Visual and Olfactory Cues used by the Apple Clearwing Moth, *Synanthedon myopaeformis* (Lepidoptera: Sesiidae), to locate Inflorescences of Showy Milkweed

by

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Abstract

The apple clearwing moth, *Synanthedon myopaeformis* Borkhausen (Lepidoptera: Sesiidae), is a recently introduced pest of apples in Canada. In British Columbia, adult moths are attracted to, and feed upon, visually conspicuous and fragrant inflorescences of showy milkweed, *Asclepias speciosa* (Torrey). I analyzed visual and olfactory cues that might mediate this behaviour. Histological studies of *S. myopaeformis* eyes revealed apposition ommatidia. In electroretinograms, *S. myopaeformis* eyes were sensitive to ultraviolet (UV) and green wavelengths, indicating their potential for dichromatic vision. However, inflorescences of *A. speciosa* do not reflect UV light, and field experiments revealed that *S. myopaeformis* relies mostly on semiochemicals to locate the inflorescences.   Floral semiochemicals were analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD), GC-mass spectrometry and proboscis extension reflex bioassays. Among the > 10 candidate floral semiochemicals field-tested, phenylacetaldehyde was the most attractive semiochemical for *S. myopaeformis*. Phenylacetaldehyde shows promise as a lure to monitor or mass-trap *S. myopaeformis.*

Keywords: *Synanthedon myopaeformis*; *Asclepias speciosa*; visual and olfactory cues; insect-plant interaction; Sesiidae

Dedication

To my parents, for their unending love, encouragement and support.

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1.   Introduction

1.1. Sensory modalities used by nectar-feeding Lepidoptera

Many adult Lepidoptera feed on floral nectar to meet their energetic needs (e.g. May 1992). Long-distance orientation to flowers by both moths and butterflies can be mediated by visual and/or olfactory cues, whereas tactile and gustatory cues play a more dominant role in stimulating feeding once an insect has arrived at a flower or inflorescence, although visual cues such as UV nectar guides can also be utilized (Krenn 1998, Goyret and Raguso 2006). Visual cues include, but are not limited to spectral reflectance, intensity, contrast, and shape, but most studies have focused on spectral reflectance (Dafni and Kevan 1997).

With most visual cues inconspicuous in the dark, nocturnal moths are commonly presumed to rely on olfactory cues, whereas diurnal butterflies and moths are thought to utilize visual cues alone or in conjunction with olfactory cues (Dobson 1994). However, many studies have shown that the primary food-foraging cue can differ greatly among species. Some diurnal Lepidoptera rely on either visual (Ômura and Honda 2005, Balkenius et al. 2006) or olfactory cues exclusively (Pellmyr 1986), while others require a combination of both visual and olfactory cues (Naumann et al. 1991). Some even possess innate olfactory preferences, although visual cues are primarily used (Ômura et al. 1999, Andersson and Dobson 2003, Andersson 2003). In nocturnal moths, reliance on olfactory cues is prevalent (Brantjes 1978, Haynes et al. 1991, Tingle and Mitchell 1992, Plepys et al. 2002, Balkenius et al. 2006), although some moths use both visual and olfactory cues (Raguso and Willis 2005, Goyret et al. 2007).

1.2. Visual system of Lepidoptera

1.2.1.   Anatomy of the visual system

The visual system of moths and butterflies typically consists of three dorsal ocelli and two large compound eyes. Consisting of a single lens, ocelli are simple eyes that measure light intensity and are generally considered to be important for maintaining level flight (Wilson 1978). Compound eyes are essentially a collection of simple eyes called ommatidia that function together to form an image, and are the primary visual sensory organs. Each ommatidium consists of a dioptric and a retinular portion (Goldsmith and Bernard 1974). The dioptric region consists of a corneal lens and a crystalline cone, which is surrounded by primary/iris pigment cells (Warrant et al. 2003, Stavenga 2005). Secondary pigment cells surround the retinular region that consists of 4-16 (typically 8-9) elongated retinular cells (sensory neurons), each of which contributes a rhabdomere (densely packed, photoreceptor-containing microvilli) to form a fused rhabdom at the centre of each ommatidium (Warrant et al. 2003).

1.2.2.   Types of ommatidia

Ommatidia can be broadly classified into two groups, based on the anatomical structure of their light-gathering apparatus. These two structural groups are referred to as "apposition" or "superposition" configurations. Apposition type eyes are typical of diurnal Lepidoptera, whereas superposition eyes are typical of nocturnal Lepidoptera. In apposition eyes, each ommatidium gathers light via the dioptric region and directly focuses the light onto the underlying retinula (rhabdom), which is continuous from the base of the crystalline cone to the basement membrane (Figure 1.1; Warrant et al. 2003). In apposition eyes, the iris and secondary pigment cells help to isolate each ommatidium by absorbing light impinging from neighbouring ommatidia (Warrant et al. 2003). In superposition eyes, the dioptric region and retinula are separated by a clear zone (Figure 1.2; Warrant et al. 2003). Rhabdoms are restricted to the proximal part of the ommatidia and are sometimes connected to the crystalline cones by a thin thread known as a crystalline tract, formed by rhabdomere-less portions of retinular cells (Goldsmith and Bernard 1974, Warrant et al. 2003). The clear zone is made of secondary pigment cells, whose pigments migrate distally when in a dark-adapted state

(Warrant et al. 2003). This allows light gathered from several lenses to be focused onto a single rhabdom, thus maximizing use of the amount of light available (Land 1981, Stavenga 2005). In superposition and apposition eyes, a light reflecting layer of tracheoles at the base of the ommatidia, the "tapetum", may be present to increase light capture (Warrant et al. 2003).

1.2.3.   *Processing the information contained in light*

The dioptric region gathers light from a set visual field and focuses this light onto the retinula, where the light is absorbed by photoreceptors, which generates an action potential that travels down the axon of the retinular cell to the brain (Stavenga 2005). Lepidoptera typically have ultraviolet (UV), blue and green photoreceptors, each sensitive to a specific range of electromagnetic radiation. Some species are thought to have a fourth receptor type that typically has maximum sensitivity in the red range of the visible spectrum (Briscoe and Chittka 2001). The functional unit of a photoreceptor is the visual pigment (Kelber 2006). Visual pigments are rhodopsins which are made up of an opsin protein that is coupled to a chromophore (retinal, 3,4-dihydroretinal, and 3-hydroxyretinal) (Stavenga 2005, Kelber 2006). The spectral tuning of a photoreceptor comes from the combination of the opsin amino acid sequence and the chromophore, as well as from the presence of additional pigments (Kelber 2006).

Lepidoptera can visually process light as either a chromatic (colour-containing) or achromatic (colour-less) cue. Colour vision is used to process chromatic cues and requires two photoreceptors allowing for discrimination of light based on wavelength, not intensity (Menzel 1979, Kelber 2006). The use of achromatic cues requires only one photoreceptor and relies on differences in the intensity of light (Kelber 2006). Polarized light is considered an achromatic cue, the perception of which requires only one photoreceptor, and specific structural arrangements of the microvilli in the rhabdom (Kelber 2006, Wehner and Labhart 2006).

1.3. Olfactory system of Lepidoptera

The olfactory system of moths and butterflies consists of the palps and the antennae, with the antennae being the primary sense organs. The antennae are

covered with various chemoreceptors called sensilla (e.g. hairs, pegs, plates and pits) that detect volatile, chemical odourants that insects use as olfactory cues or signals to locate resources (Hansson 1995). There are several distinct types of sensilla (e.g. basiconic, trichoid, coelconic, and auricillic) that differ in morphology (Hansson 1995). The cuticular surfaces of sensilla contain pores through which odourant molecules enter. The sensillar lymph contains high concentrations of odourant-binding proteins (OBPs) that capture pheromone components or general odourants and transport them to odourant receptors located on the dendritic ends of olfactory receptor neurons (Pelosi and Maida 1995, Sato and Touhara 2009). Binding of the odourant molecule, or ligand, to the odourant receptor generates an action potential that is transmitted to the brain (Sato and Touhara 2009). Unlike general (e.g. plant) odour receptors, pheromone receptors are highly specific (Hansson 1995, Sato and Touhara 2009).

1.4. Pest status of *Synanthedon myopaeformis* Borkhausen

Synanthedon myopaeformis (Borkhausen), the apple clearwing moth (Alford 2007), sometimes called the small red-belted clearwing moth, is a diurnal sesiid species native to Eurasia and North Africa (Zhang 1994) that was recently introduced into Canada (Beaton and Carter 2006, Philip 2006). Prior to the development of dwarfing apple tree production systems, *S. myopaeformis* was considered a secondary pest of apple trees. With a world-wide shift to cultivation of higher-density apple plantings where scion apple varieties are grafted onto clonal dwarfing and semi-dwarfing rootstocks, *S. myopaeformis* has become a significant, yield-affecting pest wherever it occurs (Dickler 1976, Balázs et al. 1996, Ateyyat and Al-Antary 2006). This new cultivation practice causes burr knots (adventitious roots) to form at the rootstock-scion graft union, which female moths seek as preferred oviposition sites (Dickler 1976, Balázs et al. 1996, Ateyyat and Al-Antary 2006). Larvae feed between the bark and the wood and cause a slow decline in the trees, either through direct feeding damage (girdling) or by creating entry points for secondary pests or pathogens (Voerman et al. 1978, Sahinoglou et al. 1998, Ateyyat 2006, Kutinkova et al. 2006).

The economic threshold for *S. myopaeformis* is two pupal exuvia per tree in trees < 6-year-old, and 20 pupal exuvia per tree in older/larger trees (Sahinoglou et al. 1998), or 8-10 larvae per tree (Erler 2010).

1.5. Life history of *Synanthedon myopaeformis*

Their life cycle takes circa two years, with adults emerging from late May/early June until late August/early September at most latitudes (Dickler 1976, Blommers and Freriks 1988, Judd 2008).

1.5.1. Eggs

Gravid females have a complement of circa 250 small, brown, ovoid eggs (Figure 1.3) of which circa 60 are laid singly in cracks, crevices and injury points of host plant bark, preferring injury points for oviposition (Stüber and Dickler 1988). Eggs hatch within 9-14 days in the field (Stüber and Dickler 1988).

1.5.2. Larvae

Larvae are slightly dorsoventrally flattened, and cream-coloured with a sclerotized, reddish-brown head (Figure 1.4; Alford 2007). There are seven larval instars, of which the seventh instar can reach up to 20 mm in length (Stüber and Dickler 1988, Alford 2007). Instars 3-7 overwinter as a result of the long egg-laying period (Stüber and Dickler 1988). There is no obligate diapause (Stüber and Dickler 1988). Larvae burrow beneath the bark, creating irregular galleries, where they develop for approximately 20 months (Alford 2007). At the end of development (April, May, June), larvae spin a silk cocoon near the surface of the bark using frass and sawdust (Figure 1.5; Špatenka et al. 1999, Alford 2007).

1.5.3. Pupae

The pupal stage takes about one month. Pupae range in size between 10 and 13 mm in length, and are a light brown colour (Figure 1.5; Stüber and Dickler 1988, Alford 2007).

1.5.4. Adults

Adults have a wingspan of 14-25 mm, black antennae and a white border around the eyes (Figure 1.6; Laštůvka and Laštůvka 2001). The wings are transparent, with black veins and borders (Alford 2007). Males have a long, slender body that is black with a bluish sheen except for the fourth tergite which is red, and sternites 4-6 which are white (Špatenka et al. 1999), and terminates in a fan-like tuft of scales with white anal flaps below (Laštůvka and Laštůvka 2001). Females have a shorter, fatter body that is black with a bluish sheen except for the fourth tergite and sternite which are red (Špatenka et al. 1999), and also terminates in a tuft of scales (Laštůvka and Laštůvka 2001).

Adults emerge in a 50:50 sex ratio, with males emerging slightly earlier than females (Stüber and Dickler 1988, Judd 2008). Pupal exuvia are often left protruding from the tree upon moth emergence (Figure 1.7).

1.5.5. Larval host plants

Apples (*Malus* spp.) are the most common host plant, although other trees and shrubs in the Rosaceae family have been recorded as hosts, including hawthorn (*Crataegus* spp.), *Sorbus* spp., plums, cherries, apricots, peaches and almonds (*Prunus* spp.), cherry (*Cerasus* spp.), *Padus* spp., the common medlar (*Mespilus germanica*), pear (*Pyrus* spp.), and the common sea-buckthorn [*Hippophae rhamnoides* (Elaeagnaceae)] (Špatenka et al. 1999).

1.6. Efforts to control *S. myopaeformis*

Control of *S. myopaeformis* is difficult because of the cryptic feeding habit of the larvae and the long emergence period of the adults (Deseö and Miller 1985, Bosch et al. 2001, Erler 2010). In Europe, conventional control of *S. myopaeformis* has historically been based on insecticidal sprays of organophosphates (Dickler 1976, Castellari 1987, Blommers and Freriks 1988, Kilic et al. 1988, Balázs et al. 1996, Ateyyat 2006, Ateyyat and Al-Antary 2006, Erler 2010), synthetic pyrethroids (Blommers and Freriks 1988), and carbamates (Balázs et al. 1996), although benzamides (Balázs et al. 1996),

benzolylureas (Balázs et al. 1996), and *Bacillus thuringiensis* Berliner (Balázs et al. 1996, Shehata et al. 1999) have also been used. In British Columbia (BC), two spinosad products ("Entrust" and "Success") have received registration for control of *S. myopaeformis* (British Columbia Ministry of Agriculture and Lands 2011), and an insect growth regulator product "Rimon" has recently been approved for registration (G.J., personal communication).

