Plants make scents:
Variation in plant volatile organic chemical emission at multiple scales

by

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For my mom, a doctor who helps people.

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ABSTRACT

Plants make scents:
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Substantial evidence indicates that herbivore-induced plant volatile organic chemical (VOC) emission can serve as an indirect plant defense by attracting the natural enemies of herbivores to sites of plant damage. A thorough understanding of the function and evolution of plant VOC emission requires detailed knowledge of the underlying variation that influences the trait. This dissertation investigates genetic variation in plant VOC emission at multiple scales: intraspecific variation within a population, intraspecific variation among plant populations, and variation among species within a genus. I used field and common garden experiments to study the potential role of VOC emission as an indirect defense in plants of the genus *Asclepias* (milkweeds) against an important specialist herbivore, *Danaus plexippus* Linnaeus (monarch caterpillar). I found that herbivore-damaged milkweed plants attracted more natural enemies than did undamaged plants, and that VOC emission varied among plant genotypes within a single population. Although VOC concentration and blend varied among genotypes, natural enemies attacked decoy caterpillars with equal frequency on all plant genotypes. To evaluate variation in VOC emission among plant populations, I tested the hypothesis that the indirect defense would vary predictably across a latitudinal gradient. Contrary to a predicted pattern of more intense defense at low latitudes, I found that herbivore-induced VOC emission increased with increasing latitude. Finally, I used phylogenetic analyses to investigate macroevolutionary patterns in VOC emission among plant species in the genus *Asclepias*. I tested the hypothesis that this indirect defense would exhibit a pattern of phylogenetic escalation to compensate for the phylogenetic decline of direct defenses in the same system. I observed macroevolutionary escalation in the number of VOCs emitted upon herbivore damage and a directional change in herbivore-induced VOC blend, which may indicate that *Asclepias* species have evolved to target more effectively the natural enemies of their herbivores. I also observed a phylogenetic decline in total constitutive and induced VOC concentrations among *Asclepias* lineages. These experiments provide substantial evidence that plant genotype influences variation in VOC emission at multiple scales.

Chapter 1

Introduction

The emission of trace gases by plants is of broad interest to multiple groups in the scientific community because of its implications for plant defense, insect responses to chemical signals, and for its role in the dynamics of atmospheric gases (Laothawornkitkul et al. 2009). Plant volatile organic chemicals (VOCs) make important contributions to ecosystems by helping to shape both the atmosphere and the biosphere (Monson and Holland 2001). For example, VOC emissions can affect local and regional atmospheric chemistry (Fuentes et al. 2000), influencing both oxidant photochemistry and the formation of secondary organic aerosols (Tsigaridis and Kanakidou 2007). Interactions between reactive nitrogen and VOCs produce photochemical oxidants, with ozone (O_3) among the most well known examples. Both oxidant photochemistry and the formation of secondary organic aerosols affect air quality (Atkinson and Arey 2003), crop yields, and forestry yields (Ashmore 2005) and are of interest on local and regional scales. Thus, VOCs strongly link the biosphere, atmosphere, and climate (Monson and Holland 2001), and important feedbacks can occur between VOCs and climate change (Peñuelas 2008, Laothawornkitkul et al. 2009).

Moreover, abundant evidence indicates that plant emission of VOCs can serve as a plant defense by facilitating multitrophic interactions. Herbivore damage can induce higher concentrations and different blends of VOCs emitted by plants, which can attract enemies of herbivores to sites of plant damage (D'Alessandro and Turlings 2006, Heil 2008). The phenomenon of enemy attraction via herbivore-induced VOC emission has been observed in multiple tritrophic systems (Dicke et al. 1998, Shimoda et al. 1997, De Moraes et al. 1998, Thaler 1999, Turlings et al. 1990, Turlings et al. 1993, De Moraes et al. 2001) and occurs relatively commonly in nature (Vet and Dicke 1992, Dicke and van Loon 2000, Kessler and Baldwin 2001). However, the degree to which natural selection acts upon VOC emission and related plant-insect interactions remains unclear.

Selection acts upon genetic variation, a prerequisite for evolutionary change (Futuyma 1997). To determine whether VOC emission potentially is subject to selection, evaluating genetic variation for the trait is essential, especially in native populations of plants. Very few studies directly explore the role of genetic variation in observed differences in volatile emissions; thus, causes of intraspecific variation remain poorly understood (Street et al. 1997, Isebrands et al. 1999, Funk et al. 2003, Staudt et al. 2003). Of this small group of studies, even fewer consider non-cultivated plant species (Halitschke et al. 2000, Staudt et al. 2001, Delphia et al. 2009, Schuman et al. 2009, Kariyat et al. 2012), and a particular problem is that genetic variation for VOC production rarely is measured in a comprehensive fashion in natural populations of native plants (Hunter 2002). This greatly limits our ability to understand the evolution of VOC-based defenses and their role in natural plant communities. With a more thorough understanding of the genetic basis for VOC emission, we can better estimate the ability of plant populations to respond to changing environmental pressures and ask broad ecological questions about this indirect defense. My dissertation provides an initial investigation of underlying genetic variation in VOC emission at multiple scales, specifically (1) intraspecific variation within a natural population, (2) intraspecific variation among populations at the geographic scale, and (3) variation among species within a genus. These three topics comprise three dissertation chapters, which are described in more detail below.

Chapter 2: Genetic variation in plant volatile emission does not result in differential attraction of natural enemies in the field. In this chapter, I evaluate standing genetic variation in plant VOC emission within a population of native plants using four complementary field and greenhouse experiments. Genetic variation in this chapter refers to either variation among genets, which are groups of clonal plants, or variation among half-sib genetic families, which are plants that share half of their genetic material. I employed the plant *Asclepias syriaca* (the common milkweed) and its herbivore *Danaus plexippus* (monarch butterfly) as a model system. In one experiment, I used monarch caterpillar decoys constructed from non-drying modeling clay to gauge natural enemy attraction to milkweed VOC emission in the field. The aims were to determine whether VOC emission can serve as an indirect defense in this system, and whether different milkweed genotypes differentially attract natural enemies to the monarch caterpillar herbivore in the field. In another experiment, I measured constitutive and monarch caterpillar-induced VOC emission from multiple plant genotypes growing naturally in the field. Because environmental factors likely contributed to the observed variation in VOC emission phenotype among field plants, I conducted two further experiments using a common garden of milkweed plants established in the greenhouse. These experiments enabled me to examine the genetic basis for variation in VOC emission as distinct from environmental and ontogenetic factors.

Chapter 3: A genetically-based latitudinal cline in the emission of herbivore-induced plant volatile organic compounds. This chapter examines intraspecific variation among populations of plants at the geographic scale. Genetic variation in this chapter thus refers to population-level variation. A prominent hypothesis posits that plants exhibit higher levels of antiherbivore defense at low latitudes than at high latitudes as a coevolutionary response to higher herbivore pressure on low-latitude plants (Pennings et al. 2001, Pennings et al. 2009). A similar pattern has been observed for indirect defenses wherein plants enlist the aid of ants to deter herbivores (extrafloral nectaries and ant domatia) (Pemberton 1998, Chamberlain and Holland 2009, Moles et al. 2011). However, evidence for mite domatia (O'Dowd and Willson 1991) and terpenoids contained in juniper needles (Martz et al. 2009) indicates that at least some constitutive indirect defenses are more prevalent at high latitudes. I conducted the first experimental investigation of both constitutive and herbivore-induced indirect plant defense across a latitudinal gradient. I used a common garden to test whether milkweed populations vary in their VOC emissions, and whether emission varied predictably across the latitudinal gradient.

Chapter 4: Phylogenetic patterns in plant volatile emission: The potential adaptive evolution of indirect defense among milkweed species. In this chapter, I assess variation in VOC emission among plant species within a genus. Genetic variation in this chapter refers to species-level variation, measured as nucleotide substitutions per site in three non-coding regions of the plastid genome. Broad evolutionary questions can be addressed by implementing phylogenetic analyses (e.g., Farrell et al. 1991, Becerra 1997, Thaler and Karban 1997, Farrell and Mitter 1998, Heil et al. 2004), and milkweed plants comprise an exceptional system in which to apply a phylogenetic framework. Milkweeds express a well-studied arsenal of direct defenses against herbivores (Malcolm and Zalucki 1996, Fordyce and Malcolm 2000, Matter 2001, Zalucki et al. 2001, Van Zandt and Agrawal 2004, Agrawal 2005, Agrawal and Fishbein 2006, Zehnder and Hunter 2007, Vannette and Hunter 2011a, Vannette and Hunter 2011b), and several of these defense traits exhibit distinct evolutionary patterns. Cardenolides (toxic steroids), latex, and trichomes, three important direct defenses in milkweed, follow a pattern of macroevolutionary decline, whereas tolerance to herbivory (regrowth ability) exhibits macroevolutionary escalation in the same *Asclepias* species (Agrawal and Fishbein 2008). Direct defenses in this system decline over evolutionary time possibly because milkweed suffers herbivory by specialist arthropods that can exploit (Malcolm et al. 1989, Holzinger et al. 1992, Dobler et al. 2011, Helms et al. 2004, Lefèvre et al. 2010) or otherwise circumvent (Malcolm 1994) the direct defenses. In contrast, indirect defenses may be more challenging for milkweed herbivores to overcome, especially because the chemicals that they sequester seem ineffective against parasitoids (Hunter et al. 1996, Helms et al. 2004). I used a common garden of multiple *Asclepias* species to test the following two hypotheses: milkweed species vary in their VOC emissions, and VOC emission phenotype exhibits a macroevolutionary pattern of escalation to compensate for the declining efficacy of direct defenses in the same species.

In a concluding section, I place the results of my work in a broader context and consider opportunities for future work.

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Chapter 2

Genetic variation in plant volatile emission does not result in differential attraction of natural enemies in the field

Abstract

Volatile organic chemical (VOC) emission by plants may serve as an adaptive plant defense by attracting the natural enemies of herbivores. For plant VOC emission to evolve as an adaptive defense, plants must show genetic variability for the trait. To date, such variability has been investigated primarily in agricultural systems, yet relatively little is known about genetic variation in VOCs emitted by natural populations of native plants. Here, we investigate intraspecific variation in constitutive and herbivore-induced plant VOC emission using the native common milkweed plant (*Asclepias syriaca*) and its monarch caterpillar herbivore (*Danaus plexippus*) in complementary field and common garden greenhouse experiments. In addition, we used a common garden field experiment to gauge natural enemy attraction to milkweed VOCs induced by monarch damage. We found evidence of genetic variation in the total constitutive and induced concentrations of VOCs and the composition of VOC blends emitted by milkweed plants. However, all milkweed genotypes responded similarly to induction by monarchs in terms of their relative change in VOC concentration and blend. Natural enemies attacked decoy caterpillars more frequently on damaged than on undamaged milkweed, and natural enemy visitation was associated with higher total VOC concentrations and with VOC

blend. Thus, we present evidence that induced VOCs emitted by milkweed may function as a defense against herbivores. However, plant genotypes were equally attractive to natural enemies. Although milkweed genotypes diverge phenotypically in their VOC concentrations and blends, they converge into similar phenotypes with regard to magnitude of induction and natural enemy attraction.

Introduction

The emission of volatile organic chemicals (VOCs) by plants is prevalent and may serve as an important defense against herbivores (D'Alessandro and Turlings 2006, Heil 2008). Plant VOCs can attract the natural enemies of herbivores, serving as an indirect defense by reducing further herbivore damage (Dicke 1994) and ultimately improving plant fitness (Fritszche Hoballah and Turlings 2001). Moreover, herbivore damage often modifies plant VOC emission quantitatively (via increased VOC concentration) and qualitatively (in terms of the presence and ratio of chemicals within the VOC blend) (D'Alessandro and Turlings 2006, Dudareva et al. 2006), which can attract higher numbers of natural enemies (De Moraes et al. 1998, Thaler 1999, Kessler and Baldwin 2001).

Investigating quantitative and qualitative variation in VOC emission is important for a thorough understanding of VOC emission as a plant defense. Different plant species emit different VOC blends (Kesselmeier and Staudt 1999, Pichersky et al. 2006). Within species, damage by host and non-host herbivores can induce different plant VOC responses, which are distinguishable by their natural enemies (De Moraes et al. 1998, Takabayashi et al. 2006), and even herbivores of different developmental stages can induce different blends and concentrations of VOCs (Takabayashi et al. 1995). VOC emission has been shown to vary with plant genotype, though our knowledge of genetic variation in VOC emission is limited primarily to a few cultivated agricultural plants, such as maize (e.g., Turlings et al. 1998, Gouinguené et al. 2001, Degen et al. 2004, Rasmann et al. 2005), cotton (Elzen et al. 1986, Loughrin et al. 1995), wheat (Weaver et al. 2009), and rice (Lou et al. 2006). To date, few studies have reported intraspecific variation in VOC emission in natural populations of plants (Halitschke et al. 2000, Staudt et al. 2001, Hare 2007, Delphia et al. 2009, Schuman et al. 2009, Kariyat et al. 2012). This paucity of studies is surprising, given that the evolution of VOC emission as an adaptive indirect defense is contingent on heritable genetic variation for the trait. In light of recent rapid changes in climate and species distributions, and concerns about potential rates of adaptation in plant populations, characterization of intraspecific genetic variation for VOC defense among uncultivated plant species is crucial.

To assess intraspecific variation in VOC emission and its potential role in plant defense, we studied naturally growing and common garden milkweed plants (*Asclepias syriaca*). Over the course of three growing seasons, we addressed the following questions: (1) Do VOC concentration, blend, and magnitude of induction vary among milkweed genotypes? (2) Does caterpillar damage attract the natural enemies of herbivores to milkweed in the field? (3) How do VOC concentration, blend, and plant genotype influence natural enemy attraction in the field?

Methods

Asclepias syriaca is a widespread native plant in eastern North America, with a range that extends north to Canada and south to Louisiana. Milkweed ramets are clonal individuals that originate vegetatively via rhizome from one seed; a group of genetically identical ramets forms a genet. Monarchs (*Danaus plexippus*) are specialist herbivores that feed as larvae on plants in the genus *Asclepias*. Monarch larvae are attacked by a variety of parasitoids (e.g., tachinid flies and braconid wasps) and predators (ants, spiders, paper wasps, and hemipterans) (Prysby 2004, Mooney and Agrawal 2008). Some evidence suggests that VOCs mediate interactions in the milkweed-monarch system; for example, ovipositing butterflies can distinguish between the VOCs of old and young milkweed plants (Bergström et al. 1995). Other evidence indicates that VOC emission from milkweed roots can serve as a belowground indirect defense by attracting predatory nematodes (*Heterorhabditis bacteriophora*) to longhorn beetle root herbivores (*Tetraopes tetrophthalmus*) (Rasmann et al. 2011).

We wished first to determine if milkweed genets growing naturally under field conditions vary in their aboveground vegetative VOC emissions; therefore, we collected VOCs from field plants in the summer of 2008. However, field plants vary in their environments as well as their genotypes, so we used a greenhouse common garden in 2009 and 2010 to explore genetic variation in VOC emission among milkweed families. Second, we sought to determine whether monarch-induced VOC emission attracts natural enemies to milkweed plants under field conditions. Finally, we linked VOC data obtained in the greenhouse to field observations of natural enemy attraction; our aim was to quantitatively evaluate aboveground VOC emission as an indirect defense in the field.

VOC collections

In 2008, we measured milkweed VOC emission from plants growing naturally in a single population at the University of Michigan Biological Station (UMBS) in Pellston, Michigan. Genets initially were identified by location, phenology, morphology, and chemistry; subsequently, genet identities were verified by molecular markers (Kabat et al. 2010). Between July 7 and August 24, we sampled VOCs from 6 milkweed genets, with 5 undamaged and 5 monarch-damaged ramets per milkweed genet, for a total of 60 observations. Measurements from genets were randomized among sampling days. Aboveground VOC emissions were collected continuously for 12 h.

To assess the potential for genetic variation in VOC emission while excluding environmental variability inherent to field conditions, we sampled VOCs from plants grown in a common garden in the UMBS greenhouse. All seeds from a single milkweed pod are full siblings (Gold and Shore 1995) and hereafter are called “genotypes”. Seedpods were collected from adult milkweed plants that grew naturally in our experimental population at UMBS. To the greatest extent possible, we matched the common garden genotypes with those sampled in the field in 2008. However, not all field plants that we sampled produced viable seeds; three of the six 2008 field genotypes were replicated in the 2009 and 2010 common garden experiments. We supplemented the common garden experiments with an additional 3 (2009) or 4 (2010) genotypes from the same population. All common garden plants used in the 2009 and 2010 experiments were established during the summer of 2008. Seeds were washed with a solution of 5-10% bleach in water and cold stratified for four weeks on damp paper towels. The seeds were germinated on moist filter paper in Petri dishes in a growth chamber and grown in 6-inch pots containing unfertilized soil (Glacial Bay Soils, Elsie, MI, USA) in the UMBS greenhouse. The plants were fertilized with Osmocote 14N:14P:14K (Scotts, Marysville, OH, USA) at the beginning of their second (2009) and third (2010) growing seasons.

Between August 31 and September 5 2009, we sampled VOCs from 6 genotypes in the common garden, with 10 undamaged and 10 monarch-damaged individuals per genotype, for a total of 120 plants. Blocks of 20 plants (10 undamaged and 10 damaged) were sampled each day: all 6 genotypes were represented at least once every sampling day (12 plants), with 4 more genotypes chosen at random from the pool of plants to complete the sampling block (8 plants). VOCs were sampled continuously for 7 h.

We repeated the common garden experiment in 2010. Between the 2009 and 2010 growing seasons, common garden plants were overwintered outdoors under mulch at UMBS. The 2010 common garden experiment included the same 6 genotypes from the previous year, plus 1 additional genotype. We planted in excess of our needs for the experiments; thus, experimental plants in 2010 were drawn randomly from the same 2009 pool of plants and were not always the same individuals in both years. In 2010, VOCs were collected between July 13 and August 14. We sampled VOCs from 7 genotypes, with an average of 27 observations from 5 of the plant genotypes and 11-15 observations from the other 2 genotypes, which were less abundant in the common garden. We collected VOC emissions from a total of 160 plants (N=81 undamaged plants, N=79 herbivore-damaged plants). Comprising the 20-plant sampling block were 10 damaged and 10 control plants, one from each of the 7 genotypes every sampling day (14 plants), with 3 more genotypes chosen at random from the pool of plants (6 plants).

Monarch caterpillars were used to experimentally damage all treatment plants. In 2008, we raised the caterpillars from eggs that were laid in captivity by monarch butterflies obtained at our field site. In 2009 and 2010, we obtained the monarchs from Flutterby Gardens, Bradenton, FL, USA. One third-instar caterpillar was placed on the fifth leaf pair of each treatment plant. Netting was placed over each treatment leaf to restrict caterpillar movement and over control leaves without caterpillars to control for effects of netting on VOC emission. Caterpillars were allowed to feed from late afternoon until the following morning (about 18 hours) prior to VOC collection. Larvae and bags were removed before VOCs were collected. VOCs were sampled during approximately the same daylight hours on each sampling day following the previous night of herbivore damage.

Chambers were constructed from wood or PVC pipes and inert Tedlar sheeting. Soil VOCs were minimized in samples by covering the soil around the plant stems with inert or plastic material. Rotameters connected to each chamber enabled us to quantify and adjust the velocity of air flowing through the chambers (an average rate of 1-1.5 liters per minute in 2008, 2.8 liters per minute in 2009, and 1.5 liters per minute in 2010. The higher airflow in 2009 was due to pump availability, and all VOC data were corrected for flow rate prior to analysis). We collected VOCs by enclosing one plant in each chamber and collecting the gases that accumulated in the chambers. In 2008, we used a “push-pull” headspace VOC collection system, wherein a brushless single-head DC diaphragm pump (Brailsford, Antrim, NH, USA) pushed ambient air through a series of filters that preceded each chamber and a second pump pulled gases from the chamber through a VOC adsorbent. In 2009 and 2010, we used a “pull” headspace collection system, wherein a dual-diaphragm vacuum pump (Gardner Denver Thomas, Sheboygan, WI, USA) drew air from a series of separate chambers. In all years, incoming air flowed through a filter containing sodium thiosulfate that prevented ozone gas, which can react chemically with VOCs in the atmosphere (Helmig 1997), from entering chambers. After the ozone filter, air flowed through an activated carbon filter to prevent ambient VOCs from entering chambers. Thus, "clean" air flowed into the chambers; however, we also sampled VOCs from empty chambers to evaluate any remaining VOCs in the "clean" air. In 2008 and 2010, VOCs adsorbed on Super-Q (Analytical Research Systems, Gainesville, FL, USA). In 2009, based on availability of materials, VOCs adsorbed on activated charcoal (Sigma-Aldrich, St. Louis, MO, USA).

