

Patterns of Phytochemical Variation in *Mimulus guttatus* (Yellow Monkeyflower)

Liza M. Holeski · Ken Keefover-Ring ·
M. Deane Bowers · Zoe T. Harnenz ·
Richard L. Lindroth

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Abstract The search for general patterns in the production and allocation of plant defense traits will be facilitated by characterizing multivariate suites of defense, as well as by studying additional plant taxa, particularly those with available genomic resources. Here, we investigated patterns of genetic variation in phytochemical defenses (phenylpropanoid glycosides, PPGs) in *Mimulus guttatus* (yellow monkeyflower). We grew plants derived from several natural populations, consisting of multiple full-sibling families within each population, in a common greenhouse environment. We found substantial variation in the constitutive multivariate PPG phenotype and in constitutive levels of individual phytochemicals within plants (among leaves of different ages), within populations (among full-sibling families), and among populations. Populations consisting of annual plants generally, but not always, had lower concentrations of phytochemicals than did populations of perennial plants. Populations differed in their plastic response to

artificial herbivory, both in the overall multivariate PPG phenotype and in the individual phytochemicals. The relationship between phytochemistry and another defense trait, trichomes, differed among populations. Finally, we demonstrated that one of the PPGs, verbascoside, acts as a feeding stimulant rather than a feeding deterrent for a specialist herbivore of *M. guttatus*, the buckeye caterpillar (*Junonia coenia* Nymphalidae). Given its available genetic resources, numerous, easily accessible natural populations, and patterns of genetic variation highlighted in this research, *M. guttatus* provides an ideal model system in which to test ecological and evolutionary theories of plant-herbivore interactions.

Keywords Defense · Genetic variation · *Junonia coenia* · Kairomone · *Mimulus guttatus* · Phenylpropanoid glycoside · Model system

L. M. Holeski (✉) · K. Keefover-Ring · Z. T. Harnenz ·
R. L. Lindroth
Department of Entomology, University of Wisconsin,
Madison, WI 53706, USA
e-mail: holeski@wisc.edu

K. Keefover-Ring
Department of Plant Physiology, Umeå Plant Science Centre,
Umeå University,
Umeå 90187, Sweden

M. D. Bowers
Department of Ecology and Evolutionary Biology,
University of Colorado,
Boulder, CO 80309, USA

Z. T. Harnenz
Integrated Biological Sciences Summer Research Program,
University of Wisconsin,
Madison, WI 53706, USA

Introduction

Herbivory has exerted strong selection pressures on natural plant populations over both ecological and evolutionary time scales, leading to the great diversity of plant physical, chemical, and “behavioral” defenses observed today (Berenbaum and Zangerl, 2008). Plant species that are exposed to a wide variety of generalist and/or specialist invertebrate and vertebrate herbivores are likely to have evolved a diversity of defenses (Agrawal and Fishbein, 2006). In addition to evolving different mechanisms of defense, plants may maximize the effectiveness of particular defenses, while minimizing their costs, via the incorporation of intra-plant variation in defense production (Stamp, 2003). Forms of intra-plant variation in defense include differences in induction and development; genetic variation in these traits is increasingly documented in herbaceous and woody

plant species (Boege and Marquis, 2005; Barton and Koricheva, 2010).

A major goal in the study of plant-herbivore interactions is to identify general patterns in the production and allocation of plant defense traits that hold true across plant species and types of defense (Stamp, 2003). Plant secondary compounds have been characterized, and their evolution and impact on herbivores have been intensively studied in plants of the Brassicaceae (e.g., *Arabidopsis spp.*), Apiaceae (e.g., wild parsnip), Fabaceae (e.g., lima beans), Solanaceae (e.g., tobacco), Asclepiadaceae (e.g., milkweed), and Salicaceae (e.g., *Populus spp.*) among others (Berenbaum and Zangerl, 2008). Classic studies in these systems highlight the complexity of the interactions between plant secondary compounds and insect herbivores. For example, depending on the insect species and its degree of adaptation to a plant species, plant secondary compounds may act as allomones (deter herbivores) or kairomones [(attract herbivores); Harborne, 1993].