When *S. myopaeformis* populations are small, the synthetic sex pheromone of *S. myopaeformis,* (*Z,Z*)-3, 13 octadecadienyl acetate (Judd et al. 2011), can be deployed to provide effective control either through disorientation (Voerman and Van Deventer 1984, Stüber and Dickler 1987, Blommers and Freriks 1988, Kyparissoudas and Tsourgianni 1993, Trematerra 1993, Bosch et al. 2001) or mass-trapping of male moths (Trematerra 1993, Bosch et al. 2001, Aurelian 2011).

In Europe, natural enemies of *S. myopaeformis* have been identified, but occur at rates too low to be considered for biological control (Soentgen and Sengonca 1988, Stüber and Dickler 1988). Biological control of *S. myopaeformis* has been attempted with *Steinernema* and *Heterorhabditis* nematodes with limited success (Deseö and Miller 1985, Kahounova and Mracek 1991). In BC, the fungus *Metarhizium brunneum* (Petch) has been identified from cadavers of *S. myopaeformis*, and along with *Beauvaria bassiana* (Balsamo) Vuillemin are considered to be good candidates for biocontrol in the field (Cossentine et al. 2010).

Other control measures include killing larvae with wire, coatings or wrappings of tree trunks, and preventative wound healing (Ciglar and Masten 1977, Ciglar and Masten 1979, Ateyyat and Al-Antary 2006, Erler 2010). These measures can be effective, but are very labour intensive.

Liquid baits, consisting of mixtures of various juices/wines, molasses/sugar, vinegar, and brewers yeast, attract both male and female *S. myopaeformis*, and have been used to assess efficacy of treatments, especially mating disruption (Ciglar and Masten 1979, Van Frankenhuyzen and Wijnen 1979, Stüber and Dickler 1987, Blommers and Freriks 1988, Kilic et al. 1988, Kyparissoudas and Tsourgianni 1993, Onucar and Ulu 1995, Bosch et al. 2001). The use of liquid baits, like grape juice, to

mass-trap male and female *S. myopaeformis* has been investigated in BC (Aurelian 2011), but commercialization of this approach seems unlikely because the baits are messy and labour intensive to maintain. Identification of volatiles from grape juice, which appear to be as attractive as sex pheromone (Aurelian et al. 2012), seems a more promising approach. Recently, a binary combination of ethyl-2,4-decadienonate and acetic acid was shown to attract to male and female *S. myopaeformis*, albeit at lower levels than with the sex pheromone (Tóth et al. 2012).

1.7. Nectar-feeding of *S. myopaeformis*

In Europe, adult *S. myopaeformis* have been observed feeding on various flower species (Popescu-Gorj et al. 1958, Injac and Tosevski 1987). I observed the same phenomenon in BC. In an exploratory floral-choice study in apple orchards in the Similkameen Valley of BC, *S. myopaeformis* appeared to preferentially feed from inflorescences of showy milkweed, *Asclepias speciosa* (Torrey).

1.8. Distribution and biology *Asclepias speciosa* Torrey

Asclepias speciosa (Torrey) is a rhizomatous perennial herb occurring at low elevations in moist soil from south-central and south-eastern BC, east to Manitoba, and south to Missouri, Texas and California (Douglas et al. 1998). This milkweed has opposite leaves, inflorescences in an umbrella-shaped cluster, and can reach up to 1.2 m in height (Figure 1.8; Douglas et al. 1998). Flowering occurs between June and August (Burbridge 1989). The flowers are complex, with five reflexed pink to reddish-purple petals, and five erect white to pink hoods with a "horn" appendage that curves inwards to the gynostegium (Figure 1.9; Bookman 1981, Burbridge 1989). The gynostegium is comprised of two fused pistils to form a flat stigma head with five lateral stigmatal chambers formed by the fusion of five stamens (Bookman 1981). The stigmatal chambers have a narrow entrance formed by the wings of adjacent anthers (Figure 1.10; Bookman 1981). Within the stigmatal chamber are pairs of pollinia (pollen sacs) that are connected by translator arms to a corpusculum (Bookman 1981).

The flowers are fragrant and secrete copious amounts of nectar, which attracts many pollinators (Bookman 1981, Burbridge 1989). While feeding, an insect inserts its tarsus into the stigmatal chamber and when it tries to fly away, the upward pulling motion causes the corpusculum to snap onto the tarsus, thus attaching the pollinia to the insect (Burbridge 1989). Visiting insects must be sufficiently robust to pull their leg free from the flower, or risk losing life or limb (McNeil 1977). After successful pollination, hairy pods are formed which dehisce along one side at maturity to release flat seeds with tufts of silky hair (Antos et al. 1996). *Asclepias speciosa,* like other milkweeds, serves as a host-plant for monarch butterflies (*Danaus plexippus* Linnaeus), whose larvae sequester cardenolides from the milkweed latex (Ladner and Altizer 2005).

1.9. Research objectives

My observations that adult male and female *S. myopaeformis* often feed on the visually conspicuous and fragrant inflorescences of *A. speciosa* led to research aimed at determining whether specific visual and/or olfactory cue(s) might mediate their flower-finding behaviour. Identification of the cue(s) that strongly attract female apple clearwing moths might become useful in developing tools for mass trapping and thus controlling populations of *S. myopaeformis*.

The specific objectives of my research were to:

1) determine whether *S. myopaeformis* is attracted to inflorescences or foliage of *A. speciosa* (Chapter 2);

2) determine the relative importance of visual and olfactory cues used by *S. myopaeformis* in locating *A. speciosa* (Chapter 2);

3) identify candidate semiochemicals from *A. speciosa* by gas chromatographic-electroantennographic detection and gas chromatography-mass spectrometry (Chapter 2);

4) bioassay proboscis extension reflexes of *S. myopaeformis* in response to candidate semiochemicals from *A. speciosa* (Chapter 2);

5) field test selected candidate semiochemicals for attraction of *S. myopaeformis* (Chapter 2);

6) describe the microstructure of the compound eyes of *S. myopaeformis* and determine whether their eyes have apposition or superposition ommatidia (Chapter 3);

7) determine the spectral sensitivity of the compound eyes of *S. myopaeformis* using electroretinograms (Chapter 3); and

8) obtain spectral reflectance curves from *A. speciosa* (Chapter 3).

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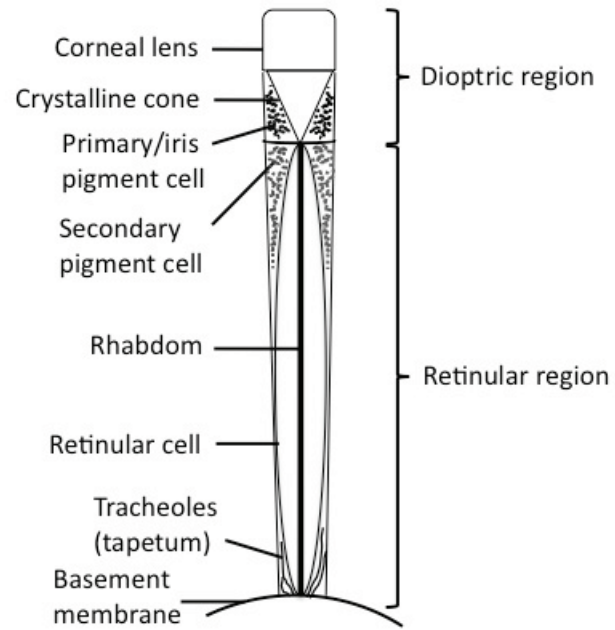
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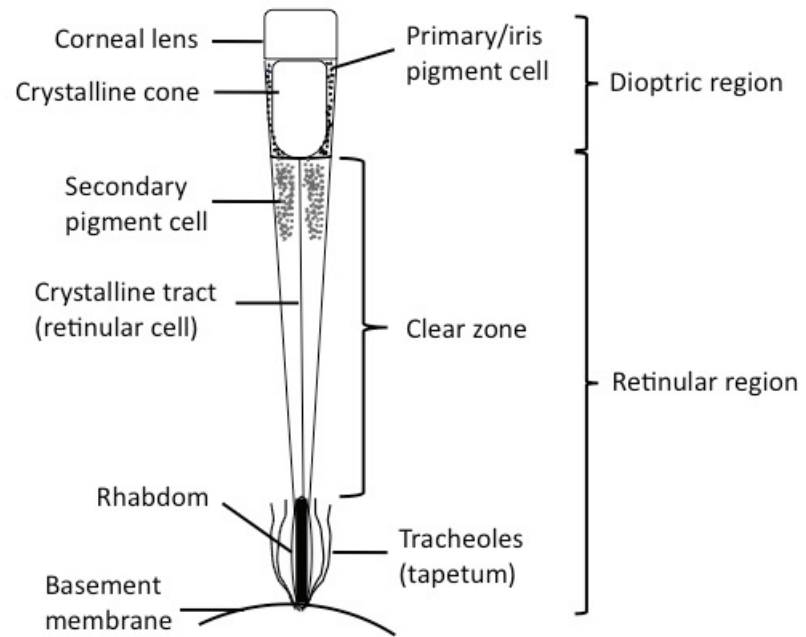
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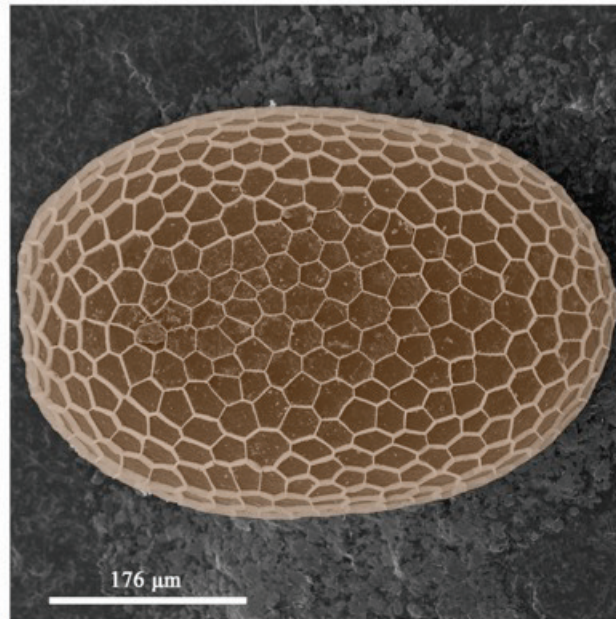
Figure 1.3. ***Artificially coloured scanning electron micrograph of an S. myopaeformis egg (Photo credit: Michael Weis).***

Figure 1.4. S. myopaeformis *larva in an apple tree (Photo credit: Mark Gardiner).*

Figure 1.5. **Cocoons and pupae of** S. myopaeformis *(Photo credit: Mark Gardiner).*

Figure 1.6. *A pair of mating* S. myopaeformis*. The larger female is on the upper right, and the smaller male is on the lower left. Note that the male has white sternites while the female does not.*

Figure 1.7 **Pupal exuvia of** S. myopaeformis *protruding from an apple tree after moth emergence (Photo credit: Sean McCann).*

