

# Optimal defense theory explains deviations from latitudinal herbivory defense hypothesis

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**Abstract.** The latitudinal herbivory defense hypothesis (LHDH) postulates that the prevalence of species interactions, including herbivory, is greater at lower latitudes, leading to selection for increased levels of plant defense. While latitudinal defense clines may be caused by spatial variation in herbivore pressure, optimal defense theory predicts that clines could also be caused by ecogeographic variation in the cost of defense. For instance, allocation of resources to defense may not increase plant fitness when growing seasons are short and plants must reproduce quickly. Here we use a common garden experiment to survey genetic variation for constitutive and induced phenylpropanoid glycoside (PPG) concentrations across 35 *Mimulus guttatus* populations over a ~13° latitudinal transect. Our sampling regime is unique among studies of the LHDH in that it allows us to disentangle the effects of growing season length from those of latitude, temperature, and elevation. For five of the seven PPGs surveyed, we find associations between latitude and plant defense that are robust to population structure. However, contrary to the LHDH, only two PPGs were found at higher levels in low latitude populations, and total PPG concentrations were higher at higher latitudes. PPG levels are strongly correlated with growing season length, with higher levels of PPGs in plants from areas with longer growing seasons. Further, flowering time is positively correlated with the concentration of nearly all PPGs, suggesting that there may be a strong trade-off between development time and defense production. Our results reveal that ecogeographic patterns in plant defense may reflect variation in the cost of producing defense compounds in addition to variation in herbivore pressure. Thus, the biogeographic pattern predicted by the LHDH may not be accurate because the underlying factors driving variation in defense, in this case, growing season length, are not always associated with latitude in the same manner. Given these results, we conclude that LHDH cannot be interpreted without considering life history, and we recommend that future work on the LHDH move beyond solely testing the core LHDH prediction and place greater emphasis on isolating agents of selection that generate spatial variation in defense and herbivore pressure.

**Key words:** biogeography; chemical defense; cline; flowering time; genetic constraint; herbivory; latitudinal herbivory defense hypothesis; life history; *Mimulus guttatus* (common monkeyflower); optimal defense theory; phenylpropanoid glycosides.

## INTRODUCTION

It is widely hypothesized that the importance and intensity of biotic species interactions increases in populations closer to the equator (Dobzhansky 1950, MacArthur 1972, Schemske et al. 2009). A predicted consequence of these intensified species interactions is the evolution of increased resistance and/or tolerance to predators, herbivores, or pathogens in lower latitude populations compared to higher latitude populations (Fischer 1960, Janzen 1970, Connell 1971, Levin 1976, Anstett et al. 2016b). In the plant–herbivore literature, this hypothesized latitudinal gradient in defense is known

as the latitudinal herbivore-defense hypothesis (LHDH; Coley and Aide 1991, Johnson and Rasmann 2011).

Empirical support for the LHDH has been mixed (Coley and Aide 1991, Coley and Barone 1996, Moles et al. 2011a). In support of LHDH, several studies have demonstrated increased herbivore damage (Coley and Barone 1996, Pennings and Silliman 2005, Pennings et al. 2009, Anstett et al. 2015, Wang et al. 2016) or physical or chemical defenses in populations closer to the equator (e.g., Anstett et al. 2015, Wang et al. 2016). However, a number of studies have also found that no association exists between either herbivore damage and latitude or defense and latitude (Andrew and Hughes 2005, Adams and Zhang 2009, Salazar and Marquis 2012). In addition, some studies have observed the opposite association, with levels of damage or defense increasing further from the equator (e.g., Adams et al. 2009, Moles et al. 2011b).

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The discrepancies among these studies may be explained, in part, by the difficulty in testing the LHDH comprehensively due to the scale of the sampling required (Anstett et al. 2015, 2016b, Wang et al. 2016). It is logistically difficult to find a widespread species that spans sufficient latitudinal breadth to evaluate the LHDH, and thus most work has involved multiple species from the same genus or family that in combination span a broad latitudinal range (Levin 1976, Rasmann and Agrawal 2011, Pearse and Hipp 2012). While different species in a family may have different levels of a defense due to spatial variation in herbivore pressure, different levels of a particular defense can also be a phylogenetic artifact or due to absence of other defenses in a particular species (Johnson and Rasmann 2011).

Inconsistent results across studies could also reflect the complex suite of selective forces that shapes spatial variation in plant defense and the logistical difficulty in disentangling the impact(s) of specific pressures (Johnson and Rasmann 2011, Moles et al. 2011a, Salazar and Marquis 2012). For instance, in addition to tolerance to herbivory, an individual plant species may have a wide arsenal of constitutive and/or induced physical and chemical resistance traits. Selection likely acts on each particular mode of defense at least partially independently, thus measuring any single defense may not adequately represent total defense investment (Berenbaum 1996). Modes of defense can also be confounded if phenotypes are measured from field-grown plants; without growing plants in a common garden environment, constitutive levels of defense cannot be distinguished from those induced by field herbivory (Rasmann and Agrawal 2011).

Several hypotheses seek to explain variation in levels of plant defense across tissues and within or among populations (Stamp 2003). One of the leading hypotheses, optimal defense theory, predicts that plants will evolve levels of defense that are positively related to levels of herbivory and negatively related to allocation or ecological costs (Rhoads 1979, Stamp 2003). Spatial variation in defense could thus be generated by differential rates of herbivory as well as by differential costs of allocating resources to defense. For example, annual plants inhabiting environments with shorter growing seasons may allocate fewer resources to defense and more resources towards rapid development and reproduction than populations inhabiting environments with longer growing seasons, even if herbivore pressure is equal across populations.

Disentangling the primary selective forces underlying spatial heterogeneity in plant defense is challenging, as the intensity of herbivore pressure will often covary with abiotic factors along an environmental gradient. Growing season length and average temperature are both thought to affect herbivory levels and typically increase as latitude decreases. Longer growth seasons lead to stable environments with less abiotic stress, more possible herbivore generations per season, and greater herbivore abundance (Janzen 1970, Roff 1980, Mitton and Ferrenberg 2012).

Higher temperatures may promote greater herbivore metabolic rates and more rapid development (Hillebrand et al. 2009, Lemoine et al. 2014). One approach to overcome these correlated patterns is to examine plant defense patterns along environmental gradients where temperature, growing season, and herbivore pressure do not covary, and in doing so, determine which factors best explain variation in plant defense. Comparative studies of elevation gradients can be an effective means to achieve this goal. Some elevation gradients mimic latitudinal gradients with higher temperatures, longer growing seasons and greater herbivore pressure at lower elevations (Pellissier et al. 2014, Rasmann et al. 2014), while others may have longer growing seasons or greater herbivore pressure at high elevations. For example, low elevation populations in the Sierra Nevada in California have shorter growing seasons than high elevation populations for some annual plant species because reduced seasonal water availability leads to early and rapid onset of terminal summer drought (Kooyers et al. 2015).