Although we took care to limit our sampling to vegetative emissions, reproductive structures on some naturally growing plants in 2008 were unavoidable; we accounted for the effect of flowers and seedpods by including them as continuous covariates in the analyses below.

We also controlled for the effect of plant biomass on VOC emission. In 2009 and 2010, we destructively harvested all aboveground vegetation in the common garden, placed the samples in a drying oven for a minimum of 24 hours, and measured the biomass of each plant. In 2008, we nondestructively estimated plant dry mass by using the stem height and number of leaves measured for each ramet in a regression equation (Eqn. 1; $R^2=0.79$, $F_{2,\,97}=177.99$, $P<0.0001$). The regression was derived previously using 100 destructively harvested ramets from the same natural milkweed population at UMBS.

Eqn. 1: $$\mathit{biomass} = (0.17 \bullet \mathit{leaves}) + (0.186 \bullet \mathit{height}) - 3.853$$

In 2008, naturally occurring insects on experimental ramets were removed at least three days before taking measurements and whole plants were netted to prevent inadvertent induction. During the 2009 growing season, aphids colonized some plants in the greenhouse. Aphid abundance was scored for each plant, and these data were used as a continuous covariate in statistical analyses. Aphids were removed from plants with a fine paintbrush 7 days before VOC collection. During the 2010 growing season, two generalist insect herbivores infested the common garden. Cabbage loopers (*Trichoplusia ni*) were removed by hand from nearly 100% of the plants. Thrips were eradicated by a combination of applying Organic Colorado Potato Beetle Beater Concentrate and moving the plants outdoors (Bonide Products, Oriskany, NY, USA).

Environmental variables

We collected data for environmental variables that can influence VOC emission (Kesselmeier and Staudt 1999). HOBO data loggers (Onset Corporation, Bourne, Massachusetts, USA) were deployed in or near chamber arrays to measure temperature and relative humidity, while photosynthetically active radiation (PAR) sensors (LI-COR, Lincoln, NE, USA) gauged ambient light levels. The sensors provided a reading every minute. The data from each sensor were integrated across minutes for the total sampling time, yielding a cumulative value for each of the variables per day. These data were used to standardize for environmental variables across days.

To account for covariance among environmental factors, we combined them using a principal components analysis (PCA), and PCA axes were used as covariates in all analyses. Both of the following criteria had to be met to retain PCA axes in analyses: the axis explained at least 10% of total variance in the PCA, and the eigenvalue of the axis was >1. In 2008, environmental data were combined into two PCA axes explaining 48.1% and 34.7% of the variation, respectively (total = 82.8%). In 2009, two PCA axes explained 70% and 22.9% of the variation (total = 92.9%). In 2010, two PCA axes explained 60.3% and 25.6% of the environmental variation (total = 85.9%).

Direct defenses

Although a hypothesized trade-off between indirect and direct plant defense rarely has been observed empirically in any plant system (Steward and Keeler 1988, Rudgers et al. 2004, but see Ballhorn et al. 2008), we wanted to account for the potential existence of such a trade-off among milkweed genotypes. We evaluated five plant quality traits that are related to direct defense (Scriber 1977, Frost and Hunter 2007, Vannette and Hunter 2011a): foliar cardenolide concentration, latex production, trichome density, foliar water content, and specific leaf area (SLA; an index of leaf toughness). Direct defense data from the 2008 field experiment included only cardenolides, trichomes, and leaf toughness. All five plant defense traits were recorded for the 2009 and 2010 common garden experiments.

Cardenolides were separated and quantified using reverse-phase high-performance liquid chromatography (HPLC; Waters, Milford, MA, USA), following established protocols (Zehnder and Hunter 2007). Fresh leaf discs were punched from the fifth pair of leaves (opposite the damaged leaf) using a hole puncher directly into methanol for cardenolide extraction. Identical control discs were taken from the same leaves, weighed fresh, dried, and reweighed. These control disks provided an estimate of sample dry mass for cardenolide analysis, foliar water content, and SLA. Cardenolide samples were run on the HPLC with digitoxin as an internal standard. We considered peaks with symmetrical absorbance between 217 and 222 nm to be cardenolides. Cardenolide concentrations were standardized for sample dry mass. Compounds present in only one plant and compounds that comprised less than 1% of the total concentration of cardenolides across all plants in an experiment were excluded from analyses. Total cardenolide concentrations were calculated as the sum of individual cardenolide peaks.

Foliar latex was collected from the holes that had been punched from leaves for cardenolide analysis. Latex flowing from the holes was absorbed onto pre-weighed cellulose discs (1 cm diameter). Discs were dried and reweighed to provide estimates of latex production. Trichomes were counted on the lower surface of control leaf discs that had been removed for cardenolide sampling (Vannette and Hunter 2011b).

To account for covariance among the direct defenses and reduce the dimensionality of the direct defense data, we combined all measured direct defense traits using PCA. In 2008, direct defense data were combined into one PCA axis explaining 41.6% of the variation. In 2009, two PCA axes explained 35.4% and 23.6% of the variation (total = 59%). In 2010, two PCA axes explained 37.8% and 22.5% of the variation (total = 60.3%).

Analysis and identification of VOCs

VOCs were eluted from adsorbents with 750 µl of dichloromethane (CH_2Cl_2) containing 1.94 µg of nonyl acetate (Sigma-Aldrich, CAS # 143-13-5) as an internal standard. We analyzed 1-µl aliquots of VOC samples with a gas chromatograph-mass spectrometer (GCMS; Agilent Technologies, Santa Clara, CA, USA) using the following GC method: injector held at 250 ^{o}C, initial column temperature at 50 ^{o}C held for 10 min, ramped at 5 ^{o}C min^{-1} to 200 ^{o}C, held for 10 min. Helium carrier gas flow was set to 1.3 ml min^{-1}.

Methods were identical for all three experiments, except for the GC column. In 2008, we used a 30 m x 0.25 mm inner diameter fused-silica column with a 5% phenyl stationary phase and 0.25 μm film thickness (Agilent Technologies, Santa Clara, CA, USA). In 2009, excess moisture was removed from eluted samples using anhydrous sodium sulfate. In 2009 and 2010, we used a 30 m x 0.25 mm inner diameter fused-silica column with a polyethylene glycol stationary phase and 0.25 μm film thickness (Restek Corporation, Bellefonte, PA, USA).

Compounds were tentatively identified with reference to the NIST (National Institute of Standards and Technology) library database, and the identity of key peaks subsequently was verified using authentic standards (Sigma-Aldrich, St. Louis, MO, USA; MP Biomedicals, Solon, OH, USA). Synthetic chemicals and VOCs collected from empty chambers were omitted from the dataset. VOC concentrations were quantified by calculating relationships among internal standard concentration, internal standard peak area, and focal compound peak area. VOC concentrations were standardized for the total volume of air sampled from each chamber and for aboveground plant biomass. Compounds emitted by only one plant and compounds that comprised less than 1% of the total concentration of VOCs across all plants were omitted. In 2008, we retained 9 compounds (90% of total emissions) in the dataset. In 2009, we retained 13 compounds (94% of the total). In 2010, we retained 26 compounds (87% of the total).

Predation and parasitism in the field

In 2010, we investigated whether the natural enemies of monarchs are attracted to induced milkweed VOCs in the field. We estimated attraction of enemies by observing damage to artificial caterpillars deployed as “decoys” (Skoczylas et al. 2007) on milkweed plants placed in a field common garden. Non-drying modeling clay was used to create decoy herbivores that mimicked the pattern and size of third-instar monarch caterpillars (Van Aken International, Rancho Cucamonga, CA, USA). Natural enemy visitation was inferred if we observed gouges or scratches in the clay (Figure 2.1; Skoczylas et al. 2007). This type of damage to clay caterpillars is likely to be caused by chewing predators such as wasps and ants, and thus underestimates natural enemy visitation (e.g., parasitoids).

We used 5 genotypes in the experiment, with an average of 40 observations for 3 of the genotypes and 12-14 observations for the other 2 genotypes, which were less abundant in the common garden. We placed decoys on a total of 144 plants (N=72 undamaged plants, N=72 monarch-damaged plants). These experimental plants were a subset of genotypes from the same pool of potted plants used for the 2010 common garden experiment; however, no individual plant was included in more than one experiment during the same year. Plants were kept in the greenhouse through damage treatment; the following morning they were transferred to a field common garden (see below). Monarch caterpillars were used to damage the treatment plants. After the live caterpillars were removed from the treatment plants, one decoy caterpillar was adhered to the fifth leaf of undamaged and monarch-damaged plants using double-sided insect monitoring cards (Whitmire Micro-Gen Research Laboratories, St. Louis, MO, USA).

Because clay caterpillars were placed near foliar damage, we cannot exclude the possibility that foraging predators used visual cues.

One week before the experiment began, we mowed the entire field within a 12.5 m x 25 m mammal exclosure at UMBS to reduce the potential for insects to congregate heterogeneously in the vegetation. For the experiment, blocks of 16 plants per sampling day were placed in the exclosure for 24 hours in a 4 plant x 4 plant array (1 m between rows, 2 m between columns). Each day, 2 undamaged and 2 monarch-damaged plants from each of 4 genotypes were placed in the field. Plant genotype and herbivore treatment were fully randomized in the arrays. The experimental plants were placed in holes dug into the ground, with the soil in each pot level with the surface of the ground. After 24 hours in the field, the plants were moved back to the lab, the decoy caterpillars were scored for damage (presence/absence), and the aboveground vegetation was harvested for biomass measurements.

We could not simultaneously measure plant VOCs and allow access to plants by natural enemies. However, we implemented this experiment concurrently with the greenhouse VOC collections in 2010 to associate specific VOC profiles with natural enemy visitation. Plants in the field were matched with plants in the greenhouse according to genotype. Every day in the greenhouse, we sampled VOCs from each of the genotypes that were represented in the field, with at least one undamaged and one monarch-damaged plant per genotype.

Statistical analyses

To assess whether VOC concentration varied between herbivore treatments and among genotypes, we used mixed model analyses of variance (SAS Version 9.2) with total VOC concentration (ln-transformed) as the response variable; herbivore treatment, plant genotype, environmental variables (PCA axes as described above), direct defense traits (PCA axes), and Julian date were independent variables; chamber was a random variable. We optimized model fits by minimizing the log likelihood and Akaike information criterion (AIC) scores. In 2008, we included the number of flowers and seedpods per plant as random variables. In 2009, we included the number of aphids per plant as an independent variable. We used Tukey's test to compare VOC concentrations among genotypes. We determined whether the magnitude of VOC induction (induced concentration minus constitutive concentration) varied among genotypes by assessing the herbivory treatment by genotype interaction term.

To evaluate whether VOC blend varied between herbivore treatments and among genotypes, we used permutational MANOVA in R (Version 2.11.1) (Oksanen 2010) to evaluate the multivariate composition of chemicals. Herbivore treatment, plant genotype, treatment by genotype interaction, environmental variables, direct defense traits, Julian date, and chamber were independent variables. Flowers and seedpods were included in the 2008 analysis, and aphid numbers were included in 2009.

To test whether natural enemies were differentially attracted to monarch-induced VOCs in the field, we used a binomial generalized linear model with logit link function (SAS Version 9.2), with presence or absence of damage to clay caterpillars as the response variable. Herbivore treatment, plant genotype, and their interaction were predictor variables.

To associate quantitative and qualitative variation in VOC emission in the greenhouse with natural enemy attraction in the field, we first calculated the mean concentrations of the 26 VOCs within herbivore treatment and plant genotype in the greenhouse in 2010. Total VOC concentration and VOC blend (represented by PCA) for each herbivore treatment within each genotype were estimated from those mean VOC concentrations. We calculated the proportion of clay caterpillars attacked on undamaged and monarch-damaged plants for each plant genotype. These proportions were arcsine-square-root transformed prior to analysis.

To determine whether natural enemies were more attracted to higher concentrations of VOCs, we evaluated a linear model (R Version 2.11.1) with mean total VOC concentration emitted per genotype (ln-transformed), herbivore damage treatment, and concentration by treatment interaction as predictors. To determine whether natural enemies responded differently to different VOC blends, we first used a PCA to combine the mean concentrations of all 26 VOCs. We then tested a linear model with proportion of attacked clay caterpillars (arcsine-square-root transformed) as the response variable; PCA axis 1 (explaining 49.1% of the variance in VOC blend), herbivore treatment, and blend by treatment interaction were predictors.

Results

VOC collections

Herbivory by monarch caterpillars induced higher concentrations of VOC emissions in all years (Table 2.1, Figure 2.2; 2008, $F_{1, 27.1}$=4.80, P=0.037; 2009, $F_{1, 64}$=8.11, P<0.01; 2010, $F_{1, 120}$=5.43, P=0.021). Total VOC concentrations differed among genets in the field in 2008 (Figure 2.3A; $F_{5, 27.4}$=2.75, P=0.039). Although VOC concentration did not vary among genotypes in the common garden in 2009 (Figure 2.3B; $F_{5, 64}$=1.30, P=0.274), plant genotype explained over 13% of the total variation in VOC concentrations in 2010 (Figure 2.3C; $F_{6, 119}$=4.32, P<0.001). Magnitude of induction was similar among genotypes in all of the experiments (treatment x genotype; 2008, $F_{5, 27.1}$=0.41, P=0.836; 2009, $F_{5, 64}$=0.64, P=0.667; 2010, $F_{6, 121}$=1.10, P=0.365).

In 2008 and 2009, VOC emissions increased with increasing temperature and light and decreasing humidity (2008, $F_{1, 28}$=6.89, P=0.014; 2009 PC2, $F_{1, 64}$=5.16, P=0.027). In 2010, VOC concentrations increased with increasing temperature, decreasing PAR, decreasing humidity inside chambers, and increasing ambient humidity (PC2, $F_{1, 109}$=7.83, P<0.01). For field plants, emissions decreased as the growing season progressed (2008, $F_{1, 21.2}$=33.95, P<0.0001); in the greenhouse, however, VOC concentrations were unrelated to Julian date (2009, $F_{1, 64}$=0.41, P=0.524; 2010, $F_{1, 111}$=2.66, P=0.106). Measures of direct defense traits were unrelated to VOC concentrations in all years (2008, $F_{1, 27.5}$=3.26, P=0.082; 2009 PC1, $F_{1, 64}$=2.38, P=0.128, 2009 PC2, $F_{1, 64}$=3.46, P=0.067; 2010 PC1, $F_{1, 124}$=0.52, P=0.471, 2010 PC2, $F_{1, 119}$=2.76, P=0.100). Neither the number of flowers ($F_{1, 26}$=3.48, P=0.073) nor the number of seedpods on a ramet ($F_{1, 26}$=0.82, P=0.374) affected VOC concentrations in 2008. Aphid densities did not significantly influence VOC concentrations in 2009 ($F_{1, 64}$=2.80, P=0.099).

VOC blend was altered by monarch damage in 2008 and 2009 (Table 2.2, Figure 2.4A,C; 2008, $F_{1, 42}$=4.50, P<0.001; 2009, $F_{1, 81}$=10.87, P<0.0001). In contrast to its strong effect in the preceding years, herbivory marginally induced different VOC blends in 2010 (Figure 2.4E; $F_{1, 144}$=1.87, P=0.054). Genetic variation in VOC blend was detected in all three years (Figure 2.4B,D,F; 2008, $F_{5, 42}$=1.64, P=0.023; 2009, $F_{5, 81}$=1.75, P<0.01; 2010, $F_{6, 144}$=3.17, P<0.0001). The change in VOC blend induced by herbivore damage was similar among genotypes in all years (treatment x genotype; 2008, $F_{5, 42}$=1.34, P=0.106; 2009, $F_{5, 81}$=0.85, P=0.739; 2010, $F_{6, 144}$=0.92, P=0.628).

Environmental variables influenced the ratio of chemicals in the VOC blend both years in the greenhouse (2009 PC1, $F_{1, 81}$=3.81, P<0.001; 2010 PC1, $F_{1, 144}$=10.14, P<0.0001, 2010 PC2, $F_{1, 144}$=7.52, P<0.0001), but not in the field plants ($F_{1, 42}$=0.66, P=0.707). Julian date influenced VOC blend in 2008 ($F_{1, 42}$=9.71, P<0.0001) and 2010 ($F_{1, 144}$=6.76, P<0.0001), but not in 2009 ($F_{1, 81}$=1.52, P=0.149). Direct defense traits were unrelated to VOC blend in 2008 ($F_{1, 42}$=1.44, P=0.180) and 2009 (PC1, $F_{1, 81}$=1.34, P=0.216; PC2, $F_{1, 81}$=1.62, P=0.112). However, direct defenses influenced VOC blend in 2010 (PC1, $F_{1, 144}$=6.65, P<0.0001; PC2, $F_{1, 144}$=2.14, P=0.031), where the first PCA axis corresponded primarily to decreasing latex, trichomes, and leaf toughness, while the second axis corresponded to increasing cardenolides and foliar water content. In 2008, the number of flowers ($F_{1, 42}$=4.41, P<0.001) and seedpods ($F_{1, 42}$=2.87, P<0.01) on ramets affected VOC blend. Aphid density did not induce any change in VOC blend among plants in 2009 ($F_{1, 81}$=0.86, P=0.553).

Using authentic standards, we identified the following three VOCs in our samples: β-caryophyllene in 2008 and 2010, cis-3-hexen-1-ol in 2009, and nerolidol in 2010. These three compounds have been associated with herbivore damage in other plant systems (Turlings et al. 1990, Wiens et al. 1991, Rasmann et al. 2005, Ruther and Kleier 2005, Kessler et al. 2006).

Predation and parasitism in the field

The proportion of decoy caterpillars attacked by natural enemies was over 2.5-fold higher on herbivore-damaged milkweeds than on control plants (χ^2_1=6.42, P=0.011; Figure 2.1B). Natural enemies more frequently visited both damaged (R^2=0.80, $F_{1,3}$=11.64, P=0.042) and undamaged (R^2=0.94, $F_{1,3}$=45.17, P<0.01) plants that emitted higher concentrations of VOCs (Figure 2.5A). Natural enemies also were differentially attracted to different VOC blends emitted by damaged (R^2=0.82, $F_{1,3}$=13.48, P=0.035) and undamaged (R^2=0.82, $F_{1,3}$=13.93, P=0.034) plants (Figure 2.5B). All plant genotypes were equally attractive to natural enemies in the field (χ^2_4=4.72, P=0.317).

Discussion

We detected genetic variation in both total concentration (Figure 2.3) and blend (Figure 2.4B,D,F) of constitutive and induced plant VOCs emitted by individuals from a single population of native milkweeds. Additionally, herbivore damage consistently induced increases in VOC emission (Figure 2.2) and changes in VOC blend (Figure 2.4A,C,E). Although we detected genetic variation in VOC emissions, the relative change induced by herbivory in both total concentration and VOC blend was similar among milkweed genotypes. Finally, herbivore damage attracted higher numbers of natural predators to decoy monarch caterpillars in the field (Figure 2.1B). Predator attraction was associated with both VOC concentration and blend (Figure 2.5); however, decoy caterpillars on the different milkweed genotypes were attacked with equal frequency.

Genetic variation in VOC emission

Our understanding of genetic variation in plant VOC emission remains incomplete. Studies to date have focused mainly on agricultural systems and other commercial plants (e.g., Gouinguené et al. 2001, Degen et al. 2004, Lou et al. 2006, Kappers et al. 2010), but these studies are limited to plants that have been subjected to artificial selection. Investigating variation in VOC emission among native plants that have evolved in a natural context reveals more about the potential evolution of this trait as an indirect defense in response to natural levels of herbivory. Our report of genetic variation in VOC emission by milkweeds joins a small number of other studies reporting genetic variation in VOC emission in natural plant populations (Halitschke et al. 2000, Staudt et al. 2001, Hare 2007, Delphia et al. 2009, Schuman et al. 2009, Kariyat et al. 2012).

