# Detection of Iridoids in Snapdragons and Methods to Observe Effects of Toxic Nectar on Pollination Behavior of Bumblebees

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## Abstract

Plants produce a vast array of secondary chemical defense compounds that are either toxic or deterrent to herbivores and microbial pathogens. Production of secondary compounds often increases following herbivore attack, resulting in an inducible chemical defense response. Application of jasmonic acid, a plant hormone released in response to herbivore attack and wounding, often triggers a similar response and is frequently used in experiments to simulate herbivory and induce production of plant chemical defenses. The role of secondary compounds as defense agents against herbivores is clearly established. More recently, however, these defense compounds have been reported from floral nectar of many flowering plant families, leading to the term 'toxic nectar' to describe such nectar. At first glance, this appears counteradaptive, given that nectar is a reward plants commonly provide to pollinators in exchange for pollination services. In this study, we sought to examine the role of iridoids, a type of secondary defense compound, in nectar of the common snapdragon, *Antirrhinum majus* (family Plantaginaceae) by assessing iridoid content in plant tissues and quantifying the effect of toxic nectar on visitation behavior of the bumblebee *Bombus impatiens*, a pollinator of snapdragons. We used high performance liquid chromatography (HPLC) to analyze plant tissues for iridoids and designed three experiments to test bee responses to toxic nectar. We confirmed the presence of iridoids in leaf and floral tissues but were unable to extract sufficient nectar for HPLC analysis. Application of JA did not increase iridoid levels in plant tissue. Attempts to measure bee responses to manipulated nectar were unsuccessful. Despite the use of outdoor and indoor enclosures, captive and wild bees, and natural and artificial flowers, bees would not forage. The presence and function of secondary compounds in snapdragon nectar remains unresolved. We suggest improvements to future experimental designs that may help answer these questions.

## Introduction

Plants have evolved various defenses via mutation and natural selection to avoid being eaten by herbivores or infected by microbial pathogens. These defenses include physical barriers on the exterior of the plant that reduce consumption or infection, and chemical compounds within plant tissue that reduce the digestibility of plant tissue, or deter consumption because of their repellent or toxic effects on herbivores and pathogens, even at low concentrations (Howe and Westley 1988).

Collectively, plants produce a vast array of chemical defense compounds that are organized into chemical classes based on structure. These classes include alkaloids, cardenolides, glucosinolates, terpenoids and others. Individual plant families usually specialize in producing a particular class of compounds. For example, members of the cabbage family (Brassicaceae) are characterized by sulfur-containing glucosinolates whereas plants in the potato family (Solanaceae) specialize in the production of heterocyclic nitrogen-containing alkaloids (Bernays and Chapman 1992; Howe and Westley 1988).

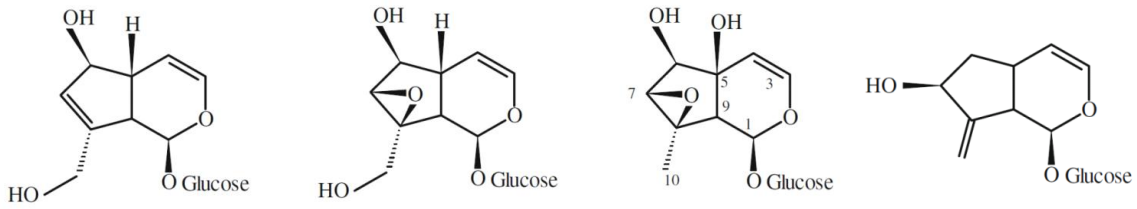
Defense compounds are commonly referred to as secondary compounds or secondary metabolites because they do not appear to be essential for plant growth and reproduction (Bernays and Chapman 1994; Howe and Westley 1988; Osbourn and Lanzotti 2009). Instead, these compounds are either toxic to herbivores or deter them because of their distastefulness (Rosenthal and Burnbaum 1991). Levels of secondary compounds in plant tissue vary with plant part and plant condition. Toxin concentrations are typically highest in the more vulnerable plant parts such as tender new shoots and seeds (Bernays and Chapman 1994). In addition, constitutive baseline levels of toxins present in plants not under attack are typically very low. Toxin levels can increase significantly within hours or days following attack, however, a process known as induction (Bernays and Chapman 1994). Jasmonic acid (JA) is one plant hormone released in response to herbivore attack and wounding and its release triggers a signaling pathway that leads to increased production of a number of plant defense compounds (Thaler 1999). Exogenous application of JA to leaf tissue can induce production of plant chemical defenses and is commonly used by researchers to experimentally induce defenses in a controlled fashion (Thaler 1999).

The role of secondary compounds as defense agents against herbivores is clearly established (Bernays and Chapman 1994). More recently, however, these defense compounds have been reported from floral nectar of many angiosperm families (Detzel and Wink 1993; Adler 2000; Adler and Irwin 2005; Kessler and Baldwin 2006; Gegear et al. 2007; Hölscher et al. 2008), leading to the term 'toxic nectar' to describe defense such nectar. At first glance, this appears counteradaptive, given that nectar is a reward plants commonly provide to pollinators in exchange for pollination services. Various hypotheses have been proposed to explain the presence of toxins in nectar, however. For example, toxic nectar may encourage visits from specialist pollinators that sequester the toxins for their own defense, deter nectar robbers, prevent microbial degradation of nectar, or alter pollinator behavior in a way that enhances plant fitness (Adler 2000; Adler and Irwin 2005; Heil 2011). Despite the wealth of proposed hypotheses, few experiments have been performed to tests these hypotheses (but see Detzel and Wink 1993; Gegear et al. 2007).