In this study, we test the predictions of the LHDH by exploring patterns of genetic variation in plant resistance traits and herbivory in annual *Mimulus guttatus* populations. Our study uses a common garden experiment leveraging widespread sampling along a latitudinal gradient in combination with two elevational gradients in order to tease apart the effects of temperature, growing season length, and levels of herbivory on genetically based phenotypic variation in a suite of phytochemical defenses, phenylpropanoid glycosides (PPGs), across populations. First, we characterize range-wide patterns of variation and covariation of constitutive and induced levels of PPGs. Second, we evaluate whether constitutive or inducible levels of individual PPGs or total PPGs increase with decreasing latitude, consistent with the LHDH. Third, by using elevation transects to examine the relationships between temperature, growing season length and PPG levels, we test for patterns consistent with optimal defense theory. Finally, we test whether herbivory levels vary with latitude in the field and thus assess the potential influence of herbivore pressure on our observed spatial patterns in plant defense.

## METHODS

### *Study system*

*Mimulus guttatus* is a model species for ecological genetics with populations found from northern Mexico to central Alaska and from sea level to 3000 m in elevation (Vickery 1978, Wu et al. 2008). Across its range, *M. guttatus* has adapted to environments that vary drastically in the timing and the length of the growing season (Hall and Willis 2006, Lowry et al. 2008, Hall et al. 2010, Kooyers et al. 2015). For annual populations of this species, growing season timing is determined by water availability. Their habitats are typically temporary streams, seepy meadows, and rock walls where water is

continually available only for short time intervals each year. Growing seasons begin with winter/spring rainfall or with spring snowmelt and last until terminal summer droughts. Annual monkeyflower populations in the Central Valley of California and in high elevation populations in the Cascades have growing seasons lasting only two months or less, while annual populations in the Willamette Valley and coastal mountains of Oregon have growing seasons of about five months. Populations with short growing seasons employ a rapid growth and reproduction life history strategy, whereby plants develop and flower more quickly than plants from populations with longer growing seasons (Kooyers et al. 2015).

*Mimulus guttatus* has a substantial physical and chemical defense arsenal for resistance to herbivores. Phenylpropanoid glycosides (PPGs) are the dominant bioactive secondary compounds in *M. guttatus* implicated in plant defense against herbivores (Holeski et al. 2013, Keefover-Ring et al. 2014). PPGs are synthesized via the shikimic acid pathway, which is the source of a wide array of secondary compounds across higher plant species (Knaggs 2003, Fraser and Chapple 2011). PPGs typically consist of caffeoyl or hydroxytyrosol moieties bonded to a central  $\beta$ -glucopyranose sugar (Mølgaard and Ravn 1988). PPGs act as generalist herbivore feeding deterrents (Cooper et al. 1980, Mølgaard 1986, Rotter and Holeski 2016) and as specialist herbivore feeding stimulants (Holeski et al. 2013; M. Rotter and L. M. Holeski, *unpublished data*), and the production of these compounds is genetically variable within and among natural populations (Holeski et al. 2013, 2014).

#### *Sampling design and common garden experiment*

We sampled populations along elevational gradients in the Sierra Nevada Mountains and Cascade Mountains in order to disentangle growing season length from temperature (sampling described in Kooyers et al. 2015). In the Sierra Nevada, growing seasons are short in low elevation populations in the Central Valley, but are considerably longer in the snowmelt-fed populations at higher elevations. The opposite pattern is observed in the Oregon Cascades; low elevation populations have long temperate growing seasons while high elevation populations have much shorter growing seasons.

We conducted a common garden experiment utilizing seed from 35 of the populations that were initially described in Kooyers et al. (2015). For this study, we focused on populations that were either sampled at low elevation along a latitudinal gradient stretching from southern California to central Oregon (about half its latitudinal range) or that were sampled along elevation gradients in the central Sierra Nevada or the Oregon Cascades (Fig. 1). Population climatic data and growth season data were also obtained as in Kooyers et al. (2015) (Data S1). We used 6–10 lines from each population; each line was bred via selfing and single seed descent for two generations under greenhouse conditions. Seeds

were planted in Fafard 3B soil in 2.5" square pots, cold stratified for 1 week at 4°C, and germinated on a mist bench. Germination status was recorded daily. After 1 week on the mist bench, plants were sorted into treatment groups (damaged or control) and randomized across 40 flats. Flats were rotated every 3–4 d to minimize variation due to spatial heterogeneity in greenhouse conditions. Greenhouse conditions were 16 h days (19.4°C) and 8 h nights (17.2°C). Plants were bottom-watered and were not given supplemental fertilizer. We applied a damage treatment by punching a hole in each first true leaf with a one-hole paper punch after the second true leaves had unfolded (~15–20 d after germination). A small number of plants affected by greenhouse pests were replaced. Plants were surveyed daily to determine time to first flower from germination (hereafter, flowering time). For phytochemical analysis, we collected both true leaves from the second, third, and fourth nodes of each plant after those from the fifth node were unfolded and expanded. Leaves were flash frozen then freeze dried using a pre-chilled FreeZone 4.5 System (Labconco, Kansas City, Missouri, USA).

#### *Phytochemical analysis*

Plant tissue was ground and PPGs were extracted as described previously (Holeski et al. 2013, 2014). We quantified PPGs via high-performance liquid chromatography (HPLC; Agilent 1260 HPLC with a diode array detector and Poroshell 120 EC-C18 analytical column [4.6 × 250 mm, 2.7  $\mu$ m particle size]; Agilent Technologies, Santa Clara, California, USA) maintained at 30°C.

HPLC run conditions included a binary mobile phase gradient with 3.1 mmol/L phosphoric acid as mobile phase A, and acetonitrile as mobile phase B at a constant total flow rate of 0.75 mL/min. The gradient for each run consisted of B initially set at 5%, 15% at 5.5 min, 16% at 7.5 min, 13% at 9 min, 20% from 10 to 12 min, 60% at 13 min, and 95% from 14 to 15 min. From 15 to 16.5 min, mobile B returns to 0% to re-equilibrate the column. We injected 3 mL of the standards and samples, monitored ultraviolet signals at 274 (catechol) and 340 nm (PPGs), and used a diode array detector to collect ultraviolet data from 190 to 400 nm. We calculated PPG quantities as verbascoside equivalents, using a standard solution of pure verbascoside (Santa Cruz Biotechnology, Dallas, Texas, USA) as described previously (Holeski et al. 2013, 2014).

