Floral and honeydew foraging ecology of select mosquito species

by

Daniel Peach

B.Sc. (Dist.), Simon Fraser University, 2012

Associate of Arts, Douglas College, 2008

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Name:	**Daniel Peach**
Degree:	**Doctor of Philosophy**
Title:	**Floral and honeydew foraging ecology of select mosquito species**
Examining Committee:	**Chair:** Jim Mattsson Associate Professor
	Gerhard Gries Senior Supervisor Professor
	Carl Lowenberger Supervisor Professor
	Margo Moore Supervisor Professor Emerita
	Sheila Fitzpatrick Examiner Research Scientist Agriculture and Agri-Food Canada
	Woodbridge Foster External Examiner Faculty Emeritus Department of Entomology The Ohio State University
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Abstract

Both male and female mosquitoes exploit a wide variety of plant sugar resources, including floral nectar and aphid honeydew, as important sources of carbohydrates. Mosquitoes are generally considered nectar thieves that do not pollinate the flowers they visit, and volatile semiochemicals are believed to be the primary driver of mosquito attraction to plant sugar sources. Using the northern house mosquito, *Culex pipiens*, and its nectar host the common tansy, *Tanacetum vulgare*, we showed mosquito-induced seed-set. We found that semiochemicals from *T. vulgare* flowers are attractive to *Cx. pipiens* and the yellow fever mosquito, *Aedes aegypti*, that visual and olfactory inflorescence cues in combination attract more mosquitoes than olfactory cues alone, and that plant CO_2 enhances the attractiveness of a 20-component synthetic blend of tansy inflorescence odourants. This blend included 9 odourants found in human odour, which are also attractive. Electroretinograms revealed that *Cx. pipiens* eyes can sense ultra-violet (UV) wavelengths, with peak sensitivity at 335 nm. Experiments found that UV inflorescence cues of *T. vulgare* and the common hawkweed, *Hieracium lachenalii*, enhance the attractiveness of inflorescence odour to female *Cx. pipiens* through floral patterns of UV-absorption and UV-reflection. We then established the attraction of *Ae. aegypti* to honeydew odourants from the green peach aphid, *Myzus persicae*, and the pea aphid, *Acyrthosiphon pisum*, feeding on fava bean, *Vicia faba*. We collected and analyzed headspace odourants from honeydew of *A. pisum* feeding on *V. faba*. An 8-component synthetic blend of these odourants and synthetic odourant blends of crude and sterile honeydew we prepared from literature data all attracted female *Ae. aegypti*. The synthetic blend containing microbial odour constituents proved more effective than the blend without these constituents. Our data support the hypotheses that mosquitoes are pollinators, that the entire inflorescence Gestalt of olfactory, CO_2 and UV cues is more attractive to mosquitoes than floral odourants alone, that olfactory cues attract mosquitoes to honeydew, and that microbe-emitted volatiles play a role in mosquito attraction to honeydew.

Keywords: Mosquitoes; *Aedes aegypti*; *Culex pipiens*; Pollination; Chemical Ecology; Sensory Ecology

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List of Acronyms

CHD	Crude Honeydew
CO_2	Carbon Dioxide
CSB	Complete Synthetic Blend
DP	Daniel Peach
ERG	Electroretinogram
GC-MS	Gas Chromatography-Mass Spectrometry
HSV	Headspace Volatiles
IHE	Inflorescence-Hour Equivalents
PSB	Partial Synthetic Blend
SFU	Simon Fraser University
SHD	Sterile Honeydew
UV	Ultra-violet

Chapter 1.

Mosquito phytophagy – sources exploited, ecological function, and evolutionary transition to haematophagy[1]

[1]The corresponding manuscript is in review in *Entomologia Experimentalis et Applicata* with the following authors: Peach, D.A.H., and Gries, G.

Introduction

To complete a gonotrophic cycle, the females of many mosquito species obtain vertebrate blood, a behaviour known as haematophagy; however, for a very long time mosquitoes have also been known or suspected to consume plant liquids (Ficalbi 1899; Swammerdam 1758). It is now recognized that sugary plant liquids provide essential food for adult male and female mosquitoes (Foster, 1995; Stone & Foster, 2013; Nyasembe & Torto, 2014) which are considered at least partly phytophagous (Stone *et al.* 2018). Recently eclosed mosquitoes cannot survive long without consuming sugary plant liquids (Foster 1995). These sugary plant meals provide fuel for flight and enable blood-feeding and mating (Foster 1995). Blood-fed but sugar-deprived mosquitoes lay fewer eggs (Foster 1995), have lower energy stores for overwintering (Foster 1995), and are less able to mate (Stone *et al.* 2009, 2011). Populations of even highly synanthropic mosquitoes may not be able to persist without phytophagy, even when vertebrate blood is readily available (Stone *et al*., 2009).

Phytophagy is a key element of mosquito ecology and understanding it is critical to combatting mosquito-borne diseases (Ferguson *et al.* 2010). Nonetheless, many questions regarding interactions between mosquitoes and plants remain. In this review, we will summarize current knowledge about mosquito phytophagy and outline future research needs. For interactions between mosquitoes, plants and pathogens, we refer the reader to comprehensive and recent reviews by Stone and Foster (2013) and Stone *et al.* (2018).

1.1. Is the term “sugar-feeding” appropriate?

Plant sugars provide adult mosquitoes with vitally important energy for flight and survival (Foster 1995). However, conceptualizing mosquitoes simply as plant “sugar-feeding” is overly reductive. Mosquitoes also require non-energy nutrients including some amino acids, salts and vitamins (Rivera-Pérez *et al.*, 2017) that occur at levels up to 2.8 µmol/mL in nectar or other plant-derived fluids (Baker & Baker 1973; Nicolson & Thornburg 2007). Mosquitoes acquire these types of nutrients as part of their larval diet or from blood-feeding but also from plant-derived products (Rivera-Pérez *et al.* 2017). Amino acids added to synthetic nectar, in doses greater than are present in the field, enhanced the survival of adult *Culex quinquefasciatus* females (Vrzal *et al.* 2010), and multi-vitamins added to a 10% sucrose or 10% glucose solution increased survivorship for adult anopheline males in some species (Phasomkusolsil *et al.* 2017). Adult *Aedes aegypti* and *Culex pipiens* survived longer when they ingested the protein-rich nectar of *Impatiens walleriana* instead of a 10% sucrose solution or other plant nectar with lower protein content (but unknown sugar content) (Chen & Kearney, 2015). Polyphenols added to the diet of *Ae. aegypti* enhanced autophagy in midgut cells, decreased midgut microbiota, and increased mosquito longevity (Nunes *et al*. 2016). Moreover, adult female *Ae. aegypti* lived longer when they consumed aqueous extracts of pollen which can be present in nectar (Todd & Vansell 1942) instead of a 10% sucrose solution, and some females even laid eggs when fed an aqueous extract of corn pollen, but not when fed a 10% sucrose solution (Eischen & Foster 1983).

Phytophagy provides mosquitoes not only with energy but also with nutrition, to an extent that they can develop fertile eggs (Eischen & Foster, 1983). Therefore, the term “phytophagy”, or “host-plant feeding”, should be used to describe the acquisition of both carbohydrates and non-energy nutrients. The relative contribution of non-energy plant nutrients to nutrient provisioning of mosquito populations is not yet known.

1.1.1. Floral nectar

Floral visitation by mosquitoes dates at least to the Cretaceous (Hartkopf-Froder *et al.* 2012) and many extant species visit a diverse array of inflorescences (see

appendix A). Floral nectar is the most important and most heavily utilized component of the phytophagous diet of adult mosquitoes (Foster 1995, 2008; Stone & Foster, 2013; Nyasembe & Torto, 2014). Volatile floral and nectar semiochemicals (message-bearing chemicals) guide mosquitoes to nectar sources (Foster 2008; Nyasembe & Torto 2014) and help them discern inflorescences with varying nectar content (Manda *et a*l., 2007; Schlein & Müller 2008; Gouagna *et al*. 2010; Nyasembe *et al*. 2018; Gouagna *et al*. 2010; Nikbakhtzadeh *et al*. 2014; Chen & Kearney 2015; Nikbakhtzadeh *et al.* 2016; Yu *et al*. 2017, 2018). However, only few floral semiochemicals that attract mosquitoes have been identified. They include alcohols, aldehydes, fatty acids, fatty acid derivatives, ketones, phenols and terpenes (Nyasembe & Torto 2014). The components that mosquitoes exploit to discern inflorescences and their nectar content are not known.

Semiochemicals are shared between plants and vertebrates (Lutz *et al.* 2017; Nikbakhtzadeh, Mahmood R Terbot *et al.* 2014; Peach *et al.* 2019b) but the underlying mechanisms of resource discrimination by mosquitoes are not known. Findings that the same set of semiochemicals guides mosquitoes to different resources (Nikbakhtzadeh, *et al.* 2014; Peach *et al.* 2019b) is evolutionarily significant. The concept that pollinators forage primarily for resources, not flowers specifically, has found support (Hoffmeister & Junker 2017) and may be applicable also to mosquitoes. Shared resource cues imply that mosquitoes may forage for resources in general, whether vertebrate or plant, and that vertebrate hosts (e.g., humans) are sometimes simply more enticing (and more rewarding) resources than others. Irrespectively, semiochemicals shared between resources cannot be resource indicators. Investigating cues that attract non-anthropophilic mosquitoes to inflorescences and to amphibian or avian hosts, or even to annelids (Reeves *et al*. 2018), may reveal the specific semiochemicals that serve as resource indicators.

Visual cues such as colour and contrast play a role for host-foraging mosquitoes, with dark colours usually being most attractive (Brown 1951, 1954; Sippell & Brown 1953; Wen *et al.* 1997; Chambers *et al*. 2013; Breugel *et al*. 2015). Visual floral cues are also thought to help attract nectar-foraging mosquitoes (Clements 1999). Light-coloured flowers were most often frequented by mosquitoes (Sandholm & Price 1962; Magnarelli 1977 1979), but the visual characteristics of those flowers were not measured. In contrast, oxeye daisies, *Leucanthemum vulgare,* placed behind glass to eliminate odor cues, failed to attract mosquitoes, whereas inflorescences in the

presence of floral scent, with and without visual cues, strongly attracted mosquitoes (Jepson & Healy 1988). In the context of host-foraging, visual cues become attractive to mosquitoes when gated by olfactory cues (van Breugel *et al*. 2015). This concept may also apply to nectar-foraging mosquitoes. In the presence of floral scent, non-occluded inflorescences of common tansy, *Tanacetum vulgare*, were more attractive to *Ae. aegypti* than occluded inflorescences (Peach *et al.* 2019b). The odor or CO_2 of a human observer may also have affected the responses of mosquitoes when they learned to associate light and dark shapes with sugar resources (Bernáth *et al.* 2016) and when they learned to prefer dark artificial flowers over light-coloured alternatives (Dieng *et al.* 2018).

The many visual cues that attract pollinators and other floral visitors include inflorescence shape, colour, and colour patterns (Orbán & Plowright 2014; Brodie *et al*. 2015). The circular "bullseye" pattern on many flowers attracts pollinators and serves as a nectar guide, orientating insects to the centre of a flower once they have arrived (Free, 1970; Dinkel & Lunau, 2001). Bullseye patterns are often present in the UV range (Horovitz & Cohen, 1972), and have been implicated in pollinator attraction in several systems (Horth *et al.* 2014; Koski & Ashmann 2014; Orbán & Plowright 2014). Many insects including mosquitoes sense UV light (Muir *et al.* 1992; Briscoe & Chittka 2001; Shimoda & Honda 2013) and can read the pattern of UV-absorptive and -reflective petals.

Host body heat is a well known close-range attractant to host-foraging mosquitoes (Bowen 1991; Olanga *et al.* 2010; van Breugel *et al.* 2015; Zermoglio *et al.* 2017), suggesting that floral heat too may affect nectar-foraging mosquitoes. A variety of mechanisms, including thermogenesis (Seymour & Schultze-Motel 1997), focusing solar radiation (Hocking & Sharplin 1965), and heat production by microbial metabolism of floral nectar (Herrera & Pozo 2010), all enable inflorescences to become and stay warmer than their environment. Elevated inflorescence temperatures increase respiration and CO_2 production (Seymour *et al*. 2003, 2015; Seymour & Matthews 2006), enhance semiochemical dissemination (Meeuse & Raskin 1988), and generate a direct energy reward for pollinators (Seymour *et al.* 2003). Mosquitoes have been observed basking in the warm centres of heliotropic paraboloid-shaped flowers in the Canadian high arctic (Hocking & Sharplin, 1965), lending support to the concept that they do respond to thermal inflorescence cues.

CO_2 is another potential cue for plant-foraging mosquitoes. When diurnal photosynthesis ceases at dusk, plants become net CO_2 producers (Allen 1971; Amthor 2000; Chapman et al. 1954). While this transition occurs during the peak plant-foraging time of many mosquito species (Andersson & Jaenson 1987; Clements 1999), their activity is still thought to be endogenously regulated (Clements 1999). Vegetative CO_2 emission also results from increased respiration during thermogenesis (Seymour *et al.* 2003; Seymour & Matthews 2006). Whether the rhythmic CO_2 pulses from some orchids (Hew *et al.* 1978) enhance attraction of mosquitoes is not known.

1.2. Plant-derived food sources sought and consumed by mosquitoes

1.2.1. Floral nectar

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1.2.2. Extrafloral/extrasoral nectaries

Extrafloral/extrasoral nectaries (EFNs) too provide sugar for mosquitoes but their visitation is harder to track than floral visitation. Nonetheless, there are many reports of mosquitoes feeding from EFNs. EFNs provide a survival benefit to mosquitoes (Gary & Foster 2004), but little is known about the cues that attract mosquitoes to EFNs.

EFN semiochemicals attract parasitoid wasps (Röse *et al.* 2006; Géneau *et al*. 2013). Of six headspace volatiles (benzaldehyde, benzyl alcohol, linalool, 1-octanol, two

unknowns) originating from EFN nectar of Fava bean, *Vicia faba,* most were also found in leaves, but one of the two unknowns was specific to EFN nectar (Hoffmeister & Junker 2017). Benzaldehyde, benzyl alcohol, and linalool are floral odorants attractive to mosquitoes (Jhumur *et al.* 2007; Yu *et al.* 2015), whereas 1-octanol causes flight aversion (von Oppen *et al.* 2015). Visual cues associated with EFNs are speculated to add to the attractiveness of EFNs (Hoffmeister & Junker 2017).

1.2.3. Fruit and seedpods

The fruit-feeding behaviour of mosquitoes has been known or suspected for a long time (Swammerdam 1758). In the late 19th and early 20th centuries, many mosquito researchers fed their laboratory mosquito colonies on various fruit such as apples, bananas, pear, plum, dates, and wet raisons (Smith 1904; Howard *et al.* 1912; Bates 1949; Chapman 1962). Other authors reported field observation of mosquitoes feeding on fruit, including apples (Joseph 1970; Theobald 1901), grapes, peaches and watermelons (Joseph 1970), and possibly poke berries (Joseph & Bickley, 1969 in Joseph 1970). Traps baited with cantaloupe, *Cucumis melo cantalupensis*, did capture mosquitoes but much fewer than in CO_2-baited traps (Reisen *et al*. 1986). In semi-field conditions, mango and guava nectar were generally not very effective attractants for *Ae. aegypti* but did attract small numbers of male mosquitoes (Fikrig *et al*. 2017).

The predilection for wild mosquitoes to feed on damaged, decaying or fermenting fruit (Theobald 1901; Joseph 1970) has been validated by several studies. In Israel, *Aedes albopictus*, *Cx. pipiens*, and *Cx. perexiguus* were observed feeding on fermenting liquid from seed pods of the carob tree, *Ceratonia siliqua*, previously damaged by moth larvae (Müller *et al*. 2010). Some individuals pierced plant tissue to feed, while others fed on over-ripe, damaged sabra, *Opuntia ficus-indica*. Field experiments revealed mosquito attraction to damaged *C. siliqua* seed pods with fermenting liquid but not to intact pods, as well as to damaged pomegranate, *Punica granatum*, and to intact *O. ficus-indica*, *Ficus carica*, *Eriobotyra japonica*, and *Rubus sanctus* (Müller *et al.* 2011). *Culex. pipiens pallens* did feed on seed pods of white pear, *Pyrus bretschneideri*, and paper mulberry, *Broussonetia papyrifera*, but lived longer when feeding on select experimental flowers, and when feeding on *P. bretschneideri* seedpods instead of on a sucrose solution (Yu *et al*. 2016). In a follow-up study, mosquitoes discerned between several decaying fruit and seedpods (Yu *et al.* 2017). Females of *Cx. pipiens pallens* showed the greatest

preference for decaying seed pods of *B. papyfera*, whereas males showed comparable preference for *B. papyfera* and decaying fruit of peach, *Amygdalus persica*, and melon, *Cucumis melo*. Artificial apple and cherry scents were attractive to *Ae. aegypti* (D.A. Carlson, unpubl. data, in Foster & Hancock, 1994) and synthetic strawberry flavouring was used as a mosquito attractant (Yee & Foster 1992). Oranges and watermelons were as attractive to *An. arabiensis* as a 10% sucrose solution (Tenywa *et al*. 2017).

Fresh or rotting/overripe mango, guava, honey melon, plums, nectarines, prickly pear cactus, as well as red wine and millet beer, have all been used in attractive toxic sugar baits (Fikrig *et al.* 2017; Fiorenzano *et al.* 2017; Scott-Fiorenzano *et al*. 2017) which are designed to attract and kill mosquitoes. Whether the semiochemicals that attract mosquitoes originate from these resources themselves or from resource-dwelling microbes is not known but would make an intriguing study. Moreover, the semiochemicals mediating the attraction of mosquitoes remain largely unknown, as do the semiochemicals causing differential attraction of specific mosquito species (Fikrig *et al.* 2017).

1.2.4. Plant tissues

Mosquitoes have been observed to occasionally feed directly on the tissue of damaged plants (de Meillon *et al.* 1967; McCrae *et al*. 1969; Foster 1995; Stone & Foster 2013) and intact plants (Schlein & Müller 1995; Qualls *et al*. 2013), obtaining sugar and non-energy nutrients from tissue fluids or the phloem sap (Stone & Foster 2013). This behaviour may occur only when other resources are not available (Muller & Schlein, 2005), or when plants are injured or stressed (Stone & Foster 2013). Although plant tissue is not an ideal diet, it is widely available and can provide sufficient nutrition for a female mosquito to survive sufficiently long to complete at least one gonotrophic cycle (Qualls *et al.* 2013). The underlying mechanisms that attract mosquitoes to plant tissue are barely understood but recent electrophysiological recordings revealed several classes of plant chemicals that may be involved as well as species-specific sensitivity of mosquitoes to these chemicals (Nyasembe *et al.* 2018). Differential odour profiles of damaged and intact plants (Smith & Beck 2013; Beck *et al*. 2015; Copolovici & Niinemets 2016), and of water-stressed and well-watered plants (Copolovici & Niinemets 2016; Salerno *et al*. 2017), may help mosquitoes select and feed on damaged or stressed plants (Junnila *et al.* 2010; Stone & Foster 2013), which may offer more

nutritional benefits to mosquitoes than healthy plants (Nunes *et al.* 2016). Applying molecular techniques, such as DNA barcoding, to identify plant genes in mosquito samples, holds great promise to determine the type of plant tissue mosquitoes feed on as well as the relative frequency of their plant feeding (Nyasembe *et al.* 2018).

1.2.5. Honeydew

Honeydew is excreted by aphids, coccids, and other hemipterans feeding on plant sap. It is a food-source exploited by many insects including ants, honeybees (Auclair 1963), and mosquitoes (Haegar 1955; Burkett *et al.* 1999; Russell & Hunter 2002; Gary & Foster 2004). Both honeydew and floral nectar contain various sugars and amino acids (Auclair 1963; Hussain *et al.* 1974; Blüthgen *et al.* 2004; Pozo *et al.* 2014). The composition of honeydew varies with the species and the age of the insect producing it, and the host plant it is feeding on (Fischer *et al*. 2002; Blüthgen *et al.* 2004; Pringle *et al*. 2014).

Captures of mosquitoes in a sand pine, *Pinus clausa*, infested with aphids, but not in another nearby pine void of aphids, were attributed to honeydew on the infested pine (Clouse *et al*. 1997). In Florida, *Ae. taeniorhynchus, Ae. sollicitans*, and *An. atropos*, were all observed feeding on honeydew from unspecified aphids residing on the leaves of Spanish needle, *Bidens* spp*.* (Haegar 1955). In North Central Florida, almost 60% of wild-caught *Anopheles quadrimaculatus* and 31% of *Culiseta melanura* tested positive for honeydew-feeding (Burkett *et al*. 1999), whereas in Canada aedine mosquitoes had little evidence for honeydew-feeding (Russel & Hunter 2002). Intriguingly, neurones on the labella of *An. gambiae* can sense melezitose (Kessler *et al.* 2015), a main sugar component of some honeydews (Fischer & Shingleton 2001; Blüthgen *et al.* 2004).

Honeydew odorants guide many insects to honeydew itself or the insect expelling it (e.g., Hung *et al*. 2015). Odorants in honeydew expelled by scale insects on New Zealand's South Island attract the common yellow jacket, *Vespula vulgaris*, which is invasive in New Zealand (Brown *et al.,* 2015). Microbes dwelling in aphid honeydew produce semiochemicals that attract the predatory hoverfly *Episyrphus balteatus* (Leroy *et al.,* 2011). Field observations that neither *Culex pipiens* nor *Aedes albopictus* responded to honeydew-soiled plants (Schlein & Müller 2008; Müller *et al*. 2011) were

attributed to exogenous microbes and their semiochemicals still absent from that honeydew. A role of microbe-derived honeydew semiochemicals was evident in a recent study (Peach *et al*. 2019a) showing that synthetic semiochemical blends of microbe-infested honeydew were more attractive than those of sterile honeydew. The same study also demonstrated anemotactic attraction of *Ae. aegypti* to bean plants, *Vicia faba,* soiled with honeydew from pea aphids, *Acyrthosiphon pisum,* and green peach aphids, *Myzus persicae.* Several types of honeydew may have more nutritional value than certain types of floral nectar to at least some mosquito species. *Anopheles gambiae* survived better on mealybug honeydew than on floral and extra-floral nectar of several plants (Gary & Foster 2004), and *Cx. quinquefasciatus* survived longer on aphid-infested plants than on aphid-free plants (Patterson *et al.* 1969). In the diet of some mosquitoes, honeydew may play a particularly important role because it may be available at times when widely-used sources of plant-derived food, such as floral nectar, are absent. The nutritional benefit honeydew provides, its attractiveness relative to other plant-derived food sources, and the circumstances that prompt its consumption are all yet to be studied.

1.2.6. Ant regurgitate

As a form of remarkable kleptoparasitism, regurgitate of *Cremastogaster* spp. ants becomes a food source for *Malaya* spp. mosquitoes (Clements, 1999). When a female mosquito inserts her proboscis into the mouth of an ant, she induces trophallaxis and then feeds upon the ant's regurgitate (Edwards 1932). Repeated encounters and mutual disturbance of *Hodgesia* mosquitoes and ants at damaged-plant-tissue feeding sites (McCrae *et al.* 1969), or mosquito consumption of honeydew in the presence of ants (Clouse *et al.* 1997), may indicate events or circumstances that have given rise to the evolution of this form of kleptoparasitism. Whether also mosquito males kleptoparasitize ants, and the cues mosquito females exploit to locate these ants, has yet to be investigated.

1.2.7. Sweet food waste

Consumption of sweet food waste by laboratory-reared mosquitoes (Dieng *et al.* 2017) increased their longevity (Dieng *et al.* 2017). The phenomenon is analogous to wild mosquitoes feeding on honeydew or on damaged plant tissue (de Meillon *et al.*

1967), and indicates that proper sanitation and disposal of food waste is one tactic to help curtail mosquito populations.

1.2.8. Summary

Mosquitoes commonly exploit plant semiochemicals to locate plant-based food sources. Foraging mosquitoes also respond to visual plant cues (e.g., floral UV pattern), vegetative CO_2 and thermal inflorescence cues. Plant-based food sources most attractive to mosquitoes offer often, but not always, rich rewards of sugar or non-energy nutrients (Chen & Kearney 2015; Nikbakhtzadeh *et al.* 2016; Yu *et al.* 2016). Neither the airborne semiochemicals that attract mosquitoes to plant resources nor the non-volatile phagostimulants that induce probing and feeding have been intensely studied. Plant semiochemicals that effectively attract mosquitoes in the laboratory may not be equally effective in more complex field settings.

1.3. Do mosquitoes have a functional role as pollinators?

Floral visitation by mosquitoes is wide-spread and well-documented (Foster 1995). However, the functional role of mosquitoes visiting inflorescences has hardly been studied. Mosquitoes are considered nectar thieves (consuming nectar without transferring pollen), nectar robbers (piercing through inflorescences to access nectar; Inouye 1980), or legitimate pollinators. As nectar thieves and nectar robbers, mosquitoes have adverse impact on the reproductive fitness of plants *(Irwin et al*. 2010; Zhang *et al.* 2014).

1.3.1. Instances of nectar-theft

Claims that mosquitoes are floral nectar thieves are supported only by few observations (Otienoburu *et al.* 2012; Pansarin & Pansarin 2017; Smith & Gadawski 1994). *Aedes provocans* feeding on nectar of pin cherry, *Prunis pensylvanica*, was deemed nectar theft because they hardly accumulated pollen on their body and failed to contact floral pistals (Smith & Gadawski 1994). Mosquitoes feeding on common milkweed, *Ascelpias syriaca,* and on the orchid *Epidendrum avicula*, were also considered nectar thieves due to their small body size (Otienoburu *et al.* 2012; Pansarin & Pansarin 2017). Mosquitoes were observed nectar robbing the stinking-bean trefoil,

Anagyris foetida (Ortega-Olivencia *et al.* 2005), and possibly the creeping thistle, *Cirsium arvense* (Britten 1937).

1.3.2. Evidence for pollination

Pollination by mosquitoes is unequivocal. According to various studies, *Cx. pipiens* transfer pollen between inflorescences of the mosquito flower, *Lopezia racemosa* (Müller 1873); *Cx. pipiens* and *Cs. annulata* pollinate the Spanish catchfly, *Silene otites* (Brantjes & Leemans 1976); *Cx. pipiens* pollinate the common tansy, *Tanacetum vulgare* (Peach & Gries 2016), yarrow, *Achillea millefolium* (D.P., unpubl. data), and carry pollen of Canada goldenrod, *Solidago canadensis*, which may also be pollinated by *Cx. tarsalis* and *Cs. incidens* (Peach & Gries 2016).

The small northern bog orchid, *Platanthera obtusata,* is pollinated by *Aedes* spp. (Raup 1930; Twinn *et al*. 1948; Hocking *et al.* 1950; Stoutamire 1968; Thien 1969; Thien & Utech 1970; Gorham 1976), taxonomically unspecified mosquitoes (Dexter 1913), and by *Ae. campestris* in the Yukon Territory (D.P., pers. obs. 2017). *Aedes* spp. also pollinate other orchids including the palegreen orchid, *Pl. flava* (Luer 1975; Stoutamire 1971), the northern green orchid, *Pl. hyperborea* (D. Saville, pers. comm. in Catling & Catling 1991), and possibly the slender bog orchid, *Pl. stricta* (Patt *et al.* 1989). Moreover, *Aedes spp.* along with *An. anulipes* and possibly *Culex spp.* pollinate the green labellum orchid, *Pterostylus procera* (Bartareau & Jackes, 1994), possibly the nodding greenhood orchid, *Pt. falcata* (Coleman 1934; Hyett 1960), as well as the pointed greenhood orchid, *Pt. acuminate* (Coleman 1934). Orchids in New Zealand are visited by small taxonomically unspecified Culicidae (Thomson 1927). *Aedes* spp., probably *Ae. impiger* and *Ae. nigripes* (Hocking & Sharplin, 1965; Wood *et al.* 1979), contribute to the pollination of the white mountain-avens, *Dryas integrifolia,* in the Canadian high arctic (Kevan 1972). *Culex* spp. and *Armigeres* spp. were deemed exclusive pollinators of *Burmannia lutescens*, *Gnetum cuspidatum*, and *Sciaphila secundiflora* because of a morphological congruence between their proboscis and the corolla tube length of these plants (Kato 1996).

Conceptually, the pollination function of mosquitoes may take one of three forms. Mosquitoes may be (1) somewhat specialized pollinators or co-pollinators together with small moths; (2) co-pollinators together with other dipterans (myophily), and (3)

generalist pollinators. Mosquitoes are exclusive pollinators of *B. lutescens* (Kato 1996). Together with moths, they co-pollinate the orchids *Pl. obtusata* and *Pl. flava* (Stoutamire 1968; Voss & Riefner 1983) and the catchfly *S. otites* (Brantjes & Leemans 1976). Together with flies they co-pollinate the mosquito flower, *L. racemosa* (Müller 1873; Eyde & Morgan 1973), the short-lipped greenhood, *Pt. procerea* (Bartareau & Jackes, 1994), and *S. secundiflora* (Kato 1996). As generalist pollinators, mosquitoes together with many other insects contribute to the pollination of tansy, *T. vulgare* (Peach & Gries 2016), yarrow, *A. millefolium* (DP, unpubl. data), and *D. integrifolia* (Kevan 1972).

Interestingly, some mosquito-pollinated orchids are visually inconspicuous and scentless to humans. Both mosquitoes and lepidopterans can sense CO_2 which some orchids emit in rhythmic pulses (Hew *et al.* 1978). CO_2 pulses might serve as foraging cues to mosquitoes visiting *P. obtusata* (Stoutamire 1968). CO_2 also enhanced attraction of *Ae. aegypti* to tansy odorants (Peach *et al*. 2019b). Growing gregariously may be advantageous for plants as their mosquito or lepidopteran pollinators can access them by short flights or walks (Brantjes & Leemans 1976).

1.3.3. Summary

Mosquitoes are nectar thieves but also pollinators for many plants. Compared to other insects, mosquitoes may be less effective at carrying and transferring pollen, but by virtue of large numbers they may assume an important pollination role (Larson *et al.* 2001).

1.4. Predation risk of plant-foraging mosquitoes

Blood-feeding mosquitoes are often killed by their vertebrate hosts (Corbet & Downe 1966; Edman & Kale 1971; Edman *et al.* 1984) but nectar-feeding mosquitoes too are subject to increased predation risk. Predators such as goldenrod crab spiders, *Misumena vatia*, ambush mosquitoes visiting flowers (Peach & Gries, 2016). Predators have both a direct and an indirect impact on pollinators in that they reduce their numbers and modulate the energy they invest in predator avoidance (Reader *et al.* 2006), thereby possibly reducing their fitness (Reader *et al.* 2006).

1.5. The role of microbes in attracting mosquitoes to plant resources

Insect-microbe inter-kingdom signalling is widespread (Davis *et al.* 2013) and also involves mosquitoes. Mosquitoes respond to microbial semiochemicals or CO_2 when they seek vertebrate hosts (Verhulst *et al*. 2009, 2010; Busula *et al*. 2017; Takken & Verhulst 2017), floral nectar (DP, unpubl. data), aphid honeydew (Peach *et al.* 2019b) and oviposition sites (Ponnusamy *et al.* 2008).

Microbes commonly inhabit inflorescences (Endo *et al*. 2011; Aleklett *et al.* 2014; Ushio *et al.* 2015) and their nectar (Álvarez-Pérez *et al.* 2012; Fridman *et al.* 2012), and produce semiochemicals that help attract insect pollinators (Pozo *et al.* 2014; Rering *et al.* 2018). For instance, the presence of the nectar specialist and nectarivorous yeast *Metschnikowia reukaufi* increases the number of bumblebee visits to inflorescences of the stinking hellebore, *Helleborus foetidus* (Herrera *et al.* 2013). Odorants of *M. reukaufi* alter the floral scent composition of the sticky catchfly, *Silene caroliniana* (Golonka *et al.* 2014). 3-Methyl-1-butanol as one of these microbial attractants is also produced by the human skin microbe *Staphyloccocus epidermis* (Verhulst *et al.* 2009, 2010). Inflorescence-dwelling microbes also generate heat (Herrera & Pozo 2010) and CO_2 (Smallegange *et al.* 2010) which are both attractive to mosquitoes.

Microbe-mediation is likely also responsible for the attraction of mosquitoes to rotting and fermenting fruit (Theobald 1901; Joseph 1970; Müller *et al*. 2010; Müller *et al.* 2011; Yu *et al.* 2017), and to fruit previously been fed upon by hymenopterans (Joseph 1970) that vector semiochemical-emitting microbes between food sources (Davis *et al.* 2012).