Figure 1.8. *Photograph of* A. speciosa *showing the umbrella-shaped inflorescence (Photo credit: Mark Gardiner).*
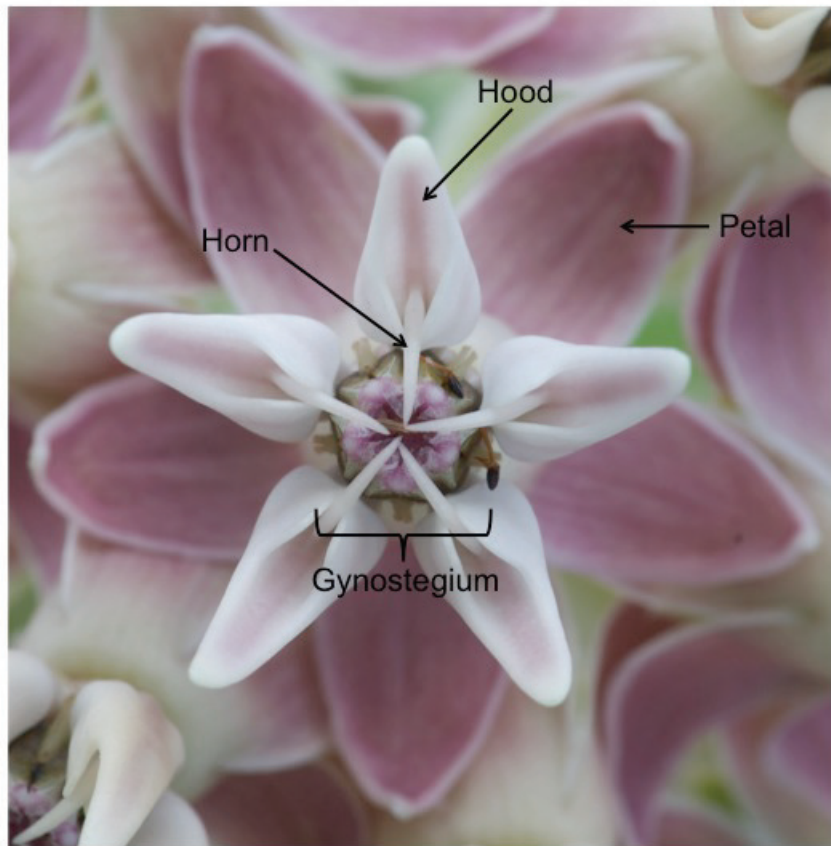
Figure 1.9. ***Front-view photograph of an*** A. speciosa *flower showing the petals, hoods, horns and gynostegium (Photo credit: Mark Gardiner).*
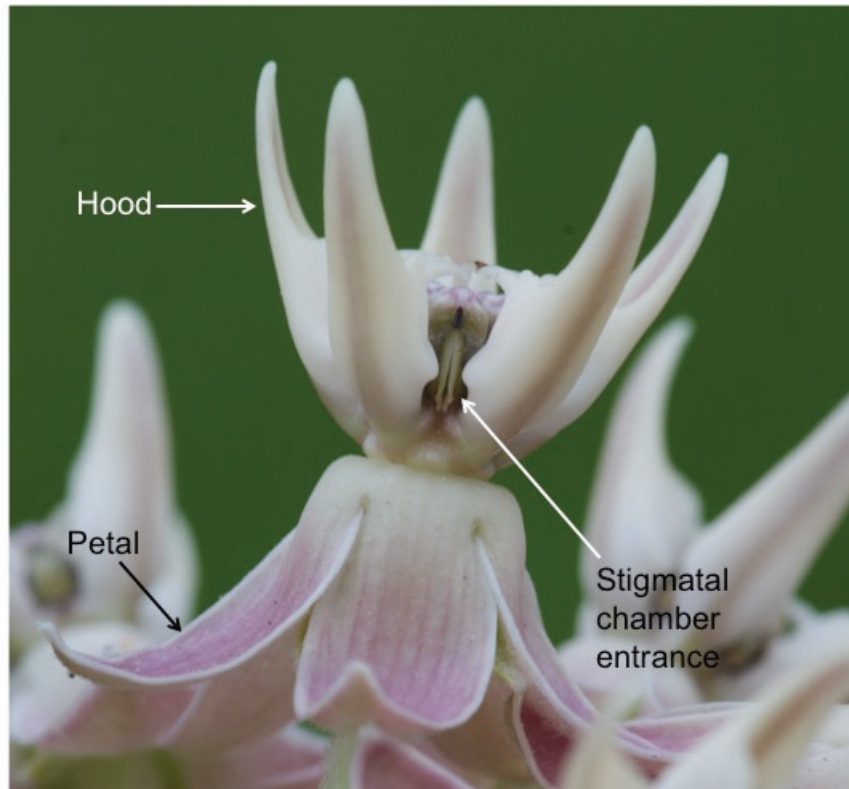
Figure 1.10.   Side-view photograph of an A. speciosa flower showing the reflexed petals, upright hoods and a stigmatal chamber entrance (Photo credit: Mark Gardiner).

2. Phenylacetaldehyde attracts male and female apple clearwing moths, *Synanthedon myopaeformis*, to inflorescences of showy milkweed, *Asclepias speciosa*[*]

2.1. Abstract

Synanthedon myopaeformis (Borkhausen) (Lepidoptera: Sesiidae) is a diurnal clearwing moth native to Eurasia that was recently introduced into British Columbia (BC) and Ontario, Canada, where it has become a serious pest in apples orchards. In BC, these moths commonly feed on nectar of inflorescences, particularly that of showy milkweed, *Asclepias speciosa* (Torrey). We investigated the relative importance of visual and olfactory cues, and the key floral semiochemical(s) mediating attraction of *S. myopaeformis* to *A. speciosa*. In field experiments, inflorescences left exposed or enclosed in dyed-green cheesecloth bags induced similar visitation rates by moths, indicating olfactory cues are attractive. Among the > 10 floral odourants that elicited responses from moth antennae in coupled gas chromatographic-electroantennographic detection analyses, phenylacetaldehyde induced the most frequent proboscis extension reflexes among intact male and female moths. Among eight floral odourants that were field-tested singly, phenylacetaldehyde attracted 35 times more male and female moths than any other candidate semiochemical. Attractiveness of phenylacetaldehyde could not be enhanced by admixture with other floral odourants indicating that it alone may mediate attraction of *S. myopaeformis* to inflorescences of *A. speciosa*. The potential use of phenylacetaldehyde as a bait to monitor or mass-trap populations of male and female *S. myopaeformis* should be investigated.

2.2. Introduction

Floral nectar is a primary energy source for many species of Lepidoptera. Butterflies and moths locate rewarding inflorescences via innate and learned behaviours (Balkenius & Kelber, 2006; Riffell et al., 2008), often utilizing visual and/or olfactory cues associated with the flowers. The use of multi-modal cues may afford a foraging insect with a more accurate search image, but may force it to spend too much time and energy looking for a very specific image and is therefore considered costly (Balkenius et al., 2006), indicating that prioritizing one sensory mode may be beneficial.

The relative importance of visual and olfactory cues in locating floral resources has been well studied in social bees, e.g., *Apis melifera* (L.) and *Bombus* spp., but hardly examined in butterflies and moths. With visual cues mostly inconspicuous at night, nocturnal moths are commonly presumed to rely on olfactory cues, whereas diurnal butterflies and moths are thought to utilize visual cues on their own or in conjunction with olfactory cues.

Visual rather than olfactory cues typically, but not always, facilitate nectar foraging in diurnal butterflies and moths. Visual cues are exploited by the butterfly *Vanessa indica* (Herbst) (Ômura & Honda, 2005), the sphingid moth *Macroglossum stellatarum* (L.) (Balkenius et al., 2006), and pierid butterflies, even though the latter have an innate preference for olfactory cues (Ômura et al., 1999; Andersson, 2003). Several nymphalid butterflies exhibit an innate preference for olfactory cues over colour cues upon emergence, but switch preference after foraging experience (Andersson, 2003; Andersson & Dobson, 2003). Both visual and olfactory cues are needed to elicit feeding responses in the moth *Zygaena trifolli* (Esper) (Naumann et al., 1991), whereas fritillary butterflies require only olfactory cues to locate inflorescences of the bugbane, *Cimicifuga simplex* (Wormskjold) (Pellmyr, 1986).

Olfactory rather than visual cues facilitate nectar foraging in many species of nocturnal geometrid (Brantjes, 1978), noctuid (Brantjes, 1978; Haynes et al., 1991, Tingle & Mitchell, 1992; Dobson, 1994; Plepys et al., 2002b), and sphingid moths (Brantjes, 1978; Dobson, 1994; Balkenius et al., 2006). The sphingid moth *Manduca*

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sexta (L.)*,* in contrast, responds best to a combination of visual and olfactory cues (Raguso & Willis, 2005; Goyret et al., 2007).

Sesiids, including the apple (or small red-belted) clearwing moth, *Synanthedon myopaeformis* (Borkhausen) (Lepidoptera: Sesiidae), are mostly diurnal with many species feeding on inflorescences of various plants (Špatenka et al., 1999; Laštůvka & Laštůvka, 2001). Native to Eurasia and Northern Africa (Zhang, 1994), *S. myopaeformis* was recently introduced into Canada (Beaton & Carter, 2006; Phillip, 2006) where it has rapidly infested all apple orchards in the Similkameen Valley of British Columbia (BC) (Judd et al., 2011). Apple clearwing larvae develop in stems of rosaceous trees, primarily apple (Špatenka et al., 1999). Adults emerge from June to late August at most latitudes, with males emerging slightly before females (Dickler 1976, Blommers & Freriks 1988, Stüber & Dickler 1988, Judd 2008). Adults fly during the warmest and brightest time of day (Ciglar & Masten, 1979; Castellari, 1987; C.E. unpublished data). In Europe, adult apple clearwings have been observed to nectar feed (Popescu-Gorj et al., 1958; Injac & Tosevski, 1987) and to respond to a plant-derived blend of ethyl-2-4-decadienoate and acetic acid (Tóth et al., 2012). In apple orchards in the Similkameen Valley of BC, we have observed the moths feeding on inflorescences of several plant species that vary in appearance and scent. In an exploratory floral preference study, we showed that showy milkweed, *Asclepias speciosa* (Torrey) (Apocynaceae), is a preferred nectar resource for *S. myopaeformis* in BC (C.E. et al. unpublished data). *Asclepias speciosa* has visually conspicuous, highly scented, and nectar-rich inflorescences. Our objectives were to (1) test the hypothesis that *S. myopaeformis* responds to both visual and olfactory cues of these inflorescences, and (2) determine the key floral semiochemical(s) mediating this attraction.

2.3. Materials and methods

2.3.1. *Cultivation of* A. speciosa

To obtain potted plants for field experiments, rhizomes (root pieces) of *A. speciosa* were purchased from commercial nurseries (Sevenoaks Native Nursery, Albany, OR, USA; Willamette Gardens, Corvallis, OR, USA), stored at 5°C, and planted

at bi-weekly intervals in 7.6-L round, soft plastic pots (E2HMP600, Eddi's Wholesale Garden Supplies Ltd., Langley, BC, Canada), using a 50:50 soil: Sunshine #4 potting mix (Eddi's Wholesale Garden Supplies Ltd.). Plants were grown in a greenhouse at Simon Fraser University under a L14:D10 cycle at circa 22°C and transported to Summerland, BC in late May where they were placed in a screen-house under ambient conditions. Potted 2010-plants were overwintered outside under sawdust for the 2011 field season.

2.3.2.   Collection and identification of floral odourants

Floral odourants of *A. speciosa* were collected *ex situ* and *in situ*. For *ex situ* collections, inflorescences (with a few green leaves) were cut and placed in a 250-mL Erlenmeyer flask containing water, and transferred into a cylindrical Pyrex® glass chamber (34 × 12.5 cm). A water aspirator drew charcoal-filtered air at 0.7-0.9 L min^{-1} for 72 h through the chamber and through a Pyrex® glass column containing 500 mg of Porapak-Q (50-80 mesh, Waters Associates, Inc., Milford, MA, USA). Volatiles were desorbed from the Porapak-Q volatile trap with 2 mL of pentane.

For *in situ* volatile collections, each of five intact inflorescences (with a few green leaves) was enclosed in a plastic oven bag (LOOK!, imported by Reckitt and Coleman Canada, Toronto, ON, Canada) that was tightly sealed around the plant stem and a Porapak-Q volatile trap which was connected to a water aspirator drawing air at 0.5-0.7 L min^{-1} through the bag for 24 h (Figure 2.1 a). One corner of the bag was removed to facilitate airflow. Air was also drawn through an empty oven bag as a control. Odourants were desorbed from each Porapak-Q trap with 2 mL of pentane, and an internal standard (dodecan-1-yl acetate, 100 μg) was added for quantification of those odourants (μg/inflorescence/24 h) that elicited responses from moth antennae (see below).

2.3.3.   Derivatization of acid floral odourants

A 50-μL sub-sample of a Porapak-Q headspace volatile extract was treated with 10 μL of BSTFA (bis(trimethylsilyl)trifluoroacetamide) for derivatization of acids to trimethylsilyl esters for identification using gas chromatography-mass spectrometry (see below).

2.3.4. Gas chromatographic-electroantennographic detection (GC-EAD) and GC-mass spectrometric (MS) analyses of floral odourants

Aliquots (2 µL) of Porapak-Q extract were analyzed by coupled gas chromatographic-electroantennographic detection and GC-mass spectrometry, with procedures and equipment previously described in detail (Arn et al., 1975; Gries et al., 2002). Here, the Hewlett-Packard 5890A gas chromatograph was fitted with either a DB-5 (J&W Scientific, Folsom, CA, USA) or Z5 (Phenomenex, Torrance, CA, USA) column (30 m × 32 mm ID). Helium was used as the carrier gas (35 cm sec^{-1}) with the following temperature program: 50°C for 1 min, 20°C min^{-1} until 280°C (10 min). The injector port and flame ionization detector (FID) were set at 250°C. Odourants that elicited responses from several male and female *S. myopaeformis* antennae, and two potential acid odourants which chromatographed poorly, were further analyzed by a Saturn 2000 Ion trap GC-MS operated in full-scan electron impact mode and fitted with a DB-5 column (50 m or 30 m × 25 mm ID). Helium was used as the carrier gas (35 cm sec^{-1}) with the following temperature program: 50°C for 5 min, 10°C min^{-1} until 280°C (10 min). The injector port and ion trap were set at 250°C. Odourants were identified by comparing their retention indices (Van den Dool & Kratz, 1963) and mass spectra with those reported in the literature [benzaldehyde, benzyl alcohol, phenylacetaldehyde, methyl benzoate, 2-phenylethanol, methyl salicylate, benzyl tiglate, α-farnesene, dendrolasin, benzyl benzoate (Adams, 1989); 3*E*-4,8-dimethyl-1,3,7-nonatriene, benzyl iso-valerate, benzoic acid, phenylacetic acid (McLafferty, 1994-1998)] and with those of authentic standards.

2.3.5. Sources of floral odourants

The following compounds were purchased: benzaldehyde (≥99% chemically pure), phenylacetaldehyde (≥90%), methyl salicylate (≥99%), benzyl benzoate (≥99%), benzoic acid (≥99.5%), and phenylacetic acid (≥99%) from Sigma-Aldrich (St. Louis, MO, USA); benzyl alcohol (≥99%) and methyl benzoate (≥98%) from Fischer Scientific (Fair Lawn, NJ, USA); 2-phenylethanol (≥99%) from Fluka (Buchs, Switzerland); and α-farnesene (~77%) from Treatt (Lakeland, FL, USA). 3*E*-4,8-Dimethyl-1,3,7-nonatriene (~92%), benzyl iso-valerate (~92%) and benzyl tiglate (~92%) were synthesized in the

Gries-lab according to standard procedures [3*E*-4,8-dimethyl-1,3,7-nonatriene (Leopold, 1986 & 1990), benzyl iso-valerate and benzyl tiglate (Solomons, 1992)].

