Multimodal mate-finding and floral foraging behaviour of *Aedes* and *Culex* mosquitoes

by

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Bachelor of Science (Honours), Simon Fraser University, 2018

Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Pest Management

in the
Department of Biological Sciences
Faculty of Science

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SIMON FRASER UNIVERSITY

Spring 2022

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Abstract

I investigated mate location and nectar foraging behaviour in the diurnal yellow fever mosquito, *Aedes aegypti*, and the crepuscular house mosquito, *Culex pipiens*. High-speed video recordings revealed that incident light reflects off the wings of swarming *Ae. aegypti* males. In behavioural experiments, LED assemblies flashing light at the wingbeat frequency of females (665 Hz) mediated swarm and mate recognition at long range, whereas play-back of wingbeat sound (665 Hz) mediated mate recognition at short range. As predicted by the sensory drive theory, light flashes had no signal function for swarming *Cx. pipiens*. All five milkweed species/varieties tested attracted *Cx. pipiens*. Phenylacetaldehyde and benzaldehyde were the key floral semiochemicals emitted by showy milkweed, *Asclepias speciosa.* Combining floral attractant of *A. speciosa* with those of four other plant species did not result in a super-flower blend that was more attractive to *Ae. aegypti* than the *A. speciosa* floral blend on its own.

Keywords: *Aedes aegypti; Culex pipiens;* nectar foraging; multimodal communication; mate-location behaviour

Dedication

I dedicate this thesis to my mother, Natalie, who has supported me in all of life's endeavours, and my partner-in-Stardew Diana, who makes me strive to be better.

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Introduction

1.1. General ecology of mosquitoes

1.1.1. Mosquito life cycle

Most mosquitoes follow a similar life cycle (Figure 1). Gravid females lay their eggs in aquatic environments but the micro-habitats they prefer for oviposition, the number of eggs they deposit, and the oviposition behaviour all differ between species. Females of *Aedes* spp., e.g., lay some 100 eggs above waterlines (Joy et al. 2010). Within 5 days of hatching, larvae develop into motile pupae which become adults within 2–3 days (Centers for Disease Control and Prevention [CDC] 2020). Soon after eclosion, both males and females seek inflorescences to obtain nectar (Fosters 1995). Within four days of eclosion, females engage in host-seeking behaviour and attempt to take a bloodmeal (Davis 1984). Blood-fed females become inactive for about 48 h (Jones 1981). Mating typically takes place after the first bloodmeal (Teesdale 1955) and occurs in swarms formed around a swarm marker (Downes 1969). Soon after mating, females lay eggs (Shroyer & Sanders 1977).

1.1.2. Mosquitoes as vectors

Aedes aegypti, Anopheles gambiae and *Culex pipiens* vector various pathogens (Table 1.1) which can cause deadly and debilitating diseases, including dengue fever, malaria and West Nile fever These diseases have widespread global impacts. For example, half the global human population is estimated to live in areas where dengue virus transmissions may occur (Brady et al. 2012; World Health Organization [WHO] 2021a), which are responsible for roughly 20,000 deaths annually (Stanaway et al. 2016). Approximately 390 million people are infected with dengue yearly, and around 96 million resultingly fall ill (Bhatt et al. 2013). After recovery from the disease, lingering effects such as weakness and trouble working can manifest (Tiga et al. 2016). Similar to dengue, nearly 50% of the global human population is at risk of malaria, which leads to

more than 200 million cases and 400,000 deaths yearly (WHO 2021b). Additionally, complications from malaria can include anemia, hypoglycemia, pulmonary edema, organ failure and low weights of newly-born babies (Mayo Clinic 2012).
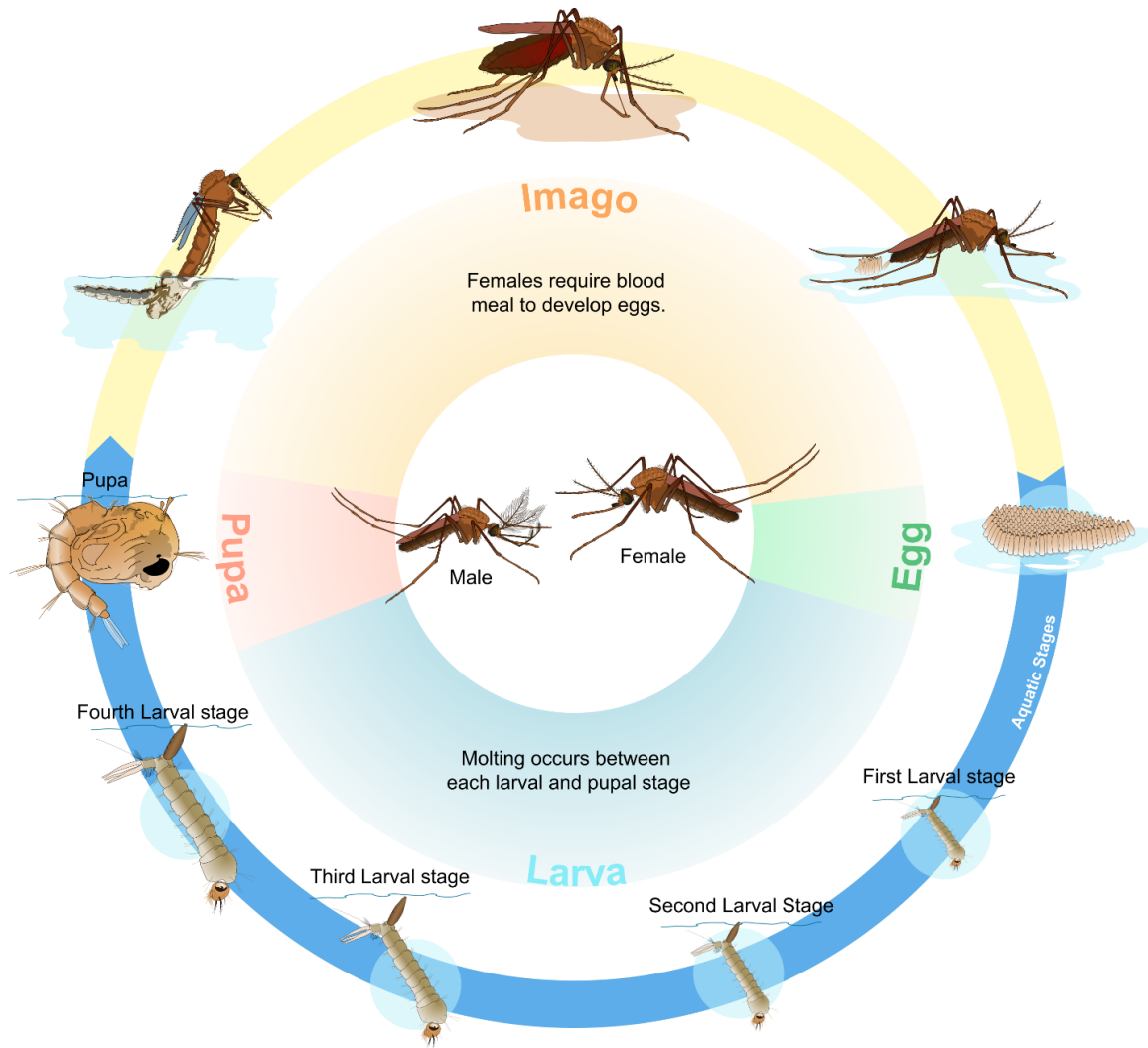
Figure 1.1. Generalized life cycle of a mosquito. Adapted from Villarreal (2010).

Mosquito-borne illnesses are also a major socio-economic burden. Direct costs include ambulatory and hospital care and indirect costs include productivity losses. In 2000, it was estimated that malaria decreased economic growth in African countries by 1.3% annually; this led to a 32% lower GDP than otherwise expected for those countries (WHO 2003). Additionally, the annual global costs resulting from dengue illness were estimated to be $8.9 million USD in 2013 (Shepard et al. 2016).

Table 1.1. A non-exhaustive list of mosquito-borne pathogens and their mosquito vectors

Pathogen	Disease caused	Mosquito species	Source
Chikungunya virus	Chikungunya	*Aedes aegypti*	WHO (2020)
Dengue virus	Dengue	*Aedes aegypti*	WHO (2021a)
Filarial worms	Lymphatic filariasis	*Aedes, Anopheles* & *Culex* spp.	WHO (2020)
Japanese encephalitis virus	Japanese encephalitis	*Culex* spp.	WHO (2020)
Plasmodium falcipuarum	Malaria	*Anopheles gambiae* & other *Anopheles* spp.	Molina-Cruz et al. (2016)
Plasmodium vivax	Malaria	*Anopheles darlingi* & other *Anopheles* spp.	Laporta et al. (2015)
West Nile virus	West Nile fever	*Culex pipiens* & other *Culex* spp.	Kilpatrick et al. (2007)
Yellow fever virus	Yellow fever	*Aedes aegypti*	WHO (2020)
Zika virus	Zika fever	*Aedes aegypti*	WHO (2020)

1.2. Mosquito swarming behaviour

The males of many mosquito species form mating swarms around conspicuous markers (Downes 1969). A swarm can be defined as an assembly of individuals all independently responding to the same marker (Downes 1969). There are also swarms of female mosquitoes. Females of the woodland floodwater mosquito, *Ae. hexodontus,* utilize the same swarm markers as males but fly at a lower height and in a different pattern (Downes 1958 in Downes 1969). Males and unmated females of the southern house mosquito, *Cx. quinquefasciatus,* respond to the same markers and display similar swarming patterns (Gibson 1985). Both males and females of the yellow fever mosquito,

Ae. aegypti, swarm around vertebrate hosts (Jones 1981). This behaviour differs from that of other species that form distinctive mating swarms in areas without host presence (Charlwood & Jones 1979). Swarms may consist of just a few individuals (Marchand 1983) or of hundreds or thousands of individuals (Marchand 1983; Yuval & Bouskila 1993). In fact, a single male exhibiting swarming behaviour may be considered a "swarm" (Downes 1969; Marchand 1983). Dense swarms of mosquitoes have sometimes been mistaken for columns of rising smoke (Knab 1906). Swarming is energetically expensive (Yuval et al. 1994) and exposes individuals to predation (Yuval & Bouskila 1993) but it is also efficient for attracting and locating mates, particularly in those species that cover a wide spatial scale (Klassen & Hocking 1964).

1.2.1. Swarm formation

Swarm markers provide visual reference points (Downes 1969; Gibson 1985) and include prominent environmental landmarks such as church steeples, corn stalks or tree tips (Knab 1906), the heads of human observers (Corbet 1964), dark cloth (Ikeshoji 1985), or possibly even electrostatic potential (Maw 1962). Larger swarm markers tend to produce larger swarms (Downes 1969). Males of *Ae. aegypti* and the Asian tiger mosquito, *Ae. albopictus,* are attracted to the same vertebrate cues as females, and form mating swarms around hosts possibly to intercept females seeking a bloodmeal (Hartberg 1971; Gubler & Bhattacharya 1972 in Bargielowski et al. 2013). Certain swarm sites are consistently sought year after year (Diabate & Tripet 2015). Active swarms of the West African mosquito, *An. melas*, can be relocated by transferring a swarm marker to a new location (Charlwood & Jones 1980). Whereas visual markers are often the focal point of swarms (Gibson 1985), the African mosquitoes *An. gambiae* and *An. arabiensis* prefer swarming in flat, open areas without distinct visual swarm markers (Marchand 1983).

Swarm formation and persistence are affected by ambient illumination, temperature, season, and host presence. Swarm formation usually starts in response to changes in ambient illumination (Reisen et al. 1977; Charlwood & Jones 1980). Swarming of *Cx. quinquefasciatus* can experimentally be induced by reducing ambient illumination to 1 lux (Gibson 1985). Swarms of the common house mosquito, *Cx. pipiens pallens,* form at about 20 lux, become more active as light levels decrease, reach maximum size at about 2 lux, and break up at 0 lux (Omori 1954). Swarms of *An. arabiensis* and *An.*

gambiae form only at dusk despite similar light levels during dawn (Marchand 1983). Swarming of *Ae. aegypti* males can be induced by the onset of the photophase or the presence of a host (Cabrera & Jaffe 2007). Males and females of *Ae. aegypti* exhibit a bimodal peak of flight activity, with one peak in the morning and another in the afternoon, apparently induced by the photoperiod (Teesdale 1955; Taylor & Jones 1969; Trpis et al. 1973; Jones 1981; Lees et al. 2014). Swarm formation of *Cx. pipiens pallens* is faster below 20° C but does not occur at temperatures below 13–14° C (Omori 1954). Swarms of *Cx. pipiens pallen*s persist for the longest time in July (Omori 1954), with the onset of swarming starting progressively earlier towards winter (Omori 1954).

The persistence of swarms varies between species and is affected by the mating status of males, the presence of females in the swarm, and by ambient conditions. Males of *Ae. aegypti* swarm for 24–35 minutes (Cabrera & Jaffe 2007) or longer when females are present (Cabrera & Jaffe 2007) or following copulation (Nijhout & Craig 1971). Males of the western encephalitis mosquito, *Cx. tarsalis,* reportedly swarm continuously from 1 h before sunset until darkness, and also from 1 h before sunrise to 1 h after sunrise (Reisen et al. 1985). Swarms of *An. gambiae* form about 10 min after sunset and last for about 30 min (Marchand 1983). In laboratory settings with the light intensity set to 3 lux, males of *An. gambiae* could be kept swarming for 1.5 h (Charlwood & Jones 1980).

The mechanisms by which sympatric mosquito species remain reproductively isolated are known for some but not all species. For example, males of *An. coluzzii* and *An. melas* preferentially swarm within human dwellings and salt-processing areas, respectively (Assogba et al. 2013). Other mosquito species may utilize the same marker but form swarms at different heights or at different times a day (Charlwood et al. 2002). In species that form species-specific swarms at the same time of day (e.g., *An. coluzzii* and *An. Melas)* (Assogba et al. 2014), or both at the same time of day and at the same marker (e.g., *Ae. aegypti* and *Ae. albopictus*) (Hartberg 1971; Gubler & Bhattachaya 1972 in Bargielowski et al. 2013), chemical and/or acoustic mate recognition signals (Roth 1948; Nijhout & Craig 1971; Pennetier et al. 2010) may serve as reproductive isolating mechanisms.

1.2.2. Acoustic signals

Male mosquitoes have long been known to rely on acoustic signals for mate location/recognition (e.g., Johnston 1855). In early studies, tuning forks have been used to generate sound frequencies that induced mate-seeking behaviour (e.g., Roth 1948, Mayer 1874). Although female mosquitoes were thought not to sense male wingbeat sound for mate recognition (e.g., Downes 1969; Cabrera & Jaffe 2007), it is now well accepted that females possess functional sound receptors (Johnston's organ) (Göpfert & Robert 2000), recognize and respond to the males' wing beat sounds (Ikeshoji 1981), and that they may use male wing beat sound for mate selection (Cator et al. 2010). Although the Johnston's organ of females is less sensitive than that of males, it is still among the most sensitive sound receptors in insects (Göpfert & Robert 2000). Reports that acoustic trap baits attract mosquito females (Ikeshoji et al. 1985), and that female *Uranotaenia* spp. exploit frog calls to locate host frogs (Borkent & Belton 2006), provide further evidence that females can sense certain frequencies of sound.

Male mosquitoes respond to a wide range of sound frequencies (Roth 1948). Males of *Ae. aegypti* are attracted to sound frequencies ranging between 256–512 Hz (Roth 1948). This broad range of sensitivity is advantageous, as the wingbeat frequency of conspecific females changes in accordance with female age, body size and ambient temperature (Belton 1994). As a result, wingbeat sound cues or signals on their own seem insufficient to play a role in reproductive isolation (Roth 1948).

Sound cues/signals are perceived over relatively short distances (Wishart & Riordan 1959; Cator et al. 2009). In cage experiments, *Ae. aegypti* males took flight and pursued a vibrating 480 Hz tuning fork (Roth 1948). Exposed to a 480-Hz frequency, they also grasped and seized other males and attempted to copulate with them (Roth 1948). Swarming males of *Cx. quinquefasciatus* respond to sounds of 500–600 Hz, which are within the wingbeat frequency range of conspecific females (Gibson 1985). These males responded by slowing their flight and swarming over a smaller area, apparently attempting to locate the source (Gibson 1985). Males of *An. gambiae* and *An. coluzzi* that are exposed to wingbeat frequencies of conspecific females show similar behaviours, and phonotactically approach the sound source (Simões et al. 2017).

Mixed swarms of *An. gambiae* and *An. arabiensis* occur (Marchand 1983) but hybrids are extremely rare, suggesting that reproductive 'barriers' exist (Marchand 1983). Differential wingbeat frequency alone is not likely an adequate reproductive

barrier, because many species have overlapping wingbeat frequencies (Roth 1948). As close relatives, *Ae. aegypti* and *Ae. albopictus* exhibit similar behaviours (Bargielowski et al. 2015b), produce overlapping wingbeat frequencies (Brogdon 1994), and form mating swarms around vertebrate hosts (Hartberg 1971; Gubler et al. 1972 in Bargielowski et al. 2013). However, *Ae. albopictus* males do not copulate with *Ae. aegypti* females (Nijhout & Craig 1971), possibly because of a contact pheromone present on females of *Ae. albopictus* but not *Ae. aegypti* (Nijhout & Craig 1971). Additionally, in areas where *Ae. aegypti* and *Ae. albopictus* populations overlap, *Ae. aegypti* males may have evolved to be more selective for conspecific females (Bargielowski et al. 2015a).

Multiple mosquito species, including *Ae. aegypti*, *Cx. quinquefasciatus, An. gambiae* and *Toxorhynchites brevipalpis,* engage in 'harmonic convergence' prior to mating (Gibson & Russell 2006; Cator et al. 2009; Warren et al. 2009; Pennetier et al. 2010). In this courtship ritual, male–female pairs of flying mosquitoes attempt to match harmonic components of their flight tone ('harmonic convergence') (Cator et al. 2009). Males and females of *Ae. aegypti* modulate their second and third harmonic, respectively, to a shared 1200 Hz frequency (Cator et al. 2009). After successfully pairing, the pair leaves the swarm to complete copulation (Cator et al. 2011). Harmonic convergence has adaptive significance and fitness consequences. It conveys information about the male's quality (Cator & Harrington 2011), helps avoid same-sex pairings (Gibson & Russell 2006), and may serve as an interspecific mating barrier (Ritchie & Immonen 2010). It also facilitates assortative mating between the closely related M- and S- forms of the *An. gambiae* complex (Pennetier et al. 2010), which are now recognized as distinct species (*An. coluzzii* and *An. gambiae* s.s.; Ranford-Cartwright et al. 2016). Sons of *Ae. aegypti* pairs that converged at harmonic frequencies prior to mating had increased mating success and, in turn, their offspring were more likely to converge prior to mating (Cator & Harrington 2011). Mated *Ae. aegypti* females are less likely to attempt harmonic convergence (Cator et al. 2009), possibly because they generally use the sperm only from their first mate for egg fertilization (Christophers 1960).

In field studies with *Cx. tarsalis* and *Ae. albopictus*, sound-baited traps captured many males and reduced insemination rates of *Cx. tarsalis* females to near zero (Ikeshoji et al. 1985; Ikeshoji & Ogawa 1988). As wingbeat frequencies overlap between species, males of multiple species could be attracted to, and captured in, the same trap (Diabate & Tripet 2015). Moreover, sound baits could be coupled with chemosterilants to sterilize mosquito populations (Ikeshoji & Yap 1987). Combining wingbeat sound baits

for attraction of males with semiochemical baits for attraction of (gravid) females may allow monitoring of both male and female mosquito populations (Johnson & Ritchie 2016) Both tactics could also be deployed together with attract & kill tactics such as toxic sugar baits (Beier et al. 2012) to reduce mosquito populations.

There is controversy as to whether male mosquitoes can, or cannot, discriminate between the wingbeat sound of female and male conspecifics. Males were considered "deaf" to their own wingbeat sound (Tischner & Schief 1955 in Cabrera & Jaffe 2007) and were shown to be attracted to female, but not male, artificial wingbeat sound (Johnson & Ritchie 2016; Menda et al. 2019). Exposed to wingbeat sound of females, "hypersexual" *Ae. aegypti* males attempt to mate with males, or even speakers and tuning forks (Roth 1948; Nijhout & Craig 1971) but males were not attracted to wingless females (Roth 1948). Similarly, males attempted to copulate with newly eclosed males whose wingbeat sound resembled that of females (Roth 1948). Conversely, harmonic convergence of in-flight male–female pairs is reliant upon males sensing their own wing beat sound and adjusting it to achieve harmonic convergence with the flight tone of females (Cator et al, 2009; Warren et al. 2009; Pennetier et al. 2010). The data in combination then suggest that males can sense their own wing beat sound but that they are not attracted to it. Whereas wingbeat sound is essential for mate attraction and recognition in *Ae. aegypti,* it seems immaterial in the winter marsh mosquito, *Culiseta inornata.* Here, males with or without their flagellum ablated are still attracted to dead or wingless females and attempt to copulate with them (Kliewer et al. 1966), suggesting that pheromones play a larger role than sound in mate location or recognition (Kliewer et al. 1966). The findings that taxon-specific cues mediate mate location behaviour indicate that mosquitoes are a not a monolithic group and that their sexual communication systems cannot be generalized across taxa.

1.2.3. Visual cues

In the context of mosquito mate location, sound is the most well-known and well-studied sensory modality, but vision may be as important (Gries et al. 2017). Visual mate location signals consist of light flashes reflecting off wings of in-flight females (Gries et al. 2017). Assemblies of LEDs flashing light at various frequencies are more attractive to *Ae. aegypti* males than LEDs emitting constant light (Gries et al. 2017), with flash frequencies resembling female wingbeat frequencies being most attractive to males.

Similar phenomena have been reported for house flies, *Musca domestica,* vinegar flies*, Drosophila melanogaster,* soldier flies, *Hermetia illucens,* and common green bottle flies, *Lucilia sericata* (Eichorn et al. 2017; Gries et al. 2017), suggesting that this vision-based mate recognition system is highly conserved in Diptera. In *Ae. aegypti,* the attractiveness of light flash signals could be enhanced by swaying the LED assembly and by emitting blue instead of white light from LEDs (Gries et al. 2017).

Both visual and olfactory cues play a role during nectar-foraging and host-foraging of mosquitoes (Sippell & Brown 1953; Kawada et al. 2005; van Breugel et al. 2015; Peach et al. 2019). Conceivably then, there may also be an interplay of multi-modal signals, such as visual and sound signals, during mate location. The range over which visual signals attract mosquitoes extend up to 19 m (Bidlingmayer & Hem 1980) but this range may be contingent upon the type of stimuli that are tested. Irrespectively, visual signals have a larger recruitment range then wing beat sound signals which are effective only at close range (≤25 cm) (Wishart & Riordan 1959; Hoy 2006). Utilizing both long-range visual signals and short-range sound signals for mate location seems most efficient.

1.2.4. Chemical signals

Claims that mosquitoes emit volatile pheromones (Cabrera & Jaffe 2007) have been contested in the literature. However, contact pheromones have been documented in multiple species, including *Ae. albopictus* (Nijhout & Craig 1971), *Cs. inornata* (Kliewer et al. 1966) and *Deinocerites cancer* (Provost & Haegar 1967).

In the study by Cabrera & Jaffe (2007), female *Ae. aegypti* exposed to odors from (*i*) swarming males, (*ii*) other females, or (*iii*) a rat took flight in response only to male or female odors, suggesting both male and female *Ae. aegypti* release a volatile pheromone that remained unidentified. Later, three compounds emitted by in-flight *Ae. aegypti* were identified (Fawaz et al. 2014): 2,6,6-trimetyhlcyclohex-2-ene-1,4-dione ('ketoisophorone'; produced by males and females), 2,2,6-trimetyhlcyclohexane-1,4-dione (saturated analog of ketoisophorone; produced by females), and 1-(4-ethylphenyl) ethanone ('ethanone'; produced by males and females). Both ketoisophorone and its saturated analog induced "excited" flight in females, ethanone attracted females to the olfactometer port baited with it, and ketoisophorone induced swarming by males but not

females. In all bioassays, responses of mosquitoes were dependent on pheromone dose. When traps were baited with ketoisophorone and ethanone and tested alongside BG sentinel traps under semi-field conditions, they did not capture more mosquitoes than unbaited control traps, and the saturated analog of ketoisophorone was repellent. Additional work is needed to determine how these three compounds interact with visual and acoustic mate location signals.

More recently, an aggregation pheromone blend has been identified in two African mosquitoes, *An. gambiae* and *An. arabiensis* (Mozūraitis et al. 2020). During swarming in the laboratory, males emitted relatively large amounts of acetoin, sulcatone, octanal, nonanal and decanal (Mozūraitis et al. 2020). When tested in a wind tunnel, a synthetic blend of these compounds increased flight activity and extended swarming in *An. gambiae,* and increased the incidence of mating in *An. arabiensis, An. gambiae* s.s., *An. coluzzi, An. funestus* and *An. merus* (Mozūraitis et al. 2020).

Contact pheromones have been implicated to play a role in mate recognition. While *Ae. albopictus* males are attracted to the wingbeat sound of female *Ae. aegypti* (Nijhout & Craig 1971), they did not attempt to copulate with these heterospecific females but flew away following physical contact with them (Nijhout & Craig 1971). The contact pheromone seems to be sensed by a tarsal receptor (Nijhout & Craig 1971). There is also evidence for a contact pheromone in *Cs. inornata* (Lang 1977). Males of *Cs. inornata* attempt to copulate with legs ablated from females but do not respond to legs ablated from males (Lang 1977).