#### *Summarizing range-wide variation in PPG production*

Before testing adherence to the LHDH, we conducted several analyses to identify correlations between individual PPGs and to determine whether individual PPGs were induced by the damage treatment. To detect whether production of an individual PPG was correlated with production of other PPGs, we calculated pairwise correlations between log-transformed secondary compound levels using the Pearson product-moment coefficient. Because

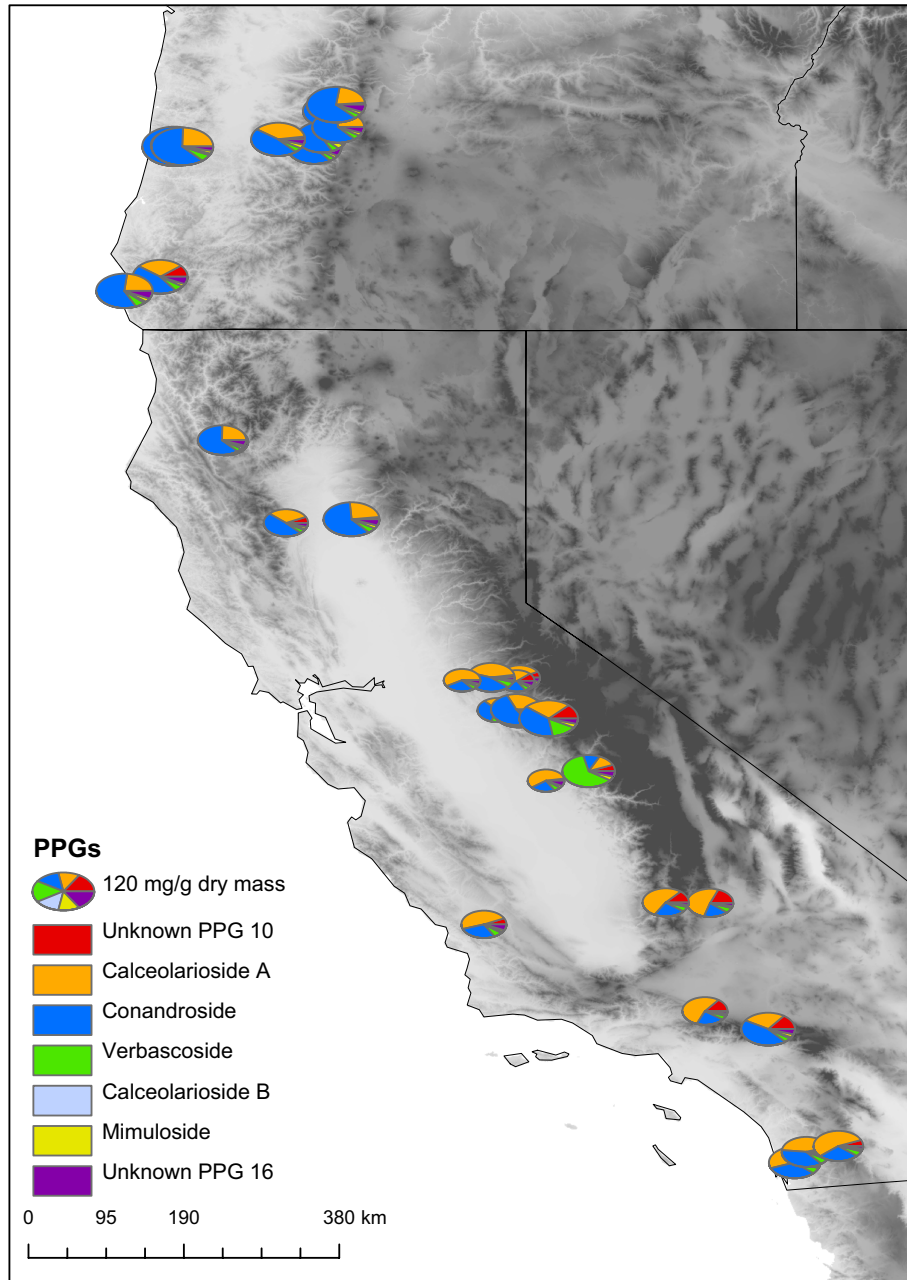


FIG. 1. Absolute and relative concentrations of phenylpropanoid glycosides (PPGs) across sampling locations of *Mimulus guttatus*. Each population sampled is represented by a pie chart with the chart area proportional to the total PPG concentration detected. Individual PPGs are represented by different colored pie slices. White to black gradient represents elevation from low to high elevation, respectively. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

there were highly significant correlations between PPGs, we reduced the dimensionality of the data set by conducting a principal component analysis on  $z$ -score transformed PPG levels using the `prcomp()` call in R 3.0.1 (R Foundation for Statistical Computation, Vienna, Austria). To determine whether damage treatment induced greater production of PPGs, we used a linear mixed model (LMM) approach implemented using the restricted maximum likelihood model in the `lme4` package in R

(Bates et al. 2015). Univariate models for each PPG were run independently as was a univariate model for total PPGs (the sum of all PPGs produced for an individual). Damage treatment (either damaged or undamaged) was modeled as a fixed variable, while populations, line (nested within population), HPLC run and flat were modeled as random variables. Statistical significance of damage treatment was determined by ANOVA using a Wald chi-square test with one degree of freedom implemented in the

car package (Fox et al. 2013). Model coefficients were used to determine whether damage treatment induced or reduced the production of individual PPGs.

*Detecting latitudinal and elevational clines  
in defense compounds*

We used the same LMM approach described in *Summarizing range-wide variation in PPG production* to investigate the presence of latitudinal gradients in PPGs. We log-transformed three PPGs (verbascoside, mimuloside, and unknown PPG 16) prior to analysis. Individual PPGs as well as untransformed Chem PC1, Chem PC2, and total PPGs were used as response variables in univariate LMMs. To identify potential latitudinal gradients in defense compounds, we modeled damage treatment and latitude as fixed factors, and population, line (nested with population), HPLC run, and flat as random variables. Statistical significance was determined through ANOVA. The results support the LHDH if there is a significant association between an individual PPG and latitude where PPG production increases as latitude decreases.

Clinal PPG patterns could be attributed to selection or could reflect population structure due to strictly demographic processes such as drift–migration balance (Vasemagi 2006, Keller et al. 2009). We used data from three microsatellite markers (AAT296, AAT217, and AAT278; Kelly and Willis 1998) and four exon-primed intron containing markers (MgSTS571, MgSTS617, MgSTS474, and MgSTS278; Lowry et al. 2008) previously genotyped on different plants from the same lines used in this experiment to test whether associations between latitude and PPG concentrations persisted after controlling for population structure (Kooyers et al. 2015). Amplification details and summary statistics for these markers are included in Kooyers et al. (2015). We extracted genotype data from three to nine lines (mean 5.8) per population for 22 populations (127 total lines reflecting 580 individuals in our data set). There was <6.5% missing data at each marker. As reported in Kooyers et al. (2015), there was significant signal of isolation by distance that could generate clinal patterns in functional traits.