A leading hypothesis for the adaptive function of toxic nectar is that it may alter pollinator behavior by deterring pollinators from lingering at flowers and consuming all nectar in one visit (Adler and Irwin 2005). This would shorten the time pollinators spend at individual flowers, thereby increasing the number of visitations each flower receives. This could lead to enhanced pollination success and increased plant fitness. Consequently, plants that incorporate toxins in nectar may be favored by natural selection.

Iridoids belong to the class of secondary compounds known as monoterpenoids. There are over 1000 known iridoid compounds reported from more than 50 plant families (Bernays and Chapman 1994; Osbourn and Lanzotti 2009), where they occur as glycosides (bound to a sugar, most commonly glucose; Taskova et al. 2011). Iridoids predominate as secondary defense compounds in most members of the plant family Plantaginaceae, including *Plantago* (plantains) and former members of the Scorphulariaceae such as *Antirrhinum* (snapdragons), *Penstemon* (beardtongue) and *Veronica* (Albach et al. 2005). Aucubin (Fig. 1a) and catalpol (Fig. 1b) are the predominant iridoids produced by narrow-leaved plantain, *Plantago lanceolata*, and serve as broad-spectrum defenses against both pathogens and herbivores (Bowers et al. 1992; Taskova et al. 2011). Bowers and colleagues found that total iridoid levels within *P. lanceolata* increased with plant age, and that the major iridoid present changed over time (Klockars et al. 1993; Fuchs and Bowers

2004). Catalpol was present at high concentrations in new leaves whereas aucubin predominated in older leaves. Iridoid production was also only weakly induced by caterpillar herbivory, and this effect was only detectable in young plants (<5 weeks of age; Fuchs and Bowers 2004).




a) aucubin b) catalpol c) antirrhinoside d) antirrhide

**Figure 1.** Chemical structures of four iridoid glycoside compounds: a) aucubin and b) catalpol found in *Plantago lanceolata*; c) antirrhinoside and d) antirrhide found in *Antirrhinum majus* (redrawn from Beninger et al. 2008)

*Antirrhinum* and other members of the tribe Antirrhineae are characterized by the iridoid antirrhinoside (Fig. 1c), a compound rarely found in plants outside this tribe (Albach et al. 1995). Antirrhinoside is structurally similar to catalpol (Fig. 1b), and along with antirrhide (Fig. 1d), comprise the two major extractable iridoids of the common snapdragon, *Antirrhinum majus* (Høgedal and Mølgaard 2000; Beninger et al. 2007). Snapdragons are native to parts of southern Europe, western Asia, and northern Africa. The species *Antirrhinum majus* is a common bedding plant available from plant nurseries and is becoming a model organism for investigating plant development at the molecular level due to its natural variation in growth habit and the large number of cultivars that have been generated by plant breeders (Coen and Meyerowitz 1991; Beninger et al. 2007). Høgedal and Mølgaard (2000) reported that iridoids were present in snapdragon leaf tissue, and that levels of antirrhinoside decreased after flowering while levels of antirrhide increased. In addition, they showed that iridoid concentration varied diurnally and seasonally, indicating a highly dynamic defense system. Beninger et al. (2007) followed up with a more detailed study of the distribution of *A. majus* iridoids. They reported that antirrhinoside is likely transported in the phloem and is found in highest concentrations (up to 20% of dry weight tissue) in young leaves, buds and flowers. In neither study,

however, did researchers sample nectar directly, leaving open the question of whether, and at what level, iridoids are found in the nectar of *A. majus* and if so, what effect they have on their pollinators.

Snapdragons possess a unique, zygomatic (tube-like) flower shape with two asymmetrical "lips" in the shape of a dragon's mouth, thus earning snapdragons their namesake (Fig. 2). Snapdragons bloom throughout the summer but the lips of the flower remain closed. As a result only visitors with sufficient weight and/or strength can depress the bottom lobe and access the nectar at the base of the flower. Bumblebees (genus *Bombus*) are robust insects capable of this feat and consequently are among their main insect pollinators (Hickey and King 1981). When crawling between the closed lips of the tube-shaped flowers, bees brush against the pollen-laden stamens located on the upper lip of the flower and similarly deposit pollen on the adjacent female reproductive structures.

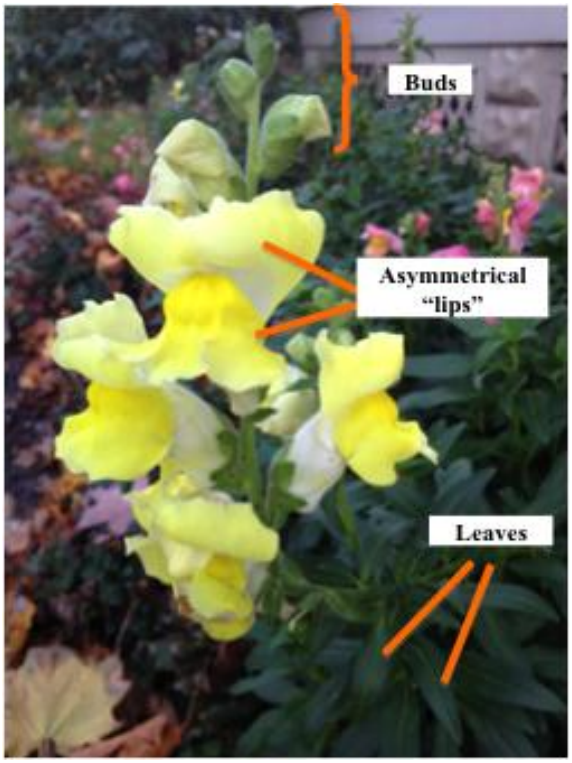



**Figure 2.** Photograph of "Rocket Mix" snapdragon plant showing buds, flowers with asymmetrical "lips" and leaves.

Bumblebees are important insect pollinators that regularly collect plant nectar to satisfy their energy requirements and to feed developing offspring. They exhibit *trap*

*lining*, a foraging behavior where workers visit flowers in a particular order and revisit the same flowers on a later flight after allowing time for flowers to replenish their nectar supply (Kearns and Thomson 2001). The frequency and regularity of their floral visitation suggest that it should be relatively easy to detect changes in behavior if nectar becomes distasteful or toxic with the addition of plant secondary compounds. Their reliable foraging behavior, coupled with the ease with which bumblebees can be observed at close range without being disturbed, makes them excellent model organisms for testing the adaptive function of toxic nectar.