1.3. Multimodal cues guide nectar- and host-foraging mosquitoes

Mosquitoes exploit multimodal resource cues when they forage for floral nectar and vertebrate hosts. Blends of semiochemicals (message-bearing chemicals) emanating from floral resources and vertebrate hosts share constituents (Peach et al. 2019a), which supports the concept that insect hematophagy has evolved from phytophagy (Waage 1979; Lehane 2005). However, the relative attractiveness of nectar- and host-associated semiochemicals differs in accordance with the age of foraging females (Foster & Takken 2004; Peach et al. 2019a). Visual cues add to the attractiveness of inflorescences. Lighter-coloured inflorescences appear to entice more mosquito visitation (Sandholm & Price 1962; Magnarelli 1977), and UV patterns modulate the

attractiveness of inflorescences (Peach et al. 2019b). Many inflorescences exhibit UV patterning (Horovitz & Cohen 1972) which helps attract and guide pollinators to nectar and pollen sources (Horth et al. 2014; Koski & Ashmann 2014). Nectar-foraging mosquitoes are attracted to a bimodal complex of semiochemical and visual inflorescence cues (Peach et al. 2019a) but responses to visual cues appear to be "gated" by floral semiochemicals that must be present for visual cues to be effective (Jepson & Healy 1988). Furthermore, the spectral composition of visual inflorescence cues alters the foraging responses of mosquitoes, as experimentally shown by presenting stimuli with or without UV wavelengths present (Peach et al. 2019b). Both nectar- and host-foraging mosquitoes prefer UV-dark and dark-coloured objects (Brown 1951; Brown 1954; Chambers et al. 2013; Peach et al. 2019b). Nectar-foraging mosquitoes appear to integrate multimodal inflorescence cues (Peach et al. 2019b) in a manner similar to host-seeking (van Breugel et al. 2015).

Host-seeking mosquitoes tend to prefer dark-coloured objects (Brett 1938; Brown 1951; Brown 1954) indicative of prospective hosts. Exposed to CO_2, female mosquitoes search for dark-coloured objects (van Breugel et al. 2015). They locate moving hosts more readily than stationary hosts (Sippell & Brown 1953) and are strongly attracted to objects with contrasting black and white colouration (Sippell & Brown 1953). This latter phenomenon is exploited in the design of BG sentinel traps that exhibit a contrasting black and white pattern to increase attraction of mosquitoes (Kröckel at al. 2006). Gravid females also respond to visual cues when they seek oviposition sites, using both UV and human-visible wavelengths (Snow 1971; Hoel et al. 2011). Light traps exploit mosquito vision for attraction (Bradley & McNeel 1935), with the colour of traps affecting their attractiveness (Barr et al. 1963; Hoel et al. 2011).

Host-seeking behaviour by female mosquitoes exemplifies the exploitation of multimodal host cues. When females detect a CO_2 plume, which may have originated from a potential host up to 37 m away (Gillies & Wilkes 1969), they are activated, follow it upwind, and become strongly attracted to host visual cues (van Breugel et al. 2015) that they can detect at a distance of 5-19 m (Bidlingmayer & Hem 1980). Once in visual range of a host, mosquitoes leave the CO_2 plume and fly towards the host guided by visual host cues (van Breugel et al. 2015). Close to the host, cues such as skin semiochemicals (Lacey et al. 2014), moisture (Wright & Kellogg 1962) and heat (van Breugel et al. 2015) inform decisions to land on the host. All cues are effective at specific

ranges and are utilized in a stepwise procedure; however, certain steps can be skipped if an attractive cue is encountered by chance (van Breugel et al. 2015).

Multisensory modalities are also involved in mosquito mate location and mate recognition behaviour but whether and how acoustic signals (Roth 1948; Belton 1994; Cator et al. 2009), visual signals (Gries et al. 2017), and pheromonal signals (Cabrera & Jaffe 2007; Fawaz et al. 2014; Mozūraitis et al. 2020) interact has not yet been studied. Analogous to host foraging cues, mate location and mate recognition signals may be used in a stepwise manner, with specific signals being effective at specific distances. Visual signals likely mediate long-range attraction (Bidlingmayer & Hem 1980), as may pheromones, whereas acoustic signals are effective only at close range (~25 cm, Wishart & Riordan 1959).

1.4. Mosquito floral foraging preferences

Nectar-foraging is essential for mosquito survival. Without carbohydrate (sugar) resources, mosquito populations eventually collapse even when provided with ample blood resources (Stone et al. 2009). Males feed exclusively on sugar resources such as nectar, whereas females typically feed on both sugar resources and blood. Females of anthropophilic mosquitoes such as *Ae. aegypti* can survive and reproduce exclusively feeding on blood (Edman et al. 1992). Males frequently nectar-feed to obtain sugar as the energy source for flight (Nayar & Handel 1971) and swarming (Grimstad & Defoliart 1974).

Nectar-foraging mosquitoes discriminate between floral plants (Gadawaski & Smith 1992). The relative attractiveness of floral species may be related to their nectar composition which varies with inflorescence age, and weather and soil conditions during inflorescence growth (Beutler 1953). Floral attractiveness may also be affected by the ratio of sugar types present in floral nectar (Grimstad & Defoliart 1974). Floral preferences by mosquitoes are not fixed and change over time in accordance with inflorescences senescing over time (Sandholm & Price 1962), with older or wilting inflorescences being less often visited and fed on by mosquitoes (Grimstad & Defoliart 1974). Preferences for floral plants may differ between species and populations of mosquitoes (Sandholm & Price 1962; Grimstad & Defoliart 1974). Mosquitoes are attracted to specific floral semiochemicals (Vargo & Foster 1982; Jepson & Healy 1988; Jhumur et al. 2008; Otienoburu et al. 2012), some of which are shared with vertebrate

hosts (Peach et al. 2019a). Shared components were attractive to older host-seeking *Ae. aegypti* (Peach et al. 2019a).

1.5. Applied aspects

As mosquitoes continue to develop insecticide resistance and non-target effects of chemical pesticides become widespread, the need for select mosquito control measures has become more urgent (Fiorenzano et al. 2017). These measures often target specific aspects of mosquito ecology, such as floral foraging, mate location, and oviposition.

In sterile insect technique (SIT) programs, mass-reared and irradiation-sterilized males (Benedict & Robinson 2003) must remain competitive with their wild counterparts (Benedict & Robinson 2003; Alphey et al. 2010). Many of these sterile males must mate with wild females to effectively reduce mosquito populations (Zheng et al. 2015). However, if mass-reared and sterilized males are consistently smaller and less able than wild males to locate and mate with wild females (Benedict & Robinson 2003; Andreasen & Curtis 2005), then SIT programs are destined to fail (Reisen et al. 1982). Studying all facets and intricacies of mate location and courtship behaviour of mosquitoes would help determine whether sterilized and wild males will be equally competitive, and the release of sterile males has prospect of curtailing mosquito populations.

Toxic sugar baits (TSBs) address the mosquitoes' quest for carbohydrates. TSBs generally consist of a liquid sugar-based solution laced with an insecticide (Lea 1965). TSBs placed near aquatic habitats of mosquito larvae have the potential to reduce alighting incidences by female mosquitoes on humans (Hossain et al. 2014). The type of sugar source is correlated with its attractiveness to mosquitoes (Müller et al. 2010, 2011). Mosquitoes could be enticed to feed on TSBs, if their sugar composition was more appealing than that of natural sugar resources (Vargo & Foster 1982; Fiorenzano et al. 2017). TSBs applied to foliage significantly reduced the survivorship of *Ae. albopictus*, *Cx. nigripalpus* and *Ochlerotatus taeniorhynchus* in small-scale cage experiments. However, in large-scale cage experiments, TSBs on foliage reduced the survivorship only of *Ae. albopictus* (Xue et al. 2006), due possibly to the sucrose solution being outcompeted by more attractive floral sources present in the same cage (Fiorenzano et al. 2017). Obviously, more attractive TSBs need to be designed. As blends of synthetic floral semiochemicals can be as attractive to mosquitoes as the inflorescences themselves (Vargo & Foster 1982; Otienoburu et al. 2012), it may be

possible not only to develop synthetic floral baits that mimic the scent of specific inflorescences but also to 'engineer' a "superflower" blend that combines the most attractive floral semiochemicals from multiple species of plants. Moreover, as mosquito species exhibit distinct preferences for specific floral semiochemicals (Manda et al. 2007; Nikbakhtzadeh et al. 2014), it follows that a superflower blend containing diverse floral semiochemicals may be appealing to multiple mosquito species.

1.6. Research objectives

Mate location and mate selection behaviour in diurnally-active yellow fever mosquitoes, *Ae. aegypti*, take place in mating swarms but the mechanisms (such as visual, acoustic and pheromonal signals) underlying swarm formation and long-range detection of females by males remain largely unexplored. Objectives of Chapter 2 were to (1) investigate through high-speed video recordings whether visual (wing light flash) signals are produced by swarming *Ae. aegypti* mosquitoes, and (2) bioassay in behavioural experiments whether visual, acoustic and pheromonal signals are (interactive) mate recognition signals at specific spatial scales. Objectives of Chapter 3 were to (1) compare the attraction of *Cx. pipiens* to inflorescences of four *Asclepias* milkweed species (which are reportedly frequented by nectar-foraging mosquitoes), (2) identify the floral semiochemicals of the most attractive species, (3) explore whether a synthetic blend of these semiochemicals attracts mosquitoes, and (4) determine whether the blend attractiveness can be enhanced by addition of floral semiochemicals from unrelated plants that are proven effective mosquito attractants.

1.7. References

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Chapter 2.   Long- and short-range bimodal signals mediate mate location and recognition in yellow fever mosquitoes

*A near identical version of this chapter has been pre-printed in *bioRxiv* with the following authors: Elton Ko*, Adam J. Blake, Chiara Lier, Stephen Takács, and Gerhard Gries. EK and GG discussed and planned the experiments. AJB wrote code for data analyses and graphs, and assisted in the calibration of LEDs, and in statistical analyses. ST assisted in generating acoustic signals and in measuring the sound intensity of earbud speakers. EK and CL ran bioassays, acquired and analyzed data and performed colony maintenance. EK and GG wrote the initial draft of the article, and all authors reviewed and approved of the final draft.

Introduction

Searching for a blood meal, female mosquitoes exploit multiple vertebrate host cues including CO_2, body odor, moisture, as well as visual and heat contrast (Gibson & Torr 1999). To locate a host, female mosquitoes are guided by these chemical and physical cues in sequential and interactive processes (Bidlingmayer 1994; McMeniman et al. 2014; Breugel et al. 2015). Exhaled in the breath of a potential host, CO_2 context-dependently (Gillies 1980) promotes host-seeking (Eiras & Jepson 1994; Healy & Copland 1995), elicits upwind flight toward the CO_2 source (Geier et al. 1999; Dekker & Cardé 2011), and enhances mosquito attraction to warmth (Kröber et al. 2010; Maekawa et al. 2011). In addition to CO_2, breath volatiles and numerous odorants emanating from bacteria on vertebrate skin (Kanda et al. 1990; Knols et al. 1997; Gallagher et al. 2008) guide host-foraging mosquitoes. The relative importance of host cues depends on the spatial scale, with some cues (thermal, skin odors, visual, moisture) being most important at close range (Khan & Maibach 1966; Browne & Bennett 1981; Lacey et al. 2014; Breugel et al. 2015).

Nectar-foraging mosquitoes also exploit multimodal cues to locate floral resources (Peach et al. 2019). Females of the yellow fever mosquito, *Aedes aegypti,* and the Northern house mosquito, *Culex pipiens*, respond more strongly to a cue complex of tansy, *Tanacetum vulgare*, inflorescences, consisting of CO_2, olfactory and visual cues, than to inflorescence odor alone (Peach et al. 2019). During floral foraging, floral odor likely acts as a long-range attractant, whereas visual cues are utilized at closer ranges.

Whereas the multimodal sensory cues that guide foraging mosquitoes to host and nectar resources have been intensely studied, the mechanisms underlying mate location

and recognition in mosquitoes are not fully understood. Many mosquito species form 'mating swarms' dominated by males (Downes 1969) that independently respond to 'swarm marker' objects in the environment (Downes 1969), such as trees, corn stalks, telephone poles (Knab 1906) or just black cards (Charlwood & Jones 1980). In *Ae. aegypti,* the vertebrate host itself serves as the swarm marker (Hartberg 1971). Swarming behaviour may expose mosquitoes to increased predation (Yuval & Bouskila 1993) and is energetically costly (Yuval et al. 1994), but it expedites mate location which is challenging for species with widespread larval habitats (Downes 1969).

In the context of swarming, mosquitoes respond to acoustic, visual and pheromonal signals or cues from conspecifics (Belton 1994; Fawaz et al. 2014; Gries et al. 2017). As shown for several species, swarming males recognize the wingbeat frequency of conspecific females that enter a swarm (Belton 1994; Charlwood & Jones 1979; Gopfert et al. 1999). The sound-receiving Johnston's organ in the males' antennae is attuned to the females' wingbeat frequencies (Belton 1994; Gopfert et al. 1999) which are attractive to males (Gibson et al. 2010). After successful coupling with the female, the male-female pair leaves the swarm to mate (Howell & Knols 2009). In *Ae. aegypti*, courtship precedes coupling and entails harmonic convergence of both male and female wingbeat frequencies (Cator et al. 2009). Analogous behaviour has been reported in the elephant mosquito, *Toxorhynchites brevipalpis* (Gibson & Russell 2006), the southern house mosquito, *Culex quinquefasciatus* (Gibson 1985), and the African mosquito, *Anopheles gambiae* (Pennetier et al. 2010).

Males typically detect the wingbeat sound of females only at close range (Wishart & Riordan 1959), indicating that physical mate location signals other than sound function at a longer range, as recently shown for several dipterans, including mosquitoes (Eichorn et al. 2017; Gries et al. 2017). Males of the common green bottle fly, *Lucilia sericata*, distinguish between the rates of light flashes reflected off the wings of in-flight female and male flies, and are most strongly attracted to flash frequencies (178 Hz) characteristic of young females (Eichorn et al. 2017). Similarly, 8-LED 'mating swarm' mimics of *Ae. aegypti* flashing white or blue light at the wing beat frequency of females (665 Hz) attract conspecific males (Gries et al. 2017). As thin-film reflectors (Sivinski et al. 2004; Eichorn et al. 2017), sun-exposed mosquito wings also reflect UV wavelengths which could be even more attractive than the previously tested white or blue lights (see above). As mosquitoes can sense UV light (Muir et al. 1992) and behaviourally respond

to it when they seek floral nectar (Peach et al. 2019) or oviposition sites (Snow 1971), it is conceivable that UV light reflections play a role in the context of mate recognition.

Volatile or contact sex pheromones have been hypothesized to contribute to mate location and recognition in mosquitoes (Kliewer et al. 1966; Nijhout & Craig 1971; Cabrera & Jaffe 2007) but supportive evidence for such pheromones remains scant (Fawaz et al. 2014). Females of *Ae. aegypti* reportedly produce a 3-component sex pheromone blend comprising 2,6,6-trimethylcyclohex-2-ene-1,4-dione ('ketoisophorone'), 2,2,6-trimethylcyclohexane-1,4-dione (the saturated analogue of ketoisophorone), and 1-(4-ethylphenyl) ethanone ('ethanone') (Fawaz et al. 2014). In laboratory, but not field settings, ketoisophorone alone elicited swarming-like flight by males. Both ketoisophorone and its saturated analogue prompted "excited flights" by females, whereas ethanone attracted females (Fawaz et al. 2014).

All the visual, acoustic or pheromonal mate location or recognition signals of mosquitoes described above were studied focused invariably on a single sensory modality, discounting possible interactions between signals and their potential function at spatially different scales. Furthermore, specifics of light flash signals on mate attraction such as the number of mosquitoes in a mating swarm generating these signals, or the most attractive wavelengths of these signals, have not yet been tested experimentally. Conceivably, large mating swarms with many mosquitoes 'flashing lights' are more attractive than small ones. Conversely, one would predict that mosquitoes swarming at dusk when light flash signals are less conspicuous may not rely on visual signals for mate location or recognition.

Working with diurnal *Ae. aegypti*, we tested six hypotheses (H): (H1, H2) Swarming mosquitoes produce light flashes, and the attractiveness of a mating swarm (i.e., array of light-flashing LEDs) is dependent upon both swarm size (i.e., number of LEDs in array) and the spectral composition of wing flashes (i.e., light emitted by LEDs); (H3) wingbeat light flashes and sound of *Ae. aegypti* females are long- and short-range male attraction signals, respectively; (H4) swarm pheromone of *Ae. aegypti* females increases the attractiveness of their wingbeat light flashes and sound; and (H5) wingbeat light flashes of *Ae. aegypti* males attract mate-seeking females. Working with *C. pipiens* as a model species for nocturnal mosquitoes, we further tested the hypothesis (H6) that dusk-swarming *C. pipiens* do not exploit wingbeat light flashes for mate attraction.

2.1. Methods and Results

H1: Swarming mosquitoes produce light flashes, and the attractiveness of a mating swarm (i.e., array of light-flashing LEDs) is dependent upon swarm size (i.e., number of LEDs in array)

High-speed video recordings of *Ae. aegypti* males swarming in a laboratory setting revealed light flashes reflecting off their wings (Fig. 2.16–2.1.8, 2.21), as also evident by rapid changes of wing-reflected light intensity over time (Fig. 2.1). The flight of *Ae. aegypti* males appears to contain a second harmonic of 1854 Hz (Fig. 2.1, C), which roughly corresponds to the third harmonic (1995 Hz) of the estimated female wingbeat frequency of 665 Hz. This corroborates previous evidence of the "harmonic convergence" phenomenon (Cator et al. 2009; Gibson et al. 2010; Pennetier et al. 2010). The degree of wing-reflected light intensity changes is reduced by the highly reflective abdomen of *Ae. aegypti*. Unlike blowflies (Eichorn et al. 2017), the strength of these flashes did depend on viewing angle. As compared with other insects, the amplitude of the wing movement forward and backward is small (Bomphrey et al. 2017), presenting very little wing surface to reflect light when viewed from the side. While high-speed video recordings of *Ae. aegypti* males swarming in outdoor settings were not obtainable, we documented the 'wing flash phenomenon' with other dipterans swarming in a sunlit courtyard (Fig. 2.7, 2.19–2.20. Ablated wings of *Ae. aegypti* males reflected broadly between 300-700 nm, with greater proportional reflection of wavelengths above 500 nm (Fig. 2.8), likely due to dark brown hairs on the wings (Carpenter & LaCasse 1955).

To determine the effect of *Ae. aegypti* swarm size on swarm attractiveness, we assembled LEDs in an array, released groups of 50 males into mesh cages for each bioassay, and video recorded their alighting responses (as a measure of attraction) on each of two LED arrays (Fig. 2.2B) that differed in the number of LEDs flashing blue light (Fig. 2.14) at 665 Hz (the wing beat frequency of females) (Table 2.1). Mosquito contacts and landings on LEDs, or on a stalk within 2.5 cm of an LED, were recorded as alighting responses. When given a choice between a 1-LED array and an 8-LED array, males alighted more often on the latter (F = 67.529, p < 0.0001; Fig. 2.3, Exp. 1). In contrast, 4- and 8-LED arrays prompted similar numbers of alighting responses by males (F = 0.64, p = 0.64; Fig. 2.3, Exp. 2). However, 16-LED arrays received three times more alighting responses than 8-LED arrays (F = 22.63, p = 0.001; Fig. 2.3, Exp. 3). These data in combination support the hypothesis that swarm size affects its attractiveness to mate-seeking males.

H2: The attractiveness of a mating swarm (i.e., array of light-flashing LEDs) is dependent upon the spectral composition of wing flashes (i.e., light emitted by LEDs)

To determine whether the attractiveness of mating swarms depends upon the wavelength of light reflected off the wings of swarming mosquitoes, we offered groups (n = 10) of 50 males a choice between two 8-LED arrays (Fig. 2.2B) flashing (665 Hz) either blue light (422 nm) or UV light (360 nm) (Table 2.1; Fig. 2.14). Video-recordings revealed that males alighted similarly often on the UV LED array and the blue LED array (n = 10, F = 3.84, p = 0.081; Fig. 2.9, Exp. 4)*,* suggesting that the short-wave spectral content of wing flashes does not modulate the attractiveness of mating swarms.

H3: Wingbeat light flashes and sound of Ae. aegypti females are long- and short-range male attraction signals, respectively

To determine whether wingbeat light flashes of females (665 Hz) are long-range male attraction signals, we ran an experiment of identical design in both small and large spatial settings (mesh cage, room; Fig. 2.2A,B,D,E), offering groups (n = 10) of 50 males a choice between two 8-LED arrays separated by 15 cm (mesh cage) or 164 cm (room) (Table 2.1). The LEDs of array 1 flashed blue light at 665 Hz, whereas the LEDs of array 2 emitted constant blue light. Each LED in both arrays was coupled with an earbud speaker (Fig. 2.2G) broadcasting female wingbeat sound (665 Hz). In the cage setting, where males are already near mating swarms (i.e., LED arrays) and can hear the wing beat sound, the type of visual stimulus (flashing or constant light) had no effect on alighting responses by males (n = 10, F = 0.86, p = 0.86; Fig. 2.4, Exp. 5). Conversely, in the room setting, where males still needed to locate mating swarms (i.e., LED arrays), LED arrays flashing blue light prompted 5.8-times more alighting responses by males than LED arrays emitting constant blue light (n = 10, F = 30.43, p = 0.001; Fig. 2.4, Exp. 6). The data of both experiments combined support the hypothesis that wingbeat light flashes of females attract males at long-range.

   To confirm that wingbeat sound of females (665 Hz) is a short-range male attraction signal (see above), we offered groups (n = 10) of 50 males in the mesh cage setting a choice between two 8-LED arrays fitted with earbud speakers that broadcasted either the females' wingbeat sound (array 1) or white noise (control stimulus; array 2) (Table 2.1). The LEDs of both arrays flashed blue light (665 Hz). In this cage setting, where males are already near mating swarms (i.e., LED arrays) and can distinguish between

arrays with or without wingbeat sound, arrays with wingbeat sound prompted 5-times more alighting responses by males (n = 10; F = 19.87, p = 0.001; Fig. 2.4; Exp. 7).

To ascertain that the white noise had no repellent effect on the males' responses in experiment 7, speakers of array 2 were kept silent in follow-up experiment 8 which otherwise was identical (Table 2.1). Similar to data obtained in experiment 7, arrays with wingbeat sound prompted 4.9-times more alighting responses by males (n = 10, F = 39.97, p = 0.0001; Fig. 2.4, Exp. 8). The data of both experiments combined support the hypothesis that the wingbeat sound of females attracts males at close range.

To further investigate whether males can indeed distinguish between the wing beat sounds of females and males and are attracted only to the sound of females, we offered groups (n = 10) of 50 males a choice between two 8-LED arrays flashing blue light at 715 Hz (the wing flash frequency of males), with earbud speakers of array 1 emitting male wingbeat sound (715 Hz) and speakers of array 2 broadcasting white noise (Table 2.1). Fewer alighting responses by males on arrays coupled with male wing beat sound (n = 10, F = 5.49, p = 0.043; Fig. 2.5, Exp. 9) indicate that males are put off by their own wingbeat sound, obviously distinguishing it from that of females.

H4: Swarm pheromone of Ae. aegypti females increases the attractiveness of their wingbeat light flashes and sound

To test whether the swarm pheromone component ketoisophorone increases the attractiveness of the females' wingbeat light flash and sound signals, we released groups (n = 9) of 50 males into a room and offered them a choice between two well-spaced 8-LED arrays each fitted with 8 earbud speakers (Fig. 2.2G, Table 2.1). All 16 LEDs flashed blue light (665 Hz) and all earbud speakers broadcasted corresponding wingbeat sound (665 Hz). The randomly assigned treatment array was baited with ketoisophorone. Video recording revealed similar numbers of alightings by males on arrays with or without pheromone (n = 9, F = 0.076, p = 0.79; Fig. 2.10, Exp. 10), indicating no effect of female pheromone on mate-seeking males.

H5: Wing beat light flashes of Ae. aegypti males are attractive to mate-seeking females

To determine whether wing beat light flashes of *Ae. aegypti* males (715 Hz) attract mate-seeking females, we ran a small-space (cage) experiment, offering groups (n = 13) of 50 females a choice between two 8-LED arrays separated by 15 cm (Table 2.1). All LEDs of

array 1 emitted constant white light (Fig. S8), whereas all LEDs of array 2 flashed white light at 715 Hz. Video-recordings revealed that females alighted more often on arrays with flashing lights than on arrays with constant light (n = 13, F = 4.94, p = 0.046, Fig. 2.6, Exp. 11).

H6: Dusk-swarming C. pipiens do not exploit wingbeat light flashes for mate attraction

To determine whether dusk-swarming *C. pipiens* use wingbeat light flashes as mate attraction signals, we ran three experiments (Exps. 12-14) in the mesh cage setting, one of which (Exp. 14) under dim light (1 lux) (Table 2.1). In each experiment, we offered groups of 50 2- to 7-day-old *C. pipiens* males a choice between two 8-LED arrays separated by 15 cm. All LEDs of array 1 emitted constant white light (Fig. 2.14), whereas all LEDs of array 2 flashed white light at either 350 Hz (Exp. 12, n = 10) or 550 Hz (Exp. 13, n = 9; Exp. 14, n = 10), two previously reported wingbeat frequencies of female *C. pipiens* (Belton & Costello 1979; Gibson 1985)*.* In all three experiments, very few males alighted on arrays (Fig. 2.11), revealing no effect of light cues on male attraction, and not warranting statistical analyses of data.