Studies to date have highlighted numerous cases of evolution and co-evolution of defenses, but often are largely idiosyncratic. Broad, cohesive patterns underlying these processes have not been easy to identify. The search for general patterns will be aided by: 1) broadening our perspective to incorporate multivariate suites of defenses (Agrawal and Fishbein, 2006; Carmona et al., 2011); 2) including studies of more plant families, genera, or species (Berenbaum and Zangerl, 2008); and 3) incorporating evaluations of the molecular genetic architecture of defenses to facilitate inferences about past selection events (Rausher, 1996; Anderson and Mitchell-Olds, 2011).

The availability of sequenced genomes for plants in several of the historically-studied families already have led to rapid advances in our knowledge of plant-insect interactions at the molecular and genetic level, and have aided our search for widespread patterns underlying defense production and diversification (Stotz et al., 1999; Mitchell-Olds, 2001; Reymond et al., 2004). For example, use of genomic resources in *Arabidopsis thaliana* allowed Schenk et al. (2000) to detect substantial coordination between the jasmonate and salicylate signaling pathways, two damage-induced pathways previously thought to act antagonistically. Our ability to connect phenotypic trade-offs (e.g., between a reproductive fitness trait and a defense trait, or between two defense traits) with genetic architecture (e.g., are genes that influence the traits pleiotropic or in linkage disequilibrium?) should provide insight into the evolutionary processes that led to these trade-offs, as well as to their future evolutionary trajectories (Rausher, 1996).

We posit that, given its available genetic resources and numerous, easily accessible natural populations, *Mimulus guttatus* (Phrymaceae) is an ideal study system in which to link the genetic architecture of plant defenses, including

secondary compounds, to processes of plant-herbivore interactions. These features would allow us to directly test ecological and evolutionary theories of plant-herbivore interactions (Wu et al., 2008; Holeski et al., 2010). To our knowledge, however, *M. guttatus* is the only plant species with a sequenced genome for which the principal secondary compounds have not been published.

Recent research by our group has shown that the dominant bioactive secondary metabolites of *Mimulus* consist of a suite of phenylpropanoid glycosides (PPGs, also called caffeoyl phenylethanoid glycosides; K. Keefover-Ring et al., unpublished data). PPGs are synthesized via the shikimic acid pathway and typically consist of caffeoyl and hydroxytyrosol moieties bonded to a central β -glucopyranose sugar. In some cases, the core glucose has a second sugar attached (Mølgaard and Ravn, 1988). In other plant species, PPGs act as generalist herbivore feeding deterrents (Mølgaard, 1986).

The purpose of this paper is to describe patterns in the production of PPGs that are likely of considerable ecological and evolutionary relevance, rather than to present formal identifications of these compounds. Here, we describe two experiments designed to examine patterns of phytochemical levels in *M. guttatus* and to illustrate the biological effects of one of these phytochemicals, verbascoside, on a specialist insect herbivore that commonly feeds on *M. guttatus*. Experiment 1 was a greenhouse common garden experiment that used multiple *M. guttatus* populations derived from natural populations spanning a range of habitats. Experiment 2 was a feeding trial that examined the effects of purified verbascoside on buckeye caterpillar (*Junonia coenia*) performance. We used the results to address the following questions: 1) Is phytochemistry genetically based and variable within and among populations? 2) Is phytochemistry variable within individual plants among leaves of different ages? 3) Is phytochemistry plastic in response to foliar damage? 4) What is the relationship between production of trichomes and concentrations of PPGs? 5) What are the effects of the PPG verbascoside on performance of a specialist herbivore?

Methods and Materials

Yellow monkeyflower [*M. guttatus*; Phrymaceae (Beardsley and Olmstead, 2002)] ranges from Mexico to Alaska in western North America and typically inhabits wet areas such as stream banks. It is a self-compatible, hermaphroditic plant that reproduces by a mixture of outcrossing and self-fertilization. Local populations differ extensively in morphology, life history, selfing rate, and levels of herbivory (Fenster and Ritland, 1994; Carr and Eubanks, 2002; Kelly and Arathi, 2003; Hall and Willis, 2006). *Mimulus guttatus* populations can be perennial or facultatively annual. Perennial populations occur in habitats with year-round moisture,

whereas annuals exist in habitats with more ephemeral water sources such as snowmelt or seeps, which typically dry out by late summer (Lekberg et al., 2012).

