

A Genetically-Based Latitudinal Cline in the Emission of Herbivore-Induced Plant Volatile Organic Compounds

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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 compared to high latitudes as an adaptive plant response to higher herbivore pressure on low-latitude plants. To date, this prediction has not been examined with respect to volatile organic compounds (VOCs) that many plants emit, often thus attracting the natural enemies of herbivores. Here, we compared genetically-based constitutive and herbivore-induced aboveground vegetative VOC emissions from plants originating across a gradient of more than 10° 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 influenced emissions. Monarch damage increased plant VOC concentrations and altered VOC blends. We found that genetically-based induced VOC emissions varied with the latitude of plant population origin, although the pattern followed the reverse of that predicted—induced VOC concentration increased with

increasing latitude. This pattern appeared to be driven by a greater induction of sesquiterpenoids at higher latitudes. In contrast, constitutive VOC emission did not vary systematically with latitude, and the induction of green leafy volatiles declined 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.

Keywords Common milkweed *Asclepias syriaca* · Herbivory · Indirect defense · Latitudinal gradient · Monarch butterfly *Danaus plexippus* · Volatile organic compounds

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; Menéndez et al. 2008; Voigt et al. 2003). Ecological processes important at the local scale can retain their importance at the macroecological scale, and it is necessary to consider multitrophic 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; Thompson 2005; Zangerl and Berenbaum

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2003). 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; Rasmann and Agrawal 2011; Schemske et al. 2009). 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, 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 (Chamberlain and Holland 2009; Moles et al. 2011a; Pemberton 1998) occur more frequently on plants at low latitudes. These patterns contrast with leaf domatia associated with mites, which occur more frequently in temperate than tropical systems (O'Dowd and Willson 1991; Weber et al. 2012).

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 (De Moraes et al. 1998; Dicke et al. 1998; Thaler 1999) and appears to be common in nature (Farmer 2001; Kessler and Baldwin 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 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 and Materials

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) (Mooney and Agrawal 2008; Prysby 2004). 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). We have shown that feeding damage by monarchs attracts predators to sites of milkweed herbivory and causes increased attacks to models of caterpillars (Wason 2012).

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° of latitude (>1,500 km) from the southernmost population in Knoxville, Tennessee to the northernmost population in Quebec City, Quebec, Canada. Using up to 10 seeds 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-l 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 (Fig. 1). A population in Pennsylvania (40.33°N) and a population in New Hampshire (43.7°N) were excluded from the experiment due to low germination rates.

Volatile Collections Experimental monarchs were obtained from a commercial monarch breeder (Flutterby Gardens, Bradenton, Florida, USA) and raised indoors (about 100 m from the experimental plants) on *A. syriaca*. Experiments were conducted between July 22 and July 29, 2009. One 3rd-instar caterpillar was placed on one of the leaves in the 5th leaf pair of each treatment plant. Damaging leaves of the same developmental stage controlled as much as possible for

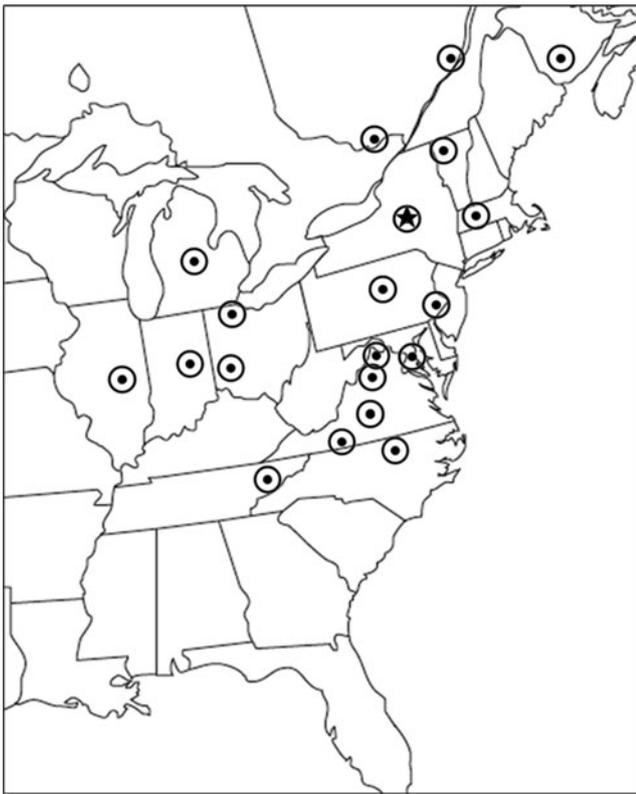


Fig. 1 The locations of 20 *Asclepias syriaca* populations from which seeds were collected to establish an experimental common garden in Ithaca, NY, USA. The star indicates the location of the common garden

