and *P. exclamans* where venom sacs are also the source of a sex pheromone (Post and Jeanne 1983, Post et al. 1984).

The alarm behaviour of vespines has been studied both qualitatively (see above and Table 1) and quantitatively. Qualitative studies have ascribed an intrinsic alarm-inducing capacity to venom sac extracts and, in some cases, to a few specific compounds therein. Quantitative studies have measured the intensity of alarm responses as a function of several factors such as dose of alarm pheromone or social state of the test individual(s). In quantitative studies with *Vespa crabro*, *Paravespula vulgaris* (=*Vespula vulgaris*), *Dolichovespula media* Retzius, and *Dolichovespula saxonica,* Moritz and Bürgin (1987) measured changes in CO_2 concentration as a proxy for metabolic rate in response to triggers of defense behaviour and in relation to the group size of wasps. Contrary to honeybees, metabolic rates of wasps exposed to alarm pheromone decreased with increasing group size (Moritz and Bürgin 1987). This effect was less obvious in wasps with heavier body weights. A potential explanation for this phenomenon is that other pheromones may alter the wasps' behavior, enhancing or attenuating the alarm response, as previously discussed (Francke et al. 1979). A caveat of conducting these types of metabolic studies is that insects may respond to a vast array of compounds; in the absence of a clearly defined stereotyped alarm response, it is difficult to quantify the alarm-inducing capacity of a compound on the basis of increased metabolic rate of wasps (Moritz and Bürgin 1987).

Within vespines, alarm-inducing chemicals identified thus far are volatile molecules of five to 10 carbon atoms, except for some long-chain alkanes reported in the oriental hornet, *Vespa orientalis* L., which have minimal alarm-inducing capacity (Saslavaski et al. 1973). In *V. orientalis,* a set of ketones has the greatest alarm-inducing effect but these ketones were not identified in venom sac extracts. As the long-chain alkanes had minimal alarm-inducing effects, they do not challenge early predictions on the nature and size of alarm pheromones (Wilson 1965, Saslavaski at al. 1973, Landolt et al. 1998, Bruschini et al. 2010). Discounting spiroacetals, higher-molecular-weight and thus less volatile components in venom sacs may mark perpetrators or enhance the effect of alarm-inducing compounds. This concept is exemplified in European

honeybees, *Apis mellifera* L., where (Z)-11-eicosen-1-ol enhances the effect of the volatile alarm pheromone component isopentyl acetate (Boch et al. 1962, Pickett et al. 1982). The less volatile components may help direct and coordinate a defense, as observed in the red ant, *Solenopsis saevissima* (Smith), where a combination of trail and alarm pheromone components first alarms and then recruits defending ants to the source of danger (Wilson 1965).

1.6. **Inter-specific roles of alarm pheromones**

Cross recognition of alarm pheromones between wasp species has been noted repeatedly. Southern yellowjackets, *Vespula squamosa*, are facultative social parasites of the eastern yellowjacket, *V. maculifrons*, and display alarm behaviour when exposed to venom extract of their host (Landolt et al. 1995). This response is partially explained by the presence of MBA, the alarm pheromone of southern yellowjackets, in the venom sac of both species (Heath and Landolt 1988; Landolt et al. 1995). However, MBA at biologically relevant amounts does not elicit alarm behaviour of eastern yellowjackets.

The occurrence of the same alarm pheromone components in several species could be due to a limited number of compounds with characteristics that meet the requirements of an alarm pheromone, such as low molecular weight and high volatility (Wilson 1965). Alternatively, southern yellowjackets as social parasites of eastern yellowjackets might benefit from detecting alarm signals from their host nest mates (Landolt et al. 1995). Similarly, *Polistes sulcifer* as an obligate social parasite of *Polistes dominulus* exploits chemical signals of its host in that it causes temporary havoc in the nest, which facilitates parasitism by increasing intra-nest conflict during the “invasion” (Bruschini and Cervo 2011). Differences in the chemical profiles of both species include slightly larger amounts of MBA and significantly larger amounts of *N*-3-methylbutylpropanamide (MBP) in the social parasite, and significantly larger amounts of (*E,E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane in the host (Bruschini et al. 2008).

In the *Ropalidia flavopicta* species “complex”, venom sac extract of undescribed species C elicits alarm behaviour in undescribed species A (Fortunato et al. 2004), likely because both species share many constituents in their venom sacs (Fortunato et al.

2004). The same phenomenon may explain cross recognition of venom sac extracts by the social wasps *Polistes exclamas* and *P. fustactus*, although venom sac constituents are yet be analyzed (Post et al. 1984).

Heterogeners nesting in groups could potentially benefit from eavesdropping on alarm pheromones from nearby nests. Working with *Polybia occidentalis* and *Mischocyttarus immarginatus* Richards, London and Jeanne (1996) tested this hypothesis but found no evidence for cross-recognition of alarm pheromones between these genera.

1.7. Exploitation of alarm pheromones as cues for deception

As described in the preceding section, alarm pheromones and alarm behaviour are not in a static evolutionary phase but are, and will continued to be, under significant pressure to adapt to the current ecological context. The evolution of these pheromones could potentially lead to a reversal of their original alarm function. The chemical mimicry of the wasp-pollinated orchid *Dendrobium sinense* Tang & Wang is a perfect example. This orchid disseminates the alarm pheromone component (Z)-11-eicosen-1-ol of the honeybees *Apis mellifera* and *A. cerana* F. as an allomone to attract the bees' hornet predator *Vespa bicolor* F. for pollination purposes (Brodmann et al. 2009).

1-Methylbutyl-3-methylbutanoate plays a role in predator-prey interactions (Ono et al. 2003). The compound is produced by the giant Japanese hornet *Vespa mandarinia* Smith which preys on nests of the hornet *Vespa simillima xanthoptera* Smith. The predatory giant hornet uses this ester to mark prospective prey nests for raiding. Once *V. simillima xanthoptera* hornets detect the kairomone they respond defensively and fight to prevent predation. Ono et al. (1995, 2003) suggest that detection of this kairomone is an adaptation to escape predation.

Alarm pheromones of eusocial wasps do not seem to fall under the red queen hypothesis; however, indirect and tangential evidence (as presented above) warrants the study of rate change for genes associated with the production and detection of alarm

pheromone components of eusocial insects. We would expect to observe a higher rate of change in alarm pheromone genes for those lineages where alarm pheromones are exploited by antagonistic organisms.

Our understanding of the underlying genetic mechanisms for the evolution of eusocial traits is increasing. Not limited to eusocial insects, we know that pheromone blends can be encoded by a single gene or multiple genes (Symonds and Elgar 2008). However, testing for changes in the relative proportion of alleles between sister taxa requires the identification of those genes responsible for the heritable effects described above, which to our knowledge has not yet happened (Smith et al. 2008). The field is young and few examples of gene-controlled production or detection of pheromones have been published; however, much is to be gained by studying the genetic mechanisms of chemical communication (Smith et al. 2008).

1.8. **Natural occurrence of vespid alarm pheromone components in other systems**

N-3-Methylbutylacetamide (MBA), an alarm pheromone component of *V. squamosa* and *V. maculifrons,* has been described as a component of alarm and sex pheromones in other organisms. In the desert cockroach, *Therea petiveriana* (L.), MBA functions as an alarm pheromone (Farine et al. 2002). In males of the tephritid fruit flies *Dacus tryoni* (=*Bactrocera tryoni* (Froggatt)) and *D. neohumeralis* (=*Bactrocera neohumeralis* (Hardy), MBA along with MBP serves as a major sex pheromone component (Bellas and Fletcher 1979).

2-Methyl-3-buten-2-ol (MBO), the alarm pheromone of the European hornet, *Vespa crabro* L., has been reported as an aggregation pheromone of several bark beetles including *Ips erosus* Wood & Bright, *Ips typographus* Wood & Nright, *Ips nitidus* Eggers, *Ips shangrila* Cognato & Sun, and *Pteleobius vittatus* (F.) (Giesen et al. 1984, Schlyter et al. 1987, Klimetzek et al. 1989, Zhang et al. 2009a,b). MBO is also described as a sex pheromone component of the triatomine bug *Rhodnius* prolixus Stål (Pontes et al. 2008). Pentan-2-ol and 3-methylbutyl-1-methybutanoate, alarm pheromone components of the giant Japanese hornet, have been reported as pheromones in five

and two insect taxa, respectively, whereas 3-methylbutanol, also an alarm pheromone component of the giant Japanese hornet, is a pheromone component in at least nine species, ranging from assassin bugs to Bengal tigers (www.pherobase.com).

MBA and MBP as well as other volatiles in the venom sacs of Japanese hornets also occur in volatile blends of sherry, tobacco, wines and other food and cosmetic products (Farine et al. 2002, Ono et al. 2003). Most of these compounds, such as acetates and C5 alcohols, are present in volatile blends of fermented products, which explains previous descriptions of venom sac scent as being “redolent of fermenting wine” (Maschwitz 1964a, Aldiss 1983). This observation, coupled with the recent discoveries that eusocial wasps vector brewer’s yeasts (Stefanini et al. 2012) and respond to volatiles of both brewer’s yeast and epiphytic fungi (Davis et al. 2012, Vásquez et al. 2012, Brown et al. 2014, Olofsson et al. 2014), support the concept of an interwoven evolutionary natural history between insects and microorganisms. Indeed, one might wonder to what extent these microorganisms could mediate vespine behaviour.

1.9. **Lack of evidence is not evidence of lack**

Reports of pheromone-mediated defense behaviour in all four vespine genera imply widespread occurrence of alarm pheromones within the taxon. Within the genus *Vespula,* however, there is no such evidence for several species including *V. pensylvanica* (de Saussure), *V. atropilosa* (Sladen) and *V. vulgaris* (=*V. alascensis* (L.)) (Akre 1982).

Experimental data do not support the occurrence of pheromone-mediated alarm behaviour in the independent-founding polistine wasps *Belonogaster petiolata* (De Geer) and *Mischocyttarus immarginatus* (Keeping 1995, London and Jeanne 1996). Observations suggest further that also the swarm-founding polistine wasps *Pseudochartergus fuscatus* (=*Protopolybia fuscatus* (Fox)) and *Stelopolybia testacea* (=*Polybia emaciata* Lucas) (Jeanne 1970) exhibit no pheromone-mediated alarm behaviour. This may also apply to *Parachartergus colobopterus* (Licht.) whose workers defend the nest by bending the gaster forward and spraying venom towards moving

objects (Jeanne and Keeping 1995). The Stenogastrinae as the most primitive of social wasps appear to lack alarm pheromones or alarm behaviour, based on behavioural experiments (Dani et al. 1998).

Even if pheromone-based defense behaviour could not be demonstrated in a particular species, this type of behaviour may still exist. Some type of bioassays simply failed to reveal defense behaviour in some species now known to display pheromone-based nest defense (Freisling 1943, Maschwitz 1964a, Batra 1980, Bruschini et al. 2010). This apparent controversy emphasizes the need for designing species-specific bioassays that take life history traits of the target species into account (Hölldobler and Wilson 1990, Reed and Landolt 2000). Of particular interest in this context are *Vespula* congeners of underground-nesting yellowjackets where substrate vibrations may function as a cue for danger (Figure 1) and where alarm pheromones may play only a minor role in inducing defense behaviour (Akre 1982). If so, these phenomena may point to either the evolution or loss of alarm pheromone systems in multiple lineages (Landolt et al. 1998).

Polistines build an envelope around their nest and thus are thought to be less reliant on alarm pheromones (Landolt et al. 1998), which would explain the lack of alarm pheromones in *Ropalidia sumatrae* (Webe) (Fortunato et al. 2004). However, this train of thought does not hold true for *Polistes* spp that lack a nest envelope but exhibit pheromone-mediated alarm behaviour (Bruschini et al. 2010).

Direct observation of pheromone release by eusocial wasps are missing (Landolt et al. 1998; Bruschini et al. 2010). It has been hypothesized that wasps spray targets with the content of their venom sacs including the alarm pheromones; however, heretofore there are no empirical data on how these described alarm pheromones are being released (Landolt et al. 1998). The alarm signalling process itself remains unknown, although Reed and Landolt (2000) could demonstrate that wasps applied “something” to corks that then elicited alarm behaviour; however, the previously reported alarm pheromones could not be extracted from these corks. Data most strongly supporting a pheromone-release phenomenon were collected with *Polybia occidentalis* (Landolt et al. 1998). When the researchers exhaled onto a wasp, they observed her

alarm response spreading away and being repeated by all workers within 1.5 s, the explanation being that the first wasp emitted an alarm signal that was sensed by her neighbour that then signalled herself, causing a chain reaction (Landolt et al. 1998).