The purpose of this study was twofold: (1) to examine iridoid levels in snapdragon tissues from uninduced and induced plants, and (2) to quantify visitation behavior of a bumblebee pollinator offered nectar with and without iridoids. More specifically within these aims, we set out to address the following questions surrounding toxic nectar within the snapdragon-bumblebee system:

a) Are iridoids present in the nectar of snapdragon flowers, and how does this compare to levels in other plant tissues?
b) Does iridoid content in leaf and/or floral tissue increase following application of jasmonic acid?
c) Does the presence of iridoids in plant nectar alter bumblebee pollinator behavior in a way that is consistent with enhanced plant fitness?

We addressed the first two questions by harvesting plant tissues from control and experimental plants and analyzing tissue for iridoid content using high performance liquid chromatography. We addressed the third, adaptive function hypothesis, question by experimentally manipulating nectar composition of intact and artificial flowers and observing visitation patterns by bumblebees. Based on previous analyses of iridoid content in snapdragons (Høgedal and Mølgaard 2000; Beninger et al. 2007) and the role of jasmonic acid in inducing plant defense responses (Thaler 1999) we hypothesized that iridoids would be present in leaves, buds and floral tissue, and that iridoid concentration would increase in all tissue types after treatment with jasmonic acid. In accordance with the adaptive function hypothesis, we predicted that the presence of iridoids in floral nectar would decrease the duration of bumblebee visits to flowers, and increase the frequency of

visitation. To our knowledge, this study is the first to examine whether the presence of iridoids in snapdragon nectar affects the behavior of bumblebee pollinators.

## Methods and Results

Study Site

Fieldwork for this study was conducted at Pierce Cedar Creek Institute (PCCI), Barry County, MI. Greenhouse and laboratory chemical work was performed at Kalamazoo College, Kalamazoo County, MI. All work was conducted during the summer of 2012.

Iridoid Content of Snapdragons

*Methods* – Ninety-six "Rocket Mix" snapdragons were purchased from a local plant nursery and repotted individually into 10 cm (4") plastic pots containing potting soil on June 19, 2012. Plants were immature at this time; the majority had not yet flowered and were less than 0.5 m tall. Individual pots were arranged into groups of 12 similar plants based on appearance of each plant, including the color, height, and stage of development. Plants 0.4 m or taller were grown indoors under two grow lights (80-100 lm/W each) on a 14 h light: 8 h dark cycle. Plants shorter than 0.4 m were grown in a separate part of the room under one grow light on the same light cycle. Due to the physical arrangement of the pots and lamps, each plant had roughly equal light exposure. All plants were watered daily and treated weekly with a 10-10-10 N-P-K fertilizer solution to enhance growth. Plants were grown indoors in order to control light exposure and amount of water each plant received, as well as to prevent herbivores from feeding on plant tissue, as this could have affected expression of iridoids in the plant tissue.

We initially attempted to collect nectar for iridoid analysis from snapdragon flower heads directly using 1.0 μL glass microcapillary tubes (Sigma-Aldrich, St. Louis, MO). Flower heads were probed in anticipation of collecting 20 μL of nectar, the minimum amount needed for HPLC analysis. Despite two weeks of adequate watering and controlled light exposure, nectar amounts harvested from plants were negligible. As a result, the nectar could not be directly analyzed by HPLC as we had planned. Our next course of action was to test for the presence of iridoids by harvesting plant tissue from different parts of the plant.

To test for the presence of iridoids in plant tissues, while also examining the effects of simulated herbivory on iridoid concentrations throughout the plant, we selected six pairs of snapdragon plants as test subjects. One plant in each pair served as a control plant, while the other was designated the experimental plant. Individuals within each pair were as similar as possible in color, height, and stage of development. To simulate herbivory, we prepared a 0.5 mM solution of jasmonic acid (Sigma-Aldrich) in water by first dissolving 10.5 μL JA in 12.5 μL acetone and diluting this in 100 mL distilled water. This was called the treatment solution. A control solution was also prepared by mixing 12.5 μL acetone in 100 mL distilled water. Solutions were stored in plastic spray bottles and kept at 4 °C until use. Each plant received five sprays with the corresponding JA or control solution once daily over the course of 7 days. To be consistent among all treatments, spray was applied directly to the surface of the largest leaves from all pairs because these leaves had the greatest surface area to absorb JA.

After one week of daily treatment, the leaves, buds, flowers and post-bloom plant material (seeds, dried flowers, etc.) were cut from each plant, stored in paper envelopes and frozen at -80 ˚C for one day to ensure that tissue was dead before drying. Tissues were then dried in their respective envelopes at 50 ˚C for three days to remove water from the tissue. Dried tissue was ground to a fine powder using a mortar and pestle and powdered samples were stored in airtight glass vials for later chemical analysis.