In all three years of experiments, the ratio of chemicals emitted by milkweed varied among plant genotypes (Table 2.2, Figure 2.4B,D,F). We consider this strong evidence for genetic variation in the blend of VOCs emitted by plants in our milkweed population. In contrast, the evidence for genetic variation in total VOC concentration was weaker (Table 2.1, Figure 2.3). In 2008, we observed differences in constitutive and induced VOC concentration among genets in the field (Figure 2.3A), though we cannot separate the effects of genotype and environment on VOC emission from those data alone. We detected variation in VOC concentration among milkweed genotypes in the 2010 common garden (Figure 2.3C), which provides the strongest evidence for a genetic component to total VOC emission. However, we observed no significant variation in total VOC emissions among genotypes in the 2009 common garden experiment (Figure 2.3B). We note that plants were younger in 2009 and volatile emissions lower. Moreover, our ability to detect genetic variation in total VOC emission in 2010 was based on the inclusion of an additional milkweed genotype (genotype J) that was not present in 2009 (Figure 2.3B,C). Interestingly, this additional genotype (half sibs from a single seedpod) originated from the same milkweed genet as genotype G, which was used in both common garden experiments. Due to the pollinium reproductive strategy in milkweed (Gold and Shore 1995), genotypes G and J differed only in their pollen donor. Thus, our results suggest that pollen donation may be an important determinant of the variability in VOC emission among milkweed offspring (Mazer et al. 1986, Andersson 1990).

Other sources of variation in VOC emission

As expected, environmental variables (Kesselmeier and Staudt 1999) and seasonality (Hare 2010) markedly affected VOC emission (Tables 2.1 and 2.2); however, the relationship between direct defense traits and VOCs remains unclear. We found that direct defenses were correlated only with VOC blend in 2010 (Table 2.2). It is possible that infestation by pests in the greenhouse may have altered the expression of direct defenses, which potentially may have influenced the emitted VOC blends. This explanation might also account for the marginally non-significant effect of experimental monarch herbivory on VOC blend in 2010 (Table 2.2). Addressing these hypotheses would require further study. To date, efforts to correlate direct and indirect defenses have not revealed a consistent pattern; some evidence supports a trade-off (Ballhorn et al. 2008), whereas some studies show that direct and indirect defenses are expressed independently (Steward and Keeler 1988, Kahl et al. 2000, Rudgers et al. 2004). For the most part, it appears that direct and indirect defenses in milkweed operate independently, for VOCs emitted both aboveground (reported here) and belowground (Rasmann et al. 2011).

VOC emission as a defense against herbivores

Our results are consistent with the hypothesis that milkweed VOCs can function as a defense against herbivores. Foliar damage by monarch caterpillars consistently induced higher total concentrations of milkweed VOCs (Table 2.1, Figure 2.2) and altered the VOC blend emitted by milkweed plants (Table 2.2, Figure 2.4A,C,E). In field trials, natural enemies attacked decoy caterpillars more frequently on damaged than on undamaged plants (Figure 2.1B). Moreover, both induced and undamaged plants emitting higher total concentrations of VOCs elicited higher rates of natural enemy visitation (Figure 2.5A). Natural enemies also appeared to distinguish among different VOC blends in the field, attacking decoy caterpillars with increasing frequency on plants emitting volatile blends characterized by PCA axis 1 (Figure 2.5B).

Determining the relative importance of plant VOC concentration and blend in attracting herbivore enemies remains a persistent problem. In some cases, natural enemies may respond more strongly to VOC blend than to changes in VOC concentration

(Bruce et al. 2010). In contrast, higher total concentrations of VOCs have been shown here and elsewhere (Gols et al. 2003, Turlings et al. 2004) to increase recruitment of natural enemies. Until VOC concentration and blend can be manipulated independently, a task that is especially difficult in field experiments, clear conclusions cannot be drawn regarding whether natural enemies respond more strongly to VOC concentration or VOC blend. For example, in the 2010 experiment described here that links VOC emission to natural enemy attraction in the field, the PCA axis loadings for VOC blend indicate that all but two compounds increase in concentration along the first PCA axis (including β-caryophyllene and nerolidol). Further investigation is required to determine whether the natural enemies are responding to either an increase in total VOC concentration, or an increase in one or a few important chemicals associated with herbivore damage, or a specific combination or ratio of compounds within the VOC blend.

Ecological consequences of genetic variation in VOC emission

Surprisingly, despite the genetic differences in milkweed VOC concentration and blend, decoy caterpillars were attacked with similar frequency on all milkweed genotypes. This result is puzzling because we expected that the genetic variation in emission phenotype would scale up to genetic variation in natural enemy attraction. Discrimination by natural enemies among VOC emissions from plants of different genetic backgrounds has been shown in other systems (Krips et al. 2001, Fritzsche Hoballah et al. 2002, Kappers et al. 2010), but in laboratory olfactometers rather than in the field. In the milkweed system, the effect of herbivore damage on VOC emission may overwhelm more subtle differences among plant genotypes.

The consistency across genotypes in terms of relative change induced by herbivory may contribute to the resolution of the “reliability-detectability problem” (Vet et al. 1991). Natural enemies contend with the reliability-detectability problem in locating their herbivore prey: although signals from herbivores are reliable, selection on herbivores minimizes their detectability; conversely, constitutive signals from plants are highly detectable but are less reliable indicators of the presence of prey (Vet et al. 1991). One solution to this problem that benefits both the plant and the natural enemy is the induction of plant VOCs by herbivores. However, variability among genotypes in their VOC induction responses may compromise their reliability in signaling the presence of herbivores (Gouinguené et al. 2001, Degen et al. 2004). The same herbivore-induced signal across genotypes would appear to be a reliable indicator of herbivore attack, and natural enemies may select against the emission of novel signals by specific genotypes. The result would be a lack of genotype by damage interactions on VOC concentration, blend, or attraction of enemies, which is precisely what we observed with our milkweed experiments, where decoy caterpillars were equally likely to incur damage on any of the experimentally induced milkweed genotypes.

Conclusion

For VOC emission to evolve as an adaptive plant defense against herbivores, natural selection must act upon intraspecific genetic variation for the trait in natural populations. Such intraspecific variation potentially can be observed in the following aspects of an emission phenotype: (1) total VOC concentration, (2) VOC blend, (3) magnitude of induction, and (4) natural enemy attraction. Our results indicate that constitutive and induced VOC concentrations and blends differ among plant genotypes. However, the relative change in emissions induced by herbivores was the same for all plants, and decoy caterpillars deployed on different milkweed genotypes were attacked with equal frequency. That natural enemies did not exhibit differential attraction to different milkweed genotypes was unexpected and requires further investigation.

In summary, milkweed genotypes diverge phenotypically in their VOC concentrations and blends, but appear to converge into similar phenotypes with regard to magnitude of induction and natural enemy attraction. We illustrate that aboveground VOC emission from milkweed induced by monarchs potentially serves as a plant defense, although the evolutionary and fitness consequences of natural enemy attraction have yet to be addressed.

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Table 2.1 Concentration of milkweed volatile organic chemical (VOC) emissions. Mixed model for the effects of herbivore treatment (undamaged or damaged by monarch caterpillars), plant genotype, direct defenses (including total cardenolide concentration, latex production, trichome density, foliar water content, and leaf toughness), environmental variables (temperature, photosynthetically active radiation, and relative humidity), and Julian date on concentration of VOCs emitted by milkweed. Significant *P*-values are bold.

	2008 Field plants	2009 Greenhouse	2010 Greenhouse
Treatment	**0.037**	**<0.01**	**0.021**
Genotype	**0.039**	0.274	**<0.001**
Treatment x Genotype	0.836	0.667	0.365
Direct Defenses	PC1=0.082	PC1=0.128, PC2=0.067	PC1=0.471, PC2=0.100
Environmental Variables	PC1=**0.014**	PC1=0.656, PC2=**0.027**	PC1=0.403, PC2**<0.01**
Julian date	**<0.0001**	0.524	0.106

Table 2.2 Blend of milkweed volatile organic chemical (VOC) emissions. PerMANOVA (permutational multivariate analysis of variance) for the effects of herbivore treatment (undamaged or damaged by monarch caterpillars), plant genotype, direct defenses (including total cardenolide concentration, latex production, trichome density, foliar water content, and leaf toughness), environmental variables (temperature, photosynthetically active radiation, and relative humidity), and Julian date on the multivariate blend of VOCs emitted by milkweed. The analysis in 2008 includes 9 VOCs, 13 VOCs in 2009, and 26 VOCs in 2010. Significant *P*-values are bold.

	2008 Field plants	2009 Greenhouse	2010 Greenhouse
Treatment	**<0.001**	**<0.0001**	*0*.054
Genotype	**0.023**	**<0.01**	**<0.0001**
Treatment x Genotype	0.106	0.739	0.628
Direct Defenses	PC1=0.180	PC1=0.216, PC2=0.112	PC1**<0.0001**, PC2=**0.031**
Environmental Variables	PC1=0.707	PC1**<0.001**, PC2=0.131	PC1**<0.0001**, PC2**<0.0001**
Julian date	**<0.0001**	0.149	**<0.0001**

Figure 2.1 Induced volatile organic chemical (VOC) emissions from milkweed plants attract higher natural enemy attack rates on monarch caterpillar prey in the field. (A) Example of a “decoy” monarch caterpillar made from non-drying modeling clay and damaged by a natural enemy. Dashed white circles indicate gouges in the decoy caused by a natural enemy. (B) Mean proportion (+1 SE) of damaged decoy monarch caterpillars on undamaged (open bars) and monarch caterpillar-damaged (solid bars) potted milkweed plants placed in the field.

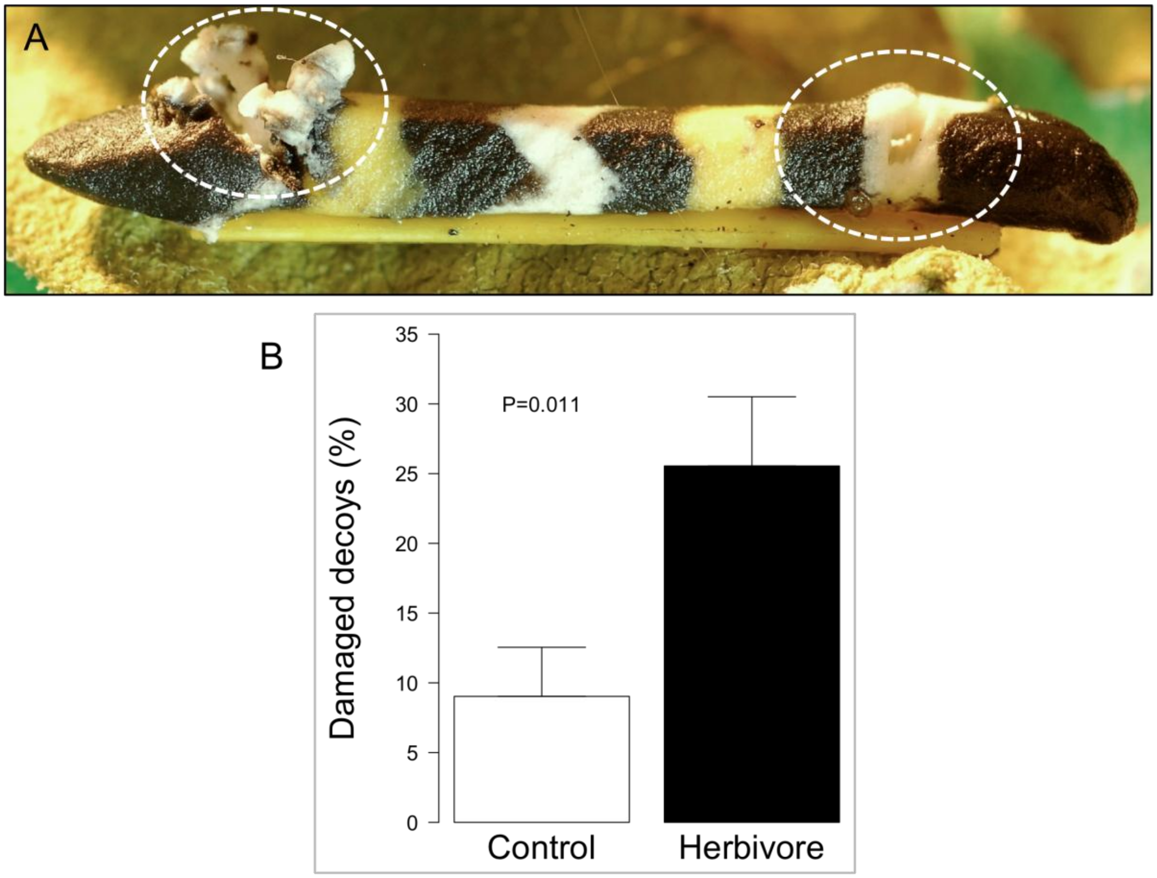


Figure 2.2 Monarch herbivory induces higher concentrations of volatile organic chemicals (VOCs) emitted by milkweed. Mean total VOC concentration (+1 SE) emitted by undamaged (open bars) and monarch caterpillar-damaged (solid bars) milkweed plants. (A) Naturally growing milkweed plants in the field in 2008. (B) Milkweed plants grown in a greenhouse common garden in 2009 (C) and in 2010.

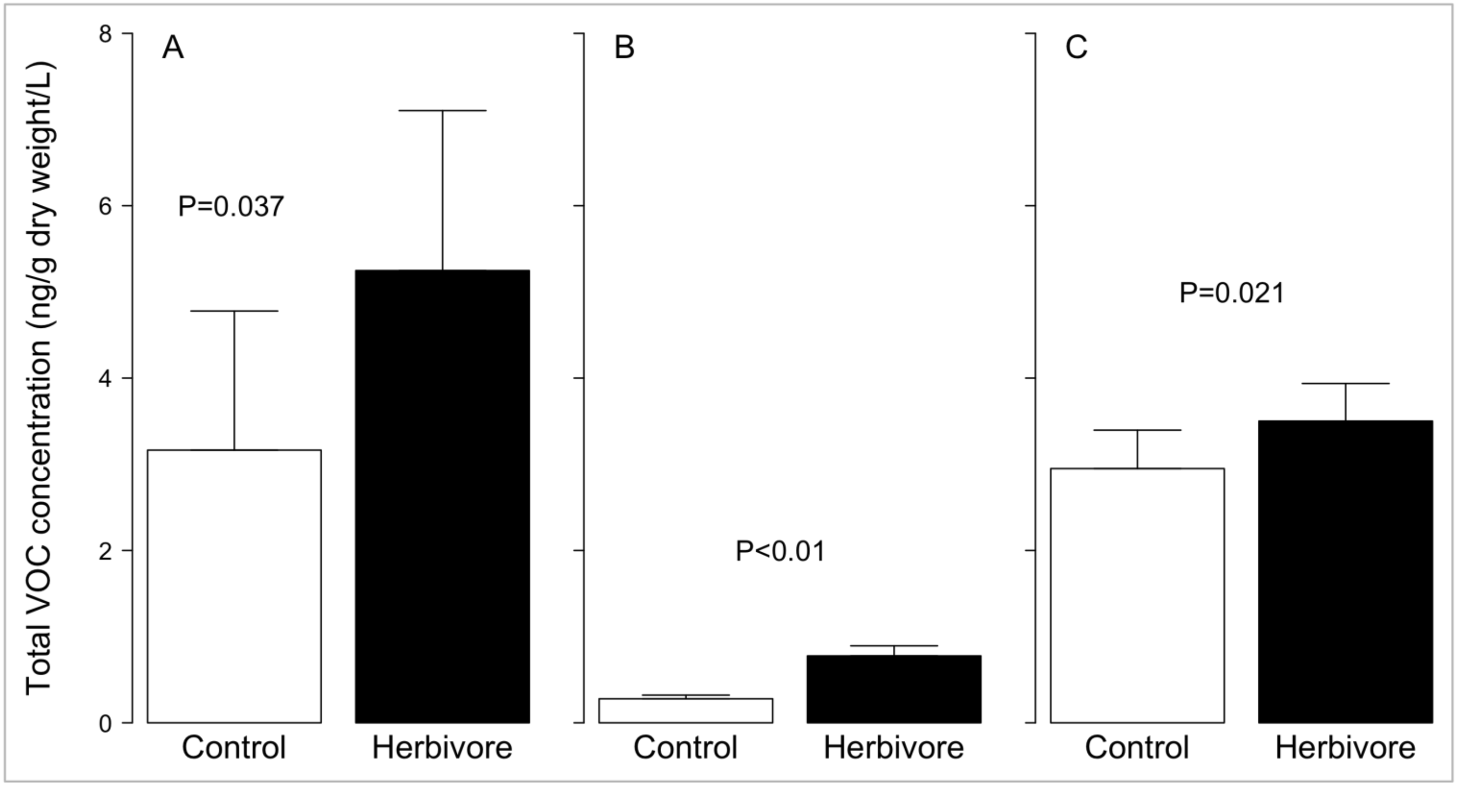


Figure 2.3 Milkweed genotypes vary in their constitutive and monarch-induced volatile organic chemical (VOC) emissions. Mean total VOC concentration (+1 SE) emitted by undamaged (open bars) and monarch caterpillar-damaged (solid bars) milkweed genotypes (labeled as letters on the x-axis). Different letters above bars indicate significant differences in total VOC concentration among milkweed genotypes within an experiment. (A) Naturally growing milkweed plants in the field in 2008. (B) Milkweed plants grown in a greenhouse common garden in 2009 (C) and in 2010.

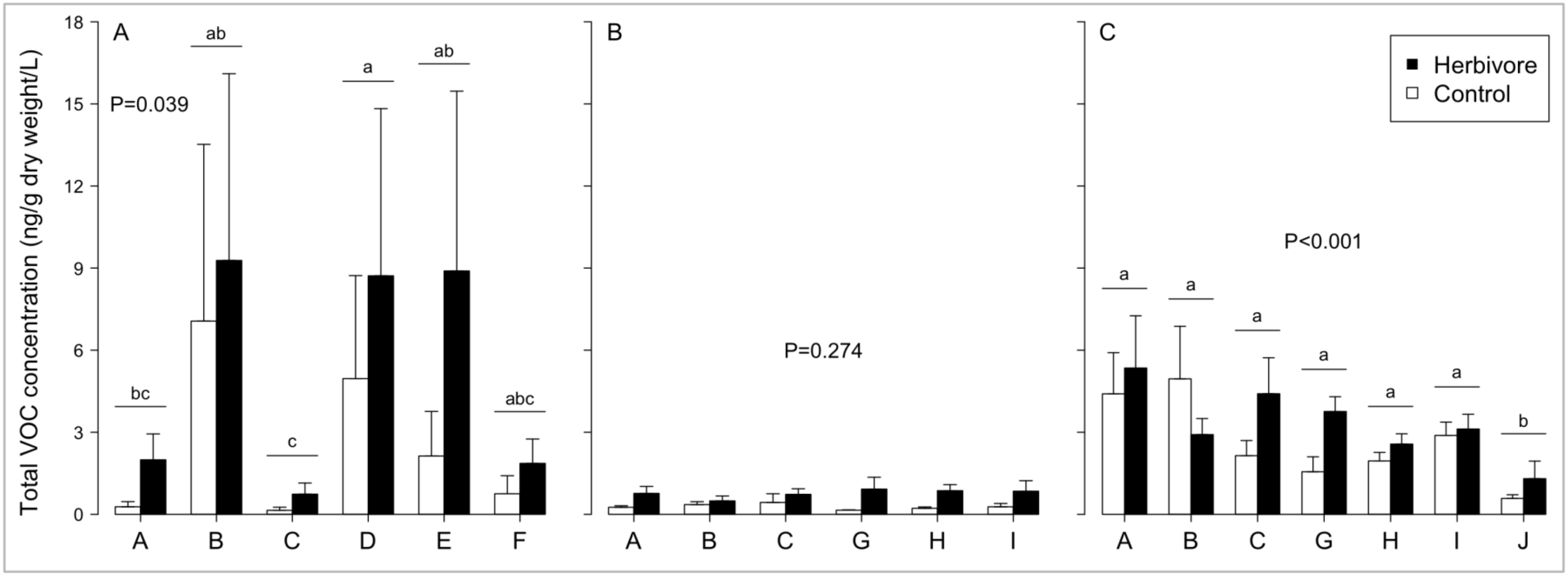


Figure 2.4 Milkweed genotypes vary in the volatile organic chemical (VOC) blends they emit, and herbivore induction alters the emitted blend. The principal components analyses depicted here indicate the multivariate composition of VOC blends emitted by undamaged (open circles) and monarch caterpillar-damaged (solid circles) milkweed plants (A) growing naturally in the field in 2008; (C) grown in a greenhouse common garden in 2009; (E) grown in a greenhouse common garden in 2010. Principal components analyses indicate the VOC blends emitted by milkweed genotypes (represented by different letters) sampled in (B) 2008; (D) 2009; and (F) 2010. The analysis in 2008 includes 9 VOCs, 13 VOCs in 2009, and 26 VOCs in 2010. Note that the scale of the axes differs among plots.