To determine whether or not PPG clines were artifacts of demographic processes, we included a measure of genetic distance as a covariate in the LMMs described above. We performed a principal coordinates analysis using the *ade4* and *ade4* libraries in R. The first two principal coordinate axes were included as fixed effects in LMMs and are referred to as genetic PC1 (10.6% variation) and genetic PC2 (7.1% variation) in the results. All plants from a maternal line were given the same genetic PC values. If associations between PPG concentration and latitude are products of natural selection, the effect of latitude will remain significant despite the inclusion of the genetic covariates. However, if the presence of genetic covariates removes the effect of latitude, the possibility that original association between latitude and PPG concentration reflects processes other than natural selection cannot be excluded.

*Using elevational transects to disentangle environmental variables that latitudinally covary*

To better understand the selective pressures that create associations between PPGs and latitude, we examined associations between a number of abiotic factors and PPG concentrations along two elevation gradients that disentangle temperature, precipitation, and growing season length. Abiotic factors included annual temperature, annual precipitation, seasonality, elevation, latitude, growing season length, and a climate PC axis. The climate PCA was performed incorporating all 19 BIOCLIM variables (Hijmans et al. 2005) as well as annual aridity and July evapotranspiration variables from Consultative Group on International Agricultural Research–Consortium for Spatial Information (CGIAR-CSI; Zomer et al. 2008) using the *prcomp()* call in R. We extracted PC1 (termed climate PC1; 70.5% of total variance) for each population. Higher values of climate PC1 were associated with higher annual temperature and lower annual precipitation. We calculated growing season length index as the relative amount of precipitation during the growing season divided by the start date of the growing season. Start date of the growing season was defined as the date minimum temperature averaged 4°C over the last 100 yr. This index is an excellent representation of the relative growing season lengths of most annual *M. guttatus* populations (N. J. Kooyers, *personal observation*). However, we reduced our data set to include only populations that occur in the Sierra Nevada and Cascade Mountain ranges (27/35 populations) because this metric of growing season length was not appropriate for populations in Southern California. These data sets were used in LMMs with PPG levels as univariate response variables; damage treatment and an abiotic factor as fixed factors; and population, line (nested with population), HPLC run and flat as random variables. Statistical significance of each fixed effect was determined by ANOVA. If the cost of defense production is an important determinant of variation in defense, we expect that populations with shorter growing seasons should have lower concentrations of PPGs. In addition, we expect to find stronger correlations between growing season length and individual PPG concentrations than between other abiotic variables and PPG concentrations.

If allocation costs lead to reduced PPG concentrations in environments with short growing seasons, then reduced PPG levels may be a component of a rapid growth and reproduction syndrome often observed for annual *M. guttatus* populations. We investigated potential correlations between production of PPGs and flowering time, a key trait associated with rapid development in annual populations (Hall and Willis 2006, Kooyers et al. 2015). We calculated pairwise correlations between line means, pooling individuals in both the damaged and undamaged treatments since these treatments had little impact on the results of this analysis. A significant positive correlation between flowering time and a PPG level

(e.g., later flowering time associated with higher PPG concentration) suggests that an allocation cost exists for either producing a particular PPG or for flowering earlier.

#### *Field herbivory observations*

In April 2016, we surveyed foliar damage within 13 low elevation populations across the latitudinal transect. A single observer (N. Kooyers) recorded the percentage of damaged area on every pair of true leaves for 8–12 plants per population. We used LMMs to model the association between latitude and foliar damage. Damage was log-transformed to improve model fit. Latitude, leaf pair, and latitude  $\times$  leaf pair interaction were treated as fixed effects in the model; population and plant (nested within population) were used as random effects. This model explicitly controls for differences due to variation in developmental states between plants. This was necessary as the growing season in low latitude populations begins earlier than for high latitude populations, and leaf damage accumulates on foliar tissue throughout the growing season. The significance of fixed effects was determined as above. The LHDH predicts that there should be higher herbivory at low latitudes. As a parallel analysis, we averaged the foliar damage on the first two pairs of true leaves. When we used this average as a response variable in an LMM, the relationship between latitude and leaf damage was qualitatively similar to the full model (data not shown).

## RESULTS

### *PPG levels are highly variable across the range of annual Mimulus guttatus*

PPGs accounted for a large percentage of dried leaf mass across samples in our experiment, making up an average of 8% of dried leaf mass (minimum 3.4%, maximum 37.1%, Table 1). We found no evidence that the damage treatment induced greater PPG levels (Table 1; Appendix S1: Table

S1). Rather, mechanically damaging plants actually reduced concentrations of several PPGs including conandroside ( $\chi^2 = 5.62$ ,  $P = 0.02$ ), calceolarioside B ( $\chi^2 = 8.16$ ,  $P = 0.004$ ), and unknown PPG 16 ( $\chi^2 = 8.44$ ,  $P = 0.004$ ). Variation in each PPG was geographically structured (Fig. 1; Appendix S1: Table S1). Conandroside and calceolarioside A made up the majority of the total PPGs in almost all populations, composing 47.5% and 33.2% of total PPGs on average. The rest of the PPGs generally made up <10% of total PPGs, with the exception of one high elevation population that had an unusually high absolute and relative concentration of verbascoside (SHL; 62% of total PPGs). We found extensive correlations between the PPGs, independent of damage treatment, which largely separated PPGs into two different groups (Fig. 2). These groups were well-summarized by principal component analysis. Chem PC1 (35% variation) was strongly associated with variation in calceolarioside B, conandroside, mimuloside, and unknown PPG 16 where high values were associated with high concentrations of each compound. Chem PC2 (20% variation) was associated with on calceolarioside A, verbascoside, and unknown PPG 10, where higher values indicated high concentrations of calceolarioside A and unknown PPG 10, but lower levels of verbascoside (Appendix S1: Table S2). Geographic regions (either Southern California, Sierra Nevada, or Cascade) clustered together along Chem PC1 and Chem PC2 axes, indicating that there is geographic structure in PPG arsenal composition (Appendix S1: Fig. S1).

### *Latitudinal clines in PPGs are common but vary in directionality*

The LHDH predicts that there should be higher levels of defense in lower latitude populations. To test the LHDH, we explored the relationship between latitude and total PPGs as well as the relationships between latitude and the level of each PPG using models that control for damage treatment, spatial location in the common

TABLE 1. Summary statistics of phenylpropanoid glycoside (PPG) concentrations in the damaged and undamaged treatment.