Experiment 1—Patterns of Variation in M. guttatus Phytochemistry This experiment involved plants derived from 12 natural populations in California, Oregon, or Washington, U.S.A, and British Columbia, Canada. Seed collected from at least 10 plants per natural population was grown in the greenhouse at the University of Wisconsin (UW), Madison (Fafard 3B potting soil, 16 hdays with high pressure sodium supplemental lighting, bottom-watered daily, and fertilized weekly with Blossom Booster, J.R. Peters, Allentown, PA, USA). Within each population, we randomly assigned one plant from each maternal, field-collected population to be the maternal or paternal plant in a mating pair. We then manually pollinated each pair unidirectionally. The seed resulting from these crosses, comprising 2–5 full-sibling families within each population and 33 families in total, was used in our experiment.

We germinated seed from each of the full-sibling families, derived from each of the 12 natural populations, in the UW greenhouse using the conditions described above. Twelve d after seeding, each experimental plant (10 per family) was transplanted into its individual 2-in pot. Plants were randomized in their position within a flat, and flats were rotated daily on the greenhouse bench in order to minimize environmental effects. Half of the plants of each family were control plants, while half were subjected to simulated insect-chewing damage by punching two holes about 6 mm in diameter on both leaves of the 2nd true leaf pair (counting up the main stem from the cotyledon) as soon as its 3rd leaf pair was unfurled (Holeski, 2007; Holeski et al., 2010). For plants in both groups, when the leaves of the 6th leaf pair were fully expanded we performed a 5th leaf trichome count on a defined area of 1 cm² on the basal central part of the adaxial side of the leaf (Holeski, 2007; Holeski et al., 2010). We harvested the 2nd, 3rd, 4th, 5th, and 6th leaf pairs of the plant and kept each pair separately. Because these leaves were collected at the same time from each plant, the 2nd leaf pair is the oldest physiologically, and the 6th leaf pair is the youngest so we use “leaf age” as synonymous with “leaf pair”. We flash-froze tissue using liquid nitrogen, freeze-dried and finely ground the tissue in a small-capacity ball mill (dental amalgamator with steel bearings), and stored the samples at –20 °C. The 6th leaf pair of most plants weighed less than 3 mg dry weight and was excluded from chemical analysis.

We employed UV spectroscopy and liquid chromatography-mass spectrometry (LC-MS) to tentatively identify 7 compounds, each present in at least one of each of the 12 populations, as phenylpropanoid glycosides (PPGs). We employed carbon (¹³C-) and proton (¹H-) NMR to confirm

the identities of verbascoside and conandroside (K. Keefover-Ring et al., unpublished data). NMR analysis for the remaining compounds is currently in progress, and will be published elsewhere (K. Keefover-Ring et al., unpublished data). PPGs in dried, ground samples were extracted in methanol with 10 min of sonication followed by 12 h at 21 °C in the dark. A 100 µl aliquot of each methanol extract was vacuum dried and redissolved in 100 µl of a water spiked with a catechol internal standard. We quantified PPGs via high performance liquid chromatography using a Hewlett Packard 1090 HPLC with a diode array detector and Vydac C18 analytical column (4.6×250 mm, 5 µm particle size; W.R. Grace & Co., Columbia, MD, USA) maintained at 30 °C. HPLC run conditions included a binary mobile phase gradient with 3.1 mM phosphoric acid as mobile phase A, and acetonitrile as mobile phase B at a constant total flow rate of 0.75 mlmin⁻¹. The gradient for each run consisted of 14 % B for the first 17 min, increased to 30 % B from 17 to 35 min, returning to 14 % B for 10 min to re-equilibrate the column. We injected 20 µl of the standards and samples, monitored UV signals at 274 (catechol) and 340 nm (PPGs), and used the DAD to collect UV data from 190 to 400 nm. We calculated all sample PPG quantities as verbascoside equivalents after normalization using the catechol internal standard peak area. The linear calibration curve was produced using a standard solution of pure verbascoside isolated from *Plantago lanceolata* by M.D. Bowers. We assessed phytochemical concentrations in the 2nd, 3rd, 4th, and 5th leaf pairs ($N=237$, 113, 236, and 310, respectively) and expressed individual and total PPG concentrations as mgg⁻¹ dry weight.