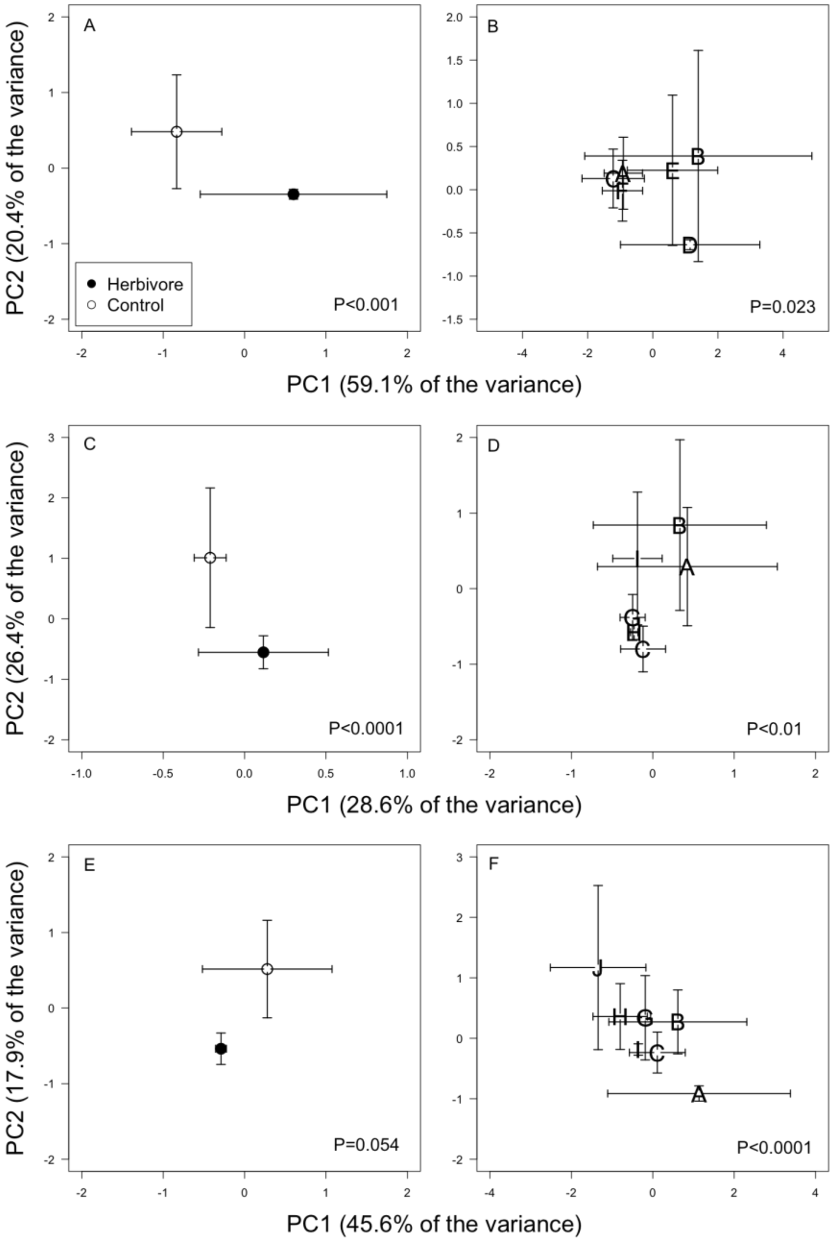
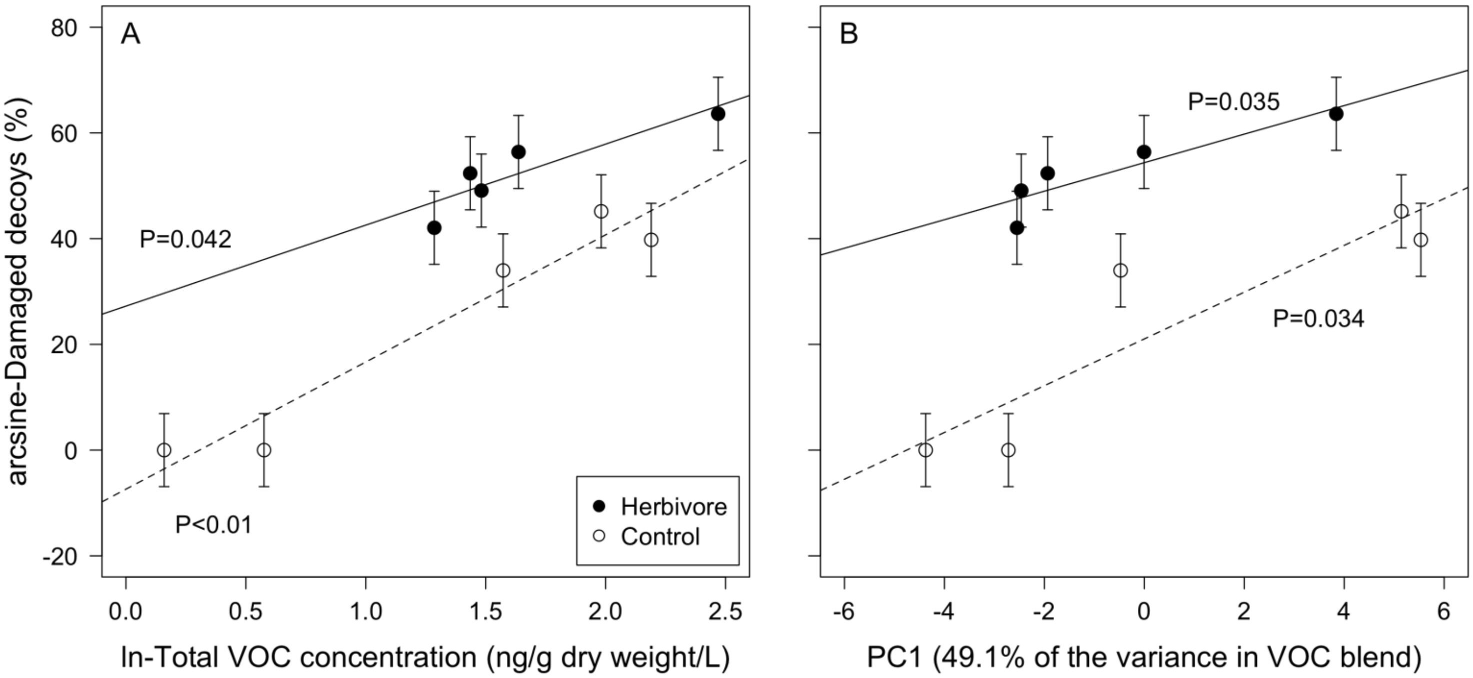


Figure 2.5 Natural enemies of monarchs are attracted to higher concentrations and a specific blend of volatile organic chemicals (VOCs) emitted by milkweed. Mean arcsine-square-root transformed proportion (±1 SE) of damaged decoy monarch caterpillars on undamaged (open circles) and monarch caterpillar-damaged (solid circles) milkweed plants placed in the field as a function of (A) mean ln-transformed total VOC concentration or (B) the first axis of a PCA representing the multivariate blend of 26 VOCs emitted by milkweed plants in the 2010 greenhouse common garden. Proportion of damaged decoys, mean VOC concentration, and PC1 score are pooled by plant genotype.



Chapter 3

A genetically-based latitudinal cline in the emission of herbivore-induced plant volatile organic compounds

Abstract

The existence of predictable latitudinal variation in plant defense against herbivores remains controversial. A prevailing view holds that higher levels of plant defense evolve at low latitudes than at high latitudes as an adaptive plant response to higher herbivore pressure on low-latitude plants. To date, this view has not been examined with respect to volatile organic compounds (VOCs) that many plants emit, often to attract the natural enemies of herbivores. Here, we compare genetically-based constitutive and herbivore-induced aboveground vegetative VOC emissions from plants originating across a gradient of more than 10^{o} of latitude (>1,500 km). We collected headspace VOCs from *Asclepias syriaca* (common milkweed) originating from 20 populations across its natural range and grown in a common garden near the range center. Feeding by specialist *Danaus plexippus* (monarch) larvae induced VOCs, and field environmental conditions (temperature, light, and humidity) also impacted emissions predictably. Monarch damage increased plant VOC concentrations and altered VOC blends. We found that genetically-based induced VOCs varied with the latitude of plant population origin, although the pattern followed the reverse of that predicted—induced VOC concentration increased with increasing latitude. In contrast, constitutive VOC

emission did not vary systematically with latitude. Our results do not support the prevailing view that plant defense is greater at lower than at higher latitudes. That the pattern holds only for herbivore-induced VOC emission, and not constitutive emission, suggests that latitudinal variation in VOCs is not a simple adaptive response to climatic factors.

Introduction

Geographic patterns in species interactions are of broad interest when considering the adaptability of species to environmental pressures such as climate change and the introduction of non-native species. Until recently, a common assumption has been that, at large scales, climate exerts the major constraint on species distributions, overwhelming the relatively minor influence of biotic interactions (Araújo and Luoto 2007). However, recent work has revealed the importance of geographic variation in trophic interactions when predicting species responses to environmental perturbations (Davis et al. 1998, Voigt et al. 2003, Menéndez et al. 2008). Ecological processes important at the local scale can retain their importance at the macroecological scale, and it is necessary to consider multi-trophic interactions in tandem with abiotic constraints on individual species (Araújo and Luoto 2007).

Herbivory is an important interaction between plants and animals that can vary at large geographic scales (Coley and Barone 1996, Zangerl and Berenbaum 2003, Thompson 2005). For example, latitudinal variation in plant defense traits may influence future interactions between plants and herbivores under climate change (Engelkes et al. 2008). Plant and animal species from a variety of habitats tend to have lower palatability and higher levels of defensive traits at low latitudes (Coley and Barone 1996, Pennings et al. 2001, Schemske et al. 2009, Rasmann and Agrawal 2011). One prominent hypothesis asserts that higher levels of herbivory at low latitudes explain these observed patterns in plant palatability and defense. The hypothesis posits that greater herbivory selects for greater plant defense at low latitudes, producing a cline of increasingly strong plant defense with decreasing latitude (Pennings et al. 2001, Pennings et al. 2009). However, the prevalence and basis of latitudinal patterns in plant defense remain controversial (Moles et al. 2011a, b).

Although many studies have investigated latitudinal gradients in plant defense traits that directly deter herbivore feeding, little work has been conducted on geographic patterns of indirect plant defenses. Indirect defenses exploit higher trophic levels—the predators and parasites of herbivores—that reduce herbivory (Dicke 1994). The few studies that have evaluated indirect plant defense at the geographic scale corroborate the general patterns observed for direct defenses across latitude. Ants facilitate reductions in herbivory to a greater degree at low latitudes (Chamberlain and Holland 2009), and ant domatia (Chamberlain and Holland 2009) and extrafloral nectaries (Pemberton 1998, Chamberlain and Holland 2009, Moles et al. 2011a) occur more frequently on plants at low latitudes. These patterns contrast with leaf domatia associated with mites, which appear to occur more frequently in temperate than tropical systems (O'Dowd and Willson 1991).

Plant emission of volatile organic compounds (VOCs) can serve as an indirect plant defense by attracting the predators and parasites of herbivores. The phenomenon of enemy attraction via herbivore-induced VOC emission has been observed in multiple tritrophic systems (Dicke et al. 1998, De Moraes et al. 1998, Thaler 1999) and appears to be common in nature (Kessler and Baldwin 2001, Farmer 2001). Plant VOC emission has been shown to vary on regional scales (Geron et al. 2000, Halitschke et al. 2000, Staudt et al. 2004), although very few studies have investigated patterns of VOC emission across latitude (Martz et al. 2009), and no previous study has employed experimental approaches to address latitudinal variation in herbivore-induced VOC emission.

Our aim was to address the following questions: (1) Do the concentration and blend of plant VOC emissions vary among milkweed populations? (2) Do VOC emissions vary predictably in concentration and blend with respect to latitude? (3) Does the magnitude of induction vary predictably with latitude? Here, we used *Asclepias syriaca* (common milkweed) and the specialist herbivore *Danaus plexippus* (monarch caterpillar) to test the hypothesis that milkweeds show a gradient in genetically-based constitutive and herbivore-induced VOC emission, such that plants originating from low latitudes emit higher VOC concentrations than do plants originating from high latitudes.

Methods

Asclepias syriaca is a widespread native plant in eastern North America, ranging north to Canada and south to Louisiana. Monarch butterflies (*D. plexippus*) are specialist herbivores that feed as larvae on plants in the genus *Asclepias*. Larvae are attacked by a variety of parasitoids (e.g., tachinid flies and braconid wasps) and predators (ants, spiders, paper wasps, and hemipterans) (Prysby 2004, Mooney and Agrawal 2008). Some evidence exists to suggest that VOCs serve a defensive function in the milkweed-monarch system. For example, ovipositing butterflies can distinguish between the VOCs of old and young milkweed plants (Bergström et al. 1995), and entomopathogenic nematodes are attracted to the herbivore-induced VOCs emitted by milkweed roots (Rasmann et al. 2011). Results described in Chapter 2 indicate that feeding damage by monarchs attracts predators to sites of milkweed herbivory and causes increased damage to caterpillar mimics.

Common garden

The milkweed plants used in this experiment were grown in a common garden near the center of their natural range in Ithaca, New York, as described in Woods et al. (2012). Briefly, the common garden contained individuals grown from seeds collected from 22 natural plant populations, spanning 10^{o} of latitude (>1,500 km) from the southernmost population in Knoxville, Tennessee to the northernmost population in Quebec City, Quebec, Canada. From each of 5 milkweed pods collected from the 22 populations, we established a common garden in a plowed field in early June 2008. Each seedling was placed in a 4-liter plastic pot filled with field soil and sunk into the ground about 1 m from surrounding plants in a completely randomized design. In 2009, VOCs were collected from plants originating from 20 of the 22 populations (Figure 3.1).

VOC collections

Experimental monarchs were obtained from a commercial monarch breeder (Flutterby Gardens, Bradenton, FL, USA) and raised indoors (about 100 meters from the experimental plants) on *A. syriaca*. Experiments were then conducted between July 22 and July 29, 2009. One third-instar caterpillar was placed on one of the leaves in the fifth leaf pair of each treatment plant. Damaging leaves of the same developmental stage controlled as much as possible for any effect of plant source/sink dynamics on VOC emission (Halitschke et al. 2000). Netting was placed over each treatment leaf to restrict caterpillar movement, and caterpillars were allowed to feed from late afternoon to the following morning (about 18 hours). Netting also was placed over one of the leaves in the fifth leaf pair on each control (undamaged) plant to control for any effect of netting on VOC production.

We collected VOC emissions from control and herbivore-damaged plants originating from multiple sites each day, such that “collection day” was not confounded with latitude. Our sampling system facilitated VOC collection from up to 23 chambers per day. Chambers constructed from PVC pipes and inert Tedlar sheeting were placed around entire plants (one plant per chamber) growing in the common garden. Plastic sheets covered the soil around plant stems to minimize collection of soil volatiles. Air was drawn into each chamber through a sodium thiosulfate filter and an activated carbon filter. Sodium thiosulfate absorbs ozone gas, which can react with VOCs in the atmosphere (Helmig 1997), while activated carbon collects ambient VOCs. Thus, “clean” air flowed into each chamber; however, we sampled VOCs from eight empty chambers to evaluate any remaining VOCs in this “clean” air.

Dual-diaphragm vacuum pumps (Gardner Denver Thomas, Sheboygan, Wisconsin, USA) pulled air from each chamber through a charcoal adsorbent (Sigma-Aldrich, St. Louis, MO, USA), which trapped plant VOCs that accumulated in each chamber. Rotameters connected to each chamber enabled us to adjust and quantify the velocity of air flowing through the chambers (average = 1.5 L per minute). Whole-plant

VOCs were sampled continuously for 5 h during approximately the same daylight hours on each sampling day (generally between 14:00 and 19:00) following the previous night of herbivore damage. We took care to sample vegetative VOCs by avoiding flowering plants. By the end of the experiment, we had collected VOC emissions from 81 plants (control N=36 plants, herbivore-damaged N=45 plants) originating from 20 different milkweed populations.

We controlled for the effect of plant biomass on VOC emission. We measured stem height and number of leaves from each plant in the field and applied a regression equation (Eqn. 1; R^2=0.6019) to estimate the aboveground biomass of each experimental plant. The equation was derived using 100 ramets from a natural population of *Asclepias syriaca* plants at the University of Michigan Biological Station in Pellston, Michigan.

Eqn. 1: $$\textit{biomass} = (0.092 \bullet \textit{leaves}) + (0.101 \bullet \textit{height}) - 0.480$$

Environmental variables

We collected data on environmental variables that may influence VOC emission under field conditions (Kesselmeier and Staudt 1999). Data loggers (Onset Computer Corporation, Bourne, MA, USA) were deployed on each sampling day to measure temperature and relative humidity, while photosynthetically active radiation (PAR) sensors (LI-COR, Lincoln, NE, USA) gauged ambient light levels. Two sensors measuring each of the three environmental variables every minute were placed near the collection chambers every day. The data from each sensor were integrated across minutes for the 5 sampling hours, yielding a cumulative value for each of the variables per sampling day. The data from one humidity sensor was omitted due to malfunction; the remaining data were used to standardize environmental variation across days. To account for covariance among these environmental factors, we combined the factors using a principal components analysis (PCA). The first PCA axis explained nearly 85% of the variation in the data from the sensors and this axis was used as a covariate in all subsequent analyses of VOC concentrations.

Analysis and identification of VOCs

VOC samples were eluted from charcoal traps with 750 µl of dichloromethane (CH_2Cl_2) containing 1.94 µg of nonyl acetate (Sigma-Aldrich, St. Louis, MO, USA; CAS # 143-13-5) as an internal standard. Moisture in the samples was removed using anhydrous sodium sulfate. We analyzed 1-µl aliquots of VOC samples using a gas chromatograph-mass spectrometer (GCMS; Agilent Technologies, Santa Clara, CA, USA) on a 30 m x 0.25 mm inner diameter fused-silica column with a polyethylene glycol stationary phase and 0.25 μm film thickness (Restek Corporation, Bellefonte, PA, USA). We used the following GC method: injector held at 250 ^oC, initial column temperature at 50 ^oC held for 10 min, ramped at 5 ^oC min^{-1} to 200 ^oC, held for 10 min. Helium carrier gas flow was set to 1.3 ml min^{-1}.

Compounds were tentatively identified with reference to the NIST (National Institute of Standards and Technology) library database, and the identity of key peaks subsequently was verified using authentic standards (Sigma-Aldrich, St. Louis, MO, USA; MP Biomedicals, Solon, OH, USA). VOC concentrations were quantified by calculating the relationships among internal standard concentration, internal standard peak area, and focal compound peak area. Synthetic chemicals and any VOCs collected from empty chambers were omitted from the dataset. Compounds emitted by only one plant and compounds that comprised less than 1% of the total concentration of VOCs across all plants also were omitted, leaving 16 compounds (comprising about 80% of total emissions) in the dataset from the 81 independent plant samples. Peak concentrations were standardized for the total volume of air sampled from each chamber and for aboveground plant biomass.

