

**VISUAL AND OLFACTORY SENSORY SYSTEMS EMPLOYED BY
MONARCH BUTTERFLIES (*DANAUS PLEXIPPUS*) TO LOCATE THEIR
MILKWEED HOST PLANTS**

by

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Abstract

Monarch butterflies (*Danaus plexippus*) are well known to depend almost exclusively on milkweed (genus *Asclepias*) host plants for oviposition sites, as their larvae need to ingest compounds critical for adult butterfly chemical defense against predators. Many phytophagous insects make fast and accurate decisions on the quality of host plants, and whether or not to accept them as oviposition sites. We were interested in the cues that attract monarchs (males and females) to milkweed, while in flight, before contact stimulation can occur. We developed a novel flight apparatus to test monarch attraction behaviour, under a number of different experimental protocols, to both milkweed and control stimuli in order to identify the cues necessary or sufficient for monarch attraction to milkweed. Monarchs were found to be attracted to milkweed stimuli when the visual image alone of milkweed was available or when olfactory cues alone were available. Attraction behaviour was maximized when both cues were available. It was also discovered that attraction to milkweed was significantly diminished when ultraviolet reflection from milkweed leaves was prevented from being detected by monarchs in flight.

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Chapter I.

General Introduction

Animals are adapted to particular ecosystems that provide food, optimal places for finding mates and breeding, and suitable areas to rear young. In many instances, animals are born in these very ecosystems that meet all of these needs, but in others, they must search out and locate suitable habitats that may be scattered throughout large regions of space. Within an ecosystem, an animal needs to be able to locate and differentiate objects in their environment in order to survive, and multiple sensory systems are employed in order to determine what and where these objects are, and how to react accordingly. One of the well known problems associated with detecting relevant information, however, is that there is far more information that is not relevant to an animal. How animals detect targets of interest in the face of countless distracters in the environment has been studied extensively in animals, of which insects are a model system (Bernays 2001).

Two vital aspects of insect sensory behaviour are the detection of mates and conspecifics often through the use of pheromones, and the detection of suitable host plants for egg laying. A pheromone is a unique chemical or cocktail of chemicals, specific to a species, which mediates behavioural responses such as flying towards potential mates from a distance. Two defining features of insect pheromones are that they are usually multicomponent blends and are composed of various isomers that can lead to different behavioural responses (Silverstein and Young, 1976; Tillman *et al.*, 1999). Male moths will fly in a wind tunnel or in the field up a female-produced pheromone concentration gradient, and this has been demonstrated extensively in the

tobacco hawk moth, *Manduca sexta* (Tumlinson *et al.*, 1989; Tumlinson *et al.*, 1994). However, pheromones serve other functions in addition to locating potential mates. The honey bee (*Apis mellifera*) queen emits pheromones to control the social organization of her hive, while other bees in the colony utilize pheromones to signal each other and coordinate attacks on intruders (Naumann *et al.*, 1991). Many species of ants use pheromones as a warning to other colonies and predators, as well as to direct other workers to a food source far from the colony (Hangartner 1969). The common theme in pheromone research is the need for animals to locate and differentiate conspecifics, as well as to locate objects of interest, primarily food resources that are widely distributed spatially in the environment. Another important resource that is also widely distributed and crucial to many insects' survival is host plants on which to lay their eggs.

Phytophagous insects often possess multiple metamorphic stages and must seek out part of a habitat to lay eggs that will provide optimal conditions for their young, such as proximity to food sources. This is because their larval forms are restricted in mobility and access to food is limited. In many insects, eggs are laid either in the vicinity or directly onto a particular host plant, which is often specific to their species. How insects locate very specific plants in nature has received increased attention in the scientific community due to its relevance to agriculture. Many insects are pests to food and other crops, and understanding how these insects precisely locate and are attracted to specific plant hosts may help to eradicate certain pests through applications of biologically safe control strategies. The gypsy moth, *Lymantria dispar*, after its accidental introduction to North America from Europe and Asia, has become the largest defoliator of trees and has developed a vast range of over 300 species of hosts leading to severe impacts on local

ecosystems (Hermes 2003). The diamondback moth, *Plutella xylostella*, is one of the most destructive insect pests to some of the most economically and biologically important crops, and was the first insect to develop resistance to DDT (Sarfraz *et al.*, 2005). Both the gypsy and diamondback moth pose a large threat to a number of different plant species they utilize as hosts, but there exists many different pests that have adapted to very specific hosts and pose just as large a threat to agriculture and forestry. One such example is the Colorado potato beetle, *Leptinotarsa decemlineata*. Uncontrolled populations can completely decimate potato crops, as both larvae and adult forms feed almost exclusively on this one crop (Hare 1990). Another example is the Emerald ash borer, *Agrilus planipennis*, an invasive beetle species which only infests ash trees (*Fraxinus* Sp.) (Haack *et al.*, 2002).

In many of the above cases, much of the management strategies have focused on insecticides, herbicides, insect viruses, and parasitoids, but many of these control strategies either is not sufficient to stop outbreaks, or often leads to adverse impacts on other moths and butterflies, some of which are already threatened. It is clear that host plant availability is the key to regulating the presence of pests and it is our belief that understanding the interactions between insects and their host plants may lead to more efficient, biologically safe, control strategies.

In addition to insects locating host plants, there exist multiple insect-plant interactions in nature. For example, different nectar-bearing plants often attract potential pollinators through a variety of means. Specific visual stimuli and olfactory scents often attract only one or a few species of moths and butterflies as pollinators, and depending on the insect and plant, either of these cues, or both, may be paramount in ultimately

attracting a specific pollinator to the plant (Kevan and Baker 1983). Some plants, in addition to visual or olfactory cues, have co-evolved with insects specialized structures to attract only one pollinator. The sphinx moth, *Xanthopan morgani*, has the longest known proboscis and pollinates an orchid in Madagascar with a 25-30cm spur that is structured to guide the proboscis into its nectary (Kevan and Baker 1983). Another complex insect-plant interaction is the plant release of volatiles following herbivory. Plants normally release small quantities of volatile chemicals to recruit potential pollinators, however, when a plant is damaged by an herbivore, there occurs a substantial release of additional volatiles. The chemical composition of these volatiles depends upon the species of plant as well as the insect herbivore, and can attract specific predators of the herbivores (Paré and Tumlinson 1999). Cabbage plants respond to the cabbage white butterflies' (*Pieris brassicae*) larval enzymatic secretions during herbivory by releasing volatiles, which attract parasitic wasps (*Cotesia glomerata*) that specifically target these butterflies (Mattiacci *et al.*, 1995).

All of these plant-insect interactions involve a level of specificity in which plants possess specific signals, whether they be visual, olfactory, or structural, that attract insects. Another plant-insect interaction is the sequestering of unpalatable toxic substances from host plants by moths and butterflies. Many of these insects possess aposematic, or conspicuous, coloring that acts as a warning signal to predators that they are toxic. One of the most well known examples of such chemical defense is the monarch butterfly, which possess bright orange, conspicuous colouration. It has been well established that adult monarchs whose larvae feed on poisonous milkweed plants obtain chemical defenses from them (Brower 1984). A number of cardenolides, in the

form of cardiac glycosides, have been identified in monarchs, as well as in their milkweed host plants of the genus *Asclepias*, which are cardio-active substances that induce severe vomiting in bird predators following consumption, and cause a visually conditioned avoidance behaviour towards prey with similar colourations (Ackery and Vane-Wright, 1984; Nishida 2002). Studies have shown that when monarch larvae were not reared on milkweed host plants, birds, after initial reluctance, would consume them (Brower, Brower, and Corvino, 1967; Brower 1972). Thus the unpalatability of monarchs depends upon their access as larvae to milkweed host plants.

It is thus crucial for monarchs to be able to successfully locate milkweed host plants in their environment to lay eggs on in order to ensure offspring survival. In addition to their importance for larval survival, milkweed clusters in the field have been observed to play a role in breeding, as males often patrol the areas of milkweed patches where female monarchs lay their eggs (Zalucki 1993). These observations suggest both sexes are attracted to areas of milkweed. Much research of host plant identification and attraction in monarchs and other insects has focused on postalightment oviposition behaviour, and the oviposition stimulants released from milkweed hosts after landing. It has been found that after landing, forelegs, midlegs, and antennae are used to assess host suitability, with the midleg tarsal claws rupturing leaf surfaces to release chemicals perceived by antennae (Haribal and Renwick 1998). Some of these *contact* oviposition stimulants have been identified from a series of milkweed plants, a family of compounds known as flavonol glycosides (Haribal and Renwick 1996, Haribal and Renwick, 1998). However unlike other butterflies, female monarchs have been observed to generally oviposit eggs on the first milkweed leaf she alights on (Bergstrom *et al.*, 1995), indicating

monarchs are receiving information regarding milkweed host suitability well before landing. We were interested in understanding which cues are being utilized by monarchs of both sexes before landing on their milkweed hosts.

We constructed a novel flight apparatus to test experimentally monarch attraction to milkweed while *in flight*, in order to elucidate the cues used by monarchs to locate their host plants. We hypothesize that monarchs use a visual-olfactory cue synergism to locate milkweed, which is used by some other insects such as the hawkmoth, *Manduca sexta* (Raguso and Willis, 2002). In their study, Raguso and Willis (2002) showed hawkmoths repeatedly passed over and hovered over paper flowers visually modeled after their pollinated-flowers of choice, and this hovering behaviour was replicated using olfactory cues from their flowering plants. However, the strongest attraction was observed in hawkmoths where floral fragrance and visual display were presented in combination (Raguso and Willis, 2002). In other plant-visiting Lepidoptera, visual and olfactory cue combinations elicit stronger behavioural responses than either cue administered alone, however this is not always the case (Raguso and Willis, 2002). Bumblebees will readily visit artificial flowers where olfactory cues have been removed (Chittka *et al.*, 2001), whereas some species of moth require only visual cues to be attracted to nectar-bearing plants (Kelber and Pfaff 1997). We found that monarchs of both sexes were selectively attracted to young milkweed over a variety of different controls, with visual and olfactory cues available alone, or in combination. Young milkweed leaves were used in all experiments based on previous findings that female monarchs preferentially oviposit on the young growing tips of their host plants (Bergstrom *et al.*, 1995). In addition, we determined that UV light reflected from young

milkweed leaves may be an important signal when combined with olfactory and visible-light spectrum cues for locating milkweed hosts in their environment. This was interesting in light of a previous study suggesting that monarchs are attracted to young milkweed via differential volatile cues emitted from young versus old leaves (Bergstrom *et al.*, 1995).

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Chapter II

Visual and olfactory cues used by monarch butterflies (*Danaus plexippus*) to locate milkweed host plants

Introduction

Insect-plant interactions are complex and varied, and include examples such as predators and parasites using herbivory-induced plant-released volatiles to locate prey (Turlings *et al.*, 1995), attraction of potential pollinators via volatiles and visual cues, and attraction of phytophagous insects to their host plants for egg-laying. Many phytophagous insects make fast and accurate decisions on the quality of host plants, and whether or not to accept them as oviposition sites (Bernays 2001). Monarch butterflies (*Danaus plexippus*) are well known to depend almost exclusively on milkweed (genus *Asclepias*) host plants for oviposition sites, as their larvae need to ingest cardiac glycosides critical for adult butterfly chemical defense against predators (Brower 1969; Rothschild 1970). Female monarch butterflies generally lay eggs on the first milkweed plant they land on (Bergstrom *et al.*, 1995), indicating specific cues have already been processed regarding host plant quality *before* landing. Although female monarchs have been observed to dip their antennae and drum leaf surface with their forelegs and midlegs after landing, and diverse contact oviposition stimulants, detected through contact chemosensory receptors on these appendages, have been identified (Haribal and Renwick, 1998; Haribal and Renwick 1996), there has been little attention given to the sensory information received by monarchs, while in flight, *before* contact-stimulated oviposition cues are available.