2.2. Discussion

The wing light flash-guided mate location and recognition system of *Ae. aegypti* takes place in a swarm context but otherwise resembles that of other dipterans. This remarkable mate recognition system hinges upon the immense processing speed of dipteran photoreceptors (Burkhardt 1977; Miall 1978) and was only recently discovered in the common green bottle fly, *L. serricata* (Eichorn et al. 2017)*.* Ever since, the same type of system has been shown to occur in other dipterans, including house flies, *Musca domestica*, black soldier flies, *Hermetia illucens,* and *Ae. aegypti* (Gries et al. 2017).

     The system in green bottle flies depends upon both the frequencies of light flashes caused by moving wings being sex- and age-specific, and the ability of male bottle flies to recognize the light flash frequency of young female flies that are prospective mates (Eichorn et al. 2017). A single LED flashing white light at the wingbeat frequency of young females (178 Hz) is sufficient to attract and prompt alighting responses by males (Eichorn et al. 2017). In *Ae. aegypti*, however, mate location typically takes place in a swarm context (Hartberg 1971; McClelland 1959), and a single light-flashing LED is not attractive to males or females (Gries et al., unpublished). To present a 'mating swarm'

and to test its attractiveness to males, we built assemblies of 8 LEDs (Fig. 2.2) and offered groups of males a choice between two assemblies that emitted either constant light or light flashing at one of eight frequencies (430, 480, 500, 545, 665, 800, 950 Hz) (Gries et al. 2017). In these experiments, males invariably alighted more often on flashing-light LEDs than on constant-light LEDs (Fig. 2.12; adapted from Gries et al. 2017), suggesting that mate-seeking males may respond to flashing lights of swarming males to locate swarms. However, the effect of wingflash light signals on the responses of males in this previous study (Gries et al. 2017) was tested in the absence of wingbeat sound and in a relatively small space. To reveal the effects of light and sound signals [which are perceived at long and short (< 25 cm) range, respectively] at different spatial levels, we ran experiments in both a large setting (2.25 × 2.1 × 2.4 m high) and a small setting (61 × 61 × 61 cm). Our selection of the female (rather than the male) wingbeat light flash and sound frequency (665 Hz each) as test stimuli for the response of males was guided by four considerations: (1) even though females do not form mating swarms on their own, multiple females may concurrently be present in a mating swarm sought after by males. For example, in *Anopheles stephensi mysorensis*, as many as 23% of swarm mates were found to be females (Quraishi 1965); (2) males ought to be able to recognize females approaching a swarm, or flying well apart within a swarm, at a distance greater than the hearing range for wingbeat sound (15-25 cm) (Wishart & Riordan 1959); (3) light flash frequencies covering the range produced by females (665 Hz) and males (715 Hz) were both highly and almost equally attractive to males (Fig. 2.12); and (4) mate location in *Ae. aegypti* may also occur in a context other than mating swarms (Roth 1948; Nijhout & Craig 1971).

Our data show that flashing lights (665 Hz) are long-range signals that attract males to mating swarms or to mates (Fig. 2.4). In a large-space setting, LED assemblies flashing light at 665 Hz and emitting wingbeat sound (665 Hz) prompted 5.8-times more alighting responses than LED assemblies emitting constant light and wingbeat sound (665 Hz) (Fig. 2.4, Exp. 6). Conversely, in a small space setting, when wingbeat sounds were present, flashing lights had no apparent signal characteristics. Each of two LED assemblies producing either flashing or constant light induced similar numbers of alightings by males (Fig. 2.4, Exp. 5).

Our data (Fig. 2.4, Exps. 7, 8) also confirm that the wingbeat sound of females is a close-range signal to mate-seeking males. (Wishart & Riordan 1959; Hoy 2006; Cator et al. 2011). When offered a choice between two LED assemblies, both flashing light (665

Hz) but only one emitting female wingbeat sound, males alighted 5.0- and 4.9-times more often on assemblies emitting female wingbeat sound than on assemblies that emitted white noise or were silent (Fig. 2.4, Exps. 7, 8). Conversely, the wingbeat sound of males (715 Hz) was off-putting to mate-seeking males (Fig. 2.5, Exp. 9), corroborating previous conclusions that males distinguish between wingbeat sounds of females and males (Belton 1994; Johnson & Ritchie 2015).

The attractiveness of light flash mate location signals – tested in small-space bioassays in the absence of sound signals – is modulated not only by the flash frequency (Fig. 2.12) but also by the number of signals (i.e., mosquitoes in mating swarms, or LEDs in assembly) and the wavelengths of flashing lights. Increasing the number of LEDs in assemblies increased the number of mosquitoes alighting on assemblies (Fig. 2.3, Exps. 1-3), suggesting that larger mating swarms, or swarms containing a higher percentage of females, are more attractive to mate-seeking males. LED assemblies emitting UV light were as attractive to males as blue-light LED assemblies (Fig. 2.9, Exp. 4) which were more attractive than white-light LED (Fig. 2.13) assemblies. Whether equivalent physical characteristics of visual mate location signals affect the behaviour of females is not yet known. However, our findings that females, on average, alighted more often on LED assemblies flashing light at the male wingbeat frequency (715 Hz) than on LED assemblies emitting constant light (Fig. 2.6, Exp. 11), suggest that females may recognize a mating swarm, in part, based on the flashing lights 'produced' by swarming males.

With convincing data showing that visual and acoustic signals contribute to long- and short-range mate location in *Ae. aegypti* (Fig. 2.4), there was ample incentive to also test the effect of a chemical signal, the female-produced pheromone (Fawaz et al. 2014), on responses of males. We predicted that female pheromone presented in combination with light and sound signals would modulate the behaviour of males. However, the synthetic pheromone component ketoisophorone added to the bimodal complex of visual sound signals failed to express any additive or synergistic effect on the responses of males (Fig. 2.10, Exp. 10). It is conceivable, though, that the still-air setting of this experiment, with pheromone dissemination being entirely reliant on diffusion without forming a discrete pheromone plume, was not conducive for male attraction. Alternatively, in the absence of air current, pheromone may have built up in the room, ultimately disorienting males rather than guiding them to the pheromone source.

The sensory drive theory predicts functional links between signal design and presentation such that the conspicuousness of signals is maximized relative to environmental conditions and background noise (Endler 1992). Previous reports in the literature and our data on *Ae. aegypti* and *C. pipiens* are in complete agreement with these predictions. The onset of the photophase induces swarm formation by male *Ae. aegypti* in both laboratory and field settings (Hartberg 1971; Cabrera & Jaffe 2007) but swarm formation has also been observed during an afternoon peak flight activity (McClelland 1959). With incident light reflecting off the wings of swarming males, their swarm becomes a visual beacon for other males and females in search for mates. As more mosquitoes enter the swarm, the "firework" of light flashes becomes larger and more attractive (Fig. 2.3; Fig. 2.16–2.21). The conspicuousness of the swarm display is further enhanced 3- to 4-times when putting the light flash LED assembly on an oscillating shaker table (Gries et al. 2017), mimicking a swarm gently swaying in the wind. In contrast, visual mate location systems hinging on incident sunlight reflecting off the wings of in-flight dipterans, as shown for bottle flies, house flies and black soldier flies (Eichorn et al. 2017; Gries et al. 2017), as well as yellow fever mosquitoes (Gries et al. 2017; also shown in this study), would not be expected to evolve in crepuscular mosquito species such as *C. pipiens* that swarm at dusk when sunlight is absent and illumination is dominated by diffuse light from the horizon (Können 1985). As predicted, LED assemblies flashing light at the reported wingbeat frequencies of *C. pipiens* (350 Hz, Belton & Costello 1979; 550 Hz, Gibson 1985) had no signal characteristics for bioassay mosquitoes and prompted hardly any behavioural responses (Fig. 2.11; Exps. 12–14).

In conclusion, we describe that mate location or recognition in *Ae. aegypti* is mediated, in part, by long-range wingbeat light flash signals and by short-range wingbeat sound signals. The attractiveness of the light flash signals is dependent upon both the number of light flashes (i.e., mosquitoes in the swarm) and the wavelengths of the flashing light (i.e., light reflected off wings). As both male and female *Ae. aegypti* respond to light flash signals, these signals apparently contribute to the processes of forming and locating mating swarms. Moreover, with males and females having significantly different wingbeat frequencies (Cator et al. 2009; Brogdon 1994), and thus light flash frequencies, the flash frequency could also facilitate long-range recognition of prospective mates. Our data address knowledge gaps as to how male and female *Ae. aegypti,* and possibly the sexes of other (diurnal) mosquitoes, find each other (Howell &

Knols 2009). Elucidating the mate location and courtship biology of mosquitoes will inform quality assessments of males that are mass-reared and released in sterile insect release tactics. Successful integration of these tactics into mosquito vector control programs (Alphey et al. 2010; Lees et al. 2015; Yakob & walker 2016) hinges on sterile and transgenic males effectively competing with wild males for access to females.

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2.4. Method Details

2.4.1. Rearing of experimental insects

Aedes aegypti mosquitoes were reared in the insectary of the Burnaby campus of Simon Fraser University (SFU) at 23–28 °C, 40-60% RH, and a photoperiod of 14L:10D. Adult mosquitoes were kept in mesh cages (30 × 30 × 46 cm high) provisioned with a 10-% sucrose solution *ad libitum* and allowed to blood-feed on the arm of GG or Regine Gries once a week. Three days after blood-feeding, gravid females were offered an oviposition site consisting of a 354-mL water-filled paper cup (Solo Cup Company, Lake Forest, IL, USA) lined with a paper towel (Kruger Inc., Montreal, QC, CA). For storage, egg-lined towels were inserted into Ziploc bags (S.C. Johnson & Son, Inc., Racine, WI, USA) kept at 23–28 °C. To initiate a new generation of mosquitoes, towels were transferred to a glass dish (10 cm diam × 5 cm high), containing water enriched with brewer's yeast (U.S. Biological Life Sciences, Salem, MA, USA). After egg hatching, 1st instar larvae were transferred to water-filled trays (45 × 25 × 7 cm high) and provisioned with NutraFin Basix tropical fish food (Rolf C Hagen Inc., Montreal, QC, CA). Using a 7-mL plastic pipette (VWR International, Radnor, PA, USA), pupae were transferred to water-filled Solo Cups covered with a mesh lid and fitted with a sucrose solution-soaked cotton ball to sustain adult mosquitoes eclosing over the course of 72 h. These mosquitoes were then released into mesh cages (30 × 30 × 46 cm high) and separated by sex for use in bioassays when they were 2–7 days old (males) or 5–10 days old (females).

The rearing protocol for *C. pipiens* resembled that for *Ae. aegypti* except that *(i)* rooms were kept at 23–26 °C, (*ii*) gravid females were offered a glass dish (10 cm diam × 5 cm high) as oviposition site, and (*iii*) egg rafts – rather than egg-lined towels – were transferred to water-filled trays for larval development. Only 2- to 7-day-old males were tested in bioassays.

2.4.2. High-speed videography

Swarming of *Ae. aegypti* males and of taxonomically unidentified dipterans was video-recorded using a Photron FASTCAM NOVA S16 high-speed camera (Photron USA Inc., San Diego, CA 92126, USA) fitted with a Nikon NIKKOR AF-S Micro lens (105 mm, f/2.8 AF) for close-up shots, or a Nikon NIKKOR Telephoto Zoom lens (AF 35–80 mm, f/4-5.6D) for wide angle shots (both lenses: Nikon Canada Inc., Mississauga, ON, CA). For

further magnification, a clip-on macro filter (DCR-250 Super MacroScan Conversion lens, Yoshida Industry Co., Ltd, Tokyo, JP) was attached to the 105-mm micro lens. The high-speed camera was connected via an ethernet cable to an ASUS laptop computer (ASUS Canada, Markham, ON, CA) running Photron FASTCAM Viewer 4 (PFV4) (Photron USA, Inc., San Diego, CA 92126, USA). Videos were recorded at 3,000, 5,000 or 10,000 frames per second (fps), with shutter speeds of 1/6,000, 1/10,000 or 1/30,000 s. Videos were downloaded from the camera as mRAW files, and converted with PFV4 to MP4 files.

To film swarming behaviour of *Ae. aegypti* males, 100 males were released into a mesh cage (12 × 12 × 12 cm) (BioQuip Products Inc., Rancho Dominguez, CA 90220, USA), and exposed to host cues (CO_2 released from dry ice; EK's hand or forearm) and mate recognition signals (female wingbeat sound [665 Hz] played back from a smartphone (Fig. 2.21)). The cage was illuminated from above with an LED light source (AOS Offboard LED light, AOS Technologies AG, Baden-Daettwil, CH) mounted on a friction arm (Vitec Imaging Solutions Spa, Cassola, IT). One mesh wall of the cage was replaced with plastic wrap to facilitate filming.

Dipterans swarming in a sunlit courtyard on the Burnaby campus of SFU were video recorded at 16:00 on 01 September from an open, second-story window overlooking the courtyard. Because the dipterans were swarming too high above ground and too far away from the window, voucher specimens could not be obtained for identification and deposition in a museum collection.

To quantify the light intensity of the insects across their flight paths, frames of the video were imported into FIJI as 12-bit Tiff images in a manner that preserved sensor linearity (Schindelin et al. 2012). We used the plugin TrackMate to follow the position of the insect over their flight path with a circular region of interest with a diameter encompassing both the wingspan and body length of the insect (Tinevez et al. 2017). The mean pixel value was recorded from within this circular region of interest for each frame of the flight path. These intensity data were then imported into R 3.6.2 (R Core Team 2020) to calculate fast Fourier transformation periodograms.

2.4.3. Spectroscopy of wing reflections

Wings of *Ae. aeygpti* males were ablated from live insects and mounted on an insect pin. These wings were illuminated by a xenon light source (HPX-2000, Ocean Optics,

Dunedin, FL, USA) via a fibre optic cable, and their reflections were sampled with a cosine corrector connected to a spectrophotometer (HR-4000, Ocean Optics) fitted with a fibre cable. To capture both the minimal and the maximal possible reflection, wings were positioned either edge on with respect to the cosine corrector, or at an angle where their specular reflection was directed at the center of the cosine corrector. For comparison with the wing reflections, the spectra of the xenon light source were also measured using a square of aluminum foil positioned in a similar way to the specularly reflecting mosquito wing.

2.4.4. LEDs

Spectra of white LEDs (5218268F, Dialight, London, UK), blue LEDs (TLHB5800, Vishay Intertechnology, Malvern, PA, USA) and UV LEDs (EOLD-355-525, OSA Opto Light GmbH, Berlin, DE) (Fig. S8) were recorded with a spectrophotometer (HR-4000, Ocean Optics) and SpectraSuite software (Ocean Optics). The photon flux of each LED was sampled at a distance of 5 cm from the cosine corrector connected to the sampling fibre of the spectrometer. This allowed use to vary the amperage supplied to the LEDs in order to achieve an intensity of $2e^{15}$ photons/cm^2/s. Using a lathe, the lens of each LED was flattened to widen the angle of emitted light. The frequency (Hz) and the duty cycle (set to 3%) of each LED were verified using an oscilloscope (Gould 20Ms/sec Digital Recording Oscilloscope, Gould Electronics GmbH, Eichstetten am Kaiserstuhl, DE).

2.4.5. Design of LED arrays

LED arrays consisted of up to 16 LEDs arranged in a three-dimensional circular shape (~15 cm diam) (Fig. 2.2B). Each LED was mounted upward-facing 18–23 cm above ground on a separate, rigid stalk which was attached to a ring stand, the base of which was covered with Cheesecloth (Cheesecloth Wipes, VWR International, PA, USA) to minimize visibility (Fig. 2.2B). Each LED was connected to one channel of a 16-channel pulse generator (5-Volt, 2-Amp) designed and built by the Science Technical Centre at SFU to allow independent control of test variables for each LED, including duty cycle, frequency (Hz), amperage and periodicity.

2.4.6. General design of small-space behavioural experiments

We ran behavioural bioassays with mosquitoes in a mesh cage (61 × 61 × 61 cm) (BioQuip Products, Inc., CA, USA) (Fig. 2.2A,B), with the cage bottom and the front and side walls covered with cheesecloth to minimize stray light entry and light reflectance. A lamp fitted with an LED bulb (Feit Electric, Pico Rivera, CA, USA; Fig. 2.15) was placed above the rear edge of the cage to provide illumination during bioassays. For each 20-min bioassay, we placed two LED arrays (see above) 15 cm apart from each other in the centre of the cage (alternating their position between replicates) and released 50 2- to 7-day-old sexually mature males (Exps. 1–10, 12–14), or 50 5- to 10-day-old sexually mature females (Exp. 11), into the cage. To video-record alighting responses of mosquitos on LEDs, we placed an AKASO EK7000 action camera (AKASO, Frederick, MD, USA) on top of the cage (Fig. 2.2C). Mosquito contacts and landings on LEDs, or on a stalk within 2.5 cm of an LED, were recorded as responses. During bioassays, rooms were maintained at a temperature of 23–28 °C and 40-60% relative humidity. After each bioassay, the camera was stopped and the cage was opened to release the mosquitoes which were then euthanized with an electric fly swatter (Guangzhou Sidianjin Trading Co., Guangzhou, CN). To optimize the responsiveness of *Ae. aegypti* females and males in all bioassays (see also below), we tested them only on sunny or overcast (but not rainy) days and only during the light phase of their photoperiod (14L:10D).

2.4.7. General design of large-space behavioural experiments

In a cubicle (2.25 × 2.1 × 2.4 m high; Fig. 2D) of the insectary illuminated by ceiling fluorescent lighting (F32T8/SPX50/ECO, General Electric, Boston, MA, USA; Fig. 2.15), two LED arrays were placed on a counter 164 cm apart from each other, and 43 cm and 30 cm, respectively, away from the back and side walls of the cubicle (Fig. 2.2E). For each bioassay, 50 2- to 7-day-old sexually mature males were released into the cubicle through the cubicle door. Their alighting responses on LEDs were video recorded with an AKASO action camera placed in a metal sieve (shielding the camera's electromagnetic field) (Fig. 2.2F) mounted on a ring stand 42 cm above each LED array. During bioassays, rooms were kept at 23-28 °C and 40-60% relative humidity. After 20 min of recordings, the cameras were turned off, all mosquitoes were euthanized with an

electric fly swatter, and the position of LED array 1 and 2 was reversed for the next replicate.

2.4.8. Wingbeat sound cues

To determine the effect of mosquito wing beat sound on LED-alighting responses of bioassay mosquitoes, we used Audacity 2.3.2 (Audacity Team 2019) to prepare eight sound files (see Supplementary Material) with paired channels, one of which was randomly assigned to the treatment stimulus and the other to the control stimulus. Treatment stimuli consisted of wingbeat sound characteristic of *Ae. aegypti* females (665 Hz) or males (715 Hz), whereas control stimuli consisted of white noise (sound that covers the entire range of audible frequencies). Audio tracks of wing beat frequencies or white noise were played in parallel, Doppler-shifting upwards, holding steady, or Doppler-shifting downwards to silence, each of these three phases lasting 7 s. The intensity level of the wing beat sound and the white noise control stimulus were each adjusted to 10 dBL above background (SPL = 45 dBL), measured 2.5 cm away from each sound-emitting earbud speaker (RPHJE120K, Panasonic, Osaka Prefecture, JP), using a 1551-C sound level meter fitted with a Type 1560-PB microphone (General Radio Company, Concord, MA, USA). Earbud-emitted sound was not audible to human hearing at 50 cm away from the source. Each headphone pair played back either an artificial tone (665 Hz, 715 Hz), white noise, or was kept silent, depending on the array (treatment or control) and the experiment. Sound files were played using MPV media player (mpv n.d.).

To reduce the directionality of sound stimuli, we removed the rubber tip from each earbud. On both arrays, each of eight LEDs was paired with a single upward-facing earbud which was attached with a twist tie to the LED-carrying stalk 2 cm below the LED (Fig. 2.2G). Earbud wires on the cage floor were covered with cheesecloth and routed out of the cage through a mesh sleeve. Each pair of earbuds (one earbud being assigned to the treatment array and the other to the control array) was plugged into a separate USB sound card (C-Media HS-100B Chipset, TROND, Shenzhen, CN) which, in turn, was plugged into a 4-port USB hub (Qicent, Shenzhen, CN) (Fig. 2.2H). Connecting only two soundcards to each of four USB hubs helped avoid latency of playback recordings. The USB hubs were plugged into a Raspberry Pi 3 B+ computer

(Cana Kit Corporation, North Vancouver, BC, CA), running Raspbian 10 (Raspberry Pi Foundation, Cambridge, UK).

2.4.9. Specific experiments

H1: The attractiveness of a mating swarm (i.e., array of light-flashing LEDs) is dependent upon swarm size (i.e., number of LEDs in array)

To determine the effect of LED numbers in array (i.e., 'mosquito swarm size') on array attractiveness (Exps. 1–3; n = 10 each; Table 2.1), we presented groups of 50 *Ae. aegypti* males each with a choice of two LED arrays that differed in number of LEDs. Specifically, we tested arrays with eight *vs* one LED (Exp. 1), eight *vs* four LEDs (Exp. 2), and eight *vs* 16 LEDs (Exp. 3). Each LED in each array flashed blue light at 665 Hz.

H2: The attractiveness of a mating swarm (i.e., array of light-flashing LEDs) is dependent upon the spectral composition of wing flashes (i.e., light emitted by LEDs)

The effect of LED wavelength (UV or blue) on LED-alighting responses by *Ae. aegypti* males was tested by offering groups of 50 males each a choice between two 8-LED arrays flashing either UV or blue light at 665 Hz (the light flash frequency of flying females) (Exp. 4, n = 10; Table 2.1). The amperage supplied to LEDs was modulated to an equal photon flux from the blue and UV LEDs.

H3: Wingbeat light flashes and sound of Ae. aegypti females are long- and short-range male attraction signals, respectively

To test whether wingbeat light flashes (665 Hz) of *Ae. aegypti* females are long-range male attraction signals, we ran a two-choice experiment in both a small setting (61 × 61 × 61 cm; Exp. 5, n = 10; Fig. 2.2A,B) and a large setting (225 × 210 × 240 cm high; Exp. 6, n = 10; Fig. 2.2D,E; Table 2.1). In each experiment, we offered groups of 50 males each a choice between two 8-LED arrays which were separated by 15 cm (Exp. 5) or 164 cm (Exp. 6). In both experiments, the LEDs of array 1 emitted blue light flashes of 665 Hz, whereas the LEDs of array 2 emitted constant blue light. Each LED in both arrays was coupled with an earbud speaker emitting the females' wingbeat sound (665 Hz).

To test whether wingbeat sounds (665 Hz) of *Ae. aegypti* females are short-range mate recognition signals for males, we ran a small setting experiment, offering males a

choice between two 8-LED arrays separated by 15 cm. The LEDs of both arrays emitted blue light flashes at 665 Hz. The earbud speakers of array 1 emitted female wingbeat sound (665 Hz), whereas speakers of array 2 emitted white noise (Table 2.1; Exp. 7, n = 10). To determine whether white noise may have had a repellent effect on the males' responses in Experiment 7, speakers of array 2 were kept silent in follow-up experiment 8 (n = 10) which otherwise was identical (Table 2.1). To further investigate whether mate recognition cues of males deter males, we offered groups of 50 males each a choice between two 8-LED arrays emitting blue light flashes at 715 Hz (the wing flash frequency of males), with earbud speakers of array 1 emitting male wing beat sound (715 Hz) and speakers of array 2 broadcasting white noise (Table 2.1; Exp. 9, n = 10).

H4: Swarm pheromone of Ae. aegypti females increases the attractiveness of their wingbeat light flashes and sound

To test whether the swarm pheromone component ketoisophorone increases the attractiveness of the females' wingbeat light flashes and sound, we ran a large setting (room) experiment (Fig. 2.2D,E; Table 2.1; Exp. 10, n = 9), offering groups of 50 males each a choice between two 8-LED arrays separated by 164 cm. All LEDs and earbud speakers of both arrays emitted blue light flashes (665 Hz) and the corresponding wingbeat sound (665 Hz). The bases of both arrays were fitted with a filter paper-lined watch glass which was treated with either ketoisophorone (300 µg) in pentane-ether (30 µl) (array 1) or a pentane-ether control (30 µl) (Fig. 2I). The solvent was allowed to evaporate completely prior to the onset of each bioassay.

H5: Wing beat light flashes of Ae. aegypti males are attractive to mate-seeking females

To determine whether wing beat light flashes of *Ae. aegypti* males are attractive to mate-seeking females, we ran a small-setting (cage) experiment, offering groups of 50 females each a choice between two 8-LED arrays separated by 15 cm and deprived of all earbud speakers. All LEDs of array 1 emitted constant white light, whereas all LEDs of array 2 emitted white light flashes (715 Hz) (Table 2.1; Exp. 11, n = 13).

H6: Dusk-swarming C. pipiens do not exploit wingbeat light flashes for mate attraction

To determine whether dusk-swarming *C. pipiens* use wingbeat light flashes as mate recognition cues, we ran three small-setting experiments, offering groups of 50 2- to 7-

day-old males a choice between two 8-LED arrays separated by 15 cm and deprived of all earbud speakers. All LEDs of array 1 emitted constant white light, whereas all LEDs of array 2 emitted white light flashes at either 350 Hz (Exp. 12, n = 10) or 550 Hz (Exps. 13, 14, n = 10 each; Table 2.1), two previously reported wingbeat frequencies of female *C. pipiens* (Belton 1979; Gibson 1985). Experiments 12 and 13 followed the 'general design of small-space behavioural experiments' (see above).