1.10. Overlap of humans and wasps: nuisance and invasive species

The nesting and feeding habits of yellowjackets and hornets have often brought them into close contact with humans, which usually had adverse effects on both parties (Day and Jeanne 2001, Ono et al. 2003, D'Adamo and Lozada 2005). Mostly because of their sting, yellowjackets and hornets are considered a nuisance all around the world (Day and Jeanne 2001). Between the physiological consequences of venom (hyper)sensitivity and the psychological aspects associated with insect venom, the latter ultimately affects the lives of most people (Schmidt 1986b).

With recent inadvertent introductions of exotic wasps into new ecosystems, the conflict with humans has been exacerbated (D'Adamo et al. 2004). The introduction of *Vespula vulgaris* and *V. germanica* to New Zealand has been extremely detrimental and caused a major disruption of native forest ecosystems (Brown et al. 2014). To minimize the risk associated with wasp-human overlap, including mitigating the ecological effects of invasive wasps, a better understanding of vespine communication systems is necessary (Wilson 1965, Reed and Landolt 2000, Brown et al. 2014).

Studying the chemical ecology of eusocial wasps allows us to develop improved baits for monitoring and potentially managing pestiferous wasp populations (Landolt 1998). Furthermore, it allows us to understand evolutionary relationships using chemotaxonomy by comparing phenotypic traits like volatiles in venom sacs (Bruschini et al. 2007). This could prove helpful in comparative studies on the origin of social behaviour in other taxa.

1.11. **Conclusion**

Alarm pheromones of vespids have been studied for more than 50 years. In the process, we have learned that worker wasps protect the super-organism, and we have become interested in the proximate and ultimate causes of nest defense, from their evolutionary history to their physiological and psychological effects on victims. Early predictions on the nature of alarm pheromone components still stand true today; however, we now know that single components are not silver bullets in nest defense. They interact with other components in venom sacs, enhancing defense behaviour in some systems and suppressing aggression in others. It is not known yet how individuals process these potentially conflicting messages that allow them to distinguish friend from foe. Furthermore, pheromone-mediated nest defense was once thought to be a universal behaviour, at least within vespines, but is now understood it to be a patchy phenomenon. Hypotheses have been presented to explain the lack of nest defense in some taxa, but have yet to be tested in representative species. Observing the effects of pheromones within and between species has allowed investigators to elucidate the complex evolutionary history between social parasites and their hosts. Interactions between heterogeners have been recorded, and beautiful examples of eavesdropping and the evolution of kairomones and allomones have been showcased. We begin to grasp the complexity of alarm pheromones and their place in the natural history of eusocial organisms, including the role of these pheromones in the evolution of eusociality and their potential relation in inter-kingdom communication. Newly acquired knowledge will enhance our ability to mitigate the ecological impact of invasive wasps in integrated wasp management programs.

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1.12. **References**

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Table 1.1. List of species with documented pheromone-mediated alarm behaviour, describing the pheromone source, the component(s) identified, and the bioassay used for testing defense behaviour.

Species	Pheromone components[1]	Type of bioassay	Ref.[2]
Vespa crabro	2-methyl-3-buten-2-ol	Filter papers that were impregnated with treatment stimuli (venom sac extracts or synthetic candidate pheromone component) or with a solvent control stimulus were presented sequentially (by random assignment) to a single lab colony. Numbers of hornets buzzing, flying defensively, and rushing out of the nest in response to test stimuli were recorded.	(1)
Vespa mandarinia	pentan-2-ol; 1-methylbutyl-3-methylbutanoate; 3-methyl-1-butanol	Filter papers that were impregnated with treatment stimuli (venom sac extracts or synthetic candidate pheromone components) or with a solvent control stimulus were placed sequentially (by random assignment) in front of a single feral nest. Numbers of hornets rushing out of the nest in response to test stimuli were recorded.	(2)
Vespa orientalis	N/A	"Sentry" venom droplets excreted in the presence of danger were observed.[3] Venom sac extract or synthetic compounds (artificial ketones and naturally occurring C10-14 alkanes) were presented to queen-less and queen-right nests, and to individual hornets, recording their responses.[4]	(3,4)
Provespa anomala	N/A	Squashed venom sacs were presented to a single feral nest after a "light physical disturbance" of the nest. In random sequential tests, the number of wasps attempting to attack a stick impregnated with squashed venom sacs or a control (lemon juice) were counted.	(5)
Dolichovespula saxonica	N/A	Squashed workers or body parts of workers were mounted on a stick (treatment stimulus). In sequential tests, the treatment stick or an empty or citrus oil-covered control stick were presented to a single feral nest and numbers of workers responding to treatment or control sticks were recorded	(6)

Vespula squamosa	*N*-3-methylbutylacetamide	In sequential tests, captures of wasps in traps that were unbaited or baited with venom sac extract[7], synthetic *N*-3-methylbutylacetamide[8], or head extracts[9] and that were placed at a distance of 1 m[9] or 2 m[7,8] from one[7], three[8] or four[9] feral nests were recorded.	(7,8,9)
Vespula maculifrons	*N*-3-methylbutylacetamide	Filter paper was impregnated with the treatment stimulus (venom sac extract or synthetic candidate pheromone components) or with a solvent control stimulus. By random sequential assignment, the test stimulus was then placed on an adhesive-coated black sphere at 1-m distance from 2 feral nests, counting wasp captures on spheres. The same bioassay was repeated with synthetic *N*-3-methylbutylacetamide as the treatment stimulus, testing the response of one feral nest.	(10)
Vespula germanica	N/A	A worker wasp was held with forceps, lightly squeezing her at the nest entrance, and counting the number of wasps coming out of the nest. The nest's response was compared to a forceps alone stimulus.	(11)
Vespula vulgaris	N/A	A worker wasp was held with forceps, lightly squeezing her at nest entrance, and counting the number of wasps coming out of the nest. The nest's response was compared to a forceps-alone stimulus[11]. In two-choice bioassays, nests were presented with two stimuli. The treatment stimulus consisted of whole crushed wasps, gasters, venom apparatus, whole venom sacs, crushed venom sacs or venom sac extracts, and the control stimulus was "nothing" or the equivalent amount of solvent. Each treatment was applied to a cotton roll suspended by a thread from a side arm of a T-shaped apparatus, placed in front of nests. Wasps coming out of the nest and examining and hitting the rolls were counted. The proportion of hits on the treatment roll was compared per test[12].	(11,12)

[1]References: (1) Veith et al. 1984; (2) Ono et al. 2003; (3) Ishay et al. 1967; (4) Saslavaski et al. 1973; (5) Maschwitz & Hanel 1988; (6) Maschwitz 1984; (7) Landolt & Heath, 1987; (8) Heath & Landolt, 1988; (9) Landolt et al. 1999; (10) Landolt et al. 1995; (11) Maschwitz 1964a; (12) Aldiss 1983

Figure 1.1. **Nest of *Vespula squamosa* before (a) and after (b) physical disturbance by substrate vibrations.**

Chapter 2.

Assessing a method for rearing North American yellowjackets[1]

[1]The corresponding manuscript has been submitted for peer review to the Journal of the Entomological Society of British Columbia, with the following authors: Sebastian Ibarra Jimenez, Nathan T. Derstine, and Gerhard Gries

2.1. Abstract

Studying yellowjackets is challenging due to their cryptic nesting behaviour, short field season, and extreme variation in population density. Developing or perfecting techniques for rearing yellowjackets would greatly increase the opportunity of studying the communication ecology of yellowjackets and the evolution of eusociality in the Hymenoptera. Our objective was to assess a method for rearing the five *Vespula* congeners *V. acadica* (Sladen), *V. alascensis* Packard, *V. atropilosa* (Sladen), *V. germanica* (F.), and *V. pensylvanica* (de Saussure). In early spring of 2014, we collected queens of each of the five species from the field and placed them singly in a plywood nest box connected to a mesh cylinder that served as a foraging arena and provided constant access to water and food (honey, live flies, and live caterpillars). For each queen, we recorded nest initiation, the attachment site of the nest pedicel, and the stage of nest development at the end of the experiment, 9 weeks after the last collection date of queens. Queens of *V. germanica* (n=18), *V. alascensis* (n=11), *V. acadica* (n=4), *V. pensylanica* (n=23) and *V. atropilosa* (n=11) had nest inititation rates of 61%, 50%, 25%, 17%, and 0%, respectively. The mean number of nest cells built by queens of *V. germanica*, *V. alascensis*, *V. acadica*, *V. pensylvanica*, and *V. atroplisoa* were 21.6 ±

4.6, 17.8 ± 6.3, 8.0, 26.5 ± 8.3, and 0, respectively. Two *V. germanica* queens and one *V. pensylvanica* queens established nests that produced a few worker wasps. While our rearing method compares favorably to, and in some aspects improves, previous rearing methods, further refinements are needed to generate the large numbers of wasp workers that are essential for experimental testing of hypotheses pertinent to life history traits of yellowjackets.

Key Words: Yellowjacket, *Vespula,* rearing, nesting

2.2. **Introduction**

Yellowjackets and hornets are intensely studied because they can be (*i*) invasive and pestiferous species in many ecosystems (Landolt 1998, D'Adamo et al. 2001, Day and Jeanne 2001, Landolt et al. 2005, Brown 2014), (*ii*) potential biological control agents (Hoffmann et al. 2000), (*iii*) vectors of microorganisms (Davis et al. 2012, Stefanini et al. 2012), and (*iv*) threats to citizens with venom (hyper)sensitivity (Nakajima 1986, Schmidt 1986, Ono et al. 2003). Furthermore, wasps are model organisms for studying the evolution of eusociality (Landolt et al. 1998) and chemotaxonomy (Bruschini et al. 2007). However, studies of wasps are challenging due to a short field season, extreme variation in wasp population densities, and the often cryptic nesting behaviour of wasps (Edwards 1980).

There are seven accounts of establishing vespine nests in the laboratory. Ishay et al. (1967) reared *Vespa orientalis* L. with field-collected nests and overwintered gynes in their "Vespiaries", but did not comment on the success rate of either method. Ross et al. (1981) attempted to rear nests of five *Vespula* species under laboratory conditions, and recorded the percent of nest initiation for each of these species. None of the nests developed beyond the emergence of the first workers. Following up on the work by Ross et al. (1981), Matthews et al. (1982) reared 14 nests of *V. maculifrons*, five nests of *V. germanica*, one nest of *V. vulgaris* (=*V. alascensis*), and one nest of *V. vidua* under environmentally controlled conditions. All of these nests progressed to producing at least two queen larvae. Using the method by Ross et al. (1981), Ross (1983) reared and studied queen foraging behaviour of *V. germanica*, *V. vulgaris* (=*V. alascensis*) and *V.*

maculifrons, using 3-5 nests of each species. Vetter and Visscher (1995) successfully reared nests of *V. pensylvanica* (de Saussure) from field-collected gynes, reporting the first and only account of laboratory-reared *Vespula* nests from spring-captured queens through to males and gynes. In New Zealand, Leathwick (1997) reared one *V. germanica* nest and two *V. vulgaris* nests which produced workers. Finally, Hoffmann et al. (2000) mated gynes and males from two feral *Vespa crabro* L. nests and over-wintered five mated gynes, of which one established a nest that produced next-generation gynes.

Both transplanting feral nests into research areas and *in situ* observations and experimentation are means of studying eusocial wasps (Spradbery 1973, Edwards 1980, Akre et al.1980, Akre 1982) and advancing our understanding of their ecology. However, transplanting feral nests, particularly those of underground-nesting species such as *V. alascensis*, V. pensylvanica, V. germanica and alike, can damage the brood comb and nest envelope, impact the behaviour of nest mates, and lead to the loss of queens (Vetter and Visscher 1995).

A consistent supply of wasp nests would greatly benefit the study of vespine ecology, particularly the biology and ecology of the nest as a super-organism (Wilson 1971, Moritz and Bürgin 1987). This is most obvious in studies of alarm pheromone systems among social wasps, where the presence of the nest is essential to observe nest defense behavior. Of the nine species of yellowjackets and hornets that reportedly use alarm pheromones, pheromone components have been identified for only three species (Maschwitz 1964a,b; Saslvasky et al. 1973, Veith et al. 1984, Maschwitz 1984, Maschwitz and Hanel 1988, Heath and Landolt 1988, Landolt et al. 1995, Landolt et al. 1999, Ono et al. 2003), and the pheromone effect has often been tested with only a single nest.

Our objective was to assess a method for rearing *Vespula* congeners targeting for diversity *V. acadica* (Sladen), *V. alascensis* Packard, *V. atropilosa* (Sladen), *V. germanica* (F.), and *V. pensylvanica* (de Saussure).