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 hr). Netting also was placed over one of the leaves in the 5th leaf pair on each control (undamaged) plant to control for any effect of netting on VOC production. We excluded natural aboveground herbivory from experimental plants by placing netting on all plants after removing any observed herbivores. The enclosure netting remained on plants for 3 days prior to any exposure to experimental damage and subsequent VOC sampling. Natural herbivore damage by chewing herbivores was negligible in the common garden (Woods et al. 2012); nonetheless, we took care to sample plants that showed little to no natural damage. Furthermore, root herbivores (*Tetraopes tetraophthalmus*) were excluded from all plants in the common garden. The *Tetraopes* beetle lays eggs in hollow grass stems (McCauley 1983), and hatching larvae overwinter in the soil and feed on *Asclepias* roots (Davis 1984). To prevent colonization by these beetles, the common garden was established on a mowed site, and landscape fabric prevented both grass growth and herbivory by *Tetraopes*.

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, WI, 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 min). Whole-plant VOCs were sampled continuously for 5 hr 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. We had planned to sample three genotypes from each of 20 plant populations, with three replicate plants per genotype, for a total of 180 plants. However, three replicates were not available for every genotype, and by the end of the experiment, we had sampled VOCs from a total of 160 plants. Because compounds emitted by only one plant and compounds that comprised less than 1 % of the total concentration of VOCs across all plants were omitted from the analyses (see *Analysis and Identification of Volatile Compounds* in the *Methods and Materials* section), many of the original plant replicates were excluded. Thus, the results presented here include VOC collections from 81 plants (control $N=36$ plants, herbivore-damaged $N=45$ plants) originating from the 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 (Eq. 1; $R^2=0.6019$, $F_{2, 97}=177.99$, $P<0.001$) to estimate the aboveground biomass of each experimental plant. The equation was derived using 100 ramets from a natural population of *A. syriaca* plants at the University of Michigan Biological Station in Pellston, Michigan.

$$\text{biomass} = (0.092 \cdot \text{leaves}) + (0.101 \cdot \text{height}) - 0.480 \quad (1)$$

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 Volatile Compounds 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 \times 0.25 mm inner diam 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 $^\circ\text{C}$, initial column temperature at 50 $^\circ\text{C}$ held for 10 min, ramped at 5 $^\circ\text{C min}^{-1}$ to 200 $^\circ\text{C}$, 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 peaks subsequently was verified where possible using authentic standards (Sigma-Aldrich, St. Louis, MO USA; MP Biomedicals, Solon, OH, USA). We obtained specific authentic standards based on the plant VOCs that have been commonly reported in the literature. The identity of 12 peaks remained unverified. The chemical classes of those unknown peaks (green leafy volatiles, monoterpenes, and sesquiterpenes), however, were estimated tentatively according to the number of carbons in the compounds proposed by the MS and peak retention times. Green leafy volatiles (GLVs) were peaks with 6–8 carbons; monoterpenes/monoterpenoids contained 10–12 carbons; sesquiterpenes/sesquiterpenoids contained 15–16 carbons. Our estimates took into account the top three identifications proposed by the MS. One of the peaks could not be identified to chemical class, because the MS did not provide a consistent molecular formula for the peak.

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 (see Supplemental Material Online Resource 1). The peaks omitted from the analyses did not show a latitudinal pattern in concentration ($P > 0.05$ for control, herbivore-damaged, and combined plants; see Supplemental Material Online Resource 2). 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. VOC concentrations were \ln -transformed prior to all analyses, and all 16 peaks were analyzed statistically (not just the 4 peaks that were identified). To address the first question, we employed mixed model analysis of variance (SAS Version 9.2, SAS Institute, Cary, NC, USA) 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 (E. L. Wason, *unpublished data*) 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, R Foundation for Statistical Computing, Vienna, Austria) 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 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 permutational MANOVA. Herbivore treatment and latitude were specified as independent variables in the analysis.