Metabolites and semiochemicals of microbes dwelling in or on nectar, pollen, honeydew, fruit, or other types of host-plant food could inform mosquitoes about the nutritional quality of a resource. Mosquitoes can acquire microbes from floral nectar or floral nectar surrogates (Maier *et al*.1987; Kenney *et al*. 2017) and transmit them between nectar-sources (Kenney *et al.* 2017), as many other insects do (Ushio *et al.* 2015).

1.5.1. Summary

Microbe-derived semiochemicals guide mosquitoes to vertebrate hosts, floral nectar, aphid honeydew and suitable oviposition sites. Few studies have addressed "signalling" between plant-dwelling microbes and mosquitoes.

1.6. The evolution of haematophagy in mosquitoes

Haematophagy by insects is thought to have arisen multiple independent times (Lehane 2005) and to have evolved from either entomophagy or phytophagy involving an association between either ancient insect prey and vertebrates, or plant matter and vertebrates (Lehane 2005). This association is further thought to have eventually led to accidental feeding on vertebrates, subsequent physiological adaptation by mosquitoes to process blood meals, and finally to the evolution of associations between the now haematophagous mosquitoes and their vertebrate hosts (Lehane 2005).

According to the rare field observations of mosquitoes engaging in entomophagy, mosquitoes fed on a cicada, the chrysalis of a butterfly, and on small dipterans (Howard *et al*. 1912). However, Downes (1958) considers the former two instances accidental and the latter a misinterpretation of Hagen (1883). According to another field report (Eliason 1963), *Culex tarsalis* females fed on the dry remains of an insect that had impacted on a car window. Entomophagy by female mosquitoes has more often been observed in the laboratory. Females of *Ae. aegypti* and *Cx. tarsalis* feeding on various soft-bodied lepidopteran larvae experienced mixed effects on their survival and egg development (Harris & Cooke 1969; Harris *et al.*1969). In Y-tube-olfactometer bioassays, female but not male *An. stephensi* were attracted to insect larvae, likely in response to larval respiratory CO_2 (George *et al.* 2014; Martel *et al.* 2011).

Anthophilous nematocerans such as early mosquitoes were possible pollinators of primitive angiosperms (Labandeira 1997; Larson *et al.* 2001). Fossil evidence of floral visitation by mosquitoes in the mid-Cretaceous (Hartkopf-Froder *et al.* 2012), and genetic evidence for rapid radiation in mosquito diversity corresponding with the appearance and radiation of angiosperms (Reidenbach *et al.* 2009), all suggest an ancient relationship between mosquitoes and plants. Phytophagy (e.g., consumption of host-plant nectar, fruit, tissue) is considered one possible diet from which haematophagy

evolved in mosquitoes (Foster 1995; Lehane 2005; Mattingly 1965; Pawlowski *et al.* 1996), and possibly other haematophagous nematoceran dipteran families (Mattingly 1965). The elongate mouthparts of mosquitoes may have first arisen as a means of reaching the base of tubular corollas to obtain nectar (Foster, 1995; Larson, Kevan & Inouye, 2001). Primarily frugivorous noctuid moths (*Calyptera* spp.) appear to be in the process of evolving haematophagy (Bänziger 1975, 1979; Zaspel *et al.* 2007; Hill *et al*. 2010; Zaspel *et al*. 2012). This evolutionary process may be linked to differences in sensillum numbers between haematophagous and non-haematophagous individuals and chemoselectivity towards vertebrate-related odorants (Hill *et al.* 2010).

Plant-feeding mosquito ancestors that possessed elongate sucking mouthparts would have been pre-adapted to haematophagy, requiring only an impetus to be in continual association with vertebrate hosts and to accidentally bite them (Lehane 2005). Attractive odorants shared between floral and vertebrate headspaces, as well as CO_2 being a resource indicator of both vertebrate hosts (Gillies 1980) and floral nectar (Peach *et al.* 2019b), all provide evidence of intriguing overlap in those cues that mosquitoes exploit to locate food plants and vertebrate hosts. This overlap in foraging cues may have been a contributory cause for the shift from phytophagy to haematophagy and may also support the argument that phytophagy pre-empted haematophagy in ancient mosquitoes or their ancestors (Peach *et al.* 2019b).

The ability of female mosquitoes feeding on laboratory-reared lepidopteran larvae to develop and lay eggs (Harris & Cooke 1969) has received much attention. However, these females were provisioned with a sugar source in the form of honey water and honey water controls were not run. Furthermore, many mosquito species in the laboratory or field require a meal of plant fluids to maximize egg production or even to develop eggs (O'Meara 1987). Moreover, when *Ae. aegypti* females were provisioned with pollen, or an aqueous extract thereof, they were able to develop and lay eggs without consuming vertebrate blood (Eischen & Foster 1983).

The ancient mecopteran-like insects currently believed to be the ancestors of the diptera possessed mandibular mouthparts and may have been entomophagous (Waage 1979), comparable to the modern-day entomophagous insects that also feed on aphid honeydew or nectar from extra-floral nectaries (Heil 2015; Way 1963). Fossil records of early mosquitoes are sparse (Borkent & Grimaldi 2004, 2016; Briggs 2013; Poinar *et al.*

2000) and lack useful information, although it does seem that vertebrate blood-feeding mosquitoes existed at least 46 million years ago (Greenwalt *et al.* 2013). The appearance of lepidopterans in the fossil record prior to the currently accepted arrival date of angiosperms (Eldijk *et al.* 2018) also raises the intriguing possibility that nectar-like substances may have been sufficiently common to allow for adaptive radiation based on plant-derived food-sources prior to the appearance of floral nectaries.

Changes in dietary regimes in mosquitoes may be the result not of single but multiple transitions, such as from entomophagy to phytophagy and then to combined phytophagy and haematophagy. They may also include the loss of adult feeding, as seen in some sister taxa of the Culicidae (Grimaldi & Engel 2005), and subsequent re-acquisition of adult feeding, possibly in different dietary regimes. Ultimately, additional fossil specimens are needed to fully elucidate mosquito evolution.

1.6.1. Summary

Haematophagy in mosquitoes likely evolved from either entomophagy or phytophagy. Entomophagy by female mosquitoes has been observed in the laboratory but not in the field. Mosquito phytophagy is ancient. Overlap in vertebrate host and floral cues that foraging mosquitoes exploit to locate resources may be part of the underlying mechanisms that facilitated the adoption of haematophagy to the phytophagous diet.

1.7. Conclusion

Many aspects of the phytophagous foraging ecology of mosquitoes remain unexplored or underexplored. Field studies ought to investigate (*i*) the interaction between mosquitoes and plants (e.g., pollination), (*ii*) the effect of phytophagy on the vectorial capacity of mosquitoes, (*iii*) the mechanisms by which mosquitoes discern sources of plant-derived nutrition, and (*iv*) the semiochemical and visual cues that attract mosquitoes to these resources. As mosquitoes are not monolithic, a better understanding of species-specific foraging tactics and dietary needs may tailor and optimize efforts for mosquito control. We should also acknowledge that mosquitoes are often viewed through an anthropocentric lens that is focused on their haematophagy and disease transmission. Adopting the paradigm that mosquitoes are first and foremost

phytophagous may offer new avenues for research and ultimately control of mosquito populations.

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Chapter 2.

Nectar thieves or invited pollinators? A case study of tansy flowers and common house mosquitoes[1]

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Introduction

Floral visitation by nectar-foraging mosquitoes is well documented (Andersson & Jaenson 1987; Grimstad & DeFoliart 1974; Magnarelli 1977, 1978, 1983; Sandholm & Price 1962). Hundreds of nectar-feeding mosquitoes have been collected around specific plants over just a few nights (Andersson & Jaenson 1987; Yee *et al.* 1992). Despite all these accounts, mosquitoes are generally considered nectar thieves (Foster 1995; Foster & Hancock 1994; Inouye 2010; Smith & Gadawski 1994; Zhang *et al.* 2014) that consume nectar as legitimate pollinators do but without effectively transferring pollen between inflorescences (Inouye 1980), essentially ‘‘cheating’’ by reaping rewards without providing any pollination service. In a primary account of mosquitoes being nectar thieves, Smith & Gadawski (1994) report that early-spring mosquitoes, *Aedes provocans*, rarely carry pollen when they visit flowers and consume nectar of Canada plum, *Prunus nigra*, and pin cherry, *P. pensylvanica*. Similarly, mosquitoes are deemed not able to transfer pollen of the common milkweed, *Asclepias syriaca* (Otienoburu *et al.* 2012), a plant they are known to visit (Grimstad & DeFoliart 1974). Mosquitoes have also been observed nectar robbing which involves accessing floral nectar through a hole in the side of the flower, thus avoiding the acquisition or deposition of pollen (Inouye 1980). This form of floral larceny by mosquitoes has been observed in the stinking bean trefoil, *Anagyris foetida* (Ortega-Olivencia *et al.* 2005), and may also occur in the creeping thistle, *Cirsium arvense* (Britten 1937).

Floral visitation by nectar thieves or other floral larcenists can impact the reproductive fitness of plants through direct or indirect effects (Irwin *et al.* 2010), including fewer floral visits and pollen deposits by legitimate pollinators (Irwin *et al.* 2015; Irwin & Brody 1999; Norment 1988), changes in the behaviour of pollinators such as longer floral visits (Lara & Ornelas 2001, 2002; Zhang *et al.* 2014), and shifts in the

composition of pollinator communities (Hazlehurst & Karubian 2016). Fewer visits by legitimate pollinators may adversely affect plant fitness (Irwin & Brody 1999), whereas prolonged visits may have a positive effect on plant fitness (Lara & Ornelas 2002).

The alternative concept that mosquitoes are pollinators rather than nectar thieves is supported by several reports, as follows: (1) *Cx. pipiens* transferring pollen of the mosquito flower, *Lopezia racemosa* (Müller 1873); (2) *Cx. pipiens* along with *Culiseta annulata* and some nocturnal lepidopterans contributing to the pollination of the Spanish catchfly, *Silene otites* (Brantjes & Leemans 1976); (3) *Aedes* spp. pollinating the small northern bog orchid *Platanthera obtusata* (Stoutamire 1968; Thien 1969), the palegreen orchid *Pl. flava* (Luer 1975; Stoutamire 1971), the Northern green orchid *Pl. hyperborea* (D. Saville, pers. comm. in Catling & Catling 1991), and possibly the slender bog orchid *Pl. stricta* (Patt *et al.* 1989); (4) *Aedes* spp. adding to the pollination of white mountain-avens, *Dryas integrifolia* (Kevan 1972); (5) unspecified mosquitoes transferring pollen of the Asian skunk cabbage *Symplocarpus renifolius* (Uemura *et al.* 1993); and (6) *Anopheles annulipes*, *Aedes* spp., and probably *Culex* spp. pollinating the green labellum orchid *Pterostylus procera* (Bartareau & Jackes 1994), some of which probably also pollinating the nodding greenhood orchid *Pt. falcata* (Coleman 1934; Hyett 1960) as well as the pointed greenhood orchid *Pt. acuminata* (Coleman 1934).

These reports made us wonder whether mosquitoes, in general, are really just nectar thieves. In the light of field observations that *Cx. pipiens* visit tansies, *Tanacetum vulgare* (Andersson & Jaenson 1987; Ardo 1968; Bro-Larsen 1948), we tested the hypothesis that *Cx. pipiens* indeed pollinate *T. vulgare*.

2.1. Materials and methods

2.1.1. Rearing of experimental mosquitoes

We reared *Cx. pipiens* at temperatures of 22–26 C, 40–60 % RH, and a photoperiod of 14L:10D. We maintained mixed groups of males and females in mesh cages (30 x 30 x 46 cm high) provisioned ad libitum with a 10 % sucrose solution. DP fed females once a week on his arm (males do not take blood meals), after which they were given access to a glass dish (10 cm diameter x 5 cm high) containing circa 300 mL of water in which to oviposit. We transferred egg rafts to water-filled trays (45 x 25 x 7

cm high), provisioned larvae with NutriFin Basix tropical fish food (Rolf C Hagen Inc., Montreal, Canada), and transferred pupae via a 7-mL plastic transfer pipette (VWR International, Pennsylvania, USA) to water-containing, 354-mL cups (Solo Cup Company, Illinois, USA) covered with a mesh lid. We collected emergent adults via aspirator and kept them for 1–3 days prior to bioassays in similar cups that we provisioned with a moist cotton wick (Richmond Dental Supplies, North Carolina, USA) but no food.

2.1.2. Field collection of flower-probing mosquitoes

During July and August 2015, we collected mosquitoes on the Burnaby campus of Simon Fraser University (SFU). We chose three densely stocked patches of tansy, *T. vulgare* (each patch circa 30-m^2), one thinly stocked patch of ox-eye daisy, *Leucanthemum vulgare* (approximately 12 plants over 30-m^2), and one 50-m^2 patch of yarrow, *Achillea millefolium*. All patches were sufficiently small and in close proximity to each other, allowing us to sample the entirety of each patch, proceed to the next, and repeat the process several times for the duration of each sampling survey. We ran sampling surveys during the scotopic periods of (1) 00:00–01:00 h on each of 31 July and 04 August, (2) 22:30–23:00 h on each of 11 and 18 August, and (3) 21:00–22:00 h on 22 August. Illuminating plants with a headlight, we aspirated mosquitoes while they were probing for nectar and then placed each mosquito in a separate sealable sandwich bag, which we stored in a cooler on ice packs. After terminating each survey, we transferred the bags into a freezer for overnight storage and examined all specimens the following day. Using a microscope and applying guidelines provided by Wood *et al*. (1979), Belton (1983), and Thielman & Hunter (2007), we identified each mosquito to species and recorded the number and distribution of pollen grains found on its body.

2.1.3. Pollen abundance and distribution on mosquito bodies

To investigate further whether mosquitoes indeed accumulate pollen grains on their bodies while visiting an inflorescence (cluster of composite flowers arranged on a stem; Fig. 1), we exposed mosquitoes to blooming inflorescences in the laboratory. Specifically, we placed a mixed-sex group of 60 *Cx. pipiens* in a mesh cage (30 x 30 x 46 cm high) that enclosed two field-collected inflorescences of either *T. vulgare* or *A. millefolium*, or one inflorescence of *Solidago canadensis*. All inflorescences were in peak

bloom (with many but not all florets open) and were stored in an 80-mL beaker of water. After 2 h of exposure, we removed the inflorescences and placed the cage overnight in a freezer to kill the mosquitoes. The next day, we examined the mosquitoes under a microscope, recording the number and distribution of pollen grains on their bodies.

2.1.4. Pollination experiments

We obtained seeds of *T. vulgare* from Richters Herbs Nursery (Otto Richter and Sons Limited of Goodwood, Ontario, Canada) and raised plants in a greenhouse at SFU, watering them every 2–4 days and fertilizing them once a week.

We ran two pollination experiments (Fig. 2.1). Experiment 1 consisted of three treatments: (1) cross-pollination, (2) self-pollination, and (3) a blank control. For the cross-pollination treatment, we inserted 60 mixed-sex mosquitoes into a cage (30 x 30 x 46 cm high) and gave them access for 2 h to two inflorescences (each with 20–50 % of their florets open) that we previously had (1) excised from greenhouse-grown or in situ field plants (based on availability) and (2) kept in water-filled 4-mL vials. After 2 h of access, we transferred the mosquitoes to, and kept them for 48 h in, a pollinator exclusion bag (3.78 L) enclosing one inflorescence of another living (potted) greenhouse-grown plant in full bloom. For the self-pollination treatment, we kept a mixed-sex group of 60 mosquitoes without prior exposure to any inflorescence in a pollinator exclusion bag (3.78-L) enclosing one inflorescence of a living (potted) greenhouse-grown plant in full bloom. The control treatment consisted of one inflorescence of a living (potted) greenhouse-grown plant in a pollinator exclusion bag without mosquitoes present. We did not provide mosquitoes with water or sugar water in any of these treatments. We collected inflorescences from treatment and control bags after seed set and counted the resulting seeds under a microscope.

Pollination experiment 2 (Fig. 2.1) consisted of a modified cross-pollination treatment and a control treatment. For the cross-pollination treatment, we kept a mixed-sex group of 60 mosquitoes without water or sugar water for 3 days in a pollinator exclusion bag (3.78-L), enclosing one experimental inflorescence of a living (potted) greenhouse-grown plant and two inflorescences excised from a different plant, retaining each cut inflorescence in a water-filled, 20-mL vial. The control treatment was identical

except that mosquitoes were absent from the pollinator exclusion bag. We counted seed set of experimental inflorescences from treatment and control bags under a microscope.

2.1.5. Statistical analyses

We used JMP version 11 (SAS Institute Inc.) for data analysis. We analysed data of pollination experiment 1 with a single-factor, complete randomized design ANOVA followed by Tukey's honest significant difference (HSD) test, and data of pollination experiment 2 by the Student's t-test.

2.2. Results

2.2.1. Field collection of flower-probing mosquitoes

We field-collected a total of 182 mosquitoes (49 females and 133 males) while probing inflorescences. Twenty-seven percent of these mosquitoes (12 females and 37 males) carried pollen (Table 2.1), which was identified as Anthemis type Asteraceae pollen (Moore *et al.* 1991). Pollen was present on eight out of 39 female and 33 out of 125 male *Cx. pipiens*, one out of two male *Cx. tarsalis*, and one out of six female *Culiseta incidens* collected from *T. vulgare* (Table 2.1). Two female *Cx. pipiens* (one carrying pollen) were engorged with old blood, and 10 female and three male *Cx. pipiens* were replete with a ''clear fluid'', which was likely nectar. However, these samples are biased toward mosquitoes that were on inflorescences and thus are not representative of the general population of mosquitoes in the field.

We recorded as many as 39 grains of pollen on a male *Cx. tarsalis* probing *T. vulgare* inflorescences and 30 pollen grains on each of several specimens of *Cx. pipiens*. All of the three female and two of the three male *Cx. pipiens* that we collected while probing *A. millefolium* florets carried pollen, with a mean pollen load of 109 grains and a maximum pollen load of 325 grains. We also collected one female and one male *Cs. incidens* on *A. millefolium* inflorescences but neither carried any pollen (Table 2.1). We collected a single male *Cx. pipiens* and a single male *Cs. incidens* from *L. vulgare*, the former carrying one grain of pollen.

Other floral visitors that we observed on *T. vulgare* either during nocturnal collections or diurnal visits included the common earwig, *Forficula auricularia*, the goldenrod crab spider, *Misumena vatia*, the stink bug, *Chlorochroa rossiana*, the European honey bee, *Apis mellifera*, a *Bombus* sp., a *Lygus sp*., as well as representatives of diverse insect taxa including Formicidae, Nabidae, Chrysopidae, Syrphidae, Vespidae, and many other bees, weevils, dipterans, and lepidopterans. It is noteworthy that on two occasions we observed goldenrod crab spiders, *Misumena vatia*, consuming mosquitoes that they had captured while visiting *T. vulgare* inflorescences.

2.2.2. Pollen abundance and distribution on mosquito bodies

When given access to inflorescences of *T. vulgare*, *A. millefolium* and *S. canadensis* in a laboratory experiment, specimens of *Cx. pipiens* carried pollen grains on all parts of their body, the greatest numbers on their mouthparts (proboscis and palps), antennae, legs, and abdomen (Fig. 2.2; Table 2.2). This distribution of pollen closely mirrors the distribution of pollen found on specimens field-collected from *T. vulgare* and *A. millefolium* (Table 2.1). Mosquitoes visiting *T. vulgare* inflorescences in greenhouse settings inadvertently picked up pollen on their proboscis and palps while probing or feeding, on their antennae while probing or drumming antennae on florets, and on their legs and abdomen while walking across florets (Fig. 2.2). Specimens actively transferred pollen from body parts to their legs while using their legs to clean their probosces, palps, and their pollen-covered antennae (Fig. 2.2d). We observed that mosquitoes alighted on flower heads, probed with their proboscis around florets, fed, and then walked or flew over to other florets or flower heads, or alighted and rested on the cage wall. The alighting of one mosquito on a flower head occasionally disturbed the probing of other mosquitoes, causing them to re-locate.

2.2.3. Pollination experiments

Cx. pipiens pollinated *T.vulgare* in pollination experiment 1 ($F_{2,38}$ = 6.69, P = 0.003) (Fig. 2.3), where the cross- and self-pollination treatments yielded a total of 394 and 99 seeds, respectively, whereas the control treatment yielded only 13 seeds. Mosquito-mediated self-pollinated inflorescences (n = 13) and cross-pollinated inflorescences (n = 12) had a mean successful seed set per inflorescence of 0.22% (95 % CI: 0–0.61 %) and 0.97 % (95 % CI: 0.57–1.37 %), respectively. Control

inflorescences (n = 16) without exposure to mosquitoes had a mean successful seed set per inflorescence of 0.018 % (95 % CI: 0–0.37 %). Mean successful seed set per inflorescence differed significantly between (1) cross-pollinated inflorescences and control inflorescences (Tukey's HSD, P = 0.003) and (2) cross- and self-pollinated inflorescences (Tukey's HSD; P = 0.03). However, there was no significant difference in mean seed set per inflorescence between control inflorescences and self-pollinated inflorescences (Tukey's HSD; P = 0.73).

Culex pipiens pollinated *T. vulgare* also in pollination experiment 2 (t ratio19 = 2.78, P = 0.006) (Fig. 2.3). The mean seed set per inflorescence between mosquito-mediated cross-pollinated inflorescences (mean: 12.5 %; 95 % CI 6.0–18.9 %), and control inflorescences (mean: 0.06 %; 95 % CI: 0–6.8 %) differed by 12.44 % (95 % CI: 3.1–21.8 %). The cross-pollination treatment (n = 11) yielded a combined total of 3939 seeds, whereas the control treatment (n = 10) yielded a total of only 14 seeds.

In the course of both experiments, we did not quantify mosquito mortality but noticed that it was minor.

2.3. Discussion

Our data show that (1) *Cx. pipiens* mosquitoes frequently visited *T. vulgare* inflorescences in the field, (2) field-collected specimens of *Cx. pipiens* probing *T. vulgare* for nectar carry pollen on various parts of their bodies, (3) laboratory reared specimens of *Cx. pipiens* that are given access to *T. vulgare* inflorescences in a laboratory setting pick up *T. vulgare* pollen, and (4) laboratory-reared specimens of *Cx. pipiens* effectively transfer *T. vulgare* pollen between inflorescences of the same or different plants, resulting in seed set. These data in combination support the conclusion that *Cx. pipiens* serves as a pollinator of *T. vulgare*.

Culex pipiens, *Cx. tarsalis*, and other species of mosquitoes have been recorded visiting, or nectar-feeding on, many species of plants including *T. vulgare*, *A. millefolium*, and *Leucanthemum vulgare*. Such records stem from geographically diverse areas of the world, including Europe (Andersson & Jaenson 1987), eastern North America (Haegar 1955; Knab 1907), central North America (Grimstad & DeFoliart 1974; Philip 1943; Sandholm & Price 1962; Smith & Gadawski 1994), and northern North America

(Gorham 1976; Hocking *et al.* 1950; Kevan 1972). Here we show that *Cx. pipiens*, *Cx. tarsalis* and *Cs. incidens* visit flowers in the Pacific region of North America. We particularly note that large numbers of *Cx. pipiens* nectar forage on *T. vulgare*.

In Sweden, Andersson & Jaenson (1987) collected many specimens of *Cx. pipiens* and *Cx. torrentium* as well as a few specimens of many other mosquito species while feeding on *T. vulgare* and occasionally on *A. millefolium*. Importantly, 75% of the males and 78% of the females that they collected from *T. vulgare* inflorescences tested positive for fructose, indicative of nectar-feeding. In Denmark and Sweden, Ardo (1958) and Bro-Larsen (1948), respectively, observed *Cx. pipiens* probing *T. vulgare* inflorescences. In Russia (near Moscow), Kupriyanova & Vorotnikova (1967) (in Vinogradova 2000) report *Cx. pipiens* feeding on inflorescences of *Tanacetum* and *Achillea*. In Wisconsin, Grimstad and DeFoliart (1974) noted *Cx. pipiens* visiting A. *millefolium* inflorescences. In contrast, *Cx. tarsalis* has previously been observed visiting inflorescences only of goldenrods, *Solidago* spp. (Philip 1943; Sandholm & Price 1962). In our study, pollen was present on mosquitoes collected from *T. vulgare*, *A. millefolium*, and *L. vulgare*.

The numbers of pollen grains found on field-collected mosquitoes, or on laboratory-reared mosquitoes given access to field-collected *T. vulgare* inflorescences in a laboratory setting, are in agreement with the numbers of pollen grains found on other dipterans pollinating Asteraceae (Johnson & Midgley 1997). The number of pollen grains on mosquitoes also compared well with those found on lepidopterans and coleopterans, but was lower than those on most hymenopterans, such as bees (Orford *et al.* 2015).

Both the amount and distribution of pollen on an insect's body are indicative of an insect's potential efficiency as a pollinator. The distribution of pollen is affected by a variety of factors including floral morphology, grooming behaviour, or behaviour during floral visits (Freitas 1997; Ish-Am & Eisikowitch 1993). A substantial number of *the Cx. pipiens* specimens that we field-collected while probing inflorescences of *T. vulgare* and *A. millefolium*, or that we laboratory-reared and then exposed to field-collected inflorescences of *T. vulgare*, *A. millefolium*, or *S. canadensis*, carried pollen, the largest proportion of which were on their antennae, mouthparts, and legs. Similarly, 77 % of *Aedes* spp. sampled on Ellesmere Island in the Canadian arctic carried pollen (Kevan 1972). Following exposure to *Silene otites*, *Cx. pipiens*, and *Cs. annulata* carried an

average pollen load of 211 grains per individual, mostly on their legs (Brantjes & Leemans 1976). A significant percentage of *Aedes canadensis* (8.5 %) and *Ae. communis* (15 %) captured over a 1-month period in Wisconsin carried orchid pollinia attached to their eyes (Thien 1969). Accumulation of pollen on the forelegs of mosquitoes engaged in grooming behaviour resembles the same phenomenon observed in pollen-grooming bees, a behaviour which is argued to have contributed to the evolution of pollen-collecting behaviour (Jander 1976).

While pollen on an insect's body does not prove a pollinator function of the insect, it does imply the potential for pollination. Our data not only show that *Cx. pipiens* carries pollen, but also show that *Cx. pipiens* indeed pollinates *T. vulgare*. In pollination experiment 2, pollen transfer by *Cx. pipiens* resulted in a mean seed set per inflorescence of 12.5 %, and in the highest instance yielded a level of seed set approximating 44 %. This impressive pollination effect compares favourably with the 48 % level of seed set obtainable by artificial pollination of *T. vulgare* (Lokki *et al.* 1973), but it likely overestimates the degree of mosquito pollination that may occur in the field.

Deviating levels of mean seed set per inflorescence recorded for the cross-pollination treatments of pollination experiments 1 and 2 may be attributed to at least two factors. In pollination experiment 2, mosquitoes had unlimited access to pollen and could transfer pollen for 3 days, whereas in pollination experiment 1 only those pollen grains a mosquito could accumulate during 2 h, or carry on its body, were available for pollination.

While already impressive, the mosquito-mediated seed set in experiment 2 may still be an underestimate of the mosquitoes' pollinator potential. Blooming of Asteraceae inflorescences is a complex phenomenon as each floret within a composite flower has its own phenology (Neff & Simpson 1990). As the opening of florets on *T. vulgare* composite flowers commences at the margin and then moves inwards, only a certain number of florets are receptive to pollination at any one time. Yet, *T. vulgare* inflorescences in experiments 1 and 2 were exposed to mosquitoes for only a limited time, likely resulting in much fewer pollen transfers, and lower seed set, than could be expected had inflorescences been exposed to mosquitoes for the entire blooming period, as would be the case with in situ plants.

The contribution of an insect as a pollinator of a plant depends on both the likelihood of the insect effecting pollination during a visit and the insect's frequency of visitation. In many systems, it is the frequency of visitation that determines the importance of a pollinating species to the pollination of a plant (Bosch & Blas 1994; Cane & Schiffhauer 2003; Olsen 1997).

Frequency of visitation taking primacy over degree of pollination during a single visit in determining the contribution to pollination of a flower by a floral visitor is expected when there is no co-specialization between a plant and its floral visitors (Waser *et al.* 1996). Many plants visited by mosquitoes indeed attract generalist pollinators (Foster 1995). While Asteraceae inflorescences are deemed specialized, they also share many attributes with less specialized floral forms, thus possibly resulting in relationships with pollinators that are similar to those of more generalized inflorescences (Neff & Simpson 1990). We predict that *Cx. pipiens* and other mosquitoes may function as generalist pollinators of some flowering Asteraceae based on their floral morphology.

Floral scent is known to play a key role in the pollination syndromes of plants (Knudsen & Tollsten 1993; Fenster *et al*. 2004), and mosquitoes have been shown to be attracted to the floral semiochemicals of the plants they visit (Vargo & Foster 1982; Jepson & Healy 1988; Healy & Jepson 1988; Mauer & Rowley 1999; Otienoburu *et al*. 2012; Nyasembe *et al*. 2012). When floral semiochemicals play an essential role in attracting pollinators, they are subject to pollinator-mediated selection pressures and likely function to mediate co-specialization between a plant and a pollinator, or a guild of pollinators (Fenster *et al.* 2004; Knudsen & Tollsten 1993; Whitehead & Peakall 2009). Whether these floral semiochemicals are exploited as foraging cues by nectar-thieving mosquitoes, or serve as a means by which plants attract mosquito pollinators, remains to be investigated.

There is at least some evidence that floral semiochemicals attract mosquito pollinators. *Cx. pipiens* is attracted to floral semiochemicals of *S. otites* (Jhumur *et al.* 2007, 2008), a member of the carnation plant family that they are known to pollinate (Brantjes & Leemans 1976). A four-component blend of floral semiochemicals (acetophenone, linalool oxide, phenylacetaldehyde, and phenylethyl alcohol) produced by *S. otites* elicits significant attraction of *Cx. pipiens* in behavioural bioassays (Jhumur

et al. 2008). Conceivably then, *T. vulgare*, or other Asteraceae, may use floral semiochemicals rather than floral morphology to co-specialize with mosquito pollinators.

When mosquitoes respond to floral semiochemicals of a plant such as *A. syriaca* that they cannot pollinate (Otienoburu *et al.* 2012), they may simply exploit floral information that is targeted to potential pollinators. Alternatively, the plants that mosquitoes can or cannot pollinate may overlap in the composition of their floral bouquet, prompting attraction of both legitimate pollinators and nectar thieves. The extent and specificity of mosquito attraction to floral semiochemicals are yet to be thoroughly investigated.

2.4. Conclusion

Mosquitoes are generally considered nectar thieves rather than pollinators of plants (Foster & Hancock 1994; Inouye 2010; Smith & Gadawski 1994; Zhang *et al.* 2014). Contrasting with this prevailing opinion, we present data showing conclusive evidence for pollination of *T. vulgare* by *Cx. pipiens*, and potential pollination of additional Asteraceae, including *A. millefolium*, by several other mosquito species. This evidence coupled with literature records of confirmed and probable pollination by mosquitoes (Bartareau & Jackes 1994; Brantjes & Leemans 1976; Catling & Catling 1991; Coleman 1934; Hyett 1960; Kevan 1972; Müller 1873; Patt *et al.* 1989; Stoutamire 1968, 1971) suggests that mosquito pollination is more widespread than previously thought. Furthermore, given the current knowledge of mosquito attraction to floral semiochemicals, including that the floral semiochemicals of a plant mosquitoes are known to pollinate, an olfaction-based, mosquito-specific pollination syndrome seems possible.