Statistical analyses

We tested (a) whether total constitutive and induced VOC emissions varied among milkweed populations, (b) whether such variation was predictable with respect to latitude, and (c) whether the magnitude of induction varied predictably with latitude. To address the first question, we employed mixed model analysis of variance (SAS Version 9.2) to investigate total VOC emissions, specifying herbivore treatment, site of origin, and PCA axis 1 (environmental variables) as independent variables, with chamber as a random factor. We also included genotype nested within population origin as a random factor to account for local genetic variation in VOC emission, which has been observed in milkweed (see Chapter 2) and other plant systems (e.g., Delphia et al. 2009, Schuman et al. 2009). Second, we used weighted linear regression in the statistical package R (Version 2.11.1) to explore patterns in total VOC emission across latitude. Control and herbivore-damaged plants were regressed separately against latitude. Total VOC concentration was averaged for all plants within a site; therefore, site averages used in the analysis were weighted by 1/variance. Weighting required that we omit sites containing single samples, which yielded N=14 sites for control and N=17 sites for herbivore-damaged treatments. We verified the appropriateness of the linear model by evaluating the data using a post-hoc weighted quadratic model, and assessed the relative fit of both models by calculating Akaike Information Criterion (AIC) scores and implementing a likelihood ratio test. To address the third question, we used a general linear model to test whether the slopes of the weighted linear regressions between VOC emission and latitude differed between the control and herbivore-damaged treatments; a significantly steeper positive slope in the damaged treatment would demonstrate an increase in the magnitude of VOC induction with latitude.

The effects of herbivory, plant origin, and latitude on the multivariate VOC blend were evaluated with perMANOVA (permutational MANOVA; Oksanen et al. 2010). First, to investigate whether VOC blends varied among plant populations, we specified herbivore treatment, population origin, PCA axis 1 (environmental variables), and chamber as independent variables. Second, to determine whether VOC blends varied systematically across latitude, the concentration of each individual compound within the blend was summed for all plants within a treatment within a site. The sums for the 16 peaks, with N=17 population origins for control and N=19 population origins for herbivore-damaged treatments, were used in the perMANOVA. Herbivore treatment and latitude were specified as independent variables in the analysis.

Results

Monarch caterpillar damage induced a 62% increase in VOC emission, on average, from common milkweed plants (Figure 3.2; $F_{1,\,55.9}$=4.05, P=0.049). The plant population from which seeds were collected accounted for over 41% of the variation in total VOC emission among plants in the common garden ($F_{19, 46.8}$=3.02, P=0.001), indicating a likely genetic basis for variation in VOC emission among milkweed populations. Environmental variables (PCA axis 1) also influenced VOC emission among plants ($F_{1, 45.1}$=12.12, P=0.001). Based on PCA axis loadings, VOC emission increased with increasing temperature (Figure 3.3; $F_{1, 79}$=4.841, P=0.03) and PAR and declined with increasing humidity (data not shown).

Total VOC emission from caterpillar-damaged milkweeds increased with increasing latitude, with latitude explaining over 54% of the variation in induced VOC emission across the gradient (Figure 3.4A; $F_{1, 15}$=19.68, P<0.001). Visually, it appears as if a quadratic model could better explain the trend in Figure 3.4A, but a quadratic model ($F_{2, 14}$=9.26, P<0.01) was no improvement over the linear model (AIC, linear=56.3, quadratic=58.2; log likelihood, P=0.79) in this weighted regression. Constitutive VOC emission did not vary predictably with latitude (linearly or quadratically, Figure 3.4B; linear $F_{1, 12}$=1.69, P=0.22; quadratic $F_{2, 11}$=0.932, P=0.42). The slope of the clines across latitude differed between constitutive and herbivore-induced emissions (damage by latitude interaction $F_{3, 27}$=17.56, P<0.0001), indicating that the magnitude of induction varied predictably with latitude, but in the opposite direction to our prediction.

Herbivory by monarch caterpillars also altered milkweed VOC blend ($F_{1, 80}$=2.76, P=0.002). Although it exhibited no systematic change with latitude ($F_{1, 35}$=1.34, P=0.18), VOC blend differed among milkweed populations (Figure 3.5; $F_{19, 80}$=1.28, P=0.02). Temperature, humidity, and PAR (PCA axis 1) marginally influenced VOC blend among populations ($F_{1, 80}$=1.80, P=0.05). Four of the 16 VOCs emitted by milkweed plants were positively identified using authentic standards. Three of these compounds have been shown to increase in concentration following herbivore damage or have been implicated in plant defense: 1,8-cineole (Wiens et al. 1991, Kessler et al. 2006), cis-3-hexen-1-ol (Ruther and Kleier 2005, Kessler et al. 2006), and β-caryophyllene (Wiens et al. 1991, Rasmann et al. 2005).

Discussion

To our knowledge, this experiment is the first to investigate intraspecific variation in induced indirect defenses across a latitudinal gradient. Based on previous studies of direct (Coley and Barone 1996, Pennings et al. 2001) or constitutive indirect defenses (Chamberlain and Holland 2009), we had predicted that the concentration of herbivore-induced VOCs would decline with increasing latitude. Surprisingly, we found that they increased with latitude, while constitutive VOCs emitted by undamaged plants showed no geographic pattern (Figure 3.4). Because constitutive and induced VOCs behaved differently, it appears that variation in abiotic factors such as light, temperature, or photoperiod cannot alone drive variation in plant VOC emissions with latitude. Overall, monarch herbivory induced higher concentrations (Figure 3.2) and a different blend ratio of VOCs emitted by milkweed plants. The composition of the VOC blend also varied among milkweed populations (Figure 3.5), although we observed no systematic change in VOC blend with latitude.

The ecological implications of variation in VOC concentration and blend remain poorly understood. For example, higher concentrations of VOCs have been shown to increase recruitment of natural enemies (Gols et al. 2003, Turlings et al. 2004), while other work has indicated that natural enemies can respond more strongly to VOC blend (Bruce et al. 2010). Disentangling the influence of VOC concentration from that of blend on natural enemy attraction remains challenging. Manipulating one without compromising the fidelity of the other presents a complicated problem, especially in field experiments that incorporate natural plant VOC emissions. We suggest that it is reasonable and informative to employ VOC concentration as a metric for the strength of VOC emission as an indirect defense.

In light of substantial evidence linking VOC emission with predator and parasitoid attraction in other plant species, along with increasing evidence in the milkweed system (Rasmann et al. 2011), our results contradict the current view that plants have evolved stronger defenses at low latitudes in response to greater herbivore pressure at low latitudes. Milkweed defenses in general may be part of a growing group of cases that challenge the conventional view (Moles et al. 2011a, b). In related work with milkweeds, Woods et al. (2012) measured direct defense traits of plants from the same common garden that we utilized for the experiment described here. Similarly, they found that milkweed latex production increased with latitude, and that greater latex production corresponded with greater resistance to monarch larvae. In contrast, cardenolides and foliar trichomes did not vary systematically with latitude. Outside of the common garden, Hunter et al. (1996) reported an increase in foliar cardenolides with latitude in natural milkweed populations.

A spatial survey of natural herbivore pressure may help to explain why milkweed defenses do not follow the predicted trend. Woods et al. (2012) surveyed herbivores and herbivory in milkweed common gardens replicated across latitude and found that the highest levels of herbivore abundance and damage occurred at the center of the *Asclepias syriaca* range. Sites at the northern and southern latitudinal extremes showed reduced herbivore diversity and leaf damage relative to the range center. While these observations may be consistent with a quadratic model for induced VOCs across latitude, Woods et al. (2012) suggest that the observed pattern of natural herbivory reflected differences in plant size rather than variation in defense traits. Furthermore, by employing replicated assays of natural aphid colonization and a bioassay of monarch performance in the common garden, they found that higher-latitude plants were more resistant to herbivory. Consistent with these findings, our results provide stronger support (lower AIC score) for a linear than quadratic increase in induced VOC concentration with latitude. Although the spatial surveys conducted by Woods et al. (2012) may explain why milkweed defenses do not decrease systematically with latitude, they fail to explain why certain milkweed defenses, including latex and induced VOCs, should increase with latitude.

The unexpected latitudinal pattern that we found for milkweed VOC emission may be driven by a variety of potential mechanisms, and we discuss four possibilities here. First, total VOC emission increases with increasing temperature (Figure 3.3), and average annual temperature decreases with increasing latitude. High-latitude plants might compensate for low VOC emission at low temperatures by emitting higher concentrations of VOCs per unit damaged plant material relative to low-latitude plants. Second, VOC emission may be associated with latex production in milkweed. Latex in most plant species is derived from terpenoids (Agrawal and Konno 2009), and increased VOC emission at high latitudes may relate to higher latex production by high-latitude milkweed plants (Woods et al. 2012). Woods et al. (2012) argue that the observed gradient in herbivore resistance driven by latex suggests that protection against generalist herbivores is important at high latitudes. If generalist predators and parasites likewise are important at high latitudes, increased total VOC concentrations may attract generalists more effectively than subtle changes in VOC blend, to which specialists may be more attuned. Third, high VOC concentrations at high latitudes may reflect a cline in the prevalence of the enemies of herbivores. Plants may invest more in herbivore-induced VOCs at high latitudes, where stronger signals are required to attract natural enemies over longer distances due to a lower abundance of natural enemies. Lower VOC concentrations (weaker signals) may be effective at low latitudes, where the abundance of natural enemies likely is greater. Although a rigorous assessment of latitudinal variation in monarch parasitism has yet to be conducted, parasite diversity, parasite attack rates, and predator pressure generally appear to be higher at low latitudes for monarchs (Prysby 2004) and in other systems (Dyer and Coley 2004). Fourth, although unlikely, preferential feeding by experimental monarch larvae on high-latitude plants could have produced the pattern in VOC emission. Concentrations of herbivore-induced VOC emission have been shown to correlate positively with the amount of herbivore damage on plants (Rodriguez-Saona et al. 2009). Although we were unable to quantify monarch damage on our experimental milkweeds, previous results in the same common garden have shown that higher-latitude plants are more resistant to monarch damage than lower-latitude plants (Woods et al. 2012), suggesting that greater amounts of herbivore damage at higher latitudes cannot explain greater VOC emission at higher latitudes.

While constitutive indirect plant defenses associated with ant mutualists may be stronger at low latitudes in some other systems (Pemberton 1998, Chamberlain and

Holland 2009, Moles et al. 2011a), a growing body of evidence contradicts the prevailing view that plants are better defended at low latitudes. For example, total constitutive terpenoid concentration (mono- and sesquiterpenoids extracted from needles) in juniper (*Juniperus communis*) increases with increasing latitude in Finland (Martz et al. 2009). Mite domatia, another mode of indirect plant defense, tend to occur more often in temperate than tropical plant species (O'Dowd and Willson 1991). A recent study used standardized methods of data collection with 301 plant species at 75 sites around the world to test the hypothesis that defense expression declines with increasing latitude (Moles et al. 2011b). The data provided little support for the hypothesis; in fact, the data indicated that several plant resistance traits actually increase with increasing latitude. Similarly, a meta-analysis revealed that chemical defenses are higher at high latitudes (Moles et al. 2011a). The herbivore-induced emission of VOCs from milkweeds reported here appears to support this alternative view.

We report an intraspecific pattern for VOC emission revealed in a common garden experiment. This study compares a defense trait in a single plant species distributed across a broad geographic range; however, most latitudinal studies, including some of those cited here for indirect defense (O'Dowd and Willson 1991, Pemberton 1998, Chamberlain and Holland 2009), compare defense traits across different plant species. Unlike intraspecific studies, interspecific comparisons potentially confound plant species distributions and phylogenetic constraints with latitudinal patterns in plant defense. In addition, the common garden experiment described here was appropriate for evaluating genetically-based patterns in plant VOC emission. In contrast, non-experimental field surveys of plant defenses in natural populations cannot disentangle local environmental conditions (e.g., climatic factors and local adaptation of herbivores) from genetic effects on defense phenotype.

In addition to the observed cline with latitude, we found that plants from different populations varied in their VOC concentrations and blends. Surprisingly few other studies have quantified geographic variation in vegetative plant VOC emissions. Differences in VOC emission were found for *Quercus ilex* at two different sites in France (Staudt et al. 2004) and for *Nicotiana attenuata* from three sites in the southwestern United States (Halitschke et al. 2000). Geron et al. (2000) modeled regional variation in VOC production by extrapolating constitutive emissions from individual tree species to the regional scale. Our study is the first comprehensive empirical demonstration of geographic variation in vegetative plant VOCs.

Our results suggest that herbivore contributions to plant VOC emission may vary predictably at regional scales. Our findings may have additional relevance beyond plant-herbivore defense theory, with applications in atmospheric science and human health. Plant VOCs strongly link the biosphere, atmosphere, and climate and affect air quality (Monson and Holland 2001). Plant VOCs affect crop and forestry yields (Ashmore 2005), with important feedbacks on climate change (Laothawornkitkul et al. 2009). We observed a significant pattern of increasing concentrations of herbivore-induced VOC emission with increasing latitude, and this phenomenon warrants further exploration. Because this pattern holds only for herbivore-induced VOC emissions (not constitutive emission), we suggest that latitudinal variation in VOCs is not a simple adaptive response to climatic factors. Investigating potential drivers of the cline in VOC emission may explicate contradictory latitudinal patterns and improve regional VOC emission inventories.

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Figure 3.1 The locations of 20 *Asclepias syriaca* populations from which seeds were collected to establish an experimental common garden in Ithaca, NY, USA. The star indicates the location of the common garden.

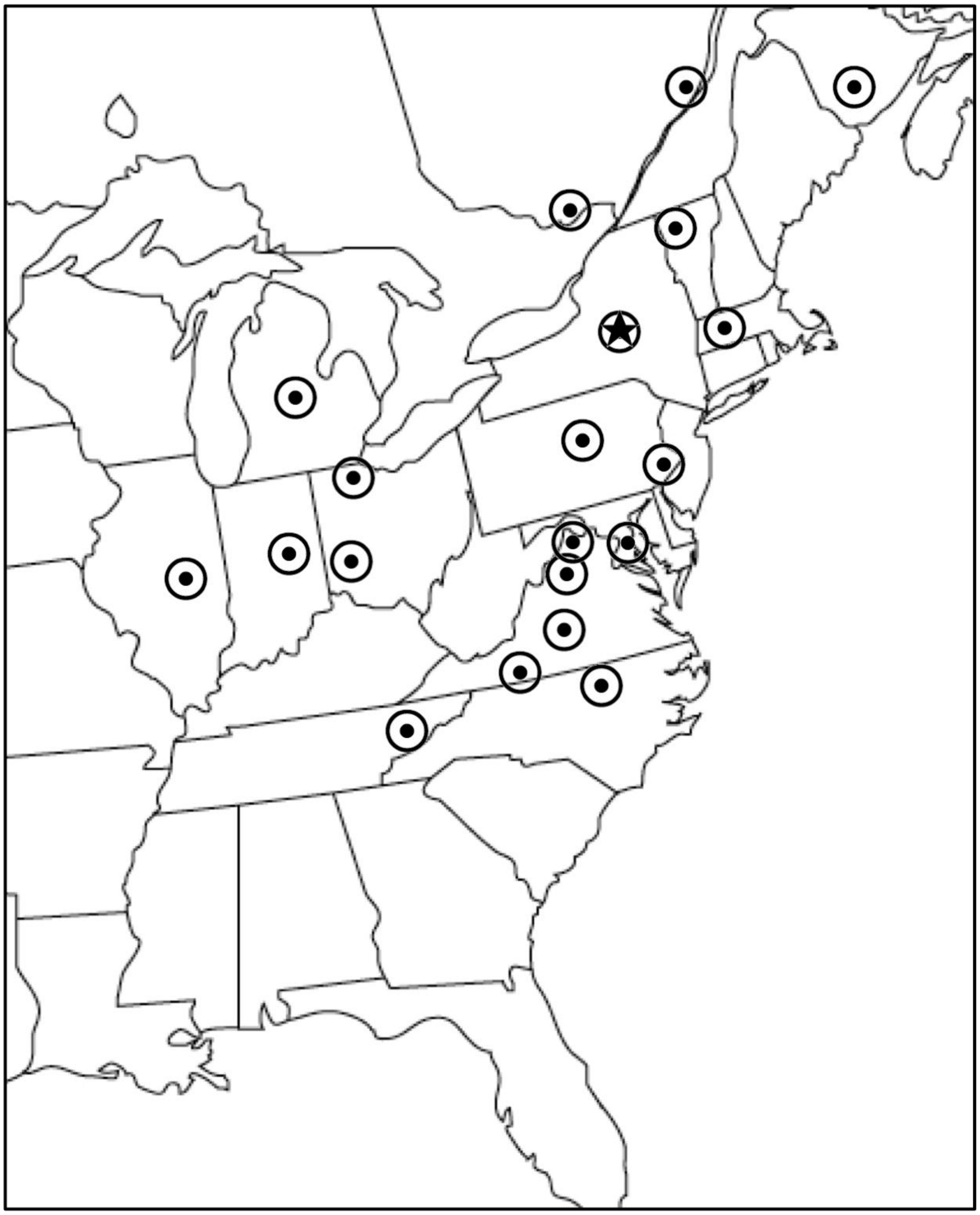


Figure 3.2 Monarch caterpillar damage induced higher concentrations of volatile organic chemicals (VOCs) emitted by milkweed plants originating across a broad latitudinal gradient. Mean VOC emission (+1 SE) from undamaged (open bars) and caterpillar-damaged (solid bars) *Asclepias syriaca* plants growing in a common garden in Ithaca, NY.

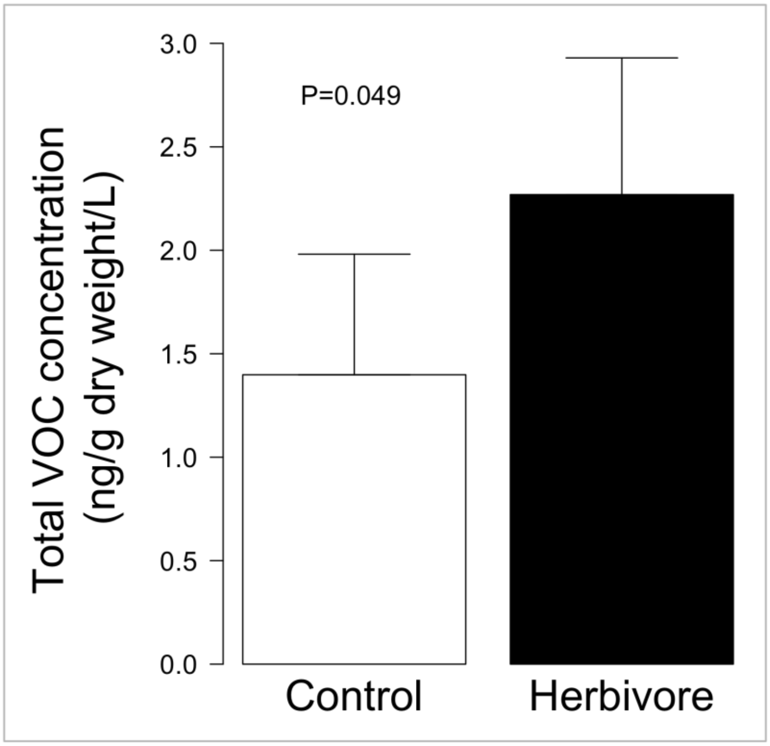


Figure 3.3 Higher temperatures correlate with higher constitutive and herbivore-induced volatile organic chemical (VOC) concentrations emitted by milkweed plants. The effect of temperature on VOC emission from all *Asclepias syriaca* plants growing in a common garden in Ithaca, NY. Temperatures were recorded once per minute during five sampling hours. The temperature data were integrated, yielding a cumulative value for each sampling day.

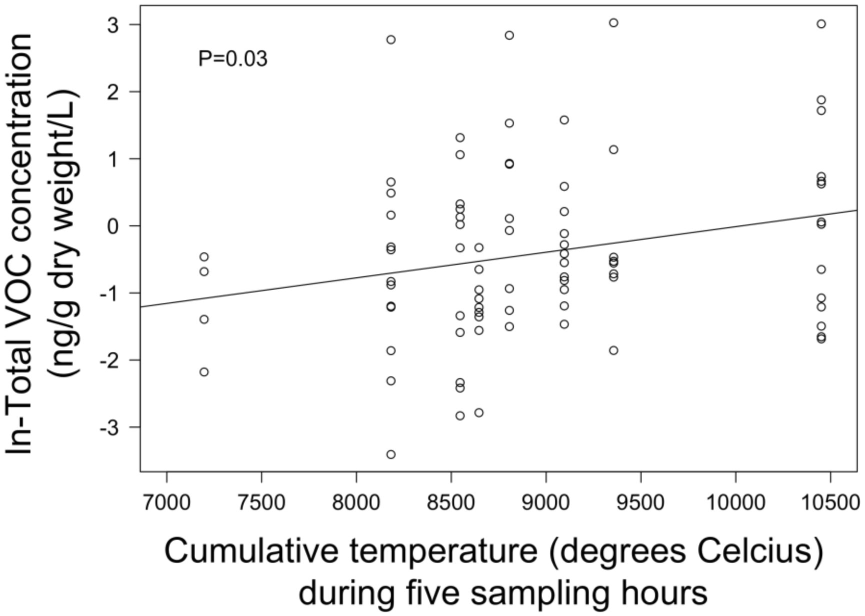


Figure 3.4 Monarch-damaged milkweed plants emit higher volatile organic chemical (VOC) concentrations as their latitude of origin increases; however, constitutive VOCs are emitted at similar concentrations by milkweed plants across latitude. Total concentration (ln-transformed) of VOC emissions (±1 SE) from *Asclepias syriaca* plants originating from 20 populations across 10^{o} of latitude. Experimental plants were grown in a common garden in Ithaca, NY. Best-fit lines were derived from weighted regressions for both treatments. (A) Emissions from caterpillar-damaged plants (N=17 sites). (B) Emissions from undamaged plants (N=14 sites).