In addition, we used weighted linear regressions and permutational MANOVAs to investigate latitudinal patterns in the types of chemicals emitted by milkweed across latitude. The three chemical classes observed in milkweed emissions (GLVs, monoterpenes, and sesquiterpenes) were regressed separately against latitude; control and herbivore-damaged plants were evaluated separately for each chemical class. As described above, the concentration of VOCs was averaged for all plants within a site. We weighted the analyses by $1/\text{variance}$, except for GLVs emitted by control plants, where single replicates at some sites across latitude prevented weighted regression. The sample size decreased markedly in some of these regressions because each analysis included a subset of the data, and not all chemical classes were produced equally by all plant populations.

Results

Monarch caterpillar damage induced a 62 % increase in VOC emission, on average, from common milkweed plants (Fig. 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 (Fig. 3; $F_{1, 79}=4.841$, $P=0.03$) and PAR, but 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 (Fig. 4a; $F_{1, 15}=19.68$, $P<0.001$). Visually, it

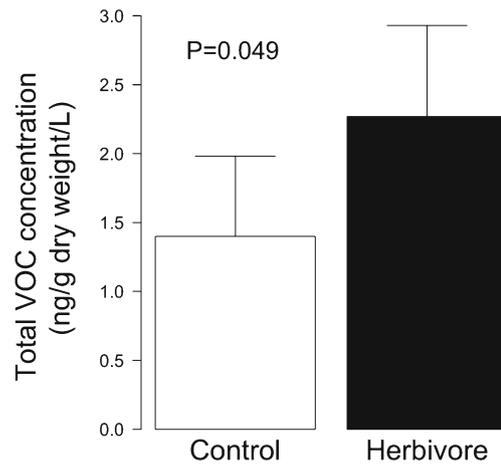


Fig. 2 Mean volatile organic compound (VOC) emission (+1 SE) from undamaged (control; $N=36$) and caterpillar-damaged (herbivore; $N=45$) *Asclepias syriaca* plants growing in a common garden in Ithaca, NY

appears as if a quadratic model could better explain the trend in Fig. 4a, but a quadratic model ($F_{2, 14}=9.26$, $P<0.01$) was a poorer fit than the linear model according to AIC scores (AIC, linear=56.3, quadratic=58.2) and no improvement using log likelihood ($P=0.79$) in this weighted regression. Constitutive VOC emission did not vary predictably with latitude (linearly or quadratically, Fig. 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 $F_{3, 27}=17.56$, $P<0.001$), indicating that the magnitude of induction varied predictably with latitude, but in the opposite direction to our prediction.

Which classes of VOC were responsible for the latitudinal trend in induced VOC emission? In damaged milkweed plants, GLV ($F_{1, 5}=22.91$, $P<0.01$) and sesquiterpene ($F_{1, 7}=28$, $P=0.001$) emissions varied systematically across latitude. Sesquiterpene emissions from damaged plants increased with increasing latitude, thus appearing to drive the observed latitudinal pattern in total induced VOC emission. In contrast, GLV emissions from damaged plants decreased with increasing latitude, counter to the pattern observed for total VOC emissions. Undamaged milkweed emitted GLVs ($F_{1, 4}=1.61$, $P=0.27$) and sesquiterpenes ($F_{1, 4}=0.88$, $P=0.40$) in similar concentrations across latitude. Monoterpene concentrations showed no pattern across latitude in either constitutive ($F_{1, 10}=1.02$, $P=0.34$) or induced ($F_{1, 14}=2.05$, $P=0.17$) emissions.

Herbivory by monarch caterpillars also altered milkweed's 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 (Fig. 5; $F_{19, 80}=1.28$, $P=0.02$). Temperature, humidity, and PAR (PCA axis 1) marginally influenced VOC blend among