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2.6. Tables

Table 2.1 Number of *Culex* (*Cx.*) *pipiens*, *Cx. tarsalis* and *Culiseta* (*Cs.*) *incidens* field-collected while they were visiting inflorescences of *Tanacetum vulgare* and *Achillea millefolium* (both Asteraceae), and the abundance and distribution of Asteraceae

Criteria recorded	*T. vulgare*			*A. millefolium*	
	Cx. pipiens	*Cx. tarsalis*	*Cs. incidens*	*Cx. pipiens*	*Cs. incidens*
Total number of mosquitoes collected	164	2	6	6	2
Number of mosquitoes carrying pollen	41	1	1	5	0
Mean number of pollen grains on pollen-carrying mosquitoes	9.4	39	9	108.8	–
Mean % of pollen grains on body parts					
Head	0.4	0	0	0.3	–
Proboscis and palps	37.5	41.0	0	22.3	–
Antennae	33.3	25.6	55.6	50.6	–
Wings	0.9	10.3	0	0.4	–
Legs	15.1	2.6	33.3	9.2	–
Thorax	1.5	12.8	0	1.8	–
Abdomen	11.3	7.7	11.1	15.5	–

Table 2.2 Accumulation and distribution of pollen on *Culex pipiens* after 2 h of experimental exposure to inflorescences of *Tanacetum vulgare*, *Achillea millefolium*, and *Solidago canadensis* (all Asteraceae)

Criteria recorded	Plant species		
	T. vulgare (n = 2)	*A. millefolium* (n = 2)	*S. Canadensis* (n = 2)
Total number of mosquitoes observed	124	107	126
Number of mosquitoes carrying pollen	59	37	43
Mean number of pollen grains on pollen-carrying mosquitoes	194.9	100.8	163.4
Mean % of pollen grains on body parts			
Head	1.9	1.5	1.5
Proboscis and palps	20.0	25.5	19.5
Antennae	23.8	26.5	20.4
Wings	1.0	2.7	1.4
Legs	37.1	34.1	41.4
Thorax	6.0	4.4	7.5
Abdomen	10.2	5.3	8.3

2.7. Figures

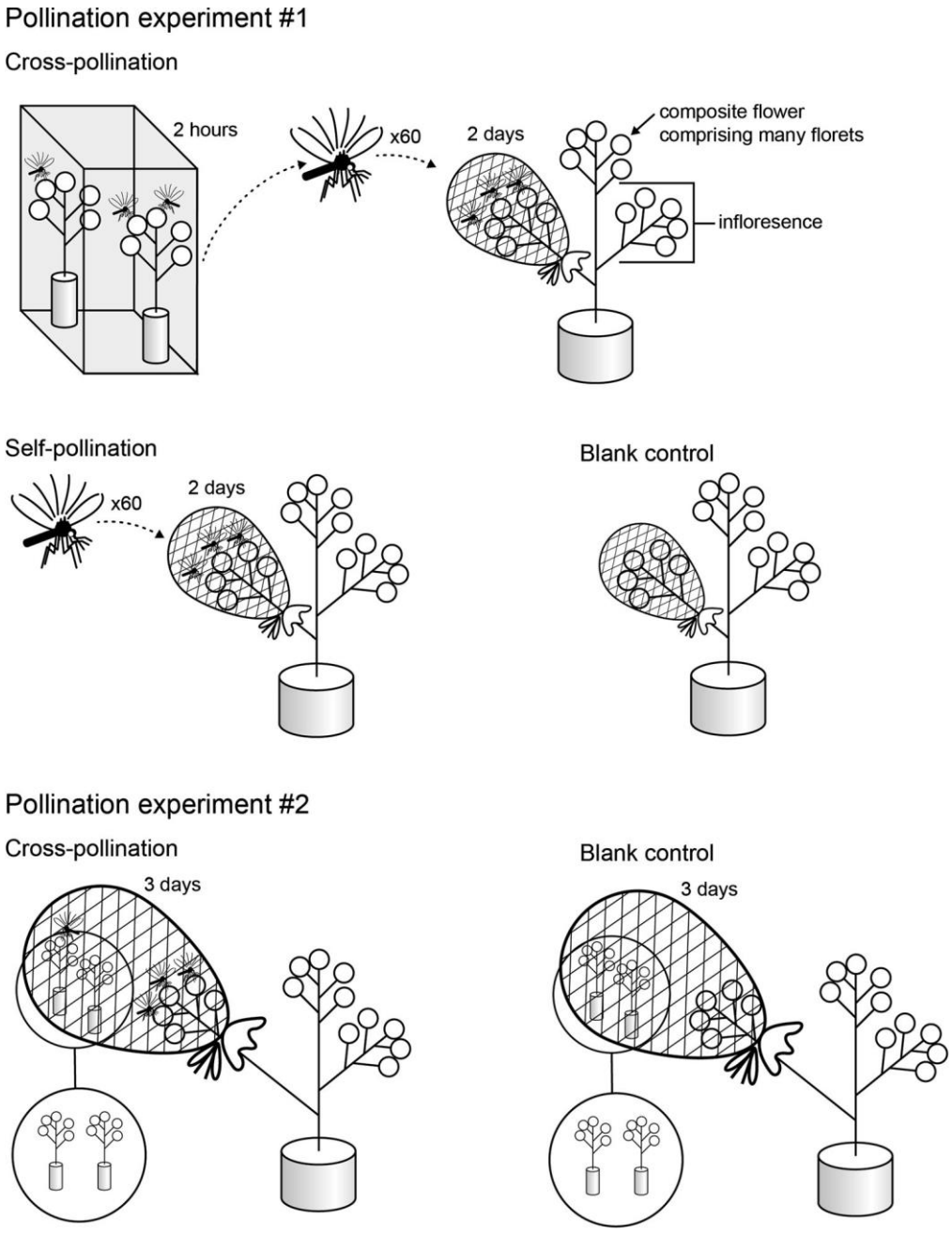
Pollination experiment #1
Cross-pollination
2 hours
x60
2 days
composite flower
comprising many florets
infloresence
Self-pollination
x60
2 days
Blank control
Pollination experiment #2
Cross-pollination
3 days
Blank control
3 days

Figure 2.2.1 **Graphical illustrations of the experimental design used for pollination experiments 1 and 2. In experiment 1, the crosspollination treatment (n = 12) consisted of 60 male and female mosquitoes (*Culex pipiens*) that were exposed for 2 h to two excised inflorescences of tansies (*Tanacetum vulgare*) in a cage, and then transferred, and kept for 2 days, in a mesh pollinator exclusion bag (3.78 L) enclosing the blooming inflorescence of a living (potted) greenhouse-grown tansy. The self-pollination treatment (n = 13) consisted of 60 male and female mosquitoes that were kept for 2 days in a pollinator exclusion bag enclosing the blooming inflorescence of a living (potted) greenhouse-grown tansy. The control treatment (n = 16) was identical to the self-pollination treatment except that mosquitoes were absent. In experiment 2, the cross-pollination treatment (n = 11) consisted of 60 mosquitoes that were kept for 3 days in a pollinator exclusion bag enclosing (1) the blooming inflorescences of a living (potted) greenhouse-grown tansy and (2) two blooming inflorescences excised from a separate plant and retained in water-filled 20-mL vials. The control treatment (n = 10) was identical to the cross-pollination treatment except that mosquitoes were absent.**

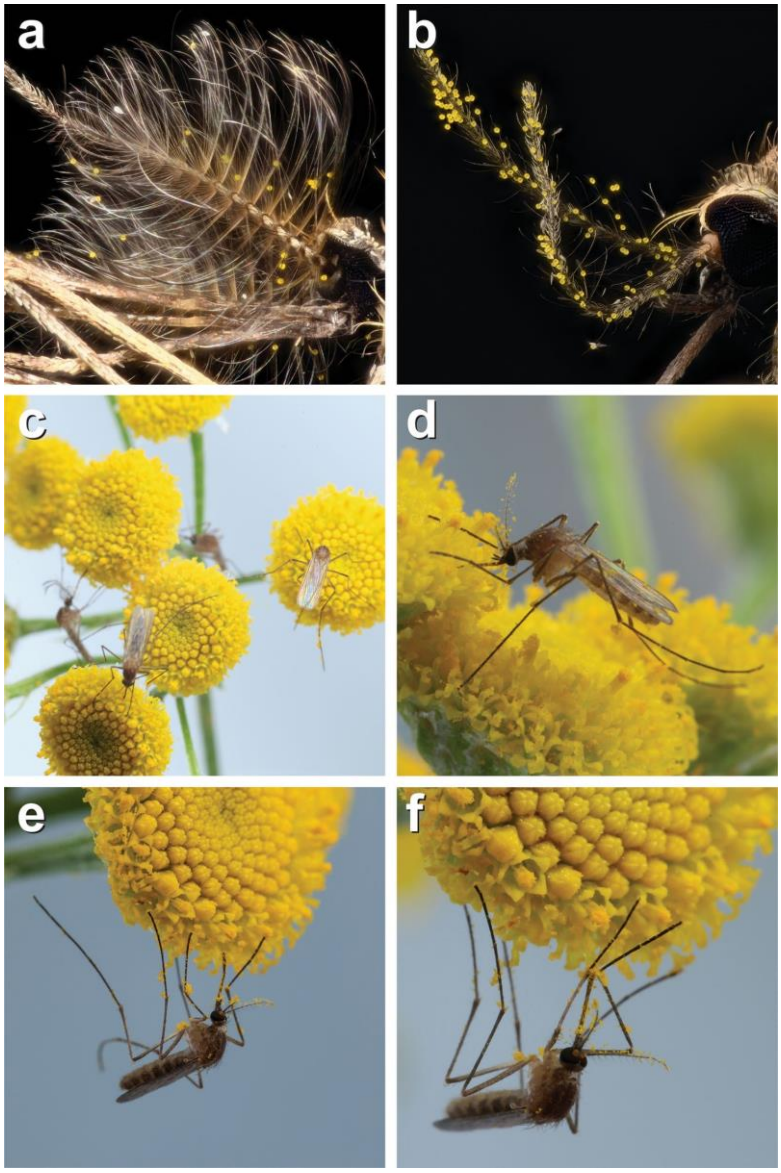

Figure 2.2 **(a) Pollen-carrying male *Culex tarsalis* field-collected from inflorescences of *Tanacetum vulgare*; (b) pollen-carrying female *Cx. pipiens* field-collected from inflorescences of *Achillea millefolium*; (c) male and female *Culex pipiens* feeding on inflorescences of greenhouse-grown *Tanacetum vulgare*; (d) pollen on the antennae of a feeding female; (e) female with numerous pollen grains on her proboscis, palps, antennae, legs, and thorax; (f) the same female as in (e) using her forelegs to remove pollen from her proboscis.**

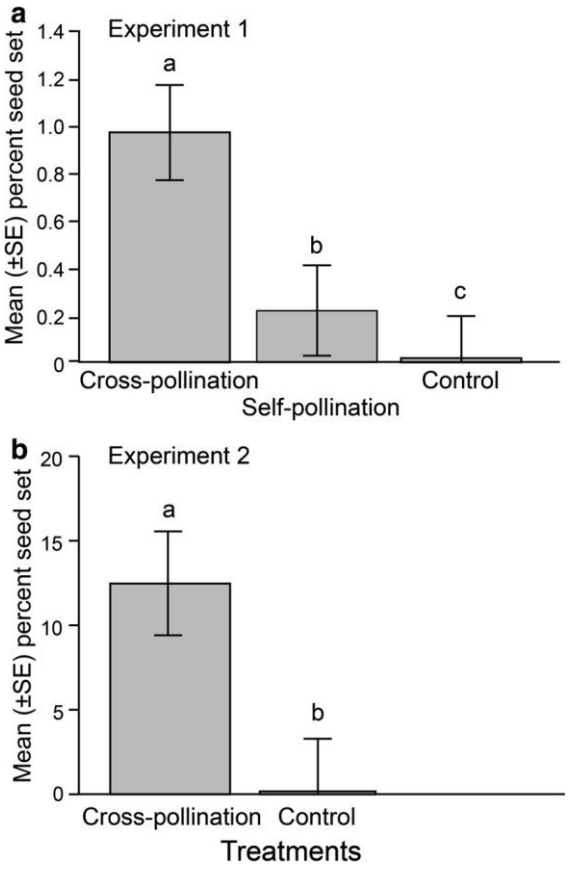

Figure 2.3 **(a) Mean (±SE) percent seed set per inflorescence of *Tanacetum vulgare* in pollination experiment 1 (see Fig. 1 for design) in relation to treatments of cross-pollination or self-pollination by *Culex pipiens*, or a control treatment with no exposure of inflorescences to *Cx. pipiens* (ANOVA: $F_{2,38} = 6.69$, P = 0.003). Different letters on bars denote significant differences in seed set between treatments (Tukey's HSD; P<0.05); (b) mean (±SE) per cent seed set per inflorescence of *T. vulgare* in pollination experiment 2 (see Fig. 1 for design) in relation to a cross-pollination treatment by *Culex pipiens* or a control treatment with no exposure of inflorescences to *Cx. pipiens*. Different letters on bars denote significant differences in seed set between treatments (one-way t test: $t\ ratio_{19} = 2.78$, P = 0.006)**

Chapter 3.

Multimodal floral cues guide female mosquitoes to tansy inflorescences[1]

[1]The corresponding manuscript is published in *Scientific Reports* (2019, Volume 9: 3908) with the following authors: Peach, D.A.H., Gries, R., Zhai, H., Young, N., and Gries, G.

Introduction

Females of many mosquito species require the nutrients obtained from a vertebrate blood meal for egg development. However, both male and female mosquitoes also consume plant sugars, primarily as floral nectar (Clements 1999; Foster 1995), that provide essential energy for flight and survival (Clements 1999; Foster 1995; Stone *et al.* 2009), thus enabling populations even of highly synanthropic mosquitoes to persist (Stone *et al.* 2009). As pollinators (Peach & Gries 2016; Stoutamire 1968) or nectar thieves (Smith & Gadawski 1994), mosquitoes seek the inflorescences of many plant species (Andersson & Jaenson 1987; Grimstad & DeFoliart 1974), responding to floral semiochemicals (message-bearing chemicals) that apparently guide them to floral resources (Nyasembe & Torto 2014).

Mosquitoes use olfactory, visual, and thermal cues to locate vertebrate hosts, including humans. Important olfactory cues are CO_2 (Gillies 1980), L-lactic acid (Acree *et al.* 1968) and other carboxylic acids (Cork & Park 1996; Smallegange *et al.* 2009). CO_2 also attracts, or prompts host-seeking behaviour of, other haematophagous insects including tsetse flies (*Glossina* spp.), kissing bugs, biting midges (*Culicoides* spp.) and black flies (Diptera: Simuliidae) (Guerenstein & Hildebrand 2008). In mosquitoes, CO_2 interacts with other host cues (McMeniman *et al.* 2014; van Breugel *et al.* 2015); however, CO_2 originates not only from vertebrate hosts but also from plants that emit CO_2 as a metabolite of cellular respiration (Amthor 2000). During diurnal photosynthesis, plants are net CO_2 sinks but at dusk cease photosynthesis and become net CO_2 producers, thus increasing ambient CO_2 concentrations (Allen 1971; Amthor 2000; Chapman *et al.* 1954). The plants' transition from net CO_2 sinks to net CO_2 producers coincides with peak nectar foraging activity of many mosquito species (Andersson & Jaenson 1987; Clements 1999). Plant CO_2 mediates insect attraction in many plant-

insect interactions (Guerenstein & Hildebrand 2008) and serves as a foraging cue for nectar-feeding insects (Goyret *et al.* 2008a) and the phloem-feeding haematophagous sand fly, *Phlebotomus papatasi* (Schlein & Jacobson 2008).

The role of visual inflorescence cues for mosquito attraction has barely been explored. Mosquitoes frequent mostly light-coloured inflorescences (Clements 1999; Foster 1995), or dark inflorescence mimics in the presence of a human observer (Dieng *et al.* 2018), but the underlying mechanisms are not known (Clements 1999; Dieng *et al.* 2018). Light-coloured and strongly-scented inflorescences are often pollinated by crepuscular or nocturnal moths (Baker 1961; Faegri & Van Der Pijl 1979; Haber & Frankie 1989; Jürgens 2004) and sometimes are co-pollinated by mosquitoes (Brantjes & Leemans 1976). Interactive effects between visual and olfactory cues have been studied in plant-heteroceran systems (Raguso & Willis 2002, 2005), as have been innate colour and odour preferences that experimentally can be manipulated via reward-based learning (Cunningham *et al.* 2004; Goyret *et al.* 2008b). The olfactory cues of oxeye daisies, with or without intact visual cues, suffice to attract mosquitoes (Jepson & Healy 1988) that learn to associate artificial visual cues with the nutrient quality of sugar rewards (Bernáth *et al.* 2016). However, visual cues mediate other plant-pollinator interactions (Nuttman *et al.* 2006), and guide host-foraging mosquitoes, provided they have been impelled by elevated levels of CO_2 (van Breugel *et al.* 2015). The concept of CO_2-"gated" activity may be applicable not only to host-foraging but also to nectar-foraging mosquitoes (Syed & Leal 2007) but remains to be studied in this context to fully understand the inflorescence cue complex.

Interestingly, some human-headspace semiochemicals (1-octen-3-ol, nonanal, specific carboxylic acids) attractive to host-seeking mosquitoes (Smallegange *et al.* 2009; Syed & Leal 2009; Takken & Kline 1989) are also present in the odour bouquet of inflorescences frequented by nectar-foraging mosquitoes (Nikbakhtzadeh *et al.* 2014; Nyasembe & Torto 2014). How frequently semiochemicals are shared by human host and plant resources remains unknown.

Various species of mosquitoes frequent the inflorescences of common tansy, *Tanacetum vulgare* (Andersson & Jaenson 1987; Grimstad & DeFoliart 1974; Peach & Gries 2016), likely in response to floral odour (Clements 1999; Foster 1995). Working with the tansy-pollinating (Peach & Gries 2016) northern house mosquito, *Culex pipiens*

L., and the yellow fever mosquito, *Aedes aegypti* (L.), as model species, we tested the hypotheses (1) that tansy-foraging females, analogous to human host-foraging females, exploit a multimodal complex of CO_2, semiochemical and visual floral cues, and (2) that key floral semiochemicals are shared with human hosts.

3.1. Materials and methods

3.1.1. Rearing of experimental mosquitoes

We reared *Cx. pipiens* and *Ae. aegypti* at 23-26 °C, 40-60% RH, and a photoperiod of 14L:10D. We maintained mixed groups of males and females in mesh cages (30 × 30 × 46 cm high) provisioned *ad libitum* with a 10% sucrose solution. We fed females once per week on the arm of DP. For oviposition, gravid females were given access to water in a circular glass dish (10 cm diameter × 5 cm high) (*Cx. pipiens*) or in a 354-mL cup (Solo Cup Company, Lake Forest, IL 60045, USA) with paper towel lining (Kruger Inc., Montréal, QC H3S 1G5, Canada) (*Ae. aegypti*). We transferred egg rafts of *C. pipiens* to water-filled trays (45 × 25 × 7 cm high), and paper towel strips with *Ae. aegypti* eggs to circular glass dishes (10 cm diameter × 5 cm high) containing water and brewers yeast (U.S. Biological Life Sciences, Salem, MA 01970, USA). Two to four days later, we transferred the dish contents to water-filled trays (45 × 25 × 7 cm high). We provisioned larvae with NutriFin Basix tropical fish food (Rolf C. Hagen Inc., Baie-D'Urfe, QC H9X 0A2, Canada), and transferred pupae via a 7-mL plastic pipette (VWR International, Radnor, PA 19087, USA) to water-containing 354-mL Solo cups covered with a mesh lid. we collected eclosed adults via aspirator and placed them in similar cups, along with a cotton ball soaked in a 10% sucrose solution.

3.1.2. Behavioural bioassays

We ran all behavioural bioassays in translucent mesh cages (77 × 78 × 104 cm) wrapped with black cloth except for the top to allow illumination from ambient fluorescent light. We kept cages at 23-26 °C, 40-60% RH, still air, and a photoperiod of 14L:10D. For each 24-h bioassay, we released 50 virgin, 1- to 3-day-old (unless otherwise stated), 24-h sugar-deprived females of *Cx. pipiens* or *Ae. aegypti* from a Solo cup into a cage. We randomly assigned the treatment and the control stimulus to adhesive-coated (The Tanglefoot Comp., Grand Rapids, MI 49504, USA), custom-made delta traps (9 cm × 15

cm) placed on each of two stands spaced 30 cm apart inside the cage. We wore latex gloves (Microflex Corporation, Reno, NV 89523, USA) during preparation of test stimuli.

3.1.3. Effect of olfactory and visual inflorescence cues on female mosquito attraction

We collected blooming inflorescences from *in-situ* tansy on the Burnaby campus of Simon Fraser University (SFU) and from potted, greenhouse-grown plants. The treatment stimulus consisted of one tansy inflorescence with 10-15 composite flowers cut from the plant bearing it. The control stimulus consisted of the stem of an inflorescence (with composite flowers excised and removed) cut from another plant. Because cut surfaces emanate “green leaf volatiles” and control plants had additional cuts due to the excision and removal of composite flowers, we inflicted cuts also on the stem of treatment plants. We covered all cut surfaces with petroleum jelly to minimize the release of green-leaf volatiles, inserted the treatment and the control plant into separate water-filled, parafilm-covered 4-mL vials, and placed each vial horizontally into a trap. We ran two experiments in parallel to rigorously study the effects of olfactory and visual inflorescence cues on mosquito attraction. To test the effect of olfactory cues, we occluded both the treatment and the control inflorescence by three layers of cheesecloth (VWR International, Radnor, PA 19087, USA) with a mesh size sufficiently wide to permit odourant dissemination. To test for an interactive effect between olfactory and visual cues, we occluded one inflorescence with three layers of cheesecloth and placed the other on top of the cheesecloth layers. To compare head-to-head the relative attractiveness of inflorescences presenting both visual and olfactory cues, or just olfactory cues, we occluded one of the two inflorescences with cheesecloth.

3.1.4. Capture and attractiveness of headspace floral odourants

We inserted 5-10 inflorescences into a 250-mL water-filled beaker which we then placed into a Pyrex® glass chamber (34 cm high × 12.5 cm wide). A mechanical pump drew charcoal-filtered air at a flow of 1 L min^{-1} for 24-72 h through the chamber and through a glass column (6 mm outer diameter × 150 mm) containing 200 mg of Porapak-Q™ adsorbent (Byrne *et al.* 1975). We desorbed floral odourants captured on Porapak-Q with 2 mL each of pentane and ether and bioassayed aliquots of Porapak-Q headspace volatile (HSV) extract for mosquito attraction. The treatment stimulus

consisted of a 1-mL HSV extract aliquot [equivalent to the amount of odourants emanating from one or two blooming tansy plants per hour for 24 h, or approximately 240 inflorescence-hour-equivalents (IHE); 1 IHE = the amount of odourants released from one inflorescence during 1 h of odourant capture], emanating from a horizontally-placed 4-mL glass vial with a 2-mm hole in its lid. In the control stimulus, the HSV extract aliquot was replaced with the corresponding amount of pentane and ether (1:1 mix).

3.1.5. Identification of floral odourants in HSV extracts

After adding octyl acetate as an internal standard to HSV extract, we analyzed 2-µl aliquots by gas chromatography-mass spectrometry (GC-MS), operating a Saturn 2000 Ion Trap GC-MS fitted with a DB-5 GC-MS column (30 m × 0.25 mm i.d.; Agilent Technologies Inc., Santa Clara, CA 95051, USA) in full-scan electron impact mode. We used a flow of helium (35 cm s^{-1}) as the carrier gas with the following temperature program: 50 °C (5 min), 10 °C min^{-1} to 280 °C (held for 10 min). The temperature of both the injector port and ion trap was 250 °C. To reveal the presence of low-molecular-weight carboxylic acids (which chromatograph poorly), we converted carboxylic acids to the corresponding silylated derivatives (which chromatograph well). To this end, we treated a 100-µL aliquot of HSV extract with BSTFA (10 µl; *N,O*-bis(trimethylsilyl)trifluoroacetamide) and TMCS (10%; trimethylchlorosilane; both Pierce Chemical Co., Rockford, IL 61101, USA) and after 5 min without any work-up analyzed 2-µl aliquots by GC-MS. We identified odourants in HSV extract by comparing their retention indices (RI; relative to *n*-alkane standards (van Den Dool & Kratz 1963)) and their mass spectra with those reported in the literature (Adams 1989) and with those of authentic standards (Table 3.1).

3.1.6. Preparation of a synthetic floral odourant blend

We prepared a synthetic blend of floral odourants (Table 1) including all those odourants present at >1.25% in floral HSV extract. The quantity and ratio of odourants in this synthetic blend matched those found in HSV extract. Moreover, we prepared a second synthetic blend (Table 3.1) consisting of only those floral odourants that are also found in headspace volatiles of human skin, breath, or skin microbiota (Table 3.1).

3.1.7. Attractiveness of synthetic floral blends to female mosquitoes (1- to 3- or 5- to 6-day-old)

We tested the attractiveness of synthetic floral blends using the two-choice general bioassay design described above. In three sets of two parallel experiments, we tested a complete synthetic blend (CSB) of all floral odourants (Table 3.1) or a partial synthetic blend (PSB) comprising only those floral components also found in headspace volatiles of human skin, breath, or skin microbiota (Table 3.1) each *versus* a solvent control. We prepared the complete blend at approximately 240 IHEs dissolved in pentane/ether (1 mL; 1:1), and disseminated it from a horizontally-placed, 4-mL glass vial with a 2-mm hole in the lid. The control stimulus consisted of the equivalent solvent mixture (1 mL) disseminated from the same type of dispenser.

3.1.8. Measurements of tansy CO_2 emissions in the field and laboratory

We measured CO_2 concentrations from a single cut tansy inflorescence weighing 3.6 g with a Q-Trak 7575-X air quality monitor (TSI Inc., Shoreview, MI 55126, USA) set to take readings every second and to average them in 1-min intervals. To track changes in ambient CO_2 around *in-situ* tansies, we placed the monitor circa 5 cm above ground in a patch of tansies on the Burnaby campus of SFU, taking measurements from 20:30 to 22:30 h on 18 August 2015, with civil dusk occurring at circa 21:00 h.

3.1.9. Effect of trace CO_2 on female mosquito attraction

Using the two-choice general bioassay design described above, and running two experiments in parallel with both *Cx. pipiens* and *Ae. aegypti*, we tested the effect of CO_2 on mosquito attraction. To provide a neutral stimulus, both traps in each experiment were fitted with a horizontally-placed, 4-mL glass vial containing pentane and ether (1 mL; 1:1) which were dispensed through a 2-mm hole in the lid. The test variable in one experiment consisted of a mixture of medical-grade air containing 1% CO_2 (Praxair Inc., Mississauga, ON L5B 1M2, Canada) which amounts to a CO_2 concentration about 10× that near a single cut tansy inflorescence (see results), or comparable to that near a single intact tansy plant at the time when it is a net CO_2 producer. To make sure that mosquitoes were not just responding to the flow of a gas mixture, the test variable in the

parallel experiment consisted of medical grade air (Praxair Inc.). we delivered each test variable at the same flow rate [5000 µL min^{-1}] through copper tubing (1.5 m × 2 mm i.d.) and aluminum tubing (0.5 m × 0.5 mm i.d.) to the respective delta trap and recorded the number of mosquitoes captured in each trap after 2 h.

3.1.10. Effect of tansy floral odourant blend on attraction of mosquitoes to CO_2

Using the two-choice general bioassay design described above, we tested whether floral odourants enhance attraction of *Cx. pipiens* and *Ae. aegypti* to CO_2. In each experiment, we delivered a mixture of medical-grade air containing 1% CO_2 to both the treatment and the control trap (as described above), baited the treatment trap with the complete blend of floral odourants (CSB; as described above), and fitted the control trap with a solvent control (as described above).

3.1.11. Statistical analyses of data

We used SAS statistical software version 9.4 (SAS Institute Inc., Cary, NC 27513, USA) for data analyses, excluding from analyses experimental replicates with no mosquitoes responding. We used a binary logistic regression model with a logit link function and a Firth bias correction factor to compare mean proportions of responders between test stimuli, with overdispersion corrected for using the Williams method where appropriate (Exp. 9). We analyzed differences between experiments using non-adjusted least squares means. We worked with back-transformed data to obtain means and confidence intervals. We analyzed vegetative CO_2 emission with autocorrelated linear regression to obtain concentration changes over time.

3.2. Results

3.2.1. Effect of olfactory and visual tansy inflorescence cues on female mosquito attraction

In two-choice laboratory experiments with a paired-trap design, traps baited with a non-occluded (i.e., fully visible) inflorescence captured more female *Ae. aegypti* ($z = 5.5$, $P < 0.0001$) and *Cx. pipiens* ($z = 12.8$, $P < 0.0001$) than traps fitted with a non-

occluded stem of an inflorescence (Fig. 3.1; Exps. 1, 4), indicating that olfactory and/or visual inflorescence cues attract females of both mosquito species. Our findings that occluded intact inflorescences, but not just their stems, continued to attract both *Ae. aegypti* ($z = 5.6$, $P < 0.0001$) and *Cx. pipiens* ($z = 10.9$, $P < 0.0001$) (Fig. 3.1; Exps. 2, 5) provide strong evidence that olfactory inflorescence cues suffice to attract females of both mosquito species. However, traps baited with a non-occluded intact inflorescence captured more female *Ae. aegypti* ($z = 7.6$, $P = 0.014$) and *Cx. pipiens* ($z = 4.1$, $P < 0.0001$) than traps fitted with an occluded intact inflorescence (Fig. 3.1; Exps. 3, 6), revealing an additive effect between olfactory and visual inflorescence cues on mosquito attraction.

3.2.2. Identification of tansy floral odourants in head space volatile (HSV) extracts

HSV extract contained 20 floral odourants (each > 1.25%) including acids, mono- and sesquiterpenes, ketones, alcohols and bifunctional compounds (Fig. 3.2). Nine of these odourants (butanoic acid, 2-methylpropionic acid, 2-methylbutanoic acid, 3-methylbutanoic acid, benzoic acid, hexanoic acid, (–)-α-pinene, benzaldehyde, acetophenone) are also found in HSVs of human skin, breath, or skin microbiota.

3.2.3. Attractiveness of tansy HSV extract and synthetic floral blends to female mosquitoes

HSV-baited traps indeed captured more female *Ae. aegypti* ($z = 7.4$, $P < 0.0001$) and *Cx. pipiens* ($z = 7.7$, $P < 0.0001$) than corresponding control traps (Fig. 3.3; Exps. 7, 9). Moreover, CSB-baited traps captured more female *Ae. aegypti* ($z = 4.8$, $P < 0.0001$) and *Cx. pipiens* ($z = 9.9$, $P < 0.0001$) than control traps (Fig. 3.3; Exps. 8, 10), indicating that the CSB contained the critically important floral odourants that attracted mosquitoes to HSV extract or to the odour bouquet of intact inflorescences (Fig. 3.2, Table 3.1).

To gauge the relative attractiveness of the CSB and the PSB, we tested them in sets of parallel experiments *versus* a solvent control. Taking into account that response preferences to the CSB (“floral nectar scent”) and the PSB (“vertebrate host scent”) may shift more strongly with aging female *Cx. pipiens* than with aging female *Ae. aegypti* that are aggressive daytime biters (Yasuno M 1990), we tested groups of both young (1- to

3-day old) and old (4- to 5-day-old) female *Cx. pipiens*. As expected, CSB- and PSB-baited traps each captured more young female *Ae. aegypti* than control traps (z = 8.6, $P < 0.0001$; z = 5.5, $P < 0.0001$) (Fig. 3.4; Exps. 11, 12). In contrast, only CSB-baited traps, but not PSB-baited traps, captured more young female *Cx. pipiens* than control traps (z = 4.7, $P < 0.0001$; z = -1.3, $P = 0.2$) (Fig. 3.4; Exps. 13, 14). Conversely, PSB-baited traps, but not CSB-baited traps, captured more old female *Cx. pipiens* than control traps (z = 2.3, $P = 0.02$; z = 1.4, $P = 0.17$) (Fig. 3.4, Exps. 15, 16). The combined data of experiments 13-16 reflect a resource preference shift from nectar to vertebrates by aging female *Cx. pipiens.*

3.2.4. Measurements of tansy CO_2 emissions in the field and laboratory

To track changes in ambient CO_2 around *in-situ* tansies, we placed an air quality monitor in a patch of tansies and took measurements from 20:30 to 22:30 h, with civil dusk occurring at circa 21:00 h. The ambient CO_2 concentration in this patch significantly increased around civil dusk at a rate of 10.4 ppm $hour^{-1}$ (Fig. 3.5). To measure CO_2 emission directly from tansies, we field-collected a single inflorescence with 26 composite flowers during midday, inserted it into a water-filled vial, placed the vial in a 3.9-L Plexiglass chamber without natural light, inserted the monitor probe through a port in the chamber, and took CO_2 measurements for four hours. During these measurements, the CO_2 concentration increased at 1.19 ppm min^{-1} (Fig. 3.6), corresponding to 5 µL of CO_2 min^{-1} emitted by the inflorescence.