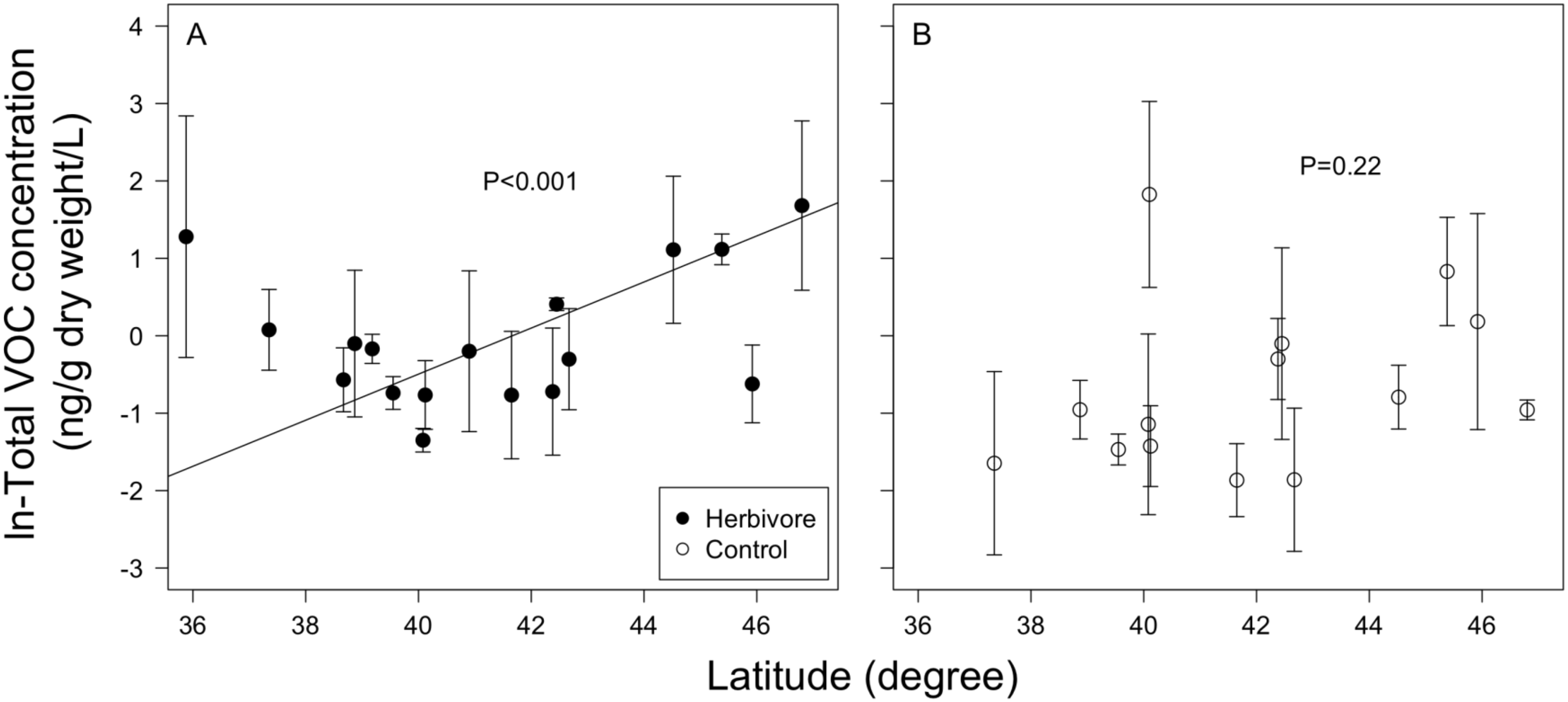
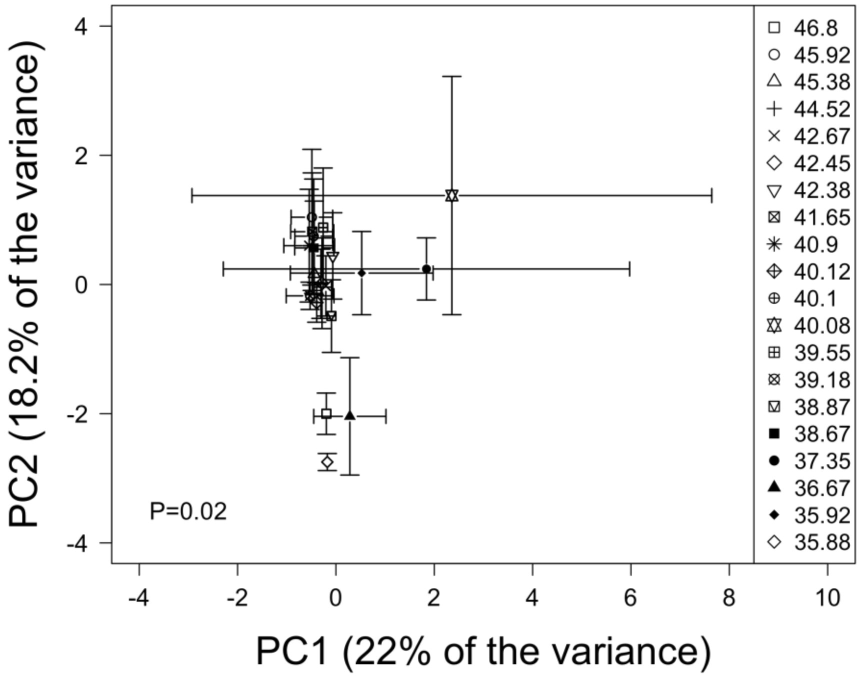


Figure 3.5 Milkweed plants originating from different latitudes emit different volatile organic chemical (VOC) blends. The principal components analysis of 16 VOCs emitted by *Asclepias syriaca* plants indicates the difference in composition of VOC blend among plant populations. Data were normalized, and standard error is shown for all 20 plant populations. The legend indicates the latitude of origin of each plant population represented in the analysis. Plants originated from 20 populations across 10^{o} of latitude and were grown in a common garden in Ithaca, NY.



Chapter 4

Phylogenetic patterns in plant volatile emission:
The potential adaptive evolution of indirect defense among milkweed species

Abstract

The application of a phylogenetic framework to plant-herbivore interactions has facilitated tests of longstanding predictions about plant defense trait evolution, though very few studies have applied phylogenetic analyses to indirect plant defenses. Volatile organic chemical (VOC) emission can serve as an indirect plant defense by attracting the enemies of herbivores to sites of plant damage. We used ten plant species in the genus *Asclepias* (milkweed) to test the hypothesis that plant VOC emission induced by herbivores exhibits a pattern of macroevolutionary escalation to compensate for a reported macroevolutionary decline in the expression of direct plant defenses. Consistent with our prediction, we observed phylogenetic escalation in the number of VOCs emitted by *Asclepias* species in response to herbivore damage. We also observed evidence of directional change in the VOC blends emitted by *Asclepias* during the evolution of the genus. However, in contrast to our expectation, total VOC concentrations in undamaged and herbivore-damaged plants exhibit a macroevolutionary decline with the evolution of *Asclepias* lineages. The decline in VOC concentrations may have allowed *Asclepias* species to conserve resources and/or minimize detection by mobile herbivores and ovipositing insects. The concomitant escalation in the number of VOCs emitted by

induced plants, along with the directional change in blend, may indicate that *Asclepias* species have evolved to more effectively target the natural enemies of herbivores. Although our results must be interpreted cautiously due to low taxonomic sampling and sample size, the novel patterns reported here can inform further macroevolutionary hypotheses regarding indirect and multivariate plant defense.

Introduction

The increasing availability of inexpensive methods of constructing phylogenies has accelerated the application of phylogenies to a broad range of ecological questions (Silvertown and Dodd 1996, Becerra 1997, Becerra and Venable 1999, Cavendar-Bares et al. 2004). For example, phylogenies have been instrumental in examining evolutionary theories of plant defense and testing predictions about defense trait evolution (Farrell et al. 1991, Thaler and Karban 1997, Berenbaum 2001, Rudgers et al. 2004, Agrawal et al. 2009a,b). Furthermore, phylogenetic techniques can highlight macroevolutionary patterns among a diversity of plant defenses, lending insights into covariation among defensive traits (Rudgers et al. 2004) and potential multivariate defense strategies (Agrawal and Fishbein 2006).

Although the phylogenetic approach has facilitated investigations of some modes of indirect defense (Heil et al. 2004, Rudgers et al. 2004), quantitative phylogenetic inquiry remains rare for volatile organic chemical (VOC) emission among plant species (Wink 2003). The emission of VOCs induced by herbivore damage can serve as an indirect plant defense by attracting the natural enemies of herbivores to sites of attack (Dicke 1994, D’Alessandro and Turlings 2006, Heil 2008). A phylogenetic analysis may enable the application of a broader perspective to persistent questions regarding VOC emission, such as whether the trait evolved as an adaptive plant defense against herbivores (Peñuelas and Llusià 2004, Heil and Karban 2010).

We investigated VOC emission in a phylogenetic framework using milkweed species in the genus *Asclepias*. This system provides an excellent opportunity to consider the evolution of multivariate plant defense (Agrawal and Fishbein 2006) by comparing VOC traits with previously published work on direct defenses among the same plant species. Recent work with *Asclepias* species (milkweeds) has revealed that prominent chemical and physical defense traits that directly repel herbivores (cardenolides, latex, and trichomes) exhibit a macroevolutionary decline, while tolerance to herbivory (plant regrowth ability) shows a phylogenetic pattern of escalation (Agrawal and Fishbein 2008). Over evolutionary time, the specialist herbivores of *Asclepias* have developed the ability to detoxify (Vaughan and Jungreis 1977, Holzinger et al. 1992), sequester (Malcolm et al. 1989, Dobler et al. 2011), and exploit as "medicine" (Lefèvre et al. 2010) the cardenolide defenses that milkweeds produce. Herbivores also can circumvent latex and trichome defenses by modifying their feeding behavior (Malcolm 1994). Simply put, the utility of direct defenses may have diminished over evolutionary time. We hypothesized that, to compensate for the declining efficacy of direct defenses, *Asclepias* species have increased their expression of indirect defense; thus, we predicted that herbivore-induced VOC emission follows a phyletic pattern of escalation.

Methods

The genus *Asclepias* contains about 130 species that are distributed across North America and the Neotropics (Fishbein et al. 2011, Rasmann and Agrawal 2011). About a dozen specialist herbivores feed on milkweeds, including the widespread *Danaus plexippus* (monarch butterfly). Female *D. plexippus* butterflies oviposit on *Asclepias* individuals, and hatched larvae feed on the plants. *Asclepias* species exhibit a suite of chemical and physical traits that are effective against herbivores, including toxic steroids called cardenolides (Malcolm 1994, Malcolm and Zalucki 1996, Dussourd and Hoyle 2000, Agrawal 2005), sticky and often potently toxic latex (Malcolm and Zalucki 1996, Zalucki and Malcolm 1999, Dussourd and Hoyle 2000), and hairy trichomes (Agrawal 2004, Agrawal 2005). Milkweed species vary in their relative expression of these plant defenses (Agrawal 2004, Zehnder and Hunter 2007).

Although direct defenses have been studied extensively in milkweeds, less is known about VOC emission among *Asclepias* species. Monarchs can use aboveground VOCs to select among milkweed oviposition sites (Bergström et al. 1995), and belowground VOC emission can serve as an indirect defense against longhorn beetle herbivores (*Tetraopes tetrophthalmus*) by attracting predatory nematodes (*Heterorhabditis bacteriophora*) (Rasmann et al. 2011). Results described in Chapter 2 suggest that monarch predators are attracted to the aboveground milkweed VOCs induced by monarch herbivory.

For this study, we sampled VOCs from 12 *Asclepias* species (*A. asperula*, *A. eriocarpa*, *A. erosa*, *A. exaltata*, *A. fascicularis*, *A. hirtella*, *A. incarnata*, *A. purpurascens*, *A. speciosa*, *A. syriaca*, *A. verticillata*, and *A. viridis*). We selected these species to maximize both variation in direct defense phenotype and distribution across the *Asclepias* phylogeny (A.A. Agrawal, *personal communication*). Seeds of all species except *A. syriaca* were obtained from commercial seed suppliers (Butterfly Encounters, San Ramon, CA, USA; Georgia Vines, Claxton, GA, USA; Everwilde Farms, New Auburn, WI, USA). Seeds of *A. syriaca* were collected from adult plants growing naturally at the University of Michigan Biological Station (Pellston, MI). All of these *Asclepias* species occur naturally throughout the United States, with the range of some species reaching Canada and Mexico.

All seeds were washed with a solution of 5-10% bleach in water, scarified, and cold stratified for four weeks on damp paper towels. The seeds were germinated on moist filter paper in Petri dishes in a growth chamber, transferred to 6-inch pots containing unfertilized potting soil (Glacial Bay Soils, Elsie, MI, USA), and grown in a greenhouse (Matthaei Botanical Gardens, Ann Arbor, MI). The seedlings were maintained for about twelve weeks before the start of the experiment. Stratification occurred through May, seedling germination and growth during the summer, and VOC collection during September 16–23 of 2010.

Monarch larvae were used to experimentally damage all treatment plants. Monarchs obtained from Flutterby Gardens (Bradenton, FL, USA) were raised from eggs and maintained on a diet of *A. syriaca* leaves in a growth chamber. One third-instar caterpillar was placed on each treatment plant in the greenhouse and was allowed to feed from late afternoon until the morning immediately prior to VOC sampling (about 18 hours). Netting was placed over entire plants to restrict caterpillar movement to treatment plants and over each control plant without caterpillars to control for effects of netting on

VOC emission. Because plant morphology varied widely among milkweed species, and larvae had access to entire plants, we were unable to quantify herbivore damage; we consider herbivory as either present (when a caterpillar was placed on the plant and consumed plant tissue) or absent (when the plant was not exposed to an experimental herbivore) in statistical analyses. Larvae and netting were removed before VOCs were sampled. VOCs were collected during approximately the same daylight hours on each sampling day following the previous night of herbivore damage (generally between 10:30 and 17:30).

VOC collection chambers were constructed out of plastic 2-L bottles (modified to have a volume of 1.65 L). Dual-diaphragm vacuum pumps (Gardner Denver Thomas, Sheboygan, WI, USA) were connected to chambers by inert tubing, allowing us to sample multiple plants simultaneously. Adjustable rotameters were connected to each chamber and ensured that air flowed at a constant velocity of 1.5 L per minute. We sampled VOCs from whole plants by enclosing one plant in each chamber and collecting gases from the chambers by drawing air continuously for 7 h through VOC traps that contained SuperQ adsorbent (Analytical Research Systems, Gainesville, FL, USA). Before entering the chambers, incoming air flowed through a filter containing activated carbon, which prevented ambient VOCs from entering the chamber, and a filter containing sodium thiosulfate, which prevented the passage of ozone gas that can react with VOCs in the atmosphere (Helmig 1997). Thus, “clean” air flowed into each chamber; nonetheless, we also sampled VOCs from empty chambers to evaluate any volatiles that were collected from the “clean” air. Plastic sheets covered the soil around plant stems to prevent collection of soil volatiles.

The system enabled simultaneous volatile collection from up to 22 plants plus one empty control chamber on any given day. We therefore randomized species among sampling days with the added constraint that one control and one treatment plant of a given species were sampled on each sampling day. Based on germination success and the removal of individual plants that emitted just one VOC peak (see below), samples sizes varied among milkweed species (Table 4.1). We standardized VOC emissions for aboveground plant biomass in all statistical analyses; experimental plants were destructively harvested, placed in a drying oven for a minimum of 24 hours, and weighed.

VOCs were eluted from the SuperQ adsorbent with 750 µl of dichloromethane (CH_2Cl_2) containing 1.94 µg of nonyl acetate (Sigma-Aldrich, CAS # 143-13-5) as an internal standard. We analyzed 1-µl aliquots of VOC samples on a 30 m x 0.25 mm inner diameter fused-silica column with a polyethylene glycol stationary phase and 0.25 µm film thickness (Restek Corporation, Bellefonte, PA, USA) in a gas chromatograph-mass spectrometer (GCMS; Agilent Technologies, Santa Clara, CA, USA). We used the following GC method: injector held at 250 ^{o}C, initial column temperature at 50 ^{o}C held for 10 min, ramped at 5 ^{o}C min^{-1} to 200 ^{o}C, held for 10 min. Helium carrier gas flow was set to 1.3 ml min^{-1}.

VOC concentrations were quantified by calculating relationships among internal standard concentration, internal standard peak area, and focal compound peak area. Concentrations were standardized for the total volume of air sampled and for aboveground plant dry mass. Omitted from the dataset were synthetic chemicals, VOCs collected from empty chambers, compounds emitted by only one individual plant, and

VOCs that contributed <1% of the total VOC concentration across all plants within a species. Omissions from the dataset substantially reduced the number of replicates available for statistical analyses, and two species, *A. hirtella* and *A. viridis,* were excluded from analyses due to low sample size and an unusually high number of singleton compounds emitted by individual plants (Table 4.1). The number of VOCs emitted by the remaining ten plant species ranged between 1 and 6 compounds.

In statistical analyses, we compared total VOC concentration, number of VOCs emitted, magnitude of induction, and multivariate VOC blend among *Asclepias* species. For each of these variables, we analyzed three different sets of VOC data: VOCs from undamaged plants, VOCs from herbivore-damaged plants, and combined VOCs from damaged and undamaged plants. Total VOC concentrations were log-transformed to improve normality. Standard error was incorporated into phylogenetic analyses and was derived for each dataset using the following method: the standard deviation of a given trait was calculated by combining all replicates of all plant species within the dataset; the standard error subsequently was calculated for each species by dividing the standard deviation of the entire data set by the square root of the sample size of each species. This conservative method enabled us to estimate the measurement error associated with each trait value, even for instances in which the sample size = 1 (Ives et al. 2007, Revell and Reynolds 2012).

Additionally, for total concentration and number of VOCs, we compared the magnitude of VOC induction among species. The magnitude of induction was calculated as the difference between the mean concentration (or number) of VOCs in herbivore-damaged plants minus the mean concentration (or number) of VOCs in undamaged plants, and the difference was divided by the undamaged mean, which yielded the relative change in the VOC trait induced by herbivory. Standard error was not incorporated for the magnitude of induction dataset.

To compare VOC blend among milkweed species, we used principal components analysis (PCA) to combine all emitted compounds into multivariate axes. We then averaged PCA axes scores within each *Asclepias* species to compare blends among species using phylogenetic methods. A PCA axis was included in the analyses if it explained >10% of the total variance in the PCA, and the eigenvalue of the axis was >1. In the data set for undamaged plants, three PCA axes explained 20.9%, 14.5%, and 10.6% of the variation in VOC blend (total = 46%). In the data set for damaged plants, four axes represented 19.2%, 13.4%, 11.5%, and 10.4% of the variation (total = 54.4%). In the combined data set, two PCA axes explained 18.5% and 15.2% of the variation (total = 33.7%).