Historically, much of the research on measuring monarch attraction to milkweed has considered either qualitative observations of monarch behaviour, or the counting of

eggs deposited by females on certain milkweed species versus control plants. We were interested in investigating both male and female monarch attraction to their host plants before landing, as male monarchs often “patrol” milkweed patches in search of mates, while females, in addition to mating, deposit their fertilized eggs in these patches (Zalucki 1993). Since we could not use traditional egg counting strategies to measure male and female attraction to their milkweed host plants, we constructed a novel flight choice apparatus that allowed flying monarchs to choose between spatially separated milkweed and control plants while *in flight*. A variety of different experimental protocols were employed to investigate the role of visual and olfactory information used by in-flight monarchs to differentiate milkweed hosts from non-hosts.

There have been conflicting reports as to which sensory cues are predominant in locating host plants in moths and butterflies. Urquart (1960), through observations in the field, believed visual cues were the most crucial sensory signals used by monarchs for host plant choice, whereas other studies have shown insects utilize plant volatiles for attraction, localization, and aggregation around host plants (Loughrin et al., 1995; Bolter et al., 1997; Jonsson and Anderson, 1999). In *Manducta sexta*, the combination of both visual and olfactory cues from flowers was significantly more attractive than either cue alone (Raguso and Willis 2002). In our study, we examined the influence of both visual and olfactory cues in monarch-host plant interactions, as well as the role of ultraviolet (UV) light, in an effort to understand the mechanisms by which monarchs locate and are attracted to young milkweed host plants. Young milkweed plants were used in all experiments, as female monarchs preferentially oviposit on the young growing tips of their host plants as opposed to older leaves (Bergstrom *et al.*, 1995).

Most insects including monarch butterflies can detect UV light, and it has been shown that insects may use this to signal to detect conspecifics and assess mate quality (Brunton and Majerus 1995; Papke et al., 2006). Many flowering plants, in addition to using chemical cues to attract pollinators, have also evolved complex colourations that match the spectral sensitivities of insect eyes (Chittka and Menzel 1992). Some plants have evolved UV-reflecting pigments, believed to be additional signals to birds and insects, such as bees, to locate and determine plant nectar loading capacity (Kevan et al., 2001). The purpose of this study was to determine how monarchs are attracted to milkweed using our novel flight apparatus. Our results demonstrate a potential multisensory process by which flying monarchs are attracted to milkweed, which is maximized when olfactory and UV visual cues reflected from leaves are both available.

Materials and Methods

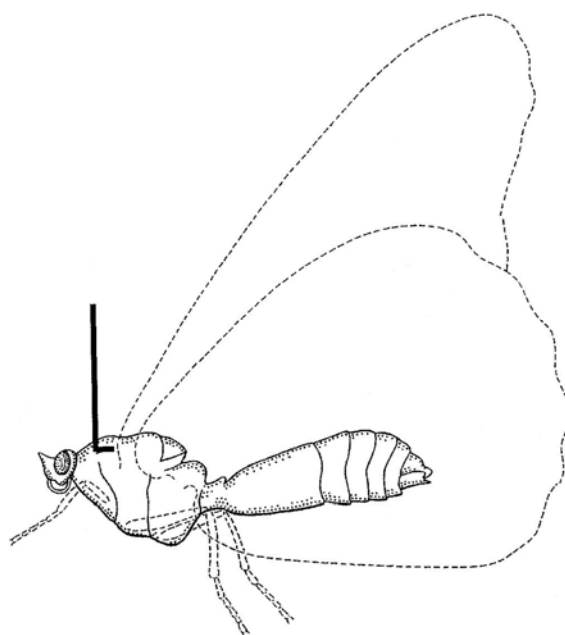
Animals

Monarch butterfly chrysalids were obtained from a commercial breeder (Magical Monarchs, Toronto, ON) and housed in 0.31m³ cages until adult emergence. Three to four days after emergence, butterflies were placed into a jig that gently restrained them but left their dorsal thorax exposed. Hair was removed from this area using a small insect pin, and a 24-gauge needle was inserted just through the chitin in the anterior midline region of the dorsal thorax. A 0.2 mm diameter, 3cm-long stainless steel wire, bent 90° 2mm from the end to form an “L-shaped” hook, was inserted into this hole so that it was oriented vertically from the thorax (Figure 1). This stalk allowed easy tethering to the flight apparatus (see Mouritsen and Frost 2007). A mixture of melted beeswax and violin rosin (1:1 ratio) was used to cement the stalk in place. After surgeries, butterflies were allowed to recover for at least 2 days before they were used in experimental protocols. Butterflies that did not fully recover within 2 days were not used in experiments. All experiments were carried out in the Animal Care Facility in the Department of Psychology at Queen’s University and approved by Queen’s University Animal Care Committee as compliant with the Canadian Council of Animal Care Guidelines.

Plants

The common milkweed, *Asclepias syriaca*, was used in all experiments testing monarch’s attraction to milkweed. Plants used in experiments were grown from seeds (Richter’s Herb Specialists; Goodwood, ON, Canada) in a greenhouse within the Phytotron at Queen’s University, Kingston and kept under natural light and temperature conditions.

Figure 1. Schematic diagram of monarch butterfly with stainless steel stalk implanted through an incision of the dorsal thorax. Stalks were held in place with a 1:1 ratio of melted beeswax and violin rosin, and butterflies were allowed 2-3 days recovery before flight in the choice apparatus.



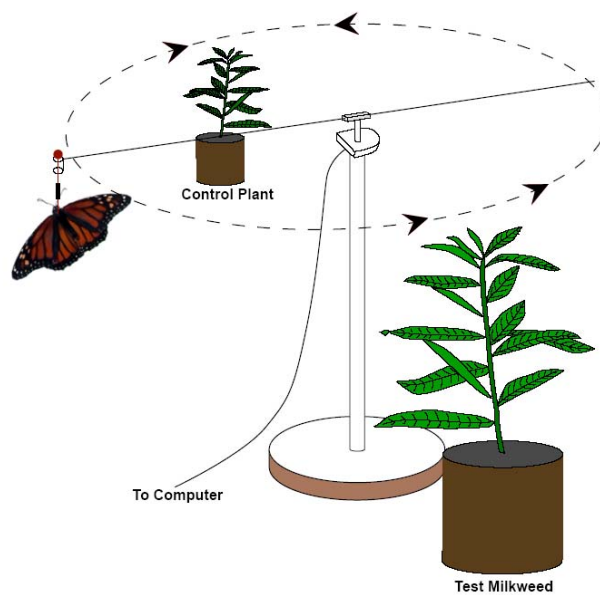
Additional *A. syriaca* used in spectral and olfactory experiments were collected fresh from the field in the Kingston, ON area within one hour of testing. Bugleweed (*Lycopus americanis*) was collected from the field and reared under the same conditions.

Flight Choice Apparatus

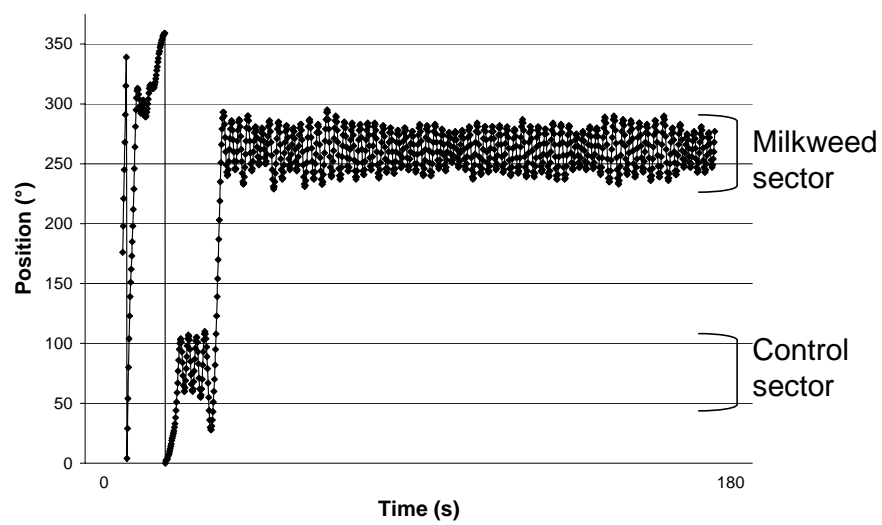
Our custom made flight choice apparatus allowed us to quantitatively measure monarch butterfly flight behaviour in response to various stimuli (Figure 2A). The apparatus consisted of two 70-cm long light-weight aluminum wire arms (forming a 140-cm diameter) that extended horizontally from a low friction bearing and optical encoder (US Digital E5S-360-250). Butterflies were individually attached, via the implanted stalk and silicon rubber tubing, to one end of the flight apparatus arms, while a small counterbalancing weight corresponding to the weight of each butterfly, was affixed to the end of the other arm. A small swivel bearing made from a bead and two supporting loops of fine piano wire allowed the butterfly to freely rotate around the vertical axis and change direction at the end of the flight apparatus arm. If the butterfly flew approximately tangentially to the circumferential path described by the end of the arm, it flew around in a circle. However, if the butterfly executed a turning response at the end of the arm it could change directions, and indeed could hover over a particular sector of the circle prescribed by the flight apparatus arms. Stimuli, which consisted of control and milkweed plants, were positioned on opposite sides of the circular flight path (at 90° and 270°), such that the butterfly could fly directly over and above them (2cm), but not land on them. The entire apparatus was housed in a 125cm tall, 250cm diameter cylinder made of white choroplast material.

Figure 2. A) General flight choice apparatus setup illustrating monarch affixed via stalk to swivel at the end of the flight arm. The swivel allowed complete rotation at the end of the arm, enabling monarchs to easily switch directions in the circle prescribed by the end of the wire arm. Position of flight apparatus arms by monarchs was recorded every 200 milliseconds using an optical encoder during a 3 minute experiment. Two plant stimuli were placed 180° apart within the flight apparatus enclosure: a milkweed stimulus and a control. Different experimental protocols used slight modifications to this general setup, as described in the Methods section. B) Example of flight choice behaviour record of one butterfly plotting position over a 3 minute time period. Note how monarch hovers back and forth over the milkweed plant for most of the experimental period. For consistency, apex of the milkweed stimulus was always placed at a position in the circle of 270°, whereas the control was placed in a position at 90°. C) Flight mill schematic from a top view, displaying relative positions of either a milkweed stimulus or control. Each sector (milkweed or control) was 80° in width: 4 bins of 20°.