2.3. **Methods and materials**

2.3.1. Collection of queens

We sweep-netted queens in the Greater Vancouver Area and Lillooet, both British Columbia (BC), during sunny clear days between 10:00 and 16:00 hours, capturing most queens while they were prey-hunting or collecting nectar from English hedge laurel, *Prunus lauresianus*. Between 20 March and 15 May 2014, we collected a total of 66 yellowjacket queens [*V. acadica* (4), *V. alascensis* (10), *V. atropilosa* (11), *V. germanica* (18), and *V. pensylvanica* (23) (Table 1)], 55 of which in Vancouver, and 11 of which during a 2-day trip (13-15 May) to Lillooet. We immediately placed captured queens singly into glass jars (0.3-0.5 L) containing foliage of *P. lauresianus* or Western-red cedar, *Thuja plicata*, on which they commonly rest. Whenever possible, we kept jars in a cool and dark area for <2 h before placing them in rearing units (see below) that we kept inside a fenced area of SFU's insectary annex. This approach minimized the queens' stress of confinement.

2.3.2. Rearing units

Nest-rearing units resembled those described by Ross et al. (1981) but had several modifications (Figure 1). Each unit consisted of a plywood box nesting cavity (15 cm high × 15 cm wide × 30 cm long) with one side panel hinged for periodic observations, a few twigs (surrogate roots) hot-glued to the roof of the box as potential sites for nest pedicel attachment, and a 2.5-cm hole in the top (dorsal) panel of the nest box. The dorsal hole provided entry into a mesh screen cylinder (15 cm diam × 20 cm tall), the top and bottom of which was hot-glued to a Petri dish (15 cm diam) for rigidity and stability. A hole (5 cm diam) in the bottom Petri dish of the cylinder corresponded with the dorsal hole of the nest box, allowing the foundress and potential workers to exit the box and to enter the mesh cylinder for foraging. The top Petri dish of the cylinder had one hole (3 cm diam) to accommodate an inverted 50-mL falcon tube with a cotton-filled pipet tip containing the water supply, and a second hole (2 cm diam) that was plugged with a cork or rubber stopper and allowed intermittent insertion of live flies and cabbage

looper larvae (see below) as food sources. The top of the falcon tube was cut off to replenish water as it was consumed or evaporated (Figure 1).

Some of the most vigorous nests of *V. vulgaris* and *V. germanica* we observed in the field were built in straw composts, as previously reported (Spradbery 1971). Therefore, we lightly packed the 6.5-L nest box with (untreated) organic animal bedding straw for insulation. We supplied the mesh cylinder with decaying wood and filter paper to encourage pulp gathering for nesting material.

To prevent predation by ants, we placed each rearing unit on a brick in a water-filled tray on a table about 1 m above ground in a south-facing, rain-sheltered area.

2.3.3. Food provisioning of queens

Starting on the day of capture, we fed each queen daily with (*i*) honey and/or corn syrup (Akre 1976, Ross et al. 1981) that we smeared on the mesh cylinder of the rearing unit, (*ii*) 3-5 common house flies, *Musca domestica* L., or the bottle flies *Lucilia sericata* (Meigen) or *Phormia regina* (Meigen) Akre 1976), and (*iii*) 5-10 2nd or 3rd instar larvae of the cabbage looper, *Trichoplusia ni* (Hübner), the latter also used as prey for yellowjackets by Vetter and Visscher (1995), although these were rarely consumed in our study. To reduce disturbance of nesting behaviour, we checked for nest initiation only once per week, always when the queen was foraging in the mesh cylinder.

2.3.4. Statistical analyses of data

We analysed all data with the statistical software R (version 3.1.3). We used a Pearson's X^2 test, binomial exact test, or Fisher's exact test (FET) depending on the data constrains and the specific hypothesis, to test for a difference in (*i*) nest initiation rate between species (X^2 test of independence or FET), (*ii*) site of pedicel attachment between species (FET), and (*iii*) for a deviation from a 50/50 chance of pedicel attachment to the twig or nest box roof for each species (binomial exact test or X^2 goodness of fit test), addressing the question whether queens have an innate preference for root-like substrates to attach the nest pedicel. We performed an ANOVA

to test for differences in the mean number of cells built by queens of the five species we studied.

2.4. **Results**

2.4.1. Percent nest initiation

There was a significant difference in the proportion of queens between the five species that initiated a nest (p=0.0013, FET; Table 1). Queens of all five species, except *V. atropilosa,* initiated a nest. Queens of *V. germanica, V. alascensis, V. acadica* and *V. pensylvanica* had nest inititation rates of 61%, 50%, 25% and 17%, respectively. The effect of collection date on nest initiation could not be tested statistically because dates could not be assigned to those wasps that failed to iinitiate a nest. Of the queens we had captured on 15, 17, and 29 April 2014 (57% of the total), six, three, and three, respectively, initiated a nest.

2.4.2. Attachment site of nest pedicel

There was no significant difference in the proportion of queens that attached the nest pedicel to a twig or the roof of the nest box (Figure 2a,b,c) (p=0.77, FET), between the four species which initiated a nest (Table 1). Of the nest-initiating queens, three of four *V. pensylvanica* queens, three of five *V. alascencis* queens, and five of 11 *V. germanica* queens attached the nest pedicel to a twig. The single nest-initiating *V. acadica* queen did the same.

Within each of the three species (*V. alascensis, V. germanica, V. pensylvanica*) where more than one queen initated a nest, there was no significant deviation from a 50/50 chance in the proportion of queens that attached the nest pedicel to a twig or the roof of the nest box [*V. alscensis*: binomial exact test, p = 1.0; *V. germanica*: X^2 (1, N = 11) = 0.09, p = 0.76; *V. pensylvanica*: binomial test, p = 0.63].

2.4.3. Cells built by queens

Between the four species of queens that initiated nests, the mean number of cells they had built (*V. acadica*: 8.0; *V. alascensis*: 17.8 ± 6.3; *V. germanica*: 21.6 ± 4.6; *V. pensylvanica*: 26.5 ± 8.3; Table 1) did not differ at the time we terminated the study [$F_{(3,17)} = 0.502$, $p = 0.686$]. One queen each of *V. alscensis* and *V. germanica* constructed only the nest pedicel and quickly abandonded further attempts of nest building.

2.4.4. Workers produced

Of the 66 queens in our study, two *V. germanica* queens maintained a nest that produced two and six worker wasps, respectively, and one *V. pensylvanica* queen produced a nest from which one worker emerged.

2.5. Discussion

The differences in nest initiation rates that we observed between queens of *V. acadica*, *V. alascensis*, *V. atropilosa*, *V. germanica*, and *V. pensylvanica* (Table 1) reflect the ecological diversity of the genus *Vespula* (Akre et al. 1980, MacDonald et al. 1980, Akre 1982, Macdonald and Matthews 1984, Landolt et al. 1998).

In Pullman (Washington, USA), queens of *V. atropilosa* begin nesting on average 10 days earlier than queens of *V. pensylvanica* (Akre et al. 1976). Nests of *V. atropilosa* also decline one month earlier than the nests of most, if not all, members of the *Vespula vulgaris* group (Akre et al. 1976). We captured all queens of *V. atropilosa,* which invariably failed to establish nests (Table 1), in late spring (May 15th), possibly at a time when these queens could have had established a nest already or could have been tending a nest at an embryo stage, thereby resulting in no (repeated) nest initiation attempts in our study. However, rearing of *V. atropilosa* nests from over-wintered field-collected queen has never been attempted before and we may have simply failed to provide one or more essential requisites for successful nesting. Therefore, it remains inconclusive whether *V. atropilosa* queens cannot be reared using the method described here or whether we simply captured *V. atropilosa* queens too late in the season.

We report the first account of nest initiation in a nest box for *V. acadica* and the second account of nest initiation for a member of the *V. rufa* group, the first account being *V. vidua* (Ross et al. 1981, Matthews et al. 1982, Ross 1983). In our study, the nest initiated by one of four *V. acadica* queens stood out from all other *Vespula* nests in that the queen incorporated prey body parts in the nest (Figure 2a). Whether this is typical for *V. acadica* queens will become apparent in further rearing studies or careful inspections of feral nests.

Nest inititation rates of 61% and 50%, respectively, by queens of *V. germanica* and *V. alascensis* (formerly *V. vulgaris*) in our study (Table 1) were twice as high as those previously reported for the same two species (Ross et al. 1981) or for *V. germanica* (Leathwick 1997). Conversely, relatively fewer queens of *V. pensylvanica* initiated nests in our study (Table 1) compared to a previous study (Vetter and Visscher 1995). The underlying mechanisms contributing to this differential rearing success are difficult to determine. Unlike previous studies where rearing units resided indoors with small temperature oscillations and a constant photoperiod (Ross et al. 1981, Vetter and Vischer 1995, Leathwick 1997), we kept our nest boxes outdoors and thus exposed them to seasonal changes in photoperiod and to significant diel and seasonal temperature fluctuations. However, the straw inside the next boxes that we provided as insulation material may have been insufficient to keep *V. pensylvanica* queens warm and to induce more consistent nest building. The relatively high propensity of *V*. *germanica* queens to initiate nests irrespective of rearing conditions might be an intrinsic characteristic of V. *germanica* that may help explain why this wasp is so widely distributed and invasive in North and South America as well as New Zealand (MacDonald et al. 1980, D'Adamo 2001, Brown 2014).

The type of potential attachment sites for nest pedicels does not seem to matter critically, because the same number of queens attached the nest pedicel to the roof of the nest box or to a twig serving as surrogate root in a quasi-subterranean nest cavity. Considering, however, that the surface of roots was much smaller than the surface of nest box roofs, queens may indeed have preferred roots as potential attachment site for nest pedicels. Alternatively, the preference for pedicel attachment sites may vary

between queens. If so, providing diverse and multiple sites for pedestal attachment could help increase rates of nest initiation.

The rate of cell building reflects queen quality and varies with species (Archer 2009). In our study, we could not consistently track nest development such as cells built per day, eggs laid, and number of cells with larvae or pupae, because 35% of those queens that initiated a nest built an envelope surrounding the cells (Figure 2c). Vetter and Visscher (1995) faced the same challenge with one of the four *V. pensylvanica* nests they reared. At the end of our study, however, we did record the number of cells per nest and did not find a significant difference in the mean number of cells built between species (Table 1). Apparently, all but three nesting activities (see below) were discontinued at the same point of brood development, just before the emergence of the first worker wasps that would have continued all tasks except egg laying (Gambino and Loope 1992). Eggs and larvae died from unknown causes. How and why two *V. germanica* and one *V. pensylvanica* queens progressed to producing a few worker wasps (Figures 2d,e) remains unknown. We envision that the well-being of larvae could have been compromised by a lack of nutritional diversity. Conceivably, eusocial wasps self-medicate in that they adjust their diet, or that of their offspring, in response to pathogens, as do caterpillars of *T. ni* and *Grammia incurrupta* (Edwards) (Singer et al. 2009; Shikano and Cory 2014).

2.5.1. Conclusion

Queens of the five *Vespula* species that we attempted to rear in nest boxes differed in nest initiation rates, with *V. germanica* having the greatest success rate. Whether these differences are due to intrinsic characteristics of these species, external factors such as ambient temperature during rearing, or the quality of the queens we had collected in the spring cannot be ascertained. The high propensity of *V. germanica* queens to initiate nests may be a contributing factor to the success of *V. germanica* as one of most pestiferous and invasive wasp species worldwide.

Most nests in our study failed to produce worker wasps. We speculate that these nests succumbed to a pathogen rather than to faulty rearing methodology because all

larvae visible in those nests that ceased to develop started to die within days of each other, and showed similar signs of a fungal infection. We recommend that in future attempts to rear yellowjackets queens are allowed to forage freely as soon as they have initiated nest building. This would enhance the nutritional diversity for larval offspring, provide the essential nutrients at particular times during nest development, and possibly help curtail the effect of pathogens in the food or nest. Irrespective, perfecting techniques for rearing yellowjackets in further studies is well justified because it will greatly increase the opportunity of investigating the role of these intriguing predatory insects in ecosystems and the evolution of eusociality in the Hymenoptera.

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Table 2.1. Numbers of overwintered *Vespula* (*V*) queens field-collected between 20 March and 15 May 2014, the proportion of queens that initiated nests and attached the nest pedicel to a twig in nest box (Figure 2.1c) or the nest box roof, and the mean number of nest cells built by queens that initiated nests.