3.2.5. Effect of CO_2 on attraction of female mosquitoes

The flow of CO_2-enriched air, but not of medical-grade air, afforded more trap captures of female *Ae. aegypti* (z = 2.3, $P = 0.02$; z = 0.7, $P = 0.47$) (Fig. 3.7; Exps. 17, 18). The flow of CO_2-enriched air, but not of medical-grade air, also afforded more trap captures of female *Cx. pipiens* (z = 2.11, $P = 0.035$; z = -0.5, $P = 0.60$) (Fig. 3.7; Exps. 19, 20).

3.2.6. Effect of floral odourants on attraction of female mosquitoes to CO_2

CO_2-enriched medical-grade air in combination with the CSB afforded more trap captures of female *Ae. aegypti* ($z = 2.4$, $P = 0.016$) and *Cx. pipiens* ($z = 2.1$, $P = 0.04$) than CO_2-enriched medical-grade air alone (Fig. 3.8; Exps. 21, 22), indicating an interactive effect of CO_2 and floral odourant cues on mosquito attraction.

3.3. Discussion

These data support the hypotheses that nectar-foraging females of *Ae. aegypti* and *Cx. pipiens*, analogous to host-foraging mosquito females, exploit a multimodal complex of CO_2, semiochemical and visual floral cues, and that many key floral semiochemicals are shared with human hosts. To detect the (sometimes) subtle effects of the various floral cues, and to reveal interactions between them, it was imperative to run laboratory experiments where cues such as CO_2 could be readily manipulated. Given that even intact inflorescences that represent all the cues of the entire inflorescence Gestalt attracted only 20-70% of the bioassayed mosquitoes (Fig. 3.1), less complex combinations of inflorescence cues – expectedly – afforded lower but still significant proportions of responding insects. Below, we elaborate on these findings and offer interpretations.

A 0.03-% rise in CO_2 above ambient triggers host-seeking by mosquitoes (Eiras & Jepson 1994). Here we show that increasing CO_2-levels approximating those around *in-situ* tansy inflorescences at dusk enhance attraction of mosquitoes to floral semiochemicals, thus demonstrating an interaction between bimodal inflorescence cues. Increased CO_2 emissions from tansy inflorescences in our field patch at dusk (Fig. 3.5) may have been comparable to those from other nearby vegetation, and on their own may not have effectively guided nectar-foraging mosquitoes, but the laboratory experiments revealed that equivalent CO_2 emissions enhance the attractiveness of inflorescence odourants to foraging mosquitoes (Fig. 3.7). Plant CO_2 has previously been shown to affect insect-plant interactions (Guerenstein & Hildebrand 2008) but interactive effects were not investigated. For example, the haematophagous sand fly, *Phlebotomus papatasi*, locates sugar-rich plant tissue in response to differential CO_2 emissions from various plant tissues, including those of the mosquito host plant *Ricinus*

communis (Gary & Foster 2004; Schlein & Jacobson 2008). Similarly, CO_2 respired by the bog orchid *Platanthera obtusata* is speculated to be a short-range foraging cue for its mosquito pollinators (Stoutamire 1968). The tomato hornworm, *Manduca sexta,* exploits CO_2 emissions from inflorescences of the sacred Datura, *Datura wrightii,* to locate its nectaries (Goyret *et al.* 2008a; Guerenstein & Hildebrand 2008). Moreover, larvae of the Western corn rootworm, *Diabrotica vergifera,* find corn roots based only on their CO_2 emissions, and larvae of the cotton bollworm, *Helicoverpa armigera*, and the lesser cornstalk borer, *Elasmopalpus lignosellus,* orient towards CO_2 sources, as do some tephritid fruit flies (Guerenstein & Hildebrand 2008).

Multimodal integration of CO_2 and other sensory cues "drive" mosquito attraction to humans (Acree *et al.* 1968; Burgess 1959; Dekker *et al.* 2005; Gillies 1980; Hawkes & Gibson 2016; Healy & Copland 1995; McMeniman *et al.* 2014; Webster *et al.* 2015) and may also underlie nectar-foraging by mosquitoes. CO_2 on its own is attractive (Lorenz *et al.* 2013), and as part of the human host cue complex is thought to (*i*) initiate mosquito take-off and flight (Dekker *et al.* 2005; Healy & Copland 1995; Webster *et al.* 2015), (*ii*) enhance the attractiveness of host odourants at close range (Acree *et al.* 1968; Gillies 1980), and (*iii*) to function as an activator that impels the mosquitoes' responses to host semiochemical, visual, and thermal cues (Burgess 1959; Hawkes & Gibson 2016; McMeniman *et al.* 2014; van Breugel *et al.* 2015). These concepts appear applicable to nectar-foraging by mosquitoes. As daylight fades, plants cease photosynthesis and become net producers of CO_2. Concurrent release of CO_2 from soil microorganisms, particularly in areas with plant roots (Silvola *et al.* 1996), contributes to a significant CO_2 rise. Independent of photoperiod, some flowers even rhythmically produce elevated levels of CO_2 (Hew *et al.* 1978). We posit that a CO_2 rise from vegetated areas following dusk, or CO_2 emitted from flowers, activates the mosquitoes' responses to olfactory and possibly visual cues associated with nectar-producing inflorescences. If indeed a multimodal cue complex, rather than "just" a mono-modal cue, guides nectar-foraging mosquitoes to inflorescences, this would explain why in some reported studies floral extracts or synthetic floral odourants on their own were not effective in attracting mosquitoes (Mauer & Rowley 1999).

The semiochemicals that tansy inflorescences share with human hosts suffice to attract female *Ae. aegypti* and aged (but not young) female *Cx. pipiens* (Fig. 4) that have apparently shifted from nectar- to host-foraging. Some of these shared semiochemicals

[butanoic acid, 3-methylbutanoic acid, benzoic acid, hexanoic acid] that attract mosquitoes to human hosts and to tansy inflorescences are proven host-foraging cues for mosquitoes (Allan *et al.* 2006; Carlson *et al.* 1973; Cork & Park 1996; Puri *et al.* 2006; Smallegange *et al.* 2009), whereas other compounds [e.g., acetophenone (but see (von Oppen *et al.* 2015)), 2-methylpropanoic acid] – while associated with humans (Bernier *et al.* 2000; Cork & Park 1996; Curran *et al.* 2005; Owino *et al.* 2015; Sanchez & Sacks 2006) – have yet to be rigorously tested in a host-seeking context. Interestingly, the human host odourants lactic acid and 1-octen-3-ol enhanced attraction of *Ae. aegypti* to fruit-based toxic sugar baits (Scott-Fiorenzano *et al.* 2017) and admixture of human host odourants to plant-derived odourants increased attraction of some *Anopheles* spp. (Jacob *et al.* 2018).

Our findings that the entire inflorescence Gestalt of olfactory and visual cues is more attractive to foraging mosquitoes than floral odourants alone (Fig. 3.1) indicate that visual displays contribute to the multimodal complex of inflorescence cues that attract mosquitoes to floral nectar. Even though human-visible floral colours may not affect mosquito foraging (Clements 1999), contrast within an inflorescence, or between an inflorescence and its surrounding (van Breugel *et al.* 2015), may play a role. Moreover, ultraviolet floral reflections likely guide nectar-foraging mosquitoes, as shown in many other insect-pollinators (Koski & Ashman 2014).

Haematophagy has arisen independently several times in the Insecta (Lehane 2005), and phytophagy is one possible feeding habit from which haematophagy may have originated, at least for the Culicidae (Mattingly 1965; Waage 1979). This previously postulated concept (Lehane 2005; Mattingly 1965; Waage 1979) is supported by our findings that the same set of semiochemicals (PSB) attracts female mosquitoes to both tansy inflorescences and human hosts. An alternate explanation for shared cues between plants and vertebrates is that inflorescences “compete” with vertebrates for the attraction of mosquitoes, particularly sugar-fed females that seem to prefer human-derived over nectar-derived odourants (Hancock & Foster 1997; Foster & Takken 2004) .

I conclude that multimodal integration of CO_2 and other sensory cues that drives mosquito attraction to humans appears to also drive mosquito attraction to inflorescences. Overlapping cues between plants and vertebrates support a previously

postulated concept (Lehane 2005; Mattingly 1965; Waage 1979) that haematophagy of some mosquito taxa may have arisen from phytophagy.

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3.5. Tables

Table 3.1 **Headspace odorants and their absolute amounts present in 240 tansy inflorescence-hour-equivalents (1 IHE = the amount of odorants released from one inflorescence during 1 h of odorant capture) and tested in behavioural bioassays. Columns on the right indicate the commercial supplier and the purity of synthetic odorants. A 240-IHE synthetic blend dissolved in pentane/ether (1:1) was tested in bioassays. [1]Numbers in parentheses correspond to literature references reporting these compounds in human headspace;[2]Sigma-Aldrich (St. Louis, MO 63103, USA); [3]Treatt Plc (Lakeland, FL 33805, USA); [4]Liberty Natural Products (Portland, OR 97215, USA); [5]see Appendix B for purification procedure; [6]obtained by acetylation of corresponding alcohols; [7]see Appendix B for synthetic procedures.**

Compound	Amount (µg)	Human-shared?[1]	Supplier	Purity %
butanoic acid	2	Yes[12,13]	Sigma-Aldrich[2]	99
2-methylpropionic acid	1	Yes[12]	Sigma-Aldrich[2]	99
2-methylbutanoic acid	120	Yes[54]	Sigma-Aldrich[2]	98
3-methylbutanoic acid	240	Yes[12,13,54]	Sigma-Aldrich[2]	99
benzoic acid	2.5	Yes[53]	Sigma-Aldrich[2]	99.5
hexanoic acid	1	Yes[49,53,54]	Sigma-Aldrich[2]	99
(–)-α-pinene	13	Yes[55]	Sigma-Aldrich[2]	98
(–)-β-pinene	4	No	Sigma-Aldrich[2]	99
(–)-sabinene	7	No	Sigma-Aldrich[2]	75
(*E*/*Z*)-ocimene	5	No	Sigma-Aldrich[2]	>90
germacrene-D	7	No	Treatt[3,5]	40
benzaldehyde	43	Yes[53,55]	Sigma-Aldrich[2]	99
acetophenone	74	Yes[56]	Sigma-Aldrich[2]	99
artemisia ketone	23	No	Liberty Natural Products[4,5]	98
umbellulone	12	No	Sigma-Aldrich[2]	98
(Z)-3-hexenyl acetate	5	No	Sigma-Aldrich[2,6]	98
hexyl acetate	0.5	No	Sigma-Aldrich[2,6]	98
yomogi alcohol	0.5	No	Liberty Natural Products[4,5]	75
phenyl-2,3-butanedione	27	No	Gries-lab[7]	75
3-hydroxy-4-phenyl-2-butanone	8	No	Gries-lab[7]	25

3.6. Figures

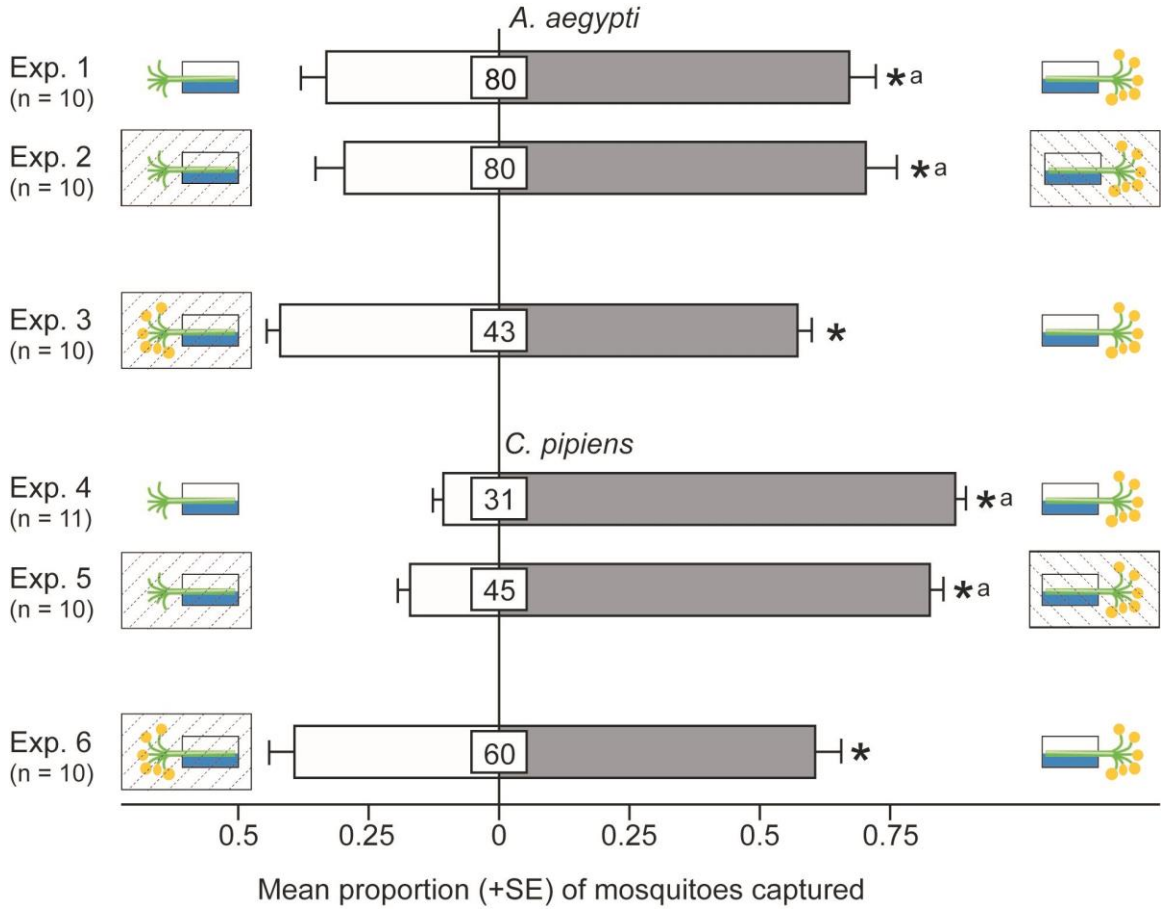

Figure 3.1 **Trap captures of 1- to 3-day-old female *Ae. aegypti* and 1- to 3-day-old female *Cx. pipiens* in response to visual and olfactory tansy inflorescence cues. A rectangular box with hatched lines indicates that the occluded inflorescence offered no visual cues. An asterisk indicates a significant preference (P < 0.01) for the specific test stimulus (binary logistic regression analyses with logit link function); the same letter on paired bars in parallel experiments indicates no difference in the mean proportion of mosquitoes responding to respective stimuli (P > 0.05); numbers within bars indicate the mean percentage of mosquitoes not captured.**

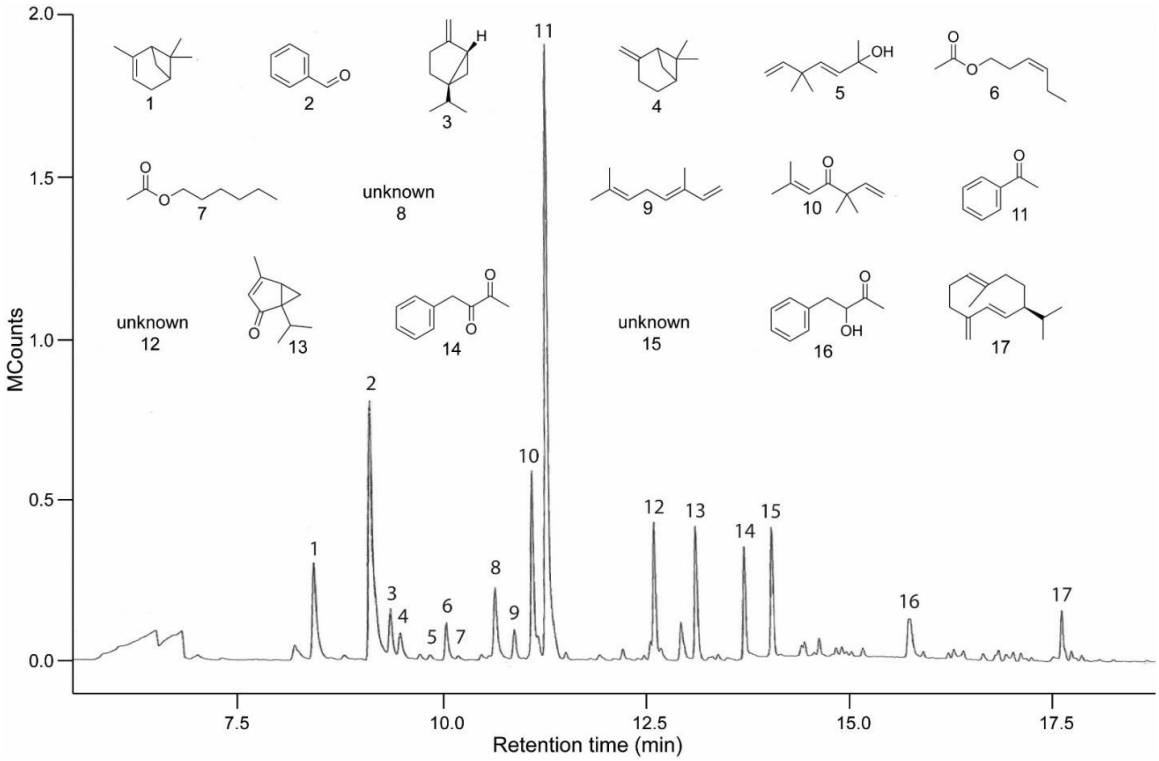

Figure 3.2 **Headspace odourants of tansy inflorescences. 1 = (–)-α-pinene; 2 = benzaldehyde; 3 = (–)-sabinene; 4 = (–)-β-pinene; 5 = yomogi alcohol (2,5,5-trimethyl-3,6-heptadien-2-ol); 6 = (Z)-3-hexenyl acetate; 7 = hexyl acetate; 8 = unknown; 9 = (E)-β-ocimene (trans-3,7-dimethyl-1,3,6-octatriene); 10 = artemisia ketone (3,3,6-trimethyl-1,5-heptadien-4-one); 11 = acetophenone; 12 = unknown; 13 = umbellulone (4-methyl-1-(1-methylethyl)-bicyclo[4.1.0]hex-3-en-2-one); 14 = phenyl-2,3-butanedione; 15 = unknown; 16 = 3-hydroxy-4-phenyl-2-butanone; 17 = germacrene-D ((E,E)-1-methyl-5-methylene-8-(1-methylethyl)-1,6-cyclodecadiene).**

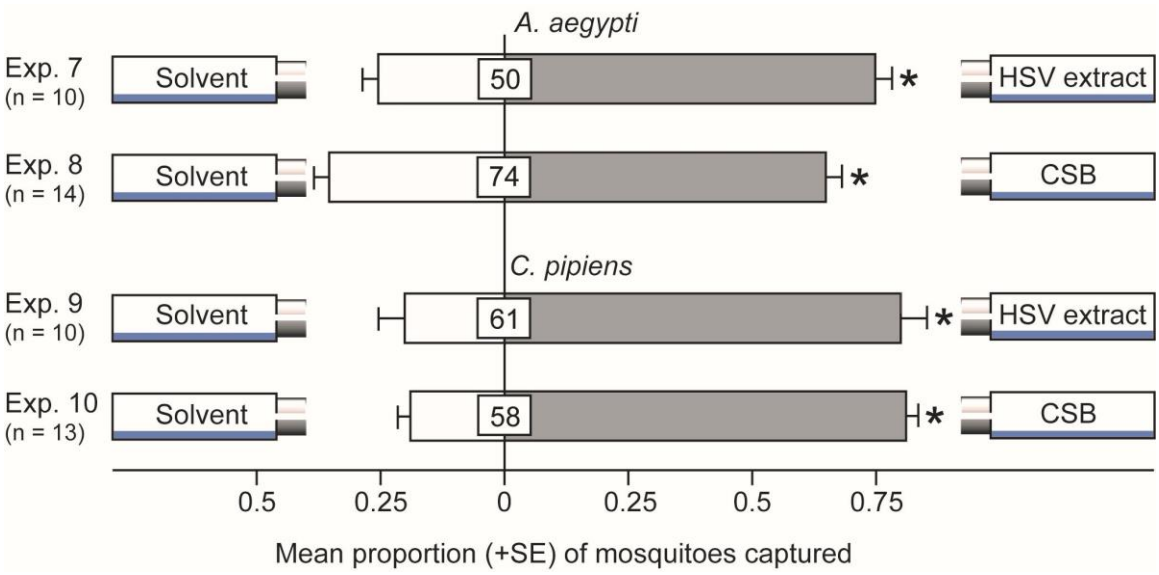

Figure 3.3 **Trap captures of 1- to 3-day-old female *Ae. aegypti* and 1- to 3-day-old female *Cx. pipiens* in response to tansy headspace volatile extract (HSV) and a complete synthetic blend (CSB) of these headspace volatiles (see Table 3.1). An asterisk indicates a significant preference ($P < 0.0001$) for the specific test stimulus (binary logistic regression analyses with logit link function); numbers within bars indicate the mean percentage of mosquitoes not captured.**

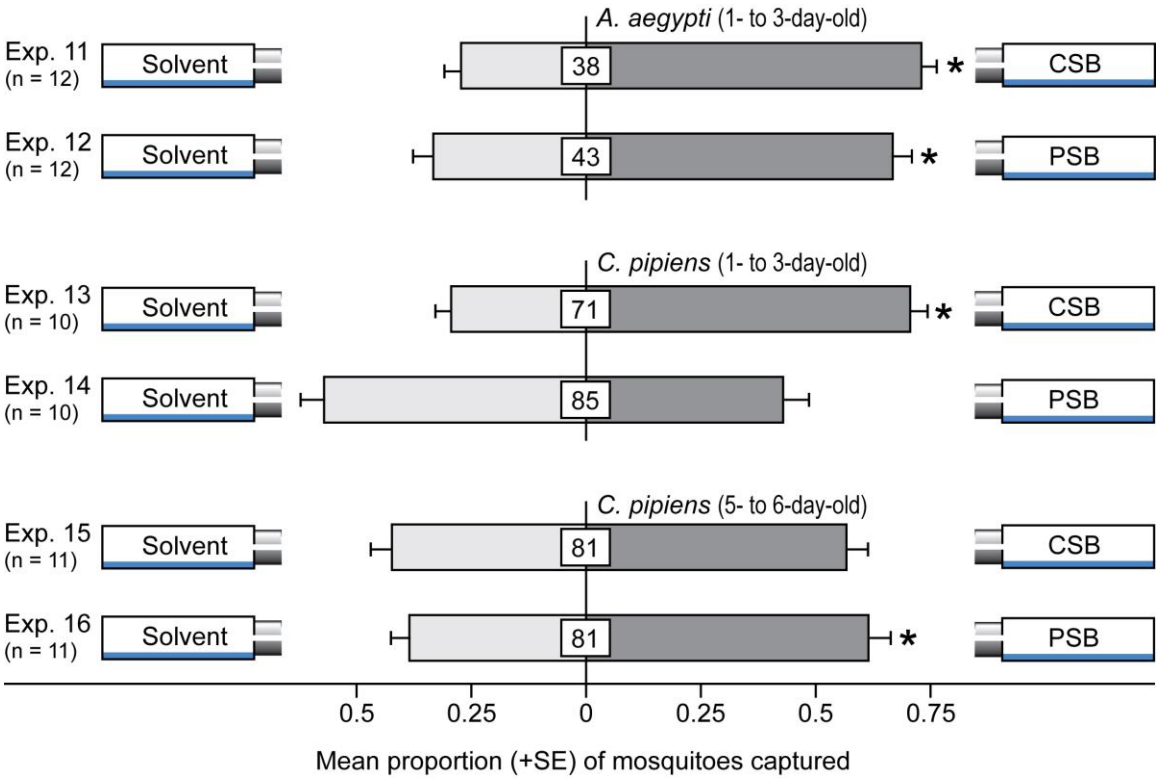

Figure 3.4 **Effects of a complete synthetic blend of tansy headspace volatiles and a partial synthetic blend on mosquito attraction. Trap captures of 1- to 3-day-old female *Ae. aegypti* and 1- to 3-day-old or 4- to 6-day-old female Cx. pipiens. An asterisk indicates a significant preference ($P < 0.05$) for the specific test stimulus (binary logistic regression analyses with logit link function); numbers within bars indicate the mean percentage of mosquitoes not captured.**

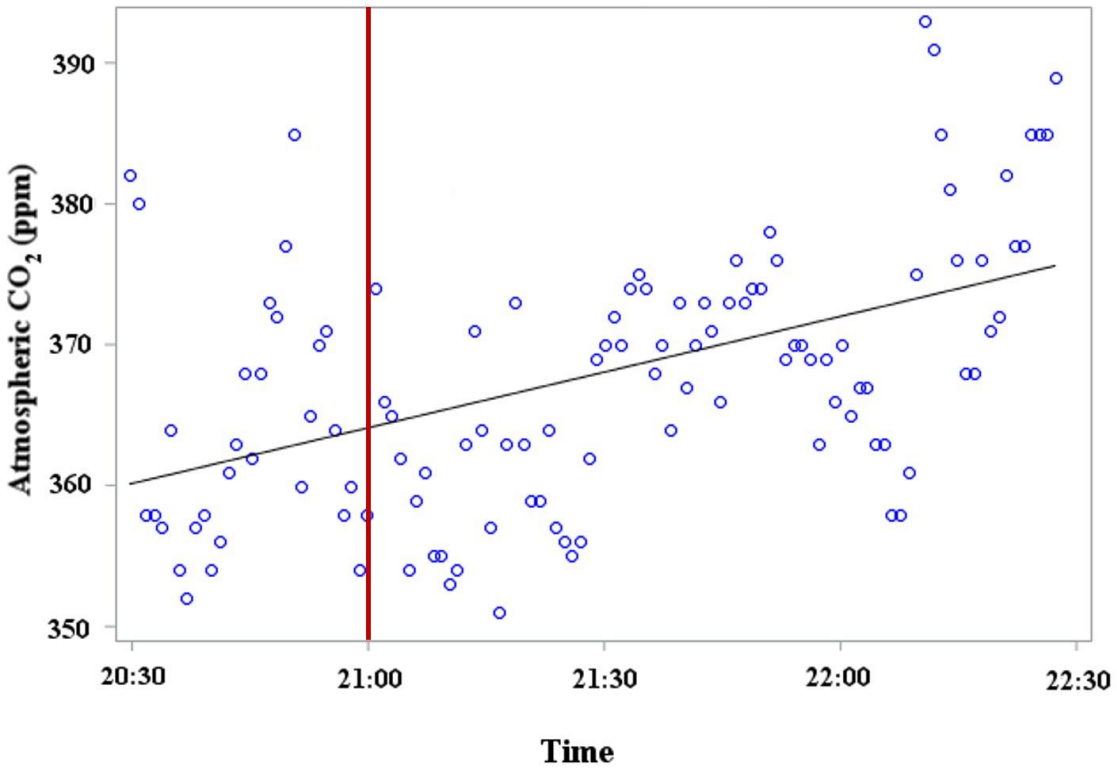

Figure 3.5 **Changes in atmospheric CO_2 concentration in a patch of tansies measured around dusk. The red vertical line represents sunset, and the solid black line represents a linear fit (y = 360 + 8.24*hours, P = 0.0032, R2 = 0.6). Measured on 18 August 2015 in a patch of tansies, *Tanacetum vulgare*, in Burnaby, British Columbia.**

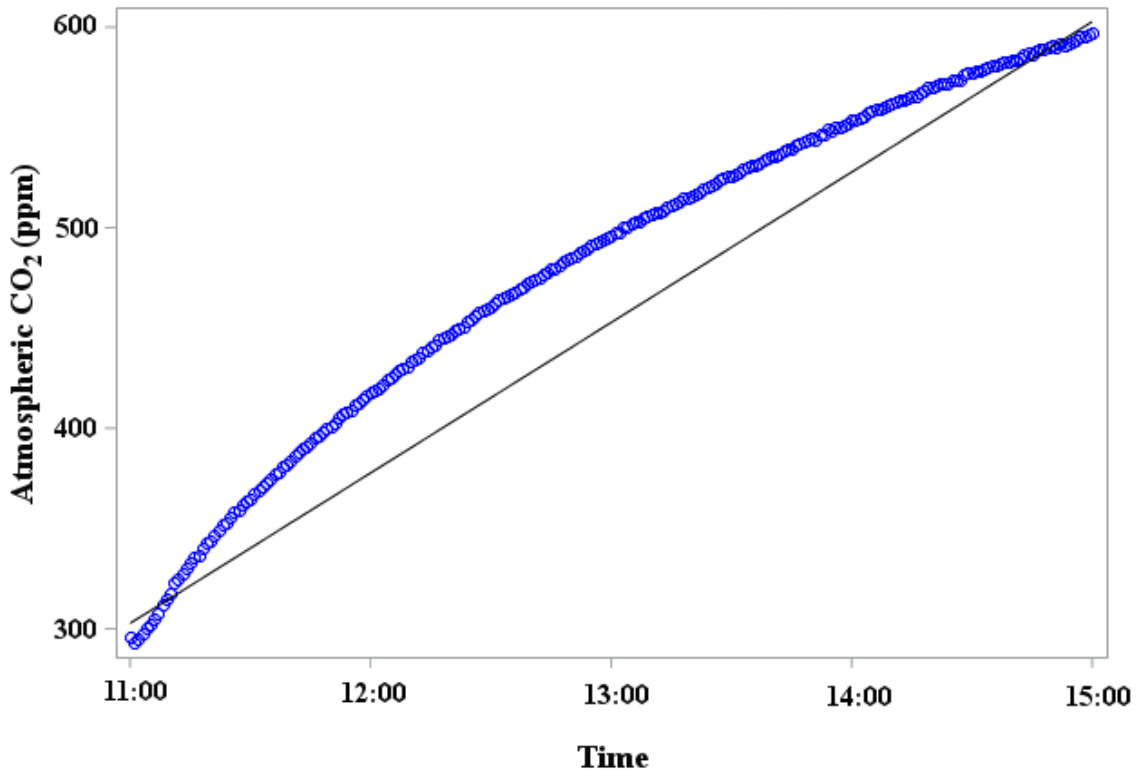

Figure 3.6 **Increase in atmospheric CO_2 measured every minute from a single tansy inflorescence (3.63 g) enclosed in a 3.9-L container. The solid black line represents an autocorrelated linear fit (y = 303 + 74.88*hours, P < 0.0001, R^2 = 0.999), corresponding to CO_2 increase of approximately 5 µL min^{-1}.**

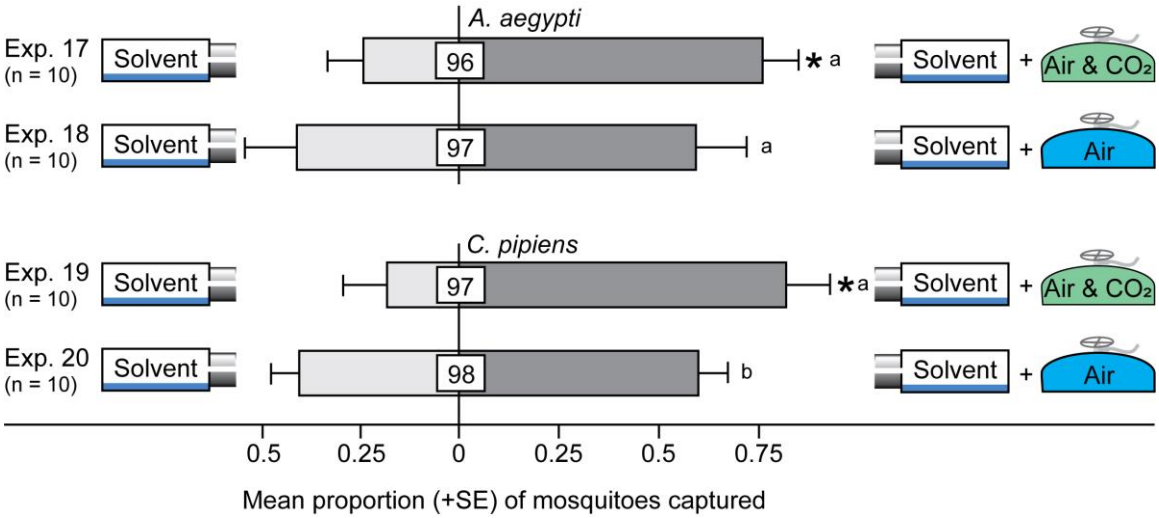

Figure 3.7 **Trap captures of 1- to 3-day-old female *Ae. aegypti* and 1- to 3-day-old female *Cx. pipiens* in response to medical-grade air (air), or medical-grade air containing 1% CO_2 (air & CO_2). In each experiment, an asterisk indicates a significant preference ($P < 0.05$) for the specific test stimulus (binary logistic regression analyses with logit link function); different letters on paired bars in parallel experiments indicate a difference in the mean proportion of mosquitoes responding to respective stimuli ($P < 0.05$). Numbers within bars indicate the mean percentage of mosquitoes not captured (= non-responders); this percentage is relatively high here because experiments 17-20 were run for only 2 h, instead of 24 h (experiments 1-16).**

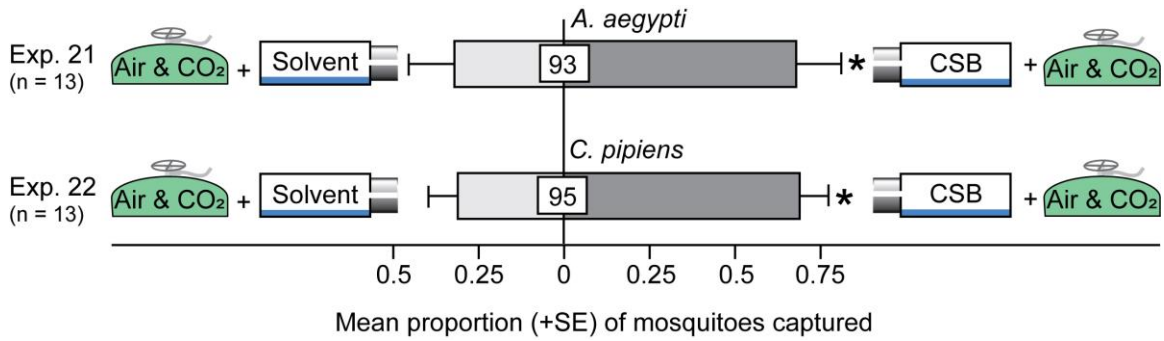

Figure 3.8 **Trap captures of 1- to 3-day-old female *Ae. aegypti* and 1- to 3-day-old female *Cx. pipiens* in response to medical-grade air containing 1% CO_2 (air & CO_2) and a complete synthetic blend (CSB) of tansy headspace volatiles (see Table 3.1). An asterisk indicates a significant preference ($P < 0.05$) for the specific test stimulus (binary logistic regression analyses with logit link function). Numbers within bars indicate the mean percentage of mosquitoes not captured (= non-responders); this percentage is relatively high because experiments 21-22 were run for only 2 h instead of 24 h (experiments 1-16).**

Chapter 4.