The phylogeny employed in these analyses was estimated by Fishbein et al. (2011) and is publicly available on TreeBASE (http://www.treebase.org). The original *Asclepias* phylogeny incorporates DNA data from three non-coding regions of the plastid genome using maximum likelihood methods and includes more than 140 species (Fishbein et al. 2011). Importantly, all phylogenetic data were employed to construct the ingroup tree of 10 *Asclepias* species that was used in the phylogenetic analyses described below. We first recreated the entire tree of more than 140 species, then pruned extraneous branches from the original phylogeny. We tested the reliability of our phylogeny by conducting a maximum-likelihood bootstrap analysis using MEGA version 5 (Tamura et al. 2011) (Figure 4.1).

We used R (Version 2.11.1) for all statistical analyses. Our primary aims were to (1) estimate the extent of any existing phylogenetic constraint and (2) determine whether a directional macroevolutionary trend exists with regard to the following four metrics of VOC emission: total VOC concentration, number of VOCs emitted, magnitude of induction, and multivariate VOC blend. We implemented a direct test of the hypothesis that *Asclepias* species have increased their expression of indirect defense to compensate for a concomitant decline in direct defenses, as reported by Agrawal and Fishbein (2008). Because the phylogenetic decline in direct defenses was shown using a gradual model of evolution (Agrawal and Fishbein 2008), we evaluated all trait data using a gradual model of evolution here, where branch lengths were proportional to the number of nucleotide substitutions per site.

We used two different methods to determine whether phylogenetic history constrains the evolution of VOC emission among these *Asclepias* species. Evidence of phylogenetic constraint, wherein the phenotypes of closely related species are more similar than expected by chance, is given by a significant phylogenetic signal (Blomberg et al. 2003, Revell et al. 2008). One method computes phylogenetic signal using Pagel's lambda (Revell 2011). When the parameter $\lambda=1$, tree topology is maintained, while $\lambda=0$ eliminates topology, causing the tree to resemble a star phylogeny (Pagel 2002). A likelihood ratio test compares a model in which $\lambda=0$ to a model in which λ has been estimated for a set of empirical data. The second method calculates the *K*-statistic of phylogenetic signal (Revell 2011). The *K*-statistic compares the variance of observed trait data to the distribution of variances resulting from multiple permutations of the trait data across the phylogeny. A phylogenetic signal exists if the variance of the empirical data is statistically less than that of 95% of the permutations (Blomberg and Garland 2002, Blomberg et al. 2003).

Finally, we evaluated whether plant VOC emission phenotype exhibits a directional trend to test the hypothesis that herbivore-induced VOC emissions would increase with the evolution of *Asclepias* species. We interpreted macroevolutionary trends as correlations between VOC trait values and the number of nucleotide substitutions per site (represented by phylogenetic branch lengths) for each *Asclepias* species. A significant correlation would indicate directional change in a trait as *Asclepias* lineages accumulate nucleotide substitutions (i.e., as the lineages evolve). Because we observed the contemporary expression of VOC emission phenotype, and did not obtain data related to ancestral VOC traits, the presence of a macroevolutionary trend served as an indirect measure allowing us to infer anagenetic change among lineages. To assess whether plant VOC emission phenotype exhibits a directional trend with the evolution of *Asclepias* species, we compared a random walk model to a model containing an additional parameter representing a directional trend (Harmon et al. 2009). We then used a likelihood ratio test to assess whether the addition of the trend parameter improved the model fit.

Results

Table 4.2 provides parameters of the best-fit models relating VOC emission to gradual evolutionary change in the genus *Asclepias*. The number of VOCs emitted by herbivore-damaged plants showed a pattern of phylogenetic escalation (β=149.58, P=0.026; Figure 4.2A) and equivocal phylogenetic signal (λ=1.530, P=0.356; K=1.224, *P*=0.0001). The blend of VOCs emitted in response to herbivore damage represented by PCA axis 4 exhibited directional evolutionary change (β=-367.40, *P*=0.008; Figure 4.2B) and explained 10.4% of the total variance in the relative concentrations of VOCs emitted by damaged plants. This same axis of herbivore-induced VOC blend also showed a strong phylogenetic signal (λ=1.392, *P*<0.0001; *K*=1.344, *P*=0.004).

Total constitutive VOC concentrations decreased with evolutionary change in the genus (β=-319.39, *P*=0.003; Figure 4.3A) and exhibited a strong phylogenetic signal (λ=1.530, *P*=0.048; *K*=1.670, *P*=0.0001). Likewise, total VOC concentrations emitted by herbivore-damaged plants showed a phylogenetic decline (β=-200.48, *P*=0.013; Figure 4.3B), though induced VOC concentration showed no significant phylogenetic signal (λ=0.385, *P*=0.679; *K*=0.980, *P*=0.070). The combined dataset showed a consistent declining trend in total VOC concentrations (β=-215.72, *P*=0.002), driven by the declines in constitutive and herbivore-damaged concentrations, and strong phylogenetic signal (λ=1.399, *P*<0.0001; *K*=1.226, *P*=0.019), as influenced by the undamaged data (Table 4.2).

We found evidence of phylogenetic constraint in herbivore-induced VOC blend represented by PCA axis 3 (accounting for 11.5% of the variation in blend) (λ=1.345, *P*<0.0001; *K*=0.972, *P*=0.043). The VOC blend emitted by undamaged plants, represented by PCA axis 3 (10.6% of the variation), showed equivocal phylogenetic signal (λ=1.450, *P*<0.0001; *K*=1.121, *P*>0.99). The change in the number of emitted VOCs induced by herbivores (i.e., the number of VOCs emitted in the magnitude of induction dataset) also showed equivocal phylogenetic signal (λ=0.767, *P*=0.351;

K=1.068, *P*=0.017). For all other metrics of VOC phenotype, we found evidence of neither phylogenetic constraint nor macroevolutionary trend (Table 4.2).

Discussion

The expression of direct defense traits in *Asclepias* has been shown to decrease as lineages evolve (Agrawal and Fishbein 2008), perhaps because the specialist herbivores that commonly attack milkweeds can sequester (Malcolm et al. 1989; Helms et al. 2004), co-opt for medication purposes (Lefèvre et al. 2010), and otherwise circumvent plant defenses such as cardenolides, latex, and trichomes. The defensive function of VOC emission occurs indirectly via the attraction of natural enemies, and parasitoids seem undeterred by sequestered defenses in milkweed herbivores (Hunter et al. 1996, Helms et al. 2004). On the basis of these observations, we predicted that the adaptations of specialist herbivores would not reduce the potency of indirect defense at the macroevolutionary scale. Instead, we predicted that measures of VOC emission would intensify as *Asclepias* lineages evolved, to compensate for the decreasing efficacy of direct plant defense traits.

Consistent with our hypothesis, the number of VOCs induced by herbivores has increased as *Asclepias* lineages evolved (Figure 4.2A, Table 4.2). This observation suggests that plants may be selected to emit increasing numbers of VOCs in response to herbivore damage, possibly to target natural enemies more effectively when herbivores are present. Herbivore damage has been shown to induce higher numbers of plant VOCs in other systems (Mattiacci et al. 1994, Scutareanu et al. 2003, Bruinsma et al. 2009), and the induction of a novel chemical can drive the attraction of higher trophic levels. For example, Rasmann et al. (2005) showed that maize roots emit (*E*)-β-caryophyllene only when damaged by herbivores, and (*E*)-β-caryophyllene was directly linked to the attraction of a nematode predator. In the case of *Asclepias*, it remains unknown whether individual novel chemicals increase the attraction of natural enemies, or whether novel chemicals contribute to a more attractive VOC blend. Unfortunately, attempts to verify the identity of VOCs emitted by the experimental plants using several authentic standards yielded no positive identifications; thus, we do not know which specific VOCs drove the observed trend.

We found that herbivore-induced VOC blend also exhibited a phylogenetic pattern among the *Asclepias* species tested here (Figure 4.2B). Notably, this macroevolutionary directional change in blend occurred only in the VOC blend emitted in response to herbivore damage (Table 4.2). It would be interesting and informative to test whether the natural enemies of *Asclepias* herbivores are more attracted to the blend that the *Asclepias* species are evolving to resemble.

Total VOC concentrations emitted by both undamaged and herbivore-damaged plants have decreased during the evolution of *Asclepias* lineages (Figure 4.3, Table 4.2). At least two potential adaptive explanations for this phylogenetic decline in VOC concentrations may apply to both constitutive and induced plant emissions, and these explanations are nonexclusive. First, the decline in total concentrations may represent a plant strategy of reduced resource investment. Plants may divert resources away from the production of constitutive VOCs that, unlike herbivore-induced VOCs (De Moraes et al. 1998, Kessler and Baldwin 2001, Pichersky and Gershenzon 2002, D'Alessandro and Turlings 2006), are not reliable indicators of the presence of prey (Vet et al. 1991).

Likewise, a cost of induced VOC emission, which has been demonstrated for plant biomass in maize (Hoballah et al. 2004), may select for a reduction in total induced concentrations in favor of resource allocation toward specific VOCs that more effectively attract natural enemies. Second, both constitutive and induced VOC emission may be associated with ecological costs. Plant emission of constitutive VOCs can facilitate the detection of host plants by herbivores (Visser 1986, Halitschke et al. 2008, Kariyat et al. 2012), in addition to potentially damaging the plant itself when produced at high quantities (Paré and Tumlinson 1999). In addition, constitutive VOCs can elicit higher rates of oviposition. For example, greenhouse and field experiments have indicated that wheat stem sawflies prefer to lay eggs on a wheat cultivar that produces higher constitutive VOC concentrations (Weaver et al. 2009). While some plant species have been shown to deter oviposition by emitting herbivore-induced VOCs (De Moraes et al. 2001, Kessler and Baldwin 2001), induced emissions also can increase visitation by herbivores and ovipositing insects (Dicke and van Loon 2000). The potential for plants to both optimize resource allocation and reduce susceptibility to herbivore damage and oviposition through reductions in total VOC concentrations thus could explain the observed pattern of macroevolutionary decline in total constitutive and induced VOCs.

Clearly, the effectiveness of herbivore-induced VOC emission as an indirect defense depends on the balance between these potential costs and the projected benefits. The results of this phylogenetic analysis provide tentative support for the prediction that plants will evolve to reduce the physiological and ecological costs of VOC emission and simultaneously optimize the attractiveness of the signal to natural enemies. *Asclepias* species appear to reduce costs through a phylogenetic decline in total VOC concentrations (Figure 4.3); these species also appear potentially to maximize the effectiveness of VOC signals through a macroevolutionary escalation in the number of VOCs emitted upon herbivore damage and a change in the herbivore-induced VOC blend (Figure 4.2).

Alternatively, the declines that we observed in total VOC emission could be related to the evolutionary decline in latex production reported for *Asclepias* species by Agrawal and Fishbein (2008). Latex in many plant species often derives from and contains terpenoids (Agrawal and Konno 2009), and a potentially adaptive decline in latex may result in a concomitant, though nonadaptive, decline in VOCs. However, evidence of a correlation between latex exudation and VOC concentration is lacking among *Asclepias* species. We examined the phylogenetically independent contrast (PIC) between the total VOC concentrations sampled here and latex exudation measured by Agrawal and Fishbein (2008) and Agrawal et al. (2008). The best-fitting PIC was not significant for either constitutive (R^2=0.17, $F_{1,7}$=1.429, P=0.27) or induced (R^2=0.14, $F_{1,7}$=1.146, P=0.32) VOC emissions among *Asclepias* species, and a similar lack of correlation between these defense traits has been observed within the species *Asclepias syriaca* (see Chapter 2).

In general, the remaining metrics of VOC emission phenotype examined among these *Asclepias* species do not appear to be constrained by shared ancestry. Although many evolutionary explanations can account for low phylogenetic signal, including divergent selection (Revell et al. 2008), VOC emission is known to be highly variable among plant species (Kesselmeier and Staudt 1999, Dudareva et al. 2004, Jürgens et al. 2006). While a heritable genetic basis has been quantified for VOC emission phenotype

(Degen et al. 2004), natural variability in the trait may contribute to the low phylogenetic signal observed for most metrics of VOC phenotype. High intraspecific variability also can obscure differences among species driven by evolutionary history (Capellini et al. 2008), and the genotypes of the plants sampled in this experiment remain unknown. In addition, we were unable to incorporate potential sources of variation such as environmental factors (temperature, photosynthetically active radiation, and humidity) into the analyses reported here. Including in such analyses not only the proximate environmental conditions, but also the environmental conditions under which these plant species evolved, would supplement our understanding of evolutionary patterns across species (Vermeij 1994).

Two caveats must be noted when interpreting the results of this phylogenetic analysis. First, we evaluated VOCs collected from ten plant species within one genus, and our conclusions are limited by low taxonomic sampling and low replication within species. Second, all of these analyses tested the gradual model of trait evolution, where trait values were evaluated with regard to root to tip branch lengths of the phylogeny. The interpretation of such results can be difficult because phylogenetic branch lengths based on different gene sequence data potentially could provide different results. In the present study, branch lengths were represented by the number of nucleotide substitutions per site in three non-coding regions of the plastid genome. The evolutionary relevance of conclusions based on correlations involving non-coding DNA data remains unclear. However, an advantage to using these data in an analysis of the genus *Asclepias* is that a similar phylogenetic analysis, but for direct plant defenses, has been conducted previously using identical phylogenetic data (Agrawal and Fishbein 2008). We therefore can test our hypotheses concerning indirect defenses against reported patterns in direct defenses in the same system, and the results can be compared and interpreted meaningfully within the context of the consistent construct.

A meaningful interpretation hinges on the evolutionary relevance of these observed macroevolutionary trends based on non-coding DNA data. Indeed, nucleotide substitution rates are known to vary among plant lineages (Wright et al. 2003) and regions of the plant genome (Wolfe et al. 1987), and some empirical data suggest that substitution rate heterogeneity in non-coding regions reflects relevant evolutionary processes. For example, variation in rates of metabolism among lineages may be linked tightly to variation in rates of nucleotide substitution (Gillooly et al. 2005). In a wide range of plant and animal species, the number of nucleotide substitutions per site has been shown to correlate linearly with protein evolution (Gillooly et al. 2007) and morphological and functional adaptations (Jobson and Albert 2002). In addition, phylogenetic analyses of latex evolution among *Asclepias* species based on either nucleotide substitution data or temporal duration provide similar results (Agrawal et al. 2008). Thus, evidence in the *Asclepias* system tentatively suggests that the results for VOC emission described here and the results for direct defenses (Agrawal and Fishbein 2008) may be robust and informative.

Future work would benefit greatly from replicated phylogenetic analyses of these plant defense data using different regions of the *Asclepias* genome to estimate phylogenetic branch lengths. Such replication would verify whether the observed macroevolutionary trends are robust to the underlying phylogenetic data. Despite these caveats, the macroevolutionary patterns we report here, which indicate an increase in the number of herbivore-induced VOCs, a directional change in VOC blend, and declines in total constitutive and herbivore-induced VOC concentrations during the evolution of *Asclepias* lineages, argue for increased attention to phylogenetic signals and trends in studies of the evolution of indirect plant defenses.

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Table 4.1 Number of volatile organic chemical (VOC) samples collected from *Asclepias* species for each of three data sets analyzed. The undamaged data set represents constitutive VOC emissions, the herbivore-damaged data set represents monarch-induced VOC emissions, and the combined data set contains all VOC samples obtained from undamaged and herbivore-damaged plants. Dashes indicate species that were omitted from analyses due to low germination in the greenhouse or an unusually high incidence of rare VOCs emitted from individual plants.

Species	**Undamaged**	**Herbivore-damaged**	**Combined**
A. asperula	3	1	4
A. eriocarpa	1	2	3
A. erosa	2	3	5
A. exaltata	3	3	6
A. fascicularis	4	3	7
A. hirtella	-	-	-
A. incarnata	1	4	5
A. purpurascens	1	2	3
A. speciosa	4	4	8
A. syriaca	1	1	2
A. verticillata	1	4	5
A. viridis	-	-	-
Total	**21**	**27**	**48**

Table 4.2 Results of phylogenetic analyses for three different sets of volatile organic chemical (VOC) data: VOCs from undamaged plants, herbivore-damaged plants, and combined data from undamaged and damaged plants. Magnitude of induction was calculated as the difference between mean VOC concentrations from monarch-damaged and undamaged plants divided by undamaged values, yielding the relative change in plant VOC emission induced by herbivory. All analyses were fit to gradual models of trait evolution, wherein phylogenetic distance was estimated by nucleotide substitutions per site. Pagel's lambda (λ) and the *K*-statistic are measures of phylogenetic signal (Pagel 2002, Blomberg et al. 2003), where λ=0 and *K*<1 indicate no signal, *K*=1 indicates Brownian motion, and λ=1 and *K*>1 indicate strong signal. β represents the magnitude of a putative anagenetic trend in VOC phenotype evolution. *P*-values are shown in parentheses, with significant values in bold. Dashes indicate a metric of VOC emission that was not applicable to the data set.

	Undamaged			**Herbivore-damaged**		
	λ	*K*	β	λ	*K*	β
Total VOC concentration	**1.530**† **(0.048)**	**1.670** **(0.0001)**	**-319.39** **(0.003)**	0.385 (0.679)	0.980 (0.070)	**-200.48** **(0.013)**
Number of VOCs emitted	1.530† (>0.99)	0.759 (0.415)	-70.90 (0.462)	1.530† (0.356)	**1.224** **(0.0001)**	**149.58** **(0.026)**
VOC blend (PC1)	1.530† (0.951)	0.733 (>0.99)	167.82 (0.167)	0 (>0.99)	0.496 (>0.99)	-65.100 (0.555)
VOC blend (PC2)	0 (>0.99)	1.390 (0.841)	33.84 (0.736)	0 (>0.99)	0.613 (>0.99)	-68.95 (0.454)
VOC blend (PC3)	**1.450**† **(<0.0001)**	1.121 (>0.99)	107.31 (0.211)	**1.345**† **(<0.0001)**	**0.972** **(0.043)**	97.79 (0.617)
VOC blend (PC4)	-	-	-	**1.392**† **(<0.0001)**	**1.344** **(0.004)**	**-367.40** **(0.008)**

Table 4.2 Continued.

	Combined			**Magnitude of induction**		
	λ	K	β	λ	K	β
Total VOC concentration	**1.399**[†] **(<0.0001)**	**1.226** **(0.019)**	**-215.72** **(0.002)**	0.023 (0.952)	0.681 (0.371)	253.72 (0.231)
Number of VOCs emitted	0.357 (0.882)	0.952 (0.197)	71.03 (0.384)	0.767 (0.351)	**1.068** **(0.017)**	72.93 (0.203)
VOC blend (PC1)	0.006 (0.992)	0.636 (>0.99)	-107.98 (0.448)	-	-	-
VOC blend (PC2)	0 (>0.99)	0.503 (>0.99)	13.96 (0.844)	-	-	-
VOC blend (PC3)	-	-	-	-	-	-
VOC blend (PC4)	-	-	-	-	-	-

[†] Values were estimated in the phytools package in R (Revell 2011), although in general, $0 \leq \lambda \leq 1$. Here, where $\lambda > 1$, phylogenetic signal remained significant when λ was constrained in a separate analysis to $0 \leq \lambda \leq 1$.

Figure 4.1 Consensus bootstrap phylogenetic tree of 10 *Asclepias* species used in the experiment, with 2 outgroup *Gomphocarpus* species, based on a subset of phylogenetic data obtained from Fishbein et al. (2011). This tree was reconstructed for reference using nucleotide sequence data from three non-coding regions of the plastid genome, employing maximum likelihood methods with 1,000 bootstrap replicates and the Tamura-Nei model in MEGA version 5 (www.megasoftware.net). Bootstrap values ≥50% are indicated at the nodes. The scale bar refers to branch lengths and indicates number of nucleotide substitutions per site. In analyses, all available phylogenetic data were used to construct the full *Asclepias* tree, and extraneous branches were pruned, leaving behind a 10-species subtree that was informed by the entirety of the nucleotide sequence data in Fishbein et al. (2011). Importantly, this tree is for illustrative purposes and does not represent the data used in the phylogenetic analyses described here; experimental results do not depend on the strength of the bootstrap values shown in the figure.