Taking into account that *C. pipiens* forms mating swarms at dusk, the room lights in follow-up experiment 14 were turned off and the bioassay cage was illuminated from behind by an LED bulb (Feit Electric, Pico Rivera, CA, USA; Fig. 2.15) set by a dimmer (TBL03, Leviton Manufacturing Company, Inc., Melville, NY, USA) to a light intensity level of 1 Lux at the cage centre (Gibson 1985). Likewise, the photon flux of LEDs in arrays 1 and 2 emitting constant light and flashing light, respectively, was reduced to $6.67e^{12}$ photons/cm^2/s in accordance with the low light level in the room. To facilitate recordings of alighting responses by mosquitoes on LEDs, we used a hunting camera (Campark Trail Camera, Campark Electronics Co., Ltd, HK) with an IR-sensitive wavelength range which mosquitoes cannot perceive.

2.4.10. Statistical analyses

We used R 3.6.2 (R Core Team 2020) to analyze behavioural data. Mean proportions of contact and alighting responses by mosquitoes were analyzed with logistic regression using generalized linear models. In order to determine whether proportions differed between arrays, we compared an intercept only model to a null model with a likelihood ratio test. We then used back-transformed coefficients from those models to obtain mean and standard errors for the proportion of mosquitos responding to each array.

Table 2.1. Details of signals [wingbeat light flash, wingbeat sound, pheromone (Phero)] tested in small-space (SS: 61 × 61 × 61 cm) and large space (LS: 2.25 × 2.1 × 2.4 m) behavioural bioassays (see Fig. 2 for experimental design) with *Aedes aegypti* (Exps. 1-11) and *Culex pipiens* (Exps. 12-14)

	Stimulus 1			Stimulus 2		
Exp. # Range	Light flash	Sound	Phero	Light flash	Sound	Phero
H1: The attractiveness of an Ae. aegypti mating swarm (array of light-flashing LEDs) depends upon the number of mosquitoes in the swarm (number of LEDs in the array)						
1 SS	1 LED (blue; 665 Hz)	Silent	No	8 LED (blue; 665 Hz)	Silent	No
2 SS	4 LEDs (blue; 665 Hz)	Silent	No	8 LEDs (blue; 665 Hz)	Silent	No
3 SS	16 LEDs (blue; 665 Hz)	Silent	No	8 LEDs (blue; 665 Hz)	Silent	No
H2: The attractiveness of an Ae. aegypti mating swarm (i.e., array of light-flashing LEDs) is dependent upon the spectral composition of wing flashes (i.e., light emitted by LEDs)						
4 SS	8 LEDs (UV; 665 Hz)	Silent	No	8 LEDs (blue; 665 Hz)	Silent	No
H3: Wing beat light flashes and sound of Ae. aegypti females are long- and short-range male attraction signals, respectively						
5 SS	8 LEDs (blue; 665 Hz)	Wingbeat sound (female: 665 Hz)	No	8 LEDs (blue; constant)	Wingbeat sound (female: 665 Hz)	No
6 LS	8 LEDs (blue; 665 Hz)	Wingbeat sound (female: 665 Hz)	No	8 LEDs (blue; constant)	Wingbeat sound (female: 665 Hz)	No
7	8 LEDs	Wingbeat sound	No	8 LEDs	White noise	No

SS	(blue; 665 Hz)	(female: 665 Hz)		(blue; 665 Hz)		
8	8 LEDs	Wingbeat sound	No	8 LEDs	Silent	No
SS	(blue; 665 Hz)	(female: 665 Hz)		(blue; 665 Hz)		
9	8 LEDs	Wingbeat sound	No	8 LEDs	White noise	No
SS	(blue; 715 Hz)	(male: 715 Hz)		(blue; 715 Hz)		

H4: Swarm pheromone of Ae. aegypti females increases the attractiveness of their wingbeat light flashes and sound

10	8 LEDs	Wingbeat sound	Yes[a]	8 LEDs	Wingbeat sound	No[b]
LS	(blue; 665 Hz)	(female: 665 Hz)		(blue; 665 Hz)	(female: 665 Hz)	

H5: Wing beat light flashes of Ae. aegypti males are attractive to mate-seeking females

11	8 LEDs	No	No	8 LEDs	No	No
SS	(blue; constant)			(blue; 715 Hz)		

H6: Dusk-swarming C. pipiens do not use wingbeat light flashes for mate recognition

12	8 LEDs	No	No	8 LEDs	No	No
SS	(white; 350 Hz)			(white; constant)		
13	8 LEDs	No	No	8 LEDs	No	No
SS	(white; 550 Hz)			(white; constant)		
14[c]	8 LEDs	No	No	8 LEDs	No	No
SS	(white; 550 Hz)			(white; constant)		

[a]ketoisophorone (300 µg) in pentane-ether (30 µl) applied onto filter paper; [b]pentane-ether (30 µl) applied onto filter paper. [c]Exp. 14 was run at low room lighting (1 lux) and with dimmed LEDs ($6.67e^{12}$ photons/cm^2/s).

A

B

Intensity (pixel value)

flash

Michelson contrast

$$\frac{\text{flash} - \text{background}}{\text{flash} + \text{background}} = 0.06$$

background

Time (ms)

C

932

1854

Spectral power
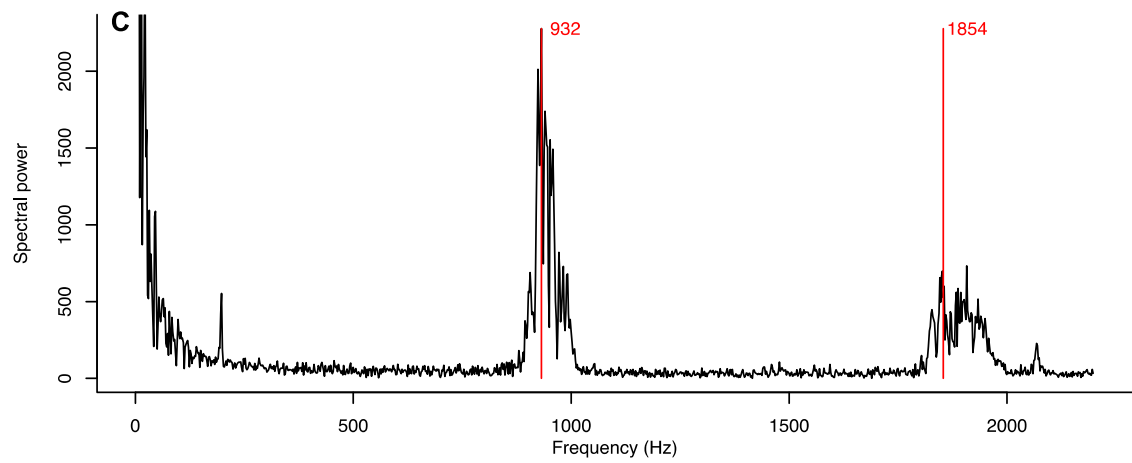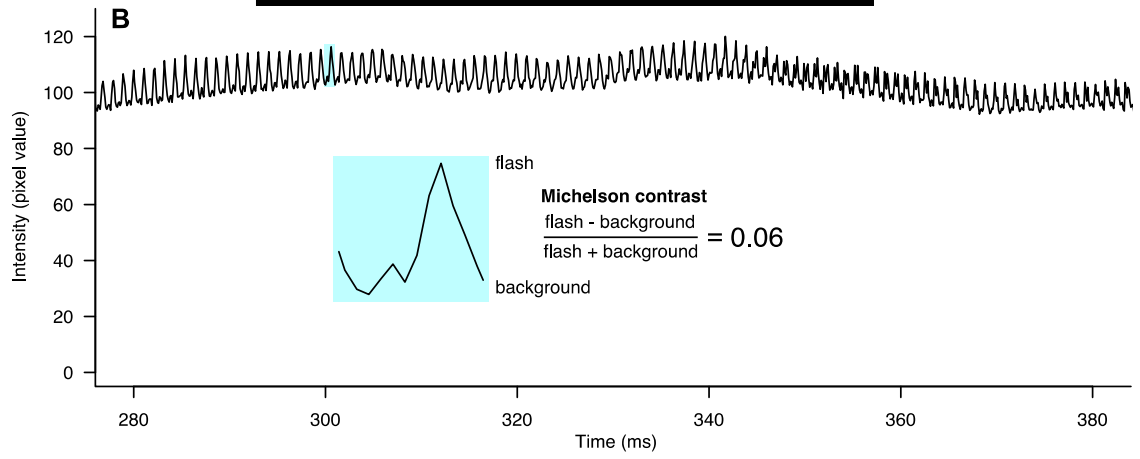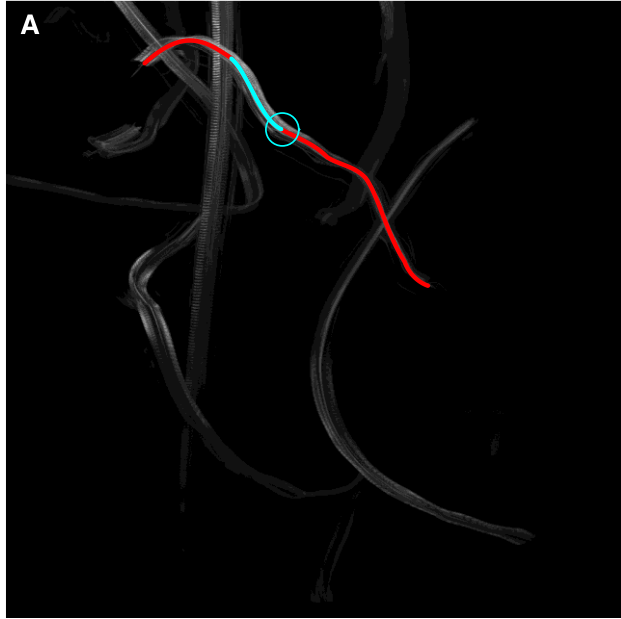
Frequency (Hz)

Figure 2.1. Contrast and frequency analyses of the wing flash series produced by a single male *Aedes aegypti* in a laboratory swarm recorded in Figure 2.16.

(A) Z-projection showing the maximum intensity in each pixel over all frames that captured the flight path of the male responding to a 665 Hz tone. The blue track shows the section of the flight path analysed in B, and the red track shows the entire flight path analysed in C. The blue circle indicates the start of the flight path shown in B, and delineates the area (tracking the flying insect) used to characterize the intensity in each frame of this section.
(B) Intensity trace showing the mean pixel value within the blue circle across each frame of this section. The two blue insets show a single flash along with a calculated Michelson contrast.
(C) Fast Fourier transform periodogram showing the relative spectral power of the wing beat frequency and its 2nd harmonic labeled in red.
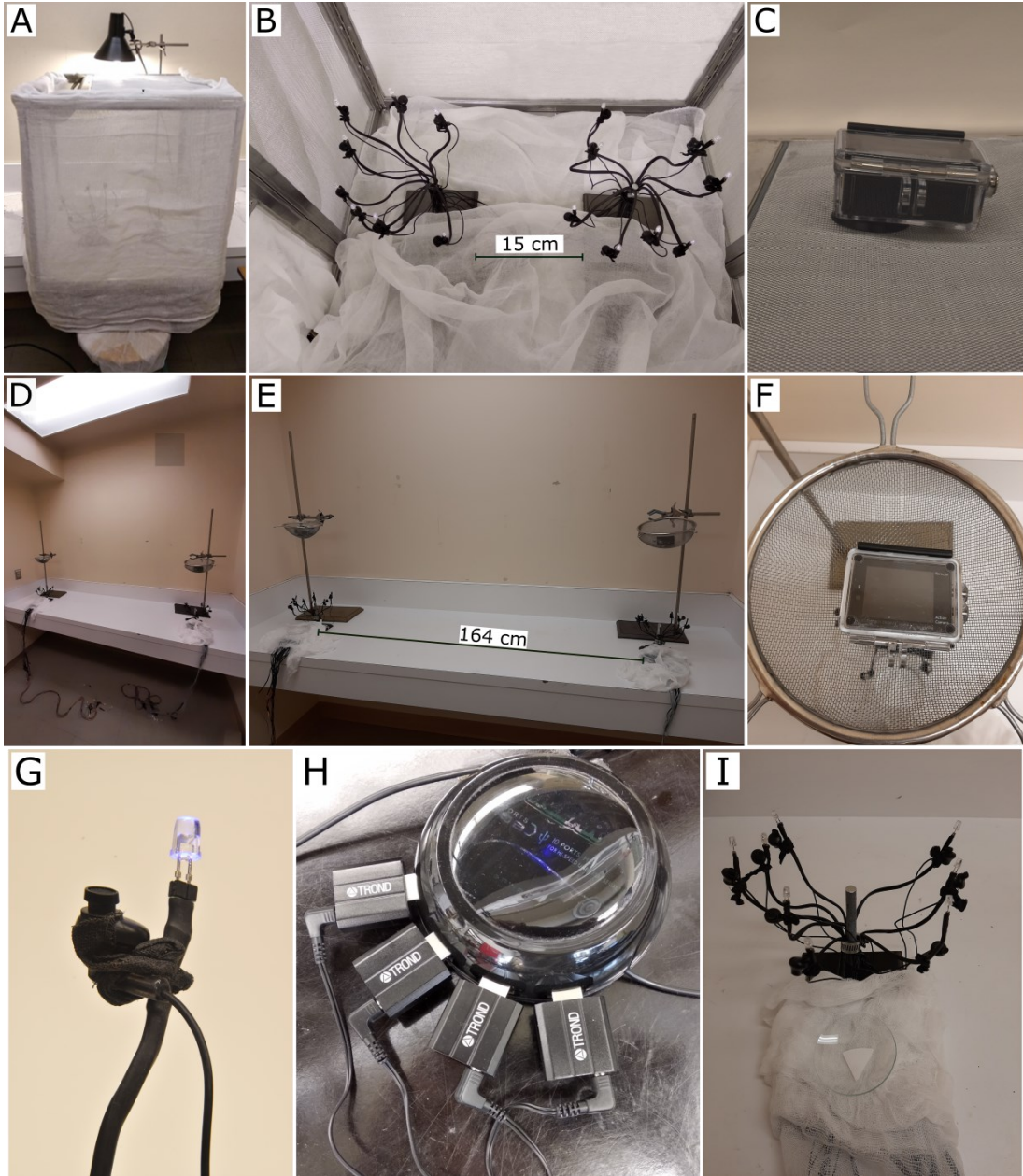
Figure 2.2. Photographs illustrating the experimental design for testing mosquitoes in behavioural bioassays

(A-C) External and internal views of the small-space bioassay arena (wire mesh cage: 61 × 61 × 61 cm), depicting two assemblies of eight light emitting diodes (LED) each (B), and a video camera on top of the cage (C) for recording alighting responses of mosquitoes on LED assemblies.

(D-F) Views of the large-space bioassay room (225 × 210 × 240 cm), with a video camera inside a metal sieve (F) positioned above each of two widely-spaced LED assemblies. The sieve blocked potential electromagnetic waves emanating from the camera. Light was provided via two fluorescent bulbs in the ceiling fixture (for spectral composition see Fig. 2.15).

(G-I) Details of the experimental design showing a paired LED/earbud speaker mounted on a single arm of the 8-LED assembly (G), the USB hub with USB sound cards driving earbud speakers (H), and a glass dish containing a piece of pheromone- or solvent-treated filter paper (I) deployed in a pheromone experiment.
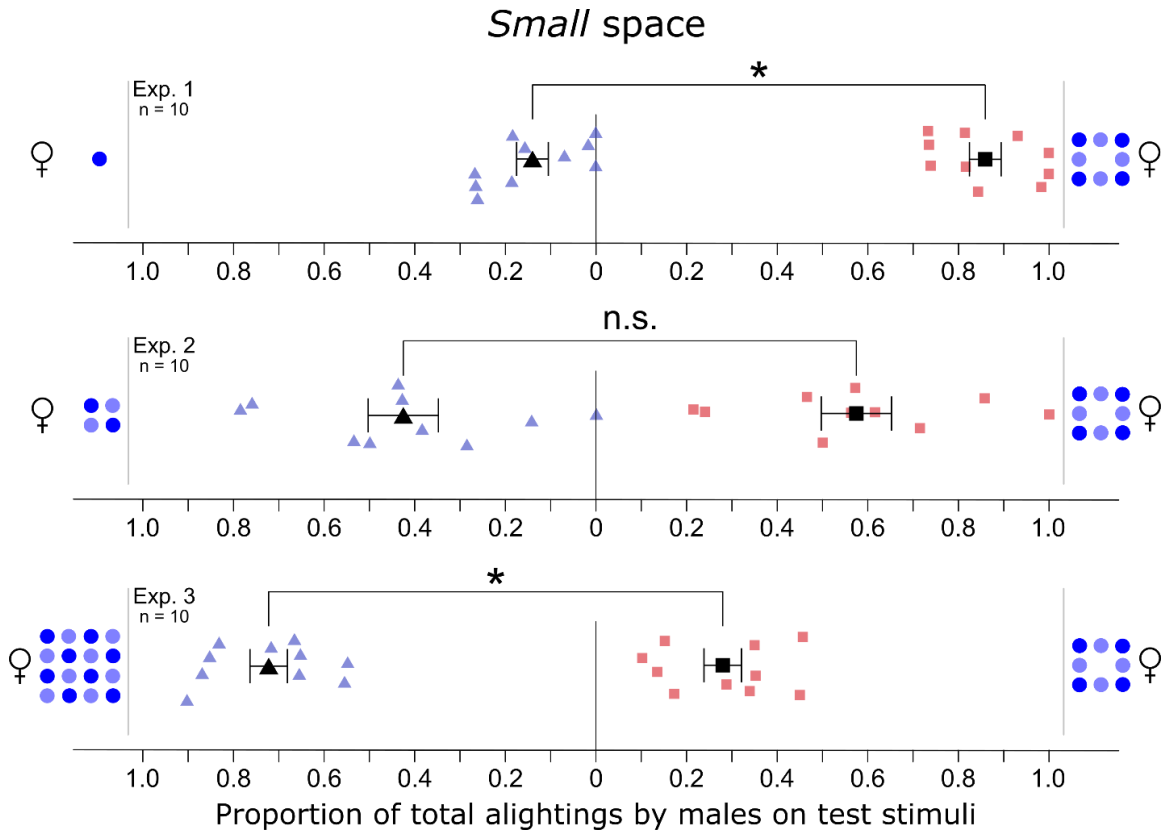
Figure 2.3. Effect of LED numbers in assemblies on alighting responses of 2- to 7-day-old male *Aedes aegypti*

Numbers of blue dots represent the number of LEDs contained within each of two LED arrays (Fig. 2B) flashing blue light at the 665-Hz wingbeat frequency of female *Ae. aegypti*. Each replicate was run with 50 males. Light blue triangles and light red squares show the data of individual replicates and black symbols the mean (± SE). An asterisk indicates a significant preference (binary logistic regression model; $p < 0.05$; n. s. = not significant).

Small space

Large space

Proportion of total alightings by males on test stimuli

Small space

Small space

Proportion of total alightings by males on test stimuli
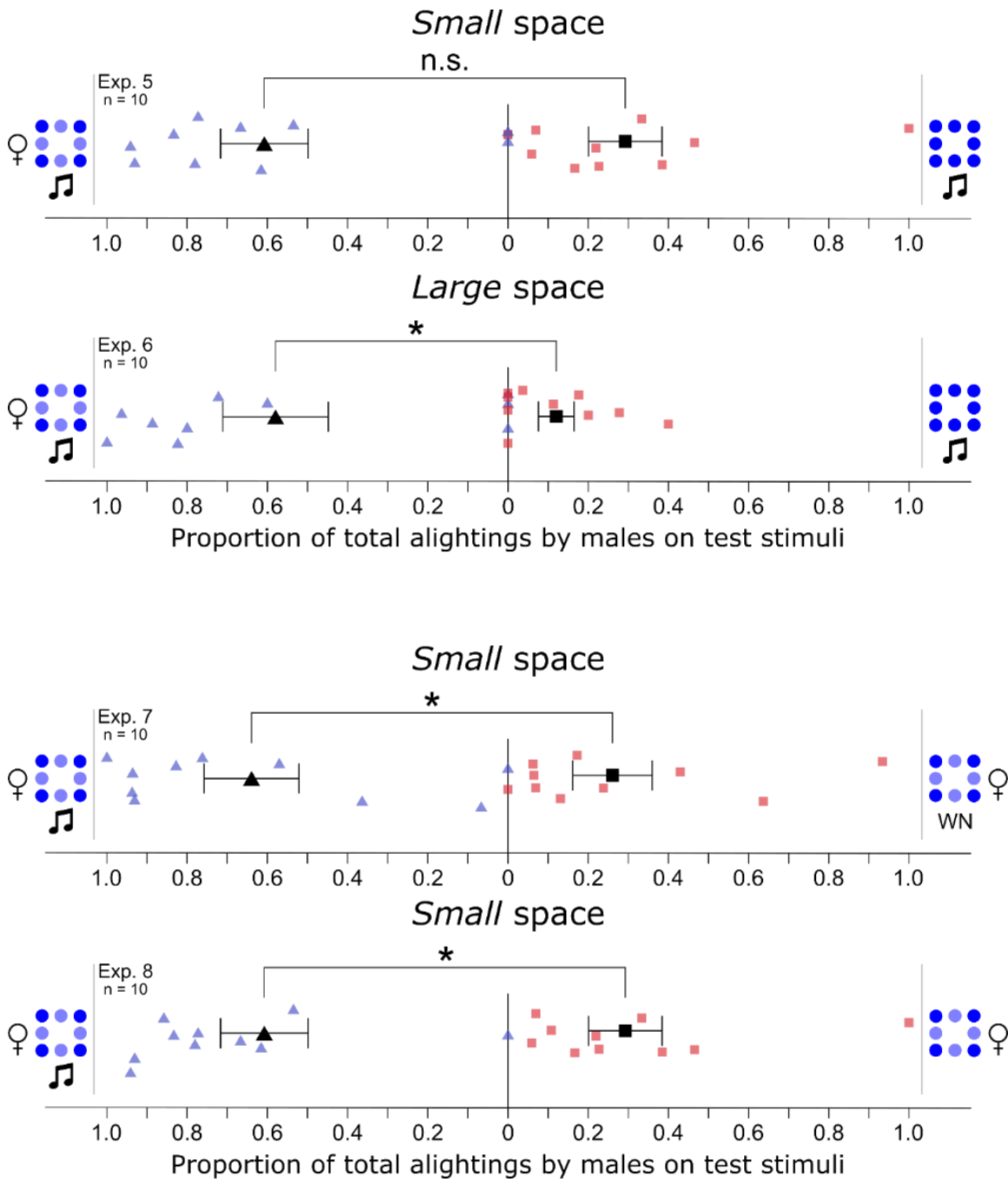
Figure 2.4. Space-dependent effects of visual and acoustic signals tested in combination on alighting responses of 2- to 7-day-old male *Aedes aegypti*

The number of blue dots represents the number of blue LEDs contained within each of two LED arrays (Fig. 2.2B), one of which was emitting light flashes (depicted as a mixture of light- and dark-blue dots) at the 665-Hz wingbeat frequency of female *Ae. aegypti,* and the other array was emitting constant light (depicted as uniformly dark-blue dots). Musical notes and WN (white noise) indicate concurrent broadcast of female wingbeat sound (665 Hz) and white noise, respectively. Light blue triangles and light red squares show the data of individual replicates and black symbols the mean (± SE). Experiments were conducted in a mesh cage [*Small* space (Fig. 2.2A,B); Exps. 5, 7, 8] or within a bioassay room [*Large* space (Fig. 2.2D,E); Exp. 6]. For each experiment, an asterisk indicates a significant preference (binary logistic regression model; $p < 0.05$; n. s. = not significant).
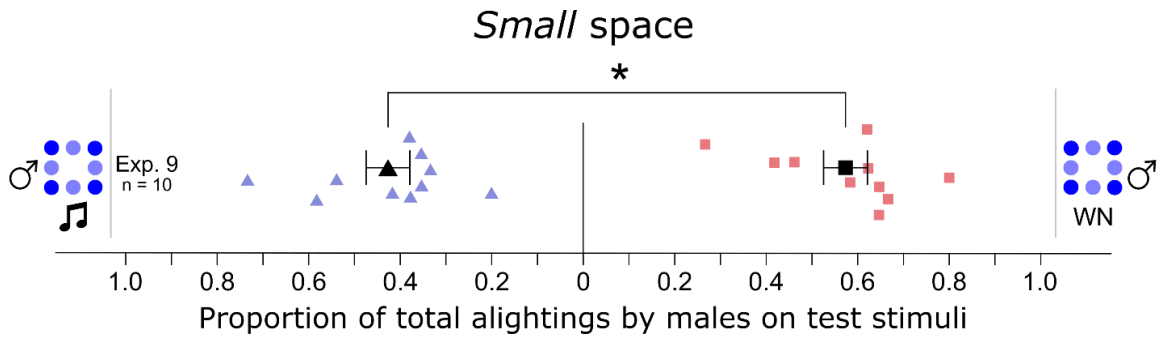
Figure 2.5. Effect of visual and acoustic signals tested in combination on the alighting responses of 2- to 7-day-old male *Aedes aegypti*

The number of blue dots represents the number of blue LEDs contained within each of two LED arrays (Fig. 2.2), with LEDs flashing light at the 715-Hz wingbeat frequency of males. The musical note and WN (white noise) indicate concurrent broadcast of male wingbeat sound (715 Hz) and white noise, respectively. Light blue triangles and light red squares show the data of individual replicates and black symbols the mean (± SE). The asterisk indicates a significant preference for WN (binary logistic regression model; p < 0.05).