We performed a series of General Linear Model ANOVAs (Minitab 14, Minitab Inc., State College, PA, USA) and a MANOVA (JMP 8, SAS Institute Inc., Cary, NC, USA) including repeated measures and nesting to evaluate differences among leaf pairs (repeated measures within individuals), individuals nested within families, families nested within populations, and populations nested within life history (annual or perennial) with respect to their effects on constitutive concentration of each PPG and on the total PPG concentration (ANOVAs) and the multivariate PPG phenotype (MANOVA), respectively. We created a Euclidean distance-based similarity matrix and used nonmetric multidimensional scaling (NMDS; Primer 5, Primer-E, Ltd., Plymouth, United Kingdom) to visualize the constitutive, multivariate PPG phenotypes (Kruskal, 1964). Two dimensional stress levels across several NMDS runs were consistently less than 0.09, suggesting the data fit the ordination well.

We used mixed-model ANOVAs with the restricted maximum likelihood function (REML; JMP 8) to evaluate the effects of 2nd leaf damage and the population x 2nd leaf interaction (all factors in the GLM ANOVA except life history also included; 2nd leaf damage was considered a

fixed factor) on each individual PPG and on total PPGs. We could not perform a single MANOVA that included all leaf pairs to assess the effects of 2nd leaf damage on the multivariate PPG phenotype, as our data were unbalanced. Thus, we performed two MANOVAs (JMP 8), one with the oldest leaves (including 8 of the 12 populations due to unbalanced data) and one with the youngest leaves (including all 12 populations). Factors included family nested within population, population, 2nd leaf damage, and the 2nd leaf damage \times population interaction (2nd leaf damage considered a fixed factor).

The distributions of each PPG (and total PPGs) were right-skewed and were log-transformed ($\log(\text{PPG} \times +1)$) prior to statistical analysis. In our analysis of trichome induction, we omitted one population that did not produce constitutive trichomes, for consistency with previous work (Holeski et al., 2010). Heritability estimates were calculated using covariances among full-siblings (Falconer and Mackay, 1996).

Experiment 2 -Feeding Trials with *Junonia coenia* We conducted feeding trials using artificial diets with varying levels of purified verbascoside to evaluate its effect on performance of the buckeye caterpillar, *J. coenia* Hubner (Nymphalidae), a common herbivore of *M. guttatus* (Levine, 1999, 2000). We also reared insects on artificial diet mixed with powdered *P. lanceolata*, which contains other feeding stimulants for *J. coenia*, as a standard against which to evaluate insect performance. Work by Camara (1997) showed that buckeye larval growth on this diet is similar to that of larvae on fresh *P. lanceolata* leaves. Typically, *J. coenia* are specialists on plants that contain iridoid glycosides, such as *P. lanceolata* (Plantaginaceae), which contain iridoid glycosides (Bowers, 1984). However, *M. guttatus*, does not contain iridoid glycosides (Hegnauer and Kooiman, 1978; S. Jensen, personal communication; Bowers, unpublished data). The discovery of verbascoside and other phenylpropanoid glycosides in *M. guttatus* (K. Keefover-Ring et al., unpublished data) suggested that *J. coenia* might use these compounds as additional feeding or oviposition cues.

Verbascoide was isolated from *P. lanceolata* leaves using a modification of previously described methods (Franzyk et al., 1998). Because there are typically many different PPGs in *M. guttatus* and they are difficult to resolve, it was efficient to obtain verbascoside from *P. lanceolata*. Approximately 100 g dried, powdered leaf material were extracted in 1 L of methanol for 24 h, filtered, and evaporated to dryness. The product was suspended in a minimal amount of 50:50 water:acetone and chromatographed on a Sephadex C18 to remove chlorophyll. The resulting fraction was evaporated to dryness, partitioned between water and butanol, and the butanol layer was evaporated to dryness. The crude residue was suspended in a minimum amount of methanol, loaded

onto a silica gel column (Merck silica gel 60), and fractionated by vacuum liquid chromatography. Fractions were eluted by a gradient of chloroform with increasing amounts of methanol in fifty fractions, each comprising 50 ml. Fractions were spotted on glass silica gel TLC plates and chromatographed before visualizing verbascoside as a bright blue spot under UV light. Pure fractions were pooled while impure fractions were subjected to a second silica gel column. This process was repeated to obtain sufficient material for the feeding experiments (see below). Identification of verbascoside was confirmed by NMR.