PPG	Undamaged treatment			Damage treatment			Damage treatment LMM			
	Range	Mean	SD	Range	Mean	SD	$\chi^2$	df	$P$	LMM Coeff.
Unknown 10	0–12.0	3.0	3.7	0–14.1	3.2	4.0	1.1	1	0.290	
Calceolarioside A	11.3–44.3	25.8	6.6	7.4–148.4	29.9	22.0	1.7	1	0.193	
Conandroside	8.6–101.4	38.8	20.9	3.0–189.4	40.8	33.0	<b>5.6</b>	<b>1</b>	<b>0.018</b>	–2.65
Verbasoside	1.8–58.4	5.9	9.5	1.8–48.6	6.1	8.3	1.0	1	0.313	
Calceolarioside B	0.2–1.6	0.8	0.3	0.1–3.6	0.8	0.6	<b>8.2</b>	<b>1</b>	<b>0.004</b>	–0.06
Mimuloside	0.8–5.7	2.1	1.1	0.8–5.2	2.1	1.2	2.3	1	0.133	
Unknown 16	1.4–6.5	3.6	1.5	1.3–6.5	3.4	1.4	<b>8.4</b>	<b>1</b>	<b>0.004</b>	–0.06
Total PPG concentration	33.8–140.4	80.1	23.2	42.6–374.1	86.4	54.1	2.3	1	0.132	

*Notes:* Range, mean, and standard deviation were calculated by pooling all individuals in a treatment. All ranges, means, and standard deviations refer to mg PPG per g leaf dry mass. Damage treatment LMM refers to a linear mixed model with damage treatment as the only fixed variable. Bold values indicate statistical significance of the damage treatment at  $P < 0.05$ . LMM coeff. is the slope extracted from the linear mixed model.

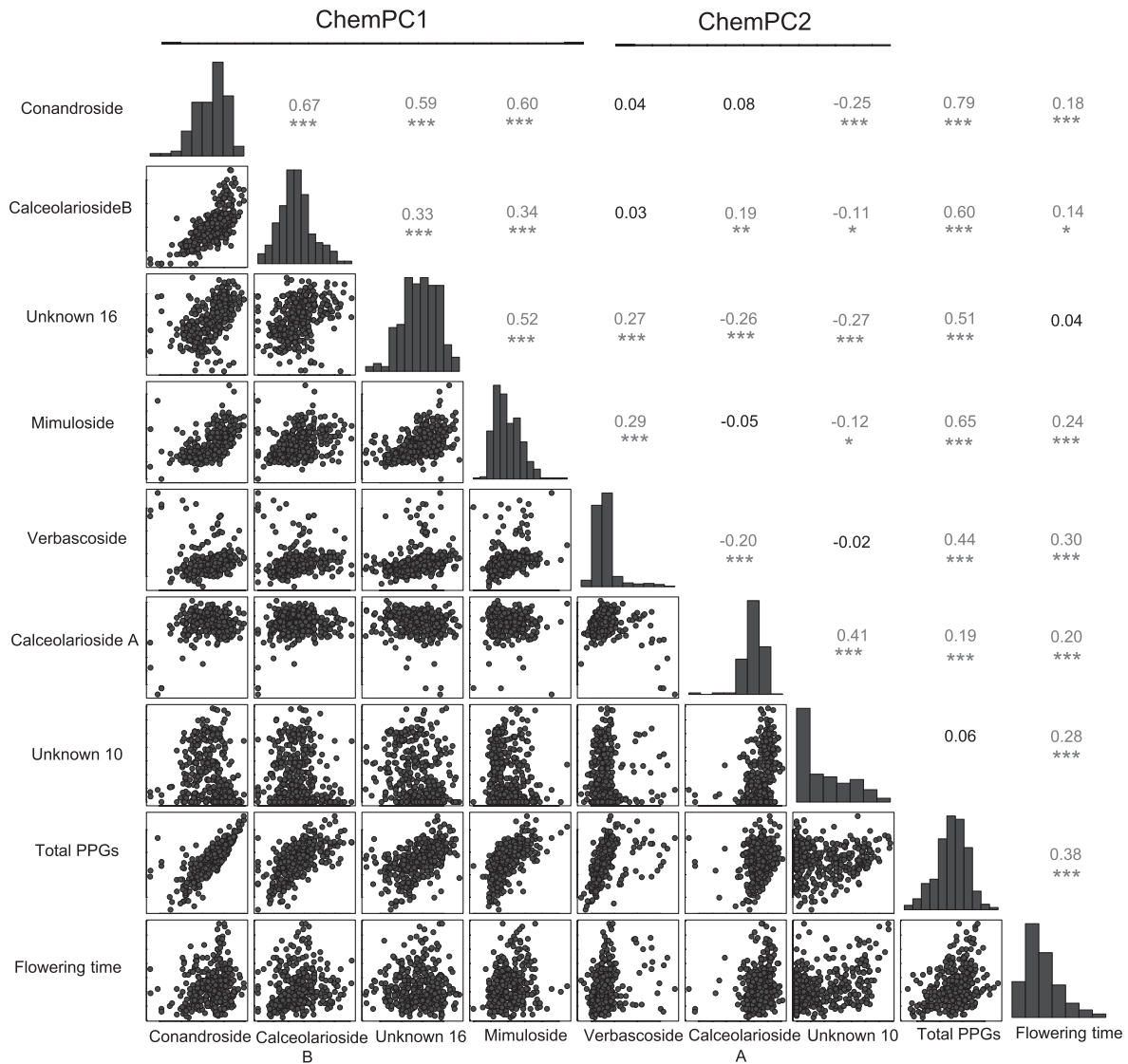


FIG. 2. Trellis plot depicting the relationships between the concentrations of individual phenylpropanoid glycosides (PPGs) as well as between PPG concentrations and flowering time. Scatterplots below the diagonal are log-transformed bivariate means for each *Mimulus guttatus* line. On the diagonal are histograms for each log-transformed PPG. Above the diagonal are Pearson correlation coefficients for each PPG–PPG correlation. Gray text indicates there is a significant correlation between PPGs, and asterisks depict  $P$  values of each bivariate correlation: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

garden, and HPLC run. There were marginal or significant relationships between individual PPGs and latitude for every PPG except verbascoside (Fig. 3; Appendix S1: Table S3). Contrary to the pattern predicted by the LHDH, the PPGs that made up Chem PC1 were present in higher concentrations in higher latitude populations (conandroside  $\chi^2 = 28.9$ ,  $P < 0.001$ ; calceolarioside B  $\chi^2 = 3.59$ ,  $P = 0.058$ ; mimuloside  $\chi^2 = 17.4$ ,  $P < 0.001$ ; unknown PPG 16  $\chi^2 = 33.9$ ,  $P < 0.001$ ). However, PPGs that made up Chem PC2, with the exception of verbascoside, had higher levels in low elevation populations (calceolarioside A  $\chi^2 = 10.8$ ,  $P = 0.001$ ; unknown PPG 10  $\chi^2 = 15.0$ ,  $P < 0.001$ ), consistent with the LHDH. Because the PPGs that made up Chem PC1 contributed

disproportionately to the total level of PPGs, there was also a relationship between total PPGs and latitude with greater levels of PPGs at higher latitudes (Fig. 3h;  $\chi^2 = 6.86$ ,  $P = 0.009$ ). To assess whether associations between latitude and PPG concentrations were artifacts of demographic processes or not, we included a measure of genetic distance between populations as a covariate in a modified model. This covariate eliminated only the association with latitude with one PPG, unknown PPG 10, although the association with latitude for mimuloside also became marginal ( $\chi^2 = 2.95$ ,  $P < 0.09$ ; Appendix S1: Table S4). It should also be noted, however, that this model has reduced power relative to the previous model due to the reduced data set; genotypic data was not previously