67
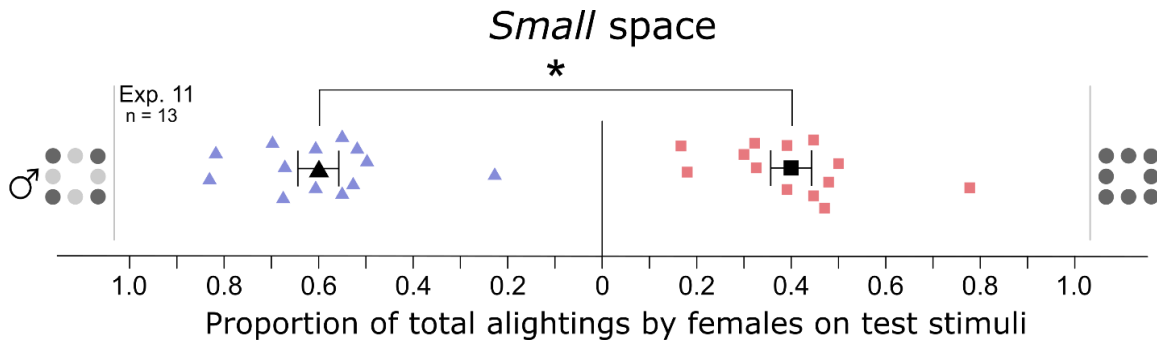
Figure 2.6. Effect of visual signals on alighting responses of 5- to 10-day-old virgin female *Aedes aegypti*

The numbers of grey dots represent the number of white LEDs contained within each of two LED arrays (Fig. 2.2), one of which was emitting light flashes (depicted as a mixture of light- and dark-grey dots) at the 715-Hz wingbeat frequency of male *Ae. aegypti,* and the other LED array was emitting constant light (depicted as uniformly dark-grey dots). The asterisk indicates a significant preference for the 715-Hz LEDs (binary logistic regression model; $p < 0.05$).

A

B

flash

Michelson contrast

$$\frac{\text{flash - background}}{\text{flash + background}} = 0.20$$

background

C

232
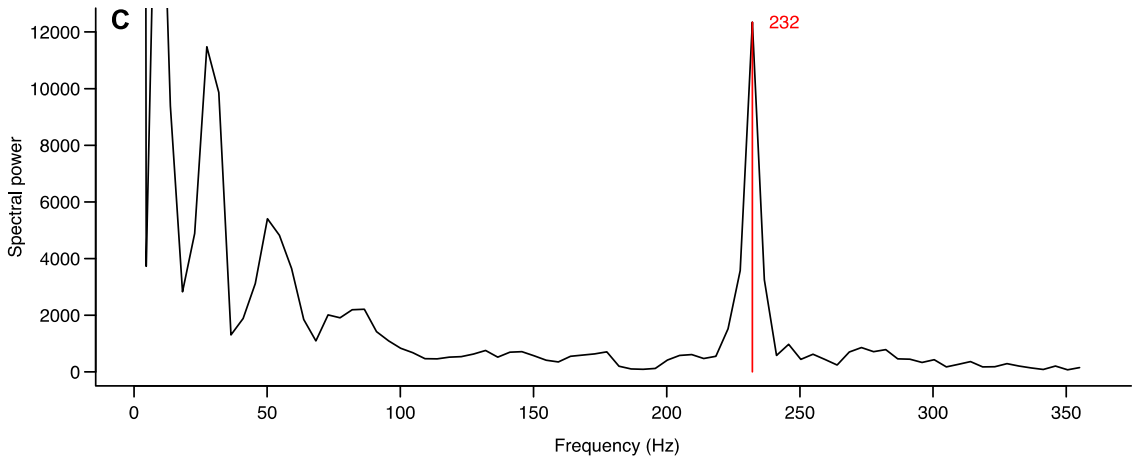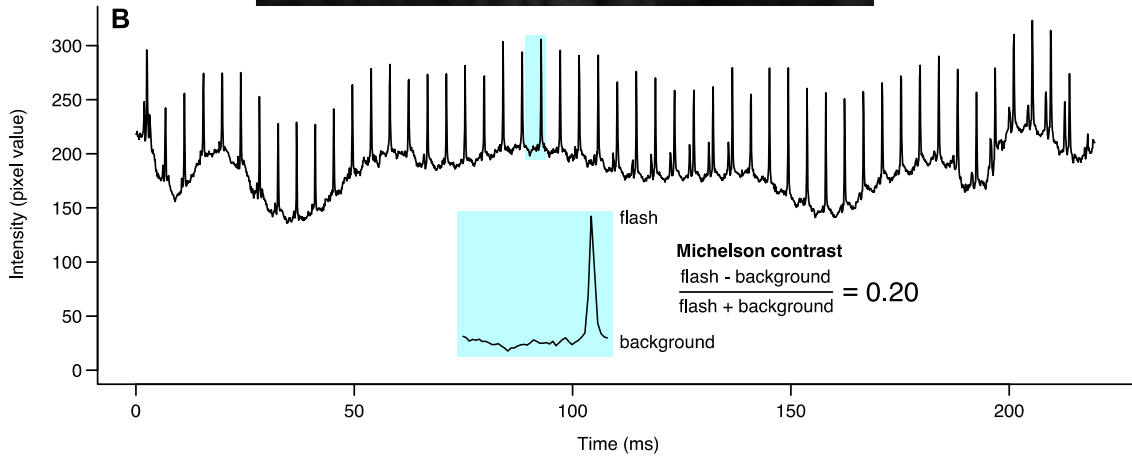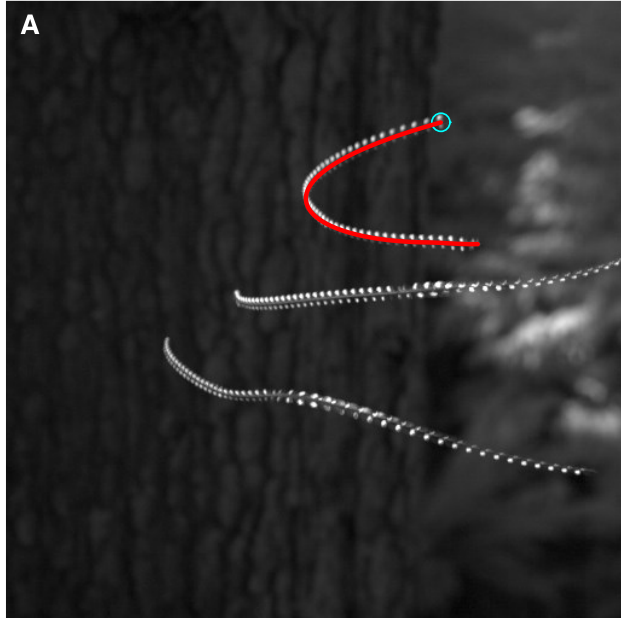
Figure 2.7. Contrast and frequency analysis of the wing flash series produced by a single insect in an outdoor swarm of midges recorded in Figure 2.19. (A) Z-projection showing the maximum intensity in each pixel over all frames that captured the insect's flight path. The blue circle indicates the start of the flight path and delineates the area (tracking the flying insect) used to characterize the pixel intensity in each frame. The red track shows the flight path that was analysed in B and C. (B) Intensity trace showing the mean pixel value within the blue circle across each frame. The two blue insets show a single flash along with a calculated Michelson contrast. (C) Fast Fourier transform periodogram showing the relative spectral power, with the wing beat frequency labeled in red. Note: the dipterans were swarming out of reach, preventing capture and identification.
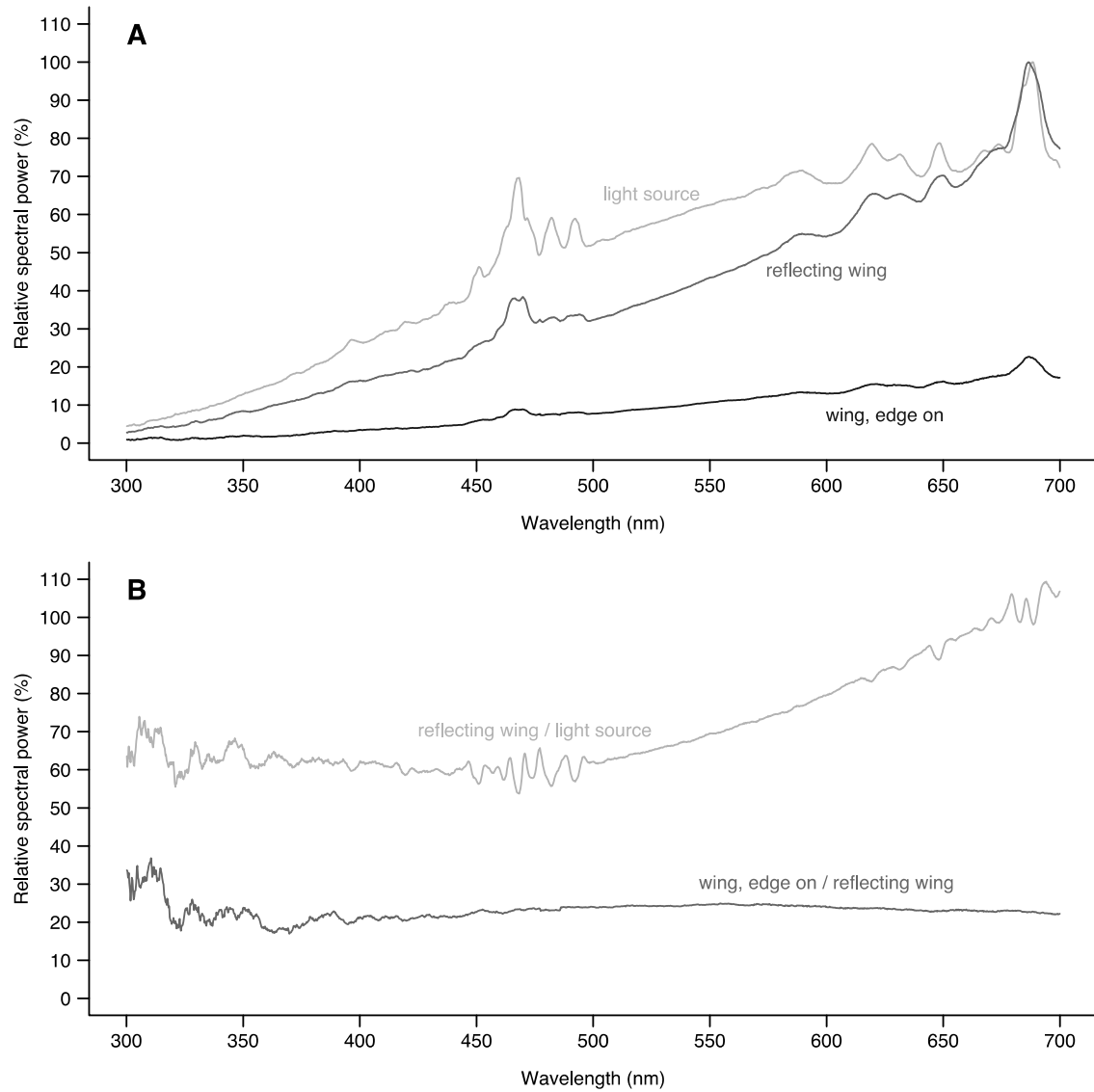
Figure 2.8. Reflections from isolated single wings of *Aedes aeygpti* males. (A) The relative spectral power of the xenon light source (as measured by the reflection of an aluminum foil square), the specularly reflecting male wing, and the same wing measured edge on. The light source and reflecting wing are normalized to their peak, whereas the edge on wing is normalized to the peak of the reflecting wing. (B) The spectral power of the reflecting wing relative to the light source, and the spectral power of the edge on wing relative to the reflecting wing.
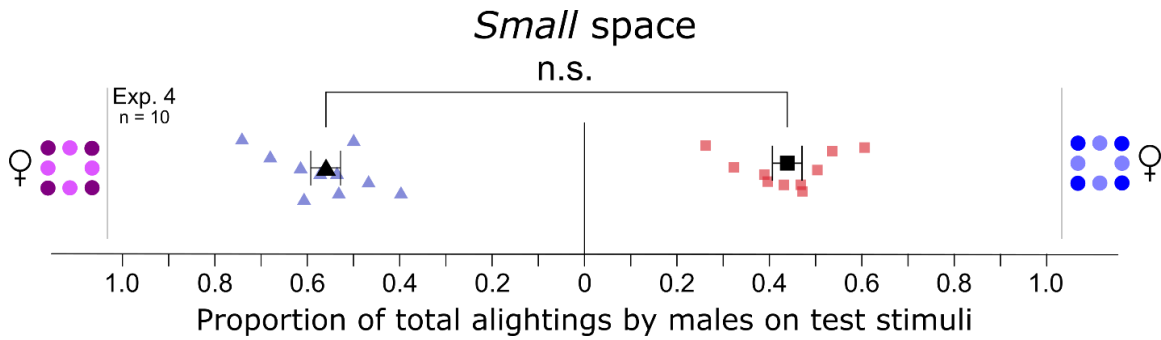
Figure 2.9. Effect of wavelength on alighting responses of 2- to 7-day old male *Aedes aegypti.* The eight purple and eight blue dots represent the number of LEDs contained within each of two LED arrays (Fig. 2.2B), one of which was flashing UV light and the other blue light at the 665 Hz wingbeat frequency of female *Ae. aegypti*. Each replicate was run with 50 males. Light blue triangles and light red squares show the data of individual replicates and black symbols the mean (± SE). There was no preference for either set of test stimuli (binary logistic regression model; p > 0.05; n. s. = not significant).
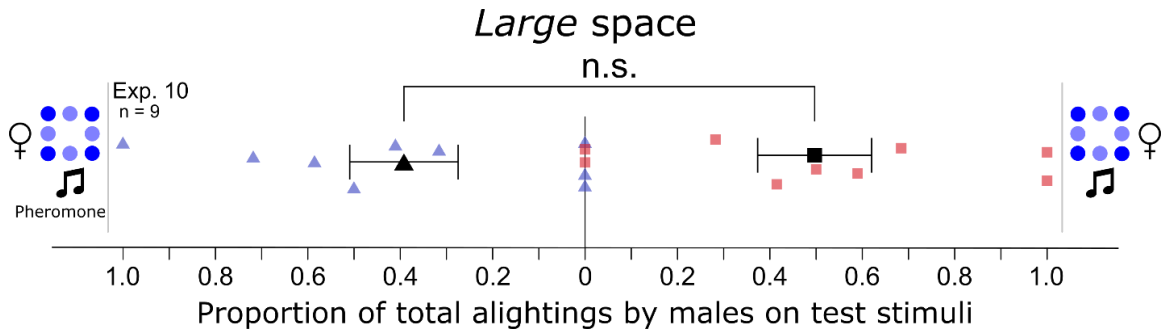
Figure 2.10. Effect of ketoisophorone on the alighting responses of 2- to 7- day old male *Aedes aegypti.* The number of blue dots represents the number of blue LEDs contained within each of two LED arrays (Fig. 2.2B), with LEDs flashing light at the 665 Hz wingbeat frequency of female *Ae. aegypti*. Musical notes indicate broadcast of female wingbeat sound (665 Hz) and 'Pheromone' indicates the presence of synthetic ketoisophorone (Fig. 2.2I), a female produced pheromone component. Light blue triangles and light red squares show the data of individual replicates and black symbols the mean (± SE). There was no preference for either set of test stimuli (binary logistic regression model; p > 0.05; n. s. = not significant).
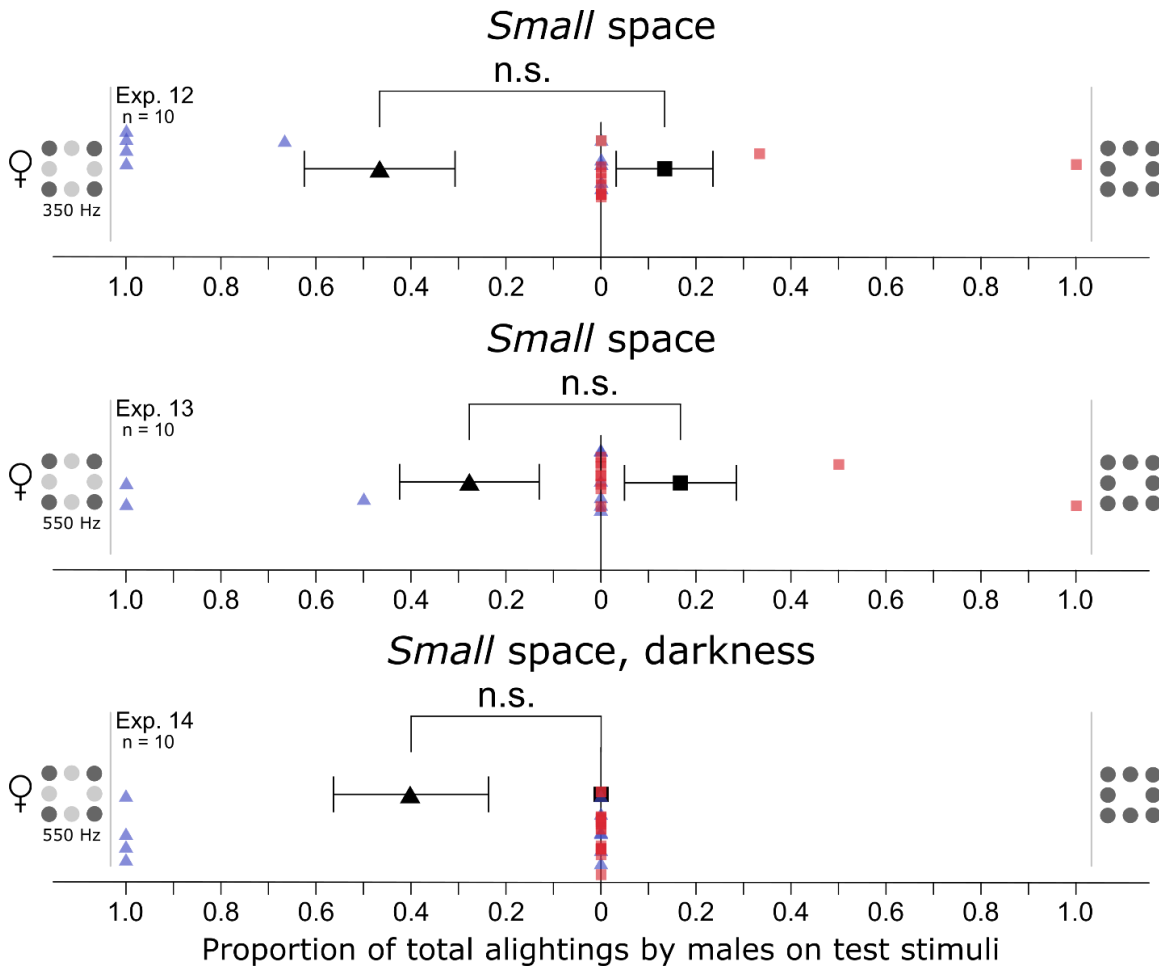
Figure 2.11. Effect of visual signals on alighting responses of 2- to 7-day-old male *Culex pipiens.* The numbers of grey dots represent the number of white LEDs contained within each of two LED arrays (Fig. 2.2B) that emitted either light flashes (depicted as a mixture of light- and dark-grey dots) at the reported wingbeat frequencies of *Cx. pipiens* females (350 Hz, 550 Hz) or that emitted constant light (depicted as uniformly dark-grey dots). Experiments 12 and 13 were run under standard illumination (see methods for detail), whereas experiment 14 was run at low light intensity (1 lux) with dimmed LEDs ($6.67e^{12}$ photons/cm^2/s). The few alighting responses (<10 total responders per replicate) recorded in these experiments did not warrant statistical analyses of data.
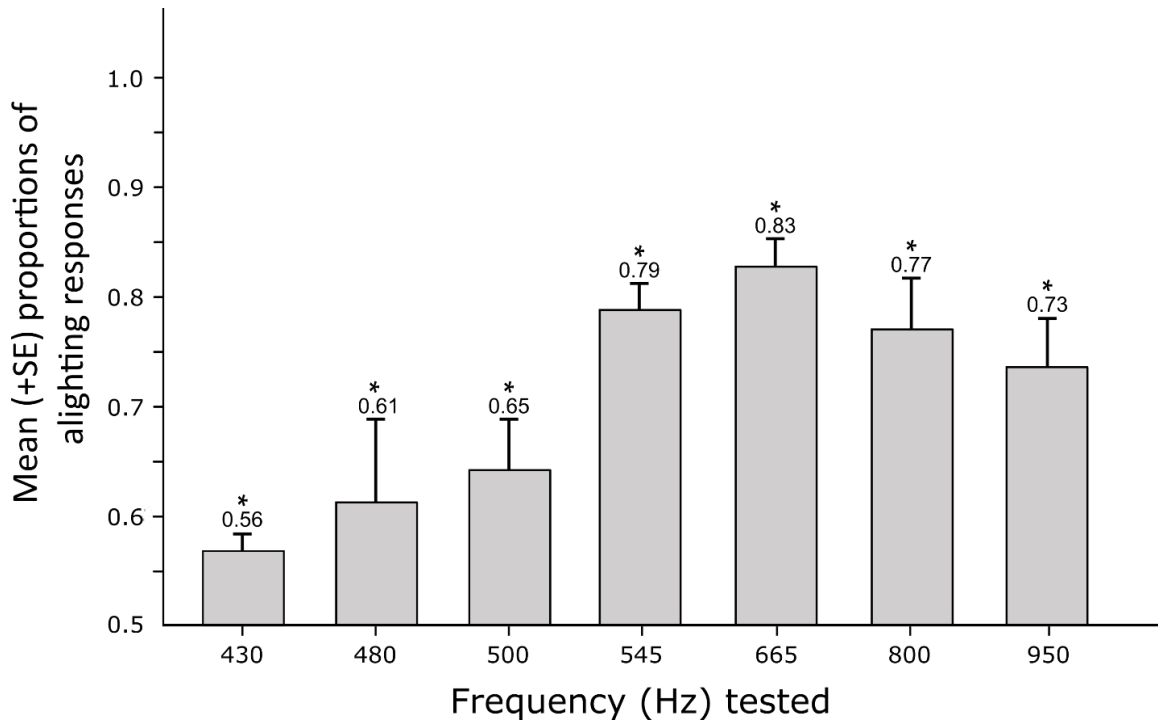
Figure 2.12. Preferential alighting by *Aedes aegypti* males on LED arrays flashing light at various frequencies. For each frequency tested, males were given a choice between two 8-LED arrays (Fig. 2.2B), one of which flashing white light at 430, 480, 500, 545, 665, 800 or 959 Hz, the other emitting constant white light. Proportional alighting responses are shown only for the flashing-light LED arrays. For each experiment, the asterisk indicates a significant preference for the flashing-light LED array over the constant-light LED array (*t*-test; $p < 0.05$). Figure adapted from Gries et al. (2017).
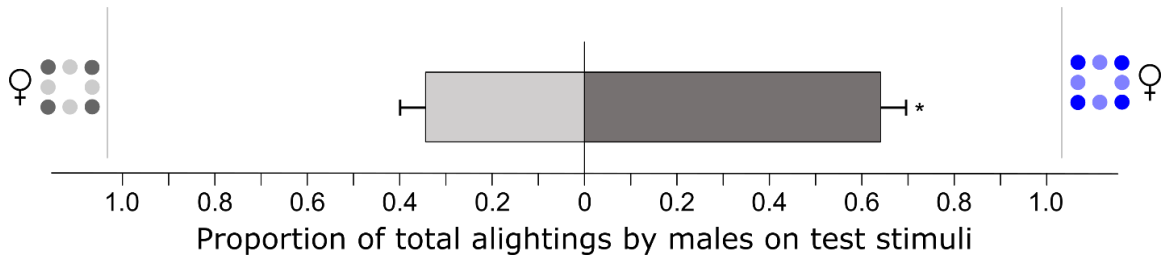
Figure 2.13. Preferential alighting by *Aedes aegypti* males on LED arrays (see Figure 2B) flashing either blue or white light at 665 Hz. The asterisk indicates a significant preference (*t*-test; p < 0.05). Figure adapted from Gries et al. (2017).
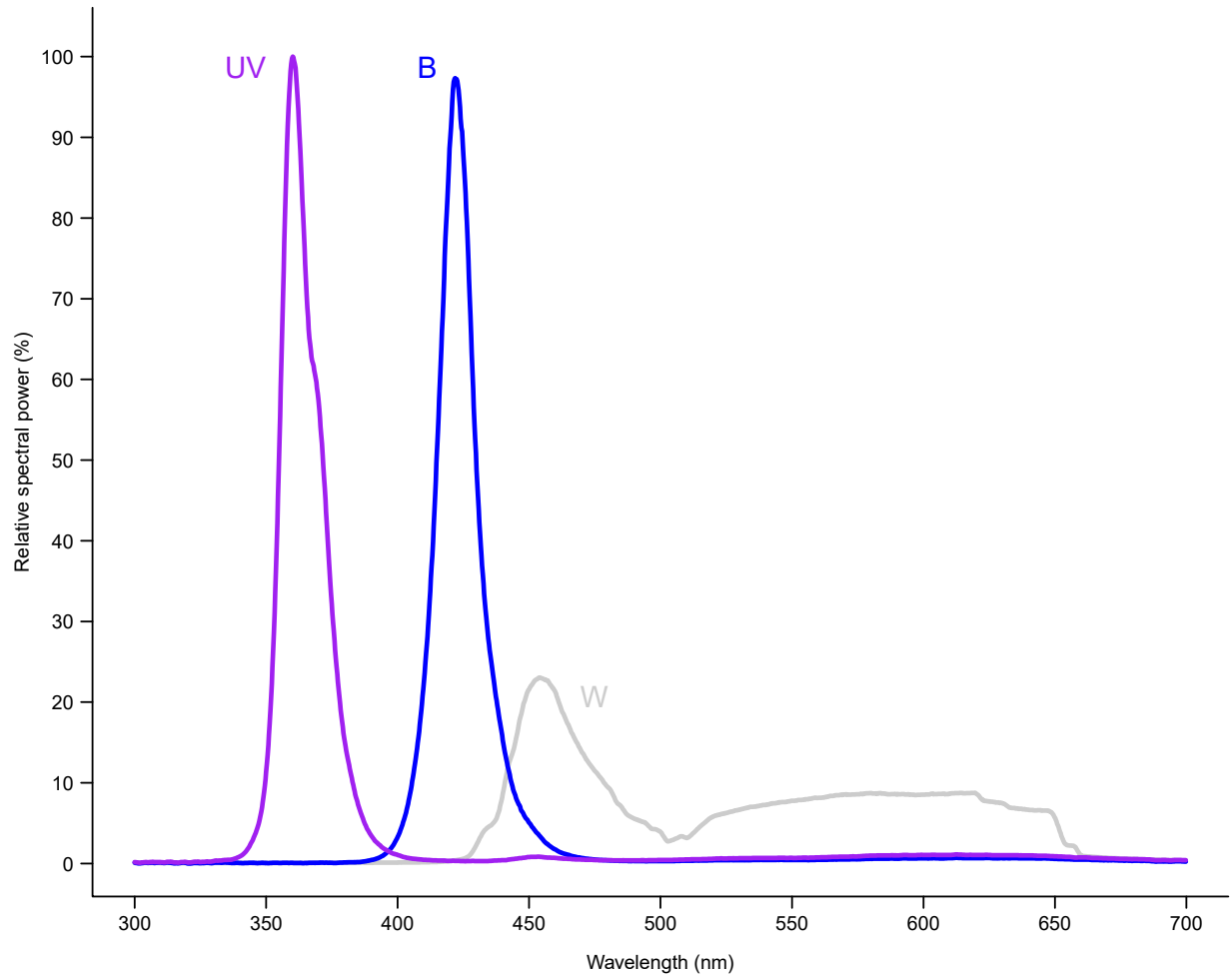
Figure 2.14. Spectra of LEDs tested in behavioural bioassays. Ultraviolet (UV), blue (B) and white (W) LEDs had intensity peaks at 360 nm, 422 nm, and at 455 nm and 620 nm, respectively. Each LED was standardized to a relative intensity of $2e^{15}$ photons/cm^2/s/nm.
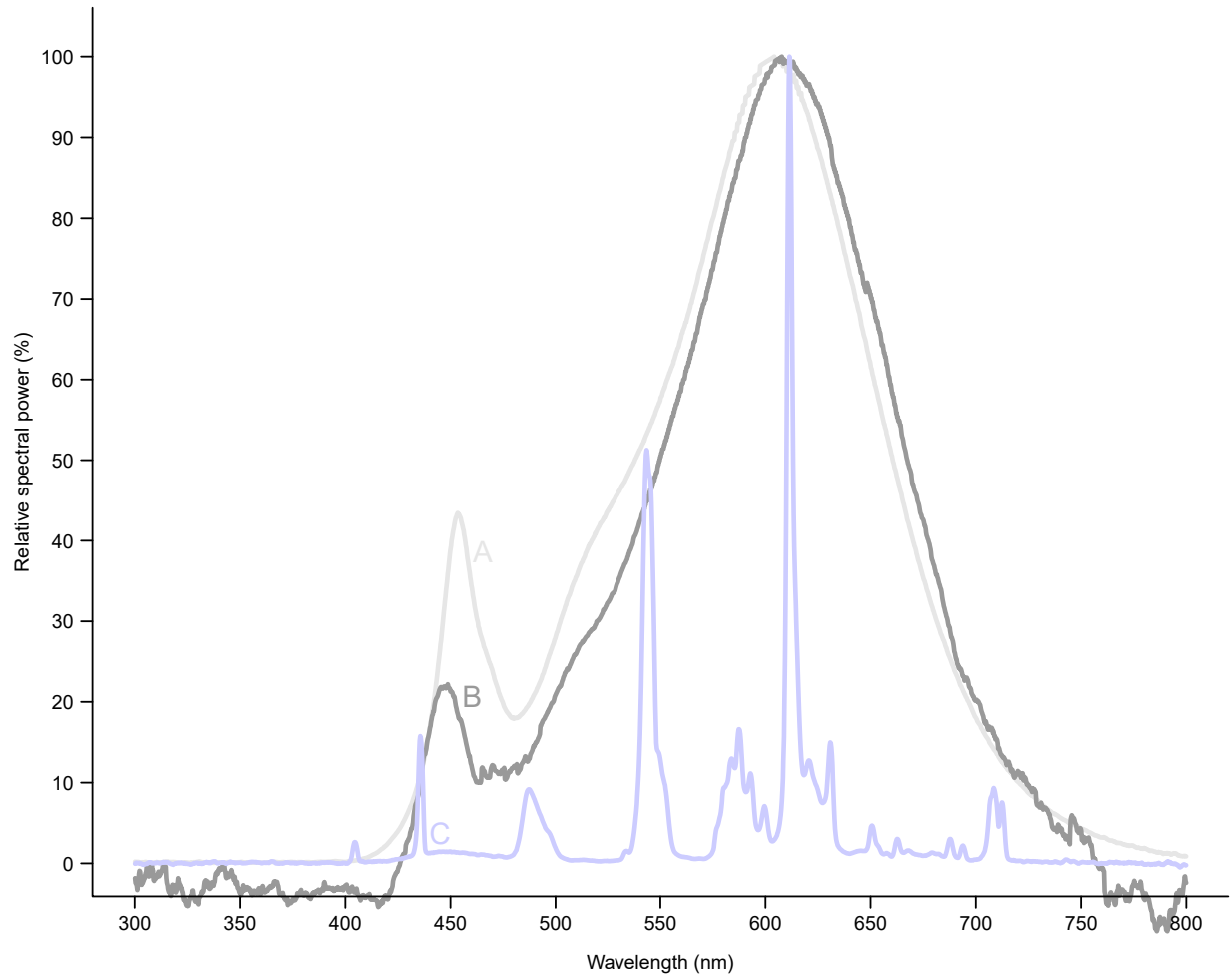
Figure 2.15. Spectra of illumination used during bioassays. Cage bioassays were illuminated with an LED bulb (Feit Electric) at full power (A) or dimmed (B). Light fixtures in the room contained two fluorescent tubes (C; General Electric). Each spectrum was normalized to its p