Eggs of *J. coenia* were obtained from a colony maintained on a standard artificial diet at the University of Colorado (Bowers, 1984; Camara, 1997). For the experiment, we placed newly hatched, unfed larvae onto one of six diets. Artificial diet was either unamended (1, Control) or amended with 2 % dry weight verbascoside (2), 4 % dry weight verbascoside (3), 7 % dry weight verbascoside (4), 11 % dry weight verbascoside (5), or powdered *Plantago lanceolata* leaves at 5 % of the dry weight of the diet (6). Because powdered leaf material was used, this diet contained 0.08 % verbascoside as well as the iridoid glycosides aucubin and catalpol, known feeding stimulants for *J. coenia* (Bowers, 1984). Verbascoide levels were chosen to reflect levels of verbascoside found in nature in some plant species (e.g., Adler et al., 1995).

Larvae were reared in groups of four, with 10 replicates of each treatment. Larvae were checked daily and food replaced as needed. On day 12, surviving larvae were weighed as a group, and the mean weight per larva was used as the dependent variable. Number of surviving larvae was also counted.

Results

Patterns of Variation in M. guttatus Constitutive Phytochemistry

The multivariate PPG phenotype differed among leaves of different age (*Wilks* $\lambda=0.184$, $F_{21, 793.07}=30.28$, $P<0.001$). Levels of each individual PPG except verbascoside also differed significantly across leaves of different age (Table 1). Across populations, concentrations of each individual PPG increased with leaf age, although several exceptions occurred, mainly in one population (Fig. 1). In one of the more striking instances of this pattern, levels of PPG 4 increased more than 10-fold between the youngest and oldest leaves collected within a population. In general, concentrations of each PPG in leaves of intermediate age (3rd and 4th leaf pairs) were intermediate to those of the youngest and oldest leaf ages (5th and 2nd leaf pairs).

We found a great deal of variation among populations in the multivariate PPG phenotype ([Figs. 2 and 3]: *Wilks* $\lambda=0.109$, $F_{70, 1616.2}=10.64$, $P<0.001$). We also observed genetic variation among populations for levels of most of the individual PPGs and for total PPGs across leaf age

Table 1 For each phenylpropanoid glycoside (PPG), the upper set of statistics shows the results of GLM ANOVAs illustrating the effects of leaf pair (repeated measure within individuals), individual nested within family, family nested within population, population nested within life history, and life history on univariate, constitutive PPG production, as well as the sum of all individual PPGs (total PPGs). The lower set of statistics provides the results of mixed-model REML analyses showing the effects of 2nd leaf damage and the population x 2nd leaf damage interaction for each univariate PPG. All factors except leaf age, life history, and 2nd leaf damage were random factors. Factors that had a significant effect on a particular PPG are shown in bold type; factors with a marginally significant effect are shown in italics