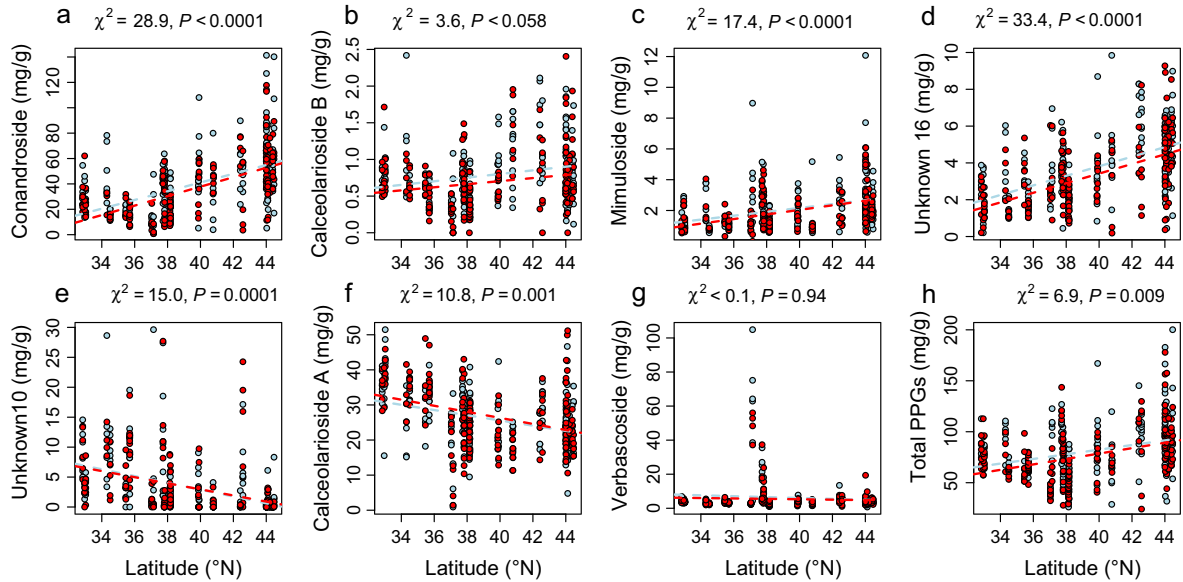


FIG. 3. Regressions of phenylpropanoid glycoside (PPG) concentrations on latitude. Red and blue points correspond to line means of plants in the damage treatment and in the control treatment, respectively. Chi-square statistic and *P* values above each graph are the fixed effect of latitude from linear mixed models described in the *Methods*. All PPG concentrations are mg PPG per g leaf dry mass. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

obtained for all lines included in this study. These results indicate that most of the associations between latitude and PPG concentrations reflect adaptive differentiation, and the differing relationships of different PPGs with latitude in turn provide mixed support for the LHDH.

*Costs of defense likely limit levels of defense*

We examined correlations between environmental factors and individual PPGs along elevation transects

with discordant gradients in growing season length to explore potential selective factors associated with spatial variation in defense. We found a significant association between growing season length and Chem PC1 ( $\chi^2 = 24.5, P < 0.001$ ; Appendix S1: Table S5) and a marginal correlation between growing season length and Chem PC2 ( $\chi^2 = 2.79, P = 0.09$ ). All PPGs except verbascoside and unknown PPG 10 were present in higher concentrations in populations with longer growing seasons (Fig. 4). Both of these outlier PPGs had higher levels (8–10× higher) in

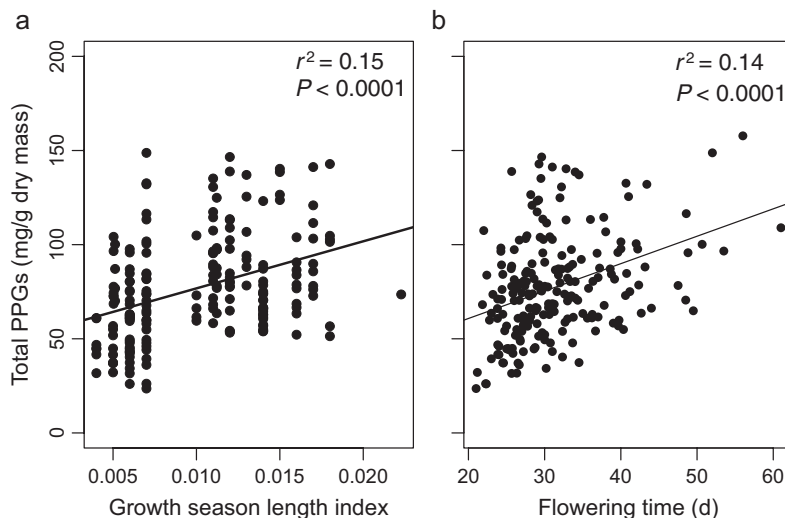


FIG. 4. (a) Regression of total phenylpropanoid glycoside (PPG) concentration and growing season length index. Growing season length index is a function of precipitation during the growing season and start date of the growing season. (b) Regression of total PPG concentration and flowering time. Points represent line means, and statistics refer to fit of the depicted regression line rather than linear mixed models presented in the *Methods*.



one or two populations (unknown PPG 10, YVO and BRC2; verbascoside, SHL) than in any other Sierra or Cascades population. Relationships between PPG concentration and other abiotic factors were generally weaker than those with growing season (Appendix S1: Table S5). For instance, there was no relationship between elevation and any individual PPG (Appendix S1: Table S5), and only two significant associations between temperature and a PPG: temperature and conandroside ( $\chi^2 = 4.07$ ,  $P = 0.04$ ) as well as temperature and unknown PPG 16 ( $\chi^2 = 9.13$ ,  $P = 0.003$ ). In both of the latter cases, higher levels of PPGs were present in regions with lower temperatures. Together, these results suggest that populations with a shorter window for growth and reproduction do not allocate as many resources to plant defense as do those with longer growing seasons.

Given the association between PPG production and growing season length, we asked whether PPGs levels were genetically correlated with other traits involved in the rapid development syndrome already described in *M. guttatus*. Concentrations of each PPG except unknown PPG 16 were positively correlated with flowering time in our common garden experiment (Fig. 2). Total PPGs was also highly correlated with flowering time ( $r = 0.35$ ,  $P < 0.001$ ) with plants producing greater total PPG concentrations typically flowering later. These results suggest that a relatively low level of herbivory resistance is associated with a rapid growth and reproduction syndrome in annual *M. guttatus* populations.