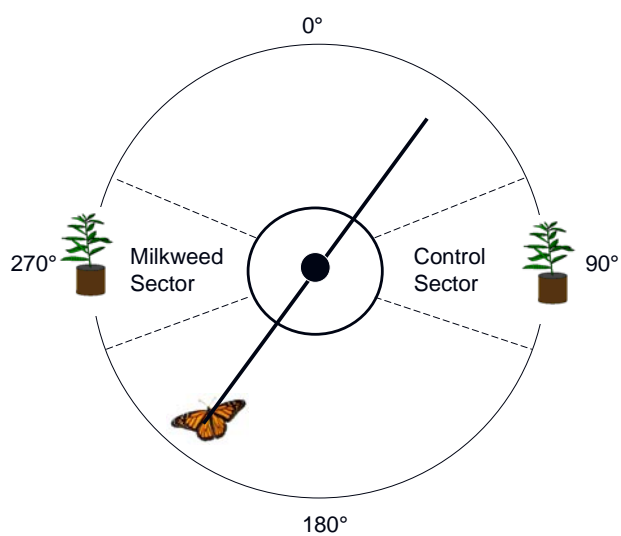
A



B



C



Data Collection and Analysis

The optical encoder fed information about each butterfly's position (1° resolution), which was recorded every 200ms, and could be used to produce individual butterfly flight choice behaviour records (Figure 2B), allowing us to reconstruct the precise location of the monarch throughout the entire experiment. This also allowed us to determine how much time a monarch spent flying through or over each position in the circular flight path. The circle prescribed by the flight apparatus arms was broken down into 18 angular position bins, each 20° wide. The position of the milkweed stimulus, called the milkweed sector of the circle, was comprised of 4 of these bins (80° total), located 180° apart from the control sector containing the control stimulus of the same dimensions (4 bins, 80°) (Figure 2C). For consistency in data collection, the apex of the milkweed stimuli was placed at a position in the circle of 270° , whereas the control was placed in a position at 90° . The optical encoder was reset between trials to reflect this positioning, as the test stimuli were constantly moved between experiments to avoid any potential conditioning of monarchs towards a particular position within the flight apparatus enclosure. Position data from each butterfly's flight record obtained from the optical encoder was grouped into the 18 angular position bins to obtain a measure of the time spent in each part of their constrained flight path. We averaged times spent in each of these bins across all butterflies for each of the experimental protocols. To establish whether monarchs were selectively more attracted to milkweed stimuli over controls, paired t-tests were performed between mean times spent within the milkweed sector compared to times spent within the control sector for all protocols. To determine if the

experimental protocols were significantly different from each other in terms of monarch attractive behaviour, we used the following equation to calculate an Attraction Index (AI)

$$AI = \frac{\text{(Average time in milkweed sector)}}{\text{(Average time in control sector + milkweed sector)}}$$

After calculation of attraction indexes for all experimental protocols, we ran a one-way ANOVA and pairwise multiple comparison procedures (Holm-Sidak method) to determine any potential significant differences between groups.

Experimental Protocols:

A. Monarch Flight Behaviour

1. Milkweed Plant and Control (Plastic Plant): Visual + Olfactory cues

A young milkweed test plant was placed on the floor of the flight choice apparatus enclosure, 180° opposite a plastic plant of similar color and size. Monarchs were tethered to the flight apparatus arm and allowed to fly for 1 minute until experimental recording began. After one minute of flying, the US digital software for recording data from the optical encoder began, and we collected data position points for 3 minutes of monarch flight. After 3 minutes, monarchs were placed back in their cages. Monarchs that did not fly for the entire length of the experiment were not included in our results, as were monarchs (n = 6) that flew around the circle continuously in one direction without initiating any turning movements via the swivel.

2. Milkweed Extract and Control Extract: Olfactory cues only

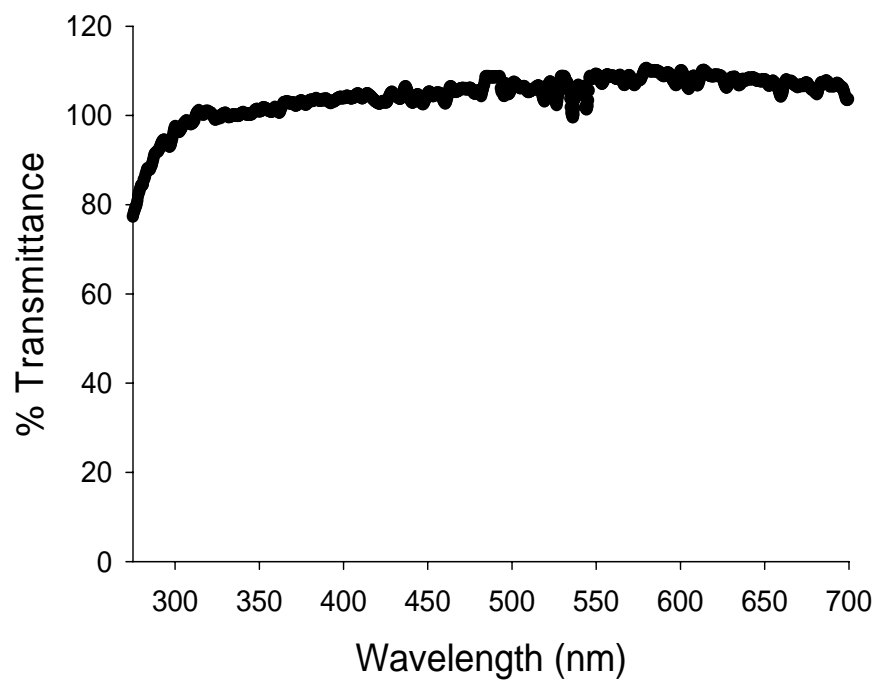
In this experimental protocol, 12 grams of fresh, young, milkweed leaves were collected and blended with 250mL of distilled water to make a milkweed “soup.” The soup was then filtered to remove solid material and the resulting liquid extract was absorbed onto a piece of cheesecloth and placed across the top of an open plastic cylinder. A small computer fan placed in the cylinder was used to direct any potential volatiles upward in the flight apparatus enclosure. A control stimulus, composed of cheesecloth soaked in distilled water and colored green, was placed on an identical cylinder with a fan, and positioned 180° away from the milkweed soup cylinder. Monarch flight behaviour was again measured for 3 minutes.

3. Milkweed Plant and Control (Plastic Plant): Visual cues only (\pm UV)

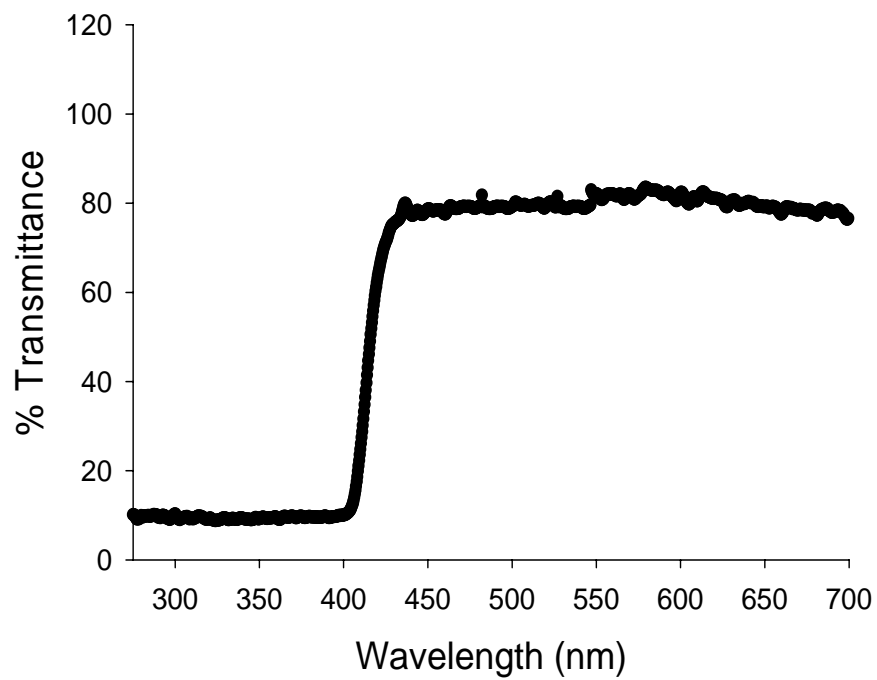
In this experimental setup, a young milkweed plant and control were placed in clear plastic cylinders with sealed Makrolon® polycarbonate lids (Sheffield Plastics, Sheffield, MA, USA) before they were placed within the flight enclosure. These lids allowed transmission of light from the UV through visible regions of the light spectrum. These experiments allowed monarchs to detect the plants visually; however, any olfactory cues released from the milkweed were substantially reduced if not eliminated. In a similar experiment investigating the effect of removing UV light, the same methodology was employed, except in this case, UV-blocking Makrolon® polycarbonate lids were placed on cylinders to block the transmission of UV light through the top of the cylinders. The relative transmission of the 2 lids used in these experiments is displayed in Figure 3 (see also Spectral Analyses in Methods).

Figure 3. Relative % transmittance of clear polycarbonate cylinder lids used in visual experiments. A) Percent transmittance of cylinder lids that transmit light in the UV range through the visible spectrum. B) Percent transmittance of cylinder lids that block light in the UV range.

A



B



4. *Milkweed vs. Control (Bugleweed– Lycopus americanus): Visual + Olfactory cues*

Here the same methodology used in the first experimental setup was employed, however, a milkweed plant was placed within the flight apparatus enclosure, and tested against a bugleweed control plant positioned 180° away within the enclosure. Bugleweed often occurs naturally in the same habitat as milkweed, and plants used were of similar size. Monarch behaviour was measured against these two naturally occurring plants for 3 minutes.

B. Spectral Analyses

Reflectance spectra from young and old, milkweed and bugleweed leaves were measured using a portable ST200 reflectance spectrometer with a USB2000 adaptor and PX2 Pulse Xenon lamp source (Ocean Optics, Dunedin, Fla., USA). Young leaves were characterized as the leaves closest to the growing tips at the top of both plants, whereas old leaves were determined to be located closer towards the ground. A modified probe was used to control distance between the light source and plant leaves undergoing spectral measurements. A total of 10 readings from the leaves of 10 plants were measured, and we calculated a mean reflectance curve for young and old leaves of both milkweed and bugleweed between 275 and 700 nanometers (nm) of the light spectrum. 95% confidence intervals were calculated and plotted every 50 nm between 325 nm and 625 nm and compared across the 4 leaf types. For visual experiments, percent transmittance of cylinder lids was determined after measuring a spectralon (Ocean Optics, Dunedin, Fla., USA) reading and a dark reading to standardize the spectralon to 100% reflectance across the spectrum. Each lid was placed on top of the standardized spectralon, and using the spectrometer, we measured the percent reflectance spectra of

the spectralon through the clear polycarbonate lids to determine the transmission spectra of the lids.

Results

I. Measuring Monarch Attraction

Milkweed vs. Control (Visual + Olfactory cues)

In this experimental setup a milkweed and control (plastic) plant were placed 180° apart, and monarch flight behaviour was measured for 3 minutes ($n = 26$) (Figure 4). Monarchs flew within the milkweed sector of the flight path for a significantly longer period of time than in the control sector (t -test, $t = 8.038$, $P < .001$, $df = 25$). In addition, monarchs spent approximately as much time flying within the control sector as they did in other positions of the flight apparatus enclosure which contained no stimuli (baseline).

Milkweed Extract vs. Control (Olfactory cues only)

When a milkweed extract and control extract were placed 180° apart on opposite sides of the flight path, monarchs spent a significantly longer period in the milkweed sector than the control sector (t -test, $t = 6.585$, $P < .001$, $df = 11$) (Figure 5), showing these monarchs were more attracted to the milkweed odour than the control. In addition, it was observed that there was a broader spatial distribution of monarch flight behaviour over time than in the previous experimental protocol (Figure 4).

Milkweed vs. Control (Visual cues only)

In this protocol, clear cylinders with sealed UV-transmitting lids containing a milkweed and control plant were placed 180° apart within the flight enclosure (Figure 6A).