Ultraviolet inflorescence cues enhance attractiveness of inflorescence odour to female *Culex pipiens* mosquitoes[1]

[1]The corresponding manuscript is in review in *PLoS ONE* with the following authors: Peach, D.A.H., Ko, E., Blake, A.J., and Gries, G.

Introduction

Plant sugar, mainly in form of floral nectar, is the essential basic food for adult mosquitoes (Foster 1995) that can serve as pollinators to the many plants they visit (Brantjes & Leemans 1976; Müller 1873; Peach & Gries 2016; Stoutamire 1968; Thien 1969). Floral semiochemicals are believed to attract mosquitoes to inflorescences (see reviews by Foster 1995, 2008; Nyasembe & Torto 2014), whereas visual floral cues were thought (Clements 1999), and recently shown (Bernáth *et al.* 2016; Dieng *et al.* 2018; Peach *et al.* 2019), to play a contributing role. Field observations suggest that mosquitoes most often visit light-coloured flowers (Magnarelli 1977, 1979; Sandholm & Price 1962) but preferential visitation to these types of flowers has yet to be rigorously tested (Clements 1999). Exclusively visual cues of oxeye daisy inflorescences did not attract mosquitoes in laboratory experiments (Jepson & Healy 1988) but olfactory oxeye daisy cues alone or in combination with visual cues did (Jepson & Healy 1988). Both the yellow fever mosquito, *Aedes aegypti* (L.), and the northern house mosquito, *Culex pipiens* L., were more strongly attracted to a combination of olfactory and visual inflorescence cues than to olfactory inflorescence cues alone (Peach *et al.* 2019), revealing a contributing role of visual cues in mosquito attraction to inflorescences.

The effect of visual cues on mosquito behaviour is evident in further studies. Southern house mosquitoes, *Cx. quinquefasciatus*, did learn to associate visual cues with palatable and non-palatable solutions of sucrose and sucrose-NaCl, respectively (Bernáth *et al.* 2016). Mosquitoes also preferred dark-coloured over white-coloured artificial inflorescences associated with sucrose solutions (Dieng *et al.* 2018); however, the presence of human observers and their associated odours (CO_2) in these experiments could have altered the preferential response of mosquitoes.

The many visual inflorescence cues that attract pollinators include inflorescence shape, colour, and colour patterns (Brodie *et al.* 2015; Dafni *et al.* 1997; Orbán & Plowright 2014). The circular "bullseye" colour pattern of many inflorescences or their UV 'bullseye' – with petals having UV-absorbing bases and UV-reflective apices – attract pollinators and guide them to the inflorescence centre (Dinkel & Lunau 2001; Free 1970; Horth *et al.* 2014; Koski & Ashman 2014; Orbán & Plowright 2014). The evolutionary "display" of inflorescences seem to factor in the UV-sensitivity (300-400 nm) of their insect pollinators (Briscoe & Chittka 2001; Shimoda & Honda 2013). Studying the sensory capabilities of mosquito photoreceptors will allow us to understand the type of visual cues and signals that mosquitoes can sense and exploit during foraging and mate location. Electroretinograms (ERGs) with compound eyes of *Ae. aegypti* revealed receptor sensitivity peaks in the UV and yellow-green wavelength ranges (Muir *et al.* 1992), implying, e.g., that UV nectar guides of inflorescences could be exploited by UV-sensitive, nectar-foraging mosquitoes. Expectedly then, UV opsins were found in *Ae. aegypti* and *Anopheles gambiae* (Hu *et al.* 2009, 2011).

The UV-sensitivity of mosquitoes is also exploited in mosquito trapping programs that deploy both UV-light and CO_2 as trap baits (Wilton 1975; Wilton & Fay 1972). Other mosquito traps exhibit visual cues that emphasize contrast (Haufe 1964; Silva *et al.* 2005) which matters to host-foraging mosquitoes (Sippell & Brown 1953; van Breugel *et al.* 2015).

Culex pipiens is a crepuscular mosquito native to temperate Eurasia and established throughout temperate North America (Darsie & Ward 2005). It vectors West Nile virus (WNV) (Hamer *et al.* 2008) and avian malaria (Kimura *et al.* 2010). *Cx. pipens* visits many flowers of the Asteraceae (Andersson & Jaenson 1987; Grimstad & DeFoliart 1974; Peach & Gries 2016), including the common tansy, *Tanacetum vulgare* (Peach & Gries 2016). To determine whether floral UV reflection and absorption patterns have a functional role in the context of nectar-foraging by mosquitoes, we used the common tansy, *Tanacetum vulgare*, which is UV-absorbing (Fig 4.1) and pollinated by *Cx. pipiens* (Peach & Gries 2016), and the common hawkweed, *Hieracium lachenalii*, which exhibits a prominent UV bullseye (Fig 4.1) and is closely related to the king-devil hawkweed, *Hieracium pratense*, which is visited by several *Aedes* spp. (Magnarelli 1979).

Our objectives were (1) to determine both the ability of *Cx. pipiens* compound eyes to sense UV light and the potential photoreceptors sensing it, (2) to biosassay the effect of visual inflorescence cues (in the presence of inflorescence odour) on attraction of *Cx. pipiens*, (3) to study the effect of UV absorption and reflection patterns in *H. lachenalii* inflorescences on attraction of *Cx. pipiens*; and (4) to determine the specific characteristics of floral UV light cues, and possible interactions with floral colour cues, that mediate attraction of *Cx. pipiens*.

4.1. Materials and Methods

4.1.1. Experimental insects

We sustained adult *Cx. pipiens* on a 10% sucrose solution, provided *ad libitum*, in mesh cages (30 × 30 × 46 cm high) maintained at 23-26 ^{o}C, 40-60% RH, and a photoperiod of 14L:10D. Once a week, we blood-fed adult females on the arm of DP. For oviposition, we gave gravid females access to water in a circular glass dish (10 cm diameter × 5 cm high). we transferred egg rafts to water-filled trays (45 × 25 × 7 cm high) and provided larvae with NutriFin Basix tropical fish food (Rolf C. Hagen Inc., Montreal, QC, Canada). We transferred pupae with a 7-ml plastic pipette (VWR International, PA, USA) to water-filled 354-ml Solo cups covered with a mesh lid (Solo Cup Company, IL, USA). We released eclosed adults into mesh cages (30 × 30 × 46 cm high), transferred virgin females via aspirator to separate water-containing Solo cups, and provisioned them with a cotton ball soaked in a 10-% sucrose solution.

4.1.2. Experimental plants

I collected inflorescences of *T. vulgare* and *H. lachenalii* from field sites in Metro Vancouver, British Columbia, Canada between June-November 2017 and 2018. we used inflorescences in experiments within two hours of collection.

4.1.3. Electroretinograms

The sensitivity of *Cx. pipiens* compound eyes to wavelengths in the UV and human-visible range (300-650 nm) was determined using electroretinogram (ERG) recordings. Each of fifteen 3- to 4-day-old *Cx. pipiens* females was first cold

anesthetized, and then immobilized ventral side up, on a piece of sticky tack. This preparation was affixed to a glass microscope slide and placed on a platform below a microscope (Wild M10, Leica Microsystems, ON, Canada). Leitz micromanipulators M (Leitz, Vienna, Austria) were used to insert glass microelectrodes into the left eye and the thorax of the immobilized female mosquito. Electrodes were formed with a micropipette puller (Model P-1000, Sutter Instrument Co., CA, USA), filled with a Ringer solution (Staddon & Everton, 1979), and fitted with a silver wire.

The mosquito eyes were adapted to darkness, green light or UV light for 45 min prior to ERGs. The adapting lights consisted of a green- and a UV light-emitting diode (LED; B5B-433-B25, UV RLT350-0.3-15; Roithner LaserTechnik, Vienna, Austria), with nominal peak wavelengths of 525 nm and 351 nm, respectively. Each LED was attached to the terminal end of the fibre optic cable delivering stimulus light and positioned such that the LED light shone on the same portion of the mosquito eye as the fiber optic cable. Each adaptation was performed on five separately prepared mosquitoes, for a total of 15 mosquitoes.

Light stimuli were generated using a 35-watt Xenon Arc light source (HPX-2000, Ocean Optics, Dunedin, FL, USA) and a fibre optic scanning monochromator (MonoScan 2000, Mikropak GmbH, Ostfildern, Germany). From this monochromator, light was transmitted through a 600-µm optical fibre (QP600-1-SR-B X, Ocean Optics, FL 32792, USA) fitted with a collimator (LC-4U-THD, Multimode Fiber Optics, Hackettstown, NJ, USA) and through a 0-2 stop circular variable neutral density wheel (fused silica (200-2500 nm); Reynard Corp., San Clemente, CA, USA) directly in front of a 20:80 beam splitter (“polka dot” 4-2001; Optometrics, Ayer, MA, USA). 20% of the light was transmitted to a calibrated cosine-corrector-fitted (CC-3-UV-S, Ocean Optics, Dunedin, FL, USA) spectrophotometer (HR-4000, Ocean Optics, Dunedin, FL, USA) to monitor and adjust the absolute irradiance of test stimuli. The remaining 80% of the transmitted light reached the eye of the test specimen via a cosine-corrector-fitted 1000-µm single fibre optic cable (PCU-1000-2-SS, Multimode Fiber Optics, Hackettstown, NJ, USA) with a Sub-Miniature-A (SMA) terminus. A custom-built programmable shutter (R. Holland, Science Technical Centre, Simon Fraser University, Burnaby, BC, Canada), located between the beam splitter and the cosine corrector, was opened for 0.5 s every 10 s to expose the eye to a test stimulus at an intensity of 1.0×10^{13} photons/cm^2/s and wavelength between 300-650 nm with a 5-nm bandwidth. The response amplitudes were

calibrated to test stimuli against an intensity–response function to determine the sensitivity of the *Cx. pipiens* compound eye to those wavelengths. The spectral sensitivities from individual compound eyes were normalized by the 97.5% quantile value of their sensitivity, and again normalized the mean spectral sensitivities for dark-, green-, and UV-adapted compound eyes in this fashion.

4.1.4. Behavioural experiments

General design

We performed experimental replicates at 23-26 ºC, 40-60% relative humidity, and a photoperiod of 14L:10D. For each replicate, we released 50 virgin, 1- to 3-day-old females starved at least 24 h into a mesh cage (77 × 78 × 104 cm high), the front and lateral sides of which were covered with black cloth to minimize stray light entry, and the top and back were left uncovered. The cage center housed two burette stands separated by 25 cm, each stand carrying a Delta trap 50 cm above the cage floor. We made traps from white or black cardstock (71.28 × 55.88 cm) (Staples Inc., MA, USA; ACCO Brands Corp., IL, USA) that were cut to size (15 × 30 cm), coated with adhesive (The Tanglefoot Company, MI, USA) on the inside, and then folded into a Delta-type trap (15 × 9 × 8 cm high).

We illuminated cages with a shop light housing (Lithonia Lighting, GA, USA) placed vertically behind each cage and fitted with both a 1.22-m 10.0 UVB fluorescent tube (Zoo Med, CA, USA) and a conventional 1.22-m fluorescent tube (F32T8/Tl835 Plus, Phillips, Amsterdam, Netherlands). We connected the housing to a timer set to the same photoperiod (14L:10D) as the room lights.

4.1.5. Specific experiments

Effect of visual inflorescence cues under UV light on female Cx. pipiens attraction (Exps 1 and 2)

In experiment 1 (Fig 4.2), treatment and control stimuli consisted of a freshly cut *T. vulgare* inflorescence with its stem inserted into a water-filled vial (4-ml) through a pre-punctured hole in Parafilm (Bemis Company Inc., WI, USA) that covered the vial opening. We placed each vial horizontally into a trap such that the inflorescence faced the light housing fitted with both a UV and a conventional fluorescent tube timed to turn

off during the scotophase (see above). To determine the (additive) effect of visual cues on the attractiveness of *T. vulgare* inflorescences, we placed the vial containing the treatment inflorescence on top of cheesecloth (Cheesecloth Wipes, VWR International, PA, USA) and occluded the vial containing the control inflorescence with cheesecloth. Experiment 2 (Fig 4.2) was identical in design except that we tested *H. lachenalii* instead of *T. vulgare* inflorescences.

Effect of visual inflorescence cues under UV-deficient illumination on female Cx. pipiens attraction (Exps 3 and 4)

The design of experiment 3 (Fig 4.2) was identical to that of experiment 1 except that we placed a sheet of polycarbonate (30.48 × 91.44 × 0.3175 cm thick; Lexan, SABIC, Riyadh, Saudi Arabia) with minimal UV transmission (S1 Fig) in front of the UV light source. This design essentially eliminated the visibility of the bullseye pattern from the inflorescence. Experiment 4 (Fig 4.2) was identical in design except that it tested *H. lachenalii* instead of *T. vulgare* inflorescences.

Effect of floral UV absorption & reflectance patterns on female Cx. pipiens attraction (Exp 5)

The design of experiment 5 (Fig 4.2) was identical to that of experiment 4 except that (1) placed each vial with its inflorescence was placed on top of black velvet (Suzhou Joytex International Co. Ltd, Jiangsu, China), (2) we deployed black instead of white delta traps, and (3) inflorescences were treated to alter their bullseye (the characteristic UV absorption and reflection pattern). The upper surface of petals of treatment inflorescences were treated with a “sunscreen mix” of UV-absorbing Parsol 1789 and Parsol MCX (50:50 w/w; Sigma-Aldrich, ON, Canada) formulated in canola oil (adapted from Koski & Ashmann 2014), and the upper surface of petals of control inflorescences with canola oil only. In addition, the receptacle of control inflorescences was treated with the “sunscreen mix” to ensure “odour symmetry” between treatment and control inflorescences.

Effect of UV light absorption, UV reflectance and colour of inflorescence model on attraction of female mosquitoes (Exps 6 and 7)

In experiment 6 (Fig 4.2), we compared the attractiveness of yellow model flower discs (2.5 cm diameter) that exhibited either a uniformly UV-dark or a uniformly UV-bright appearance. We prepared the discs from yellow printer paper (International Paper,

TN, USA), and painted treatment discs with clear nail polish (Coty Inc., NY, USA) rendering them dark in the UV range while maintaining their yellow, human-visible colouration. Using an inkjet printer, we printed control discs with a yellow ink that maintained their UV reflectance but rendered them darker to mimic the darkened appearance of nail polish-painted treatment discs. To ensure “odour symmetry” of the treatment and the control disc, we paired them using their untreated side for contact and then placed each disc pair into a black trap containing a *H. lachenalii* inflorescence which we occluded with black velvet to provide olfactory but not visual cues. In treatment and control traps, the nail polish-painted side and the yellow ink-printed side, respectively, of the paired discs leaning against the occluded inflorescence faced the trap entrance, at a 90^{o} angle relative to the trap bottom.

In experiment 7 (Fig 4.2), we explored a potential additive effect of floral colour (yellow) on the combined effect of floral odour and UV darkness on mosquito attraction. We modified the design of experiment 6 in that we prepared model flower control discs from black cardstock and model flower treatment discs from yellow printer paper, painting both discs with clear nail polish which renders them UV-dark. We also replaced black traps with white traps, and black velvet with cheesecloth.

4.1.6. Spectral analyses

The spectral reflectance of *T. vulgare* and *H. lachenalii* inflorescences was measured with a JAZ spectrometer (Ocean Optics Inc., Dunedin, FL, USA). Measurements covered a range of 300-700 nm and were corrected to absolute diffuse reflectance by a 99% Spectralon reflectance standard (SRS-99-010, Labsphere, NH, USA). Spectral reflectance measurements were acquired from *H. lachenalii* inflorescences (center and perimeter) that were (*i*) coated with canola oil (100% Pure Canola Oil, Richardson International, MB, Canada), (*ii*) coated with a sunscreen mixture (50:50 w/w Parsol 1789 and Parsol MCX, Sigma-Aldrich, ON, Canada) formulated in canola oil (60:40 w/w sunscreen mixture), or (*iii*) untreated. We also took spectral reflectance measurements of untreated *T. vulgare* inflorescences.

The absolute irradiance of 48-inch fluorescent UV bulbs (Zoo Med, San Luis Obispo, CA, USA) and conventional bulbs (Philips, Amsterdam, Netherlands) deployed in bioassays was measured, with or without a Lexan Polycarbonate filter that blocked UV

transmissions, with a calibrated spectrophotometer (HR-4000, Ocean Optics) using SpectraSuite software (Ocean Optics). Light was collected using a cosine corrector (CC-3-UV-S, Ocean Optics) placed in the center of the cage (77 × 78 × 104 cm high) at a height of 50 cm.

4.1.7. UV photography

UV photographs of *T. vulgare* and *H. lachenalii* inflorescences were taken using a custom lens mounted to an Olympus E-PM1 camera (Olympus, Tokyo, Japan) modified for spectral sensitivity covering both the UV (< 400 nm) and human-visible light range (400-700 nm) (Dr. Klaus Schmitt, Weinheim, Germany, uvir.eu). An UV/IR filter (Baader Plantarium, Mammendorf, Germany) and a U-filter (Baader Plantarium, Mammendorf, Germany) were used for human-visible and UV images, respectively.

4.1.8. Statistical analyses

We analyzed behavioural data using SAS statistical software version 9.4 (SAS Institute Inc., Cary, NC 27513, USA), excluding from analyses experimental replicates with no mosquitoes responding. We compared mean proportions of responders to paired test stimuli using a binary logistic regression model and worked with back-transformed data to obtain means and confidence intervals.

4.2. Results

4.2.1. Electroretinograms (ERGs)

In ERG recordings following dark adaptation, *Cx. pipiens* eyes (n = 5) exhibited a spectral sensitivity peak in the UV range (335 nm) and the green range (540 nm) (Fig 4.3). Adaptations of eyes to green light (n = 5) or UV light (n = 5) induced sensitivity changes to green or UV light (Fig 4.3). As expected, UV-adapted eyes became less sensitive to UV light (300 – 400 nm), whereas green-adapted eyes became less sensitive in the visual range (400 – 650 nm).

4.2.2. Behavioural experiments

Effect of visual inflorescence cues under UV light on female Cx. pipiens attraction (Exps 1 and 2)

When given a choice of either olfactory inflorescence cues alone (inflorescence under cheese cloth) or both olfactory and visual inflorescence cues (inflorescence on top of cheese cloth), female *Cx. pipiens* significantly preferred the bimodal *T. vulgare* inflorescence cue complex ($z = 2.75$, $p = 0.06$; Fig 4.4, Exp. 1) and the bimodal *H. lachenalii* inflorescence cue complex ($z = 4.44$, $p < 0.0001$; Fig 4.4, Exp. 2).

Effect of visual inflorescence cues under UV-deficient illumination on female Cx. pipiens attraction (Exps 3 and 4)

When we presented *Cx. pipiens* females with the same choices as in preceding experiments 1 and 2 but under UV light-deficient illumination, these females no longer showed a preference for the bimodal (olfactory, human-visible) inflorescence cue complex of *T. vulgare* ($z = -0.8$, $p = 0.42$; Fig 4.4, Exp 3) or of *H. lachenalii* ($z = -1.14$, $p = 0.26$; Fig 4.4, Exp 4).

Effect of inflorescence UV reflectance and absorbance pattern on female Cx. pipiens attraction (Exp 5)

Given a choice of (uncovered) inflorescences that were either uniformly UV-dark (treated with canola oil/sunscreen mix) or that still exhibited the UV bullseye (treated with canola oil control), female *Cx. pipiens* significantly preferred the former treatment ($z = 5.21$, $p < 0.0001$; Fig 4.4, Exp 5).

Effect of UV absorption, UV reflection and colour of inflorescence models on female Cx. pipiens attraction (Exps 6 and 7)

When we presented *Cx. pipiens* females (in the presence of *H. lachenalii* inflorescence odour) with a choice of yellow floral models which were either uniformly UV-bright or UV-dark, these females selected significantly more often the UV-dark model ($z = 3.31$, $p = 0.0009$; Fig 4.5, Exp 6).

When we presented *Cx. pipiens* females (in the presence of *H. lachenalii* inflorescence odour) with a choice of UV-dark floral models that were either yellow or

black (in the human-visible range), these females selected significantly more often the black model ($z = 2.58$, $p = 0.01$; Fig 4.5, Exp 7).

4.3. Discussion

These findings indicate that (1) compound eyes of *Cx. pipiens* can sense UV light; (2) visual inflorescence cues render inflorescence odour more attractive to *Cx. pipiens*; (3) the UV "bullseye" of *H. lachenalii* inflorescences (Fig 4.1b) attracts *Cx. pipiens*; (4) the UV-dark trait of inflorescences is a strong driver of *Cx. pipiens* attraction, and (5) stimuli dark in both human visible light and UV light are most attractive to *Cx. pipiens*. Below, we shall elaborate on these findings.

To determine the heretofore unknown spectral sensitivity of *Cx. pipiens* compound eyes, we conducted electroretinogram recordings, exposing eyes to 5-nm bandwidth of light in the UV and human-visible light range (300-650 nm). The recordings revealed that UV light of 335-nm wavelength and green light of 540-nm wavelength elicit the strongest receptor potentials (voltages) from *Cx. pipiens* eyes (Fig 4.3). These results indicate the presence of at least one UV-sensitive photoreceptor in *Cx. pipiens* eyes.

The spectral sensitivity of *Cx. pipiens* resembles that of other dipterans (Kirschfeld *et al.* 1977; Mellor *et al.* 1996), particularly that of the yellow fever mosquito, *Ae. aegypti,* which exhibits peak spectral sensitivity in the UV (323-345 nm) and green (523 nm) ranges (Muir *et al.* 1992). Similar to most dipterans, each ommatidium in *Ae. aegypti* contains eight photoreceptor cells (R1-8) (Hu *et al.* 2012). The six outer photoreceptors (R1-6) express a longwave-sensitive opsin (rhodopsin Aaop1) (Hu *et al.* 2012), whereas the two inner photoreceptors (R7,8) express longwave-, UV- or blue-sensitive opsins depending on the eye region (Hu *et al.* 2009, 2011, 2014). Interestingly, there is structural similarity of ommatidia in *Ae. aegypti* and *Cx. pipiens* (Sato 1957; Land e*t al*. 1999;), and similar sets of longwave-, UV- and blue-sensitive opsins are present in *Ae. aegypti* and *Cx. quinquefasciatus* (Giraldo-Calderón 2015; Giraldo-Calderón *et al.* 2017), a sister species of *Cx. pipiens* (Harbach 2012; Miller *et al.* 1996). All these facts coupled with the results of our ERG recordings (Fig 4.3) support the inference that *Cx. pipiens* and *Ae. aegypti* have similar complements of photoreceptors and comparable opsin expressions.

Following exposure to either UV light or green light, *Cx. pipiens* eyes became less sensitive to UV light and to green light (Fig 4.3), respectively. If only a single photoreceptor type were to be responsible for theses adaptions, we would expect similar sensitivity changes following pre-exposure to either UV or green light. The observed dissimilar sensitivity changes following UV or green light pre-exposure (Fig 4.3) suggest that both a green-sensitive and a UV-sensitive photoreceptor contributed to the ERG responses. Assuming this interpretation is correct, our data would provide supporting evidence that the central photoreceptors (R7, R8) of *Cx. pipiens* ommatidia express either a green or a UV opsin, unlike photoreceptors R1-6, which all express an identical green opsin in other Diptera. Our light adaptation experiments revealed no evidence for a blue-sensitive receptor contributing to the response. We expected this because the blue opsin is likely expressed at low levels in the central region of *Cx. pipiens* eyes (Hu *et al.* 2011), but photoreceptor responses may have also been affected by the green adaptation light.

The spectral sensitivity of *Cx. pipiens* eyes in the UV range (Fig 4.3) can be attributed to (*i*) the response of a UV-sensitive opsin in the central photoreceptors (R7 or R8), (*ii*) a UV-sensitizing pigment in photoreceptors R1-R6, or (*iii*) both. If *Cx. pipiens* and *Ae. aegypti* were to show similar opsin expression, then photoreceptor R7 in the central eye region (where recordings were performed) would presumably express a UV opsin with a sensitivity peak of ~330 nm. Yet, the recorded sensitivity peak (335 nm; Fig 3) may also have also originated from photoreceptors R1-R6 that - due to their abundance and size - are the main contributors to electroretinogram responses of dipteran eyes (Mellor *et al.* 1996; Minke *et al.* 1975). A UV-sensitizing pigment has been found in photoreceptors R1-6 of the common vinegar fly, *Drosophila melanogaster* (Kirschfeld *et al.* 1977), in the tiger mosquito, *Aedes albopictus* (Stavenga *et al.* 2017), but not in *Ae. aegypti* (Stavenga *et al.* 2017). Several brachyceran flies express 3-hydroxy-retinal as a UV-sensitizing pigment in their photoreceptors R1-6 (Minke *et al.* 1975). Within the Nematocera, males of black flies (Simuliidae) express a different UV-sensitizing pigment (presumably retinol) in their photoreceptors R1-6, generating a separate sensitivity peak at 340 nm (Stavenga *et al.* 2017). There is also preliminary evidence for a similar screening pigment in the Asian tiger mosquito, *Ae. albopictus* (Stavenga *et al.* 2017).

To ascertain that visual inflorescence cues contribute to the overall attractiveness of *H. lachenalii* and *T. vulgare* inflorescences, we isolated the effect of visual cues by testing inflorescences as a trap bait that were occluded, or not, with cheese cloth, presenting mosquitoes with a choice of either olfactory cues alone (inflorescence occluded) or both olfactory and visual cues (inflorescence not occluded). Significantly greater captures of *Cx. pipiens* females in traps baited with a non-occluded inflorescence (Fig 4.4, Exps 1 and 2) established a contributing effect of visual cues to the inflorescence attractiveness. These results are not surprising in light that diverse taxa of floral visitors exploit visual inflorescence cues (Brodie *et al.* 2015; Raguso & Willis 2005; Song *et al.* 2015; Weiss 1991), and that foraging mosquitoes respond to visual cues when they seek vertebrate hosts (Clements 1999). Our results also confirm previous findings that visual inflorescence cues are part of a multimodal cue complex that guides nectar-foraging mosquitoes to inflorescences (Peach *et al.* 2019). Similarly, there is synergy between visual and olfactory inflorescence cues that guide nectar-foraging wild hawkmoths (Raguso & Willis 2005). However, attraction of mosquitoes to visual inflorescence or visual vertebrate cues appears to be contingent upon the presence of other cues such as odourants or CO_2 (McMeniman *et al.* 2014; van Breugel *et al.* 2015).

To determine whether UV light contributes to the attractive effect of visual inflorescence cues, we either eliminated UV wavelengths from illuminating light sources or altered UV reflections from inflorescences. To produce UV-deficient illumination, we placed a Lexan filter in front of illumination devices, thereby effectively eliminating the UV bullseye from *H. lachenalii* inflorescences. Under UV-deficient light, female *Cx. pipiens* no longer showed a preference for inflorescences with bimodal (olfactory, human-visible) cues (Fig 4.4, Exps 3 and 4), suggesting that it is the bullseye contrast of UV-absorbed and UV-reflected light that – together with floral odourants – guide mosquitoes to inflorescences. However, uniformly UV-dark *H. lachenalii* inflorescences, following treatment with a canola oil/sunscreen mix (Exp 5), were even more attractive to *Cx. pipiens* than control inflorescences that retained the bullseye contrast (Fig 4.4, Exp 5), indicating that *Cx. pipiens* females prefer UV-dark inflorescences. These findings are surprising in light of previous reports that the treatment of silverweed cinquefoil, *Argentina anserina*, inflorescences with a sunscreen mix (that disrupted the UV bullseye) decreased insect visitation and behaviour compared to control inflorescences which

exhibited the usual UV bullseye phenotype (Koski & Ashman 2014). A potential role of UV light on attraction of mosquitoes to visual inflorescence cues could not be detected in other studies because wavelengths only in the human-visible range were considered (Bernáth *et al.* 2016; Dieng *et al.* 2018; Peach *et al.* 2019).

In choice experiments with uniformly UV-dark or UV-bright yellow or black inflorescence models (in the presence of natural inflorescence odour), *Cx. pipiens* females preferred UV-dark over UV-bright yellow models and black UV-dark over yellow UV-dark models (Fig 4.5), supporting the significance of floral UV reflectance as a visual foraging cue (see Exp. 5). Other studies have also found mosquito attraction to dark-coloured objects or to objects with light and dark contrast (Sippell & Brown 1953; van Breugel *et al.* 2015). Previous conclusions that diurnally-active dipteran pollinators prefer inflorescence patterns of UV-absorption and UV-reflection (Koski & Ashman 2014) may be attributed to the fact that pertinent experiments were performed on diurnally-active species rather than crepuscular-active nectar-foraging mosquitoes. Moreover, *Cx. pipiens* forage on many inflorescences (e.g., *Tanacetum vulgare*, *Achillea millefolium*, *Leucanthemum vulgare* (Andersson & Jaenson 1987; Peach & Gries 2016; Sandholm & Price 1962)) that are uniformly UV-dark (Arnold *et al.* 2010; Primarck 1982; Utech & Kawano 1975).

The preference of nectar-foraging *Cx. pipiens* for black UV-dark inflorescence models over yellow UV-dark models implies that attractive stimulus traits may be intensity- rather than spectrally-based, with mosquitos being attracted to models that reflect relatively little light across their entire visual range (300-600 nm). This phenomenon is reminiscent of host-foraging mosquitos that are attracted to dark objects, such as the UV-absorbing dark plumage and pelage of many avian and mammalian hosts (Burkhardt 1989; Chávez *et al.* 2003; Shekar *et al.* 2008). It seems that nectar and host-foraging mosquitoes respond to analogous but contextually different visual resource cues (Lunau & Maier 1995). This concept may also apply when mosquitoes seek resting sites.

If *Cx. pipiens* females exclusively use the outer R1-6 photoreceptors to inform orientation behaviour towards floral or vertebrate hosts, this would bypass the colour vision circuits associated with the central photoreceptors R7 and R8 and possibly explain the preference for dark objects generally and for black over yellow UV-dark

objects specifically. The R1-6 photoreceptors are thought to provide an achromatic visual channel in other flies (Sanes & Zipursky 2010) and have only a limited role in colour vision (Schnaitmann *et al.* 2013). If, like other flies, *Cx. pipiens* were to possess a UV-sensitising pigment in the R1-6 photoreceptors, these photoreceptors would be expected to have a broadband sensitivity (300-600 nm) that would only be able to distinguish among objects on the basis of intensity.