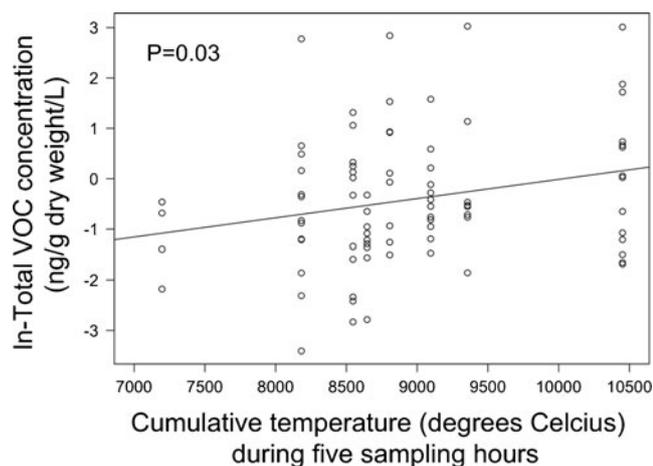


Fig. 3 The effect of temperature on volatile organic compound (VOC) emission from all *Asclepias syriaca* plants growing in a common garden in Ithaca, NY. Temperatures were recorded once per min for 5 sampling hours each day. The temperature data were integrated, yielding a cumulative value for each day

populations ($F_{1, 80}=1.80$, $P=0.05$). Monarch herbivory ($F_{1, 70}=2.83$, $P<0.01$) and latitude of origin ($F_{19, 70}=1.30$, $P=0.04$) influenced the relative concentrations of monoterpenes emitted by milkweed. The sesquiterpene blend was unaffected by monarch herbivory ($F_{1, 49}=1.15$, $P=0.33$) and marginally influenced by plant origin ($F_{18, 49}=1.54$, $P=0.059$). Relative concentrations of GLVs changed in response to herbivory ($F_{1, 25}=5.95$, $P<0.01$) but did not vary among plant populations ($F_{13, 25}=1.55$, $P=0.13$).

Four of the 16 VOCs emitted by milkweed plants were positively identified using authentic standards. Three of the compounds identified have been shown to increase in concentration following herbivore damage or have been implicated in plant defense: 1,8-cineole (Kessler et al. 2006; Wiens et al. 1991), *cis*-3-hexen-1-ol (Kessler et al. 2006; Ruther and Kleier 2005), and β -caryophyllene (Rasmann et al. 2005; Wiens et al. 1991). Of the 16 peaks, 2 were GLVs (including *cis*-3-hexen-1-ol), 10 were monoterpenes (including 1,8-cineole and eugenol), and 3 were sesquiterpenes (including β -

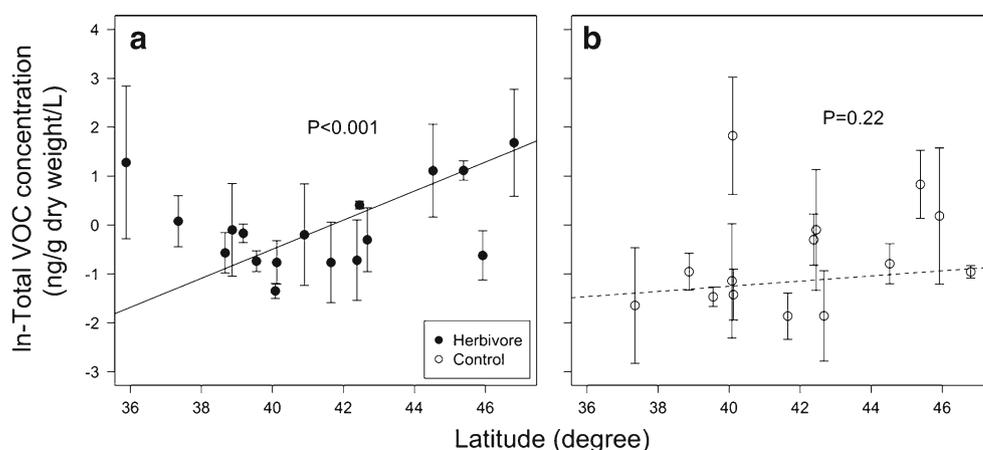
caryophyllene). The remaining compound could not be identified to chemical class, because the MS did not report a consistent molecular formula for the peak.

Discussion

This experiment investigated intraspecific variation in induced indirect defenses across a latitudinal gradient. Based on previous studies of direct (Coley and Barone 1996; Penning 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 (Fig. 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 (Fig. 2) and a different blend ratio of VOCs emitted by milkweed plants. The composition of the VOC blend also varied among milkweed populations (Fig. 5), although we observed no systematic change in VOC blend with latitude.