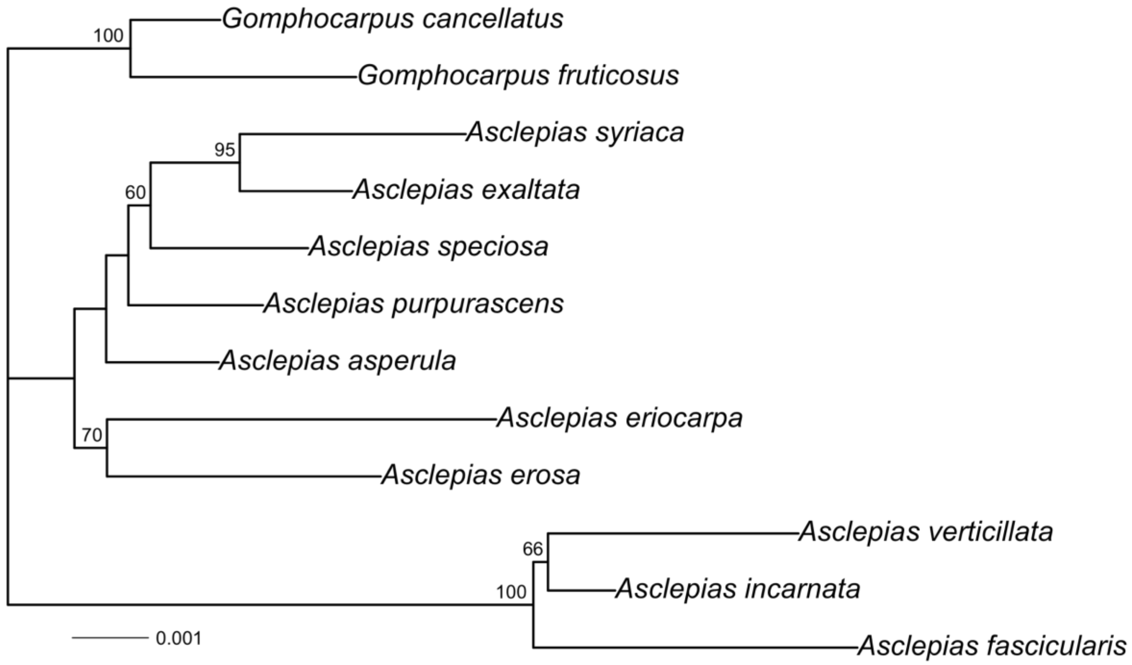


Figure 4.2 Macroevolutionary escalation in the number of volatile organic chemicals (VOCs) emitted by milkweed upon induction by monarch herbivory and directional trend in the evolution of induced VOC blend. (A) Phylogenetic escalation in the number of VOCs emitted by *Asclepias* species that were damaged by *Danaus plexippus* (monarch caterpillar) herbivores. (B) Directional change in the caterpillar-induced VOC blend represented by PCA axis 4, which explained 10.4% of the variation in the relative concentrations of VOCs emitted by damaged *Asclepias* plants. Regression lines indicate the trend in the trait (denoted as β) with respect to the root to tip branch length (nucleotide substitutions per site). Each point represents one of ten experimental *Asclepias* species; shown are mean trait values ±SE.

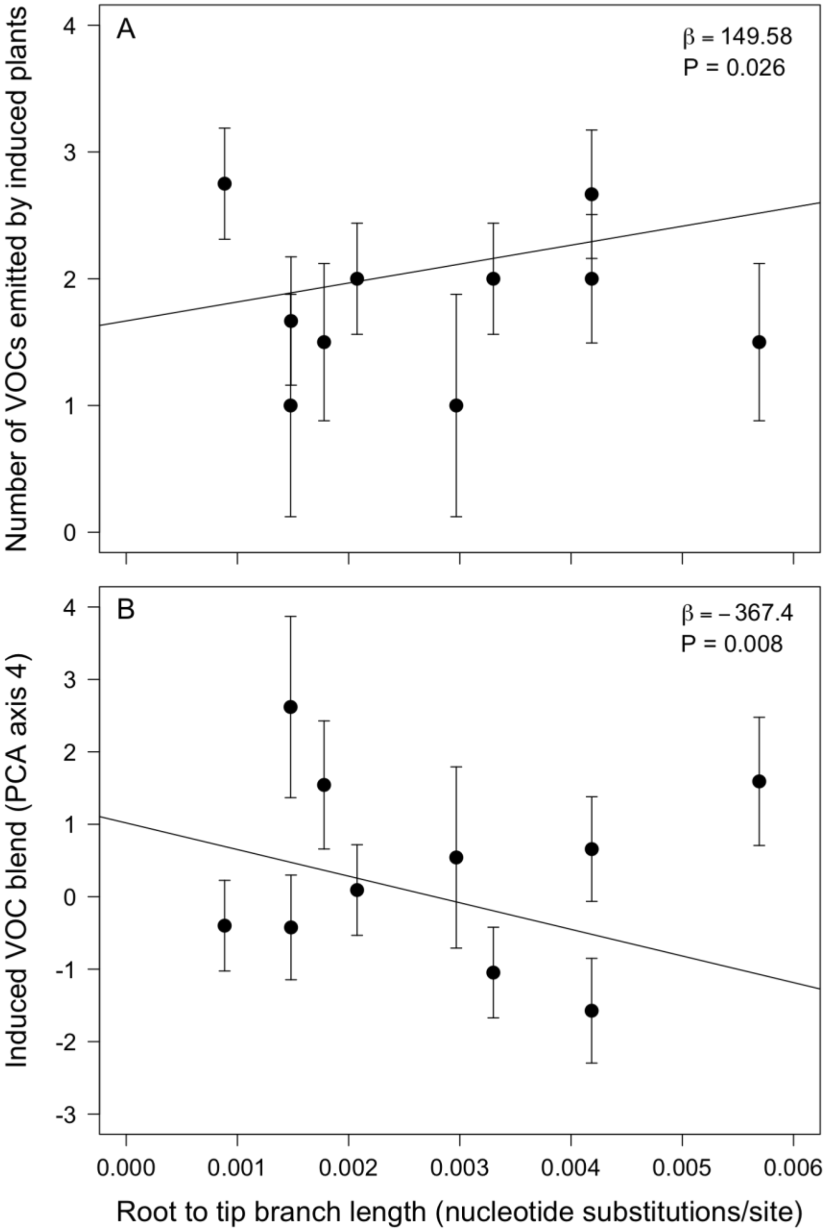
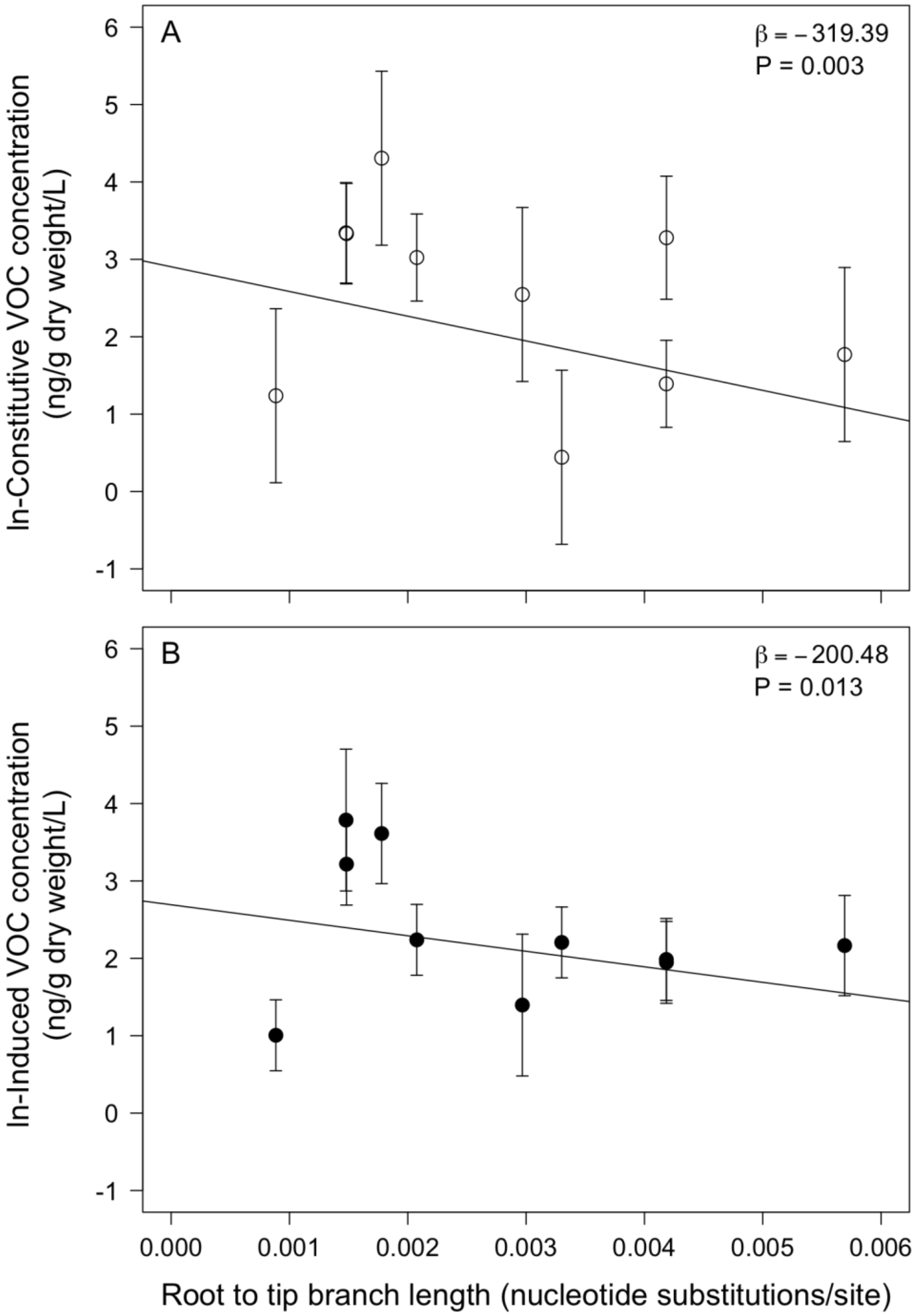


Figure 4.3 Phylogenetic decline in total concentration (ln-transformed) of volatile organic chemicals (VOCs) emitted by (A) undamaged *Asclepias* species and (B) *Asclepias* species that were damaged by *Danaus plexippus* (monarch caterpillar) herbivores. Regression lines indicate the directional trend in the evolution of the trait (denoted as β) with respect to the root to tip branch length (nucleotide substitutions per site). Each point represents one of ten experimental *Asclepias* species; shown are mean trait values ±SE. Note that some trait values are similar enough that they overlap in the figure.



Chapter 5

Conclusions and synthesis

Variation in VOC emission within and among plant taxa has important ecological implications. For example, herbivore-induced changes in VOC concentration and blend (Takabayashi and Dicke 1996, Turlings et al. 1998, Karban et al. 2000, De Moraes et al. 2001, Kessler and Baldwin 2001, Dicke et al. 2003) and diurnal cycles in emission phenotype (De Moraes et al. 1998, Shiojiri et al. 2006) can have major effects on other organisms in the community. Natural enemies that associate plant VOCs with the presence of their herbivore hosts appear to provide an indirect defense for plants (D'Alessandro and Turlings 2006, Heil 2008), and plant VOC emission can influence herbivore activity (Visser 1986, Halitschke et al. 2004, Halitschke et al. 2008, Shiojiri et al. 2006), resistance to harmful microbes (Dudareva and Negre 2005), and competitive interactions among plants (Farmer 2001, Karban et al. 2006, Kessler et al. 2006, Runyon et al. 2006, Frost et al. 2008a, Frost et al. 2008b, Karban and Shiojiri 2009). Thus, quantifying potential sources of variation in VOC emission is critical to our understanding of its potential ecological consequences.

This dissertation evaluates genetically-based variation in VOC emission in a genus of native plant at multiple scales: (1) intraspecific variation within a natural population, (2) intraspecific variation among populations, and (3) variation among species within the genus. In the majority of experiments, I controlled for environmental factors (temperature, photosynthetically active radiation, and humidity) as potential sources of variation in plant VOC phenotype. Substantial research indicates that VOC emission responds to light availability (Staudt and Seufert 1995, Schuh et al. 1997, Staudt and Bertin 1998, Halitschke et al. 2000, Sabillón and Cremades 2001, Owen et al. 2002, Gouinguené and Turlings 2002, Hansen and Seufert 2003, Staudt et al. 2003, Dindorf et al. 2006) and temperature (Staudt and Seufert 1995, Owen et al. 1997, Staudt and Bertin 1998, Komenda and Koppmann 2002, Owen et al. 2002, Staudt et al. 2003, Dindorf et al. 2006, Blanch et al. 2007, Gray et al. 2005). While the influence of environmental factors on plant VOC emission is well known, few studies account for these variables and their likely important role in plant-insect interactions in the field. By explicitly evaluating environmental controls over plant VOC emission in many of the experiments described here, I was better able to isolate the contribution of plant genotype to variability in VOC emission.

I used a combination of field and common garden experiments to investigate VOC emission in plants of the genus *Asclepias* (milkweeds) and its potential role as an indirect defense against the monarch caterpillar herbivore, *Danaus plexippus*. Recently, Rasmann et al. (2011) reported that root VOCs in *Asclepias syriaca* can serve as an indirect defense against longhorn beetle root herbivores (*Tetraopes tetrophthalmus*) by attracting their nematode predator (*Heterorhabditis bacteriophora*). Other work has shown that aboveground VOCs emitted by *A. syriaca* can affect the oviposition behavior of monarch butterflies (Bergström et al. 1995). The general results described in this dissertation indicate that aboveground vegetative VOC emission in *A. syriaca* also can play a role in indirect defense against monarch herbivores, and that plants exhibit variation for this trait at multiple scales.

Chapter 2: Genetic variation in plant volatile emission does not result in differential attraction of natural enemies in the field. Intraspecific genetic variation can be assessed using the following four metrics of VOC emission: (1) total VOC concentration, (2) VOC blend, (3) magnitude of induction, and (4) natural enemy attraction. Variation among genotypes in this chapter refers to either variation among genets, which are groups of clonal plants, or variation among half-sib genetic families, which are plants that share half of their genetic material. Induced and constitutive VOC concentrations and blends varied among plant genotypes in the field and greenhouse, although the evidence was stronger for VOC blend. The magnitude of induction (the relative change in VOC emission when plants were induced by herbivores) consistently was similar across genotypes. Unexpectedly, natural enemies attacked decoy caterpillars with equal frequency across plant genotypes that were placed in the field; this result requires further investigation. I propose the provisional hypothesis that the change in VOC emission phenotype signaling herbivory may overwhelm more subtle differences among plant genotypes. A consistent magnitude of induction among plant genotypes thus may serve as a more reliable signal to natural enemies (Vet et al. 1991, Gouinguené et al. 2001, Degen et al. 2004) and improve the effectiveness of the indirect plant defense.

Chapter 3: A genetically-based latitudinal cline in the emission of herbivore-induced plant volatile organic compounds. In this chapter, population-level variation represented genetic variation in VOC emission at the geographic scale. Across a gradient of more than 10^{o} of latitude (>1,500 km), *A. syriaca* populations differed in their total concentrations and blends of emitted VOCs. Monarch-induced VOC concentrations varied systematically across the geographic gradient, showing a pattern of increasing concentration with increasing latitude; however, neither constitutive VOC concentrations nor VOC blend varied systematically with latitude. These results are consistent with recent evidence indicating that some plant defenses tend to be more potent at high latitudes (Moles et al. 2011a, b), and contrast with a prominent prediction that higher herbivore pressure at low latitudes selects for stronger plant defense at low latitudes (Pennings et al. 2001, Pennings et al. 2009). That the latitudinal pattern was observed for induced VOCs, and not constitutive emission, suggests that latitudinal variation in VOC emission is not a simple response to climatic factors.

Chapter 4: Phylogenetic patterns in plant volatile emission: The potential adaptive evolution of indirect defense among milkweed species. The concentration and blend of VOC emissions varied among ten *Asclepias* species that were grown in a common garden. This chapter thus considers genetic variation at the level of plant species, and differences among species were calculated as the number of nucleotide substitutions per site in three non-coding regions of the plastid genome. As *Asclepias* lineages have evolved, the number of VOCs emitted by herbivore-damaged plants has increased, while blends of herbivore-induced VOCs have shown directional change. Total VOC concentrations emitted by undamaged and herbivore-damaged plants exhibit macroevolutionary declines. I propose that the phylogenetic declines in constitutive and induced VOC concentrations may reflect the evolution of reduced resource investment and minimize detection by herbivores and ovipositing insects, while the concomitant phylogenetic escalation in the number of VOCs emitted upon herbivore induction, along with the directional change in VOC blend, may indicate that *Asclepias* species have evolved to target more effectively the natural enemies of herbivores. While low taxonomic sampling and replication limit the interpretation of these results, the reported novel patterns can inform further studies.

This dissertation work not only provides evidence that plant genotype influences VOC emission at multiple scales within the same native plant system, but also addresses broader ecological and evolutionary questions that have not been investigated previously for this indirect plant defense. The work has potentially wide-ranging implications, some of which are described below.

The variation in VOC emission described here can inform future studies of VOC-mediated interactions among plants. A growing body of work has revealed that VOCs can be exploited as signals within and among plants. Most of these investigations have involved interactions between different plant species; such interactions are expected to be antagonistic. For example, a parasitic dodder (*Cuscuta pentagona*) locates host plants by detecting the VOC emissions of its victims (Runyon et al. 2006). Other plants may "eavesdrop" on the herbivore-induced signals of neighbors (Dicke et al. 2003) and prime their own defenses against future herbivory, thereby gaining a competitive advantage (Karban et al. 2000, Farmer 2001, Heil and Kost 2006, Karban et al. 2006, Kessler et al. 2006, Frost et al. 2008a, Frost et al. 2008b). However, much less is known about VOC-mediated interactions among clonal plants. Clonal network structure may lead to predictable patterns of VOC emission among plants in a population (Stuefer et al. 2004). In the context of kin selection (Hamilton 1964), plants are predicted to warn related neighbors of the presence of herbivores. Indeed, sagebrush plants exposed to VOCs emitted by genetically identical plants suffer less herbivory than sagebrush exposed to VOCs emitted by unrelated plants, suggesting that more effective communication occurs between related individuals (Karban and Shiojiri 2009). Future research can elucidate VOC-mediated interactions among plants within populations, especially among genotypes in clonal species like *Asclepias syriaca*.

Plant VOC emission influences interactions among multiple trophic levels, and the observed latitudinal pattern in herbivore-induced VOC emission is relevant when considering climate change and conservation. Recent work has emphasized the importance of considering trophic interactions when predicting species responses to environmental perturbations (Davis et al. 1998, Voigt et al. 2003, Suttle et al. 2007, Menéndez et al. 2008, Hegland et al. 2009, Berg et al. 2010, Van der Putten et al. 2010). In the face of rapid and extreme climate change, conserving species interactions may be as important as conserving individual species, especially when the possibility of habitat mismatch (Walther et al. 2002) threatens the persistence of the multitrophic interactions that sustain natural communities. Organisms are predicted to shift their geographic range in response to climate change, often toward higher latitudes (Parmesan 1996, Harrington et al. 1999, Parmesan et al. 1999, Harrington et al. 2001, Walther et al. 2002), and plants are expected to undergo genetic changes resulting from founder events during migration (Davis and Shaw 2001). The genetically-based latitudinal cline found for VOC emission contributes to our understanding of geographic patterns related to species interactions, which can aid in the implementation of effective conservation strategies.

The species studied here exhibit macroevolutionary trends in indirect defense, which complement reports of macroevolutionary trends in direct defenses observed in the same system (Agrawal and Fishbein 2008). Plant defense strategies are beginning to be considered as multivariate defense phenotypes instead of single, independent traits (Agrawal and Fishbein 2006, Agrawal 2007), and indirect defenses only recently have begun to be incorporated into established plant defense concepts (Heil 2008). More studies should examine the relationships between direct and indirect defenses to determine how plants balance the costs of implementing a combination of defenses against herbivory (Frost et al. 2008a) and elucidate the relative importance of these defense strategies under different environmental conditions. A multifaceted approach to the study of plant defense in multiple plant species will be crucial to the advancement of our ecological knowledge.

VOCs also strongly link the biosphere, atmosphere, and climate (Monson and Holland 2001), and important feedbacks can occur between VOCs and climate change (Laothawornkitkul et al. 2009). Estimates of ecosystem fluxes of VOCs, important when considering biosphere-atmosphere feedbacks and climate change (Peñuelas 2008), will gain accuracy if studies refine their models to incorporate expected variation at local, regional, and taxonomic scales.

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