Species	# Queens collected	# Queens initiating nest	Attachment site of pedicel [a]		Cells built[a] ($\bar{x}$ ± SEM)
			Twig in nest box	Nest box roof	
V. acadica	4	1 / 4 (25%)	1 / 1 (100%)	0 / 1 (0%)	8 – N/A
V. alascensis	10	5 / 10 (50%)	3 / 5 (60%)	2 / 5 (40%)	17.8 ± 6.3
V. atropilosa	11	0 / 11 (0%)	-	-	-
V. germanica	18	11 / 18 (61%)	5 / 11 (45%)	6 / 11 (55%)	21.6 ± 4.6
V. pensylvanica	23	4 / 23 (17%)	3 / 4 (75%)	1 / 4 (25%)	26.5 ± 8.3

[a] Subset of those queens that initiated nests

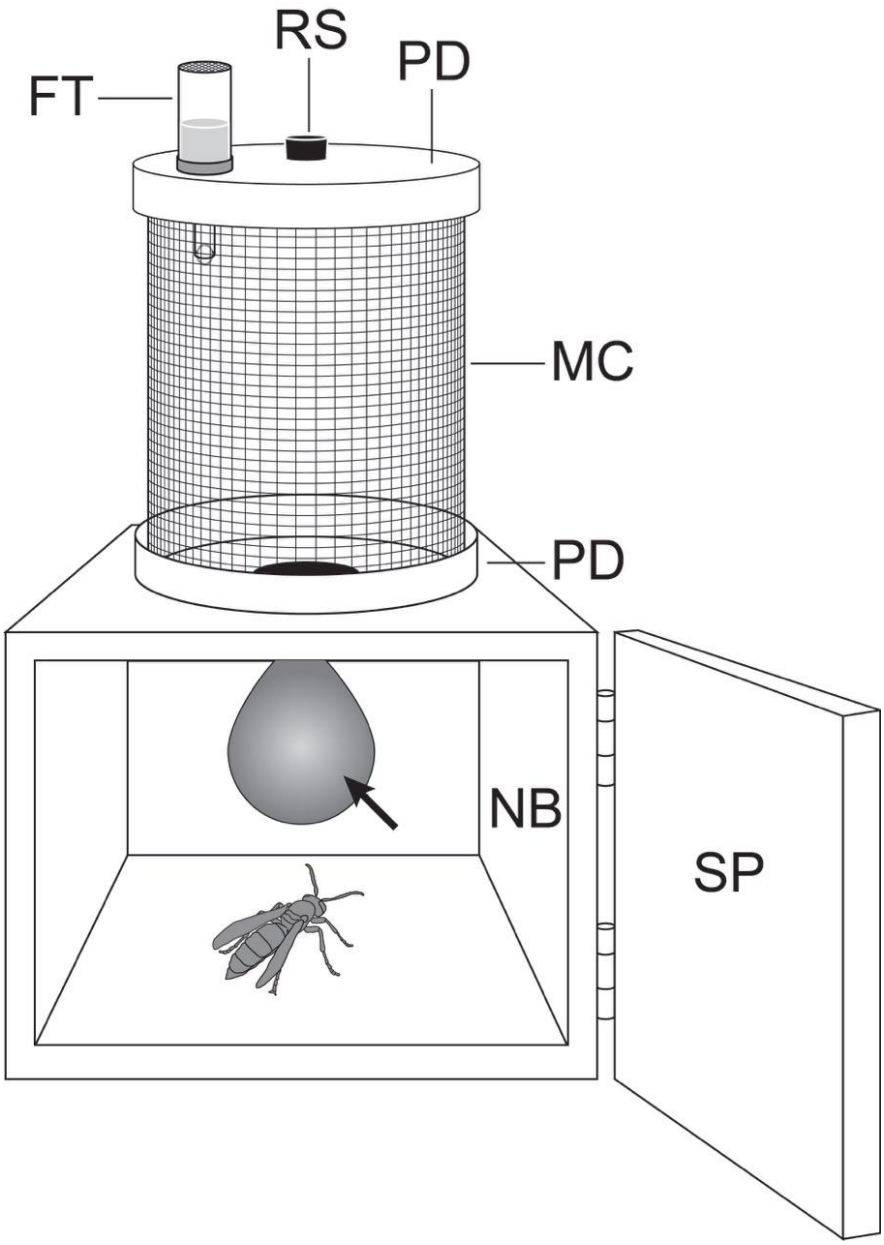

Figure 2.1. **Graphical illustration of a nest box (NB) (15 cm high × 15 cm wide × 30 cm long) with hinged side panel (SP) connected to a mesh-cylinder (MC) foraging arena (15 cm in diam × 20 cm tall), the top and bottom of which reinforced by Petri dishes (PD) for stability and to accommodate a 50-mL falcon tube (FT) with a cotton-filled pipet tip as a water reservoir. The "feeding hole" in the top Petri dish was closed with a rubber stopper (RS) and allowed intermittent insertion of live blow fly and caterpillar prey. The arrow depicts an embryo nest started by the queen.**

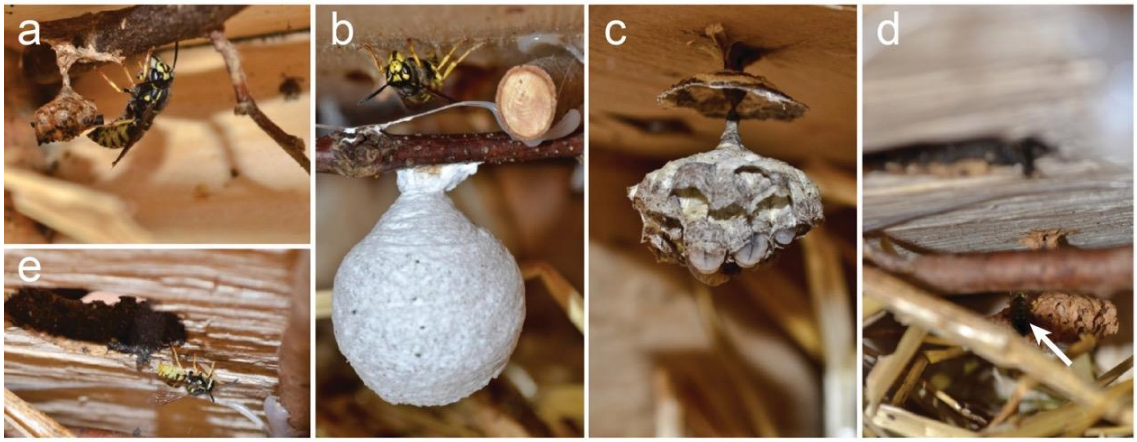

Figure 2.2. **(a) Nest of *Vespula acadica* attached to a twig (surrogate root) in the nest box guarded by the queen (note black pieces of prey incorporated into the nest); (b, c) Embryo nests of *V. germanica* with envelope (b) and of *V. pensylvanica* without envelope (c); (d, e) nest of *V. germanica* with one worker wasp (arrow) tending eggs and larvae (d) and one worker wasp wing fanning at the entrance of the nest box (e).**

Chapter 3.

Evidence for, and identification of, nest defense pheromone components of bald-faced hornets, *Dolichovespula maculata*[1]

[1]The corresponding manuscript will be submitted to a peer-reviewed journal with the following authors: Sebastian Ibarra Jimenez, Regine Gries, Huimin Zhai, Nathan Derstine, Sean McCann, and Gerhard Gries

3.1. Abstract

In eusocial insects like Bald-faced hornets, *Dolichovespula maculata* (L.), nest defense is essential because nests contain a large number of protein-rich larvae and pupae, and thus are attractive to nest predators. Our objectives were (1) to investigate whether *D. maculata* exhibit pheromone-mediated nest defense, and (2) to identify and field test the pheromone components. We tested for pheromone-mediated nest defense behaviour of *D. maculata* by (*i*) placing a paired-box apparatus near the entrance of *D. maculata* nests, (*ii*) treating both boxes with a solvent control (double-control), or, one of the two boxes with a solvent control and the other with either venom sac extract (the putative source of nest defense pheromone) or synthetic pheromone, and (*iii*) by audio-recording for 3 min the sound impulses caused by nest mates attempting to sting or strike the boxes. Compared to the double-control treatment, the number of strikes increased 27-fold when one of the two boxes was treated with venom sac extract, providing evidence for an alarm response. The box treated with venom sac extract also induced a significantly greater proportion of strikes than the corresponding control box, providing evidence for a target-oriented response. Analyzing venom sac extract by gas

chromatographic-electroantennographic detection (GC-EAD) and GC-mass spectrometry (MS) resulted in the identification of seven candidate pheromone components: (**a**) dimethylaminoethanol, (**b**) dimethylamino ethyl acetate, (**c**) 2,5-dimethylpyrazine, (**d**) *N*-3-methylbutylacetamide, (**e**) 2-heptadecanone, (**f**) (*Z*)-8-heptadecen-2-one, and (**g**) (*Z*)-10-nonadecen-2-one. Testing in paired-box bioassays blends of the nitrogen-containing volatile components **a**-**d,** the less volatile ketones **e**-**g,** or both (**a-g**), indicated that **a-d** primarily have an alarm function. The ketones **e**-**g,** in contrast, induced target-oriented responses, possibly marking potential nest predators for guided and concerted attacks, or enhancing the alarm-inducing effect of the volatile pheromone components, as shown in honey bees. Comparing the behavioural effects of venom sac extract, blends **a**-**d, e**-**g,** and **a-g**, venom sac extract was most effective in triggering the full complement of alarm and target-oriented response modes. These comparisons further revealed that a synthetic component is missing in the group of components that triggers the alarm rather than the target-oriented response.

3.2. **Introduction**

The bald-faced hornet, *Dolichovespula maculata* (L.), is a common Nearctic member of the vespine subfamily (Akre et al. 1980). Bald-faced hornets are conspicuous due to their large body size and black and white coloration. Their grey conical nests can reach an impressive size (up 35 cm wide × 60 cm long), contain 1,500-3,500 cells, and may be tended by several hundred workers (Balduf 1954; Akre et al.1980; Akre and Myhre 1992; Carpenter and Kojima 1997; Archer 2006).

The nests of eusocial insects, such as *D. maculata,* are described as super-organisms with emerging behavioural traits (Smith et al. 2008; Gardner and Grafen 2009; Nowak et al. 2010) including cooperative brood care, reproductive division of labor, and nest defense (Michener 1969; Smith et al. 2008). Nest defense is essential because nests may contain a large number of protein-rich larvae and pupae that attract nest predators (Starr 1985; McCann et al. 2014).

The successful establishment of a nest, and ultimately the fitness of the super-organism, depend – inter alia –- on the ability of nest mates to prevent nest predation

and to allow gynes and males to reproduce (Sudd and Franks 1987; McCann et al. 2014). Predatory pressures by non-human vertebrates on eusocial insects are well documented. For example, stripped skunks, *Mephitis mephitis* Shreber prey heavily on eastern yellowjackets, *V. maculifrons* (Buyson) (Preiss 1967). Raccoons, *Procyon lotor* (L.), are also common predators of North American yellowjackets (Akre et al 1980; MacDonald and Matthews 1981; Reed and Landolt 2000). In the Pacific Northwest, raccoons appear to inflict significant damage on *D. maculata* nests as evidenced by our observations that all the 15 *D. maculata* nests that we transplanted, but did not protect, succumbed to repeated attacks by raccoons (Ibarra, unpubl. obs.).

The capacity to release and sense alarm pheromones when nests are facing a threat is a major factor contributing to the success of eusocial insects (Wilson, 1965; Landolt et al. 1998). Evidence for pheromone-mediated alarm behaviour has been reported for nine species within the four genera of the vespine subfamily. When presented with venom sac extract, workers of common yellowjackets, *Vespula vulgaris* (L.), German yellowjackets, *Vespula germanica* (F.), Saxon wasps, *Dolichvespula saxonica* (F.), Oriental hornets, *Vespa orientalis* (L.), and of the nocturnal wasp *Provespa anomala* (Saussure), all display aggressive behaviour (Maschwitz 1964, 1984; Saslavaski et al. 1973; Aldiss 1983; Maschwitz and Hanel 1988).

Single- or multiple-component alarm pheromones have been identified and shown to trigger aggressive behaviour in four of the nine vespine species with well documented alarm behaviour. The European hornet, *Vespa crabro* L., displays defense behaviour when presented with 2-methyl-3-buten-2-ol (Veith et al. 1984), present in the venom sac. Workers of southern and eastern yellowjackets, *V. squamosa* (Drury) and *V. maculifrons,* display nest defense when exposed to (*N*)-3-methybutylacetamide. *Vespula maculifrons*, however, display defensive behaviour only when exposed to relatively high pheromone concentrations (Heath and Landolt 1988; Landolt et al. 1995). Finally, Giant Japanese hornets, *Vespa mandarinia* Smith, exhibit alarm behaviour in response to a 3-component alarm pheromone comprising 2-pentanol, 3-methyl-1-butanol, and 1-methylbutyl-3-methylbutanoate (Ono et al. 2003).

Several species of polistine wasps, the sister taxon of the vespines, have also been reported to exhibit pheromone-mediated nest defense (Bruschini et al. 2010) but an alarm pheromone component [(*E,E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane] has been identified only in *Polybia occidentalis* (Olivier) (Jeanne 1981; Dani et al. 2000). (*E,E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane and related spiroacetals have been isolated from multiple polistine and vespine species but bioactivity has been demonstrated only with *P. occidentalis.*

Our objectives in this study were (1) to investigate whether *D. maculata* exhibits pheromone-mediated nest defense*,* and (2) if so shown*,* to identify and field test the pheromone components.