2.3.6.   Proboscis extension reflex (PER) experiment

In August of 2011, moths were collected from the field and chilled until they were housed individually in 30-mL Solo® cups, provisioned with water and kept at room temperature next to a window for at least 24 h. Females, but not males (due to high mortality), were deprived of water for > 12 h before testing between 08:00 and 16:00 h Pacific Daylight Time. Bioassays were conducted on 11 males and 11 females, with each moth being randomly tested against 11 floral odourants [benzaldehyde, benzyl alcohol, phenylacetaldehyde, methyl benzoate, 3*E*-4,8-dimethyl-1,3,7-nonatriene, 2-phenylethanol, methyl salicylate, benzyl iso-valerate, benzyl tiglate, α-farnesene, and benzyl benzoate]. For the PER bioassay, each moth was restrained in a modified 1.5-mL microcentrifuge tube (No. 72.690, Sarstedt Inc., Montreal, QC, Canada), from which its head and antennae protruded while supported on a cotton wad (Figure 2.1 b). After the microcentrifuge tube was secured with modelling clay (Sargent Art Inc., Hazleton, PA, USA) in the centre of a white box (18 cm × 18 cm × 18 cm, without top and front), the moth was allowed to acclimate for 5 min before the PER bioassay. The box was vented (~1 m s^{-1}) into a fumehood with an 11-cm diameter tubing attached to a Variac-controlled fan (Staco Energy Products Co., Dayton, OH, USA). Antennae of the restrained moth were positioned 1 cm from a glass tube (150 × 5 mm ID), which was fitted with a Whatman #1 filter paper (1.5 cm) impregnated with 5 mg of test odourant, and which was connected to a Stimulus Controller CS-05 (Syntech Research and Equipment, Hilversum, The Netherlands) that delivered a constant stream of filtered and humidified air (500 ml min^{-1}; ADM 2000 Universal Gas Flowmeter; Agilent Technologies, Mississauga, ON, Canada) for 30 seconds. Antennae were exposed in random order to the odourant-laden air of each of the 11 test chemicals, with 1-2 min between each exposure. During exposures, partial or full extensions of the proboscis were scored as a positive response. Moths were first exposed to a control stimulus (blank filter paper), and those extending their proboscis were excluded from further testing.

2.3.7. Field experiment 1: effect of inflorescences on moth visitation

All field experiments were conducted in commercial apple orchards in the Similkameen Valley of BC. In experiment 1, one plant with inflorescences and one without were paired by height. Each of six such pairs was randomly assigned to, and placed 1 m away from the end of a row (each pair ~20 m apart) in an apple orchard. Paired plants were placed 1 m apart. The total number of moths alighting on either plant was recorded in each of three 15-min observation periods.

2.3.8. Field experiment 2: effect of visual and olfactory inflorescence cues on moth visitation

Ten potted plants with inflorescences were placed at 10-m intervals in a randomly selected row of apple trees. Inflorescences of each plant were subjected to two consecutive 20-minute treatments each, with 5 minutes between treatments. By random assignment, inflorescences were first left exposed (treatment 1) and then enclosed with a dyed-green cheesecloth bag (treatment 2) or *vice versa* (Figure 2.1 c). Cheesecloth (Natura, Home Hardware, St. Jacobs, ON, Canada) bags (26 × 16 × 18 cm tall) were four layers thick and dyed twice with a 40:1 solution of distilled water and paint (Golden Fluid Acrylics, Chromium Oxide Green #2060, Golden Artist Colors Inc., New Berlin, NY, USA). Bags were dyed green to minimize visual contrast. Enclosing inflorescences eliminated visual cues but retained olfactory cues, whereas exposed inflorescences exhibited both olfactory and visual cues. Subjecting inflorescences of the same plant to both treatments helped retain a comparable olfactory cue. The number of moths landing on treatments 1 and 2 was recorded for each plant.

2.3.9. Field experiment 3: effect of individual candidate floral semiochemicals on moth captures

In experiments 3-5, white plastic delta traps with sticky inserts (#300000075, Contech Inc., Delta, BC, Canada) were suspended at 15 m intervals (experiment 3 and 4) or 12 m intervals (experiment 5), 150 cm above ground. An 8-mL Nalgene bottle (#2006-9025, Thermo Scientific, Rochester, NY, USA) with a 6.35-mm hole in the lid was hung by wire from the inside top of each trap and baited with a liquid (2 mL) or solid

(2 g) floral odourant. Liquid odourants were pipetted onto two cotton balls (~2.4 g each) inside the bottle. Solid odourants were placed inside the bottle without cotton balls. Bottles with two clean cotton balls were used as controls. Traps were checked daily and sticky inserts were replaced as needed. Captured moths were counted and separated by sex in the laboratory using a microscope.

In experiment 3 (July 28 to August 4, 2011), eight EAD-active candidate floral semiochemicals (benzaldehyde, benzyl alcohol, phenylacetaldehyde, 2-phenylethanol, methyl salicylate, benzyl benzoate, benzoic acid, and phenylacetic acid) were selected for testing based on a minimum emission rate of 5 µg/inflorescence/24 h (see Table 2.1). Each of the eight semiochemicals and an unbaited control were tested in a completely randomized block design with five replicates.

2.3.10.   Field experiment 4: comparative attraction of moths to phenylacetaldehyde and an 8-component blend

Experiment 4 (July 29 to August 5, 2011) was designed to compare the attractiveness of phenylacetaldehyde (2 mL) against that of an 8-component blend (2 mL).   This blend consisted of the same compounds that were tested singly in experiment 3, but they were formulated at ratios found in headspace volatile extracts of inflorescences (see Table 2.1). Phenylacetaldehyde, the 8-component blend, and an unbaited control were tested in a completely randomized block design with six replicates.

2.3.11.   Field experiment 5: comparative attraction of moths to phenylacetaldehyde alone and in combination with groups of selected floral odourants

Experiment 5 (August 16-19, 2011) was designed to explore potential synergism between phenylacetaldehyde and selected groups of components, including acids (benzoic acid and phenylacetic acid), aldehydes (benzaldehyde), esters (methyl salicylate and benzyl benzoate) and alcohols (benzyl alcohol and 2-phenylethanol). Chemicals were kept in separate bottles (see above) to avoid potential chemical interactions. The six treatments, including an unbaited control, were tested using a 6 × 6 Latin square design.

2.3.12. Statistical analyses

All data were analyzed with JMP Version 8 software (SAS institute 2008). Data of experiments 1 and 2 were analyzed with a paired *t*-test. Data in experiment 3-5 were transformed to improve normality and homoscedasticity (Zar, 2010) [Exp. 3, males: square-root + 0.5, females: log + 1; Exp. 4, males and females: log + 1; Exp. 5, males and females: square root + 0.5] and analyzed by a randomized block ANOVA (Exps. 3 and 4) and by Latin square ANOVA (Exp. 5). Significant ANOVA results were followed by a *post-hoc* comparison of means using the Tukey-Kramer HSD test. The significance level of all tests was 5%.

2.4. Results

2.4.1. Identification of floral odourants

In-situ and *ex-situ* aerations had similar volatile profiles and the empty oven bag aeration showed the bag did not contribute any additional volatiles. The 12 floral odourants that elicited responses from moth antennae (Figure 2.2) were identified as benzaldehyde, benzyl alcohol, phenylacetaldehyde, methyl benzoate, 3*E*-4,8-dimethyl-1,3,7-nonatriene, 2-phenylethanol, methyl salicylate, benzyl iso-valerate, benzyl tiglate, α-farnesene, dendrolasin and benzyl benzoate. Benzyl alcohol and phenylacetaldehyde elicited the strongest responses, and benzaldehyde was the most abundant component (Table 2.1). Treatment of Porapak-Q headspace volatile extract with BSTFA revealed the presence of benzoic acid and phenylacetic acid, which due to their abundance were included in field experiments. Dendrolasin could not be purchased or be readily synthesized in quantities conducive to field testing, and therefore was excluded from volatile quantification, field experiments and the PER bioassay.

2.4.2. Proboscis extension reflex experiment

Among the 11 floral odourants identified above, phenylacetaldehyde elicited the most frequent proboscis extension reflexes from males (54.6%) and females (81.8%) (Figure 2.3). Benzaldehyde also elicited PERs from males (18.2%) and females (27.3%).   Some females (9.1%) also responded to 2-phenylethanol, benzyl iso-valerate,

α-farnesene, and benzyl tiglate, and some males (9.1%) responded to methyl benzoate and benzyl benzoate. Benzyl alcohol, 3*E*-4,8-dimethyl-1,3,7-nonatriene, and methyl salicylate elicited no PERs.

2.4.3. Field experiment 1: effect of inflorescences on moth visitation

Plants with inflorescences induced significantly more visits by moths than plants without (t = 2.63, d.f. = 5, P = 0.047). No moths visited plants without inflorescences and a mean (± SEM) of 7.33 ± 2.79 moths visited plants with inflorescences.

2.4.4. Field experiment 2: effect of visual and olfactory inflorescence cues on moth visitation

There was no significant difference (t = 1.68, d.f. = 9, P = 0.13) between the mean (± SEM) number of visits by moths on inflorescences left exposed (2.40 ± 0.70) or enclosed by dyed-green cheesecloth bags (1.00 ± 0.56).

2.4.5. Field experiment 3: effect of individual candidate floral semiochemicals on moth captures

Individual candidate floral semiochemicals differed in their attractiveness to both males and females (*males* $F_{8,32}$ = 202.09, P < 0.0001; *females* $F_{8,32}$ = 42.31, P < 0.0001). Phenylacetaldehyde attracted significantly more males and females than any of the other odourants tested (Figure 2.4).

2.4.6. Field experiment 4: comparative attraction of moths to phenylacetaldehyde and an 8-component blend

Trap captures of males and females were significantly affected by test stimuli (Figure 2.5: *males*: $F_{2,10}$ = 34.54, P < 0.0001; *females*: $F_{2,10}$ = 75.5, P < 0.0001). Phenylacetaldehyde attracted more males and females than did the 8-component blend, which in turn attracted more males and females than did unbaited traps.

2.4.7. Field experiment 5: comparative attraction of moths to phenylacetaldehyde alone and in combination with groups of selected floral odourants

Test stimuli affected trap captures of females but not males (Figure 2.6: *females* $F_{5,5,5}$ = 14.48, P < 0.0001; *males* $F_{5,5,5}$ = 1.91, P = 0.14). Phenylacetaldehyde alone was equally attractive in combination with acids or esters, and more attractive than in combination with aldehydes or alcohols.

2.5. Discussion

Our data support the conclusions that (1) male and female *S. myopaeformis* are attracted to inflorescences of *A. speciosa*, (2) olfactory cues of inflorescences play a more significant role than visual cues, and (3) phenylacetaldehyde is the key semiochemical mediating the attraction of moths.

The inflorescences, not the foliage, of *A. speciosa* are attractive to *S. myopaeformis*. This is expected because *A. speciosa* is a nectar source but not a larval host plant for *S. myopaeformis*. Although its inflorescences and foliage may share volatile components such as α-farnesene, benzaldehyde and benzyl alcohol (Andersson et al., 2002, Garlick, 2007), adult *S. myopaeformis* obviously discriminate between foliage and inflorescences using visual and/or specific olfactory cues indicative of the latter. Similarly, the butterflies *Inachis io* (L.), *Aglais urticae* (L.), *Gonepteryx rhamni* (L.), and *Heliconius melpomene* (L.) differentiate between foliar and floral odours and prefer the latter (Andersson, 2003; Andersson & Dobson, 2003).

Even though diurnal Lepidoptera may primarily use visual foraging cues to locate inflorescences (e.g., Ômura & Honda, 2005; Kelber & Balkenius, 2007) we found that similar numbers of moths contacted exposed or enclosed inflorescences of *A. speciosa*, indicating that floral scent alone can attract moths. That we did not find a statistically significant preference by moths for exposed inflorescences with visual and olfactory cues may have been due to a relatively small sample size in the respective experiment. Alternatively, by enclosing inflorescences we may have disrupted their scent plume making it more difficult for the moths to locate them (Vickers, 2000). A stronger

behavioural response to combinations of visual and olfactory cues has been observed in various diurnal and nocturnal moths (Brantjes & Leemans, 1976; Naumann et al., 1991; Balkenius et al., 2006).

As floral odour alone induced visitation by *S. myopaeformis*, we field-tested candidate floral semiochemicals that were emitted from plants at > 5 µg/inflorescence/24 h, anticipating key floral semiochemicals to be disseminated at significant quantities for attraction of insects. These included components that elicited antennal responses and proboscis extensions. Among all the candidate semiochemicals tested, phenylacetaldehyde was the key component, attracting > 35 times more moths than any other floral odourant, with no other component(s) enhancing its attractiveness. Phenylacetaldehyde is a common floral odourant and is known to attract many Lepidoptera, especially noctuid moths (Creighton et al., 1973; Cantelo & Jacobson, 1979; Honda et al., 1998; Meagher, 2002; Knudsen et al., 2006; El-Sayed et al., 2008).