Monarchs were significantly more attracted to the visual image of milkweed test plants within the milkweed sector compared to a control stimulus presented under the same conditions (t -test, $t = 5.035$, $P < .001$, $df = 10$). When cylinder lids were replaced with

Figure 4. Bar graph with standard error bars showing mean time monarchs spent in various positions within the flight apparatus enclosure. Monarch positions were recorded over a 3 minute period. In these experiments, monarchs were able to visually detect and smell both plants. Monarchs spent significantly more time over or near the milkweed sector than the control sector (for all statistics refer to Results). Black bars = milkweed sector; White bars = control sector; Gray bars = baseline (no stimuli located here) (n = 26).

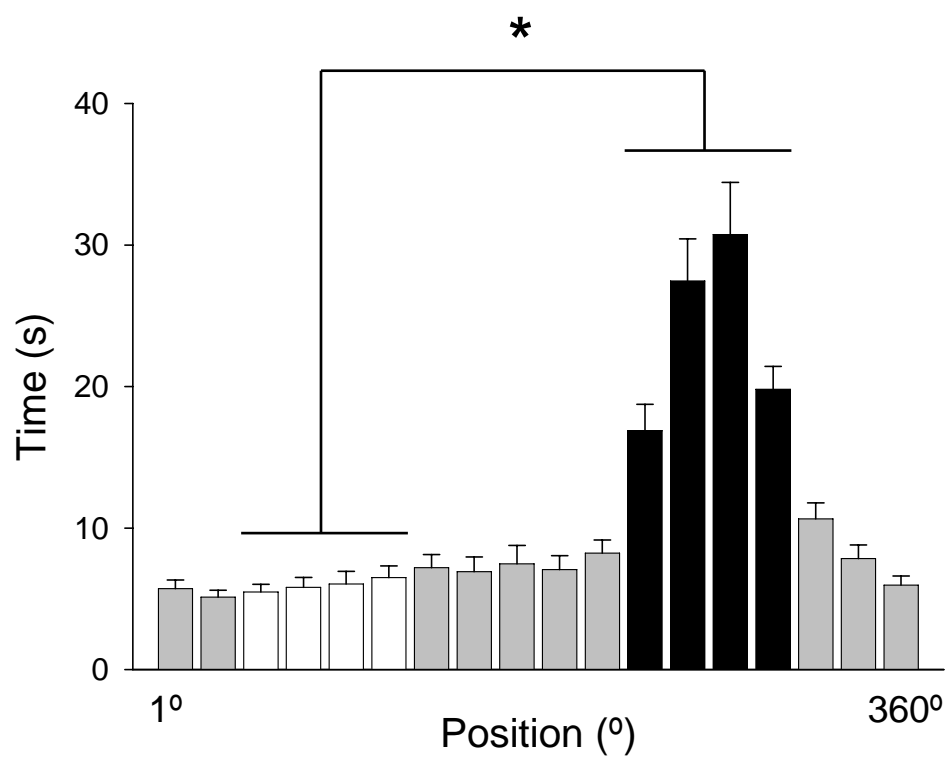
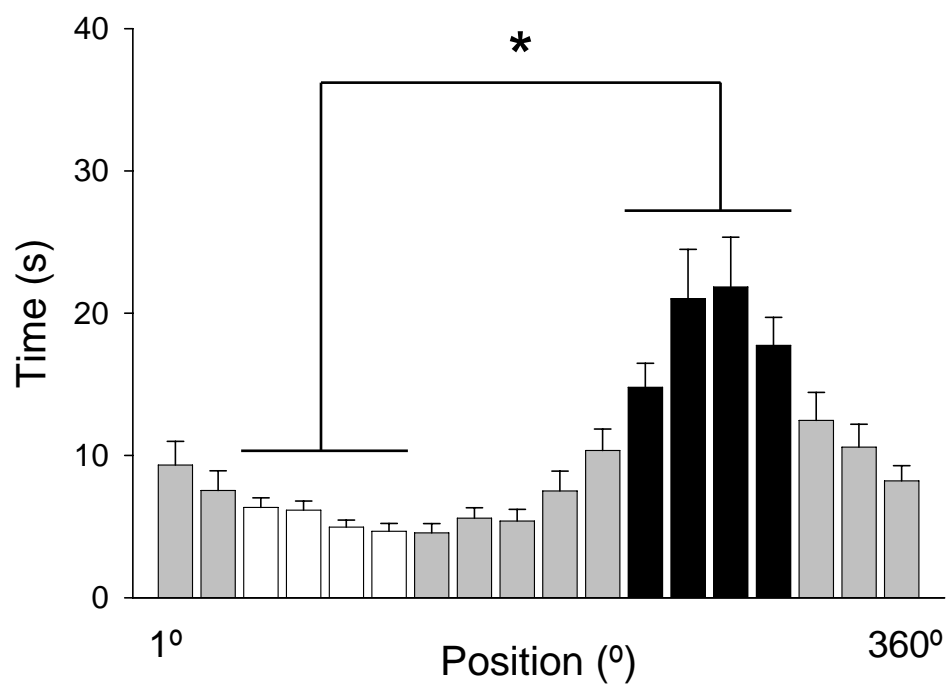


Figure 5. Bar graph showing mean time monarchs spent in various positions within the flight enclosure. In this experimental setup, a milkweed slurry, or “soup” was prepared by blending fresh, young, milkweed leaves with distilled water. For controls, only distilled water was used as a stimulus, however, the cheesecloth was colored the same green hue as milkweed soup to control for any color preferences monarchs may have had during the experiments. Monarchs were found to fly within the milkweed sector for a significantly longer period than in the control sector, showing these animals were attracted to olfactory cues of milkweed even when its visual image had been removed (n =12).



UV-blocking lids (Figure 6B), monarchs also spent a significantly longer time around the milkweed test plants within the milkweed sector when compared with control plants within the control sector (t -test, $t = 7.633$, $P < .001$, $df = 11$).

Milkweed vs. Bugleweed (Lycopus americanis)

In our final control we tested monarch attraction behaviour to either milkweed or bugleweed, using the same experimental procedure as in our first experimental protocol. Monarchs were found to spend a significantly longer time within the milkweed sector than control sector containing a bugleweed control (t -test, $t = 23.401$, $P < .001$, $df = 4$) (Figure 7), indicating a strong preference for the milkweed plant.

Figure 6. Bar graphs showing mean time spent by monarchs around milkweed host plants and controls through visual cues alone. In both sets of visual experiments, a milkweed plant was placed in a clear, sealed, plastic cylinder with a lid (Refer to Figure 3). The control (plastic) plant was placed 180° apart within the flight apparatus enclosure in a cylinder of the same materials and proportions. A) Monarchs were found to fly within the milkweed sector for a significantly longer time than controls when only the visual image of each plant was present. Lids covering cylinders in these experiments allowed transmission of light from visible range down through the UV region of the spectrum (n = 10). B) Same methodology as A, except UV-blocking lids were used to seal the plastic cylinders containing milkweed or control plants, thus substantially reducing UV cues from reaching monarchs. Monarchs were still found to fly within the milkweed sector for a significantly longer period of time than the control sector, however, this behaviour was reduced by UV removal (n = 12).

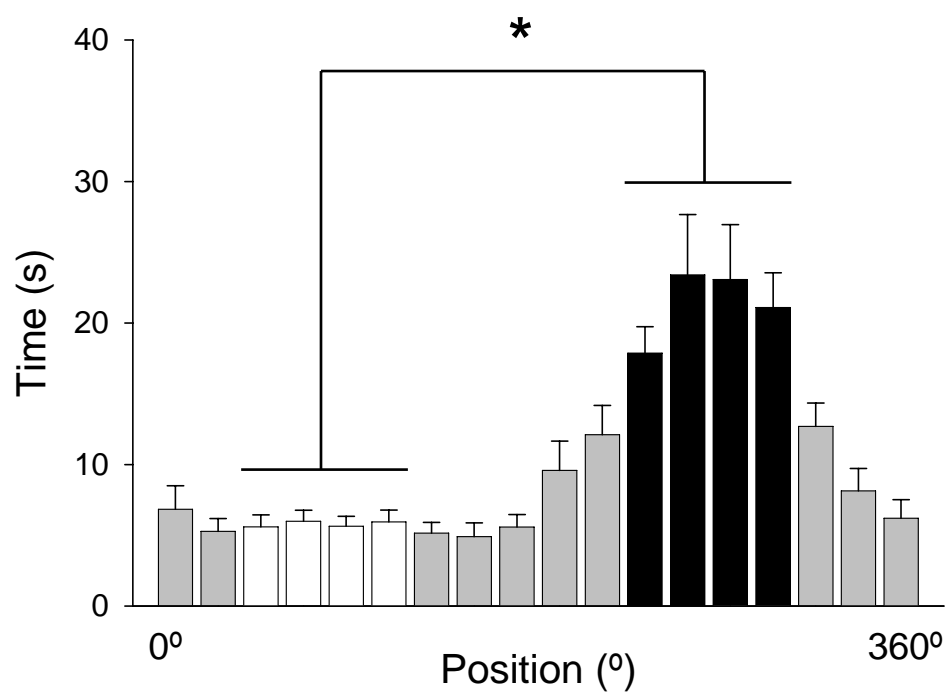
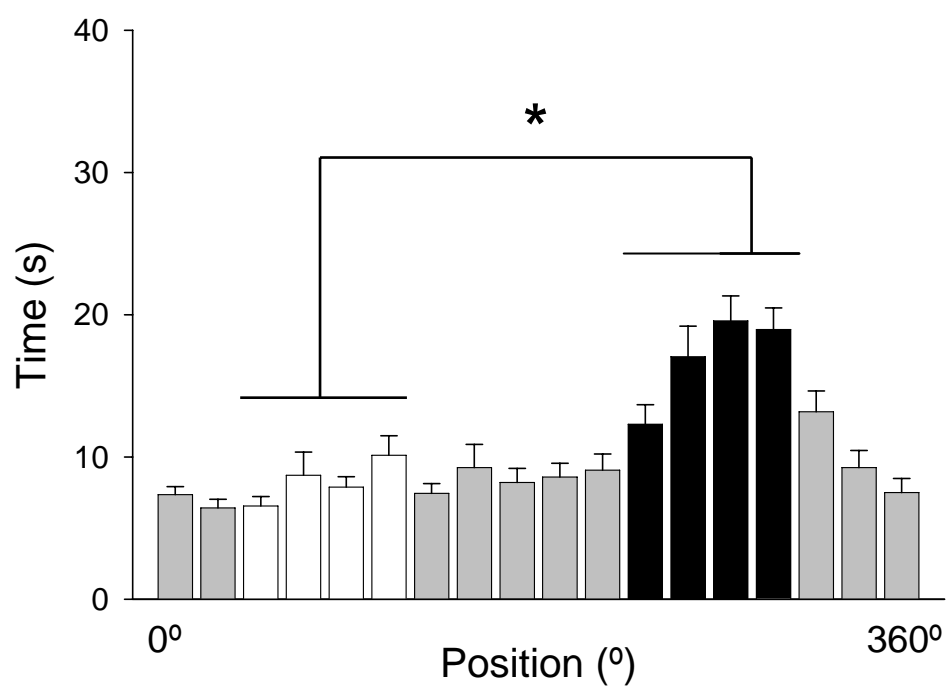
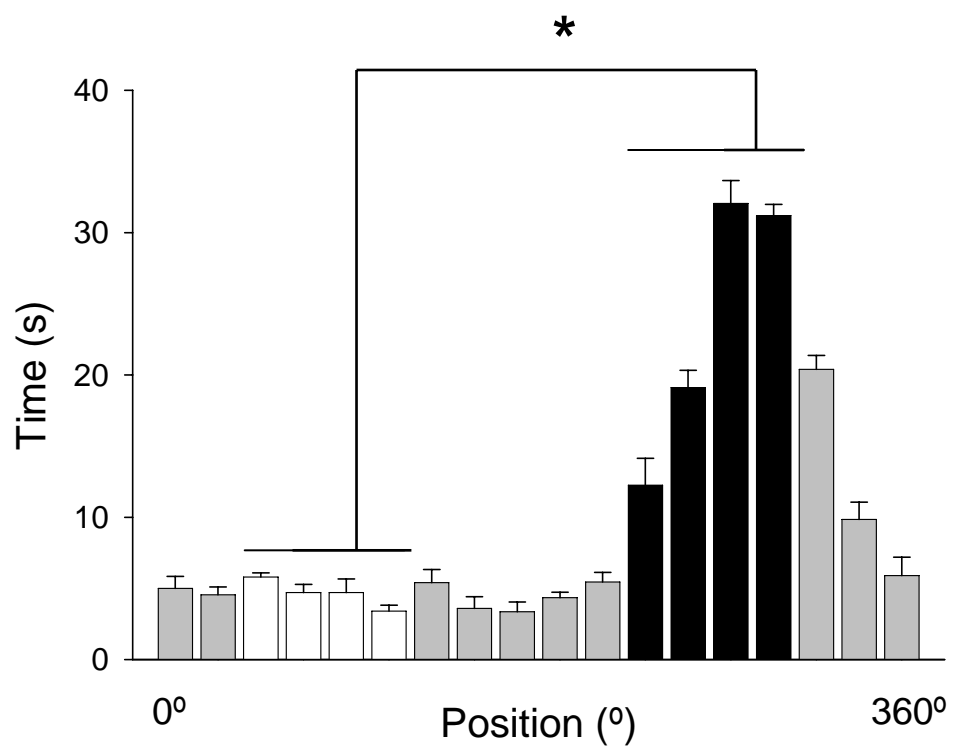
A**B**