3.3. **Methods and materials**

3.3.1. Experimental Insects

In the summer of 2013 and 2014, we located *D. maculata* nests in the Greater Vancouver Area by means of (*i*) daily surveys of suitable habitats, (*ii*) “nest-quest” advertisements in social media, bee keeping networks and supply stores, or (*iii*) direct correspondence with local parks and recreation government agencies. At dusk when all foragers had returned to their nest, we transplanted it to a fenced green house complex on the Burnaby campus of Simon Fraser University (SFU). Transplanting proceeded as follows: we soaked a cotton ball in diethyl ether (50 mL) and kept it at nest entrance until nest mates were anesthetized, as evident by cessation of buzzing activity after about 5 min. Thereafter, we replaced the cotton ball with an untreated cotton ball, carefully detached the nest from its substrate, and placed the nest in an acrylic box (30 × 30 × 46 cm) for transportation to SFU within ≤ 30 min. In the fenced green house complex at SFU, we hot-glued the top of the nest to the roof panel of another acrylic nest box (30 × 30 × 46 cm), removed the side panel facing the nest entrance to facilitate foraging by nest mates, and placed the nest box on 1-m tall tables, with ≥ 4 m between nest boxes. To protect transplanted nests from raccoon predation, we built electric fences (Electric fencing garden kit, Dare products Inc., Battle Creek, MI, USA) around tables. We allowed nests to acclimate for at least 1 week prior to any test.

3.3.2. Extraction of Venom Sacs

We sweep-netted *D. maculata* workers from the entrance of >10 nests. Following cold-euthanization of these workers, we thawed them at room temperature, pulled out the stinger with forceps, disconnected the sting apparatus from the gastro-intestinal tract, separated the venom sac, and placed it in a 4-mL glass vial containing acetonitrile (ACN) as the solvent. To facilitate pheromone extraction and analyses, we macerated submerged venom sacs with a syringe plunger (Hamilton Company, Reno, NV, USA), and filtered the supernatant through glass wool in a Pasteur pipet. We stored each extract at a concentration of 1 venom sac extract equivalent (1 VSEE) per 10 µl at –10 ºC prior to chemical analyses or use in bioassays.

3.3.3. Chemical Analysis of Venom Sac Extracts

We analyzed aliquots of venom sac extract by gas chromatographic-electroantennographic detection (GC-EAD) and GC-mass spectrometry (MS), with procedures and equipment previously described in detail (Arn et al. 1975; Gries et al. 2002). Briefly, we fitted a Hewlett Packard 5890 GC with a DB-5 GC column [30 m × 0.32 mm inner diameter (i.d.); J&W Scientific, Folsom, CA, USA], set the temperature of the injector port and flame ionization detector to 250 ºC, and operated the GC in split-less mode, using helium as the carrier gas (35 cm s^{-1}), with the following temperature program: 50 ºC for 1 min, 20 ºC/min until 280 ºC (20 min). For GC-MS analyses, we fitted a Saturn 2000 Ion Trap GC-MS with a DB-5 GC-MS column (50 m × 0.25 mm i.d.), set the temperature of the injector port and ion trap to 250 ºC, and operated the Ion Trap in full-scan electron impact mode, using Helium as the carrier gas (35 cm s^{-1}), and the following temperature program: 50º C for 5 min, 10º C/min until 280º C (20 min). For GC-EAD recordings (n = 8), we carefully pulled the antenna of a worker hornet from its head, removed the antennal tip with micro-scissors (Fine Science Tools Inc., North Vancouver, BC, Canada), and suspended the antenna between two glass-capillary electrodes (1.0 × 0.58 × 100 mm) (A-M Systems, Carlsborg, WA, USA) filled with saline solution. Volatiles in venom sac extracts that elicited responses from at least 2 antennae were considered candidate alarm pheromone components.

We identified and confirmed the structural assignment of candidate pheromone components by comparing their retention indices (Van den Dool and Kratz, 1963) and

mass spectra with those of authentic standards that we purchased [dimethylaminoethanol (>98% chemically pure); 2,5-dimethylpyrazine (>98%) (both Sigma-Aldrich Co.)], or synthesized [dimethylamino ethyl acetate: synthetized by acetylation of dimethylaminoethanol); *N*-3-methybutylacetamide (synthetized by acetylation of *N*-3-methylbutylamine); (*Z*)-8-heptadecen-2-one (see synthesis below); (*Z*)-10-nonadecen-2-one (see synthesis below)].

3.3.4. Syntheses of (*E*)- and (*Z*)-8-Heptadecen-2-one and (*E*)- and (*Z*)-10-Nonadecen-2-one

All reactions described were performed at ambient temperature and atmosphere unless otherwise specified. Column chromatography was carried out with 230-400 mesh silica gel (E. Merck, Silica Gel 60). Concentration and removal of trace solvents were done via a Buchi rotary evaporator using an acetone-dry-ice condenser and a Welch vacuum pump. Nuclear Magnetic Resonance (NMR) spectra were recorded using deuterochloroform ($CDCl_3$) as the solvent. Signal positions (δ) are given in parts per million from tetramethylsilane (δ 0) and were measured relative to the signal of the solvent (^{1}H NMR: $CDCl_3$: δ 7.26; ^{13}C NMR: $CDCl_3$: δ 77.0). Coupling constants (*J* values) are given in Hertz (Hz) and are reported to the nearest 0.1 Hz. ^{1}H NMR spectral data are tabulated in the order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet), coupling constants, number of protons. NMR spectra were recorded on a Bruker 400 (400 MHz).

Preparation of (E)-8-heptadecen-2-one.

A solution of (*E*)-7-hexadecen-1-ol (19 mg, 0.078 mmol, 1.0 eq) in CH_2Cl_2 (5 ml) was added to PCC (25 mg, 0.12 mmol, 1.5 eq.) and the reaction was stirred at ambient temperature for 2 h, then concentrated to about 1 ml. The residue was purified by flash chromatography (hexane/EtOAc = 15:1) yielding 18 mg of aldehylde. This aldehyde was dissolved in anhydrous THF (5 mL) and cooled to −78°C. After adding MeMgBr (3.0 M in Et_2O, 0.1 ml, 0.3 mmol, 4.0 eq) the mixture was stirred at −0°C for 3 h before quenching it with saturated aqueous NH_4Cl (2 mL). The aqueous layer was separated and extracted with EtOAc (5 mL). The combined organic layer was washed sequentially with water and brine, then dried over $MgSO_4$, and concentrated. The residue was used for the next step

without further purification. A solution of the prepared above alcohol (1.0 eq) in CH_2Cl_2 (5 ml) was added to PCC (24 mg, 1.5 eq.) and the reaction was stirred at ambient temperature for 2 h, then concentrated to about 1 ml. The residue was purified by flash chromatography (hexane/EtOAc = 10:1) yielding 16.6 mg (83% over 3 steps) of the methyl ketone as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.42-5.34 (m, 2H), 2.42 (t, *J* = 7.2 Hz, 2H), 2.13 (s, 3 H), 2.02-1.92 (m, 4H), 1.63-1.52 (m, 2H), 1.40-1.20 (m, 18H), 0.88 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 209.3, 130.7, 129.9, 43.8, 32.6, 32.4, 31.9, 29.8, 29.6, 29.5, 29.33, 29.29, 28.6, 23.7, 22.7, 14.1. $C_{17}H_{32}ONa$: 275.2345 (M+Na). Found: 275.2340 (M+Na).

Preparation of (Z)-8-Heptadecen-2-one.

A solution of (*Z*)-7-hexadecen-1-ol (152 mg, 0.6 mmol, 1.0 eq) in CH_2Cl_2 (15 ml) was added to PCC (258 mg, 1.2 mmol, 1.5 eq.), and the reaction was stirred at ambient temperature for 2 h, then concentrated to about 5 ml. The residue was purified by flash chromatography (hexane/EtOAc = 15:1) yielding 126 mg (83%) of aldehylde. This aldehyde was dissolved in anhydrous THF (15 mL) and cooled to −78°C. After adding MeMgBr (3.0 M in Et_2O, 0.4 ml, 1.2 mmol, 2.4 eq) added dropwise, the mixture was stirred at −0°C for 3 h before quenching it with saturated aqueous NH_4Cl (10 mL). The aqueous layer was separated and extracted with EtOAc (15 mL). The combined organic layer was washed sequentially with water and brine, then dried over $MgSO_4$ and concentrated. The residue was used for the next step without further purification. A solution of the prepared above alcohol (1.0 eq) in CH_2Cl_2 (15 ml) was added to PCC (222 mg, 1.5 eq.) and the reaction was stirred at ambient temperature for 2 h, then concentrated to about 5 ml. The residue was purified by flash chromatography (hexane/EtOAc = 10:1) yielding 89 mg (67%) of the methyl ketone as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.42-5.29 (m, 2H), 2.42 (t, *J* = 7.2 Hz, 2H), 2.14 (s, 3 H), 2.08-1.94 (m, 4H), 1.64-1.53 (m, 2H), 1.40-1.20 (m, 18H), 0.89 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 209.3, 130.2, 129.5, 43.8, 31.9, 29.7, 29.51, 29.48, 29.31, 29.30, 28.8, 27.2, 27.0, 23.7, 22.7, 14.1. HRMS: *m/z* calcd for $C_{17}H_{32}ONa$: 275.2345 (M+Na). Found: 275.2341 (M+Na).

Preparation of (E)-10-Nonadecen-2-one.

A solution of elaidic acid (85 mg, 0.3 mmol, 1.0 eq) in CH_2Cl_2 (15 ml) was sequentially added to *N*,*O*-dimethylhydroxylamine hydrochloride (44 mg, 0.45 mmol, 1.5 eq.) and 1,1′-carbonyldiimidazole (44 mg, 0.45 mmol, 1.5 eq.) at 0 °C. After 30 min, the reaction was stirred at ambient temperature overnight before quenching it with water. The aqueous layer was separated and extracted with CH_2Cl_2 (5 mL). The combined organic layer was washed sequentially with a 10-% HCl aqueous solution, a 5-% $NaHCO_3$ aqueous solution and brine, then dried over $MgSO_4$ and concentrated. The residue was used for the next step without further purification. The prepared above Weinreb amide was dissolved in anhydrous THF (8 mL) and cooled to −78 °C. MeMgBr (3.0 M in Et_2O, 0.2 ml, 0.6 mmol, 2.0 eq) was added, and the mixture was stirred at 0 °C for 5 h before quenching it with saturated aqueous NH_4Cl (2 mL). The aqueous layer was separated and extracted with EtOAc (5 mL). The combined organic layer was washed sequentially with water and brine, then dried over Na_2SO_4, and concentrated. The residue was purified by flash chromatography (hexane/EtOAc = 10:1) yielding 70 mg (83% over 2 steps) of the methyl ketone as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.41-5.34 (m, 2H), 2.42 (t, *J* = 7.6 Hz, 2H), 2.14 (s, 3 H), 2.02-1.92 (m, 4H), 1.62-1.52 (m, 2H), 1.39-1.20 (m, 20H), 0.89 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 209.3, 130.5, 130.2, 43.8, 32.6, 32.5, 31.9, 29.64, 29.56, 29.3, 29.24, 29.17, 29.14, 28.9, 23.9, 22.7, 14.1. HRMS: *m/z* calcd for $C_{19}H_{36}ONa$: 303.2658 (M+Na). Found: 303.2656 (M+Na).

Preparation of (Z)-10-Nonadecen-2-one.

A solution of oleyl alcohol (214 mg, 0.8 mmol, 1.0 eq) in CH_2Cl_2 (15 ml) was added to PCC (258 mg, 1.2 mmol, 1.5 eq.) and the reaction was stirred at ambient temperature for 2 h, then concentrated to about 5 ml. The residue was purified by flash chromatography (hexane/EtOAc = 15:1) yielding 192 mg (90%) of aldehylde. This aldehyde was dissolved in anhydrous THF (15 mL) and cooled to −78°C. After adding MeMgBr (3.0 M in Et_2O, 0.5 ml, 1.5 mmol, 2.1 eq) dropwise, the mixture was stirred at 0 °C for 3 h before quenching it with saturated aqueous NH_4Cl (10 mL). The aqueous layer was separated and extracted with EtOAc (15 mL). The combined organic layer was washed sequentially with water and brine, then dried over $MgSO_4$ and concentrated. The

residue was used for the next step without further purification. A solution of the prepared above alcohol (1.0 eq) in CH_2Cl_2 (15 ml) was added to PCC (222 mg, 1.5 eq.) and the reaction was stirred at ambient temperature for 2 h, then concentrated to about 5 ml. The residue was purified by flash chromatography (hexane/EtOAc = 10:1) yielding 180 mg (80%) of the methyl ketone as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.41-5.32 (m, 2H), 2.42 (t, *J* = 7.6 Hz, 2H), 2.14 (s, 3 H), 2.06-1.96 (m, 4H), 1.62-1.52 (m, 2H), 1.39-1.20 (m, 20H), 0.89 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 209.3, 130.0, 129.8, 43.8, 31.9, 29.83, 29.76, 29.7, 29.5, 29.32, 29.31, 29.29, 29.15, 29.10, 27.21, 23.16, 23.86, 22.7, 14.1. HRMS: *m/z* calcd for $C_{19}H_{36}ONa$: 303.2658 (M+Na). Found: 303.2654 (M+Na).