PPG	Factor	DF	F statistic	P-value	
Conandroside	Leaf pair	3	3.43	0.018	
	Individual(Family)	121	2.05	<0.001	
	Family(Population)	22	1.46	0.102	
	Population(Life history)	10	9.43	<0.001	
	Life history	1	2.96	0.114	
	Error	289			
	2nd leaf damage	1,167	2.92	0.090	
	Population*2nd leaf damage	7,170.1	2.70	0.011	
	Verbascoside	Leaf pair	3	0.57	0.663
		Individual(Family)	121	0.73	0.978
Family(Population)		22	4.81	<0.001	
Population(Life history)		10	3.49	0.006	
<i>Life history</i>		1	3.68	0.081	
Error		283			
2nd leaf damage		1,173.2	0.76	0.384	
Population*2nd leaf damage		7,177.5	0.48	0.848	
PPG 3		Leaf pair	3	11.84	<0.001
		Individual(Family)	121	0.88	0.790
	Family(Population)	22	2.70	<0.001	
	Population(Life history)	10	15.57	<0.001	
	Life history	1	0.01	0.925	
	Error	290			
	2nd leaf damage	1,176.5	0.33	0.566	
	Population*2nd leaf damage	7,180.5	2.28	0.030	
	PPG 4	Leaf pair	3	41.27	<0.001
		Individual(Family)	121	1.00	0.504
Family(Population)		22	2.89	<0.001	
Population(Life history)		10	5.01	<0.001	
Life history		1	5.09	0.045	
Error		290			
2nd leaf damage		1,174.1	2.05	0.153	
Population*2nd leaf damage		7,178.2	4.04	<0.001	
PPG 5		Leaf pair	3	39.64	<0.001
		Individual(Family)	121	0.88	0.785
	Family(Population)	22	2.56	<0.001	
	Population(Life history)	10	2.65	0.026	
	Life history	1	9.00	<0.001	
	Error	290			
	2nd leaf damage	1,152.8	0.72	0.397	
	Population*2nd leaf damage	7,156.5	1.63	0.130	
	PPG 6	Leaf pair	3	24.46	<0.001
		Individual(Family)	121	1.06	0.340
Family(Population)		22	1.46	0.100	
Population(Life history)		10	3.75	0.004	
Life history		1	3.40	<0.001	
Error		290			
2nd leaf damage		1,155.9	3.60	0.059	
Population*2nd leaf damage		7,159.6	2.14	0.042	
PPG 7		Leaf pair	3	149.64	<0.001
		Individual(Family)	121	0.86	0.830
	Family(Population)	22	1.11	0.342	

Table 1 (continued)

PPG	Factor	DF	F statistic	P-value
	Population(Life history)	10	2.00	0.077
	Life history	1	1.55	<0.001
	Error	290		
	2nd leaf damage	1,138.6	3.09	0.081
	Population*2nd leaf damage	7,139.5	2.08	0.049
Total PPGs	Leaf pair	3	32.18	<0.001
	Individual(Family)	121	2.17	<0.001
	Family(Population)	22	1.70	0.037
	Population(Life history)	10	4.55	0.001
	Life history	1	4.22	0.063
	Error	283		
	2nd leaf damage	1,169.6	2.43	0.121
	Population*2nd leaf damage	7,172.9	3.44	0.002

groups (Table 1). For example, in the oldest leaves, total PPG concentrations ranged from less than 7 mg g⁻¹ dry weight in a plant from Population 3 to over 166 mg g⁻¹ dry weight in a plant from Population 12.

The multivariate PPG phenotypes varied considerably among families nested within each population (*Wilks* $\lambda=0.334$, $F_{154, 1860.7}=2.15$, $P<0.001$). When we examined individual PPGs, we found significant genetic variation at

Fig. 1 Constitutive levels of individual phenylpropanoid glycosides (PPGs) in the youngest and oldest *Mimulus guttatus* leaves (mg PPG g⁻¹ plant dry weight). Each line connects the population mean PPG concentration in the youngest leaf to that in the oldest leaf. Note the differences in scale among y-axes. The sharp decrease in verbascoside, PPG 3, and PPG 5 concentrations from youngest to oldest leaves occurred in one population