I have shown that nectar-foraging *Cx. pipiens* females respond to both olfactory and visual inflorescence cues. UV-sensitive eyes enable *Cx. pipiens* females to detect, and discern between, floral patterns of UV-absorption and UV-reflection, with preference for inflorescences with low reflection of both human-visible and UV light. With feathers and pelts of many avian and mammalian hosts being similarly dark, foraging mosquitoes may respond to analogous but contextually different visual cues when they seek nectar and vertebrate blood resources.

4.4. References

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4.5. Figures

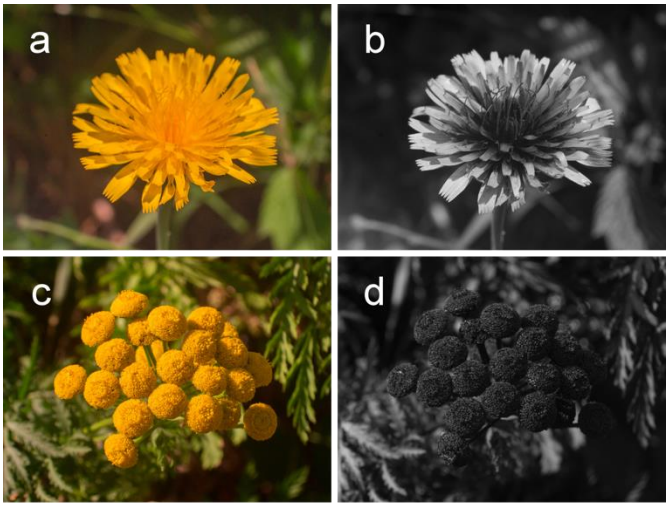


Figure 4.1 **Photographs of common hawkweed and common tansy in the human-visible light range and UV light range. Inflorescences of common hawkweed, *Hieracium lachenalii* (a,b), and common tansy, *Tanacetum vulgare* (c,d), photographed with a custom-built camera capable of taking images in the human-visible light range (a,c) and UV light range (b,d). *Hieracium lachenalii* (b) displays a prominent UV "bullseye" with UV-absorbing petal bases and UV-reflective petal apices.**

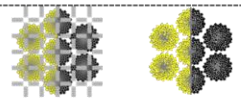
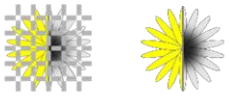
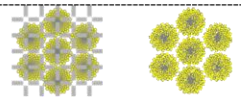
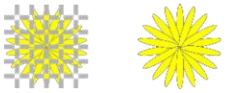
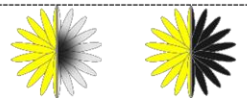
Exp. #	Stimuli tested	UV light?	Trap colour	Type of fabric	# Rep.
Effect of visual inflorescence cues under UV light					
1		Yes	White	Cheesecloth	20
2		Yes	White	Cheesecloth	20
Effect of visual inflorescence cues under UV-deficient illumination					
3		No	White	Cheesecloth	20
4		No	White	Cheesecloth	20
Effect of floral UV absorption & UV reflectance patterns					
5		Yes	Black	Black velvet	20
Effect of UV absorption, UV reflection and colour of inflorescence models					
6		Yes	Black	Black velvet	12
7		Yes	White	Cheesecloth	10

Figure 4.2 **Summary of the experimental design to test attraction of female *Culex pipiens* to inflorescences of *Hieracium lachenalii* and *Tanacetum vulgare*, or to inflorescence models. Test stimuli are presented in schematic drawings, with left and right sections presenting the human-visible and UV light image, respectively; grey and black in the UV light image indicate UV reflection (UV-bright) and UV absorption (UV-dark), respectively; hatched lines indicate that the inflorescence was covered by cheese cloth; odour from natural inflorescences was present in all experiments (see methods for details).**

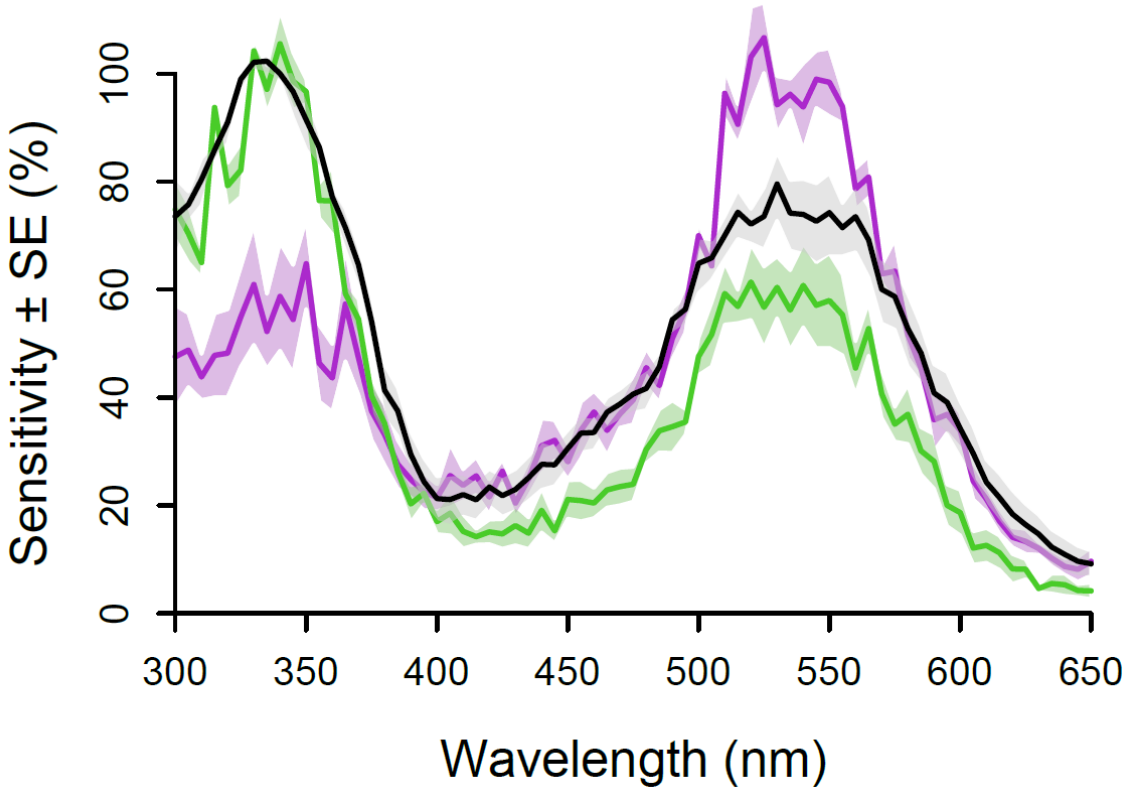


Figure 4.3 **Spectral sensitivity of *Culex pipiens* compound eyes. Electroretinograms (ERGs) showing the mean spectral sensitivity of compound eyes of 1- to 3-day-old female *Culex pipien*s that were dark-adapted (black lines; n = 5), green-adapted (green lines; n = 5), or UV-adapted (purple lines, n = 5). The shaded area around each line represents the standard error of the spectral mean.**

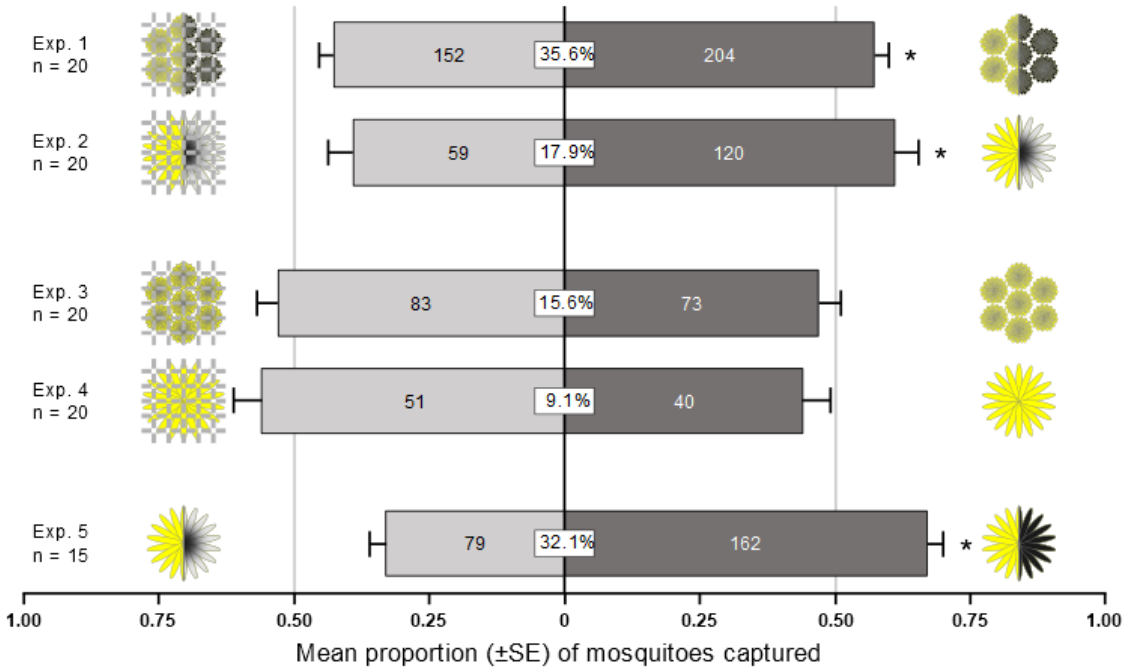


Figure 4.4 **Effect of visual and olfactory inflorescence cues on trap captures of 1- to 3-day-old female *Culex pipiens*. Inflorescences of *Hieracium lachenalii* (Exp. 1) and *Tanacetum vulgare* (Exp. 2), respectively, are shown in schematic drawings, with left and right sections presenting the human-visible and UV-light image, respectively; hatched lines indicate the inflorescence was covered by cheese cloth. Visual inflorescence cues did enhance the effect of inflorescence odour under UV light (Exps. 1, 2) but did not under UV-deficient illumination (Exps. 3, 4). Uniformly UV-dark *H. lachenalii* inflorescences (as a result of sunscreen treatment) were more attractive than inflorescences with the natural UV absorption and UV reflectance pattern (Exp. 5). Numbers in bars indicate total number of mosquitoes responding. For each experiment, an asterisk indicates a significant preference for a test stimulus (binary logistic regression model; $p < 0.05$).**

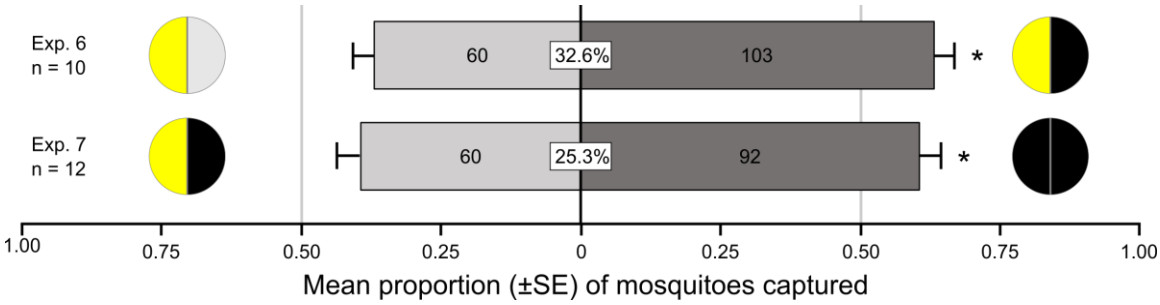


Figure 4.5 **Effect of UV absorption, UV reflection and colour of inflorescence models in the presence of inflorescence odour (occluded inflorescence) on trap captures of 1- to 3-day-old female *Culex pipiens*. Inflorescence models are shown in schematic drawings, with left and right sections presenting the human-visible and UV light image, respectively. Yellow UV-dark models were more attractive than yellow UV-bright models (Exp. 6), whereas black UV-dark models were more attractive than yellow UV-dark models (Exp. 7), indicating an interaction between UV-darkness and colour. Numbers in bars indicate the total number of mosquitoes responding. For each experiment, an asterisk indicates a significant preference for a test stimulus (binary logistic regression model; $p < 0.05$).**

Chapter 5.

Attraction of female *Aedes aegypti* (L.) to aphid honeydew[1]

[1]The corresponding manuscript is published in *Insects* (2019, Volume 10: 43) with the following authors: Peach, D.A.H., Gries, R., Young, N., Lakes, R., Galloway, E., Alamsetti, S., Ko, E., Ly, A., and Gries, G.

Introduction

Honeydew is a sugar-rich liquid (Auclair 1963) secreted by aphids and scale insects feeding on plant sap (Douglas 2009). Honeydew may be available at times or in locations when other sources of sugar, such as floral nectar, are not available or abundant. Many insects feed on honeydew, including honey bees, ants, wasps (Auclair 1963; Douglas 2009), and even blood-feeding dipterans such as deer flies (Janzen & Hunter 1998; Ossowski & Hunter 2000), black flies (Burgin & Hunter 1997a, 1997b), sand flies (MacVicker *et al.* 1990), and mosquitoes (Burkett *et al.* 1999; Gary & Foster 2004; Haegar 1955; Russell & Hunter 2002).

Plant sugar is an essential basic food for adult male and female mosquitoes (Foster 1995). Mosquito populations can persist only through ready access to plant sugar, even if they have ready access to blood (Stone *et al.* 2009). Plant sugar also enhances the vectorial capacity of mosquitoes (Gu *et al.* 2011; Stone *et al.* 2018). Mosquitoes feed on many forms of plant sugar including floral and extra-floral nectar, fruit juices, exudate from damaged plant tissue, plant sap they access with their piercing mouthparts (Foster 1995), honeydew (Burkett *et al.* 1999; Gary & Foster 2004; Haegar 1955; Russell & Hunter 2002), and even ant regurgitate (Clements 1999). Most mosquitoes extensively exploit floral nectar but also use honeydew when nectar is scarce, as do other insects (van Rijn *et al.* 2013). For some mosquitoes, honeydew provides a valuable primary plant sugar source (Burkett *et al.* 1999) .

Inflorescence odourants are the most important cues that guide mosquitoes to floral nectar (Foster 1995, 2008; Nyasembe & Torto 2014). Numerous floral and fruit odourants have been identified and eventually may be used for monitoring or controlling mosquito populations, but no study has yet addressed whether mosquitoes are attracted to honeydew. Many insects that feed on honeydew, or that consume or parasitize the

hemipteran insects that produce it, are attracted to honeydew odourants (Brown *et al.* 2015; Choi *et al.* 2004; Leroy *et al.* 2012). This may also apply to mosquitoes.

Aphid honeydew and floral nectar contain sugars and amino acids (Auclair 1963; Hussain *et al.* 1974; Pozo *et al.* 2014) that exogenous microbes metabolize, producing odourants in the process (Álvarez-Pérez *et al.* 2012; Fridman *et al.* 2012; Leroy *et al.* 2011; Stadler & Müller 1996). Mosquitoes respond to microbial odourants when they forage for hosts (Busula *et al.* 2017; Takken & Verhulst 2017; Verhulst *et al.* 2009, 2010), and seek oviposition sites (Ponnusamy *et al.* 2008). Microbial odourants emanating from aphid honeydew attract aphidophagous hoverfly predators (Leroy *et al.* 2011) and may also attract mosquitoes.

The yellow fever mosquito, *Aedes aegypti*, is a widely distributed mosquito that can vector many arboviruses including dengue, yellow fever, chikungunya, and Zika (Hayes 2009; Jansen & Beebe 2010; Monath 2001; Pialoux *et al.* 2007). In the laboratory, *Ae. aegypti* have been observed to imbibe honeydew from pea aphids, *Acyrthosiphon pisum,* and green peach aphids, *Myzus persicae,* colonizing broad beans, *Vicia faba* (DP, pers. obs.). Working with broad bean-colonizing pea and green peach aphids and *Ae. aegypti* as model organisms, we tested the hypothesis that *Ae. aegypti* females are attracted to (*i*) natural aphid honeydew odourants, (*ii*) a synthetic blend of these odourants, and (*iii*) the microbe-produced constituents of this blend.

5.1. Materials and Methods

5.1.1. Rearing of Experimental Mosquitoes

We reared mosquitoes at temperatures of 23-26 °C, a photoperiod of 14L:10D, and a 40-60% RH. We maintained adult mosquitoes in mesh cages (30 × 30 × 46 cm high) and provisioned them *ad libitum* with a 10% sucrose solution. Once a week, we fed female mosquitoes on the arm of DP, 3 days later giving them access to a water-containing 354-mL cup (Solo Cup Comp., IL, USA) with a paper towel (Kruger Inc., Quebec, Canada) lining its sides. we transferred strips of paper towel carrying *Ae. aegypti* eggs into a small circular glass dish (10 cm diameter × 5 cm high), filled with water, and inoculated with brewer's yeast (U.S. Biological Life Sciences, MA, USA). Upon larval hatching (2-4 days later), we transferred the larvae with the water to water-

filled trays (45 × 25 × 7 cm high) and provisioned them with NutriFin Basix tropical fish food (Rolf C Hagen Inc., Montreal, QC, Canada). Daily, we transferred pupae via a 7-mL plastic pipette (VWR International, PA, USA) to water-containing 354-mL Solo cups (Solo Cup Comp., Illinois, USA) covered with a mesh lid. We aspirated eclosed adults into separate Solo cups, fitted with a cotton ball soaked in a 10-% sucrose solution.

5.1.2. Rearing of Plants and Aphids

We grew fava beans from seed (Northwestern Seeds, Vernon, BC, Canada) in a greenhouse at Simon Fraser University (Burnaby, BC, Canada) under a 16L:10D light regime, watering plants every other day. We kept colonies of green peach aphids and pea aphids on fava bean plants in separate bug dorms (61 × 61 × 61 cm) (BioQuip Products, Rancho Dominguez, CA, USA) under these same conditions.

5.1.3. General Design of Y-tube Behavioural Experiments

To determine whether mosquitoes are attracted to aphid-infested or mechanically injured plants, we ran bioassays in Y-tube olfactometers (diameter: 2.5 cm; length of the main and lateral arms: 23 cm and 19 cm, respectively; angle of lateral arms: 120°) inclined at 45° (Derstine *et al.* 2017)**.** We placed the treatment and the control stimulus (e.g., a plant with or without aphid infestation) in a plastic oven bag (Reckitt Benckiser Inc., Mississauga, ON, Canada) and tightly connected the bag to a randomly assignment lateral arm of the Y-tube. A carbon filter affixed to a small opening in one corner of each bag allowed us to draw purified air through the bags and the Y-tube. For each bioassay, we placed a single, 1- to 3-day-old, 24-h sugar-deprived female mosquito into a holding glass tube (diameter: 2.5 cm; length: 26 cm) with stainless steel mesh covering both openings**.** We then attached the holding tube to the Y-tube stem via a ground glass joint. Following a 60-s acclimation period, we removed the wire mesh and initiated airflow at a rate of 4 cm s^{-1} via a mechanical pump, thus carrying volatiles towards the mosquito that could now enter the Y-tube. For each replicate, we employed a clean Y-tube, a new female mosquito, and new test stimuli. we recorded the lateral arm of the Y-tube a mosquito entered first, and considered all mosquitoes making no decisions within 5 min as non-responders, which were excluded from statistical analyses.

5.1.4. Attractiveness of Aphid-infested and Honeydew-soiled Plants

We assigned potted bean plants with 6-10 “true” leaves to a treatment or a control group and placed them in separate plastic cages (21 × 26 × 32 cm). We released 20 green peach aphids, or 20 pea aphids, onto treatment plants, but not control plants, allowing honeydew to accumulate on treatment plants over seven days. Over this time, colonies of green peach aphids and pea aphids grew to a mean size of 31 and 103 individuals, respectively. To account for the possibility that mechanical, feeding-related plant odourants, in addition to honeydew odourants, affect the mosquitoes’ responses, we mechanically injured each plant (Landolt *et al.* 1999), by cutting one leaf along its long axis, and then left the plant for 1 h prior to commencing a bioassay. In Y-tube olfactometers, we offered mosquitoes a choice between two mechanically injured bean plants (each inside an oven bag) that we had infested, or not (control), with either green peach aphids (Exp. 1) or pea aphids (Exp. 2) (Table 5.1).

5.1.5. Attractiveness of Mechanically-injured Plants

To determine whether plant odourants derived from mechanical feeding injury suffice to attract mosquitoes, we mechanically injured plants (see above), and in Y-tube olfactometers offered mosquitoes a choice between two non-infested bean plant (each inside an oven bag) that had, or had not (control), been mechanically injured (see above) (Table 5.1, Exp. 3).

5.1.6. Attractiveness of Plants in the Presence of Non-feeding Aphids

To separate effects of aphid feeding and aphid presence on attraction of mosquitoes, we offered mosquitoes a choice between two intact bean plants (each inside an oven bag) that were paired with a mesh-covered Petri dish containing, or not (control), 100 non-feeding pea aphids (Table 5.1, Exp. 4).

5.1.7. Honeydew Collection and Odourant Analysis

We collected (commonly discoloured) droplets of honeydew from plants heavily infested with pea aphids, using a 10-µL glass capillary fitted with a rubber bulb. We

collected a total of 50 µL of honeydew and expelled it into a 4-mL glass vial with a rubber septum lid. Through this lid, we inserted a carboxen-polydimethylsiloxene-coated solid-phase micro extraction (SPME) fibre (75 µm; Supelco Inc., Bellefonte, PA, USA), allowing absorption of honeydew odourants on this fibre for 24 h at room temperature. Prior to each odourant collection, we conditioned the fibre at 280 °C for 5 min in a GC injector port. We desorbed odourants from the fibre in the hot (250 °C) injection port of the gas chromatograph (GC), and analyzed odourants by GC-mass spectrometry (MS) using a Saturn 2000 Ion Trap GC-MS fitted with a DB-5 GC-MS column (30 m × 0.25 mm i.d.; Agilent Technologies Inc., Santa Clara, CA, USA) in full-scan electron impact mode. We used a flow of helium (35 cm s^{-1}) as the carrier gas with the following temperature program: 40 °C (5 min), 10 °C min^{-1} to 280 °C (held for 10 min). We identified volatiles by comparing their retention indices (RI) relative to n-alkane standards (van Den Dool & Kratz 1963), and their mass spectra with those reported in the literature (Adams 1989) and with those of authentic standards.

5.1.8. Preparation and Testing of Synthetic Honey Dew Odourant Blends

We prepared three blends of synthetic honeydew odourants. Two blends reflected the composition of crude honeydew collected and analyzed in this study (CHD_1), and in a previous study (CHD_2) (Leroy *et al.* 2011) (Table 5.2), and a third blend resembled the composition of sterilized honeydew (SHD) as previously reported (Leroy *et al.* 2011) (Table 5.2) for anemotactic attraction of mosquitoes in paired-trap experiments. All blends were dissolved in a 1-mL mixture of pentane (50%) and ether (50%), and pipetted treatment and corresponding solvent control stimuli into separate 4-mL glass vials with a 2-mm hole in the lid. We tested the CHD_1 at doses equivalent to 2.5×10^{1} µL and 2.5×10^{0} µL of crude honeydew (Exps. 5,6), the CHD_2 at honeydew equivalent doses of 2.5×10^{6} µL, 2.5×10^{5} µL, 2.5×10^{4} µL, 2.5×10^{3} µL, 2.5×10^{1} µL, and 2.5×10^{0} µL (Exps. 8-15), and the SHD at honeydew equivalent doses of 2.5×10^{6} µL and 2.5×10^{5} µL (Exps. 7, 14, 15). The dose equivalents tested in bioassays are biologically relevant, considering that 2.5×10^{1} µL of honeydew approximate the amount of honeydew produced by 25 pea aphids per day (Boullis *et al.* 2018) and that aphid infestations can reach several thousand individuals per m^2 (Elliott & Kieckhefer 2000; Sunderland & Vickerman 1980).

5.1.9. Captures of Mosquitoes in Traps Baited with Synthetic Honeydew Odourant Blends

In laboratory mesh-cage experiments**,** we tested captures of mosquitoes in traps baited with synthetic honeydew odourant blends (see below). Each cage (77 × 78 × 104 cm) was wrapped with black cloth except for the top allowing light entry from above. we provided illumination with a shop light housing (Lithonia Lighting, GA, USA) fitted with two conventional 1.22-m fluorescent tubes (F32T8/T1835 Plus, Phillips, Amsterdam, Netherlands). The cage housed two burette stands separated by 25 cm, each stand carrying a Delta trap 50 cm above the cage floor (Peach *et al.* n.d.). We prepared traps from white cardstock (71.28 × 55.88 cm) (Staples Inc., MA, USA; ACCO Brands Corp., IL, USA) that we cut to size (15 × 30 cm), coated with adhesive (The Tanglefoot Company, MI, USA) on the inside, and then folded into a Delta-type trap (15 × 9 × 8 cm high). We randomly assigned the treatment and the control stimulus (see below) to one trap in each pair. For each bioassay replicate, we released 50 1- to 3-day-old, 24-h sugar-deprived females from a Solo cup (see above) into a cage and recorded trap captures 24 h later. We ran experiments at 23-26 °C, 40-60% RH, and a photoperiod of 14L:10D, commencing the bioassay 4-6 h prior to onset of the scotophase.

We dissolved all synthetic honeydew blends in a 1-mL mixture of pentane (50%) and ether (50%), pipetted treatment and solvent control stimuli into separate 4-mL glass vials with a 2-mm hole in the lid, and randomly assigned the treatment and the control vial to one trap in each pair. We tested the CHD_1 at a dose of 2.5×10^1 µL honeydew equivalents (Exp. 5), and the CHD_2 at doses of 2.5×10^6 µL, 2.5×10^5 µL, 2.5×10^4 µL, 2.5×10^3 µL, and 2.5×10^1 µL honeydew equivalents (Exps. 6-10). To compare the relative attractiveness of crude and sterilized honeydew, we tested the CHD_2 *vs* the SHD at doses of 2.5×10^6 µL and 2.5×10^5 µL honeydew equivalents (Exps. 11, 12).

5.1.10. Statistical Analyses

We analyzed behavioural data using SAS statistical software version 9.4 (SAS Institute Inc., Cary, NC, USA), excluding experimental replicates with no mosquitoes responding. We analyzed data of Y-tube experiments (Exps. 1-4) using a two-tailed exact-goodness-of-fit test. For cage experiments 5-15, we compared mean proportions

of responders to paired test stimuli using a binary logistic regression model and worked with back-transformed data to obtain means and confidence intervals.

5.2. Results

5.2.1. Attractiveness of Plants that were Aphid-infested, Mechanically Injured, or Paired with Non-feeding Aphids

In Y-tube olfactometer experiments, plants infested with green peach aphids (Exp. 1) or pea aphids (Exp. 2) attracted 81% and 77.3% of responding mosquitoes, respectively, significantly more than aphid-free control plants (Exp. 1: $z = -2.84$, $p = 0.007$; Exp. 2: $z = -2.56$, $p = 0.017$; Fig. 5.1). Intact and mechanically injured plants were equally attractive to female mosquitoes ($z = 0.45$, $p = 0.82$; Fig. 5.1, Exp. 3), as were intact plants in the presence or absence of non-feeding pea aphids ($z = -0.85$, $p = 0.52$) (Fig. 5.1, Exp. 4).

5.2.2. Analyses of Honeydew Headspace Odourants

Desorption and GC-MS analyses of SPME collected honeydew headspace odourants consistently revealed eight compounds (Fig. 5.2; Table 2), including ketones, alcohols, acids, and aldehydes. The most abundant compounds were 3-hydroxybutanone and 3-methyl-1-butanol.

5.2.3. Attractiveness of Synthetic Honeydew Odourant Blends in Y-tube Olfactometers

The CHD_1 (a synthetic blend of crude honeydew odourants prepared according to our own data; Fig. 5.2) at a dose of 2.5×10^1 µL honeydew equivalents (Exp. 5), but not at a dose of 2.5×10^0 µL honeydew equivalents (Exp. 6), attracted significantly more mosquitoes than corresponding solvent control stimuli (Exp. 5: $z = 2.7$, $p = 0.007$; Exp. 6: $z = 0.92$, $p = 0.36$; Fig. 5.3).

The SHD (a synthetic blend of sterile honeydew odourants prepared according to literature data (Leroy *et al.* 2011)) at a dose of 2.5×10^6 µL honeydew equivalents attracted significantly more mosquitoes than the corresponding solvent control stimulus ($z = 5.2$, $p < 0.0001$; Fig. 5.4, Exp. 7).

The CHD_2 (a synthetic blend of crude honeydew odourants prepared according to literature data (Leroy *et al.* 2011)) attracted significantly more mosquitoes than the corresponding solvent control when tested at descending honeydew dose equivalents of 2.5×10^6 uL (Exp. 8: $z = 7.1$, $p < 0.0001$), 2.5×10^5 µL (Exp. 9: $z = 6.0$, $p < 0.0001$), 2.5×10^4 µL (Exp. 10: $z = 4.9$, $p < 0.0001$), 2.5×10^1 µL (Exp. 12: $z = 2.8$, $p = 0.005$), and 2.5×10^0 µL (Exp. 13: $z = 2.1$, $p < 0.039$; Fig. 5.4). Inconsistently, the CHD_2 was not attractive at a dose of $2.5*10^3$ µL honeydew equivalents (Exp. 11: $z = 1.3$, $p = 0.2$).

When the CHD_2 and the SHD were tested head-to-head at honeydew dose equivalents of 2.5×10^6 µL (Exp. 14) and 2.5×10^5 µL (Exp. 15), CHD_2 at the lower dose, but not the higher dose, attracted more mosquitoes than the SHD (Exp. 14: $z = 1.3$, $p = 0.2$; Exp. 15: $z = 6.5$, $p <0.0001$; Fig. 5.5).

5.3. Discussion

These data show that *Ae. aegypti* females anemotactically orient towards aphid-infested and honeydew-soiled bean plants and that synthetic blends of honeydew odourants are attractive to mosquitoes, particularly when they contain constituents of microbial origin.

Herbivory can induce the emission of plant defensive chemicals (Aljbory & Chen 2018; Allmann & Baldwin 2010; Hare 2011) that may be herbivore-specific (Allmann & Baldwin 2010) and attract natural enemies of the specific herbivore (Aljbory & Chen 2018; Allmann & Baldwin 2010; Hare 2011). As mosquitoes were not attracted to odourants from mechanically injured plants (Fig. 5.1, Exp. 3), or to odourants from non-feeding aphids (Fig. 5.1, Exp. 4), it follows that mosquito females responded to either aphid-induced plant defensive chemicals that signalled aphid feeding, or to honeydew odourants. As pea aphids feeding on bean plants do not prompt the emission of plant defensive chemicals (Schwartzberg *et al.* 2011), attraction of mosquitoes to plants infested with green peach aphids or pea aphids (Fig. 5.1, Exps. 1, 2) can be attributed to odourants associated with honeydew expelled by these feeding aphids.

Honeydew consumption by mosquitoes is well known but we present the first evidence of mosquitoes being attracted olfactorily to aphid honeydew. Our findings that honeydew from two aphid species induced the same attraction response by foraging

mosquitoes suggest that honeydew odourants might be generic indicators of plant-derived sugar. Attractiveness of honeydew has previously been shown in studies with the common yellowjacket, *Vespula vulgaris* (Brown *et al.* 2015), the house fly, *Musca domestica* (Hung *et al.* 2015), and the marmalade hoverfly, *Episyrphus balteatus* (Leroy *et al.* 2011). Unlike hoverflies, *Ae. aegypti* females did respond to a synthetic blend of honeydew odourants lacking constituents of microbial origin (Fig. 5.4, Exp. 1) but the dose of this synthetic blend was rather high. When we tested synthetic blends of honeydew odourants at a 10-fold lower dose, with and without the microbial odourants, mosquito females strongly preferred the more complex inclusive blend.