#### Leaf herbivory is higher in higher latitude populations

To examine the relationship between herbivore pressure and plant defense, we surveyed foliar damage in the field for low elevation populations along the latitudinal transect. After accounting for developmental state, foliar damage was strongly associated with latitude, with higher levels of damage in higher latitude populations ( $\chi^2 = 12.6$ ,  $P < 0.001$ ; Fig. 5). Average foliar damage was  $>10\times$  higher in the highest latitude population than the lowest latitude populations (Appendix S1: Table S6). We noticed little damage to stems in any population, and we could not assess damage to flowers or seeds, as most higher latitude populations had not yet flowered at the time of the surveys. We also could not assess damage if herbivores consumed entire seedlings, as is typical for herbivory by mollusks. Nonetheless, these results suggest that herbivore pressure on *M. guttatus* is stronger at higher latitudes than at lower latitudes, contrary to the pattern predicted by the LHDH.

#### DISCUSSION

Although the LHDH has been highly cited for the last 50 years, its empirical support is mixed, and few studies have attempted to examine the ecological trade-offs underlying the hypothesis. In this study, we found strong ecogeographic patterns in a suite of phytochemical

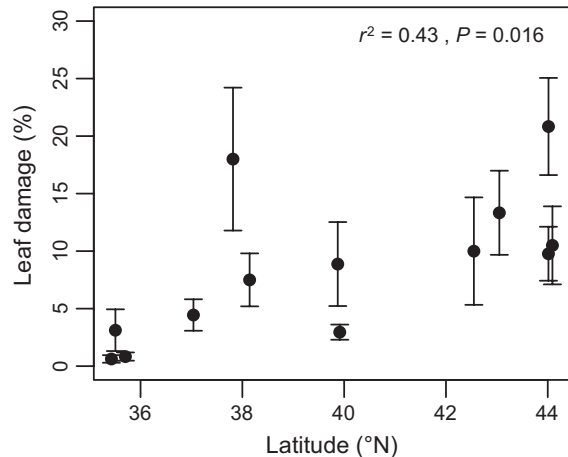


FIG. 5. Scatterplot depicting the relationship between leaf damage and latitude. Points are population averages and error bars represent standard error. Leaf damage refers to the average leaf damage on the first two pairs of true leaves of a plant. Regression statistics refer to a linear regression between population average leaf damage and latitude rather than the linear mixed model described in the *Methods*.

defense traits in *M. guttatus*. However, we found only limited support for the LHDH. Herbivore damage was highest at high, rather than low, latitudes. Moreover, concentrations of only two of seven PPGs surveyed were highest in low latitude populations. In contrast, four PPGs varied clinally in the opposite direction, and one PPG showed no relationship with latitude. Our results indicate that allocation of resources to rapid development and reproduction may be as or more important to plant fitness than allocation to herbivory resistance in populations with short growing seasons, as levels of six of seven PPGs were lower in populations with shorter growing seasons than in those with longer growing seasons. Lending further support for this hypothesis, flowering time was positively correlated with concentration of six of seven PPGs, suggesting that decreased allocation to herbivore resistance is part of the rapid growth and reproduction syndrome needed in populations with growing seasons with early terminal droughts. In summary, our results indicate that spatial variation in herbivore pressure and the cost of defense likely underlie spatial variation in plant defense; however, these selection pressures may result in ecogeographic patterns far more complex than the simple latitudinal cline predicted by the LHDH.

#### No evidence for PPG induction in response to artificial herbivory

One factor frequently hypothesized to alter or accentuate patterns of the LHDH is the presence of inducible defenses (Rasmann and Agrawal 2011, Więski and Pennings 2014, Anstett et al. 2016a). Inducible plant defenses are predicted to evolve when herbivory early in development is a reliable cue for herbivory later in

development, defenses are costly, defenses are effective against deterring herbivory, and herbivory is unpredictable between years (Zangerl and Rutledge 1996, Karban et al. 1997). We found little evidence for an inducible response in PPG concentrations to mechanical wounding. This result was unexpected, as a previous study examining population differentiation in PPG phytochemistry using a similar mechanical damage treatment found evidence of population by damage treatment effects (Holeski et al. 2013). The populations used by Holeski et al. (2013) consisted of both annual and perennial populations, while our experiment used only annual populations. Reevaluation of their data set suggests that some PPGs (calceolarioside A, unknown PPG 16, and mimuloside) were inducible in the perennial populations but not in the annual populations (Appendix S1: Table S7). Both the Holeski et al. (2013) and the present study have the caveat that herbivory was simulated via mechanical damage. While mechanical damage mimics generalized tissue loss, it does not have the same chemical signature as herbivory. Nonetheless, the apparent lack of inducible chemical defenses by annual *M. guttatus* plants in response to herbivory affords a simplified system for evaluating the LHDH.

#### *Limited evidence supporting the LHDH*

The most basic prediction of the LHDH is that herbivore pressure and plant defense should increase in populations closer to the equator. Our results were generally not consistent with this hypothesis. We found that herbivory was greater on plants found at higher latitudes than at lower latitudes even though (1) the plants sampled in higher latitude populations were younger because they were observed earlier in their growing season, and (2) these plants were genetically predisposed to producing greater constitutive levels of PPGs (Fig. 5). Furthermore, total PPG concentrations were nearly twice as high on average in high latitude, compared to low latitude, populations (Figs. 1, 3; Data S1). Our results do support the hypothesis that spatial heterogeneity in herbivore pressure drives variation in plant defense, as both herbivore pressure and total PPG concentrations are highest in high latitude populations. Studies of other plant species within temperate regions have found gradients in herbivore pressure of the same direction as ours, perhaps indicating expectations should differ for patterns only within temperate zones vs. those spanning both temperate and tropical zones (Adams and Zhang 2009, Kim 2014).

#### *Discordant latitudinal clines among PPGs*

One of the most striking results of our study was the discordance in spatial patterns between individual PPGs. The majority of the PPGs surveyed were found at higher levels in higher latitude populations, contrary to pattern predicted by the LHDH. However, the two PPGs that made up Chem PC2 were found at higher levels in lower latitude

populations, often constituting over 3.5% of dry leaf mass in populations found in Southern California. One potential explanation for the formation of discordant PPG clines is that differences in PPG composition and abundance reflect adaptation to local herbivore communities. If differences in PPG concentrations or arsenal composition are responsible for variation in resistance to different herbivore species, plant populations may adapt to produce higher levels of the PPGs that promote resistance against the most damaging and/or abundant herbivores in a community. The evolution of such geographic mosaics in plant defense driven by herbivore richness or abundance is common (Berenbaum and Zangerl 1998, Zangerl et al. 2003, Bravo-Monzón et al. 2014).