Although semiochemical blends mimicking floral bouquets can be more attractive than one or more of the single components therein (Pellmyr, 1986; Honda et al., 1998; El-Sayed et al., 2008; Stringer et al., 2008), in our study, the attractiveness of phenylacetaldehyde to *S. myopaeformis* could not be enhanced by additional floral components from *A. speciosa*. When we tested the 8-component blend versus phenylacetaldehyde alone, significantly more moths were attracted to phenylacetaldehyde. This might be attributed to a lesser amount of phenylacetaldehyde, or to the effects of antagonistic components in the blend. However, less complex blends of phenylacetaldehyde with selected groups of floral odourants also failed to enhance the attractiveness of phenylacetaldehyde alone. That mostly females responded in experiment 5 was due to their prevalence at the end of the flight season. In several noctuid moths and the small cabbage white butterfly *Pieris rapae* (L.), a single floral component is as attractive as a multiple-component blend (Haynes et al., 1991; Heath et al., 1992; Ômura et al., 1999; Plepys et al., 2002a; Dötterl et al., 2006).

The PERs by *S. myopaeformis* in response to phenylacetaldehyde exposure implies recognition of a semiochemical indicative of a food source. Because bioassayed moths were field-collected, it was not possible to infer whether the PER represents an innate or learned behaviour. However, subsequent results (G.J. unpublished data)

obtained from naïve, laboratory-reared *S. myopaeformis* yielded similar results, indicating that the PER to phenylacetaldehyde is innate. A single floral semiochemical, such as phenylacetaldehyde, may reliably indicate the location of a wide array of nectar resources, whereas other components in the floral bouquet may help foraging insects discriminate between species of flowers (Plepys et al., 2002a). This may apply to *S. myopaeformis*, as six of the eight chemicals tested elicited antennal responses but not necessarily PER responses. There is not always a correlation between strong EAD and PER responses (Ômura & Honda. 2009), but the strong antennal and PER responses of male and female *S. myopaeformis* to phenylacetaldehyde support a previous conclusion (Honda et al., 1998) that the PER bioassay is a good tool to screen candidate feeding attractants before field-testing.

Phenylacetaldehyde attracts both male and female *S. myopaeformis*, and thus is an ideal candidate for exploitation in pest management programs. It could be developed to monitor, mass-trap or attract-and-kill populations of male and female moths (Camelo et al., 2007). Combined with the sex pheromone of *S. myopaeformis*, it may be very effective in suppressing male populations while also reducing female populations. Phenylacetaldehyde has been combined with the sex pheromone of the cabbage looper *Trichoplusia ni* (Hübner), for attraction of male and female moths (Creighton et al., 1973).

Several questions need to be addressed before phenylacetaldehyde can be used operationally for monitoring and controlling populations of *S. myopaeformis*. The sticky inserts in delta traps used in our experiments were quickly covered with captured moths and needed to be replaced daily during peak flight. Operationally, this would be too labour-intensive, clearly indicating the need for a trap type with greater capture capacity. Furthermore, a floral-feeding attractant as a lure may also attract non-target species, including pollinators. Phenylacetaldehyde is attractive to various Hymenoptera and other moth species (Meagher & Mitchell, 1999; Meagher, 2002; El-Sayed et al., 2008), which we also observed in this study. Captures of non-target Hymenoptera can be reduced by trap type and colour (Meagher & Mitchell, 1999; Clare et al., 2000). Also, semiochemicals which repel bees, such as methyl salicylate, mixtures of methyl salicylate and benzyl alcohol, or of dibutylamine and benzyl benzoate (Henning et al., 1992; Sahebzadeh et al., 2009) could be added to phenylacetaldehyde baits to

discourage foraging bees from entering traps (but see Mayer, 1997). In our study, the addition of methyl salicylate and benzyl benzoate with phenylacetaldehyde did not significantly decrease trap captures of *S. myopaeformis* and captured few bees (data not shown). Once an optimal trap and non-target semiochemical repellent have been determined, the use of phenylacetaldehyde for monitoring and controlling populations of *S. myopaeformis* should be investigated.

2.6. Acknowledgements

2.7. References

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Table 2.1. ***Mean (± SEM, n = 5) amounts (µg) of odourants released from an inflorescence of*** Asclepias speciosa *during 24 h.*

Chemical	µg/inflorescence/24 h
Benzaldehyde	199.87 ± 22.81
Phenylacetaldehyde	108.05 ± 20.57
2-Phenylethanol	77.28 ± 33.39
Benzoic acid	39.97*
Benzyl alcohol	35.34 ± 5.59
Phenylacetic acid	19.99*
Benzyl benzoate	18.45 ± 10.09
Methyl salicylate	8.82 ± 2.59
Methyl benzoate	2.20 ± 0.64
3E-4,8-Dimethyl-1,3,7-nonatriene	1.61 ± 0.60
Benzyl iso-valerate	0.93 ± 0.72
Benzyl tiglate	0.53 ± 0.48
α-farnesene	0.01 ± 0.01

*Benzoic acid and phenylacetic acid do not have standard errors as amounts were based on a ratio to benzaldehyde using GC-MS.
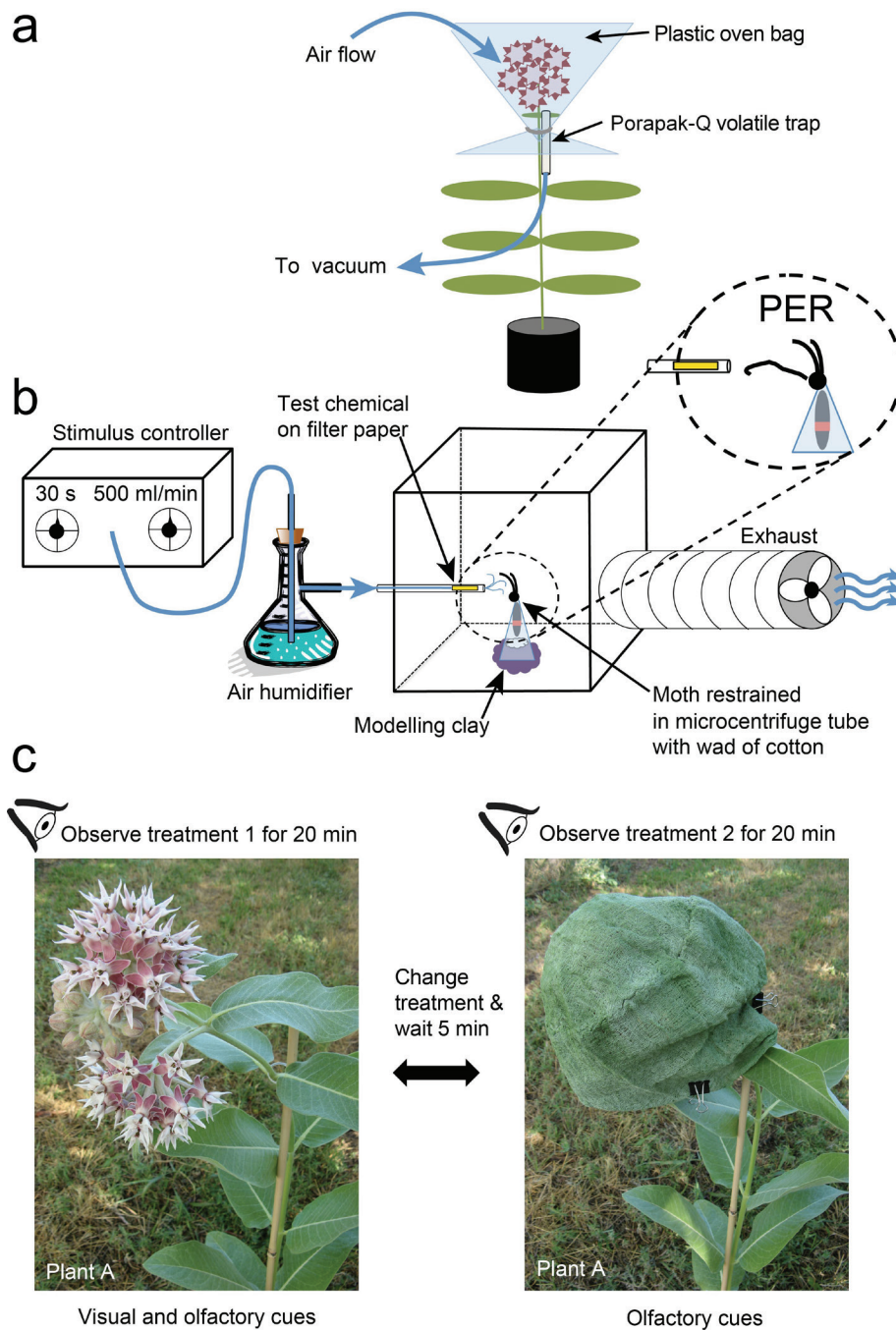
Figure 2.1. *Experimental design employed to: (a) acquire floral headspace volatiles from* in-situ *inflorescences of showy milkweed (*Asclepias speciosa*), (b) bioassay proboscis extension reflexes (PERs) of male and female* Synanthedon myopaeformis *in response to floral odourants of* A. speciosa*, and (c) field bioassay the response of* S. myopaeformis *to inflorescences of potted* A. speciosa *that were left exposed or enclosed in dyed-green cheesecloth bags.*
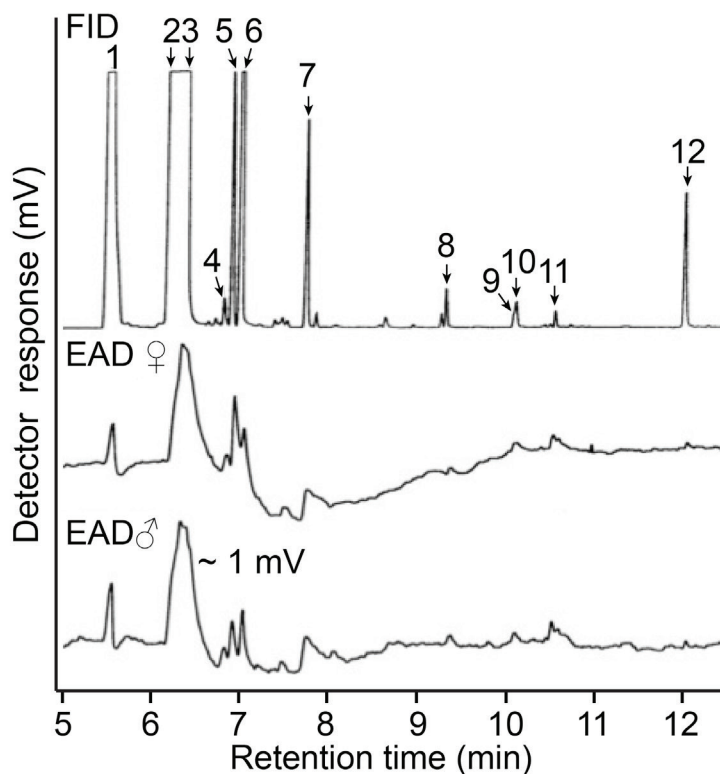
Figure 2.2. *Representative recording of the responses of a gas chromatographic flame ionization detector (FID) and electroantennographic detectors (EAD: male or female* Synanthedon myopaeformis *antennae) to aliquots of Porapak-Q extract of* Asclepias speciosa *floral headspaces. Twelve components elicited antennal responses as follows: 1 = benzaldehyde; 2 = benzyl alcohol; 3 = phenylacetaldehyde; 4 = methyl benzoate; 5 = 3E-4,8-dimethyl-1,3,7-nonatriene; 6 = 2-phenylethanol; 7 = methyl salicylate; 8 = benzyl iso-valerate; 9 = benzyl tiglate; 10 = α-farnesene; 11 = dendrolasin; 12 = benzyl benzoate.*