Although we have not yet identified all of the VOCs emitted by milkweed plants, the increasing induction of volatiles with latitude appears to be driven by sesquiterpenes, including β -caryophyllene. In contrast, induction of GLVs (including *cis*-3-hexen-1-ol) declined with latitude, counter to the trend shown for total VOC induction. Both sesquiterpenes and GLVs have been implicated in indirect plant defense (Frost et al. 2007; Rasmann et al. 2005). However, the ecological implications of variation in VOC concentration and blend are not fully 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

Fig. 4 Total concentration (ln-transformed) of volatile organic compound (VOC) emissions (± 1 SE) from *Asclepias syriaca* plants originating from 20 populations across 10° 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)



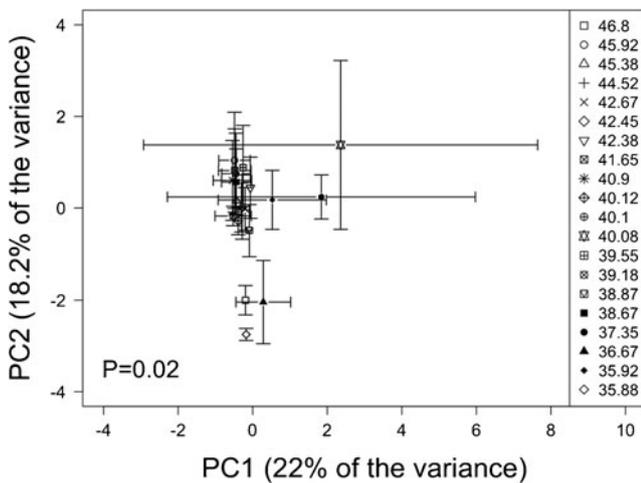


Fig. 5 Principal components analysis of 16 volatile organic compounds (VOCs) emitted by *Asclepias syriaca* plants, indicating the difference in composition of VOC blend among plant populations. Data were normalized, and standard error is shown for all 20 populations. The legend indicates the latitude of origin of the plant populations depicted. Plants originated from 20 populations across 10° of latitude and were grown in a common garden in Ithaca, NY

natural enemies can respond more strongly to VOC blend (Bruce et al. 2010). In addition, individual chemicals isolated from VOC blends can elicit varied responses in natural enemies (Das et al. 2007; Späthe et al. 2013). 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 *A. 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 (Fig. 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 induction at high latitudes may relate to higher latex production by high-latitude milkweed plants (Woods et al. 2012). This possibility assumes that the volatile profile of milkweed contains other terpenes, in addition to the β -caryophyllene we identified, that contribute to latex production in the plant. Indeed, 13 of the 16 compounds we analyzed were mono- or sesquiterpenes, and emissions of the latter increased with latitude. 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 (Prysbly 2004) and in other systems (Dyer and Coley 2004).

Fourth, we cannot exclude the possibility that preferential feeding by experimental monarch larvae on high-latitude plants could have produced the observed 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), and it remains possible that caterpillars consumed greater quantities of high-latitude plants due to their potentially higher palatability (Pennings et al. 2001, 2009). We were unable to quantify monarch damage on our experimental milkweed; thus, the influence of latitude and a potential effect of variation in herbivore damage across latitude may be confounded. However, 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 do not explain greater VOC emission at higher latitudes.

It is unlikely that uncontrolled natural herbivory produced the patterns we observed here. In the common garden used for this experiment, chewing herbivores were too few to count, and natural herbivore damage was negligible (Woods et al. 2012). In the same common garden the previous year, and in a similar common garden established in North Carolina, no correlation was observed between chewing herbivore abundance and latitude of origin (Woods et al. 2012). Additionally, we excluded natural herbivores to the greatest extent possible during our experiments, and we randomly assigned the herbivory treatments to the experimental plants. While no common garden experiment can completely control all variables, important insights remain possible despite the inherent challenges. Regardless of whether herbivore damage varies in extent across latitude and influences VOC concentration, our observation stands that high-latitude plants emit higher concentrations of induced VOCs when attacked by monarchs. The ecological implications of this gradient in VOC emission may have multitrophic ramifications in plant and insect communities, and the phenomenon should be explored further.