We used high performance liquid chromatography (HPLC) to measure iridoid content in plant tissue following a method described by Høgedal and Mølgaard (2000). Fifty mg of dried plant material was added to 10 mL of 20% methanol solution, to which 0.5 μg/mL of the internal standard (IS) p-hydroxybenzaldehyde (Sigma-Aldrich) was added. Samples were sonicated in a 55 °C water bath for 60 min and then filtered using a 0.20 μm pore size filter to remove particulate. A 20 μL volume of filtered sample was injected manually into HPLC for analysis. For post-bloom material where 50 mg of plant tissue was not available from a single plant, samples from multiple plants in the same treatment group were pooled before extraction. Very little flower tissue was available for harvest. As a result, flower tissue from multiple control and experimental plants was pooled into a single sample for analysis.

A Waters 2847 Dual Wavelength Absorbance Detector instrument was set to read samples at 205 nm, and flow rate was set to 0.5 mL/min. The column used in the analysis was a Nova-Pak reversed-phase C18 column (3.9 mm x 150 mm, 4 μm particle size; Waters Corporation, Milford, MA) and the solvent system consisted of isocratic (non-changing) 3% acetonitrile solution (Sigma-Aldrich) in Milli-Q water. Sample runtime was 25 minutes.

Chromatogram peaks were quantified by integrating area under the peak. Major peaks other than the internal standard were assumed to be iridoids but this could not be confirmed without mass spectrometry or authentic iridoid standards, neither of which was available to us. For each sample, peak areas were normalized by dividing peak area of putative iridoid peaks by area under the internal standard peak to correct for variation in sample injection volume and day-to-day variability in instrument detection capability. Peaks areas other than the internal standard were summed to yield total iridoid content per sample. Due to day-to-day variability in instrument detection we could not pool data across sample dates for analysis. Instead, we compared total iridoid content among samples run on the same date only. As a result, samples sizes were too small for meaningful statistical comparisons. Instead, we compared total iridoid content among plant tissue types and between JA and control treatments qualitatively.

*Results* – In addition to the peak for the internal standard (IS) at 24 min, each chromatogram revealed two major peaks, presumed to be iridoids (Fig. 3). Iridoids were detected in all leaf, flower and post-bloom tissue samples (Fig. 4). Due to the large difference in total iridoids detected from leaf tissue samples run on 09/05 and 09/06, it is not possible to say whether iridoid levels in post-bloom and flower material are comparable to, or higher than those of leaf tissue. It does appear, however, that treatment with JA did not elevate total iridoid content in leaf or post-bloom tissue (Fig. 4).

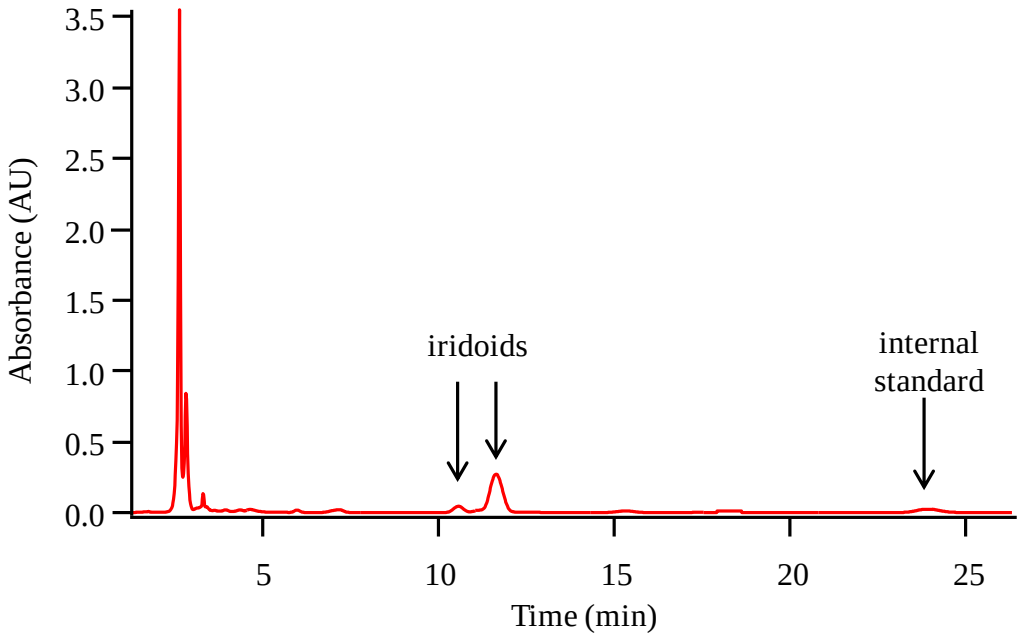



**Figure 3.** Representative chromatogram of snapdragon leaf tissue sample showing peaks for the two putative iridoids and for the internal standard, p-hydroxybenzaldehyde.