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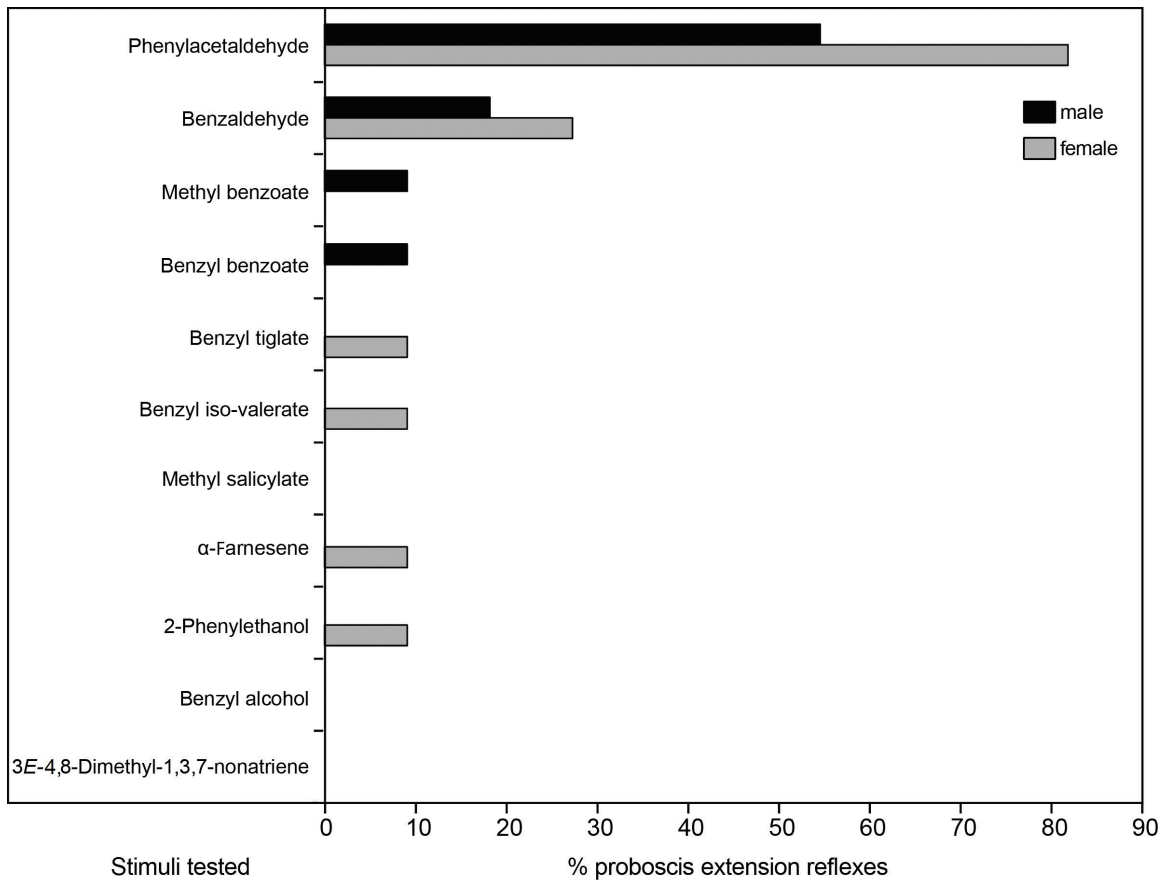
Figure 2.3. *Percentage of proboscis extension reflexes exhibited by 11 male and 11 female* Synanthedon myopaeformis *in response to exposure to each of 11 floral odourants from* Asclepias speciosa *that also elicited antennal responses (see Figure 2.2).*
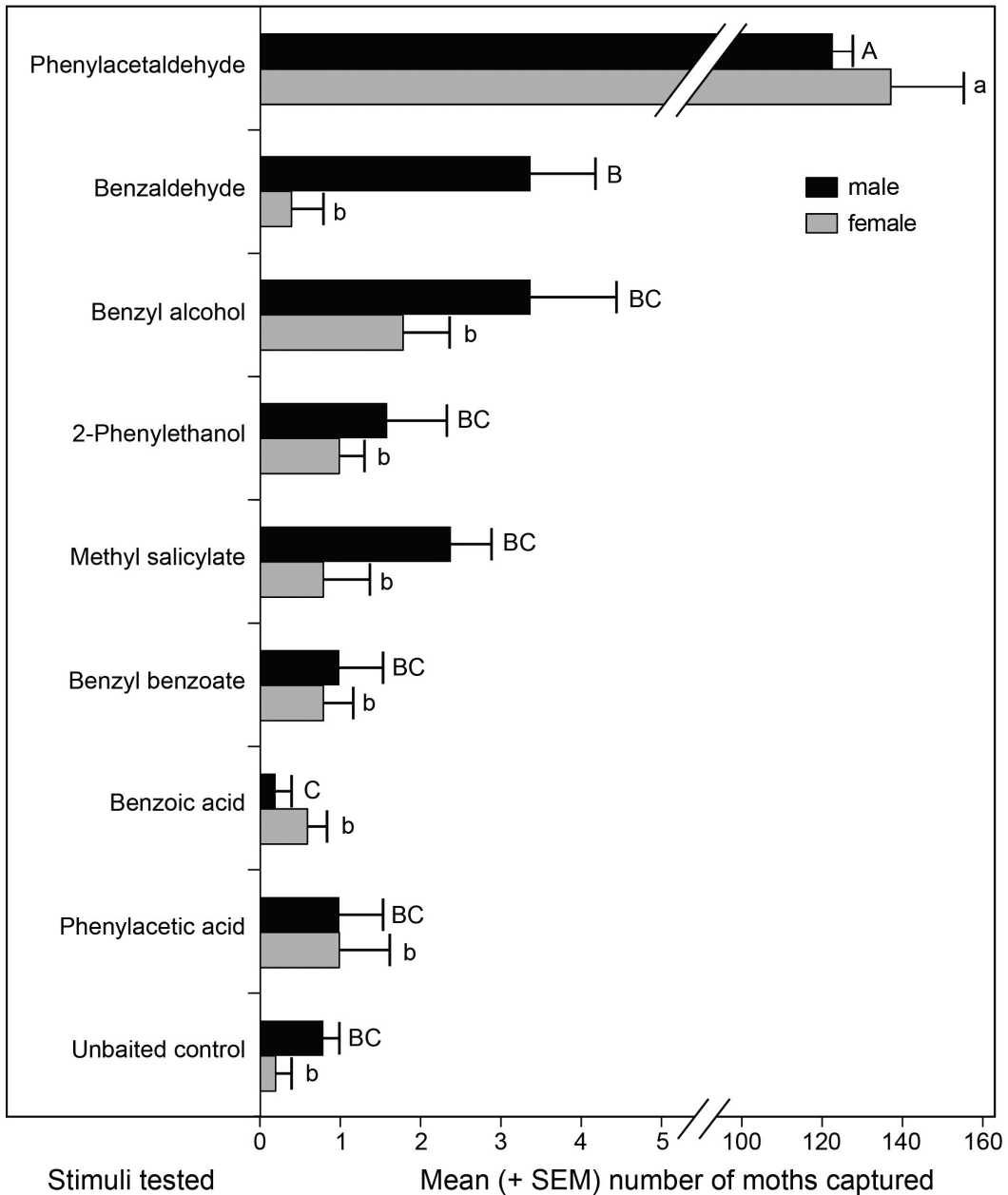
Figure 2.4. *Untransformed mean (+ SEM) number of male and female* Synanthedon myopaeformis *captured in traps baited with single floral odourants of* Asclepias speciosa. *Significant differences between captures are denoted by different upper and lower case superscript letters for males and females, respectively. All data were transformed [square-root + 0.5 (male); log + 1 (female)] before subjecting them to ANOVA followed by the Tukey-Kramer HSD test,* $\alpha = 0.05.$
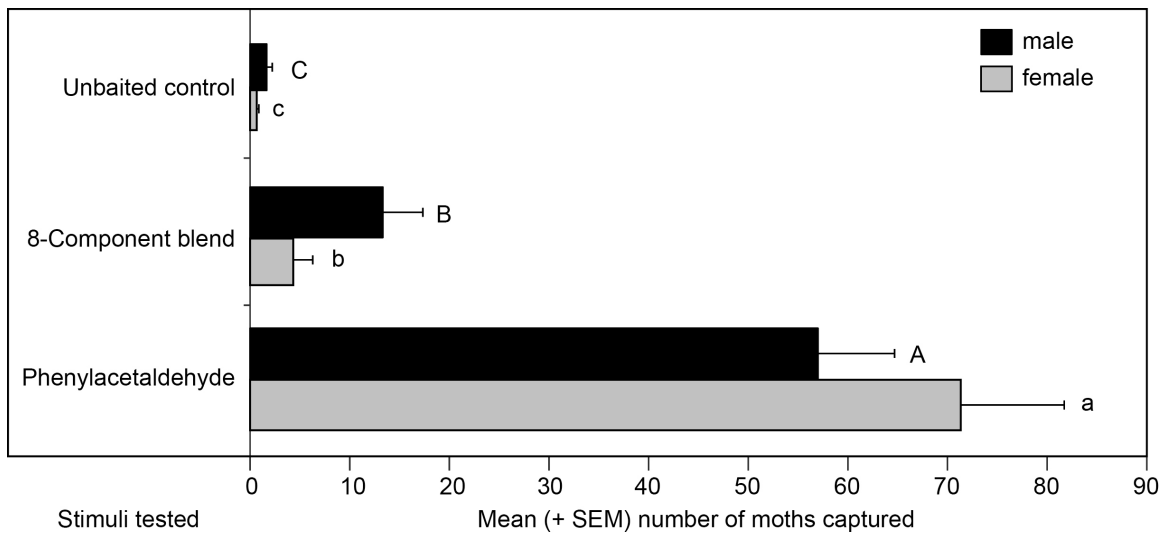
Figure 2.5. *Untransformed mean (+ SEM) number of male and female* Synanthedon myopaeformis *captured in traps baited with phenylacetaldehyde or an 8-component blend of floral odourants from* Asclepias speciosa *(see methods for detail). Significant differences between captures are denoted by different upper and lower case superscript letters for males and females, respectively. All data were log + 1 transformed and subjected to ANOVA followed by the Tukey-Kramer HSD test, α = 0.05.*
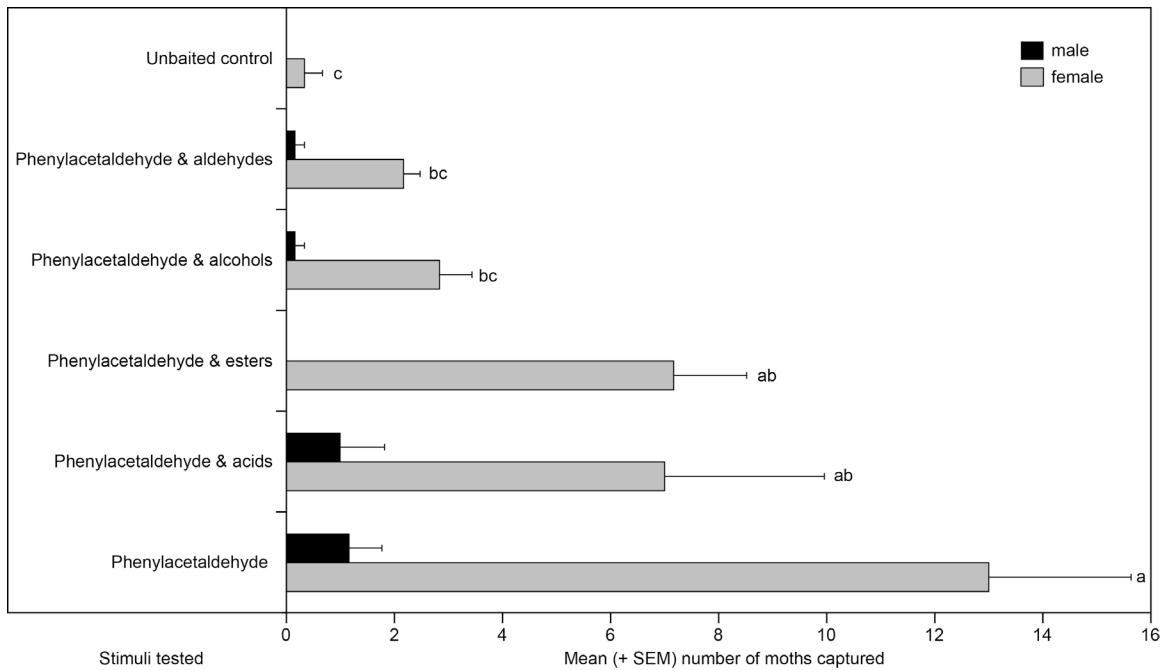
Figure 2.6. *Untransformed mean (+ SEM) number of male and female* Synanthedon myopaeformis *captured in traps baited with phenylacetaldehyde alone, and in combination with specific groups of floral odourants from* Asclepias speciosa *(see methods for detail). Significant differences between captures are denoted by different superscript letters. All data were square root + 0.5 transformed and subjected to ANOVA followed by the Tukey-Kramer HSD test, α = 0.05.*

3. Spectral sensitivity and microstructure of the compound eyes of *Synanthedon myopaeformis* (Lepidoptera: Sesiidae)*

3.1. Abstract

The apple clearwing moth, *Synanthedon myopaeformis* Borkhausen (Lepidoptera: Sesiidae), is a day-flying species that is attracted by inflorescences of many plants and commonly feeds on floral nectar. In British Columbia, this invasive species is often observed feeding on visually conspicuous showy milkweed, *Asclepias speciosa* Torrey (Apocynaceae). We measured the spectral sensitivity of the compound eyes of *S. myopaeformis* in the context of their capacity to discriminate the measured spectral reflectance from inflorescences of *A. speciosa*, and conducted a histological examination of these eyes to determine whether they possess apposition type ommatidia, as commonly observed in diurnal butterflies. Light micrographs of the compound eyes in *S. myopaeformis* revealed eucone apposition type ommatidia, which is consistent with their diurnal behaviour. When compound eyes in electroretinogram studies were exposed to monochromatic, 10-nm bandwidth light stimuli (330–700 nm and 335–695 nm), they were particularly sensitive to ultraviolet (UV) wavelengths in the 335-370 nm range and green wavelengths in the 495-560 nm range. These results support the conclusion that the compound eyes in *S. myopaeformis* have the capacity for dichromatic vision based on UV and green photoreceptors. However, spectral reflectance curves obtained from inflorescences and foliage of *A. speciosa* revealed no evidence of UV reflectance. This suggests that visual detection of these flowers may only require stimulation of a green photoreceptor, making it less likely that colour plays a primary role in the attraction of *S. myopaeformis* to *A. speciosa*.