Figure 7. Bar graph showing mean time spent by monarchs within flight apparatus enclosure at various positions. Same methodology as that shown in Figure 4, except the control plastic plant was replaced with the naturally occurring bugleweed. Monarchs were found to fly within the milkweed sector for a significantly longer period of time compared with the bugleweed sector ($n = 5$).



Comparison of Experimental Protocols: Attraction Index

We next determined which of the experiments resulted in the highest attraction behaviour of monarchs towards milkweed test plants. An attraction index was used (see Methods) that calculated the ratio of time spent in the milkweed sector to time spent around control and milkweed sector for all experimental protocols (Figure 8). The highest attraction index was found in the milkweed vs. control (plastic plant) experimental setup, when visual and olfactory cues were present, whereas the lowest attraction index was observed in the visual experiments after UV light was removed as a cue. It was found that there were no significant differences between any of the milkweed vs. control, milkweed vs. bugleweed, milkweed extract vs. control, or milkweed vs. control (UV present, visual) experimental protocols (statistics not shown). There was, however, a significant effect of experimental protocol on the attraction of monarchs to milkweed (One-way Analysis of Variance, $P = 0.01$, $F_{(4,61)} = 3.676$). Significant reductions in monarch attraction to milkweed were observed in the visual experiments where UV cues were removed compared, respectively, with: 1) milkweed vs. control protocols (*post-hoc* Holm-Sidak method, $P < 0.05$), 2) milkweed vs. bugleweed (*post-hoc* Holm-Sidak method, $P < 0.05$), 3) milkweed extract vs control extracts (*post-hoc* Holm-Sidak method, $P < 0.05$), and 4) milkweed vs. control with UV cues (*post-hoc* Holm-Sidak method, $P < 0.05$) protocols.

II. Spectral Analyses

Mean percent reflectance measurements in young and old milkweed and bugleweed leaves are illustrated in Figure 9. It was found that young milkweed leaves and old milkweed leaves reflect differential UV-light signatures. Below 500nm and within the UV region of the spectrum, young milkweed leaves display clear reflectance peaks,

Figure 8. Attraction indices for each of five experimental protocols. Monarch attraction to milkweed vs. controls was significantly diminished in visual experiments when UV signals were removed. V = visual cues; O = olfactory cues; V+O = both visual and olfactory cues were available. Control refers to protocols utilizing plastic plant, common asterisks indicate significant differences.

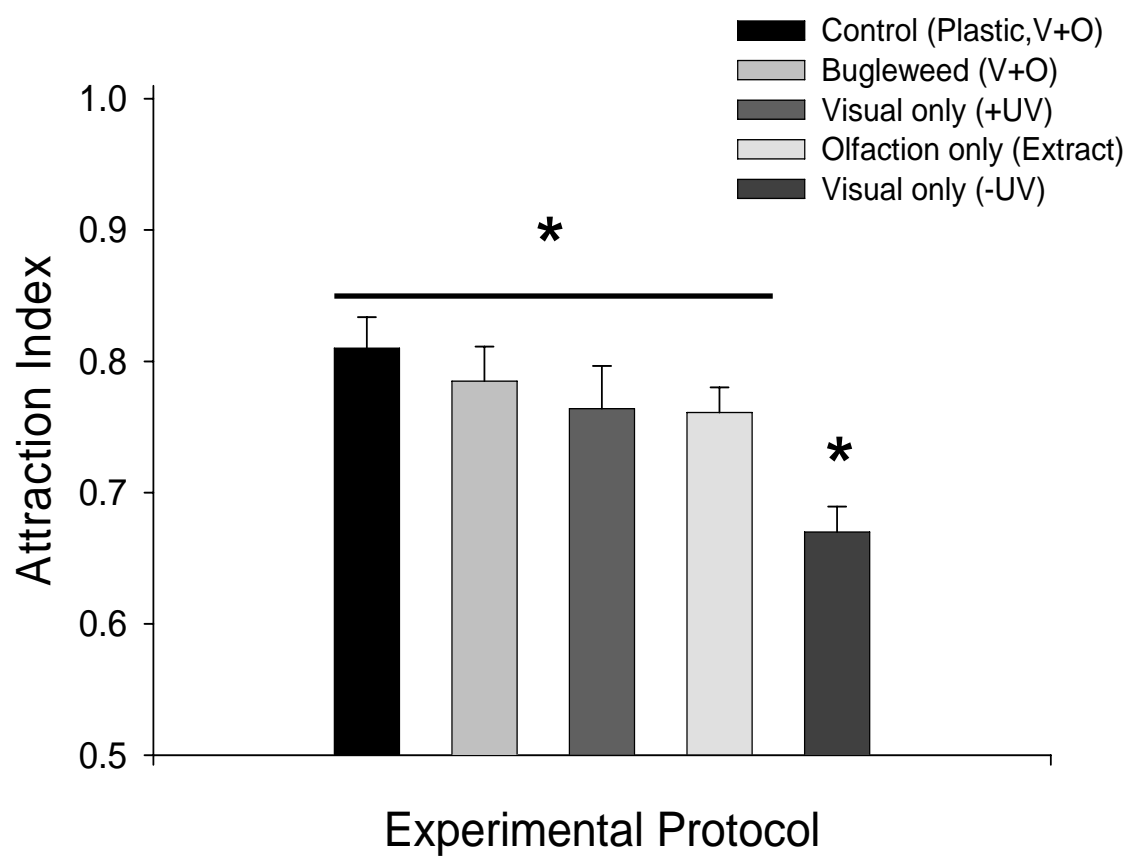
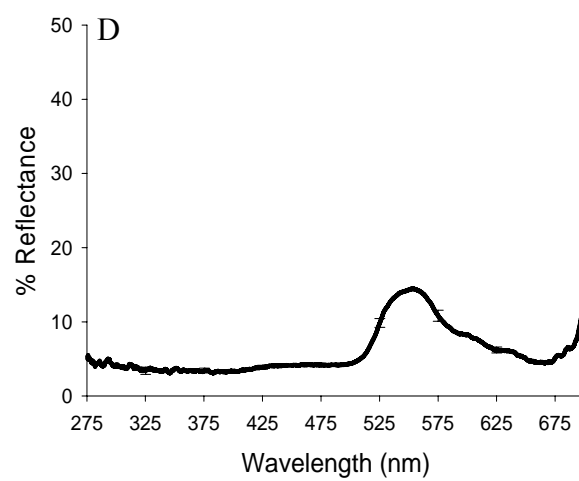
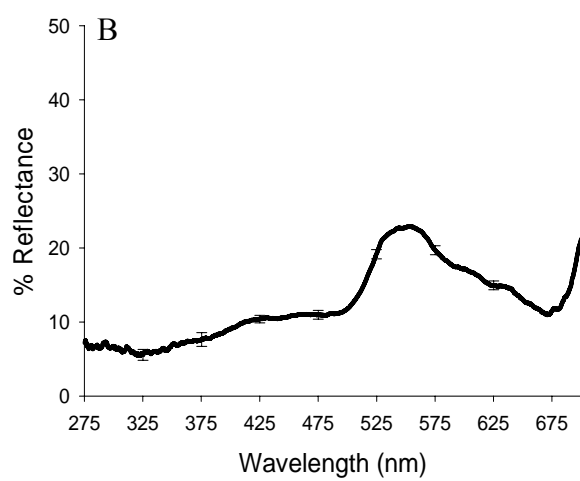
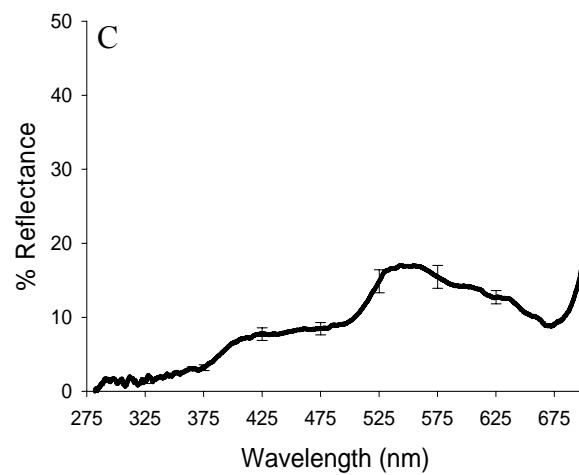
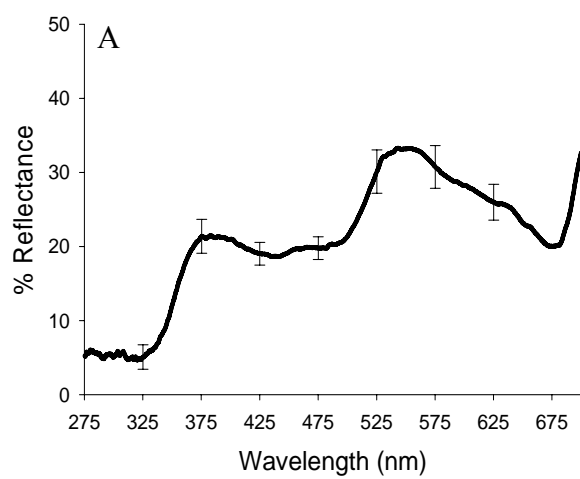


Figure 9. Spectral reflectance measurements from young and old milkweed and bugleweed leaves. A) Spectral reflectance measurement from young milkweed leaves B) Spectral reflectance measurements from old milkweed leaves C) Spectral reflectance measurements from young bugleweed leaves and D) Spectral reflectance measurements from old bugleweed leaves ($n = 10$ for all leaf types). Bars indicate 95% confidence intervals every 50nm between 275nm and 700nm, with milkweed displaying significantly larger reflectances in the UV and visible part of the spectrum in young milkweed leaves than in all bugleweed leaves.



whereas old milkweed and all bugleweed leaves displayed minimal light reflectance, as indicated by the means curves and 95% confidence intervals. We also observed an overall broader reflectance across the entire spectrum in milkweed leaves compared with bugleweed controls, as indicated by their respective peaks at approximately 560nm, indicating bugleweed leaves absorbed much more light than young milkweed leaves. Mean reflectance curve comparisons indicate these leaves have significantly different reflectance spectra across the entire insect-visible range.