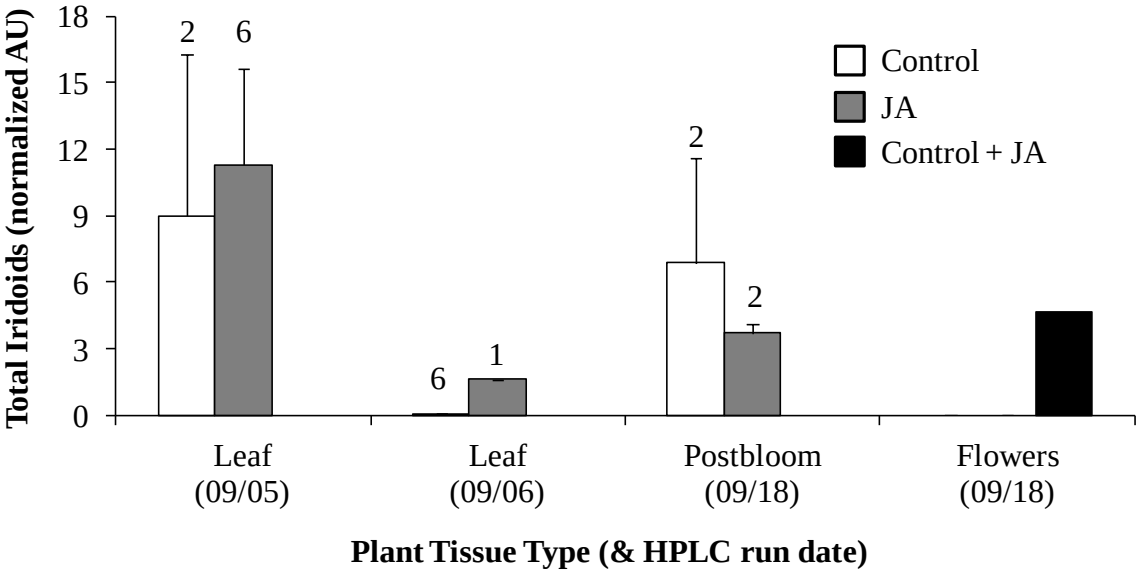



**Figure 4.** Iridoid content of plant tissue collected from snapdragons. Samples were analyzed by HPLC on various dates. Bar heights represent mean (± SEM) peak area for total iridoids detected, measured in absorbance units normalized to area under the internal standard peak. Numbers above bars represent number of samples analyzed.

Pollinator Experiments

To record behavioral responses of bumblebees to toxic nectar, we conducted three experiments in which we presented floral displays containing manipulated nectar to bees, each in a different setting. Experiments within enclosures were conducted using captive colonies of *Bombus impatiens* purchased from Koppert Biological Systems (Howell, MI). *Bombus impatiens* is the most common species of commercially available bumblebees in eastern North America and readily adapts to nesting in confinement (Kearns and Thomson 2001). The native range of *Bombus impatiens* includes southwest Michigan, where the study took place, and this species is the most common wild bumblebee at the PCCI field site (Arnosky 2008).

Two colonies of *B. impatiens* were purchased for this research, each for a separate experiment. Each colony had a queen and consisted of bees in all stages of development. The colony was contained in an insulated and ventilated plastic box, which itself was enclosed within a cardboard box. Within the hive, there were approximately 75 worker bees. Bees were provided with pollen and an artificial honey source (37% refractive index) purchased from Koppert Biological Systems.

Experiment 1: Outdoor enclosure with captive bees and snapdragon flowers

*Methods* – We constructed a large (3 m L x 3 m W x 2.2 m H) screened outdoor enclosure to accommodate a captive colony of *B. impatiens* and test plants (Fig. 5). The outdoor enclosure was erected on level ground south of the Education Building at PCCI. The frame was made from metal pipes and brackets, and the ceiling and walls were black mesh and wrap-around white mesh, respectively.

**Figure 5.** Homemade outdoor enclosure constructed at PCCI for bumblebee foraging experiment with snapdragons. A zipper door allowed persons to enter and exit the enclosure without bees escaping.

Flats containing flowering snapdragons were gradually moved into the enclosure in preparation for experiments with captive bumblebees. On June 27, 2012, a colony of *B. impatiens* was purchased from Koppert Biological Systems (Howell, MI) and placed in the enclosure on top of an inverted crate in the corner of the enclosure. The hive was covered with a solid plastic lid to protect it from the rain. The colony contained an internal honey source from which the bees could feed freely. Because of the extremely hot and dry weather, a water dispenser consisting of an inverted glass jar atop a dish containing mixture of absorbent paper and small pebbles was also placed in the middle of the enclosure. With this design, bees could lap water from the water-filled dish at the base without drowning.

*Results* – Despite the placement of 24 flowering snapdragon plants flanking the hive on either wall, in addition to honey feeders in the center of the enclosure, bees were hesitant to leave the hive. We spent much of the next few weeks attempting to get the bees to forage on the flowers. In an attempt to coax the bees out of the hive, the internal honey source was removed from the hive. This honey was used to refill the three external feeding

reservoirs placed in the center of the enclosure. These small reservoirs were made from 0.03 L (1 oz) plastic cups filled with honey solution. They had yellow plastic tops with dark nectar guides and an absorbent wick that drew up honey through the center of each lid, from which the bees could feed. This setup had been used in our preliminary studies with a "practice hive" in a 60 x 60 x 60 cm mesh enclosure at Kalamazoo College during February 2012 and was a successful feeding mechanism for captive bees. Despite a seemingly healthy colony and plenty of resources within the enclosure, few worker bees ever left the hive to forage for food and water.

After two weeks, many worker bees perished, presumably from desiccation and lack of food. There was no rainfall over the experimental period, nor was there any rainfall during the week prior to the arrival of the bees. Realizing that our colony was unhealthy and inadequate for pollination studies within the enclosure, we alter our experimental plan by setting up a new experiment in the fields of PCCI using wild bumblebees.

Experiment 2: Natural setting with wild bees and knapweed flowers

*Methods* – From opportunistic observations, it was evident that wild bumblebees in the Northeast Prairie adjacent to the outdoor bumblebee enclosure frequented spotted knapweed, *Centaurea maculosa*. Spotted knapweed (family Asteraceae) is an open-faced flower that attracts a wide diversity of flower visitors (Shuttleworth and Johnson 2009). In contrast to the zygomatic floral shape of snapdragons, knapweed produces composite flower heads containing numerous individual flowers that house nectar. Because individual flowers are so small, it is not feasible to fill each flower individually with experimental nectar solutions. Nonetheless their attractiveness to bumblebees led us to devise a means by which we could spray an iridoid-containing nectar solution directly onto the open flower head to test the effects of secondary compounds on pollinator behavior.