Figure 2.16. Males of *Aedes aegypti* swarming in a cage in a laboratory setting. Light flashes reflected from the wings of in-flight males are clearly visible. The video was recorded with a Photron FASTCAM NOVA S16 high-speed camera fitted with a Nikon NIKKOR Telephoto Zoom lens (AF 35-80 mm, f/4-5.6D) at a frame rate of 10000 fps and a shutter speed of 1/20000 s. Contrast and frequency analyses of the wing flash series produced by a single male from this video are shown in Fig. 2.1.

Figure 2.17. Close-up of a swarming *Aedes aegypti* male. Wing flashes appear with each flap of the male's wings. The video was recorded with a Photron FASTCAM NOVA S16 high-speed camera fitted with a Nikon NIKKOR AF-S Micro lens (105 mm, f/2.8 AF) at a frame rate of 3000 fps and a shutter speed of 1/6000 s.

Figure 2.18. Close-up of swarming *Aedes aegypti* males. The male flying towards the camera exhibits the 'seizing and clasping' response typical of a male that approaches a female in response to her wingbeat sound. The video was recorded with a Photron FASTCAM NOVA S16 high-speed camera fitted with a Nikon NIKKOR AF-S Micro lens (105 mm, f/2.8 AF) and a clip-on macro filter at a frame rate of 5000 fps and a shutter speed of 1/10000 s.

Figure 2.19. Wide view of swarming dipterans in an outdoor (courtyard) setting. These dipterans exhibited swarming behaviour with visible wing flashes under sunlight. Contrast and frequency analyses of the wing flash series produced by a single insect from this video are shown in Fig. 2.7. The video was recorded with a Photron FASTCAM NOVA S16 high-speed camera fitted with a Nikon NIKKOR Telephoto Zoom lens (AF 35-80 mm, f/4-5.6D) at a frame rate of 10000 fps and a shutter speed of 1/30000 s.

Figure 2.20. Wide view of swarming dipterans in an outdoor (courtyard) setting. These dipterans exhibited swarming behaviour with visible wing flashes under sunlight. The video was recorded with a Photron FASTCAM NOVA S16 high-speed camera fitted with a Nikon NIKKOR Telephoto Zoom lens (AF 35-80 mm, f/4-5.6D) at a frame rate of 10000 fps and a shutter speed of 1/30000 s.

Figure 2.21. Wide view of *Aedes aegypti* males swarming in a cage in a laboratory setting. The males are responding to a 665 Hz tone played back from a smartphone in the lower portion of the frame. The males dart towards the phone's speaker, and exhibit wing flashes under artificial light. The video was recorded with a Photron FASTCAM NOVA S16 high-speed camera fitted with a Nikon NIKKOR Telephoto Zoom lens (AF 35-80 mm, f/4-5.6D) at a frame rate of 5000 fps and a shutter speed of 1/10000 s.

Chapter 3.   Comparative attraction of mosquitoes to floral milkweed semiochemicals and an engineered 'super-flower' blend

[*]A near identical version of this chapter has been prepared for peer review with the follow authors:

Elton Ko, Mikhaela Ong, Tia Young, Courtney Hicks, Regine Gries, Santosh Kumar Alamsetti, Gerhard Gries. EK and GG discussed and planned the experiments. EK, MO, TY and CH ran bioassays, acquired and analyzed data and performed colony maintenance. RG analyzed headspace volatiles and prepared synthetic floral blends. SKA synthesized and purified test chemicals for bioassay experiments. EK and GG wrote the first draft of the article and received feedback from all co-authors.

Introduction

Feeding on sugary plant liquids such as nectar, fruit juices, plant sap, and plant exudates is vital for mosquito survival (Peach & Gries 2019; and literature cited therein). Sugary plant meals provide essential nutrients for adult mosquitoes (Foster 1995; Stone & Foster 2013; Nyasembe & Torto 2014), supply fuel for flight (Nayar & Handel 1971; Grimstad & Defoliart 1974) and enable mating and blood-feeding (Foster 1995). Blood-fed but sugar-deprived female mosquitoes are shorter-lived and less able to mate (Nayar & Sauerman, 1971; Stone et al. 2009, 2011), lay fewer eggs (Foster 1995), and have lower energy reserves for overwintering (Foster 1995). Without access to sugar resources, mosquito populations may collapse even when vertebrate blood is obtainable (Stone et al. 2009).

Floral nectar is the most heavily consumed constituent in the phytophagous diet of adult mosquitoes (Foster 1995, 2008; Stone & Foster 2013; Nyasembe & Torto 2014). Both floral and nectar semiochemicals (message-bearing chemicals) attract mosquitoes to nectar sources and allow them to discern inflorescences with varying nectar content (Peach & Gries 2019). These semiochemicals include compounds of various functional groups (e.g., aldehydes, alcohols, ketones, fatty acids, esters, terpenes) (Healy & Jepson 1988; Jhumur et al. 2007; Nyasembe & Torto 2014; Peach et al. 2019) but how they guide mosquitoes in selecting certain inflorescences is largely unknown. The same floral semiochemical may originate from multiple species of flowering plants and help attract multiple species of mosquitoes. For example, phenylacetaldehyde is a floral semiochemical of Spanish catchfly, *Silene otites* (Jhumur et al. 2006), common milkweed, *Asclepias syriaca* (Otienoburu et al. 2012), and sweet alyssum, *Lobularia maritima* (Von Oppen et al. 2015), and attracts both the northern house mosquito, *Culex pipiens,* and the yellow fever mosquito, *Aedes aegypti* (Jhumur et al. 2006, 2007).

The appeal of flowering plants to mosquitoes varies widely (Grimstad & Defoliart 1974; Gouagna et al. 2010; Nyasembe et al. 2012; Lahondère et al. 2020). Inflorescences of *A. syriaca* were frequented and probed by mosquitoes disproportionate to their abundance relative to other flowering plants (Grimstad and Defoliart 1974) but whether other milkweed species are even more attractive than *A. syriaca,* and share the *A. syriaca* semiochemicals that attract male and female *Cx. pipiens* (Otienoburu et al. 2012), is not clear. Even within the same plant genus, species can have highly contrasting appeal to mosquitoes. For example, the northern bog orchid, *Platanthera obtusata,* is visited and pollinated by *Ae. aegypti* but other *Platanthera* species are not (Lahondère et al. 2020). Preferences for floral resources may also differ among mosquito populations and may be affected by mosquito age (Grimstad & Defoliart 1974). For example, some floral semiochemicals of common tansies, *Tanacetum vulgare,* also emanate from vertebrate blood hosts (e.g., butanoic acid, hexanoic acid, benzaldehyde, acetophenone) and preferentially attract older mosquitoes (Peach et al. 2019) that are typically in host-seeking mode (Armstrong & West 1965).

Floral *bouquets* that attract mosquitoes may share semiochemicals like phenylacetaldehyde and benzaldehyde (Jhumur et al. 2007; Otienoburu et al. 2012; Lahondère et al. 2020), or – alternatively – contain rather specific and less ubiquitous semiochemicals. For example, floral *bouquets* of *T. vulgare* contain 3,3,6-trimethyl-1,5-heptadien-4-one (artemisia ketone), 2,5,5-trimethyl-3,6-heptadien-2-ol (yomogi alcohol), 4-methyl-1-propan-2-ylbicyclo[3.1.0]hex-3-en-2-one (umbellulone), and butanoic acid (Peach et al. 2019), and those of *P. obtusata* contain indane and decane (Lahondère et al. 2020), all of which are not readily found in other flowering plants. These phenomena made us wonder whether it is feasible to combine the floral semiochemicals from multiple plant species in a synthetic 'super-flower blend' that may then become more attractive to certain mosquito species or attract a wider range of species. In this spirit, we wondered about the attractiveness of a 'super-flower blend' containing not only floral semiochemicals of milkweeds but also of *T. vulgare* and *P. obtusata.*

Working with *Cx. pipiens* and *Ae. aegypti*, our objectives were to (1) test inflorescences of various milkweeds (*Asclepias* spp*.)* for attraction of mosquitoes*;* (2) determine the key floral semiochemicals in a highly attractive milkweed, and (3) explore the feasibility of a super-flower concept. *Asclepias* spp. was chosen as the model floral genus due to its known attractiveness to floral-foraging mosquitoes (e.g., Vargo & Foster 1982; Otienoburu et al. 2012)

3.2. Materials and Methods

3.2.1. Rearing of experimental insects

We reared *Cx. pipiens* in the insectary of the Burnaby campus of Simon Fraser University (SFU). Rearing rooms were kept at 23-28 °C, 40-60% RH and a photoperiod of 14L:10D. Eclosed adults were released into mesh cages (30 × 30 × 46 cm high) and provided with a 10-% sucrose solution *ad libitum*. Three days after blood-feeding on GG's or RG's forearms, mosquitoes were offered an oviposition site consisting of a glass dish (10 cm diam × 5 cm high) containing a piece of white cardstock and water. Oviposited egg rafts were transferred to water-filled trays (45 × 25 × 7 cm high) for egg hatching and larval development. Hatched larvae were provisioned with NutriFin Basix tropical fish food (Rolf C. Hagen Inc., Montreal, QC, CA). Using a 7-mL plastic pipette (VWR International, Radnor, PA, USA), pupae were transferred to water-filled 354-mL Solo Cups (Solo Cup Comp., Lake Forest, IL, USA) fitted with a mesh lid. As an energy source for eclosing adults, a cotton ball soaked in a sucrose solution was placed on top of the mesh. Within 72 h of eclosion, adults were released into mesh cages (30 × 30 × 46 cm high) and females were separated from males. Females were assumed unmated, because most females are refractory to insemination within 48–72 hours after eclosion (Gwadz & Craig 1972).

The rearing protocol for *Ae. aegypti* resembled that of *Cx. pipiens* except that gravid females were offered a water-filled 354-mL Solo Cup lined with a paper towel (S.C. Johnson & Son, Inc., Racine, WI, USA) as an oviposition site. Females 1- to 3-days-old were tested in bioassays.

3.2.2. Acquisition of milkweed (*Asclepias*) plants

Potted *Asclepias incarnata* 'Cinderella', *A. incarnata* 'Ice Ballet', *A. speciosa* and *A. syriaca* were purchased from Phoenix Perennials and Speciality Plants (Richmond, B.C., CA), and potted *A. tuberosa* were purchased from Gardenworks (Burnaby, B.C.). Plants were kept in a greenhouse and transported to the lab and insectary for headspace volatile capture and analysis and for behavioural experiments, respectively.

3.2.3. General design of behavioural experiments

All behavioural experiments were run in mesh cages (77 × 78 × 104 cm high) kept at 23–26 $^{\circ}$C, 40-60% RH and a photoperiod of 14L:10D. Except for the roof, all cage walls were covered with black cloth to minimize entry of stray light. The rooms, and hence cages, were illuminated via ceiling fluorescent lighting (F32T8/SPX50/ECO, General Electric, Boston, MA, USA). Two burette stands were placed 25 cm apart from each other in the cage centre (Exps. 1–16), or in opposite corners of the cage 61 cm apart from each other (Exps. 17–18), and fitted with a delta trap (15 × 9 × 8 cm) cut to size from white cardstock (71.3 × 55.9 cm) (Staples Inc., MA, USA) and coated with adhesive on the inside (The Tanglefoot Company, MI, USA) (Fig. 3.1A). By random assignment, one trap in each pair was baited with florets pushed through parafilm (Bemis Company Inc., WI, USA) covering the orifice of a water-filled vial (2 ml; Fig. 3.1B), whereas the other trap was fitted with the same type of vial containing water without florets. For any experiment that tested synthetic floral blends, these blends were dissolved in pentane/ether (50/50; 1 mL) and disseminated through a hole (~1 mm) in the vial lid. For each experimental replicate, 25 or 50 24-h starved, 1- to 3-day-old or 5- to 10-day-old female *Cx. pipiens* or *Ae. aegypti* were released into a cage and allowed 24 h to respond to test stimuli. Captured mosquitoes were recorded and non-responders euthanized with an electric fly swatter (Guangzhou Sidianjin Trading Co., Guangzhou, CN).

3.2.4. Syntheses of test chemicals

Preparation of lilac aldehydes

Selenium oxide (SeO_2, 11.1 mg, 0.1 mmol) and 70% aq t-butyl hydroperoxide (0.83 mL, 780 mg, 6 mmol) were added to a microwave vial followed by a solution of (–)-linalool (152 mg, 1 mmol) in anhydrous CH_2Cl_2 (2 mL). The vial was sealed and the reaction mixture stirred 20 min under microwave irradiation (250 W) at 115 °C. The two-layer reaction mixture was diluted with CH_2Cl_2 (5 mL) and washed with 2.0 M KOH (5 mL), deionized water (5 mL), and brine (5 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated, and purified using flash chromatography (hexanes/EtOAc, 5/1) to afford 6-hydroxy-2,6-dimethylocta-2,7-dienal as slightly yellow oil with a yield of 86 mg (50%). Methanol (4 mL) and sodium hydride (24 mg, 1 mmol) were then added to 6-hydroxy-2,6-dimethylocta-2,7-dienal (84 mg, 0.5 mmol), and the reaction mixture was

stirred 20 h at room temperature. The solution was washed with 1 M HCl (2 × 5 mL), sat. aq NaHCO$_3$ (5 mL), and brine (5 mL). The organic layer was dried (MgSO$_4$), concentrated, and purified using flash chromatography (hexanes/EtOAc, 20/1) to provide a mixture of lilac aldehydes yielding 50.3 mg (60%).

Preparation of phenylnitroethane

After adding nitromethane (30 mmol) dropwise to a stirred solution of arylaldehyde (1.06 g, 10 mmol), ammonium acetate (CH$_3$COONH$_4$, 770 mg, 10 mmol) and glacial acetic acid (10 mL) in an oven-dried round-bottomed flask fitted with a condenser, the mixture was refluxed for 2 days. When the reaction was complete, it was cooled to room temperature and poured into ice water. The resulting precipitate was filtered with suction using a sintered G-4 glass funnel and then concentrated and purified by flash chromatography (hexanes/EtOAc, 2/1) to provide *trans*-β-nitrostyrene (1.42 mg, 60% yield). *Trans*-β-Nitrostyrene (1 g, 6.7 mmol) was stirred into a suspension of silica gel (10 g; 60 Å, 230- 400 mesh) in a mixture of chloroform (50 mL) and isopropyl alcohol (15 mL) at 23 °C, and then cooled to 0 °C. Sodium borohydride (560 mg, 14.8 mmol) was added in four equal portions over 20 min. The resulting heterogeneous mixture was stirred vigorously 30 min at 0 °C, then warmed to 23 °C, stirred an additional 30 min at 23 °C, and then cooled again to 0 °C. Subsequently, an aqueous solution of hydrochloric acid (0.2 M) was added until no gas evolution was observed. The resulting mixture was filtered and eluted with dichloromethane (30 mL). The organic phase and the extract of the aqueous phase in dichloromethane (3 × 50 mL) were separated, dried (MgSO$_4$), and concentrated to obtain phenylnitroethane (980 mg, 97% yield).

Preparation of phenylacetaldehyde oxime

An aqueous solution of Na$_2$CO$_3$ (159 mg, 1.5 mmol, 2 mL) was slowly added to a suspension of the phenylacetaldehyde (360 mg, 3 mmol) and hydroxylamine hydrochloride (229 mg, 3.3 mol) in a 1:1 mixture of H$_2$O/methanol (4 mL). After stirring the resulting mixture 3 h at room temperature, methanol was evaporated and the aqueous phase was extracted with Et$_2$O (10 mL). The combined organic phases were washed with brine (10 mL), dried (Na$_2$SO$_4$), filtered, and concentrated *in vacuo* to give 2-phenylacetaldehyde oxime as a pale-yellow solid (369 mg, 91% yield).

Preparation of benzyl salicylate

A mixture of salicylic acid (168 mg 1 mmol), benzyl bromide (125 µL, 1.05 mmol) and potassium carbonate (276 mg, 2 mmol) in DMF (2 mL) was stirred 2 h at room temperature and then diluted with ethyl acetate (20 mL). The organic phase was washed with water (20 mL) and brine (20 mL) and then dried ($MgSO_4$), filtered, concentrated, and purified using flash chromatography (hexanes/EtOAc, 10/1) to afford benzyl salicylate with a yield of 203 mg (89%).

Purification of germacrene-D

Adapting previously reported procedures (Peach et al. 2019), germacrene-D (40% technical grade, Treat Plc, Lakeland, FL, USA) was purified to 93% using a high-performance liquid chromatograph (HPLC) (Waters HPLC system, Waters Corp, Milford, MA, USA), fitted with a C_{18} revered phase column (250 × 60 mm, 4 µ) which was eluted with acetonitrile (1 ml/min).

Purification of artemisia ketone

Adapting previously reported procedures (Peach et al. 2019), artemisia ketone (3,3,6-trimethyl-1,5-heptadien-4-one; 190 g, 90% pure) was isolated from wormwood (*Artemisia annua*) essential oil (Liberty Natural Products, Portland, OR, USA) through repetitive silica gel column chromatography using hexane and ethyl acetate (90:10) as eluents.

Purification of yomogi alcohol

Adapting previously reported procedures (Peach et al. 2019), yomogi alcohol (2,5,5-trimethyl-3,6-heptadien-2-ol) was isolated from Moroccan chamomile (*Ormensis multicaulis*) essential oil (Liberty Natural Products), which also contains 62% santolina alcohol among other compounds such as cineole and monoterpenes. Yomogi alcohol was isolated from 3 g of the essential oil by flushing the analyte twice though a silica gel column, using hexane and ethyl acetate as eluents [first flash: 80:20 (hexane:ethyl acetate); second flash: 85:15]. Further purifying the isolated yomogi alcohol (180 mg,

50% pure) by HPLC with acetonitrile and water (60:40; 1 ml/min) as eluents yielded a mixture of yomogi alcohol (75%) and santolina alcohol (25%).

Preparation of phenyl-2,3-butandione and 1-phenyl-3-hydroxy-2-butanone

Phenyl-2,3-butandione and 1-phenyl-3-hydroxy-2-butanone were prepared adapting previously reported procedures (Peach et al. 2019). Sequentially, a solution of DL-3-phenyllactic acid (166 mg, 1.0 mmol, 1.0 eq.) in CH_2Cl_2 (15 ml) was 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 mixture was stirred overnight at ambient temperature and then quenched with water. The aqueous layer was extracted with CH_2Cl_2 (15 mL). The combined organic layers were washed with a 10% HCl aqueous solution followed by a 5% $NaHCO_3$ aqueous solution and brine, and then dried over $MgSO_4$ and concentrated. The residue was used for the next step without further purification. The resulting Weinreb amide was dissolved in anhydrous THF (8 ml) and then cooled to -78 °C. Then, MeMgBr (3.0 M in Et2O, 0.6 ml, 1.8 mmol, 1.8 eq.) was added and the mixture was stirred 5 h at 0 °C before quenching it with saturated aqueous NH_4Cl (5 mL). The aqueous layer was separated and extracted with EtOAc (10 mL). Afterwards, the combined organic layers were washed sequentially with water and brine, then dried over Na_2SO_4 and concentrated. The resulting residue was purified through flash chromatography (hexane/EtOAc, 6/1), giving 87 mg (53% over 2 steps) of 1-phenyl-3-hydroxy-2-butanone as a colourless oil. A portion (49 mg, 0.3 mmol, 1.0 eq.) of this ketone-alcohol was dissolved in dry CH_2Cl_2 (10 mL), and $NaHCO_3$ (38 mg, 0.45 mmol, 1.5 eq.) was added. Lastly, Dess–Martin periodinane (190 mg, 0.45 mmol, 1.5 eq.) was added and the reaction mixture was stirred 1 h at ambient temperature, after which 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). The mixture was stirred for an additional 20 min and the resulting aqueous layer was separated and extracted using EtOAc (3 × 10 ml). The combined organic layers were washed with brine (10 mL), dried it over magnesium sulfate and concentrated. Using flash chromatography (hexane/ethyl acetate, 10/1), the mixture was purified, yielding 65 mg (75% pure) of the diketone as a yellow oil.