3.3.5. Quantitative Analyses of Venom Sac Constituents

To quantify the amount of specific venom sac constituents, we injected an authentic standard of each target compound at increasing doses (20, 40, 60, 80, 100 ng per 1 µL) into the GC, recorded the flame ionization detector (FID) counts for each dose, and fitted a least squares regression curve through the data points for each compound, using the statistical software R (version 3.1.3) or JMP 12. To calculate the amount of each target compound in venom sac extracts, we entered its FID counts per 1 µl of extract into the linear-fit-formula of the corresponding authentic standard regression curve, and then multiplied the obtained value with the number of microliters per venom sac withdrawn after the extraction procedure.

3.3.6. General Design of Field Bioassays

Field experiments were ran by placing a two-choice, paired-target apparatus near (1 m) the entrance of a *D. maculata* nest (Figure 3.1.). Each target consisted of a hollow box formed by two black-coated weigh boats (13.97 cm^2; Big Science Inc., Huntersville, NC, USA) which were conjoined with adhesive tape. Each box housed a Sony tie-clip microphone (Sony ECM T-6; Sony Electronics Inc.) and was attached to one of the two end sections of a 1-m long, tripod-mounted, horizontal arm. Each of the two microphones was connected to a stereo digital audio recorder (Edirol R-09 HR, Roland Canada Ltd., Richmond, BC, Canada). Depending on the test, both boxes were left

untreated (control *vs*. control), or one of the two boxes was treated with a test stimulus (see below). To minimize any tiring or learning effects, not more than 12 tests per nest were ran on any day and the order of tests for each nest was randomized. Because nests differed in their stage of development and their propensities to respond, and were subject to predation by raccoons, not all experiments could be completed with each nest. A new test was initiated when all nest-defending hornets had returned to the inside of the nest, which was typically 30 s to 300 s following the preceding test. Thirty seconds after placing the apparatus in front of a *D. maculata* nest with the boxes at the same height as the nest entrance, the sound impulses caused by *D. maculata* workers attempting to sting or strike the boxes were recorded for 3 min. The trials were run on clear days between 15-30 August 2013 or 2014. The audio files were opened in Audacity Sound Editor software (The Audacity Team), and the two-channel stereo file was split into two mono audio files, and saved as 16-bit wav files. Numbers of strikes or pulses per channel (target) in each file were counted automatically using SoundRuler, an open source bioacoustics tool (Marcos Griddi-Papp, UCLA, USA). Strikes were counted by SoundRuler, if they had parameter of a representative pulse (peak amplitude: 1.264 ± 0.7 V; duration: 12.63 ± 0.4 ms; interpulse interval: 300 ± 0.4 ms). Data were saved as CSV files.

Stimuli tested in Specific Experiments

We ran field experiments with six separate nests (Table 3.1.). We randomly exposed nests to a series of 2-5 experiments, as follows: Exp. 1: unbaited [ACN (50 µL)] *vs.* unbaited [ACN (50 µL)]; Exp. 2: 5 VSEEs [in ACN (50 µL)] *vs.* unbaited [ACN (50 µL)]; Exp. 3: synthetic blend of candidate alarm pheromone components at 5 VSEEs [(**a**) dimethylamino ethanol (450 ng), (**b**) dimethylamino ethyl acetate (240 ng), (**c**) 2,5-dimethylpyrazine (119 ng), (**d**) *N*-3-methylbutylacetamide (160 ng), (**e**) 2-heptadecanone (413 ng), (**f**) (*Z*)-8-heptadecen-2-one (319 ng), (**g**) (*Z*)-10-nonadecen-2-one (3527 ng) in ACN (50 µL] *vs.* unbaited [ACN (50 µL)]; Exp. 4: synthetic blend of components **a-d** at 50 VSEEs [in ACN (50 µL)] *vs.* unbaited [ACN (50 µL)]; Exp 5: synthetic blend of components **e-g** at 50 VSEEs [in ACN (50 µL)] *vs.* unbaited [ACN (50 µL)]

3.3.7. Statistical Analyses of Data

Using the statistical software R (version 3.1.3), we analyzed data for differences in (1) the number of strikes (reflecting defense intensity) between experiments 1-5, and (2) the proportion of strikes to the baited treatment box within each of experiments 2-5. For analysis 1, we log-transformed data to meet the assumption of data normality and analyzed data with a linear mixed effects model using the lme4 software package. As a few replicates, mainly in experiment 1 (Blank vs Blank), had no strikes on any of the two boxes, we added one strike to the treatment and the control box for data analyses of all experiments, thus avoiding a log of 0. In the model $Strikes = Test\ type + (1|Nest\ ID\ /\ rep)$, *strikes* are the log of total strikes recorded on treatment and unbaited control boxes, *Test type* is a fixed factor with five variables (unbaited; venom sac extract; synthetic components **a-g**; synthetic components **a-d**; synthetic components **e-g**), and *(1|Nest/rep)* is a random effect of *Nest ID* where multiple replicates were run per nest and thus "rep" is nested within "Nest ID" to account for pseudo-replication.

For analysis 2, we analyzed the data with a generalized linear mixed effects model using the software package glmer. The model fitted for each of the five experiments had the following form: $\%\ hits\ (T) = (1|Nest\ ID\ /\ rep)$ where *%hits (T)* accounts for the proportion of strikes on the treatment box and *(1|Nest/rep)* accounts for the random effect of *Nest ID*, where multiple replicates were run per nest and thus "rep" is nested within "Nest ID" to account for pseudo-replication. In each experiment, we tested for a deviation from a 50:50 proportion of strikes on the treatment and control box, thus running the model based on a binomial distribution of data.

3.4. Results

3.4.1. GC-EAD and GC-MS Analyses of Venom Sac Extract

GC-EAD analyses of *D. maculata* venom sac extracts revealed seven components (**a-g** in Figure 3.2) that consistently elicited responses from antennae of *D. maculata* workers. By comparing the retention indices and mass spectra of these EAD-active compounds with those of authentic standards, we identified five candidate alarm

pheromone components as dimethylaminoethanol (**a**), dimethylamino ethyl acetate (**b**), 2,5-dimethylpyrazine (**c**), *N*-3-methylbutylacetamide (**d**), and 2-heptadecanone (**e**).

The mass spectrum of compounds **f** and **g** each revealed a strong fragmentation ion *m/z* 58 indicative of an acyl group in C2. Considering the molecular ion of **f** (*m/z* 252) and **g** (*m/z* 280), we hypothesized that **f** and **g** were a heptadecen-2-one and a nonadecen-2-one, respectively. To determine the double bond position in each ketone, we treated a 100-µl aliquot of venom sac extract with dimethyl disulfide (DMDS) (Dunkelblum et al. 1985) and analyzed the treated extract by GC-MS. The mass spectrum of the DMDS-treated heptadecen-2-one revealed a sulfur adduct ion (*m/z* 173) indicative of a double bond in C8, and the mass spectrum of the DMDS-treated nonadecen-2-one revealed sulfur adduct ions (*m/z* 173, 201) indicative of a double bond in C10. Retention and mass spectrometric characteristics of synthetic (*Z*)-8-heptadecen-2-one (but not (*E*)-8-heptadecen-2-one which eluted later), and of synthetic (*Z*)-10-nonadecen-2-one (but not (*E*)-10-nonadecen-2-one which eluted later), were in complete agreement with those of **f** and **g**, respectively, confirming our structural assignments.

The absolute amounts of the seven EAD-active components in venom sac extracts varied greatly. (*Z*)-10-Nonadecen-2-one (**g**) as the component with the highest molecular weight and lowest volatility was most abundant and present at 705 ng per venom sac equivalent. Dimethylaminoethanol, dimethylamino ethyl acetate, 2,5-dimethylpyrazine, *N*-3-methylbutylacetamide, 2-heptadecanone, and (*Z*)-8-heptadecen-2-one were present at 90, 48, 24, 32, 83 and 64 ng per venom sac equivalent, respectively.

3.4.2. Pheromone-mediated Nest Defense

Overall Attack Intensity Based on Test Stimuli

Between experiments 1-5, there was a significant difference in the number (mean ± SE) of total strikes by *D. maculata* workers on paired boxes within the 3-min test period [$F(4,10.58) = 46.094$, $p<0.001$] (Figure 3.3). For example, when both boxes were not baited, they induced only 9.51 ± 3.5 strikes (Exp. 1) but when one of the two boxes was treated with venom sac extract, the two boxes combined induced 263.23 ± 55.72

strikes (Exp. 2), a significant 27-fold increase in number of strikes (Tukey-contrast for multiple comparisons of means; z = 11.89, $p < 0.001$). Treating one of the two boxes with a synthetic blend of components **a-g** (at 5 VSEE; Exp. 3), or components **a-d** (at 50 VSEE; Exp. 4), prompted 50.59 ± 17.30 and 37.40 ± 18.16 strikes, respectively, significantly more strikes on average than prompted by the two unbaited control boxes in experiment 1 (Exp. 3 *vs.* Exp.1: z = 5.83, $p<0.001$; Exp. 4 *vs*. Exp. 1: z = 3.99, $p<0.001$), but not significantly different from each other (Exp. 3 *vs.* Exp.4: z = -1.55, $p=0.55$). In contrast, treating one of the two boxes with a synthetic blend of components **e-g** induced only 3.22 ± 0.97 strikes, as few as the two unbaited control boxes in experiment 1 (Exp. 5 *vs.* Exp. 1: z = -0.733, $p=0.95$).

Evidence for Target-oriented Responses Based on Test Stimuli

The proportions of strikes by *D. maculata* workers on treatment boxes in experiments 1-5 is displayed in figure 3.4. Boxes treated either with venom sac extract at 5 VSEE (Exp. 2), or with a synthetic blend of components **a-g** at 5 VSEE (Exp. 3), induced a significantly greater proportion of strikes than the corresponding unbaited control boxes (Exp. 2: z = 6.492, $p < 0.001$; Exp. 3: z = 5.534, $p < 0.001$). A comparable effect was not observed when we applied a synthetic blend of components **a-d** (Exp. 4: z = 0.56, $p = 0.57$), or components **e-f** (Exp. 5: z = 0, $p = 1$), to treatment boxes. Expectedly, there was no significant preference for unbaited control boxes on the left or right site of the test apparatus (Exp. 1: z = -0.11 $p = 0.91$).

3.5. **Discussion**

We present evidence for pheromone-mediated nest defense in *D. maculata,* and report the identification and function of pheromone components contributing to the defense behaviour. This is the tenth vespine shown to display pheromone-mediated nest defense, and the fifth to have some of the nest defense pheromone components identified (Maschwitz 1964; Ishay et al. 1967; Saslavaski et al. 1973; Aldiss 1983; Maschwitz 1984; Veith et al. 1984; Landolt and Heath 1987; Heath and Landolt 1988; Maschwitz and Hanel 1988; Landolt et al. 1995; Landolt et al. 1999; Ono et al. 2003).

Nest defense behaviour by *D. maculata* entails fast and sudden attacks on a potential nest predator. In attack mode at the moment of impact, *D. maculata* workers bend their gaster forward towards the predator, causing the stinger to be driven into the predator's skin and inflicting a piercing wound. During the stinging process, venom sac content either drips or is actively sprayed over the predator's compromised skin. Concurrently, volatile components from the venom sac excretion dissipate and trigger both alarm and target-oriented responses by nest mates. The alarm response mode is convincingly demonstrated by a significant 27-fold increase in the number of strikes (sting attempts) when - compared to two untreated control boxes (Figures 3.1 & 3.3: Exp. 1) - one of the two boxes is treated with venom sac extract (Figure 3.3: Exp. 3). The target-oriented response mode caused by venom sac extract is expressed by significantly more strikes being directed toward the one box in each pair treated with venom sac extract (Figure 3.4: Exps. 3, 4).

Alarm and target-oriented responses by *D. maculata* nest mates are mediated by separate components in the venom sac. To assess the behavior-modifying effects of the nitrogen-containing compounds **a**-**d** and the ketones **e**-**g**, we exposed *D. maculata* nests to synthetic blends of **a**-**d, e**-**g** or both (**a**-**g).** Exposures of nests to the **a**-**d** blend (Figure 3.3 & 3.4: Exp. 4), or the **e**-**g** blend (Figure 3.3 & 3.4: Exp. 5), each failed to trigger the full complement of alarm and target-oriented responses equivalent to those induced by the **a-g** blend (Figures 3.3 & 3.4: Exp. 3). Specifically, the **a**-**d** blend (albeit at a 10-fold higher dose than the **a-g** blend) induced the same level of alarm response (attack intensity) as the **a-g** blend (Figures 3.3: Exps. 3, 4), but failed to trigger the same target-oriented response as the **a-g** blend (Figure 3.4: Exps. 3, 4). Apparently, at least one component of the **a**-**d** and the **e**-**g** blend must be present to trigger both response modes. Conceivably, one or more of the high-molecular-weight ketone(s) in venom sac excretions may mark potential nest predators for guided, target-oriented attacks or may enhance the alarm-inducing effect of the volatile pheromone components. The latter phenomenon is exemplified in the European honeybee, *Apis mellifera,* where (Z)-11-eicosen-1-ol enhances the effect of the volatile alarm pheromone component isopentyl acetate (Boch et al. 1962; Pickett et al. 1982).