For adaptation to herbivore communities to explain the evolution of discordant latitudinal clines, PPGs must provide an effective defense against herbivores, and the presence or absence of particular herbivores from a population should correlate with changes in PPG concentrations. Our finding that PPG concentrations were highest in populations with the greatest amount of damage in the field suggests that greater levels of PPGs did indeed evolve as a defense against herbivores. We note, however, that we did not measure PPG concentrations on the same plants surveyed for foliar damage. Thus, it is possible that plasticity could obscure the genetic clines observed in our common garden (Salazar and Marquis 2012), but unlikely given that PPG levels of high latitude populations were nearly double those in most low latitude populations. Moreover, preliminary studies suggest that almost all PPGs act as deterrents to generalist herbivores but not necessarily to specialists (Rotter and Holeski 2016). Determining the role specific herbivore species play in driving plant defense will require field experiments that manipulate the herbivore community and identify associations between fitness and plant defense.

An alternative explanation for discordant latitudinal clines in individual PPGs is the occurrence of different mechanisms for adapting to similar herbivore regimes across the latitudinal transect. If different PPGs provide interchangeable resistance to herbivory, the multivariate composition of PPGs at a given location would not matter, just the total amount of PPGs. In this scenario, some source of historical contingency, such as the relative timing or effect size with which new alleles conferring elevated defense were introduced, could account for spatial variation in PPG arsenal composition. As a consequence, the alleles responsible for elevated Chem PC2 levels would rise in frequency in southern populations, and the alleles responsible for elevated Chem PC1 levels would rise in frequency in northern populations despite similar adaptation to similar herbivore regimes occurring in both places. We view this explanation as less likely given the specificity between defense compound evolution and herbivores demonstrated by other studies (Zangerl et al. 2003, Schuman et al. 2015) and because our models indicate that PPG clines persist even after controlling for population structure.

Of the few studies that have measured specific compounds in a widespread species across large spatial gradients, at least one has reported similar discordant latitudinal clines in defense traits. Anstett et al. (2015) found a latitudinal cline in *Oenothera biennis* that supports the LHDH in one ellagitannin (oenthein A) but detected a latitudinal cline in the opposite direction in a second (oenthein B). While only oenthein A was associated with decreased herbivory, the common garden experiment was conducted at high latitude. Oenthein B could still play a role in herbivore defense at low elevation, where it is more common. Gaining a more nuanced view of spatial variation in plant defense will require further in-depth ecogeographic surveys to determine whether discordant patterns between plant defense compounds are common and reflect adaptation to complex patterns of environmental variation affecting plant fitness.

#### *Resource allocation costs explain deviations from LHDH*

An often unexamined but key selective factor in the LHDH is the cost of defense. Our results suggest that a resource allocation tradeoff likely plays a role in structuring ecogeographic variation in plant defense. In areas with short growing seasons, *M. guttatus* has evolved a rapid growth and reproduction life history strategy, with rapid flowering after germination (Kooyers et al. 2015). Our results suggest that levels of PPGs are associated with this life history strategy, as we found higher concentrations of PPGs in areas with longer growing seasons (Fig. 4a). In addition, we found that flowering time and PPGs were correlated such that plants that flowered earlier produced lower concentrations of PPGs (Fig. 4b). These results make sense in light of optimal defense theory (Rhoads 1979, Stamp 2003), which predicts that levels of defense will be negatively correlated with allocation or ecological costs. Environments characterized by short growing seasons may select against allocation to defense by annual plants, as limited resources must be devoted to rapid growth and reproduction. *Mimulus guttatus* in environments with short growing seasons may only produce one to two flowers per season (Hall and Willis 2006, Hall et al. 2010), and a difference of a few days in time to flower can drastically change fitness distributions (Weis et al. 2014). In addition, plants could temporally escape herbivores in ephemeral environments by reproducing quickly, making plant defenses less relevant.

Neither herbivore pressure nor resource allocation costs alone can explain the observed patterns of variation in total PPG concentration, as both selective agents are associated with growing season length index in our study. The LHDH predicts that herbivore pressure is higher in the tropics because temperatures are higher, because conditions are more stable, or because growing seasons are longer (Dobzhansky 1950, Schemske et al. 2009). Unlike any other study that we are aware of, our study has been able to tease apart these alternative expectations by sampling along contrasting elevation clines. We find that

herbivore pressure and levels of most PPGs increased with increasing growing season length, but generally decreased with increasing annual average temperature. These patterns suggest that growing season length is more likely to drive variation in plant defenses than temperature. This conclusion may reflect the geographic area that was the focus of our sampling: a wide latitudinal gradient covering exclusively temperate latitudes (32° N–45° N) where growing season length varies dramatically. Growing season length is generally less variable in more tropical environments and generally decreases as latitude increases: the opposite spatial pattern from our study. Thus, if growing season length is a primary determinant of herbivore pressure and plant defense, the LHDH would typically be true in studies that survey from tropical to temperate latitudes but not necessarily in studies looking at only tropical or temperate samples. We note that this study utilized an annual plant with a relatively rapid life cycle, and our findings that highlight the importance of growing season length may not be generalizable to perennials. For perennials, the strength of herbivore pressure may still be contingent on growing season length, but investment in defense compounds might also increase since a plant's total lifetime reproductive output is not wholly reliant on a single growing season. We suggest that future work on the LHDH should take life history and seasonal phenology into account, and should focus on isolating factors that influence differences in defense and herbivore pressure rather than simply testing if latitudinal clines exist.

#### *Limitations of this study*

A comprehensive evaluation of the LHDH should incorporate all defenses into the analysis and assess how these defenses interact to determine palatability to herbivores. In addition to PPGs, *M. guttatus* also has physical defenses such as trichomes that we do not measure here (Holeski 2007, Holeski et al. 2010). We also did not assess plant tolerance to herbivory and did not test how PPGs interact with each other or other defenses to influence plant palatability. Patterns of trichome density and/or tolerance may either parallel or trade off with chemical defense patterns, thus contributing toward the overall cost of plant defense (Wang et al. 2016). Previous work with trichome density has shown no correlation between constitutive PPG levels and trichome density (Holeski et al. 2013) and weak correlations between trichome density and latitude (Kooyers et al. 2015), suggesting that its inclusion would not influence our conclusions about the LHDH.

#### CONCLUSIONS

In one of the most comprehensive empirical tests of the LHDH to date, we found limited evidence for greater plant defense at lower latitudes. Predictions of optimal defense theory likely explain these results. We found that

plant defenses are generally higher in areas where herbivore pressure is higher and where the cost of allocating resources to defense is likely to be comparably low. These findings provide additional context for interpreting some of the mixed empirical results from studies testing the LHDH: associations between latitude and plant defense will be variable because the relationships between latitude and the abiotic variables that actually drive variation in plant defense and its possible costs are variable. To understand the role of biotic interactions in driving evolution, future studies must work to experimentally parse potential abiotic drivers of ecogeographic patterns as well.

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