Discussion

Our novel flight choice apparatus has demonstrated monarch butterfly attraction to milkweed in response to visual and olfactory cue combinations, visual cues alone, and olfactory cues alone. In addition, UV light reflectance from young milkweed leaves appears to play a role in either determining the suitability of host plants, or as an additional cue facilitating male and female monarch attraction to milkweed while in flight.

Host plants are critical for phytophagous insects, as their larvae are generally immobile and begin feeding off the plant on which they were laid. Thus selection of suitable hosts by ovipositing females determines viability of offspring. The larvae of monarch butterflies receive not only nourishment from milkweed hosts, but sequester compounds critical for their toxic defense against predators, making suitable milkweed hosts essential for survival. Different insects face multiple challenges and employ different strategies for locating host plants. Some species of aphids utilize only one or a few closely related host plants, many of which occur at very low abundances in the field (Hopkins *et al.*, 2002) making it difficult to locate them. How these insects, including butterflies, locate hosts in the face of thousands of different species of plants with specific sizes, shapes, colors, and volatiles is a daunting task. An early theory of host plant choice suggested that phytophagous insects are constrained by the enormous amount of information in the environment, and are thus limited in their capacity of sensory processing (Levins and MacArthur, 1969). To determine how monarchs successfully locate milkweed host plants in these conditions of vast sensory information,

we have attempted to identify which specific cues are utilized by monarchs to facilitate their localization of milkweed host plants.

There is much evidence that many insects use plant volatiles to locate and subsequently land on their hosts. Plant volatiles mediate attraction to host plants in various species of moths (Reddy *et al.*, 2004; Bengtsson *et al.*, 2006), and butterflies (Bergstrom *et al.*, 1995). In our study, flying monarchs were selectively attracted to a milkweed “soup” compared with a control “soup.” This finding clearly indicates that monarchs are able to find, or are attracted to, milkweed volatiles. The data suggest monarchs were following a concentration gradient in the vicinity of the milkweed soup, as there was a much wider flight distribution compared with the more localized flight behaviour in the visual experiments. This observation may help to explain why monarchs spent a significantly longer time within only 3 of the 4 bins of the milkweed soup sector compared with visual (+UV) experiments where monarchs were observed to display similar behaviour in all 4 bins of the milkweed sector. In an investigation of host plant volatiles consistent with our results, female monarchs, before landing to oviposit, were observed to selectively choose white strips of paper sprayed with young milkweed leaf extract over those with old leaf extract, where chemical analyses determined differences in volatile components (Bergstrom *et al.*, 1995). Thus, monarchs likely use plant volatiles as a means to locate and differentiate milkweed. We are currently investigating volatile composition released by milkweed and comparing them with those released by bugleweed plants. Not surprisingly, preliminary gas-chromatography mass spectrometry (GC-MS) analysis has yielded many differences in volatile compositions and we plan to continue this investigation to possibly identify a compound or cocktail of compounds

unique to milkweed that attract monarchs, which we plan to test with our flight choice apparatus.

Our findings that monarchs were selectively attracted to the visual image of milkweed alone compared with controls are consistent with previous studies in other insects. Female swallowtail butterflies (*Papilio aegeus*) make decisions about their host plants, of the family Rutaceae, based on colour, and prefer to lay eggs on certain host plant leaves that look green to humans by utilizing special receptors to guide host plant choice behaviour (Kelber 1999). Further evidence of visual cues guiding host plant attraction are butterflies of the genus *Lycaena*, which are believed to possess specific red pigments in order to discriminate red larval host plants (Bernard and Remington 1991). It has been suggested that plant visual stimuli are detected at a distance (several meters or more away) through contrast differences between plants of interest and the surrounding environment or vegetation (Prokopy and Owens, 1983). However, as flying insects draw nearer towards a potential host plant, additional visual cues such as spectral reflectance characteristics, are potentially perceived (Prokopy and Owens, 1983), which may have been a factor in our results given monarchs' close proximity to all stimuli within the flight choice apparatus enclosure. There is some evidence that spectral reflectance characteristics, as a specific signature of host plants, may act as a trigger stimulus for insects landing on plants (Coombe 1981). This is consistent with our visual experiment results, where flying monarchs were enclosed in a relatively small area with milkweed and control stimuli yet were able to differentiate between the two plants. Monarchs were selectively attracted to the visual image of milkweed alone compared with controls and bugleweed, and this attraction was significantly reduced when UV reflectance cues were

prevented from reaching monarchs through the use of UV-blocking lids. We found that young milkweed leaves reflect much more UV and visible light than older milkweed leaves and all bugleweed controls. This is interesting due to findings that young milkweed leaves are more attractive to ovipositing female monarchs than older milkweed leaves, one of the reasons we used only fresh young plants in our experiment, and this is possibly because of a release of certain combinations of volatiles specific to young leaves (Bergstrom *et al.*, 1995). Young leaves are also more attractive as host plants to swallowtail butterflies, as caterpillars reared on young leaves (*Citrus* sp.) grow much faster and larger, pupating earlier than those caterpillars reared on older leaves (Kelber 1999). It is believed female swallowtails make choices to oviposit on young leaves based on differences in spectral compositions of leaf types (Kelber 1999). We have shown that a UV visual component differentiating young milkweed from old milkweed and bugleweed leaves may also be involved in assessing host plant quality, in addition to volatiles.

Monarchs possess populations of UV receptors in the anatomically specialized region of the eye, known as the dorsal rim area (Sauman *et al.*, 2005; Stalleicken *et al.*, 2006). In addition to the dorsal rim area (DRA), where UV receptors detect the e-vector of polarized light for polarization navigation, there exists additional UV-sensitive receptors scattered throughout the rest of the monarch eye (Stalleicken *et al.*, 2006). The exact function of these UV cells is not known, however, given their placement on the eye, it is likely they are concerned with stimuli situated below or on the same plane of their flight, rather than the DRA, which is positioned to receive visual information from the sky. Monarchs may use these UV-sensitive cells located over the entire compound eye

but outside the DRA to detect UV reflectance from either potential nectar-bearing plants or host plants. However, it is evident that spectral quality of milkweed leaves is not the only driving force by which monarchs are attracted to milkweed, as monarchs were still able to successfully locate them even after UV signals were removed. It is very interesting how monarchs, even without olfactory cues, were still able to differentiate between milkweed and all controls. Shape, size, and color are likely to play a role as well.

Comparing ratios of time spent around milkweed vs. control plastic plants or bugleweed plants showed that either visual cues or olfactory cues by themselves were sufficient, but not necessary, to elicit an attractive flight behaviour around milkweed stimuli. It is possible given the three minute experimental window, we reached a ceiling of time spent around each stimulus. Monarchs were not flown for very long due to observations that they typically cease flight activity after a period of 4-5 minutes. Given longer flight times, monarchs may have flown in the milkweed sector for the longest relative duration during the experiments where monarchs were able to detect full visual (+UV) and olfactory cues. Although the data suggests this may be the case, we were not able to test this experimentally. Much research has pointed towards the influence of volatiles on recognizing host plants, but our results clearly indicate that without a UV-signal, monarch attraction behaviour to milkweed is significantly decreased when compared to all other experimental protocols. A visual component to host plant attraction must be involved, at least when host plants are within short distances.

Our novel flight choice apparatus has allowed us to determine how monarch butterflies are attracted to milkweed host plants. This apparatus has implications for

other flying insects, such as in pheromone research in moths and butterflies, as well as in the development of new chemical attractants for pest management. We plan to investigate some of this apparatus' diverse functions in the future.

Chapter III

General Discussion

3.1 General Findings and Interpretations

Through the application of our novel flight choice apparatus, we have experimentally established that monarch butterflies are attracted to milkweed, and this attraction is maximized when olfactory and visual cues are available. We are the first group to establish, quantitatively, monarch (male and female) attraction to milkweed during the pre-alightment phase of host plant localization, before contact oviposition stimulants are perceived by monarch contact chemoreceptors. We used young plants in all experiments, due to previous findings that female monarchs preferentially oviposit on the young growing tips of milkweed (Bergstrom *et al.*, 1995), and this behaviour has also been found in other ovipositing butterflies, as young host plant leaves provide better nourishment for larvae than older leaves (Kelber 1999). We were excited to discover differential light reflectances between old and young milkweed leaves, especially in the UV region, but also throughout the visible spectrum, as previous reports have focused on the differences in plant volatiles emitted from old and young host plant leaves. Old and young milkweed leaves have been found to possess different chemical volatiles, which are believed responsible for attracting female monarchs to oviposit on young milkweed leaves (Bergstrom *et al.*, 1995). It is possible that this UV visual signal, in addition to the olfactory signal, may aid in directing females to young milkweed leaves. In comparing the different experimental protocols we employed to measure monarch attraction to milkweed while in flight, we determined that all procedures were sufficient to elicit an attraction to milkweed over controls, however, comparison of attraction indexes show

that UV is an important signal for both male and female monarchs to identify milkweed, as well as discriminating young from old leaves (much more important for females), as monarch attraction behaviour was significantly dampened upon UV filtering. Our experimental protocols that elicited the highest level of monarch attraction to milkweed were observed when monarchs could detect the combination of visual and olfactory cues, accompanied by a strong UV signal.

Locating milkweed host plants is crucial for monarch butterfly survival, as these plants are chosen as oviposition sites by females and provide larvae with nutritional requirements, as well as the chemicals that render them toxic as larvae and adults. Male monarchs have also been found to patrol patches of milkweed in the field, presumably to locate mates (Zalucki 1993), so patches of milkweed can be thought of as a “singles bar.” Based on our results that both sexes of monarchs are selectively attracted to milkweed over controls under a variety of conditions, we have proposed a model of how monarchs locate milkweed in their natural environment. Milkweed, like virtually all plants, is not evenly distributed in the field, and as monarchs are specialists of only a few species of milkweed, a monarch’s movements, like other insect host plant specialists, must depend in some way on the dispersal of milkweed hosts throughout the environment (Mackay 1985). This dispersal leads to clumped distributions of plants, whereby insects must employ non-random, guided movement strategies to actively search out host plants, because insects will not encounter their host plants very frequently (Rogers 1972). We propose that monarchs most likely utilize olfactory cues to attract them to a general area where milkweed is located, since the spatial distribution of milkweed, as well as the geographical landscape (hills, trees, etc) could prevent monarchs from visually detecting

these plants. We believe monarchs follow a concentration gradient (implied through the broad distribution in our olfactory experiments) of as yet unidentified, milkweed volatiles to guide them to an area where milkweed clusters are found. At this stage, visual cues can now, along with olfactory cues, guide them to milkweed. A UV-signal is also likely involved, however, it is not the signal that solely determines whether a monarch will land on a milkweed plant, as multiple flowers and other vegetation possess these signals (Kevan and Backhaus 1998), and because our findings that monarchs could distinguish MW even when a UV signal was removed. We believe the UV signal from milkweed works in conjunction with olfactory and other visual cues to tell monarchs that a plant of interest is a milkweed, and it is young and fresh. Leaf structure, color, and size, most likely play a role, and UV is only one part of the spectrum; other regions may be just as important (Kevan *et al.*, 2001). Once a monarch approaches a milkweed at this stage, females, especially, have received all the sensory information required to know it is a milkweed host, and land. This is supported by observations that females regularly lay eggs on the first milkweed host plant she alights on, and do not often make mistakes (Bergstrom *et al.*, 1995). After landing, post-alightment behaviour occurs, in which monarchs drum the leaf surface with their spine-bearing tarsi and antennae before ultimately accepting the milkweed as a host and lay their eggs.