3.2.5. Specific experiments

Objective 1: Test milkweed inflorescences for attraction of mosquitoes (Exps. 1-5)

2020-Experiments 1–4 (Table 1) tested inflorescences of *A. incarnata* 'Ice Ballet' (Exp. 1), *A. incarnata* 'Cinderella' (Exp. 2), *A. tuberosa* (Exp. 3) and *A. speciosa* (Exp. 4) as a trap bait for attraction and captures of *Cx. pipiens.* As *A. incarnata* 'Ice Ballet' appeared relatively most attractive (see Results), and *A. syriaca* became available in 2021, experiment 5 (Table 3.1) tested inflorescences of *A. incarnata* 'Ice Ballet' and *A. syriaca* head-to-head. To standardize test stimuli in experiment 5, florets were tested at equal weight equivalents (six florets from *A. syriaca versus* 25-30 florets from *A. incarnata*).

Objective 2: Determine the key floral semiochemicals in a highly attractive milkweed spp.

Capture and analyses of headspace floral odorants

To collect the headspace odorants of *Asclepias* inflorescences tested in experiments 1–5, one inflorescence was put into a water-filled beaker (250 mL) which, in turn, was placed into a Pyrex glass chamber (34 cm high × 12.5 cm wide). Using a mechanical pump, charcoal-filtered air was drawn at 1 L min^{-1} for 24 h through the chamber and a glass column (6 mm outer diam × 150 mm) containing Porapak-Q^{TM} adsorbent (200 mg; 50-80 mesh, Waters Associates, Milford, MA, USA). Odorants were desorbed from Porapak-Q with sequential rinses of pentane and ether (1 mL each). After concentrating the Porapak-Q extract to 500 µL, 1-µL aliquots were analyzed by gas chromatography-mass spectrometry (GC-MS) in full-scan electron ionization mode, using a Saturn 2000 Ion Trap GC-MS or an Agilent GC-MS (7890B GC and a 5977A MSD) fitted with a DB-5 MS column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Agilent Technologies Inc.). We used helium as the carrier gas (35 cm s^{-1}) and the following temperature program: 50 $^{\circ}$C (5 min), 10 $^{\circ}$C min^{-1} to 280 $^{\circ}$C (held for 10 min). We kept the temperature of the injector port at 250 °C, the ion trap at 200 $^{\circ}$C, the Agilent MS Source at 230 °C, and the MS Quadrupole at 150 °C. Odorants in Porapak-Q extracts were identified 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 and with those of authentic standards.

Comparative attraction of Cx. pipiens and Ae. aegypti to synthetic floral semiochemicals of A. speciosa (Exps. 6, 7)

With *A. speciosa* being most odiferous (see Results), and as attractive as other *Asclepias* inflorescences to *Cx. pipiens* (see Results), we opted to use *A. speciosa* as a model species for unraveling its key floral semiochemicals that attract mosquitoes. To help decide whether to test *Cx. pipiens* or *Ae. aegypti* for this objective, experiments 6–7 (Table 3.1) compared attraction of *Cx. pipiens* and *Ae. aegypti* to synthetic floral semiochemicals of *A. speciosa* (Table 3.2), following the general experimental design described above.

Attraction of Ae. aegypti to complete and partial synthetic blends of A. speciosa inflorescences (Exps. 8-14)

With evidence that *Ae. aegypti* responded better than *Cx. pipiens* to synthetic floral semiochemicals of *A. speciosa* (see Results), we worked with *Ae. aegypti* to determine the key floral semiochemicals of *A. speciosa* inflorescences. We tested the complete synthetic blend with all floral constituents (Table 3.2; Exp. 8), and partial synthetic blends that lacked aldehydes (Exp. 9), alcohols (Exp.10), or esters and nitrogen-containing compounds (Exp. 11). To further elucidate the role of esters and of nitrogen-containing compounds, parallel experiments 12–14 re-tested the complete synthetic blend with all floral constituents (Exp. 12) and partial synthetic blends lacking either the nitrogen-containing compounds (Exp. 13) or the esters (Exp. 14). Experiments 8–14 followed the general experimental design described above.

Head-to-head attraction of Ae. aegypti to complete and partial synthetic blends of A. speciosa inflorescences (Exp. 15)

To confirm that alcohols and nitrogen-containing compounds do not contribute to the attractiveness of the *A. speciosa* floral blend (see Results), we tested attraction of female *Ae. aegypti* to the complete synthetic blend (SB) and a reduced synthetic blend (RSB) head-to-head at identical amounts (100 µg; Table 3.2), following the general experimental design described above.

Objective 3: Explore the feasibility of a super-flower concept (Exps. 16–18)

To explore whether the attractiveness of floral milkweed semiochemicals can be enhanced by adding proven-effective floral semiochemicals from select other plant species (super-flower concept), we tested two floral blends head-to-head (Exp. 16): (*i*) the proven-effective RSB of *A. speciosa* (Table 3.2) and (*ii*) the synthetic super-flower blend (SSFB) containing floral semiochemicals from *A. speciosa, A. syriaca, T. vulgare* and *S. otites* (Table 3.3). Both blends contained the same total amount of semiochemicals (100 μg) but differed in the number of blend constituents.

To further explore whether the relative attractiveness of the SSFB is contingent upon the age of bioassay mosquitoes, parallel experiments 17–18 (Table 3.1) tested the SSFB for attraction of 1- to 3-day-old female *Ae. aegypti* (Exp. 17) and 5- to 10-day-old female *Ae. aegypti* (Exp. 18). Experiments 16–18 followed the general experimental design described above.

3.2.6. Statistical Analyses

Behavioural data were analyzed using R 3.6.2 (R Core Team 2020). We analyzed mean proportions of mosquitoes captured per trap with logistic regression, using generalized linear models. To determine whether proportions of captured mosquitoes differed between traps, we compared an intercept only model to a null model with a likelihood ratio test. Afterwards, we back-transformed coefficients from those models to obtain mean and stand errors for the proportion of mosquitoes captured in each trap.

3.3. Results

Objective 1: Test milkweed inflorescences for attraction of mosquitoes (Exps. 1-5)

Traps baited with florets of various milkweed species consistently captured significantly more *Cx. pipiens* females than corresponding unbaited control traps (Fig. 3.2; *A. tuberosa*: F = 18.47, p = 0.002, Exp. 1; *A. incarnata* 'Ice Ballet': F = 36.98, p = 0.00018, Exp. 2; *A. incarnata* 'Cinderella': F = 20.66, p = 0.0014, Exp. 3; *A. speciosa*: F = 30.33, p = 0.00038, Exp. 4). Traps baited with florets of *A. syriaca* or *A. incarnata* 'Ice Ballet' captured similar numbers of female *Cx. pipiens* (F = 0.0239, p = 0.8806, Exp. 5).

Objective 2: Determine the key floral semiochemicals in a highly attractive milkweed spp.

Analyses of headspace floral odorants

Analyses of milkweed headspace floral odorants revealed compounds of diverse functional groups, including aldehydes, alcohols, acetates, esters, hydrocarbons and nitrogen-containing compounds (Table 3.4). Several odorants (benzaldehyde, benzyl alcohol, 2-phenylethyl alcohol) were present in the headspace of four out of five species. The overall amounts of odorants emanating from inflorescences differed between species. *Asclepias speciosa* with 400 µg of floral compounds in the headspace odorant extract was the most 'odiferous', followed by *A. syriaca* (310 µg), *A. incaranta* 'Cinderella' (34.3 µg), *A. incaranta* 'Ice Ballet' (14.8 µg), and *A. tuberosa* (5.4 µg).

Comparative attraction of female Cx. pipiens and Ae. aegypti to synthetic floral semiochemicals of A. speciosa

Traps baited with synthetic floral semiochemicals of *A. speciosa* (Table 3.2) attracted more female *Ae. aegypti,* but not more female *Cx. pipiens,* than unbaited control traps (Fig. 3.3; *Cx. pipiens:* F = 0.0492, p = 0.8318, Exp. 6; *Ae. aegypti:* F = 21.44, p = 0.001236, Exp. 7).

Attraction of Ae. aegypti to complete and partial synthetic blends of A. speciosa floral semiochemicals (Exps. 8–15)

Traps baited with the complete synthetic blend (SB) of *A. speciosa* floral semiochemicals (Table 3.2) statistically did not capture more *Ae. aegypti* females than unbaited control traps (Fig. 3.4; F = 1.797, p < 0.2169, Exp. 9). Traps baited with partial synthetic blends lacking either alcohols (Exp. 10) or esters and nitrogen-containing compounds (Exp. 11) captured more *Ae. aegypti* females than unbaited control traps (F = 7.981, p = 0.0199, Exp. 10; F = 6.581, p = 0.0303, Exp. 11), suggesting that neither alcohols nor esters and nitrogen-containing compounds are key floral semiochemicals of *A. speciosa*. Conversely, traps baited with a partial blend lacking aldehydes (Exp. 9) failed to capture more *Ae. aegypti* females than unbaited control traps (F = 1.797, p = 0.217).

In three follow-up parallel experiments (Fig. 3.5, Exps. 12–14), we explored whether it was the esters or the nitrogen-containing compounds (which were lumped together in

Exp. 11) that could be omitted from the floral blend without affecting its attractiveness. To this end, we re-tested the complete synthetic blend of *A. speciosa* floral semiochemicals (Exp. 12, positive control) and tested partial blends lacking either the nitrogen-containing compounds (Exp. 13) or the esters (Exp. 14). Traps baited with any one of these three synthetic blends captured more *Ae. aegypti* females than unbaited control traps ($F = 10.529$, $p = 0.01$, Exp. 12; $F = 24.708$, $p = 0.00077$, Exp. 13; $F = 11.325$, $p = 0.00832$, Exp. 14), suggesting that neither the esters nor the nitrogen-containing compounds are key floral semiochemicals of *A. speciosa*.

That odorants can be omitted from the floral blend of *A. speciosa* without affecting its attractiveness was confirmed in experiment 15. Here, traps baited with the complete synthetic blend (SB) and the reduced synthetic blend (RSB) lacking non-essential components (alcohols, nitrogen-containing compounds; see Exps. 10, 11, 13) captured similar numbers of *Ae. aegypti* females (Fig. 3.5; $F = 1.9098$, $p = 0.2003$, Exp. 15).

Objective 3: Explore the feasibility of a super-flower concept (Exps. 16-18)

Head-to-head comparison of the proven-effective RSB of *A. speciosa* (Table 3.2) and the SSFB with floral semiochemicals of *A. speciosa*, *A. syriaca*, *T. vulgare* and *P. obtusata* (Table 3.3) resulted in no preference for either lure by *Ae. aegypti* females (Fig. 3.6; $F = 0.3426$, $p = 0.577$; Exp. 16). Testing the same two floral blends head-to-head in follow-up parallel experiments with 1- to 3-day-old females (Exp. 17), and with 5- to 10-day-old females (Exp. 18), again resulted in no preference for either lure (Fig. 3.7; $F = 0.3669$, $p = 0.3669$, Exp. 17; $F = 0.7475$, $p = 0.4097$, Exp. 18), revealing no age effect of mosquitoes on their responses to floral blends.

3.4. Discussion

Our data reveal that (*i*) all four species or varieties of milkweed tested (*A. tuberosa*, *A. incarnata* 'Ice Ballet', *A. incarnata* 'Cinderella', *A. speciosa*) were attractive to *Cx. pipiens*, (*ii*) *A.* incarnata 'Ice Ballet' was as attractive as *A. syriaca* which was previously reported to strongly attract mosquitoes (Otienoburu et al. 2012); (*iii*) a synthetic floral blend of *A. speciosa* (the most odiforous milkweed in our study) attracted *Ae. aegypti*; (*iv*) aldehydes in the floral blend of *A. speciosa* are the key semiochemicals for *Ae. aegypti* attraction; and (*v*) a synthetic super-flower blend containing floral

semiochemicals of four flowering plant species including *A. speciosa* was not more attractive to *Ae. aegypti* than the synthetic *A. speciosa* floral blend.

That all milkweeds tested in our study were attractive to mosquitoes (Fig. 3.2) is due, in part, to common constituents in floral bouquets, such as phenylacetaldehyde that prevailed in floral bouquets of *A. speciosa* and *A. syriaca* (Table 3.4). Phenylacetaldehyde is also emitted from many other flowering plants (Knudsen et al. 2006; Jhumur et al. 2008; Lahondère et al. 2020) and is known to attract *Cx. pipiens* and *Ae. aegypti* (Jhumer et al. 2006, 2007). However, certain floral constituents can apparently compensate for the absence of phenylacetaldehyde because *A. tuberosa* did not produce phenylacetaldehyde but was still appealing to nectar-foraging mosquitoes (Fig. 3.2).

To determine the key floral semiochemicals that mediated mosquito attraction to highly attractive milkweed inflorescences, we selected *A. speciosa* because it emitted the largest amount of floral odorants, rivaled *A. incarnata* 'Ice Ballet' for most attractive milkweed to *Cx. pipiens* (Fig. 3.2), compared favourably to *A. syriaca* (Fig. 3.2, Exp. 5) which is very appealing to *Cx. pipiens* (Otienoburu et al. 2012), and it attracted other insect taxa such as clearwing moths in previous studies (Eby et al. 2013).

As *Aedes aegypti* responded better than *Cx. pipiens* to the synthetic floral blend of *A. speciosa* (Fig. 3.3), we opted to work with *Ae. aegypti* to determine the essential floral semiochemicals of *A. speciosa*. Testing the complete synthetic blend of *A. speciosa* floral odorants and partial blends lacking functional groups such as aldehydes and alcohols, revealed that the aldehydes were essential for mosquito attraction, whereas alcohols, esters and nitrogen-containing odorants did not contribute to, or even interfered with, the blend's attractiveness (Figs. 3.4, 3.5). These data are consistent with findings that aldehydes were the key floral semiochemicals also of *A. syriaca,* and that partial floral blends lacking alcohols increased attraction of *Cx. pipiens* to the *A. syriaca* floral bouquet (Otienoburu et al. 2012). That partial floral blends were more attractive to both *Cx. pipiens* and *Ae. aegypti* than more complex blends (Otienoburu et al. 2012; this study) seems perplexing, but similar phenomena have been reported in previous studies. Both males and females of the clearwing moth *Synanthedon myopaeformis* were more strongly attracted to phenylacetaldehyde as a single component than to an 8-component blend mimicking the floral bouquet of *A. speciosa* (Eby et al. 2013). Similarly, single floral semiochemicals were as effective as multiple-component floral blends in attracting a pierid butterfly and noctuid moths (Haynes et al. 1991; Heath et al. 1992;

Ômura et al. 1999; Plepys et al. 2002; Dötterl et al. 2006). It is conceivable that milkweeds, and possibly other flowering plants, have 'designed' their floral bouquets to appeal to many different pollinators, with some floral constituents attracting a first group of pollinators and others attracting a second and third group of pollinators while – inadvertently – compromising optimal attraction of the first group.

Our first attempt to engineer a super-flower blend containing not only floral semiochemicals of milkweed but also of *T. vulgare* and *P. obtusata* was not successful. The super-flower blend and the proven effective *A. speciosa* blend, tested head-to-head at identical amounts, were equally attractive to *Ae. aegypti* females (Fig. 3.6), irrespective of age (Fig. 3.7). There are at least three plausible explanations. First, although the super-flower blend may broadly attract more species of mosquitoes, it may not be supremely attractive to certain species of mosquitoes such as *Ae. aegypti*. As we tested the response of only *Ae. aegypti* to the super-flower blend, this possibility was not addressed in our study. Second, the floral semiochemicals we selected from *A. speciosa*, *A. syriaca*, *T. vulgare* and *P. obtusata* to make up the super-flower blend may have been incompatible and may have presented a 'mixed message' to nectar-foraging *Ae. aegypti* females, interfering with the recognition of a plant's inflorescence odor 'Gestalt'. Third, specific floral components may have been presented at a ratio not indicative of a 'natural' inflorescence and thus may have been unappealing to nectar-foraging mosquitoes. Insects recognize host plants, in part, based on specific ratios of plant odorants (Bruce et al. 2005). With benzaldehyde shared between *A. speciosa, A. syriaca* and *T. vulgare*, it may have been over- or under-represented in respective floral blends, not allowing foraging mosquitoes to associate blend components with the presence of distinct inflorescences, and thus additive nectar supplies.

In summary, all species or varieties of milkweeds tested in our study attracted *Cx. pipiens*, in part, because of overlapping constituents in their floral blends. Phenylacetaldehyde and benzaldehyde in the floral blend of *A. speciosa* were the key attractants, with other constituents not contributing to, or even interfering with, the blend's attractiveness. Our first attempt to engineer a super-flower blend by combining key floral semiochemicals from three flowering plants in a single blend for enhanced attraction of *Ae. aegypti* was not successful, but the super-flower blend still may have appeal to a wider range of mosquito taxa than the *A. speciosa* blend on its own.

3.5. References

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Table 3.1. Details of test stimuli and number of replicates (n) run in laboratory behavioural experiments (see Fig. 3.1 for design) with the mosquitoes *Culex pipiens* and *Aedes aegypti*.

Exp. # (n)	Species tested	Mosquito age (days)	Stimulus 1	Stimulus 2
O1: Test milkweed inflorescences for attraction of mosquitoes				
1 (10)	*Cx. pipiens*	1-3	*Asclepias tuberosa*	Water
2 (10)	*Cx. pipiens*	1-3	*A. incarnata* 'Ice ballet'	Water
3 (10)	*Cx. pipiens*	1-3	*A. incarnata* 'Cinderella'	Water
4 (10)	*Cx. pipiens*	1-3	*A. speciosa*	Water
5 (10)	*Cx. pipiens*	1-3	*A. syriaca*	*A. incarnata* 'Ice Ballet'
O2: Determine the key floral semiochemicals in a highly attractive milkweed spp.				
6 (8)	*Cx. pipiens*	1-3	Synthetic blend (SB)[a]	Pentane/ether
7 (10)	*Ae. aegypti*	1-3	SB	Pentane/ether
8 (10)	*Ae. aegypti*	1-3	SB	Pentane/ether
9 (10)	*Ae. aegypti*	1-3	SB *minus* aldehydes	Pentane/ether
10 (10)	*Ae. aegypti*	1-3	SB *minus* alcohols	Pentane/ether
11 (10)	*Ae. aegypti*	1-3	SB *minus* (esters & nitrogen-containing compounds)	Pentane/ether

12 (10)	*Ae. aegypti*	1-3	SB	Pentane/ether
13 (10)	*Ae. aegypti*	1-3	SB *minus* nitrogen-containing compounds	Pentane/ether
14 (10)	*Ae. aegypti*	1-3	SB *minus* esters	Pentane/ether
15 (10)	*Ae. aegypti*	1-3	SB	RSB[b]

O3: Explore the feasibility of a super-flower concept

16 (10)	*Ae. aegypti*	1-3	Synthetic super-flower blend (SSFB)[c]	RSB[b]
17 (10)	*Ae. aegypti*	1-3	SSFB[c]	RSB[b]
18 (10)	*Ae. Aegypti*	5-10	SSFB[c]	RSB[b]

[a] Synthetic blend of *Asclepias speciosa* (Table 3.2) dissolved in 1 ml of pentane-ether (50:50). [b]RSB = Reduced synthetic blend of *A. speciosa* lacking alcohols and nitrogen-containing compounds (Table 3.2). [c]Synthetic super-flower blend containing floral odorants of *Asclepias speciosa, Asclepias syriaca, Platanthera obtusata* and *Tanacetum vulgare* (Table 3.3).

Table 3.2. Composition of floral synthetic blends of milkweeds (*Asclepias* spp.) tested in experiments 6-18. Numbers in columns denote the percentage of compounds in a blend.

	Compound (%) in synthetic blend (SB)			
Odorant	*A. speciosa* (SB)	*A. speciosa* (RSB[a])	*A. syriaca* (SB)	Functional group
benzaldehyde	47	54.5	8.5	aldehyde
benzyl alcohol	6.75	N/A	0.5	alcohol
benzyl benzoate	0.2	0.25	N/A	ester
benzyl salicylate	0.2	0.25		ester
3*Z*-hexen-1-ol	0.1	N/A	N/A	alcohol
indole	N/A	N/A	0.5	N-containing compound[b]
isoeugenol	N/A	N/A	4	alcohol
methyl salicylate	1.5	2	N/A	ester
cis/*trans* ocimene	N/A	N/A	46.5	terpene
phenylacetaldehyde	37	43	31	aldehyde
2-phenylacetaldehyde oxime	0.25	N/A	N/A	N-containing compound
phenylethyl alcohol	4.5	N/A	9	alcohol
phenylnitroethane	2.5	N/A	N/a	N-containing compound

[a]RSB = reduced synthetic blend lacking alcohols and nitrogen-containing compounds; [b]Nitrogen-containing compound

Table 3.3. Composition of the super-flower blend containing odorants selected from *Asclepias speciosa, A. syriaca, Tanacetum vulgare*, and *Platanthera obtusata.* Compounds were dissolved in 1 ml of pentane/ether (50/50).

Compound	Amount (µg)	Floral origin	Reference
acetophenone	8	*T. vulgare*	Peach et al. 2019
artemisia ketone	2	*T. vulgare*	Peach et al. 2019
Benzaldehyde	18	*A. speciosa*	this study
lilac aldehyde	10	*P. obtusata*	Lahondère et al. 2020
linalool oxide (pyranoid form)	5	*P. obtusata*	Lahondère et al. 2020
phenylacetaldehyde	19	*A. speciosa*	this study
phenyl-2,3-butandione	2	*T. vulgare*	Peach et al. 2019
2-methylbutanoic acid	20	*T. vulgare*	Peach et al. 2019
3-methylbutanoic acid	10	*T. vulgare*	Peach et al. 2019
2*E*-nonenal	5	*A. syriaca*	Otienoburu et al. 2012

Table 3.4. Relative amounts of headspace volatiles captured during 24-h aerations of *Asclepias* milkweed inflorescences. Numbers in columns represent the percentage of odorants in the floral blend.