To control the density of test flowers encountered by bees in our setup, we used freshly cut stalks of knapweed flowers and presented these in orderly arrays in racks (Fig. 6). For each trial, 24 flowers from the same spotted knapweed plant were selected based on similarity in color, size and age. Stems from selected flowers were cut at a 45° angle, approximately 3 cm below the calyx. Cut stems were then inserted into wet floral foam purchased from Hobby Lobby, a local craft store. Foam was used to ensure proper

hydration of flowers during field tests. Prior to use, foam pieces were cut to fit securely in 1 oz medicine cups and soaked in water overnight.

For field tests, the wet, foam-filled cups were inserted into plant tubes arranged in two identical arrays of 12 medicine cups, each spaced about 6 cm apart from its neighbor (Fig. 6 but with 12 cups per array rather than 28 pictured here). One rack was designated the control treatment and one the experimental treatment. Cups containing the cut flowers were randomly assigned to treatment group. The flower tube racks were about the same height as the surrounding spotted knapweed patches.

**Figure 6**. Nick Smith, Dr. Ann Fraser and Mary Wald assembling arrays of spotted knapweed for use in experiment with wild bumblebees.

Once flower cups were arranged in the racks, flowers in the control array were sprayed once each with a control solution of 30% w/w sucrose in water, while flowers in the experimental treatment array were sprayed once each with a treatment solution containing 1.0 mg of the iridoid aucubin (Sigma-Aldrich) dissolved in 10 mL of the 30% sucrose solution. For each new trial, 24 spotted knapweed flowers were cut from a different plant growing in the patch.

On July 20, 2012, three observations were performed during the day to determine if the bees were attracted to the arrays as much as to the spotted knapweed in the fields. Flower arrays were placed within 3 m of a knapweed patch. The number of visits a bee

made to flowers in the control and treatments groups was recorded on a laptop computer using JWatcher, a behavioral recording and analysis software package. Keystrokes on the computer recorded arrival and departure of a focal bee to flowers, from which the frequency and duration of visits could be calculated. A trial began when a bumblebee landed on a flower within either of the treatments and continued for a maximum of 10 minutes. For each trial, the behavior of only a single, focal bumblebee was followed. Additional observation sessions were conducted through August 3, 2012.

*Results* – Although knapweed in the fields attracted many bees, few bees visited the arrays and when visits occurred during a 10-minute observation period, bees remained at a flower only briefly and did not sample multiple flowers. After many field observations that yielded no interpretable visits to the experimental arrays, we began a third pollination experiment involving artificial flowers.

Experiment 3: Greenhouse cage with captive bees and artificial flowers

*Methods* – A new *B. impatiens* research colony (also from Koppert Biological Systems) was delivered on August 17, 2012 to Kalamazoo College. The colony was placed in a BugDorm-2120 insect cage (BioQuip Products, Rancho Domingo, CA) in the college greenhouse. The BugDorm enclosure is dome-shaped tent (60 x 60 x 60 cm), two sides of which are clear plastic panels for observation of insect activity and two sides of which are white polyester netting for ventilation. The greenhouse was kept at 24 ˚C. The internal honey source was left in place for the colony to feed from and bees were further provided with pellets of fresh pollen sprinkled onto the lid of the hive every other day. Bees were able to drink from a water dish with absorbent cloth placed in the cage and were also sprayed with water once daily to reduce desiccation.

Due to space constraints, and for ease of manipulation in a controlled environment, we constructed artificial flowers in this experiment to test the effect of toxic nectar on bumblebee foraging behavior. Artificial flowers were constructed by inserting 32 yellow 200 µl plastic pipette tips (0.5 cm in diameter across the top) into the circular lid of a plastic containers (11.5 cm diameter x 7 cm height). The top of the container was coated a fluorescent blue paint known to attract bees in field sampling traps (Arnsoky 2008). The

pipette tips were packed with putty and served as wells for holding the distal 1 cm portion of the pipette tip that was cut off and placed in the putty at the top of the pipette tip (Fig. 7a). This 1 cm portion was filled with nectar solution and served as the nectary from which bees could feed. A soldering iron was used to melt holes in the lid for the 32-well arrangement (Fig. 7b).

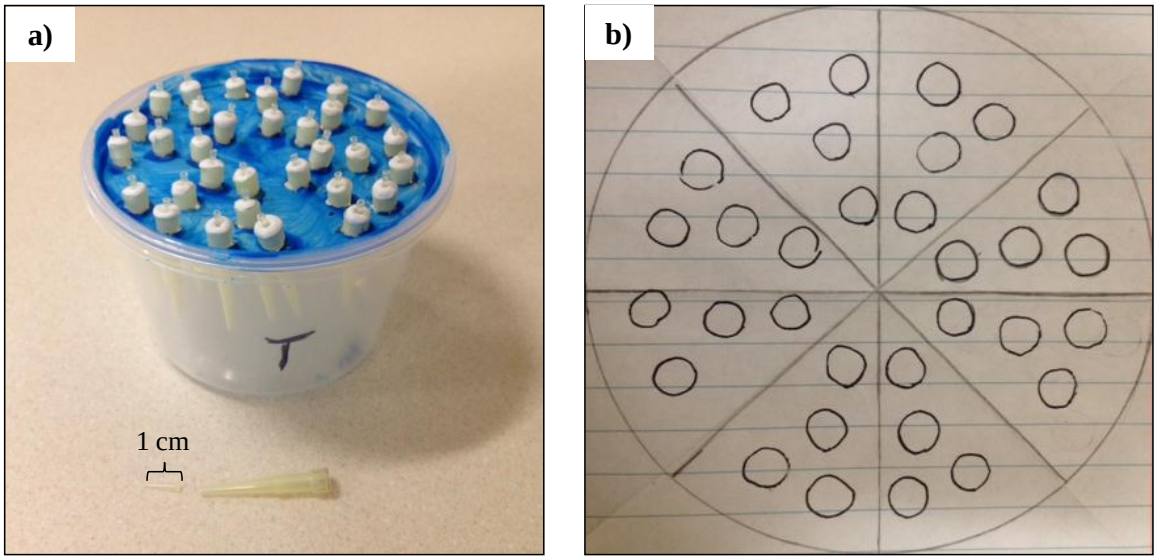



**Figure 7**. Artificial flower construction showing **a)** an assembled flower with all nectaries in place and **b)** sketch of the well template, showing spatial arrangement of the 32 nectaries on the container lid.