3.2 Possible Experimental Limitations

One possible limitation of this study was the size of the circumferential flight path prescribed by the flight choice apparatus arms. In our olfactory experiments, the volatiles may have diffused throughout the flight apparatus enclosure because it may not have been a large enough area. Although we still observed monarch attraction to milkweed

under these conditions, it would have been interesting to see if olfactory cues elicited a stronger response. However, we were reluctant to elongate the flight apparatus arms, as longer lengths lead to larger weights and more friction, and we did not believe we would then obtain a good measure of monarch flight behaviour. Another possible limitation with this study were sample sizes. Increasing some of the experimental protocol sample sizes might have resulted in more significant differences between attraction indexes in the experimental protocols that followed our observed trend. Butterfly loss, primarily through monarchs pulling out their surgically implanted stalks while in their cages, was another problem in this study, as much time was spent replacing stalks and allowing recovery before running experiments.

Lastly, we did not observe differences in attraction indices between protocols, especially when experiments were devised to remove one or more cues. We expected the largest attraction index to occur when monarchs could both see and smell the milkweed stimuli. Although our data show this trend, it was not statistically significant. One explanation for these findings could be that we reached a ceiling in amount of time monarchs spent flying around the milkweed stimulus. Flying the monarchs for longer than 3 minutes would have been ideal, however, butterflies used in our experiments were summer butterflies, not fall butterflies, and so lacked the drive to fly for extended periods of time in contrast with the fall butterflies that migrate each year to Mexico. Fall butterflies were not used in our data collection as they are not interested in mating at this point in their development, and given the large influence of milkweed on mating behaviour, we were restricted to use the summer butterflies.

3.3 Future Directions

Our novel flight choice apparatus has many potential applications for quantitative behavioural research on monarchs and other flying insects. The monarch butterfly is one of the most widely known insects, not only because of its bright warning colours, but because of its remarkable annual southern migration. This journey can be as long as 4800 km in the fall to precise over-wintering sites in central Mexico, and it is still not known how monarchs accomplish this feat, although they do not learn the route, as only one in every 5 generations makes the long journey to and from these sites (Solensky 2004). Our lab has confirmed that monarchs use a sun compass to orient themselves in a south-westerly direction (Mouritsen and Frost 2002), and other cues, such as magnetic fields, and polarized light patterns, do not seem to play a role in the migration. However others, using very small sample sizes of monarchs, have shown they may use polarized celestial patterns to navigate during migration (Reppert *et al.*, 2004) but we are more interested in how, once monarchs arrive in the general area of their over-wintering sites, they precisely locate trees that are used year after year for their over-wintering clusters. Our experiments showing the cues used by monarchs to locate their milkweed host plants may help to understand this question. We currently have collaborations with other labs that have collected samples from trees monarchs over-winter in, in an effort to isolate potential olfactory signals from the trees. We hope to test some of these compounds in our flight choice apparatus against cues from trees in the overwintering area that monarchs are not attracted to. This may help in the conservation of the monarch butterfly, as many of these overwintering sites are being cut down by illegal logging, and

the idea of “planting” a signal in a safe area to attract overwintering monarchs would be an ultimate goal.

We are also planning to investigate further the chemical composition of our milkweed “soup” through gas-chromatography mass spectrometry (GC-MS) analysis. Preliminary results show there are compounds commercially available found in this milkweed soup and not in bugleweed. We hope to test some of these compounds using our flight choice apparatus. Our apparatus could potentially be used to measure attraction of any flying insect towards any object of interest. Insect pests such as the gypsy moth, diamondback moth, and beetles could be tested against a series of compounds to develop biologically safe control strategies. However, determining which cues are used by these insects to locate their host plants must be understood before any experiments can be carried out.

Conclusions

We have developed a novel flight choice apparatus to test male and female monarch butterfly attraction to their milkweed host plants. We found monarchs are attracted to young milkweed under a number of different experimental protocols to test which cues are paramount in host plant selection and/or attraction. Both visual and olfactory cues presented alone were sufficient to elicit a hovering attraction response over the milkweed sector within our flight apparatus enclosure, however, this attraction behaviour was significantly reduced when UV cues were removed. Young milkweed leaves possessed a unique spectral peak in the UV region of the light spectrum, which was absent in both older milkweed leaves and all bugleweed controls, indicating that in addition to previous studies suggesting the young-old leave difference lies with olfactory cues, UV visual cues may also be important in guiding host plant attraction. Our apparatus can be applied to any flying insect, and we plan to investigate the influence of attractive substances on other organisms.

Appendix

Chemical Analyses

After our findings that monarchs were selectively more attracted to milkweed than bugleweed controls, we were interested in how these plants differ in order to determine what specific olfactory cues attract monarchs to milkweed. As our experiments were investigating visual and olfactory cue involvement in monarch attraction to milkweed, and spectral analyses showed differences in reflectance properties between bugleweed and milkweed, we were interested in determining differences in volatiles emitted from both plants.

Methodology

12 grams of fresh, young, milkweed or bugleweed leaves were ground up in 250mL of distilled water (as in olfactory experiments above). After filtering, a 10mL sample of this slurry was diluted in 40mL distilled water, and extracted with 50mL methyl tert-butyl ether (MTBE). The MTBE was concentrated to 0.5mL using a stream of nitrogen gas. These milkweed or bugleweed extracts (0.5g of original plant in 0.5mL of MTBE) were then run by gas chromatography-mass spectrometry (GC-MS; HP 5890 Series II Plus gas chromatograph equipped with an HP 5972 Mass selective detector, with a VOCOL™ fused silica capillary column) to identify any potential differences between the two plants. HPChem NIST software was used to analyze the chromatographs. MTBE blank samples were run with milkweed samples.

Results

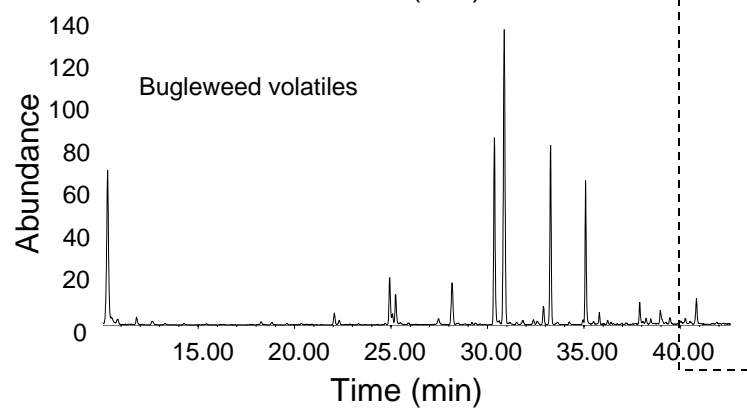
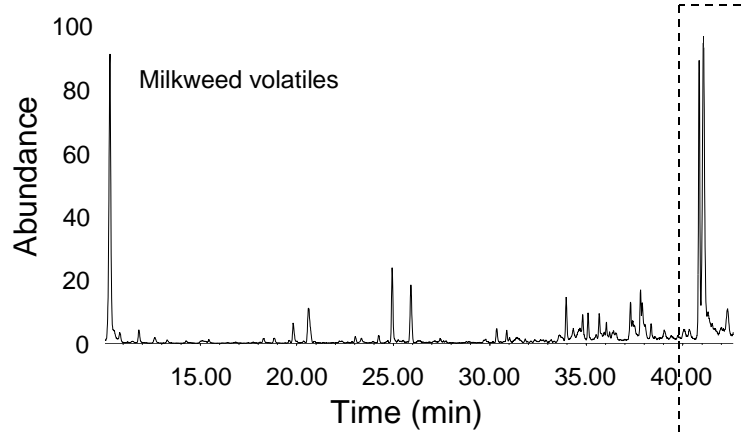
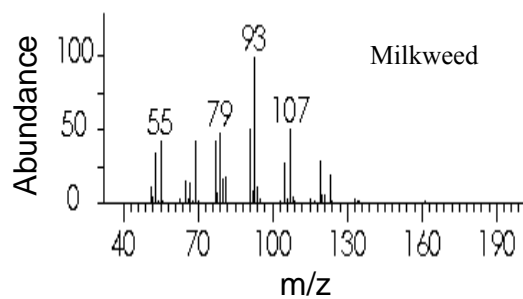
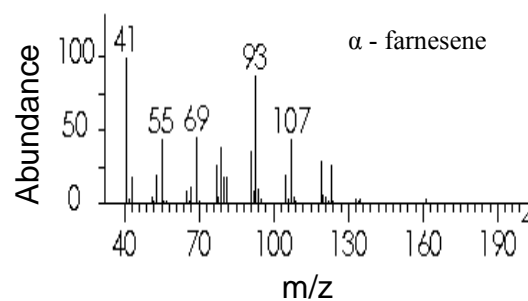
Chromatograms were examined to determine significant peaks present in the milkweed extract but not in the bugleweed extract. Similar composition between the milkweed and bugleweed extracts at early times represent the solvent. As expected, there were differences in the chemical composition of milkweed and bugleweed extracts following GC-MS analysis (Figure 11). The most significant difference was at 42 mins, where it was found that the mass spectra of this compound in the milkweed extract was absent in the bugleweed extract, and similar in composition to that of alpha-farnesene.

General Interpretations

We found distinct chemical differences in the volatiles between milkweed and bugleweed samples using GC-MS analysis. Unfortunately, we were not able to determine with high probability the chemical structures of most of these compounds, but we were able to identify, with very high probability, one volatile as α -farnesene. α -farnesene was first reported as a volatile released from the wax of apple skins, and has since been identified in a variety of different plant species, including milkweed (Bergstrom *et al.*, 1995; Yan *et al.*, 2003). An isomer of this compound has been found to be present in larger quantities in young leaves of many different insect host plants, including milkweed, apples, and cotton, which may act as a young leaf signal (Sutherland and Hutchins, 1972; Bergstrom *et al.*, 1995; Harborne 1997). This may be one reason why female monarchs tend to oviposit only on the fresh young growing tips of milkweed. The likelihood of this single compound attracting monarchs to milkweed is small and most likely involves additional volatiles for the following reasons. Previous studies examining this compounds effects

on codling moths, who utilize apples as host trees, have shown these moths have reduced responses to pure α -farnesene when compared to the entire cocktail of odours emitted from apples (Yan *et al.*, 2003). In addition, these responses to pure farnesene were only observed at specific concentrations, complicating the matter further (Yan *et al.*, 2003). Nevertheless, we plan to examine the effects of this compound on monarch butterfly attraction behaviour to milkweed using our novel flight apparatus.