Some of the odourants found in natural crude honeydew may originate from the bacterium *Staphylococcus sciuri* that is known to reside in the gut of pea aphids, to metabolize honeydew, and to produce specific odourants (Leroy *et al.* 2011). This inference is supported by findings that re-inoculation of sterilized honeydew with *S. sciuri* re-generated odourants typically associated with crude (non-sterile) honeydew (Leroy *et al.* 2011). Other odourants are likely produced by exogenous microbes that colonize and metabolize aphid honeydew over time. This would explain why freshly expelled honeydew contained only few odourants that we could detect by GC MS analysis in this study (unpubl. data). Odourants of honeydew-dwelling microbes have been implicated in attracting the black garden ant, *Lasius niger* (Fischer *et al.* 2015), and appear to contribute to the attraction of mosquitos to small quantities of honeydew that they may otherwise not be able to detect. Once mosquitoes have been attracted to, and alighted on, aphid-infested plants, they can confirm the presence of honeydew via contact chemoreceptors on their tarsi (Downes & Dahlem 1987). Well known is that mosquitoes exploit microbe-derived odourants as resource indicators when they forage for vertebrate hosts (Busula *et al.* 2017; Takken & Verhulst 2017; Verhulst *et al.* 2009, 2010) and select oviposition sites (Ponnusamy *et al.* 2008). Here we add to the knowledge base in that we demonstrate a role for microbe-derived odourants guiding mosquitoes to plant sugar sources.

Crude aphid honeydew seems to have common odour constituents. In crude honeydew of pea aphids feeding on fava beans, the same five odourants (2,3-butanedione, 3-hydroxybutanone, 3-methyl-1-butanol, 3-methylbutanoic acid, and 2-methylbutanoic acid) were found here and in a previous study (Leroy *et al.* 2011), one odourant of which (3-methyl-1-butanol) was again just recently noted (Boullis *et al.*

2018). Six odourants we identified here (2,3-butanedione, 3-methyl-1-butanol, 3-methylbutanoic acid, 2-methylbutanoic acid, 3-hydroxybutanone, and 2-ehtylhexanol) were also found in honeydew of black bean aphids, *A. fabae,* feeding on fava bean plants (Fischer *et al.* 2015), and three of these odourants (2,3-butanedione, 3-methyl-1-butanol, and 3-hydroxybutanone) were noted in honeydew from vetch aphids, *Megoura viciae,* feeding on fava beans (Leroy *et al.* 2012). At least some of these odourants may originate from microbial metabolism of honeydew amino acids (Boullis *et al.* 2018; Schulz & Dickschat 2007).

Consumption of honeydew by mosquitoes in the field (Burkett *et al.* 1999; Russell & Hunter 2002) contributes to their survival (Gary & Foster 2004) and is shown clearly by the presence of honeydew-specific sugars, such as melezitose or erlose, in the alimentary canal of mosquitoes (Burkett *et al.* 1999). However, relying solely on the presence of honeydew-specific sugars in the digestive tract of mosquitoes to gauge the extent of their honeydew consumption may lead to underestimates of this phenomenon. The constituents of honeydew change in accordance not only with the hemipteran herbivores expelling it but also the plants they feed on (Fischer & Shingleton 2001; Pringle *et al.* 2014). The importance of honeydew relative to floral nectar, preferential consumption of either sugar source by specific mosquito species, and the contribution of honeydew to the vectorial capacity of mosquitoes are all not yet known. Well established, however, is the view that the vectorial capacity of mosquitoes is reliant upon ready access to plant (floral) sugar (Stone & Foster 2013) which is why selective removal of mosquito host-plants is deemed a remedial means of shortening the longevity of mosquitoes and thus lowering their vectorial capacity (Ebrahimi *et al.* 2017). This concept, however, seems to discount the effect of alternative sugar sources, such as honeydew, on mosquito longevity (Gary & Foster 2004). Like other insects, mosquitoes may substitute aphid honeydew for floral nectar when floral nectar is scare or honeydew particularly abundant (Wäckers 2005; van Rijn *et al*. 2013).

5.4. References

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5.5. Tables

Table 5.1 **Details of treatment and control stimuli, amount of stimuli tested, type of bioassay design, and number of replicates (N) tested with yellow fever mosquitoes in experiments 1-15. [1]Fava bean plants, *Vicia faba*, infested with green peach aphid, *Myzus persicae*, or pea aphid, *Acyrthosiphon pisum*; [2]CHD1 : a synthetic blend of crude honeydew odourants prepared according to our own data (Fig. 5.2; Table 5.2); [3]SHD: a synthetic blend of sterile honeydew odourants prepared according to literature data ((Leroy et al. 2011); Table 5.2); [4]CHD2 : a synthetic blend of crude honeydew odourants prepared according to literature data ((Leroy et al. 2011); Table 5.2); [5]We mechanically injured a plant by cutting one leaf along its long axis, and then left the plant for 1 h prior to commencing a bioassay.**

Exp.	Treatment[1,2,3,4,5]	Control	Details	Design	N
Attraction of mosquitoes to plants aphid-infested, mechanically injured, or paired with non-feeding aphids					
1	*M. persicae*-infested *V. faba*	*V. faba*	Mean of 31 aphids per plant	Y-tubes	21
2	*A. pisum*-infested *V. faba*	*V. faba*	Mean of 103 aphids per plant	Y-tubes	22
3	*V. faba* (injured)	*V. faba*	Experimentally injured plant	Y-tubes	20
4	*V. faba* + *A. pisum*	*V. faba*	100 *A. pisum* in Petri dish	Y-tubes	22
Attraction of mosquitoes to synthetic honeydew odourants					
5	CHD_1	Solvents	2.5×10^{1} µL honeydew equiv.	Delta traps	15
6	CHD_1	Solvents	2.5×10^{0} µL honeydew equiv.	Delta traps	11
7	SHD	Solvents	2.5×10^{6} µL honeydew equiv.	Delta traps	12
8	CHD_2	Solvents	2.5×10^{6} µL honeydew equiv.	Delta traps	13
9	CHD_2	Solvents	2.5×10^{5} µL honeydew equiv.	Delta traps	10
10	CHD_2	Solvents	2.5×10^{4} µL honeydew equiv.	Delta traps	10
11	CHD_2	Solvents	2.5×10^{3} µL honeydew equiv.	Delta traps	15
12	CHD_2	Solvents	2.5×10^{1} µL honeydew equiv.	Delta traps	14
13	CHD_2	Solvents	2.5×10^{0} µL honeydew equiv.	Delta traps	15
Attraction of mosquitoes to odourants from honeydew-dwelling microbes					
14	CHD_2	SHD	2.5×10^{6} µL honeydew equiv.	Delta traps	26
15	CHD_2	SHD	2.5×10^{5} µL honeydew equiv.	Delta traps	15

Table 5.2 **Blends of synthetic honeydew odourants prepared according to compositions of crude honeydew collected in this study (CHD1), and in a previous study (CHD2) (Leroy et al. 2011), and of sterilized honeydew (SHD) reported in the previous study (Leroy et al. 2011). [1]Sigma-Aldrich (St. Louis, MO 63103, USA); [2]obtained by oxidation of 3-hydroxy-2-butanone; [3]Thermo Fisher Scientific (Waltham, MA, USA); [4]Fluka Chemicals Ltd. (Milwaukee, WI, USA); [5]synthesized by reduction of tiglic acid by lithium aluminum hydride; [6]synthesized by oxidation of 3-methyl-2-buten-1-ol by manganese dioxide.**

Odourants	Purity (%)	CHD1 (%)	CHD2 (%)	SHD (%)
Propanone1	99.8	-	9.25	24.62
2,3-Butanedione2	86	7.70	2.31	40.54
2,3-Butanediol1	98	3.49	-	-
3-Methylbutanal1	97	-	14.01	-
2-Methylbutanal1	>99	-	12.92	-
3-Hydroxybutanone1	98	46.38	0.78	4.77
3-Methyl-3-buten-1-ol1	97	-	0.89	5.64
3-Methyl-1-butanol3	98.5	36.82	12.32	-
2-Methyl-2-buten-1-ol5	83	-	14.41	-
3-Methyl-2-butenal6	88	-	10.73	-
Butanoic acid1	99	-	6.24	24.43
3-Methylbutanoic acid1	99	3.07	4.56	-
2-Methylbutanoic acid1	98	0.63	6.73	-
2,5-Dimethylpyrazine1	99	-	0.31	-
Limonene1	90	-	2.81	-
Benzeneethanol1	99	-	1.73	-
2-Ethylhexanol1	99	1.57	-	-
2-Phenylethyl alcohol4	98	0.35	-	-

5.6. Figures

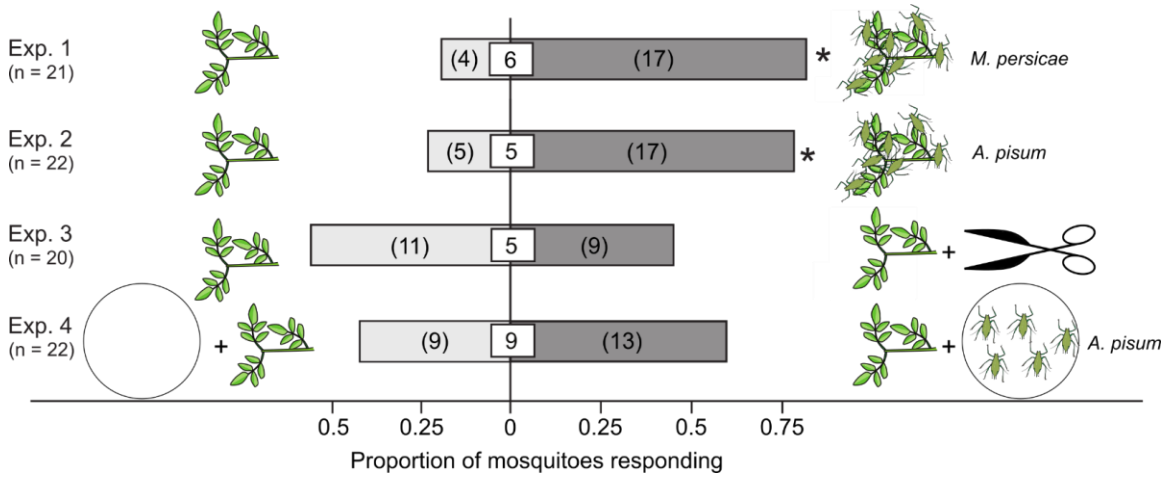

Figure 5.1 **Proportion of female yellow fever mosquitoes, *Aedes aegypti*, responding in binary choice Y-tube olfactometer experiments (N= 20-22 replicates) to fava bean plants, *Vicia faba*, that were non-infested (control) or that were (i) infested with green peach aphids, *Myzus persicae* (Exp. 1), or pea aphids, *Acyrthosiphon pisum* (Exp. 2); (ii) mechanically injured (Exp. 3), or (iii) paired with 100 non-feeding pea aphids. Numbers in parentheses represent the number of mosquitoes selecting a test stimulus, and numbers in square boxes in bars represent the number of non-responding mosquitoes. For each experiment, an asterisk (*) indicates a significant preference for a test stimulus (P<0.05; exact test of goodness-of-fit).

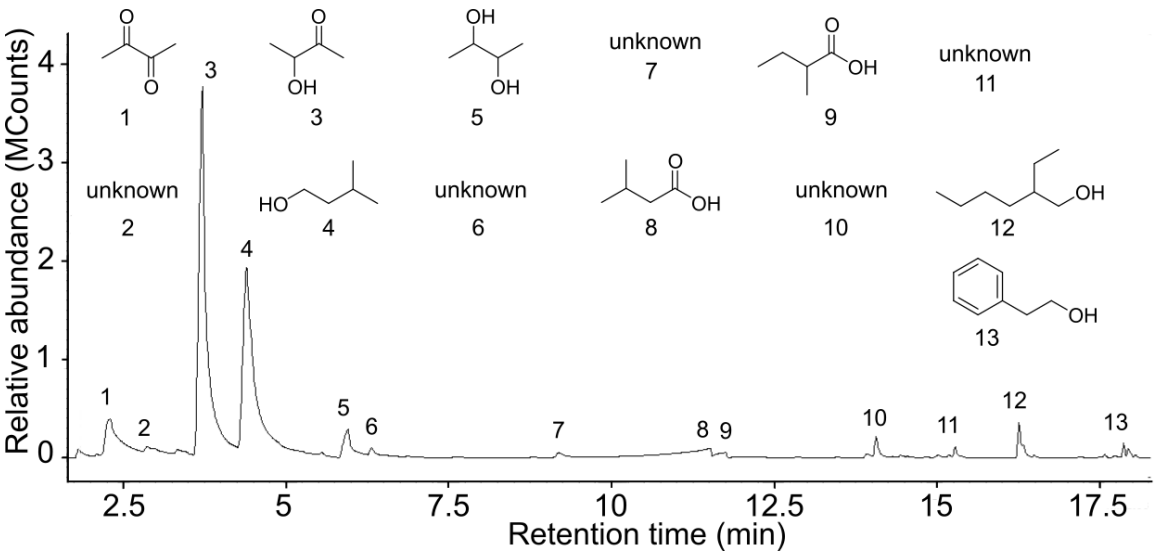

Figure 5.2 **Total ion chromotogram of pea aphid honeydew odourants collected on, and thermally desorbed from, a solid-phase micro extraction (SPME) fibre. Compound identity as follows: 1 = butanedione; 2 = unknown; 3 = 3-hydroxybutanone; 4 = 3-methylbutan-1-ol; 5 = 2,3-butanediol; 6 = unknown; 7 = unknown; 8 = 3-methylbutanoic acid; 9 = 2-methylbutanoic acid; 10 = unknown; 11 = unknown; 12 = 2-ethylhexanol; 13 = 2-phenylethanol.**

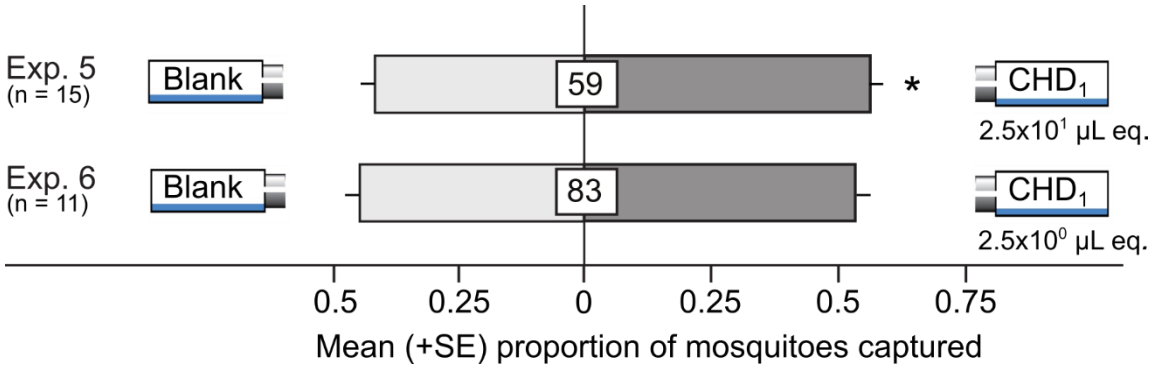

Figure 5.3 **Mean proportion (+ SE) of female yellow fever mosquitoes, *Aedes aegypti*, captured in experiments 5 and 6 in paired traps that were baited with the CHD1 (a synthetic blend of crude pea aphid honeydew odourants prepared according to our own data; Fig. 5.2; Table 5.2) or fitted with a corresponding solvent (blank) control. Numbers within bars indicate the mean percentage of mosquitoes not captured (non-responders); an asterisk (*) indicates a significant preference for a test stimulus (P<0.05; binary logistic regression); the dose of 2.5×10^{1} µL equivalents (eq.) of honeydew approximates the amount of honeydew produced by 25 pea aphids per day (Kirschfield *et al.* 1977).**

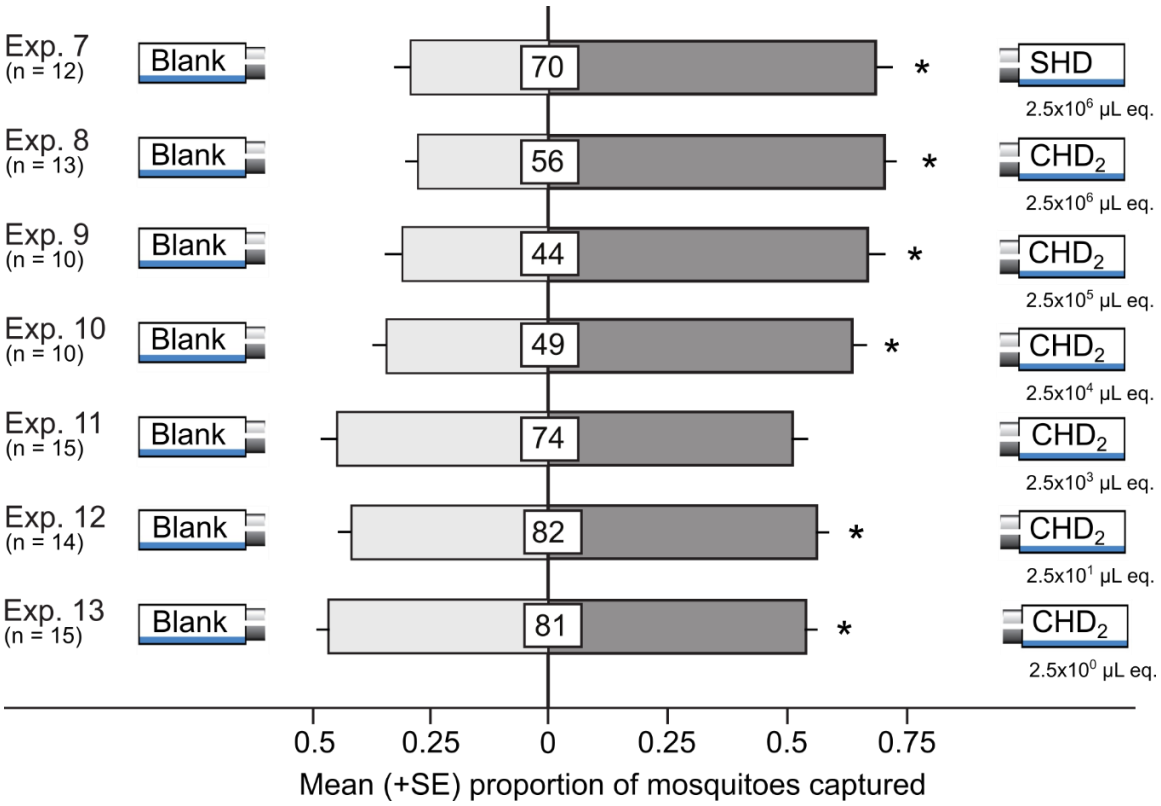

Figure 5.4 **Mean proportion (+ SE) of female yellow fever mosquitoes, *Aedes aegypti*, captured in experiments 7-13 in paired traps that were baited with the SHD (a synthetic blend of sterile honeydew-derived odourants prepared according to literature data (Leroy *et al.* 2011), Table 2) or the CHD2 (a synthetic blend of crude honeydew-derived odourants prepared according to literature data (Leroy *et al.* 2011), Table 2) at descending doses or that were fitted with a corresponding solvent (blank) control. Numbers within bars indicate the mean percentage of mosquitoes not captured; an asterisk (*) indicates a significant preference for a test stimulus ($P<0.05$; binary logistic regression); the dose of 2.5×10^{1} µL equivalents (eq.) of honeydew approximates the amount of honeydew produced by 25 pea aphids per day (Boullis *et al.* 2018).**

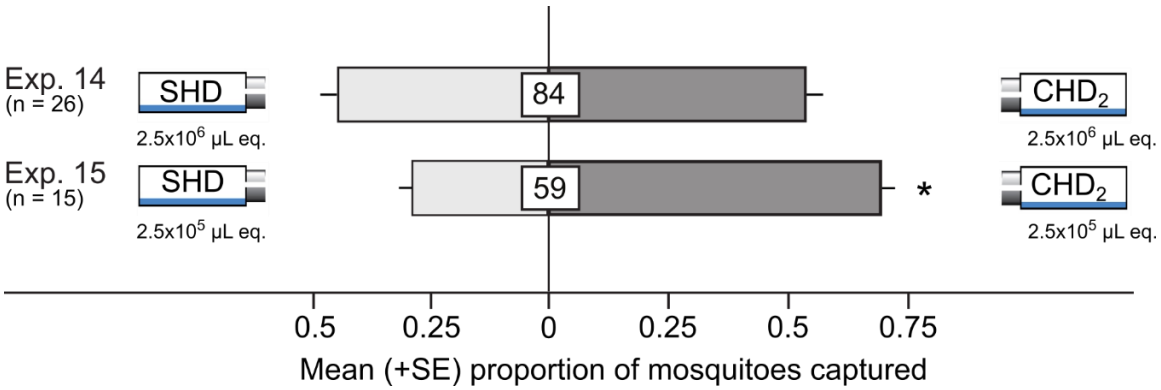

Figure 5.5 **Mean proportion (+ SE) of female yellow fever mosquitoes, *Aedes aegypti*, captured in experiments 14-15 in paired traps that were baited with the SHD (a synthetic blend of sterile honeydew-derived odourants prepared according to literature data (Leroy *et al.* 2011), Table 5.2) or the CHD2 (a synthetic blend of crude honeydew-derived odourants prepared according to literature data (Leroy *et al.* 2011), Table 5.2). Numbers within bars indicate the mean percentage of mosquitoes not captured; an asterisk (*) indicates a significant preference for a test stimulus (P<0.05; binary logistic regression).**

Chapter 6. Concluding summary

For this chapter, I review my findings in bullet form and emphasize their impact.

- *Culex pipiens* passively acquire pollen from some flowers, including tansy and other Asteraceae.
- *Cx. pipiens* can successfully transfer pollen between flowers of common tansy.
- My data support that conclusions that *Cx. pipiens* can pollinate tansy and possibly other Asteraceae, and mosquito pollination may be more common than is currently believed.

- Female *Aedes aegypti* and *Cx. pipiens* are attracted to the odours of tansy flowers.
- These mosquitoes are more attracted to tansy flowers they can see and smell compared to flowers they can only smell.
- A blend of synthetic compounds identified from tansy floral headspace is attractive to *Ae. aegypti* and *Cx. pipiens* at biologically relevant doses.
- A subset of these compounds is also known from the headspace of humans, and a synthetic blend of this subset is attractive to mosquitoes.
- Plants emit CO_2 and a flow of CO_2 at biologically relevant doses increases mosquito attraction to the complete floral synthetic blend.
- My data support the conclusions that mosquitoes use multi-modal cues, including vision and CO_2, to locate flowers, and some of these cues are shared with vertebrates.

- In the presence of UV wavelengths *Cx. pipiens* females are more attracted to tansy and hawkweed flowers that they can both see and smell.

- When UV wavelengths are filtered out, there is no preference between occluded and non-occluded inflorescences.

- When hawkweed flowers, with a UV bullseye, are treated with sunscreen to make them entirely UV-dark they are more attractive than flowers with an intact UV bullseye pattern.

- Artificial flowers, in the presence of floral odour, are more attractive when they have a UV dark bullseye than when they are entirely UV bright, and are more attractive when they are entirely UV dark than when they have only a UV-dark bullseye.

- My data support the conclusion that the visual attraction of mosquitoes to inflorescences is due to UV-dark cues, similar to mosquito attraction to dark cues when host-seeking.

- *Ae. aegypti* prefer aphid-infested plants soiled with honeydew over clean, un-infested plants. They show no preference between mechanically-damaged plants and intact plants, or between intact plants with aphids present and intact plants without aphids present.

- *Ae. aegypti* are attracted to a blend of synthetic honeydew volatiles collected from *Acyrthosiphon pisum* reared on *Vicia faba*. They are also attracted to blends of volatiles reported from microbe-contaminated honeydew and sterile honeydew in the literature.

- *Ae. aegypti* prefer the volatiles from microbe-contaminated honeydew to the volatiles from sterile honeydew.

- My data support the conclusion that mosquitoes such as *Ae. aegypti* use olfactory cues to locate aphid honeydew, and microbes present in aphid honeydew play a role in producing these attractive olfactory cues.

- Studying mosquito phytophagy has filled-in many gaps in our knowledge of mosquito ecology and sensory ecology. I provide evidence that the interactions between mosquitoes and plants are much more nuanced than previously believed, and that the sensory aspects of these interactions are similar to mosquito-vertebrate interactions in some respects.

Appendix A.

Lemongrass and cinnamon bark – Plant essential oil blend as a spatial repellent for mosquitoes in field setting[1]

[1]The corresponding manuscript is in press in the *Journal of Medical Entomlogy* with the following authors: Peach, D.A.H., Almond, M., Gries, R., and Gries, G.

Introduction

Mosquitoes transmit a plethora of pathogens that cause debilitating diseases and kill hundreds of thousands of humans annually (Stanaway *et al*. 2016, World Health Organization 2018). There are many tactics used to manage this disease burden and to control mosquito populations including vaccination against pathogens (Villar *et al*. 2015, Benelli & Mehlhorn 2016), habitat modification (Hulsman *et al*. 1989), insecticidal or larvicidal application (Conti *et al*. 2010, Bonds 2012), release of sterilized or genetically modified mosquitoes (Benedict & Robinson 2003), mass trapping, and mating disruption (Kline 2007). As mosquitoes continue to develop behavioral and physiological resistance against these tactics (Deletre *et al*. 2013, Kiplang'at & Mwangi 2014, Antonio-nkondjio *et al*. 2017, Norris & Coats 2017), there is an ongoing need for novel mosquito control technologies.

Mosquito repellents are an invaluable tool in the management of mosquitoes and the pathogens they transmit (Debboun & Strickman 2013). Topically-applied contact repellents are commonly used and can protect the people wearing them, but there is debate as to whether they are effective measures for mosquito vector management (Norris & Coats 2017). Topical repellents must be re-applied at frequent intervals to maintain an adequate level of protection, but this is not always carried out (Norris & Coats 2017). Deployment of spatial (area) repellents may provide an alternative. The vapor phase of spatial repellents generates repellency at a distance from the host, disrupting a mosquito's host-seeking behavior within a local area (Bernier *et al*. 2006). For a small space such as a room or a hut, this spatial repellent effect can reduce human-vector contact and provide a means of disease reduction for a group of people rather than a single individual (Maia & Moore 2011, Regnault-Roger *et al*. 2012,

Debboun & Strickman 2013, Deletre *et al*. 2013, World Health Organization 2013, Norris & Coats 2017, Stevenson *et al*. 2018). Spatial repellents can be disseminated from a reservoir to provide continuous protection over time without the need for frequent re-deployment (Chauhan *et al*. 2012, Norris & Coats 2017, Stevenson *et al*. 2018). Although spatial repellents rely on device-assisted dissemination and lose effective coverage outdoors due to wind (Chauhan *et al*. 2012, Norris & Coats 2017), spatial repellents still offer advantages over contact repellents in that they allow continual volatilization into the air (Norris & Coats 2017), can protect an area rather than just an individual (Achee *et al*. 2012, Norris & Coats 2017), are largely regarded as safe (Nerio *et al*. 2010, Deletre *et al*. 2013, World Health Organization 2013, United States Environmental Protection Agency 2015, Norris & Coats 2017), and can become an integral part of mosquito control programs (Achee *et al*. 2012, Regnault-Roger *et al*. 2012, Debboun & Strickman 2013, Norris & Coats 2017).

Various synthetic products are currently deployed as spatial mosquito repellents (Achee et al. 2012) but there is increasing interest in the use of plant essential oils (EOs) due to their environmental friendliness (Regnault-Roger *et al*. 2012), low cost (Maia & Moore 2011), safety of use (Regnault-Roger *et al*. 2012), availability (Maia & Moore 2011), and synergy with insecticidal permethrins *(Gross et al. 2017, Chansang et al*. 2018). EOs already serve as pharmaceuticals, detergents, cosmetics, and as cooking ingredients (Regnault-Roger et al. 2012). EOs with medicinal functions have been well studied and typically are considered low-risk and safe (Regnault-Roger *et al*. 2012, United States Environmental Protection Agency 2015). The EOs listed in Section 25(b) of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) of the United States are exempt from the registration process due to a perceived level of safety (United States Environmental Protection Agency 2015).

EOs are heterogeneous mixtures of secondary plant metabolites, containing volatile hydrocarbons and oxygenated compounds (Conti *et al*. 2010, Nerio *et al*. 2010), some of which are known mosquito repellents (Trongtokit *et al*. 2005, Campbell *et al*. 2010, Innocent *et al*. 2010, Nerio *et al*. 2010, Maia & Moore 2011, Regnault-Roger *et al*. 2012). While the repellent effect of an EO may be caused by specific metabolites (Trongtokit *et al*. 2005, Campbell *et al*. 2010, Conti *et al*. 2010, Nerio *et al*. 2010, Maia & Moore 2011, Regnault-Roger *et al*. 2012), interactions between metabolites may generate synergistic effects, resulting in greater repellency than could be ascribed to an

individual metabolite (Deletre *et al*. 2013, Kiplang'at & Mwangi 2014). Instead of presenting a single metabolite as a line of defense against herbivory, plants may have "opted" for a defensive metabolite mix that makes it harder for herbivores to overcome these defenses in the evolutionary "arms race" between plants and herbivores (Harrewijn *et al*. 1994, Naylor & Ehrlich 1997). Moreover, synergistic repellency may be found not only within the EO of a single plant species but between EOs of multiple plant species (Kiplang'at & Mwangi 2014).

Our overall objective was to assess spatial repellency effects of select EOs, and the interactions between them, on the yellow fever mosquito, *Aedes aegypti,* and local mosquito populations. In laboratory experiments, we screened EOs singly and in combinations for spatial repellent effects and then - using the Fractional Inhibitory Concentration equation - quantified EO interactions. We field-tested the most repellent EOs singly and in binary combination for their ability to express spatial repellency around a source of synthetic host attractants.

Methods

Experimental Insects

We reared *Aedes aegypti* mosquitoes (black-eyed Liverpool strain) at 23-26 °C, 40-60% RH, and a photoperiod of 14L:10D. We maintained colonies with an equal number of males and females in mesh cages (30L × 30W × 46H cm) provisioned *ad libitum* with a 10-% sucrose solution. We blood-fed females once per week by placing one of the authors' arms into a mesh cage. For oviposition, gravid females were given access to a 354-mL cup (Solo Cup Company, Lake Forest, IL 60045, USA) with a paper towel lining (Kruger Inc., Montréal, QC H3S 1G5, Canada). We then transferred paper towels carrying *Ae. aegypti* eggs into circular glass dishes (10 cm diam. × 5 cm high) containing water and brewer's yeast (U.S. Biological Life Sciences, MA 01970, USA). Two-to-four days later, we transferred the dish contents into water-filled trays (45L × 25W × 7H cm high) with NutriFin Basix tropical fish food (Rolf C Hagen Inc., Montreal, QC H9X 0A2 Canada) processed with a mortar and pestle (Coorstek Inc., Golden, CO 80401, USA). We transferred pupae via a 7-mL plastic pipette (VWR International, Allison Park, PA 15101, USA) into a water-containing 354-mL Solo cup covered with a mesh lid. Finally, we separated eclosed male and female adults via aspirator and placed

them in similar cups, along with a cotton ball soaked in a 10-% sucrose solution. We removed the cotton ball 24 h prior to bioassays and tested 20, 4- to 10-day-old, adult females in each bioassay.

As there are currently no *Ae. aegypti* present in the Greater Vancouver Area of British Columbia, Canada (Kraemer *et al*. 2015), all field experiments were tested with local mosquito populations consisting of species in the genera *Aedes, Anopheles, Coquillettidia, Culex, Culiseta,* and *Coquillettidia* (Belton 1983, Roth *et al*. 2010). This allowed us to test the effect of EOs on diverse mosquito taxa.

Plant Materials Tested for Spatial Repellency

We tested steam distilled EOs of cinnamon bark (*Cinnamomum verum*), rosemary (*Rosemarinus officinalis*), citronella (*Cymbopogon winterianus jowitt*), lemongrass (*Cymbopogum felxuosus*), geranium (*Pelargonium graveolens*), and peppermint (*Mentha piperita*) (Liberty Natural Products, Oregon City, OR 97045, USA), as well as vanillin (Sigma-Aldrich, St. Louis, MO 63146, USA) for spatial repellency towards mosquitoes.

Protocol for Testing Spatial Repellency of Plant Essential Oils in Laboratory Experiments

We followed the guidelines of the World Health Organization (WHO) for efficacy testing of spatial repellents (World Health Organization 2013), using a test apparatus (Fig. 1 (a)) modified from Grieco et al. (2005), and testing mosquitoes at abiotic conditions equivalent to those of the rearing room (23-26 °C, 40-60% RH).