Two artificial flower arrangements, each containing 32 nectaries, were prepared for each behavioral trial. One arrangement was designated the control treatment and the other the experimental treatment. To prepare the flowers, nectar solution was dispensed into each 1 cm pipette tip nectary through a fine needle attached to a 1 cc syringe. The experimental treatment solution consisted 1.0 mg aucubin dissolved in 10 mL of the 37% honey solution that had been removed from the originally purchased bee colony. The control treatment solution was similar except that it omitted the aucubin. Two drops of orange essence (Frontier, Norway, IA) were also to the honey solution prior to use in an attempt to enhance the nectar scent and its attractiveness to bees.

For testing bee foraging behavior, we set up three identical Bug-Dorm tents to hold the artificial flower treatments and test bees for each trial. Knowing that bee flowers are

visually distinct from surrounding green vegetation in the wild (Kearns and Thomson 2001), we mimicked this as best we could by placing a grass-patterned fabric on the floor of the test cage to contrast with the blue color of our artificial flowers (Fig. 8).

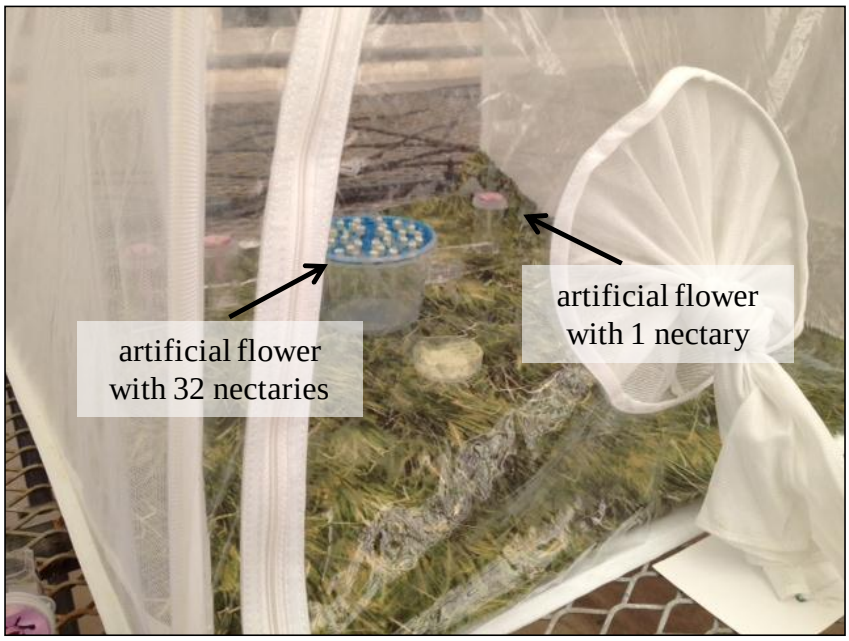



**Figure 8**. Greenhouse cage enclosure with grass pattern fabric bottom and examples of the two types of artificial flowers. The artificial flower with 32 nectaries was used in test trials.

Bumblebee test subjects were removed from the colony cage and individually marked with a unique color using a 1.0 mm fine line acrylic paint marker. To facilitate marking, we constructed a device called a "bee squeezer" (Fig. 9) to immobilize bees and reduce the need to handle bees directly. Marked bees were held in individual vials with moist paper towels overnight to induce hunger. The following day, one bee was released into each test cage with freshly prepared artificial flowers and behavior was monitored continuously for the first 20 minutes. Bees were checked periodically for the next three hours.

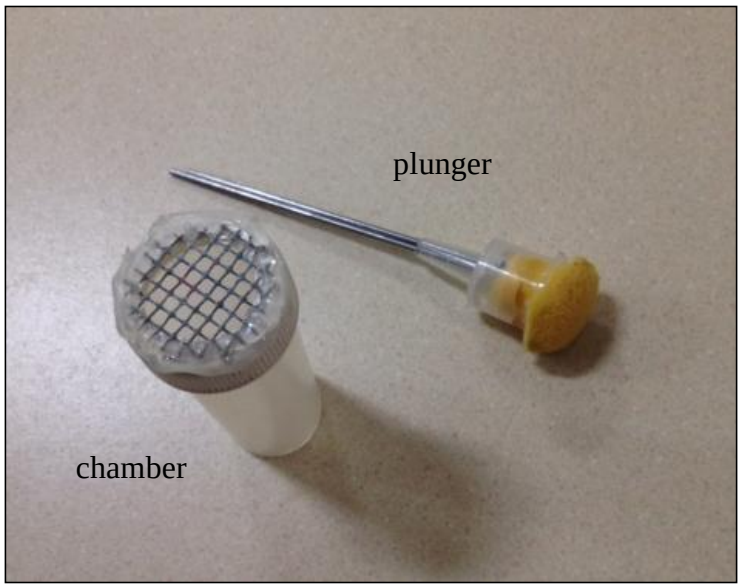



**Figure 9**. Bee squeezer parts used to immobilize bees for marking—modified from a design described in Kearns and Thomson (2001).