3.2. Introduction

Butterflies and moths have well-developed visual systems with simple and complex photosensitive organs called ocelli and compound eyes, respectively. As simple eyes, ocelli are commonly involved in monitoring light intensity levels for maintenance of level flight (Wilson 1978). Compound eyes house the primary receptors of visual stimuli and consist of many ommatidia. Ommatidia can be categorized into two basic structural forms, referred to as having apposition or superposition configurations. Both of these ommatidial types include a lens that focuses light via a crystalline cone onto the photoreceptor-containing rhabdom of the retinula cells. In the superposition ommatidium, but not the apposition ommatidium, the rhabdom is separated from the crystalline cone by a clear zone, allowing light to be focused from the lens of several ommatidia onto a single rhabdom, thus improving vision at low-light intensity (Land 1981; Stavenga 2005).

Apposition ommatidia are typically found in diurnal butterflies, whereas superposition ommatidia prevail in nocturnal moths (Yagi and Koyama 1963). Too few species have been examined to make generalizations about the ommatidial structure of day-flying moths, but given the suspected evolution of all present-day Lepidoptera from a diurnal moth with apposition ommatidia, and the presence of apposition ommatidia in many diurnally active moths examined so far, it is expected that day-active moths, such as sesiids, have apposition ommatidia (Warrant et al. 2003; Yack et al. 2007).

The apple clearwing, *Synanthedon myopaeformis* Borkhausen (Lepidoptera: Sesiidae), is a diurnal moth native to Eurasia and North Africa (Zhang 1994) that was recently introduced into Canada (Beaton and Carter 2006; Philip 2006). Vision plays a role during sexual communication of *S. myopaeformis* (Stüber and Dickler 1988), and likely plays a role in finding food. In Europe, adult *S. myopaeformis* are often seen feeding on various flower species (Popescu-Gorj et al. 1958, Injac and Tosevski 1987), and in British Columbia (BC), Canada, we observed adult *S. myopaeformis* preferentially foraging on visually conspicuous inflorescences of showy milkweed, *Asclepias speciosa* Torrey (Apocynaceae). Larvae of *S. myopaeformis* feed beneath the bark of rosaceous trees, preferably apples (Špatenka et al 1999). This species has become a serious

economic pest in commercial high-density apple plantings (Dickler 1976; Balázs et al. 1996; Ateyyat and Al-Antary 2006).

Vision is an important sensory modality used by many moths and butterflies to locate mates (reviewed by Wehner 1981), floral nectar (e.g., Ômura et al. 1999, Andersson 2003; Ômura and Honda 2005; Balkenius et al. 2006), and oviposition sites (e.g., Rausher 1978; Kolb and Scherer 1982; Vasconcellos-Neto and Monteiro 1993; Allard and Papaj 1996), but little is known about the visual systems or behaviours of diurnal flower-feeding moths, particularly those considered to be pest species.

Vision entails many aspects, including colour and shape discrimination, but colour vision has been studied most often. To be capable of colour vision, an insect must possess at least two photoreceptors and distinguish between wavelengths of light independent of their intensity (Menzel 1979). Colour vision is most often demonstrated using behavioural assays (Kelber et al. 2003) and studied physiologically using techniques such as microspectrophotometry, electroretinograms (ERGs), and intracellular recordings to identify specific photoreceptors (Briscoe and Chittka 2001).

Our objectives were (1) to determine whether the ommatidia in *S. myopaeformis* eyes have an apposition or superposition configuration, (2) to measure the spectral sensitivities of the compound eyes of *S. myopaeformis* using electroretinograms, and (3) to compare the spectral sensitivities of the compound eyes of *S. myopaeformis* in relation to the spectral reflectance curves from inflorescences and foliage of *A. speciosa*.

3.3. Methods

3.3.1. Insect rearing

In December of 2009, rootstock-scion sections of apple trees infested with larvae of *S. myopaeformis* were cut from trees removed from an orchard in Cawston, BC. Larvae were excised from infested wood, placed individually in 30-mL Solo® cups containing a pinto-based artificial diet modified from Shorey and Hale (1965), and reared in a growth chamber at 25°C under a 16:8 h L:D photoregime. Emergent moths were

transferred into clean 30-mL Solo® cups and provisioned with water *ad libitum* from a dental cotton wick.

3.3.2.   Microstructure of ommatidia in light microscopy

The microstructure of ommatidia was determined from histological sections by light microscopy. Heads were excised from live male and female *S. myopaeformis* and then split in half so each half contained one eye. Eyes were fixed in a light-adapted state, using 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer followed by 1% osmiumtetroxide in 0.1 M sodium cacodylate. After fixation, eyes were rinsed in distilled water, then dehydrated in 30%, 50%, 70% 85%, 95% and 100% dilutions of ethanol in distilled water, and ended with 100% acetonitrile as the final step. Dehydrated eyes were then embedded in a mold using a transmission electron microscopy embedding polymer (Spurr's Low Viscosity Kit, Prod. No. 18300, Pelco, Redding, CA, USA). An 8-mm diamond histo-knife (Diatome, Hatfield, PA, USA) was used in an Ultracut E microtome (Reichert-Jung, Depew, NY, USA) to cut 0.5-, 1- and 2-µm serial sections of eyes in transverse and longitudinal planes. All sections were mounted on slides or coverslips using Permount™, and some sections were stained with toluidine blue. Sections were viewed through a Zeiss AxioPhot light microscope (Carl Zeiss Canada, Toronto, Ontario, Canada) and images were captured with a microscope-mounted Nikon D700 and Capture Control Pro2 software (Nikon, Melville, NY, USA). From each of four eyes, three sections in which the full length of each ommatidium was visible were selected and the length of three ommatidia in the dorsal, equatorial and ventral region (n = 36 ommatidia per region) was measured from the top of the cornea to the basement membrane, using ImageJ software (version 1.440, National Institutes of Health, USA). The lengths of ommatidia were averaged for dorsal, equatorial and ventral regions for each section and eye. The lengths in these sections were compared by an analysis of variance (ANOVA), with eye as a blocking factor, followed by a *post-hoc* Tukey-Kramer HSD test for comparison of means. Statistical analyses were run with JMP Version 8 software (SAS institute 2008), with $\alpha = 0.05$ for all tests.

3.3.3. Spectral sensitivities of eyes in electroretinograms

3.3.3.1. Insect preparation and exposure to test stimuli

Up to two-week-old moths were fixed laterally with Tanglefoot® (Contech Enterprises Inc., Delta, BC, Canada) and modelling clay on a slide (Fig. 3.1). Antennae, legs and the left wing were removed to prevent movement and facilitate placement of the electrically-sharpened tungsten electrodes (Cool et al. 1970). Using micromanipulators (Leitz micromanipulator M; Leitz, Vienna, Austria), one electrode was inserted into the thorax of the moth and the other electrode was placed sub-corneally into the equatorial region of the compound eye, near the anterior edge to prevent shadowing. The electrical potentials generated by the eyes in response to light stimuli were pre-amplified (Syntech Auto Spike; Syntech, Hilversum, The Netherlands) and recorded with an eletroantennogram oscilloscope software program (Syntech). Recordings were obtained in a light-proof Faraday cage.

3.3.3.2. Test stimuli

A 35-watt Xenon Arc light source (HPX-2000, Ocean Optics, Dunedin, FL, USA) and a fibre optic scanning monochromator (MonoScan 2000, Mikropak GmbH, Ostfildern, Germany) were used to produce all test stimuli (Zahradnik 2012). Light stimuli consisted of 10-nm bandwidths of light tested consecutively at 10-nm increments from 330-700 nm (series 1) and 335-695 nm (series 2) in both ascending and descending order, for a total of 75, 5-nm bandwidths of light tested per eye. Every recording series had a 500-nm control stimulus at the beginning and end. The series and order to be tested first were randomly assigned, but both orders of one series were completed before the other series was initiated. Each moth was dark-adapted for 40 minutes prior to and between each of the four recordings. Electroretinograms were performed on nine males and 11 females.

A 600-μm optical fibre (QP600-1-SR-BX, Ocean Optics) attached to the monochromator was fitted with a collimator (LC-4U-THD, Multimode Fiber Optics, Hackettstown, NJ, USA) and transmitted light to a 0-2 stop circular variable neutral density wheel [Fused Silica (200-2500 nm), Reynard Corporation, San Clemente, CA, USA] directly in front of a 20:80 beam splitter ("polka dot" 4-2001, Optometrics, Ayer, MA, USA). Twenty percent of a light stimulus was transmitted to the cosine corrector-

fitted spectrophotometer (HR-4000, Ocean Optics), and was calibrated with SpectraSuite software (Ocean Optics) so that each stimulus had an intensity of 1.0×10^{13} photons/cm^2/sec. The remainder (80%) of the light stimulus was transmitted into a 1000-µm single fibre optic cable (PCU-1000-2-SS, Multimode Fiber Optics) via a collimator (LC-4U-THD, Multimode Fiber Optics) and delivered ca. 1 mm above the moth eye through a sub-multi-assembly (SMA) terminus. Situated before the collimator terminus of this cable was a programmable shutter (R. Holland, Science Technical Centre, Simon Fraser University, Burnaby, BC, Canada) which continuously intercepted any light, except for intermittent 0.5-sec intervals every 9.5 sec, during which the eye was exposed to the light stimulus.

3.3.3.3. Data analyses

For each bandwidth tested, data were averaged within eyes, normalized using the ratio to the highest response per eye (set to 1.0), and then averaged among eyes. Recordings for insects where the second 500-nm control stimulus elicited a greater response than the first control stimulus were discarded.

3.3.4. Spectral reflectance from inflorescences and leaves of Asclepias speciosa

The hoods and petals from inflorescences of *A. speciosa* (Fig. 3.4) from each of two plant specimens were excised, arranged several layers thick on a black felt background, and subjected to three spectral reflectance measurements. Leaves of two plants were measured three times *in situ* against a black felt background. Measurements for each hood, petal, and leaf sample were averaged.

Reflectance measurements employed a spectrophotometer and pulsed Xenon Arc light source (JAZ-PX, Ocean Optics) with a fibre optic reflection probe (Ocean Optics) fitted with a custom-built 45° black anodized aluminum attachment. The spectrophotometer (JAZ) was calibrated against a white standard (WS-1-SL, Ocean Optics) and dark reference prior to each sample. To obtain smooth reflectance curves, measurements were taken with the lamp flashing (10 ms), and the JAZ completing 50 scans to average with 0-boxcar smoothing (averaging of adjacent pixels).

3.4. Results

3.4.1. Microstructure of ommatidia in light microscopy

Longitudinal and transverse sections of male and female compound eyes showed no gender-specific differences in ommatidial microstructure. The convex cornea of each ommatidium has a hexagonal lens (Fig. 3.2 III) and is subtended by a corneal process (Fig. 3.2 I, II). The eucone crystalline cone has four semper cells, and is surrounded by granular pigments (Fig. 3.2 IV). The rhabdom spans the distance from the base of the crystalline cone to the basement membrane. Each ommatidium has up to 8 retinular cells, six large and two small cells (Fig. 3.2 V). Basal pigment cells occur above the basement membrane. There was no obvious tracheal bush. Ommatidia varied significantly in length ($F_{2,6}$ = 25.9, P = 0.0011), with the longest ommatidia observed in the dorsal region of the eye and the shortest ommatidia found in the ventral region of the eye (Table 3.1).

3.4.2. Spectral sensitivity of eyes demonstrated by electroretinograms

Male and female eyes exposed to 10-nm bandwidths of light in 10-nm increments from 330-700 nm (series 1) and 335-695 nm (series 2) revealed similar spectral sensitivities (Fig. 3.3). In series 1, male and female eyes were most sensitive to green light (500-560 nm), had a secondary peak of sensitivity in the near-UV light (330-370 nm) and were least sensitive to red light. Red-light sensitivity increased with increasing wavelength (660-700 nm). Similarly, in series 2, male and female eyes were most sensitive to green light (495-555 nm), secondarily most sensitive to near-UV light (335-365 nm), and least sensitive to red light, with red-light sensitivity increasing with increasing wavelength (655-695 nm).

3.4.3. Spectral reflectance from inflorescences and leaves of Asclepias speciosa

Hoods and petals of inflorescences of *A. speciosa* reflected more light than leaves, with hoods reflecting the most light (Fig. 3.4). Hoods reflected light evenly between ca. 440-560 nm, beyond which reflectance gradually increased. Reflectance

from petals was fairly even between ca. 430-550 nm, beyond which reflectance sharply increased towards ca. 650 nm, decreased to 670 nm and then again sharply increased. Leaves reflected maximally between ca. 500-580 nm, peaking at ca. 550 nm. Neither inflorescences nor leaves of *A. speciosa* reflected UV light.

3.5. Discussion

Ommatidia in compound eyes of *S. myopaeformis* contain a rhabdom that is continuous from the crystalline cone to the basement membrane, supporting the conclusion that they have an apposition configuration. Compound eyes having apposition ommatidia are present in other sesiids (Ehnbom 1948; Yagi and Koyama 1963) and while this is most common in diurnal Lepidoptera, some possess superposition ommatidia (Horridge et al. 1972, 1977; Warrant et al. 1999). Superposition eyes may have evolved from apposition eyes via a gradual shift from focal to afocal crystalline cones (Nilsson et al. 1988). To date, afocal apposition eyes have only been found in papilionid butterflies (Warrant et al. 2003). Whether *S. myopaeformis* possess afocal or focal apposition eyes remains unknown, but being phylogenetically more primitive than the papilionids (Tree of Life Web Project 2010), they have likely retained the more ancestral focal apposition eye. The longer rhabdom of ommatidia in the dorsal eye region likely enhances visual acuity in this region (Rutowski and Warrant 2002).