Alarm pheromone blends with a specific function for each component have also been reported in studies with ants. In *Bothroponera soror Emery, for example,* 2-undecanone alerts nest mates, 2-undecanol attracts them, and methyl 6-methylsalicylate elicit stinging (Longhurst et al. 1980). Similarly, *Solenopsis saevissima* (Smith) deploys a combination of trail and alarm pheromone components in response to threats (Wilson 1965, 1971).

There is evidence that one or more defense pheromone components of *D. maculata* are yet to be identified. While the presence of the **a**-**g** blend on one of the two test boxes induced significantly more strikes by *D. maculata* nest mates than did two untreated control boxes placed in front of nests (Figure 3.3: Exps. 1, 3), the **a**-**g** blend was significantly less effective in triggering strikes than venom sac extract (Figure 3.3: Exps. 2, 3). Interestingly, the **a**-**g** blend and venom sac extracts were equally effective in mediating target-oriented responses (Figure 3.4: Exps. 2, 3), implying that a pheromone component is missing for the alarm response mode rather than the target-oriented response mode. This compound might be highly volatile or unstable and thus be difficult to capture or to detect in GC-EAD or GC-MS analyses.

Some components of the *D. maculata* nest defense pheromone that we present here have previously been reported in the defense system of other insects. In response to vertebrate predation, saturniid moth caterpillars produce an exudate that contains both dimethylaminoethanol and dimethylamino ethyl acetate (Deml and Dettner 1993, 1994, 2003), implying a defensive rather than communicative function of both compounds. As precursors of neurotransmitters in vertebrates (Pfeiffer 1957), both compounds being released during a stinging event by *D. macualata* could cause a sensation of pain in the predator under attack, thus reducing the probability of nest predation (Schmidt 1986). Over evolutionary time then, these compounds may also have assumed a role in nest-defense communication, explaining their antennal activity (Figure 3.2; Wilson 1971; Hölldobler and Wilson 1990).

2,5-Dimethylpyrazine as another component in the *D. maculata* venom sac has a recruitment function in other Hymenoptera. It is deemed to be a trail pheromone component of four harvester ants, *Pogonomyrmex spp* Mayr (Hölldobler et al. 2001), the

leafcutter ant *Atta sexdens sexdens* (L.) (Morgan et al. 2006), and the ants *Daceton armiguerum* (Latreille) (Morgan et al. 1992), *Tetramorium meridonale* Emery and *Tetramorium caspitum* (L.) (Atygalle and Morgan 1984; Jackson et al. 1990). 2,5-Dimethylpyrazine was also isolated from the venom sac and the mandibular gland of the ants *Messor arenarius* (F.) and *Pachycondyla obscuricornis* Emery, respectively (Cruz-Lopez 2006; Morgan et al. 1999); however, no behavioural activity has been associated with it. In the hover wasp *Parischnogaster mellyi* (de Saussure), 2,5-dimethylpyrazine is present in the venom sac but a behavioural function is not likely because *P. mellyi* does not seem to engage in pheromone-mediated nest defense (Dani et al. 1998).

N-3-Methylbutylacetamide is an abundant alarm pheromone component of at least two species of yellowjackets, *Vespula squamosa* (Drury) and *V. maculifrons* (Buyson) (Heath and Landolt 1988; Landolt et al. 1995), occurring in the venom sac at 575 ± 60.4 and 84.2 ng, respectively (Heath and Landolt 1988, Landolt et al. 1995). Presence of *N*-3-methylbutylacetamide also in the venom sac of *D. maculata* workers, albeit at a lower amount (~32 ng), implies that yellowjackets during nest defense may "speak dialects" of a common language. Whether and to what extent these dialects are "understood" by heterospecifics may vary between specifies. For instance, *V. maculifrons* does respond, but only weakly, to the main alarm pheromone components of *V. squamosa* (Landolt et al. 1995). *N*-3-Methylbutylacetamide was also isolated from venom sacs of all the polistine and most vespine wasps studied thus far, except for any of the V*espa spp.* L. (Saslavaski et al. 1973; Aldiss 1983; Veith et al. 1984; Heath and Landolt 1988; Landolt et al. 1995; Dani et al. 2000; Bruschini et al. 2006).

There is not as much information about the occurrence and role of the *D. maculata* venom sac ketones e, f and g (Figure 3.2) in other species. The bumble-bee *Alpigenobombus wurfleini* (=*Bobmus wulferni* Rodoskowsky) uses 2-heptadecanone (e) as part of a marking pheromone deposited by labial palps (Svensson et al. 1984). In beewolves, *Philanthus basilaris* (Cresson) and *P. bicinctus* (Mickel), 2-heptadecanone seems to play a role as an attractant, marker, and sex pheromone (Schmidt et al. 1985). Curiously, (Z)-8-heptadecen-2-one (f) has been reported only in the ventral gland of hamsters, *Phodopus sungorus sungorus* (Pallas) (Burger 2001) but a biological role is not known. (Z)-10-Nonadecen-2-one (g) has been found in three arthropods. It is a trace

compound associated with the ant *Iridomyrmex humilis* (Mayr) (Cavill et al. 1980), it is part of the defensive secretion of the tenebrionid beetle *Uloma tenebrionoides* (White) (Gnanasunderam et al. 1985), and it contributes to species recognition in the European beewolve, *Philanthus triangulum* F. (Schmitt et al. 2003).

3.5.1. Conclusion

We present evidence for pheromone-mediated nest defense in *D. maculata,* the tenth such record in vespines and the fifth record where at least some of the components mediating the defense response have been identified and field tested. We show that the venom sac-derived pheromone components of *D. maculata* trigger both alarm and target-oriented responses by nest mates. The nitrogen-containing volatile components **a**-**d** appear to have primarily an alarm function, whereas the “heavier” less volatile ketones **e**-**g** appear to prompt target-oriented responses, possibly marking potential nest predators for guided and concerted attacks, or enhancing the alarm-inducing effect of the volatile pheromone components, as shown in honey bees. Some of the pheromone components reported here occur in the venom sacs of other vespids implying both a shared ancestry and the possibility of a universal nest defense language with species-specific dialects.

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Table 3.1. Number of *Dolichovespula maculata* nests transplanted for testing their nest defense responses in experiments 1-5 using the paired-target apparatus (Figure 3.1), the stimuli tested in experiments 1-5, and the number of replicates tested with each nest for its response to specific test stimuli.

	Stimuli tested in paired, two-target experiments 1-5[1]					
	Exp. 1 Blank *vs* Blank[3]	Exp. 2 Sac extract[4] *vs* Blank	Exp. 3 Synthetics[5] **a-g** *vs* Blank	Exp. 4 Synthetics[5] **a-d** *vs* Blank	Exp. 5 Synthetics[5] **e-f** *vs* Blank	
Nest ID[2]	Number of replicates					Total
1	13	7	8	5	4	37
2	12	7	9	4	5	37
3	14	35	6	4	0	59
4	0	13	0	4	0	17
5	0	7	0	0	0	7
6	2	12	6	0	0	20
All	41	81	29	17	9	177

[1]See figure 1 for a photograph of the paired-target test apparatus;
[2] Nests 1, 2, 3, 4, 5, and 6 were transplanted on 11-08-2014, 11-08-2014, 01-08-2013, 05-08-2013, 08-08 2013, and 13-08-2013 (DD-MM-YYYY), respectively;
[3]Blank treatments consisted of 50 µl of acetonitrile applied to the control target (see Figure 1);
[4]Venom sac extract was tested at 5 venom sac equivalents present in 50 µl of acetonitrile;
[5]Synthetic candidate pheromone components were: [(**a**) dimethylamino ethanol, (**b**) dimethylamino ethyl acetate, (**c**) 2,5 dimethylpyrazine, (**d**) *N*-3-methylbutylacetamide, (**e**) 2-heptadecanone, (**f**) (*Z*)-8-heptadecen-2-one, (**g**) (*Z*)-10-nonadecen-2-one]; components **a-g**, **a-d**, and **e-g** were tested at 5, 50 and 50 venom sac equivalents, respectively, in 50 µl of acetonitrile.

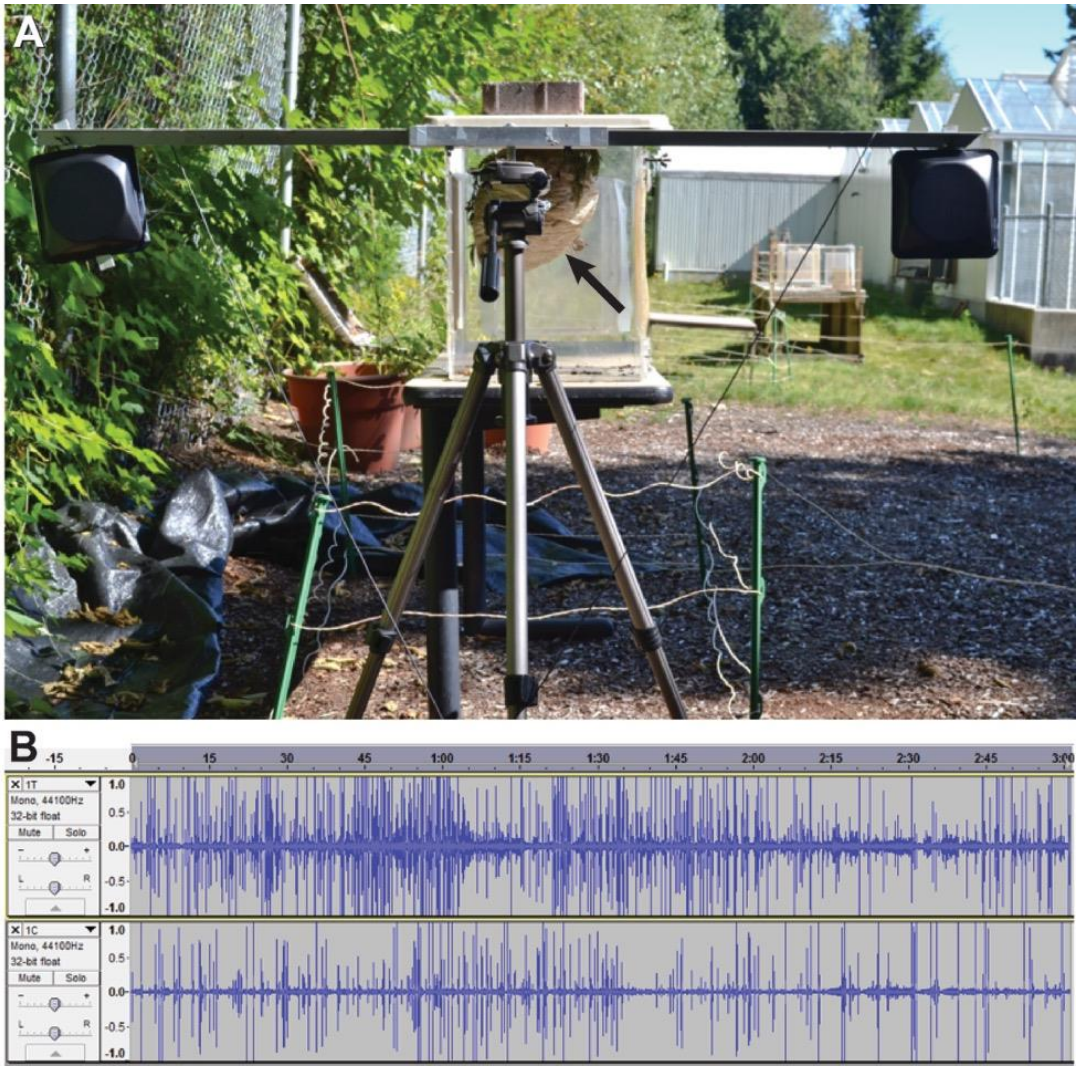

Figure 3.1. **(A) Paired-target bioassay apparatus. Each of the two plastic black-painted boxes houses a Sony tie-clip microphone (Sony ECM T-6) that is attached to a tripod-mounted, 1-m long horizontal arm and connected to a dual channel digital audio recorder (Edirol R-09 HR; not shown). The boxes are treated with test stimuli (see Table 1), and the apparatus is placed within at 1 m from the entrance of a transplanted *D. maculata* nest. (B) Example of recorded strikes caused by *D. maculata* workers hitting boxes treated with venom sac extract (top) or a solvent control (bottom). Each vertical bar is a waveform displayed by Audacity, representing a single strike on the weigh-boat box detected by the microphone inside the box. Each channel (bottom and top corresponding to the right and left box, respectively) was saved as a 16-bit wav file and exported to SoundRouler where pulses were counted automatically if they met the predetermined parameters of a representative pulse (peak amplitude: 1.264 ± 0.7 V; duration: 12.63 ± 0.4 ms; interpulse interval: 300 ± 0.4 ms)**