*Results* – Although this setup appeared promising, bees did not feed on the artificial flowers, resulting in no data collection. The bees spent most of their time on the floor or walls of the cage. Even when bees were placed on the artificial flowers, they did not drink from the nectaries. This experiment was the final attempt to test the effects of toxic nectar on foraging behavior of bumblebee pollinators.

## Discussion

Using HPLC, we were able to detect what are presumed to be iridoids in all of our snapdragon tissue samples, including floral tissue. Iridoid levels did not increase in response to exogenous application of jasmonic acid. We designed three experiments to test the effects of toxic nectar on bumblebee foraging behavior. Unfortunately, the bumblebees would not forage on any of the nectar resources we offered them.

We tentatively identified the two iridoid peaks detected by HPLC as antirrhinoside and antirrhide but cannot verify these without mass spectrometry or authentic compound standards. Identifications were based on previous studies of snapdragon iridoids by Høgedal and Mølgaard (2000) and Beninger et al. (2007), and on retention times and elution order of our peaks. Although we were successful in detecting peaks at the

appropriate wavelength and retention times, the results from our HPLC machine were not fully satisfactory. Peak areas from similar samples (i.e., of the same tissue type and treatment) were inconsistent from day to day and the cause of this inconsistency was unclear. As a result, we could not pool samples run on different days, limiting our ability to generate statistically meaningful sample sizes. Nonetheless, our results indicate that iridoids are present in floral tissue, a finding that is consistent with studies by Høgedal and Mølgaard (2000) and Beninger et al. (2007).

We found that application of JA did not have a significant effect on iridoid levels. In a study of *Plantago* by Fuchs and Bowers (2004), iridoid production increased only slightly following caterpillar herbivory. Furthermore, Rasmann et al. (2009) note that JA only partially mimics herbivory. These factors may explain the lack of response to JA in our study. In future, we would ideally use herbivores to induce iridoid defenses in our plants or mechanically damage leaves prior to spraying with JA.

Detection of iridoids in floral tissue of our snapdragons does not confirm their presence in the nectar. It may be that iridoids are in corolla tissue but not the nectar, although iridoids have been reported from nectar in at least one species, *Catalpa speciosa* (family Bignoniaceae; Stephenson 1982). Interestingly, Stephenson (1982) found that presence of iridoids in *Catalpa* nectar did not deter bumblebee foragers but did deter nectar feeding by caterpillars of a sphingid moth species that steals nectar from these flowers. This suggests that incorporation of iridoids into *Catalpa* nectar is adaptive in that it reduces nectar loss to non-pollinators.

We had intended to collect nectar directly from snapdragon flowers but could not obtain sufficient quantities using microcapillary extraction. It is unclear why nectar production was virtually nil in our plants, given that Comba et al. (1998) reported mean nectar secretion rates of up to 0.2 mg/h from garden varieties. It is possible that the Rocket Mix variety we used does not produce much nectar, or that growing conditions were such that nectar production was lower than normal. If snapdragons are used in future studies, attention should be paid to selecting a high nectar producing variety. In addition, other nectar collection approaches could be used, such as blotting nectar on to filter paper wicks, centrifuging flowers, and rinsing nectar from flowers using a solution (Kearns and Inouye 1993).

In addition to the low nectar production of snapdragon plants used in our study, we encountered further difficulties with these plants because our bumblebees did not feed on them. Our original intention was to use native hairy beardtongue, *Penstemon hirsutus,* another tube-shaped flower in the Plantaginaceae family and one that is abundant on the PCCI property. Bumblebees were often seen visiting penstemon flowers early in the season, before our study began. Due to the unusually warm weather, however, penstemon had finishing blooming prior to the beginning of our study. Future work would have to begin earlier in the season, ideally in the month of May.

The difficulties with snapdragons as a model flower, coupled with the unavailability of native penstemon during our study, led us to pursue spotted knapweed and artificial flowers as alternative models for testing the effect of toxic nectar on bumblebee foraging. Unfortunately, neither knapweed nor artificial flowers were effective in attracting bumblebees in our experimental tests. The use of artificial flowers deserves further attention, however, because it would allow researchers to control other potentially confounding variables such as flower color, scent and the presence of unknown compounds in the nectar. Future studies could try alternative artificial floral designs. Waddington (1980) and Real (1981) crafted artificial flowers for bumblebees by drilling shallow wells drilled into Plexiglas and dispensing a nectar solution into each well with a pipette. Another approach would be to train bees to forage on artificial flowers prior to use in experimental testing. Kearns and Inouye (1993) describe various ways to achieve this in their pollination manual.

Despite using multiple approaches to test the response of bumblebees to toxic nectar, we were unable to get bees to forage on the nectar sources we provided. The difficulties we experienced with the bumblebees feeding outdoors may be largely attributable to the drought conditions in the summer of 2012 in southwest Michigan. The bees appeared hesitant to leave the hive into desiccating environments. Future research should consider strategies to minimize this risk and to increase the probability of bees foraging, including performing experiments in environments where weather could be better controlled, using a greater number of bumblebee colonies, and/or using pollinators other than bumblebees.

In conclusion, our study confirmed the presence of iridoids in snapdragon tissue but could not confirm their presence in nectar directly. If iridoids are incorporated into the nectar of snapdragons, their function remains elusive. Results from this study provide valuable guidance for refining methodologies and material selection for future studies on plant and pollinator relationships with toxic nectar. We developed an interesting and cost-effective study system comprised of bumblebees and plants they frequently visit and also devised novel designs for artificial flowers. Various refinements of our techniques can inform future researchers and may be integral to subsequent investigations aimed at elucidating the role of secondary compounds in plant nectar.

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