Odorant	Percentage in blend				
	A. speciosa	*A. syriaca*	*A. incarnata* Cin	*A. incarnata* Ice	*A. tuberosa*
benzaldehyde	47	8.5	66.6	63.4	N/A
benzene-propanol	N/A	N/A	1.1	0.2	N/A
benzyl acetate	N/A	N/A	0.8	1	N/A
benzyl alcohol	6.5	0.5	28.2	27.9	N/A
benzyl benzoate	0.5	N/A	N/A	N/A	N/A
benzyl salicylate	0.2	N/A	N/A	N/A	N/A
E-caryophyllene	N/A	N/A	N/A	N/A	4
3*E*,7*E*-4,8-dimethyl-1,3,7-nonatriene	N/A	N/A	N/A	N/A	4
cis/trans-ocimene	N/A	46.5	N/A	N/A	N/A
E,E-α-farnesene	N/A	N/A	N/A	N/A	43
germacrene-D	N/A	N/A	N/A	N/A	9
3*Z*-hexenol	0.1	N/A	N/A	N/A	2
3*Z*-hexenyl acetate	N/A	N/A	N/A	N/A	20
3*Z*-hexenyl propionate	N/A	N/A	N/A	N/A	1
indole	N/A	0.5	N/A	N/A	N/A
iso-eugenol	N/A	4	N/A	N/A	N/A
linalool oxide	N/A	N/A	1	2	N/A
linalool oxide (pyranoid form)	N/A	N/A	0.2	1	N/A

methyl benzoate	N/A	N/A	1.6	N/A	N/A
methyl salicylate	2.4	N/A	0.1	0.2	1
α-pinene	N/A	N/A	N/A	N/A	2
phenylacetaladehyde	35.9	31	N/A	N/A	N/A
2-phenylethyl alcohol	4	9	0.5	3	N/A
2-phenylacetaldehyde oxime	1.3	N/A	N/A	N/A	N/A
phenylnitroethane	2.1	N/A	N/A	N/A	N/A
3*E*3,7*E*-4,8,12-trimethyl-1,3,7,11-tridecatetraene	N/A	N/A	N/A	N/A	13
Vanillin	N/A	N/A	0.1	1.2	N/A

Table 3.5. Suppliers and purities (Pur) of chemicals formulated in floral blends for behavioural responses of mosquitoes

Compound	Purity (%)	Supplier	Compound	Purity (%)	Supplier
acetophenone	99	S-A[a]	linalool oxide (pyranoid form)	98	Nippon[e]
artemisia ketone	98	Gries-lab	2-methylbutanoic acid	99	S-A
benzaldehyde	≥99	S-A	3-methylbutanoic acid	99	S-A
benzoic acid	99.5	S-A	2-methylpropionic acid	≥99	S-A
benzyl alcohol	≥95	Fisher[b]	methyl salicylate	95	S-A
benzyl benzoate	≥99	S-A	2*E*-nonenal	97	Bedoukian[c]
benzyl salicylate	≥89	Gries-lab	*cis/trans* ocimene	≥94	S-A
butanoic acid	99	S-A	phenylacetaladehyde	≥99	S-A
germacrene-D	40	Treatt[d]	phenyacetaladehyde oxime	75	Gries-lab
hexanoic acid	99	S-A	phenyl-2,3-butanedione	≥90	Gries-lab
3*Z*-hexenyl acetate	98	S-A	phenylethyl alcohol	≥97	Fluka[f]
3*Z*-hexen-1-ol	≥98	S-A	phenylnitroethane	98	Gries-lab
hexyl acetate	98	S-A	(−)-α-pinene	99	S-A
3-hydroxy-4-phenyl-2-butanone	25	Gries-lab	(−)-β-pinene	75	S-A

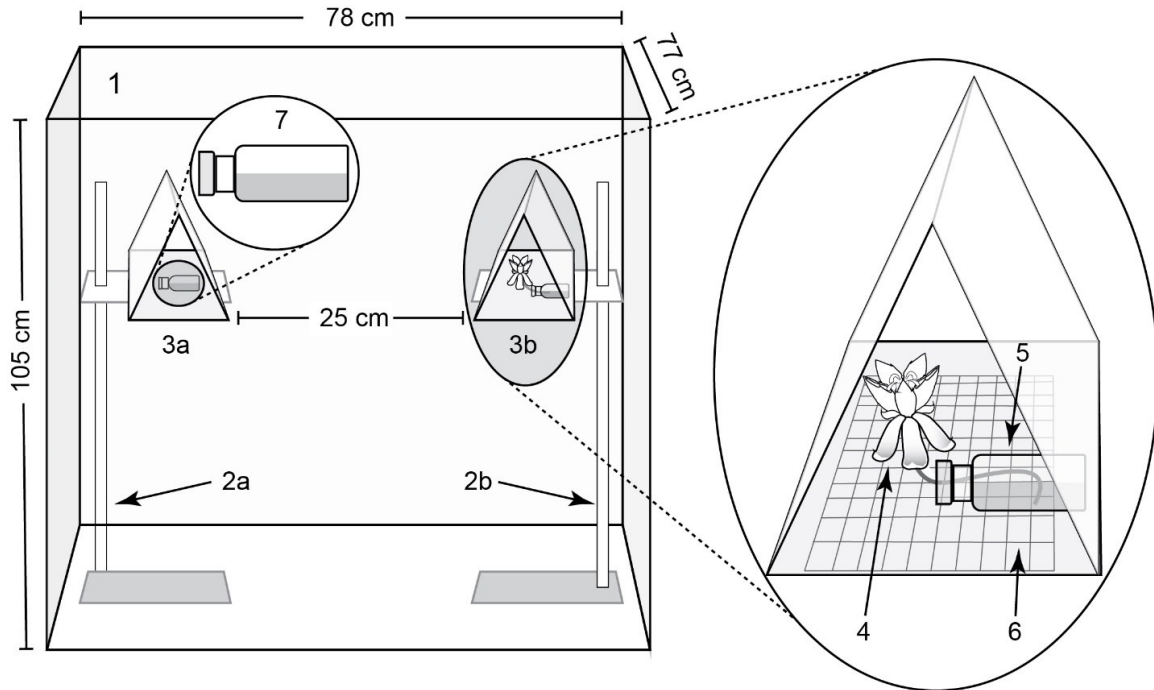|					
indole	≥99	S-A	(−)-sabinene	98	S-A
iso-eugenol	≥98	S-A	umbellulone	75	S-A
lilac aldehyde	95	Gries-lab	yomogi alcohol	≥98	Gries-lab

Figure 3.1. Schematic illustration of the experimental design used for behavioural bioassays with mosquitoes. A large mesh cage (1) was fitted with two Burette stands (2a, 2b) that supported adhesive-coated traps (3a, 3b). The treatment trap in each pair was baited with (*i*) florets of a milkweed inflorescence (4) whose stems were inserted through parafilm into a horizontally placed, water-containing vial (5) placed on a piece of cheesecloth (6) or (*ii*) a vial containing synthetic floret semiochemicals dissolved in organic solvent (pentane/ether) that were released through a hole (dimension, please) in the vial lid (6). The control trap in each pair received a vial containing either water or solvent without semiochemicals.
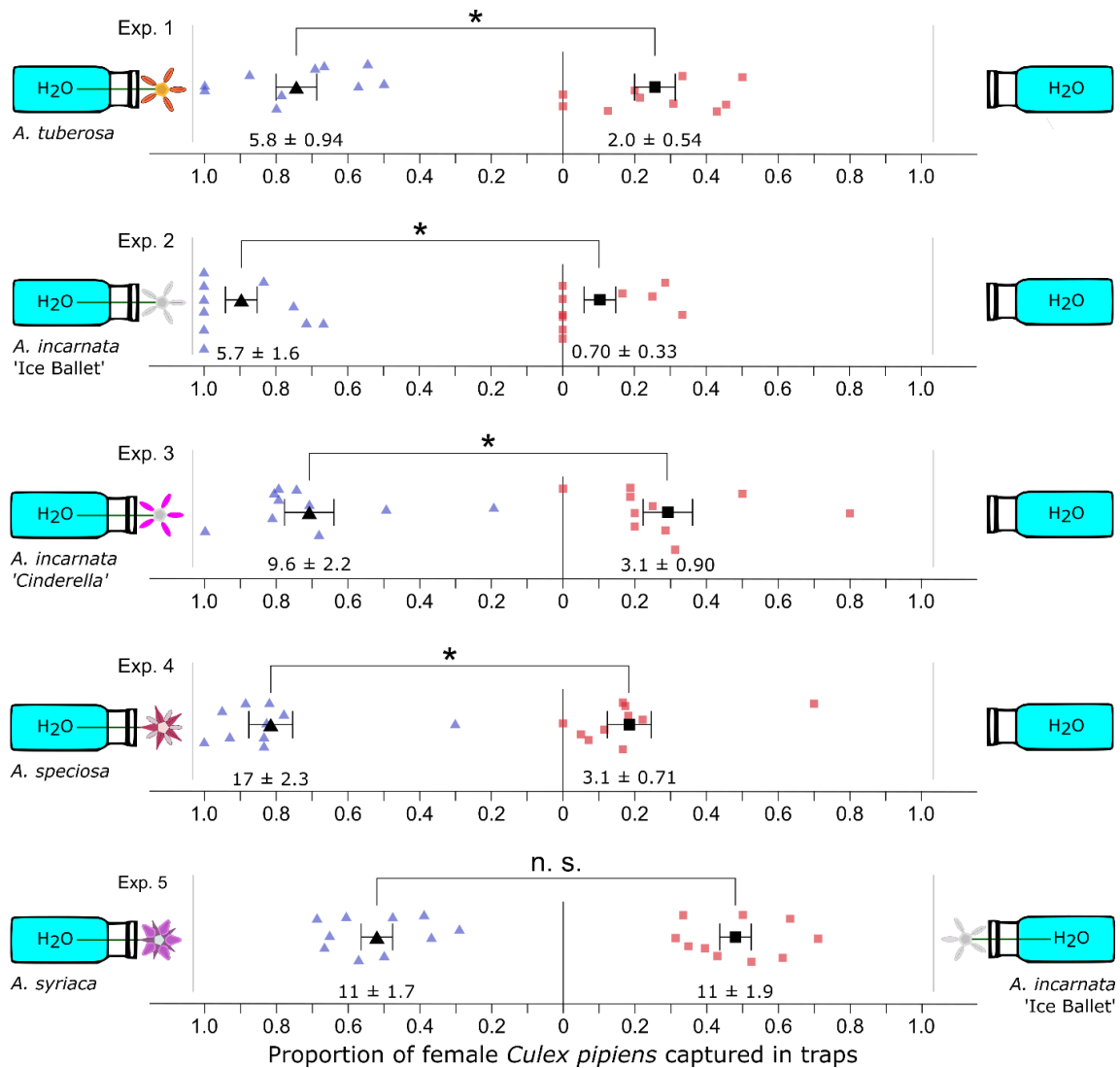
Figure 3.2. Proportions of *Culex pipiens* females captured in paired traps baited with florets of *Asclepias* milkweeds or left unbaited (water-filled vial without florets). For each experimental replicate, 50 females were released into a cage (Fig. 3.1) and allowed 24 h to respond to test stimuli. Coloured triangles and squares show the proportional data of individual replicates and black symbols the mean (± SE). The mean ± SE numbers of mosquitoes captured in traps in each experiment are listed above x-axes. An asterisk indicates a significant preference for a test stimulus (binary logistic regression model; $p < 0.05$; n. s. = not significant).
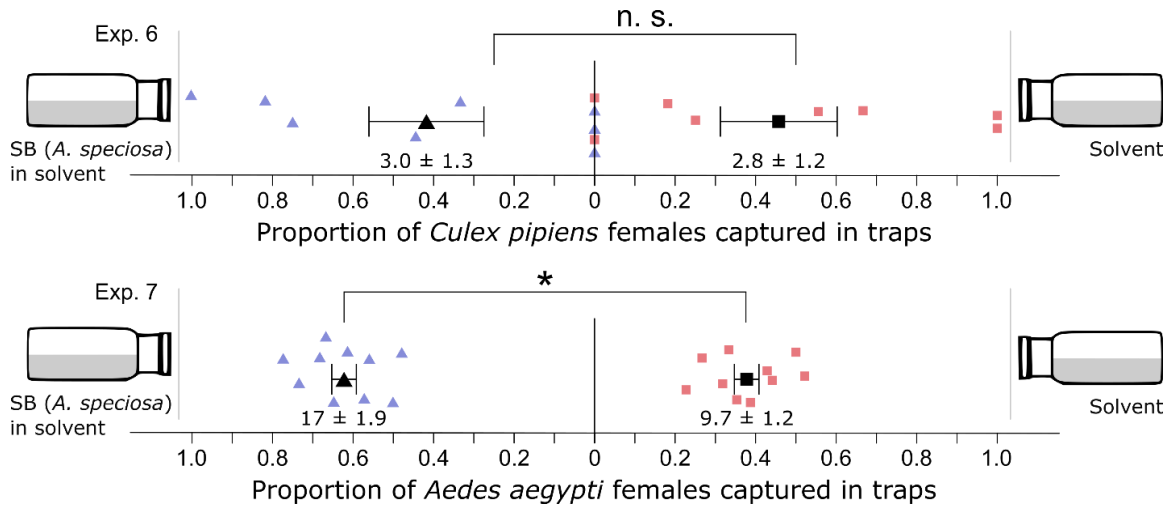
Figure 3.3. Proportions of *Culex pipiens* females (Exp. 6), and *Aedes aegpyti* females (Exp. 7), captured in paired traps baited with a complete synthetic blend (SB) of *Asclepias speciosa* floral odorants (Table 3.2) or left unbaited (solvent control). For each experimental replicate, 50 females were released into a cage (Fig. 3.1) and allowed 24 h to respond to test stimuli. Coloured triangles and squares show the proportional data of individual replicates and black symbols the mean (± SE). The mean ± SE numbers of mosquitoes captured in traps in each experiment are listed above x-axes. An asterisk indicates a significant preference for a test stimulus (binary logistic regression model; p < 0.05; n. s. = not significant).
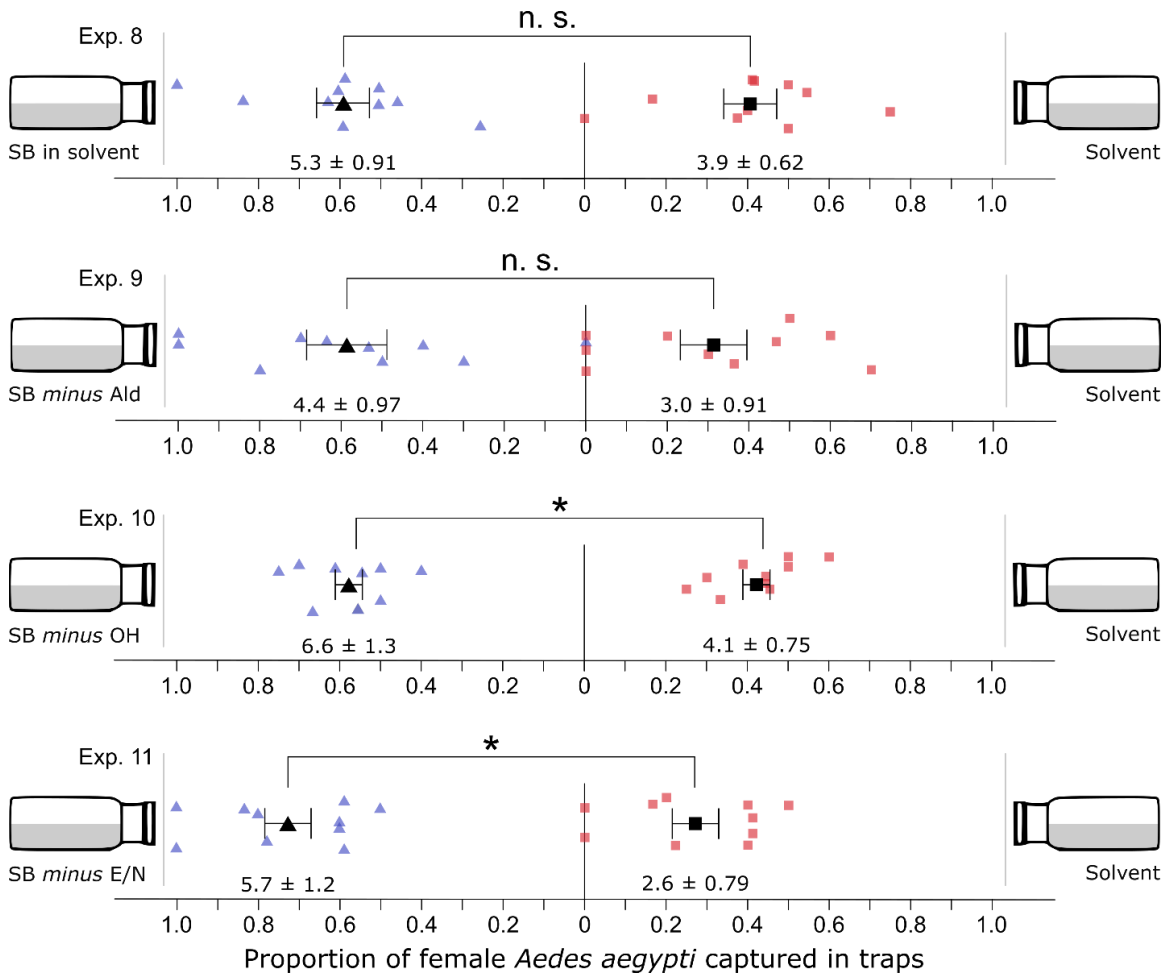
Figure 3.4. Proportions of *Aedes aegypti* females captured in traps baited with either a complete synthetic blend (SB) of *Asclepias speciosa* floral odorants (Table 3.2) (Exp. 8) or with partial blends lacking aldehydes (Ald; Exp. 9), alcohols (OH; Exp. 10), or esters and nitrogen-containing compounds (E/N; Exp. 11), all blends formulated in solvent. Vials containing only solvent served as controls. For each experimental replicate, 50 females were released into a cage (Fig. 3.1) and allowed 24 h to respond to test stimuli. Coloured triangles and squares show the proportional data of individual replicates and black symbols the mean (± SE). The mean ± SE numbers of mosquitoes captured in traps in each experiment are listed above x-axes. An asterisk indicates a significant preference for a test stimulus (binary logistic regression model; $p < 0.05$; n. s. = not significant).
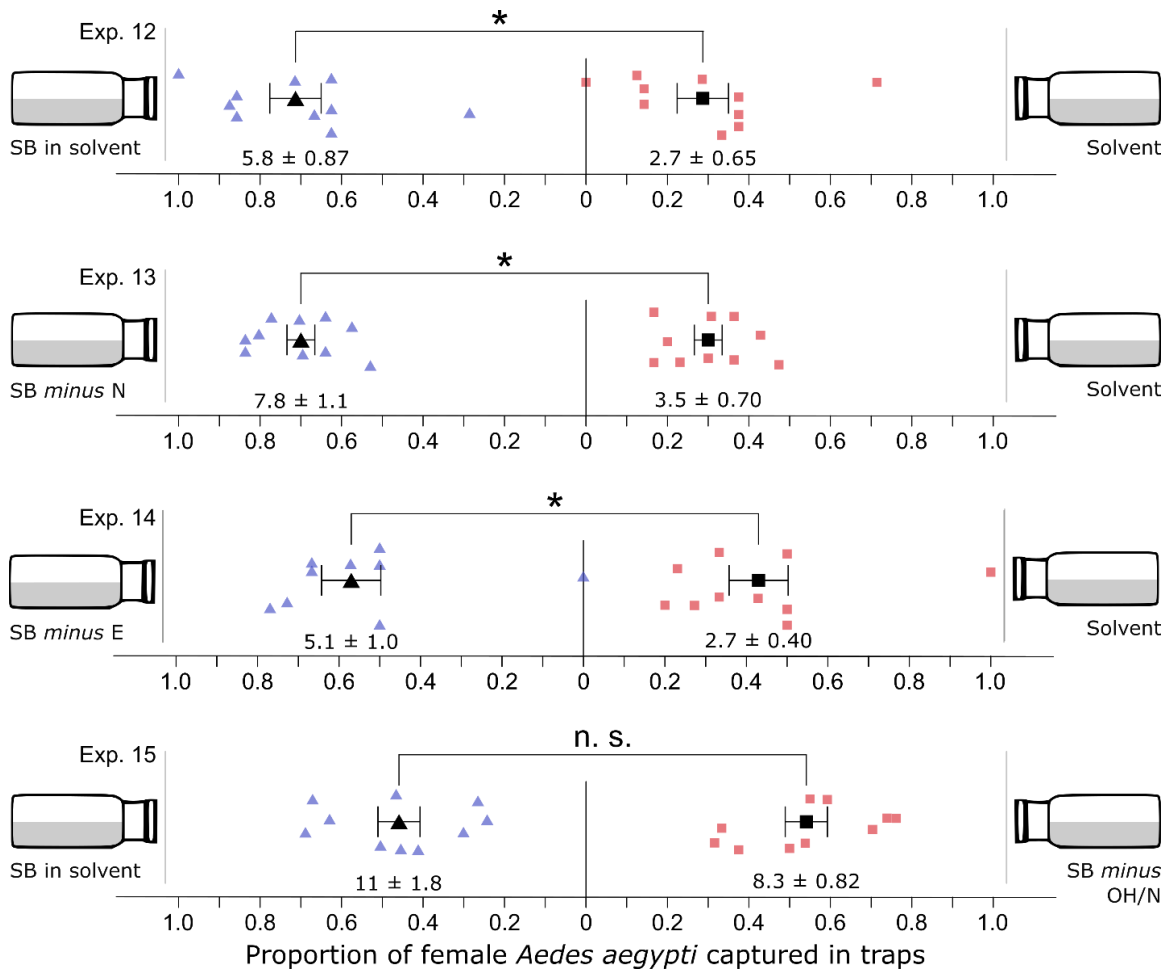
Figure 3.5. Proportions of *Aedes aegypti* females captured in traps baited with either a complete synthetic blend (SB) of *Asclepias speciosa* floral odorants (Table 3.2) (Exps. 12, 15) or with partial blends lacking nitrogen-containing compounds (N; Exp. 13), esters (E; Exp. 14), or alcohols and nitrogen-containing compounds (OH/N; Exp.15), all blends formulated in solvent. Vials containing only solvent served as controls (Exps. 12-14). For each experimental replicate, 50 females were released into a cage (Fig. 3.1) and allowed 24 h to respond to test stimuli. Coloured triangles and squares show the proportional data of individual replicates and black symbols the mean (± SE). The mean ± SE numbers of mosquitoes captured in traps in each experiment are listed above x-axes. An asterisk indicates a significant preference for a test stimulus (binary logistic regression model; $p < 0.05$; n. s. = not significant).
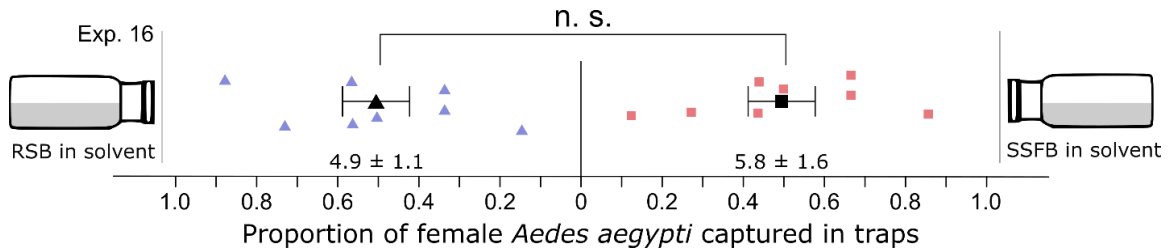
Figure 3.6. Proportions of *Aedes aegypti* females captured in traps baited with either the proven-effective reduced synthetic blend (RSB) of *Asclepias speciosa* floral odorants lacking the behaviourally benign alcohols and nitrogen-containing compounds (Table 3.2) or with a synthetic super-flower blend (SSFB) containing floral semiochemicals of *A. speciosa*, *Tanacetum vulgare* and *Platanthera obtusata* (Table 3.3), both blends formulated in solvent. For each experimental replicate, 50 females were released into a cage (Fig. 3.1) and allowed 24 h to respond to test stimuli. Coloured triangles and squares show the proportional data of individual replicates and black symbols the mean (± SE). The mean ± SE numbers of mosquitoes captured in traps are listed above the x-axis. An asterisk indicates a significant preference for a test stimulus (binary logistic regression model; n. s. = not significant).
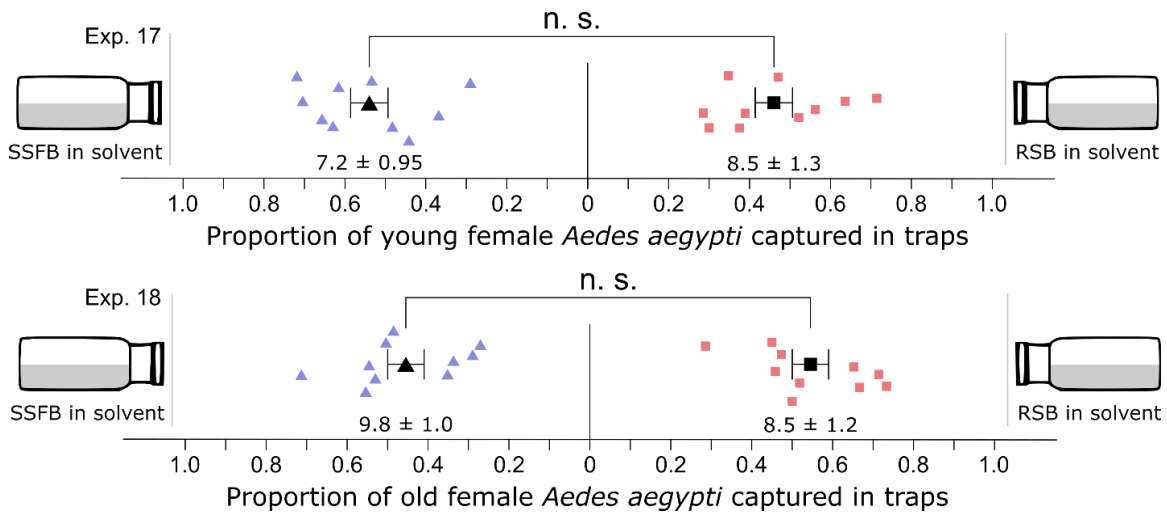
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Figure 3.7. Proportions of young (1- to 3-day-old) and old (5- to 10-day-old) female *Ae. aegypti* captured in traps baited with either the proven-effective reduced synthetic blend (RSB) of *Asclepias speciosa* floral odorants lacking the behaviourally benign alcohols and nitrogen-containing compounds (Table 3.2) or with a synthetic super-flower blend (SSFB) containing floral semiochemicals of *A. speciosa*, *Tanacetum vulgare* and *Platanthera obtusata* (Table 3), both blends formulated in solvent. For each experimental replicate, 50 females were released into a cage (Fig. 3.1) and allowed 24 h to respond to test stimuli. Coloured triangles and squares show the proportional data of individual replicates and black symbols the mean (± SE). The mean ± SE numbers of mosquitoes captured in traps in each experiment are listed above x-axes. An asterisk indicates a significant preference for a test stimulus (binary logistic regression model; n. s. = not significant).

Concluding summary

In this concluding summary, I will present, in point form, my key findings and their implications.

Chapter 2

- During mate location, *Ae. aegypti* males utilize female light flash and wingbeat sound signals at long and short range, respectively.
- Increasing the number of light flash signals increased their attractiveness to male *Ae. aegypti*.
- The wavelength (UV or blue) of the light flash signals did not affect their attractiveness to male *Ae. aegypti*.
- Male *Ae. aegypti* failed to respond to the synthetic female pheromone component ketoisophorone, when it was presented together with the bimodal complex of light and sound signals.
- Female *Ae. aegypti* were attracted to the light flash signal of males.
- Crepuscular swarming *Cx. pipiens* males did not respond to light flash signals.
- My findings support the hypothesis that *Ae. aegypti* mosquitoes utilize multimodal signals during mate-finding behavior, analogous to their use of multimodal cues during host-seeking and floral-foraging behavior. My findings have implications for the design of future mosquito control programs.

Chapter 3

- Inflorescenses of *Asclepias* spp. are attractive to female *Cx. pipiens.*
- A 10-component synthetic *Asclepias speciosa* blend attracted females of *Ae. aegypti* but not *Cx. pipiens.*
- Synthetic blends of *A. speciosa* inflorescences became more attractive to *Ae. aegypti* females when certain blend constituents (alcohols, esters, nitrogen-containing compounds) were omitted.
- A synthetic "super-flower" blend comprising select floral odorants from *Asclepias speciosa*, *Tanacetum vulgare* and *Platanthera obtusata* was not more attractive to *Aedes aegypti* females than the *A. speciosa* blend.

- This study corroborates the findings reported in previous studies that synthetic floral blends can be deployed to attract mosquitoes, and that the blends' attractiveness can be modified by the addition or subtraction of components.
- My findings have implications for the development of toxic sugar baits as tactics in mosquito control programs.