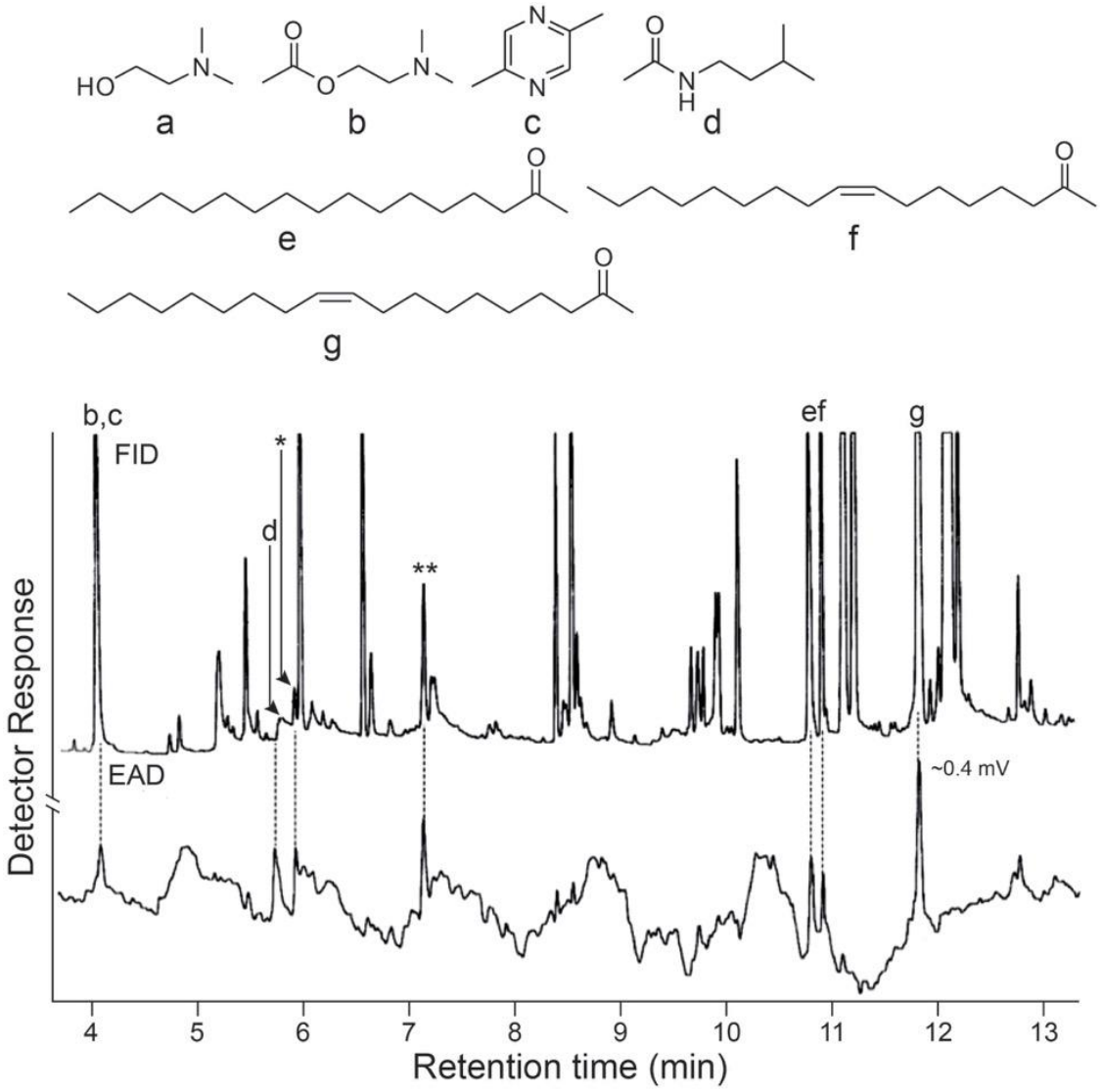

Figure 3.2. **Representative recording of the responses of a gas chromatographic flame ionization detector (FID) and an electroantennographic detector (EAD: worker *D. maculata* antenna) to aliquots of venom sac extracts of worker *D. maculata*. Components a-g that consistently elicited antennal responses were identified as follows: (a) dimethylaminoethanol (not visible in graph), (b) dimethylamino ethyl acetate, (c) 2,5-dimethylpyrazine, (d) *N*-3-methylbutylacetamide, (e) 2-heptadecanone, (f) (*Z*)-8-heptadecen-2-one, (g) (*Z*)-10-nonadecen-2-one, * = unknown; ** = 2-undecanone (originally not included in the blend and later confirmed to have no behavioral activity)**

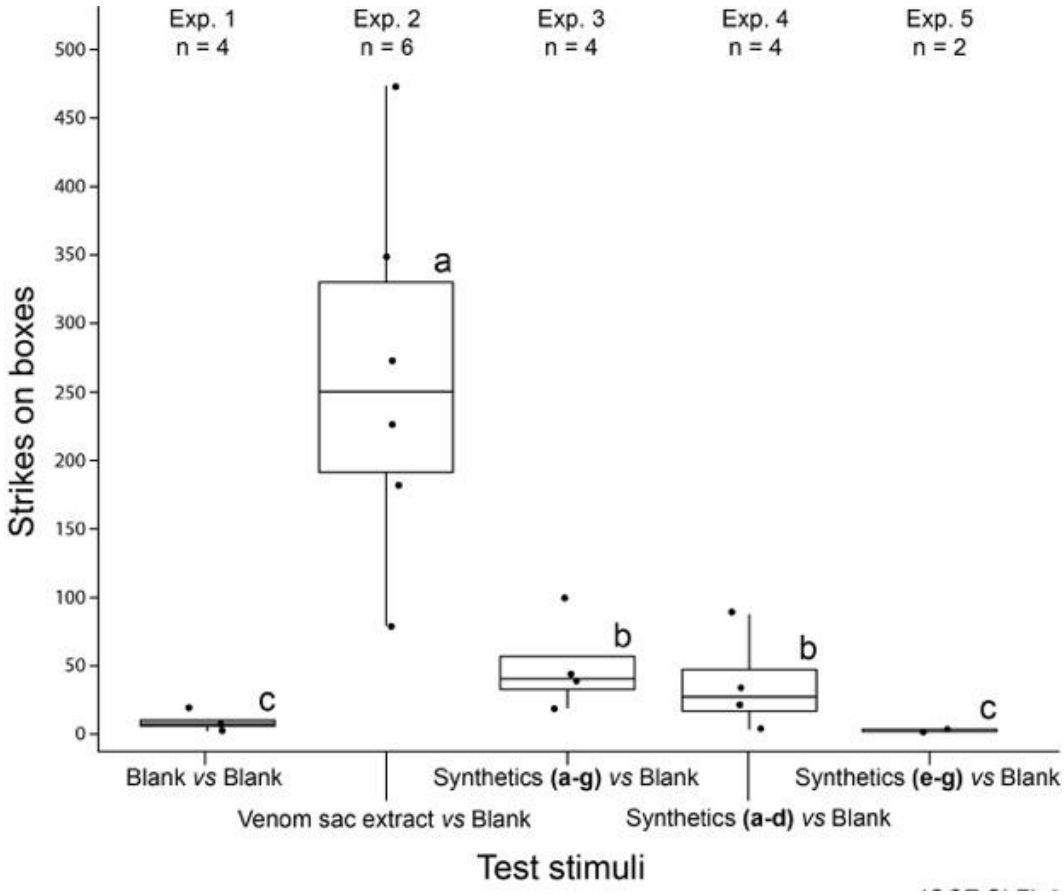

Figure 3.3. **Boxplots showing the mean, median lower and upper quartiles, and ± whiskers (minimum/maximum data points) of the number of strikes by *D. maculata* workers on paired boxes (Figure 1), two of which (Exp. 1), or one of which (Exps. 2-5) serving as unbaited controls. The identity of synthetics a-g is reported in the caption of figure 2, and more detail about test stimuli is reported in Table 1. Between experiments 1-5, there was a significant difference in the number of strikes within the 3-min test period [$F(4,10.58) = 46.094$, $p<0.001$]**

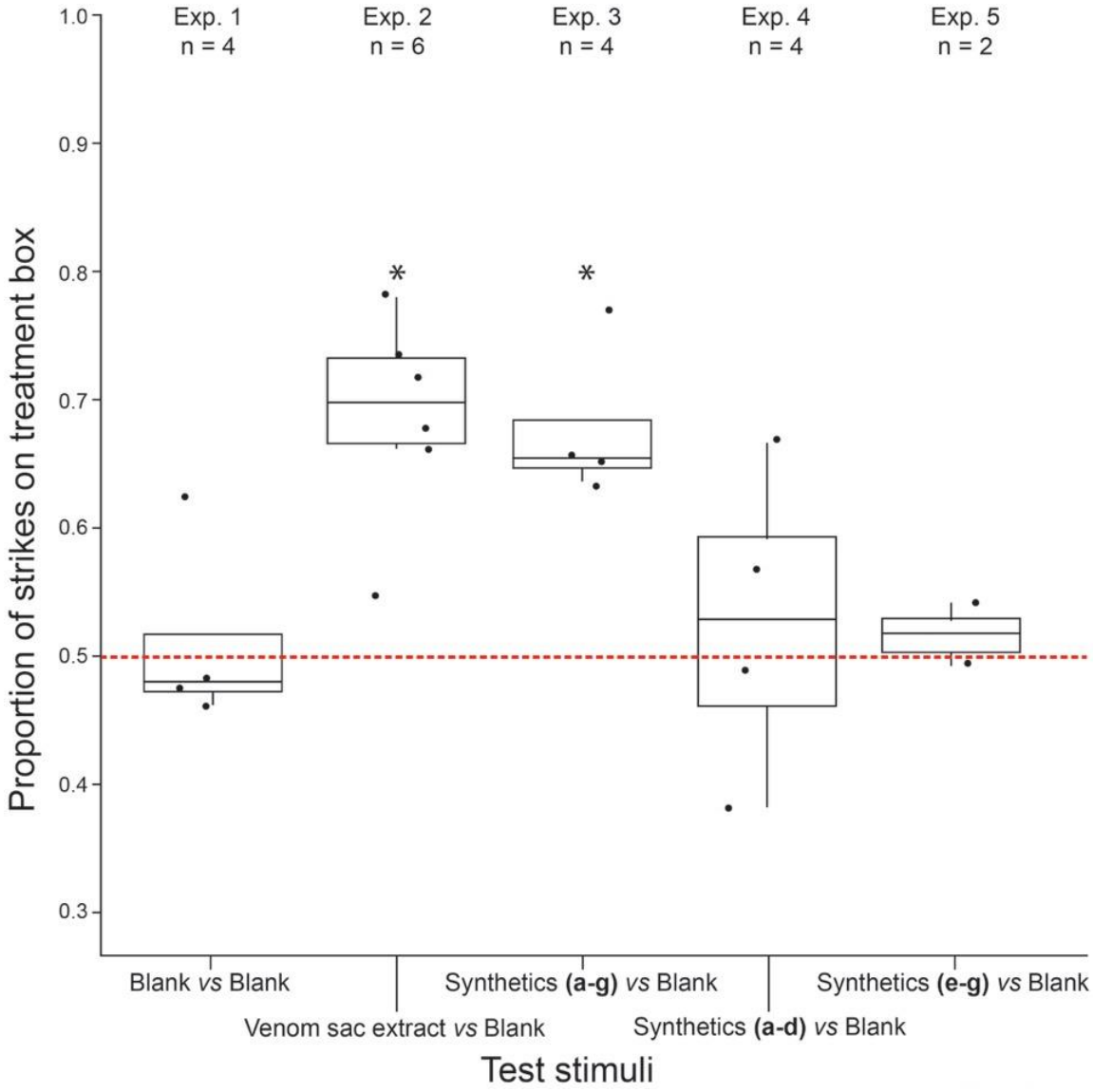

Figure 3.4. **Boxplots showing the mean, median lower and upper quartiles, and ± whiskers (minimum/maximum data points) of the proportion of strikes by *D. maculata* workers on paired boxes (Figure 1), two of which (Exp. 1), or one of which (Exps. 2-5), serving as unbaited controls. The identity of synthetics a-g is reported in the caption of figure 2, and more detail about test stimuli is reported in Table 1. The asterisk in experiments 2 and 3 denotes a significant preference for the treatment stimulus (Exp. 2: z = 6.492, $p < 0.001$; Exp. 3: z = 5.534, $p < 0.001$).**