Figure 1. A) Total ion chromatographs of milkweed (top) and bugleweed (bottom) leaves after extraction and GC-MS analysis. B) Expanded view of segment of chromatographs after 40mins of GC-MS analysis illustrating chemical composition between milkweed (top) and bugleweed (bottom) extracts. It was observed that that this was one of the major differences in chemical composition between the two plants. C) MS readings of milkweed extract compound after 40mins compared with D) the MS reading of α -farnesene. Note our compounds similar chemical composition.

A**C****D**

Literature Cited

- Ackery, P. R. and Vane-Wright, R. I. (1984). Milkweed butterflies: their cladistics and biology. Ithaca, New York: Comstock Publishing Associates, a division of Cornell University Press.
- Bengtsson, M. *et al.* (2006). Plant volatiles mediate attraction to host and non-host plant in apple fruit moth, *Argyresthia conjugella*. *Entomologia Experimentalis et Applicata* **118**, 77-85.
- Bergstrom, G. *et al.* (1995). Oviposition by butterflies on young leaves: investigation of leaf volatiles. *Chemoecology* **5**, 147-158.
- Bernard, G. D. and Remington, C. L. (1991). Color-Vision in *Lycaena* Butterflies - Spectral Tuning of Receptor Arrays in Relation to Behavioral Ecology. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 2783-2787.
- Bernays, E. A. (2001). Neural limitations in phytophagous insects: Implications for diet breadth and evolution of host affiliation. *Annual Review of Entomology* **46**, 703-727.
- Bolter, C. J. *et al.* (1997). Attraction of Colorado potato beetle to herbivore-damaged plants during herbivory and after its termination. *Journal of Chemical Ecology* **23**, 1003-1023.
- Brower, L. P., Brower, J. V., and Corvino, J. M. (1967). Plant posions in a terrestrial food chain. *Proceedings of the National Academy of Sciences of the United States of America* **57**, 893-898.
- Brower, L. P. (1969). Ecological Chemistry . *Scientific American* **220**, 22-29.
- Brower, L. P. (1972). Ecological chemistry of the palatability-cardiac glycoside spectrum in Monarch butterflies, *Danaus plexippus*, and *Asclepias* milkweeds. *American Zoologist* **12**, 712-713.
- Brower, L. P. (1984). Chemical defence in butterflies. In: *The biology of butterflies* (eds. Vanewright, R. I. and Ackery, P. R.), pp. 109-134. *Symposia of the Royal Entomological Society of London* No. 11.
- Brunton, C. F. A. and Majerus, M. E. N. (1995). Ultraviolet Colors in Butterflies - Intraspecific Or Inter-Specific Communication. *Proceedings of the Royal Society of London Series B-Biological Sciences* **260**, 199-204.

- Chittka, L. and Menzel, R. (1992). The Evolutionary Adaptation of Flower Colors and the Insect Pollinators Color-Vision. *Journal of Comparative Physiology A- Sensory Neural and Behavioral Physiology* **171**, 171-181.
- Chittka, L. *et al.* (2001). Adaptation, constraint and chance in the evolution of flower color and pollinator color vision. In: *Cognitive Ecology of Pollination* (eds. Chittka, L. and Thomson, J. D.), pp. 106-126. Cambridge University Press.
- Coombe, P. E. (1981). Wavelength Specific Behavior of the Whitefly *Trialeurodes Vaporariorum* (Homoptera, Aleyrodidae). *Journal of Comparative Physiology* **144**, 83-90.
- Haack, R. A. *et al.* (2002). The emerald ash borer: a new exotic pest in North America. *Michigan Entomological Society Newsletter* **47**, 1-5.
- Hangartner, W. (1970). Control of Pheromone Quantity in Odor Trails of Ant *Acanthomyops-Interjectus* Mayr. *Experientia* **26**, 664-667.
- Harborne, J. B. (1997). Recent advances in chemical ecology. *Natural Product Reports* **14**, 83-98.
- Hare, J. D. (1990). Ecology and management of the Colorado potato beetle. *Annual Review of Entomology* **35**, 100.
- Haribal, M. and Renwick, J. A. A. (1996). Oviposition stimulants for the monarch butterfly: Flavonol glycosides from *Asclepias curassavica*. *Phytochemistry* **41**, 139-144.
- Haribal, M. and Renwick, J. A. A. (1998). Identification and distribution of oviposition stimulants for monarch butterflies in hosts and nonhosts. *Journal of Chemical Ecology* **24**, 891-904.
- Hermes, D. A. (2003). Assessing management options for gypsy moth. *Pesticide Outlook* **14**, 14-18.
- Hopkins, G. W. *et al.* (2002). Identifying rarity in insects: the importance of host plant range. *Biological Conservation* **105**, 293-307.
- Jonsson, M. and Anderson, P. (1999). Electrophysiological response to herbivore-induced host plant volatiles in the moth *Spodoptera littoralis*. *Physiological Entomology* **24**, 377-385.
- Kelber, A. and Pfaff, M. (1997). Spontaneous and learned preferences for visual flower features in a diurnal hawkmoth. *Israel Journal of Plant Sciences* **45**, 235-245.
- Kelber, A. (1999). Ovipositing butterflies use a red receptor to see green. *Journal of Experimental Biology* **202**, 2619-2630.

- Kemp, D. J. (2006). Ultraviolet ornamentation and male mating success in a high-density assemblage of the butterfly *Colias eurytheme*. *Journal of Insect Behavior* **19**, 669-684.
- Kevan, P. G. and Baker, H. G. (1983). Insects As Flower Visitors and Pollinators. *Annual Review of Entomology* **28**, 407-453.
- Kevan, P. G. and Backhaus, W. G. K. (1998). Color vision: Ecology and evolution in making the best of the photic environment. In: *Color Vision: Perspectives from Different Disciplines* (eds. Backhaus, W. G. K., Kliegl, R., and Werner, J. S.), pp. 163-183. Berlin: De Gruyter.
- Kevan, P. G., Chittka, L., and Dyer, A. G. (2001). Limits to the salience of ultraviolet: Lessons from colour vision in bees and birds. *Journal of Experimental Biology* **204**, 2571-2580.
- Levins, R. and MacArthur, R. H. (1969). A hypotheses to explain the incidence of monophagy. *Ecology* **50**, 910-911.
- Loughrin, J. H., Potter, D. A., and Hamiltonkemp, T. R. (1995). Volative Compounds Induced by Herbivory Act As Aggregation Kairomones for the Japanese-Beetle (*Popillia-japonica* Newman). *Journal of Chemical Ecology* **21**, 1457-1467.
- Mackay, D. A. (1985). Prealighting Search Behavior and Host Plant-Selection by Ovipositing *Euphydryas-Editha* Butterflies. *Ecology* **66**, 142-151.
- Mattiacci, L., Dicke, M., and Posthumus, M. A. (1995). Beta-Glucosidase - An Elicitor of Herbivore-Induced Plant Odor That Attracts Host-Searching Parasitic Wasps. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 2036-2040.
- Mouritsen, H. and Frost, B. J. (2002). Virtual migration in tethered flying monarch butterflies reveals their orientation mechanisms. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 10162-10166.
- Naumann, K. *et al.* (1991). Production and Transmission of Honey-Bee Queen (*Apis-Mellifera* L) Mandibular Gland Pheromone. *Behavioral Ecology and Sociobiology* **29**, 321-332.
- Nishida, R. (2002). Sequestration of defensive substances from plants by Lepidoptera. *Annual Review of Entomology* **47**, 57-92.
- Papke, R. S., Kemp, D. J., and Rutowski, R. L. (2006). Multimodal signalling: structural ultraviolet reflectance predicts male mating success better than pheromones in the butterfly *Colias eurytheme* L. (Pieridae). *Animal Behaviour* **73**, 47-54.

- Pare, P. W. and Tumlinson, J. H. (1997). De novo biosynthesis of volatiles induced by insect herbivory in cotton plants. *Plant Physiology* **114**, 1161-1167.
- Prokopy, R. J. and Owens, E. D. (1983). Visual Detection of Plants by Herbivorous Insects. *Annual Review of Entomology* **28**, 337-364.
- Raguso, R. A. and Willis, M. A. (2002). Synergy between visual and olfactory cues in nectar feeding by naive hawkmoths, *Manduca sexta*. *Animal Behaviour* **64**, 685-695.
- Reddy, G. V. P., Tabone, E., and Smith, M. T. (2004). Mediation of host selection and oviposition behavior in the diamondback moth *Plutella xylostella* and its predator *Chrysoperla carnea* by chemical cues from cole crops. *Biological Control* **29**, 270-277.
- Reppert, S.M., Zhu, H., and White, R.H. (2004). Polarized light helps monarch butterflies navigate. *Current Biology* **14**, 155-158.
- Rogers, D. (1972). Random Search and Insect Population Models. *Journal of Animal Ecology* **41**, 369-383.
- Rothschild, M. *et al.* (1970). Toxic Lepidoteran. *Toxicon* **8**, 299.
- Sarfraz, M., Keddie, A. B., and Dosdall, L. M. (2005). Biological control of the diamondback moth, *Plutella xylostella*: A review. *Biocontrol Science and Technology* **15**, 763-789.
- Sauman, I. *et al.* (2005). Connecting the navigational clock to sun compass input in monarch butterfly brain. *Neuron* **46**, 457-467.
- Silverstein, R. M. and Young, J. C. (1976). Insects generally use multicomponent pheromones. ACS Symposium Series No.23 American Chemical Society. Washington, D.C. 1-29.
- Solensky, M. J. (2004). Overview of Monarch Migration. In: *The Monarch Butterfly Biology & Conservation* (eds. Oberhauser, K. S. and Solensky, M. J.), pp. 79-83. Ithaca & London: Comstock Publishing Associates, a division of Cornell University Press.
- Stalleicken, J., Labhart, T., and Mouritsen, H. (2006). Physiological characterization of the compound eye in monarch butterflies with focus on the dorsal rim area. *Journal of Comparative Physiology A-Neuroethology Sensory Neural and Behavioral Physiology* **192**, 321-331.
- Sutherland, O. R. W. and Hutchins, R. F. N. (1972). alpha-farnesene, a Natural Attractant for Codling Moth Larvae. *Nature* **239**, 170.

- Tillman, J. A. *et al.* (1999). Insect pheromones - an overview of biosynthesis and endocrine regulation. *Insect Biochemistry and Molecular Biology* **29**, 481-514.
- Tumlinson, J. H. *et al.* (1989). Identification of A Pheromone Blend Attractive to *Manduca-Sexta* (L) Males in A Wind-Tunnel. *Archives of Insect Biochemistry and Physiology* **10**, 255-271.
- Tumlinson, J. H. *et al.* (1994). Field-Tests of Synthetic *Manduca-Sexta* Sex-Pheromone. *Journal of Chemical Ecology* **20**, 579-591.
- Turlings, T. C. J. *et al.* (1995). How Caterpillar-Damaged Plants Protect Themselves by Attracting Parasitic Wasps. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 4169-4174.
- Urquhart, F. A. (1960). *The Monarch Butterfly*. University of Toronto Press.
- Yan, F. *et al.* (2003). Roles of alpha-farnesene in the behaviors of codling moth females. *Zeitschrift fur Naturforschung C-A Journal of Biosciences* **58**, 113-118.
- Zalucki, M. P. (1993). Sex around the milkweed patch: the significance of host plants in monarch reproduction. In: *Biology and Conservation of the Monarch Butterfly* (eds. Malcolm, S. B. and Zalucki, M. P.), pp. 69-76. Los Angeles: Natural History Museum of Los Angeles County.