Eyes of *S. myopaeformis* were sensitive to UV and green wavelengths, implying their potential for dichromatic vision. The potential for dichromatic vision has been found in diverse insect taxa, including the cockroach *Periplana americana* L. (Blattodea: Blattidae) (Mote and Goldsmith 1970), the owlfly *Ascalaphus macaronius* Scop. (Neuroptera: Ascalaphidae) (Gogala 1967), several species of ant (Menzel 1973; Mote and Wehner 1980; Lieke 1981), the moth *S. tipuliformis* Clerk (Lepidoptera: Sesiidae) (Karalius and Būda 2007), and the butterfly *Parantica sita* Kollar (Lepidoptera: Nymphalidae) (Eguchi et al. 1982). In most other Lepidoptera, however, three photoreceptors (UV, blue and green) are more common, with some species even possessing a fourth, red receptor (reviewed by Briscoe and Chittka 2001).

Eyes of *S. myopaeformis* were sensitive to red light increasing in wavelength from 655–700 nm. The potential to detect red wavelengths would make sense because both male and female *S. myopaeformis* have a red band around their abdomen, which males strike during courtship before females permit copulation (Stüber and Dickler 1988). An alternative, though less likely, explanation for the increase in sensitivity from 655–700 nm is that *S. myopaeformis* eyes have a near-infrared (700-1000 nm) receptor, which we did not test for in our study. To date, infrared receptors have only been found in pits on the abdomen and thorax of some species of Coleoptera (Evans 1966; Schmitz et al. 2000) and Hemiptera (Takács et al. 2009). To unequivocally reveal the specific photoreceptors in the eyes of *S. myopaeformis,* additional ERGs under adapting light or intracellular single cell recordings should be considered (Goldsmith and Bernard 1974; Kirchner et al. 2005).

With sensitivity to UV and green light, eyes of *S. myopaeformis* might readily detect plants reflecting UV and green wavelengths using colour vision. With the flowers and foliage of *A. speciosa* not reflecting UV light, *S. myopaeformis* could still detect *A. speciosa* based on contrasting light intensities reflected from inflorescences and foliage or the background using only a green receptor (Dafni and Kevan 1997). Alternatively, *S. myopaeformis* may primarily rely on olfactory cues to locate *A. speciosa,* and use vision to locate other nectar plants with corresponding visual cues.

3.6. Acknowledgements

3.7. References

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Table 3.1. *Mean (± SE) length of ommatidia in the dorsal, equatorial, and ventral regions of the compound eyes (n = 4) in* **Synanthedon myopaeformis**. *Significant differences in ommatidia length are denoted by different letters based on the Tukey-Kramer HSD test following a significant ANOVA, α = 0.05.*

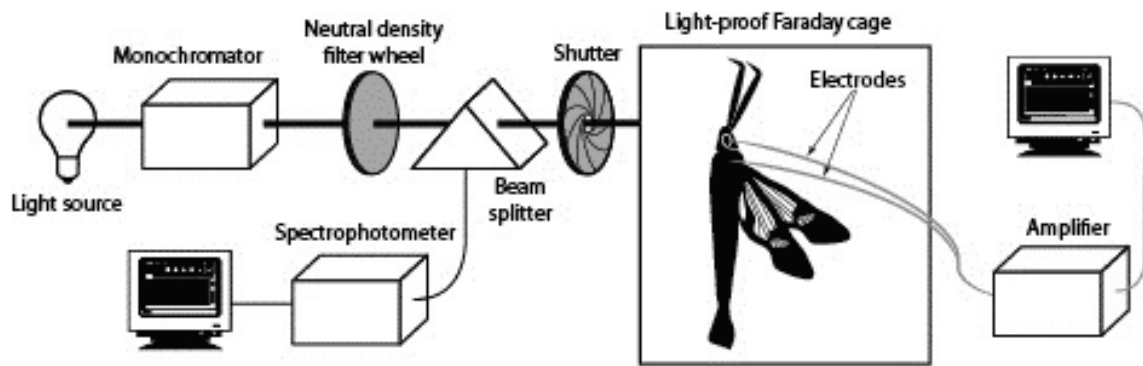| Eye region	Length (± SE) in μm
Dorsal	259.3 (± 13.7) a
Equatorial	215.1 (± 3.3) b
Ventral	117.1 (± 2.9) c

Figure 3.1. *Schematic drawing of the experimental set-up employed for electroretinogram recordings.*
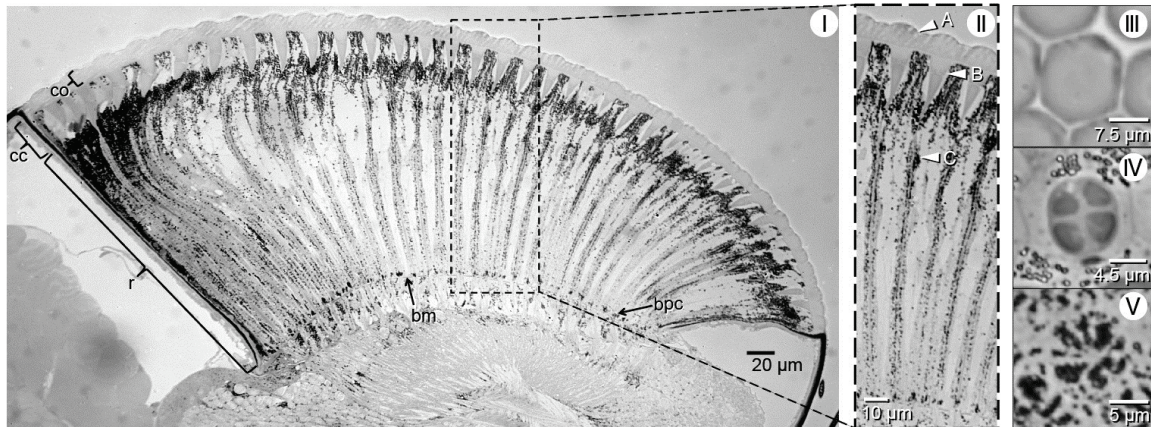
Figure 3.2. **Light micrographs of (I) a longitudinal section through a**
Synanthedon myopaeformis *compound eye revealing the cornea*
(co), crystalline cones (cc), rhabdoms (r), basal pigment cells (bpc)
and basement membrane (bm); (II) Close-up of I with transverse
sections taken at A, B and C through: the cornea (III), a crystalline
cone revealing four semper cells (IV), and a rhabdom revealing six
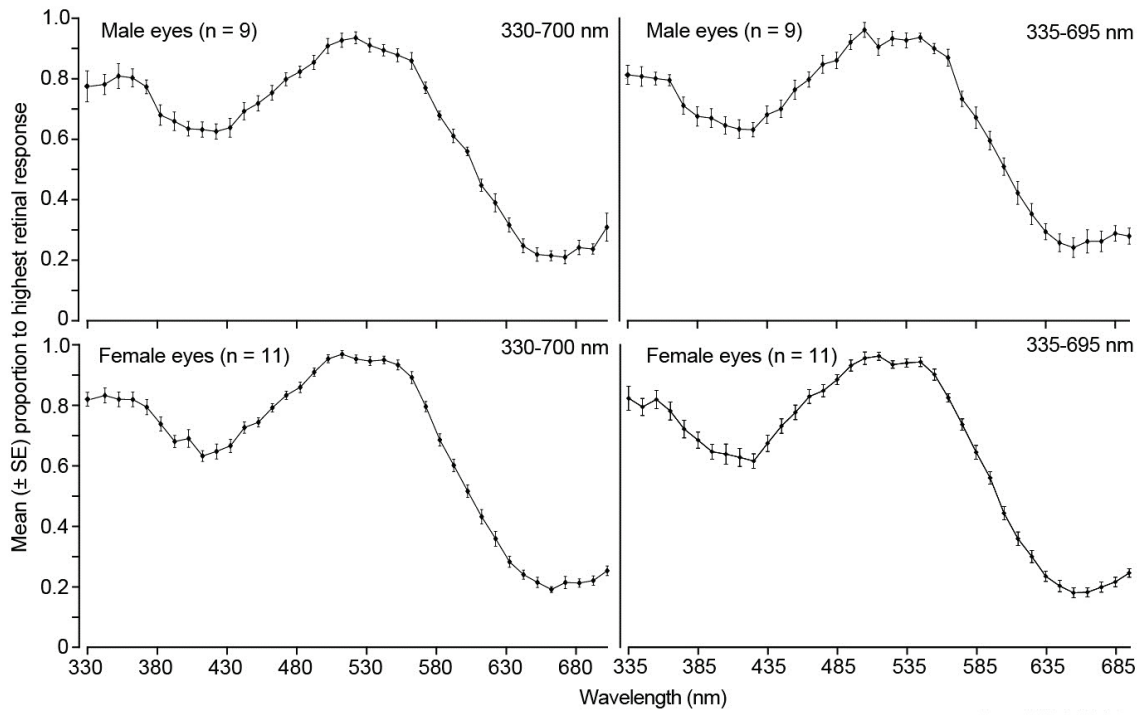*large and two small retinular cells (V).*

Figure 3.3. **Mean (± SE) retinal responses (standardized to 1.0 for the highest response for each eye) of** Synanthedon myopaeformis *eyes in* **electroretinograms using two series of monochromatic light at 10-nm bandwidths.**
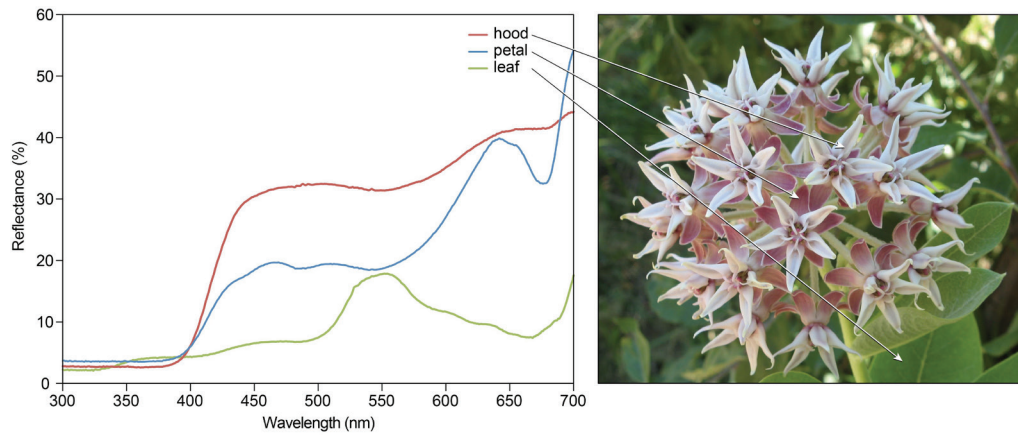
Figure 3.4. *Photograph of* **Asclepias speciosa***, and spectral reflectance curves of its inflorescences (hoods and petals) and leaves.*

4. Conclusions

This research investigated visual and olfactory cues exploited by *Synanthedon myopaeformis* to locate inflorescences of *Asclepias speciosa*. The following conclusions can be drawn from this study:

- Inflorescences, not foliage, of *A. speciosa* are attractive to male and female *S. myopaeformis.*

- The floral odour of *A. speciosa* alone is able to attract *S. myopaeformis*.

- In coupled gas chromatographic-electroantennographic detection analyses of headspace volatiles of inflorescences of *A. speciosa*, 12 components elicited responses from male and female antennae.

- Among these 12 components, plus two acid odourants, phenylacetaldehyde alone, or in combination with other floral odourants, strongly attracted male and female moths.

- Phenylacetaldehyde elicited the most frequent proboscis extension reflex responses in male and female moths, indicating an innate feeding response.

- Compound eyes in *S. myopaeformis* possess apposition type ommatidia.

- Compound eyes in *S. myopaeformis* may have the capacity for dichromatic vision with peaks of spectral sensitivity in the near UV (335-370 nm) and green (495-560 nm) regions of the electromagnetic spectrum.

- Neither inflorescences nor foliage of *A. speciosa* reflect UV light. With reflected light from inflorescences of *A. speciosa* being capable of only

stimulating potential green receptors in *S. myopaeformis* eyes, it is unlikely these moths use dichromatic vision in locating inflorescences of *A. speciosa*.

- Inflorescences and foliage of *A. speciosa* differ in colour and intensity. This difference in intensity could be exploited during foraging by *S. myopaeformis*.

4.1. Implications for pest management

Phenylacetaldehyde is a feeding attractant for both male and female *S. myopaeformis*. It could be used to develop monitoring, mass-trapping, or attract-and-kill technologies for managing moth populations in commercial apple orchards.

4.2. Future research

- As a floral odourant, phenylacetaldehyde attracts other insects, including bees. Further research should focus on determining the optimal dose of phenylacetaldehyde, trap type, trap colour and possibly the addition of a bee-repellent chemical to mitigate bee by-catch.

- To unequivocally reveal the photoreceptor system present in *S. myopaeformis*, will require additional electroretinograms under adapting light or intracellular single cell recordings.

- To demonstrate that these insects use colour vision to discriminate among potential feeding hosts would ideally require behavioural assays where moths are trained to associate a colour with a food reward. The capacity for learning in *S. myopaeformis* is unknown. An alternative method would be to conduct field experiments where chromatic and achromatic cues are tested. For example, chromatic cues (with differing spectral reflectance curve shapes) and achromatic cues (with the same

spectral reflectance curve shape but different intensities) can be painted onto objects baited with phenylacetaldehyde.