While constitutive indirect plant defenses associated with ant mutualists may be stronger at low latitudes in some systems (Chamberlain and Holland 2009; Moles et al. 2011a; Pemberton 1998), 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, occur more often in temperate than tropical plant species (O'Dowd and Willson 1991; Weber et al. 2012). 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 (Chamberlain and Holland 2009; O'Dowd and Willson 1991; Pemberton 1998), 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. Ideally, this common garden experiment would have been replicated at other sites along the latitudinal gradient to take into account local adaptation and plasticity among milkweed populations. Herbivory, light, and temperature appear to unequivocally influence plant VOC emission (Holopainen and Gershenzon 2010; Kesselmeier and Staudt 1999), and our common garden consisted of a field environment with natural vegetation, light, and temperature. The experiment serves as an intermediate strategy between a lab study (which is most common in the literature, but also the least realistic) and a totally natural study in which genotypes and environment are confounded. Although our results come from one common garden of *A. syriaca* at the center of its geographic range, we found an intriguing pattern in VOC emission that can be tested further in future experiments.

In addition to the observed cline with latitude, we found that plants from different populations substantially varied in their VOC concentrations and blends. An increasing number of studies have reported geographic variation in vegetative VOC emission from populations of cotton (Loughrin et al. 1995), *Datura wrightii* (Hare 2007), *Nicotiana attenuata*

(Schuman et al. 2009), and *Brassica nigra* (Gols et al. 2009). Likewise, differences in VOC emission were reported 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 adds to the growing evidence for geographic variation in vegetative VOCs emitted by native plant species. Furthermore, genetic variation in VOC emission has been observed within plant populations. For example, different genotypes of the native plants horsetnettle (Delphia et al. 2009) and *Nicotiana attenuata* (Schuman et al. 2009) vary in their VOC emissions, and a similar observation has been made in the milkweed system (E. L. Wason, unpublished data). While the heritability of tolerance remains unknown in this system, herbivore resistance is understood to be heritable in milkweed plants (e.g., Vannette and Hunter 2011). Different specialist herbivores, such as caterpillars (Bingham and Agrawal 2010; Vannette and Hunter 2011), aphids (Mooney et al. 2010), beetles (Agrawal 2004), and weevils (Agrawal and Van Zandt 2003) can induce different defense responses in milkweed. Because plant responses vary with herbivore species and environmental conditions, further investigation of milkweed VOC emission under different conditions would be worthwhile.

Although we have focused on the role of VOC emission in mediating tritrophic interactions, VOCs can play multiple roles in the interactions between plants and their environment. Other major functions of VOC emission include within-plant signaling, inter-plant signaling, direct defense against biotic and abiotic stresses, and synergistic defense (Heil and Karban 2010). In fact, while the role of VOCs in tritrophic interactions has been studied most extensively, the adaptive value of this function for the plant has not been demonstrated under natural conditions. Other important stresses, such as temperature, oxidation, and pathogens, may vary across latitude and provide plausible explanations for the latitudinal pattern that we observed. For example, increased VOC emission may enable high-latitude plants to shield their herbivore-induced lesions from pathogen invasion. Indeed, we observed that milkweed emits 1,8-cineole, a chemical with antifungal properties (Marei et al. 2012); *cis*-3-hexen-1-ol, which has been implicated in plant response to (Croft et al. 1993) and defense against (Arimura et al. 2001; Kishimoto et al. 2005) pathogens; and β -caryophyllene, which has been shown to directly inhibit bacterial pathogens (Huang et al. 2012); not to mention the unidentified milkweed volatiles that may exhibit activity against pathogens. We, thus, might expect that a high diversity or abundance of pathogens would correspond to higher induced plant VOC emission. However, at least for pathogens that afflict humans, the diversity and abundance of pathogens appear to decrease with increasing latitude (Guernier et al.

2004), while we observed a pattern of increasing VOC emission with increasing latitude. Unfortunately, data describing latitudinal patterns in plant pathogens are scarce. Because we observed the cline in VOC emission only for herbivore-damaged plants, it seems reasonable to correlate the pattern with biotic, instead of abiotic, stress. The data presented here indicate a clear pattern in VOC emission, but we can only speculate about the factors that drive the cline, which could involve plant signaling, tritrophic interactions (indirect defense), pathogens (direct defense), synergy among multiple plant defenses, or possibly abiotic stress.

Our results suggest that herbivore contributions to plant VOC emission may vary predictably at regional scales. 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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