In experiments 1-7 (Table 1), we tested each of the six EOs and vanillin at doses of 0.005%, 0.05%, 0.5%, 5%, or 50% (w/v) dissolved in ether to a total volume of 200 µL per test stimulus, with ether alone (200 µL) serving as the control stimulus. In experiments 8-28 (Table 2), we tested binary combinations of the materials tested in experiments 1-8, using the same concentration with equal parts of the two EOs. In experiments 29-36 (Table 3), we tested ternary combinations of select materials with equal parts of the three EOs. Based on probit dose-response curves generated from experimental data and following the WHO guidelines (World Health Organization 2013),

we then calculated spatial activity indices (SAIs) for 50% spatial repellency (RD_{50}), using the equation

$$\mathrm{SAI} = \left[\frac{(\mathrm{Nc} - \mathrm{Nt})}{(\mathrm{N})}\right]$$

where SAI is the spatial activity index, Nc is the number of mosquitoes in the control chamber, Nt is the number of mosquitoes in the treatment chamber, and N is the total number of mosquitoes released into the bioassay apparatus.

Assessing Synergistic Interactions Between Essential Oils

We assessed potential synergistic interactions between EOs using the equation for the fractional inhibitory concentration (FIC) index (Anantharaman et al. 2010, Meletiadis et al. 2010) with spatial activity index (SAI) values

$$\mathrm{FIC} = \frac{\mathrm{SAI}(\mathrm{A}+\mathrm{B})}{\mathrm{SAI}(\mathrm{A})} + \frac{\mathrm{SAI}(\mathrm{A}+\mathrm{B})}{\mathrm{SAI}(\mathrm{B})}$$

where A and B denote the two EOs tested. FIC values of ≤ 1.0, 1.0 < to ≤ 2.0, 2.0 < to ≤ 4.0, and > 4.0 indicate synergistic, additive, nix, and antagonistic interactions, respectively (Meletiadis et al. 2010).

For ternary combinations, we used a variant of the FIC equation

$$\mathrm{FIC} = \frac{\mathrm{SAI}(\mathrm{A}+\mathrm{B}+\mathrm{C})}{\mathrm{SAI}(\mathrm{A})} + \frac{\mathrm{SAI}(\mathrm{A}+\mathrm{B}+\mathrm{C})}{\mathrm{SAI}(\mathrm{B})} + \frac{\mathrm{SAI}(\mathrm{A}+\mathrm{B}+\mathrm{C})}{\mathrm{SAI}(\mathrm{C})}$$

where A, B, and C denote the three EOs tested. Here, FIC values of ≤ 1.5, 1.5 < to ≤ 3.0, 3.0 < to ≤ 6.0, and > 6.0 indicate synergistic, additive, nix, and antagonistic interactions, respectively.

Field Testing Solitary and Binary Mixtures of Essential Oils for a Repellent Effect on Mosquitoes

Drawing on results of our laboratory spatial repellency experiments, we carried out paired-trap field experiments with select EOs on the Burnaby campus of Simon

Fraser University (SFU) during August and September, 2016, and June to September, 2018. For each replicate, we placed two BG sentinel traps (Biogents AG, Regensburg, Germany) with 10-m inter-trap spacing on a lawn (Fig. 1 (b)), 3 m away from a patch of vegetation. We baited each of the two traps with both a BG lure (Biogents AG) emanating synthetic human host odorants and carbon dioxide (CO_2) emanating from dry ice. We randomly assigned the treatment stimulus and the control stimulus to a trap. The treatment stimulus consisted of a fan-driven dissemination device (Terminix Int'l Co., Memphis, TN 38103, USA) fitted with a cellulose pad infused with a single EO (3 g) and placed next (0 m) to a sentinel trap (Fig. 1 (b)). The control stimulus consisted of a Terminix dissemination device fitted with a clean cellulose pad and placed next to the other trap in each pair. We initiated and terminated replicates approximately 30 min before sunset and 60 min after sunset, respectively. We tested the repellent effect of each of three solitary EOs (cinnamon bark, lemongrass, and rosemary) (Exp. 37-39) and that of a binary mix of EOs (cinnamon bark and lemongrass) (Exp. 40), each of which scoring a RD_{50} of ≤ 3 g in laboratory spatial repellency experiments. The 3-g threshold was chosen as this amount was sufficient to saturate the cellulose pad in the Terminix dissemination device without producing an overwhelmingly strong odor.

Field Testing the Spatial Repellent Effect of a Cinnamon Bark and Lemongrass Blend on Mosquitoes

Drawing on field data that cinnamon bark and lemongrass had the relatively strongest repellent effect on mosquitoes (see Fig. 2), we then investigated the area over which a binary (1:1) blend of cinnamon bark and lemongrass grass expressed repellency (Exp. 41, 42). We followed the same protocol as described above except that we (1) placed three (instead of one) dissemination devices in a triangular configuration beside each trap (Fig. 1 (c)); (2) positioned each of the three devices 1 m (Exp. 41) and 2 m (Exp. 42) away from the central sentinel trap, and (3) infused the cellulose pad in each dissemination device with 1 g (instead of 3 g) of the EO blend. In accordance with the placement of dissemination devices at 1 m and 2 m away from the trap, we set the inter-trap spacing to 12 m and 14 m, respectively.

Statistical Analyses of Data

We used JMP version 13.0 (SAS Institute Inc., Cary, NC 27513, USA) for data analyses. We analyzed laboratory spatial repellency data using a generalized linear model (GLM) with a binomial distribution and a probit link function to calculate the RD_{50}. We analyzed field repellency data using a generalized linear model (GLM) with a binomial distribution and a logit link function.

Results

Spatial Repellency of Plant Essential Oils in Laboratory Experiments

Three of the individually tested EOs (cinnamon bark, lemongrass, rosemary) proved repellent to *Ae. aegypti,* scoring a relatively low RD_{50} and a CI not including infinity (Table 1, Exps. 1,4,6). Each of citronella, geranium, and peppermint revealed a moderate repellent effect (Table 1, Exps. 2,3,5) and vanillin showed no activity (Table 1, Exp. 7) but may enhance the effect of EOs (Tawatsin et al. 2001, Choochote et al. 2007, Nerio et al. 2010).

Binary blends of cinnamon bark and lemongrass, citronella and geranium, geranium and lemongrass, and geranium and peppermint all had a relatively low RD_{50} (Table 2, Exps. 10,14,19,20). The interactions between cinnamon bark and lemongrass, and geranium and lemongrass proved additive (FIC $1.0 <$ to ≤ 2.0), whereas those between geranium and peppermint, and citronella and geranium proved synergistic (FIC ≤ 1.0). Notably, the RD_{50} for the blend of geranium and peppermint was 16.24 mg, whereas geranium alone and peppermint alone had RD_{50} values of 37604.1 and 21565.7 mg, respectively (Table 1, Exps. 3,5). Despite the synergistic effect between peppermint and vanillin, the blend's RD_{50} (5180.25 mg) appears too high to warrant further evaluation. Blends of geranium and vanillin, lemongrass and rosemary, and lemongrass and vanillin were not repellent (Table 2, Exps. 22,24,25), and scored RD_{50} values far exceeding those when these EOs were tested singly (Table 1). Cinnamon bark and citronella were highly synergistic with a small RD_{50} when calculated (Table 2, Exp. 8); however, the raw data still indicate no repellent effect, as the low RD_{50} can be attributed to the negative (attractive) trend of the curve.

The ternary blend of cinnamon bark, geranium, and rosemary revealed a synergistic interaction, with an estimated RD_{50} of 29.50 mg (Table 3, Exp. 30). All other ternary EO blends expressed antagonism. Although the blend of cinnamon bark, rosemary, and vanillin shows a synergistic interaction with a small RD_{50} (Table 3, Exp. 33), the raw data still indicate no repellent effect, as the low RD_{50} can be attributed to the negative (attractive) trend of the curve.

Field Testing Solitary Essential Oils for a Repellent Effect on Mosquitoes

Of the three EOs (cinnamon bark, lemongrass, rosemary) tested singly in field, cinnamon bark proved repellent to mosquitoes ($z = 3.01$, $p = 0.0019$) (Fig. 2, Exp. 39), whereas lemongrass ($z = 1.23$, $p = 0.2156$) (Fig. 2, Exp. 37) and rosemary ($z = 0.90$, $p = 0.3655$) (Fig. 2, Exp. 38) had no repellent effect.

Field Testing the Spatial Repellent Effect of a Cinnamon Bark and Lemongrass Blend on Mosquitoes

The blend of cinnamon bark and lemongrass were repllent when disseminated from Terminix devices placed at 0 m ($z = 2.14$, $p = 0.0326$) (Fig. 3, Exp. 40) and 1 m ($z = 2.15$, $p = 0.0317$) (Fig. 3, Exp. 41) away from the base of the sentinel trap. When the Terminix devices were placed 2 m away from the base of the sentinel trap, the repellent effect of the blend was still apparent but statistically no longer significant ($z = 1.70$, $p = 0.0864$) (Fig. 3, Exp. 42).

Discussion

Our data show that plant essential oils (EOs) are effective at spatially repelling mosquitoes in field settings, implicating the vapor-phase of EOs as the key sensory modality affording the repellent effect. EO mixtures on their own or coupled with insecticidal permethrins (Gross et al. 2017, Chansang et al. 2018) show promise to interfere with host-seeking behavior of mosquitoes. Below we shall elaborate on these conclusions.

Even solitary EOs expressed strong spatial repellency towards mosquitoes. These results corroborate assertions made in prior literature that EOs are repellent to

mosquitoes (Tawatsin et al. 2001, Trongtokit et al. 2005, Bernier et al. 2006, Conti et al. 2010, Nerio et al. 2010, Maia and Moore 2011, Achee et al. 2012, Chauhan et al. 2012, Regnault-Roger et al. 2012, Deletre et al. 2013, Kiplang'at and Mwangi 2014, Gross et al. 2017, Norris and Coats 2017). For example, lemongrass scored the astoundingly low RD_{50} of 8.65 mg (Table 1, Exp. 4), and cinnamon bark expressed repellency in the field (Fig. 2, Exp. 39). As experiment 39 was run in British Columbia (Canada), where (sub)tropical *Ae. aegypti* is absent (Kraemer et al. 2015), the spatial repellency of cinnamon bark obviously extends to temperate-zone mosquitoes such as *Cs. incidens* which was the most prevalent species captured in our sentinel traps.

Binary combinations of select EOs enhanced the repellent effect of each solitary EO through synergistic interactions. This synergism was most evident in the RD_{50} of a geranium and peppermint EO mixture (16.24 mg; Table 2, Exp. 20) which lowered the RD_{50} of each solitary EO (geranium: 37604.10 mg; peppermint: 21565.70 mg; Table 1, Exps. 3,5) by >1000-fold. Capitalizing on such extraordinary synergistic repellency between two EOs is particularly appropriate when (*i*) the dose of each solitary oil needed to achieve the desired repellent effect is impractically high (Anantharaman et al. 2010, Gross et al. 2017, Chansang et al. 2018), (*ii*) oils can be produced from locally sourced plants to mitigate production and transportation costs for disease-burdened countries (Conti et al. 2010, Maia and Moore 2011, Regnault-Roger et al. 2012, Deletre et al. 2013, Norris and Coats 2017); (*iii*) slowing development of behavioral resistance is warranted (Pennetier et al. 2007, Regnault-Roger et al. 2012, Kiplang'at and Mwangi 2014, Chansang et al. 2018) especially when EO blends are coupled with other means of mosquito control (Pennetier et al. 2007, Kiplang'at and Mwangi 2014, Chansang et al. 2018), and (*iv*) certain species or genera of mosquitoes are indifferent to one EO but not another (Barnard and Zue 2004).

Compared to binary EO blends, ternary EO blends were often markedly less repellent to mosquitoes. This diminishing effect could possibly be explained by the dilution of the most effective EO constituent(s) in the blend. To test this inference, ternary blends would have to be prepared such that the volume of each blend constituent matches that of the corresponding constituent in binary EO blends. With carefully selected constituents, ternary blends may still be applicable as spatial repellents. For example, the ternary blend of cinnamon bark, geranium, and rosemary scored a relatively low RD_{50} and a synergistic FIC value (Table 3, Exp. 30).

The EO blend of lemongrass and cinnamon bark expressed spatial repellency even when this blend was released from dissemination devices as much as 1 m away from a source (a baited sentinel trap) of attractive host cues (Fig. 3, Exp. 41). A spatial repellency effect was still apparent when host cues and the EO blend were separated by 2 m (Fig. 3, Exp. 42), but the repellent effect was then no longer statistically significant. With a repellent radius of at least 1 m, deployment of EOs as spatial repellents in small outdoor gatherings or within small rooms and domiciles (Maia and Moore 2011, Regnault-Roger et al. 2012, Debboun and Strickman 2013, Deletre et al. 2013, World Health Organization 2013, Norris and Coats 2017, Stevenson et al. 2018) seems justified. The spatial repellency effect could further be enhanced if the key repellents in each EO were to be determined and blended in new formulations, thereby circumventing dilution effects caused by inactive blend constituents in natural plant essential oils. However, such formulations may then significantly deviate from natural EOs and likely require registration as pesticides. Alternatively, EO blends could be used in combination with insecticidal permethrins (Gross et al. 2017, Chansang et al. 2018), and be deployed in combination with tactics aimed at lowering mosquito population densities (Hulsman et al. 1989, Kline 2007, Conti et al. 2010, Bonds 2012) or reducing their vectorial capacity (Benedict and Robinson 2003, Villar et al. 2015, Benelli and Mehlhorn 2016).

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Tables

Table A1 **Plant essential oils and vanillin tested in laboratory experiments 1-7 for spatial repellency effects on *Aedes aegypti* mosquitoes. Data were recorded as spatial activity indices (SAIs; see methods) and transformed to calculate the dose (mg) needed to obtain 50% spatial repellency (RD_{50}), with the 95% confidence interval (CI) presented in log form. Note the low RD_{50} of lemongrass.**

Exp.	Essential oil	N	Slope ± SE	RD_{50} (mg)	95% CI (log)	X^2
1	Cinnamon bark	15	0.44 (±0.33)	75.92	(-0.63, 4.39)	0.15
2	Citronella	15	0.26 (±0.29)	3298.08	(0.61, ∞)	0.90
3	Geranium	15	0.20 (±0.28)	37604.1	(0.71, ∞)	0.55
4	Lemongrass	20	0.50 (±0.24)	8.65	(-0.43, 8.54)	5.17
5	Peppermint	15	0.21 (±0.30)	21565.70	(0.56, ∞)	0.54
6	Rosemary	15	0.39 (±0.29)	48.14	(-0.95, 4.32)	1.94
7	Vanillin	15	0.07 (±0.47)	2.76E+23	(1.23, ∞)	0.02

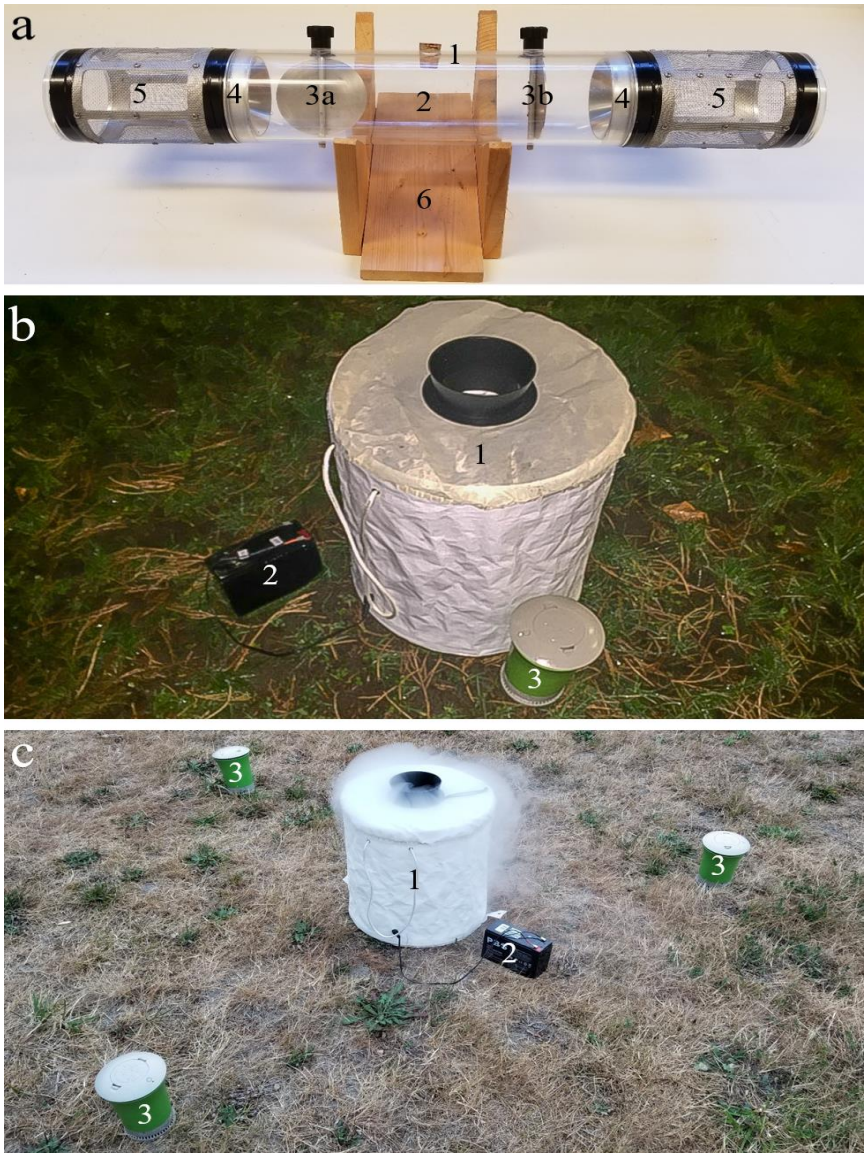
Table A2 **List of binary blends of plant essential oils and vanillin tested in laboratory experiments 9-29 for spatial repellency effects on *Aedes aegypti* mosquitoes. Data were recorded as spatial activity indices (SAIs; see methods) and transformed to calculate the dose (mg) needed to obtain 50% spatial repellency (RD_{50}), with the 95% confidence interval (CI) presented as log form. The Fractional Inhibitory Concentration (FIC) value denotes the type of interaction between oils. [1]FIC values of ≤ 1.0, 1.0 < to ≤ 2.0, 2.0 < to ≤ 4.0, and > 4.0 indicate synergistic, additive, nix, and antagonistic interactions, respectively.**

Exp.	Essential oil	N	Slope (± SE)	RD_{50} (mg)	95% CI (log)	FIC[1]	X^2
8	Cinnamon bark & Citronella	10	-0.07 (±0.36)	0	(-153.13, 125.55)	0	0.04
9	Cinnamon bark & Geranium	10	0.34 (±0.49)	1671.38	(-4.06, 10.51)	22.06	0.59
10	Cinnamon bark & Lemongrass	15	0.55 (±0.29)	11.28	(-0.68, ∞)	1.45	4.23
11	Cinnamon bark & Peppermint	15	0.35 (±0.29)	57.34	(-1.23, 4.74)	0.76	1.58
12	Cinnamon bark & Rosemary	10	0.29 (±0.46)	768.00	(-4.64, 10.41)	26.07	0.42
13	Cinnamon bark & Vanillin	10	0.39 (±0.42)	407.12	(-2.24, 7.46)	5.36	0.96
14	Citronella & Geranium	15	0.28 (±0.30)	23.35	(-1.85, 4.59)	7.70E-03	0.91
15	Citronella & Lemongrass	15	0.31 (±0.28)	136.40	(-1.90, 6.17)	15.80	1.30
16	Citronella & Peppermint	15	0.43 (±0.34)	184.97	(0.23, ∞)	6.47E-02	1.90
17	Citronella & Rosemary	10	0.20 (±0.33)	16593.20	(-9.27, 17.71)	349.74	0.38
18	Citronella & Vanillin	15	0.37 (±0.29)	59.53	(-1.11, 4.66)	1.80E-02	1.76
19	Geranium & Lemongrass	15	0.43 (±0.29)	12.12	(-0.82, 2.99)	1.40	2.43
20	Geranium & Peppermint	15	0.28 (±0.29)	16.24	(-1.82, 4.24)	1.18E-03	0.93
21	Geranium & Rosemary	10	0.25 (±0.33)	1528.67	(-5.01, 11.38)	31.80	0.62
22	Geranium & Vanillin	10	0.12 (±0.75)	1.41E+14	(-0.60, ∞)	3.74E+09	0.03
23	Lemongrass & Peppermint	15	0.39 (±0.27)	53.38	(-0.36, ∞)	6.17	2.23
24	Lemongrass & Rosemary	10	0.04 (±0.41)	1.15E+18	(-317.41, 353.54)	1.57E+18	0.01
25	Lemongrass & Vanillin	10	0.03 (±0.38)	2.31E+34	(0.77, ∞)	2.67E+33	0.01
26	Peppermint & Rosemary	10	0.27 (±0.44)	48617.50	(-8.43, 17.80)	1012.25	0.45
27	Peppermint & Vanillin	10	0.22 (±0.33)	5180.25	(-7.02, 14.67)	0.24	0.46
28	Rosemary & Vanillin	10	0.28 (±0.38)	1095.46	(-4.42, 10.50)	22.76	0.59

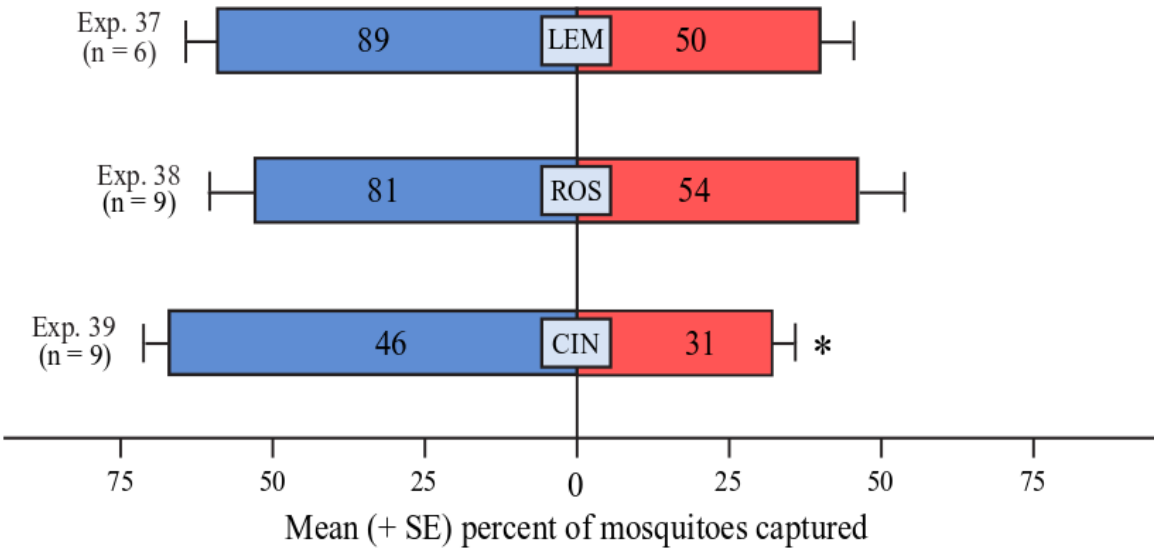
Appendix A: Table 1 List of candidate ternary blends of plant essential oils and vanillin tested in laboratory experiments 29-36 for spatial repellency effects on *Aedes aegypti* mosquitoes. Data were recorded as spatial activity indices (SAIs; see methods) and transformed to calculate the dose (mg) needed to obtain 50% spatial repellency (RD_{50}), with the 95% confidence interval (CI) presented as log form. The Fractional Inhibitory Concentration (FIC) value denotes the type of interaction between oils. [1]FIC values of ≤ 1.5, 1.5 < to ≤ 3.0, 3.0 < to ≤ 6.0, and > 6.0 indicate synergistic, additive, nix, and antagonistic interactions, respectively.

Exp.	Essential oil	N	Slope (± SE)	RD_{50} (mg)	95% CI (log)	FIC	X^2
29	Cinnamon bark/Geranium/Peppermint	10	0.13 (±0.38)	2.21E+09	(0.86, ∞)	2.93E+07	0.13
30	Cinnamon bark/Geranium/Rosemary	15	0.42 (±0.30)	29.50	(-0.78, 3.72)	1.00	2.20
31	Cinnamon bark/Lemongrass/Peppermint	15	0.29 (±0.28)	263.47	(-2.26, 7.10)	33.93	1.08
32	Cinnamon bark/Peppermint/Vanillin	10	0.38 (±0.39)	674.93	(0.05, ∞)	8.92	1.08
33	Cinnamon bark/Rosemary/Vanillin	10	-0.08 (±0.56)	0	(-157.07, 135.41)	0	0.02
34	Citronella/Geranium/Peppermint	10	0.12 (± 0.44)	1.98E+10	(0.43, ∞)	7.44E+06	0.07
35	Citronella/Peppermint/Vanillin	10	0.09 (± 0.37)	3.21E+12	(0.53, ∞)	1.12E+09	0.06
36	Lemongrass/Rosemary/Vanillin	10	0.25 (± 0.40)	9313.74	(-7.27, 15.21)	1269.70	0.43

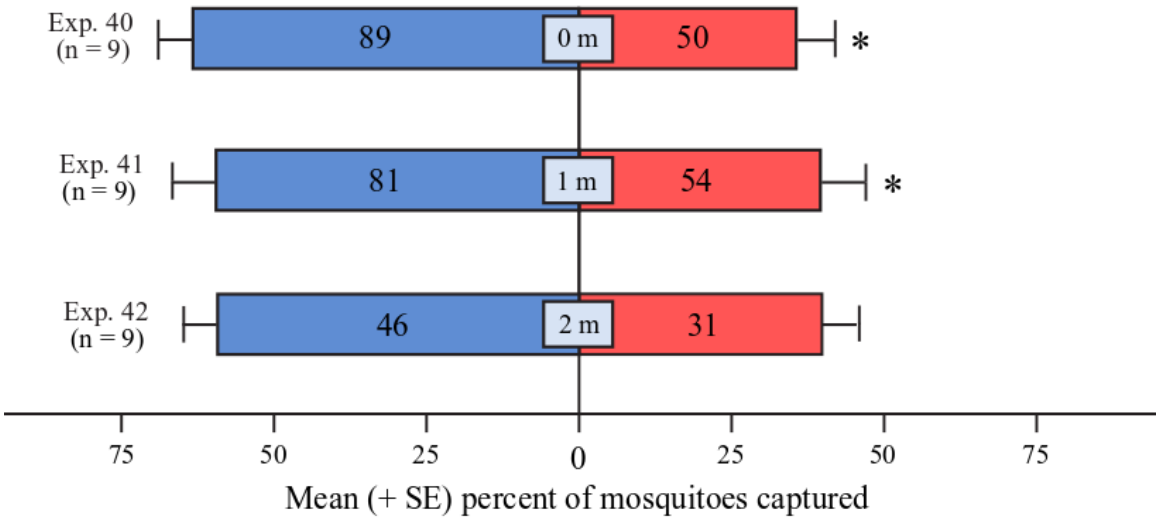
Figures



Appendix A: Figure 1 (a): Apparatus for testing spatial repellency effects of plant essential oils (EOs) singly or in combination on *Aedes aegypti* mosquitoes (Exps. 1-36). Numbers in the photograph refer to features of the apparatus, as follows: (1) entry port with cork stopper; (2) entry chamber; (3a, 3b) beveled doors; (4) funnel permitting mosquitoes to enter, but not to exit, lateral chambers; (5) lateral chamber fitted with test stimulus; (6) wooden stand supporting apparatus. (b): Layout for field testing repellent effects of EOs on host-seeking mosquitoes (Exps. 37-40). Numbers in the photograph refer to items in the layout of the treatment trap, as follows: (1) sentinel mosquito trap; (2) 12-V battery powering the sentinel trap; (3) Terminix dissemination device fitted with an EO blend. (c): Layout for field testing spatial repellent effects of two EOs (cinnamon bark and lemongrass) on host-seeking mosquitoes (Exps. 41,42). Three Terminix dissemination devices (3), each fitted with the EO blend, placed in triangular configuration around a sentinel trap (1). Note the CO_2 emanating from the trap.



Appendix A: Figure 2 Field captures of mosquitoes in paired sentinel traps (Figure 1, b) in the presence (treatment; red), or absence (control; blue), of a single essential oil (lemongrass (LEM), rosemary (ROS), or cinnamon bark (CIN)). In each of experiments 37-39, the asterisk (*) denotes a significant repellent effect for a test stimulus ($P < 0.05$, generalized linear model with a logit extension).



Appendix A: Figure 3 Field captures of mosquitoes in paired sentinel traps (Figure 1, c) in the presence (treatment; red), or absence (control; blue), of a binary blend of lemongrass and cinnamon bark disseminated from three devices that were placed at 0 m (Exp. 40), 1 m (Exp. 41), and 2 m (Exp. 42) away from the treatment trap. In each of experiments 40-42, the asterisk (*) denotes a significant repellent effect for a test stimulus ($P < 0.05$, generalized linear model with a logit extension).

Appendix B.

Preparative, Analytical, and Synthetic Procedures

Purification of Germacrene-D

Germacrene-D was purified to 93% from Treatt Plc (Lakeland, FL 33805, USA) (40% technical grade) by high-performance liquid chromatography (HPLC) [Waters HPLC system (600 Controller, 600 Delta Pump, 2487 Dual Lamda Absorbance Detector [Waters Corp. Milford, MA 01757 USA], using a C_{18} reversed phase column (Synergi-Hydro, 250 × 60 mm, 4 μ) eluted with acetonitrile (1 ml/min).

Purification of Artemisia ketone

Artemisia ketone (3,3,6-trimethyl-1,5-heptadien-4-one) is present (34%) in Wormwood (*Artemisia annua*) essential oil (Liberty Natural Products, Portland, OR 97215, USA) and was isolated (190 g, 98% pure) by repetitive silica gel column chromatography using hexane and ethyl acetate (90:10) as eluents.

Purification of Yomogi alcohol

Yomogi alcohol (2,5,5-trimethyl-3,6-heptadien-2-ol) is present (15%) in Chamomile Morocco (*Ormensis multicaulis*) essential oil (Liberty Natural Products) which also contains Santolina alcohol (62%) as well as cineole and monoterpenes. Yomogi alcohol was isolated from 3 g of the essential oil by flushing analyte twice through a silica gel column using hexane and ethyl acetate as eluents (first flash: 80:20; second flash: 85:15). This procedure yielded 180 mg of Yomogi alcohol (50% pure) which was then further purified by HPLC (see above) using acetonitrile and water (60:40; 1 ml/min) as eluents. This purification procedure resulted in a mixture of Yomogi alcohol (75%) and Santolina alcohol (25%) as by-product.

Preparation of 1-phenylbutane-2,3-dione and 1-phenyl-3-hydroxy-2-butanone

A solution of DL-3-phenyllactic acid (166 mg, 1.0 mmol, 1.0 eq.) in CH_2Cl_2 (15 ml) was sequentially added to *N*,*O*-dimethylhydroxylamine hydrochloride (195 mg, 2.0 mml, 2.0 eq.) and 1,1'-carbonyldiimidazole (324 mg, 2.0 mml, 2.0 eq.) at 0 °C. After 30 min, the reaction was stirred at ambient temperature overnight before quenching with water. The aqueous layer was separated and extracted with CH_2Cl_2 (15 mL). The combined organic layer was washed sequentially with 10 % HCl aqueous solution, 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, 0.6 ml, 1.8 mmol, 1.8 eq.) was added, and the mixture was stirred at 0 °C for 5 h before quenching with saturated aqueous NH_4Cl (5 mL). The aqueous layer was separated and extracted with EtOAc (10 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, 6/1) yielding 87 mg (53% over 2 steps) of 1-phenyl-3-hydroxy-2-butanone as colourless oil. A sample of this ketone-alcohol (49 mg, 0.3 mmol, 1.0 eq.) was dissolved in dry CH_2Cl_2 (10 ml), and $NaHCO_3$ (38 mg, 0.45 mmol, 1.5 eq.) was added followed by Dess-Martine periodinane (190 mg, 0.45 mmol, 1.5 eq.). After stirring the reaction mixture at ambient temperature for 1 h, the mixture was treated with a 10% aqueous solution of $Na_2S_2O_3$ (5 ml) and a saturated aqueous solution of $NaHCO_3$ (5 mL) and then stirred for an additional 20 min. The aqueous layer was separated and extracted with EtOAc (3× 10 ml). The combined organic layers were washed with brine (10 ml), dried over magnesium sulfate and concentrated. Purification by flash chromatography (hexane/ethyl acetate, 10/1) provided 65 mg (75 % pure) of the diketone as a yellow oil.