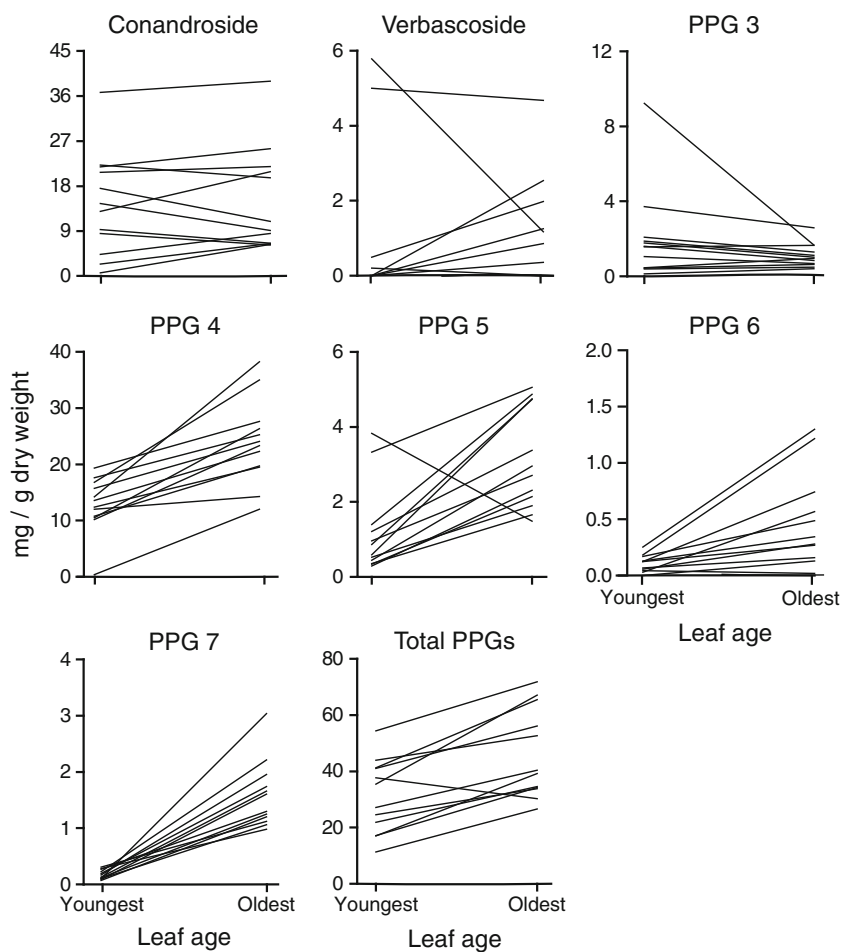
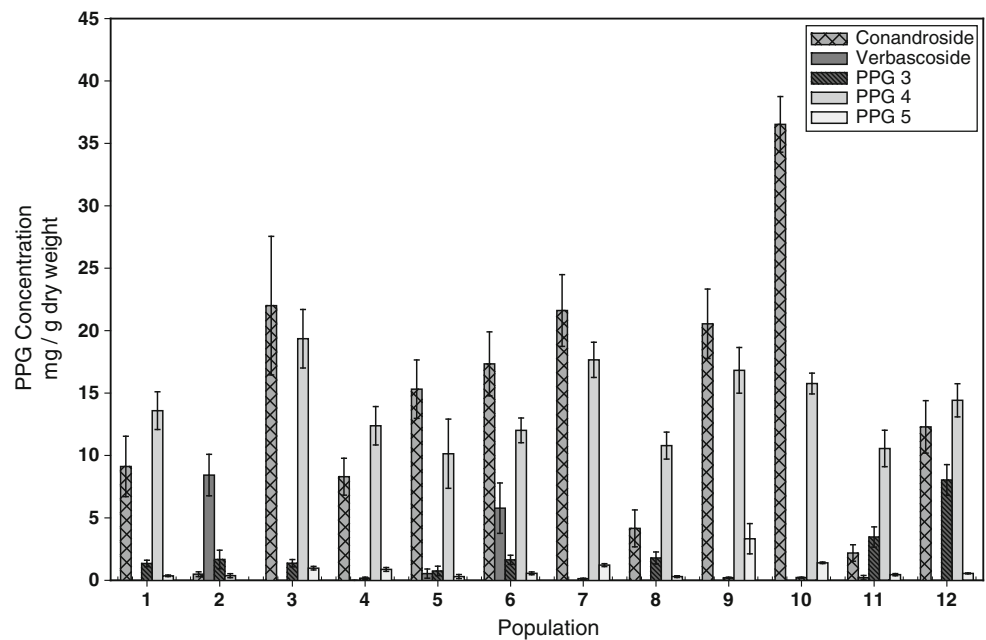


Fig. 2 Patterns in constitutive phenylpropanoid glycoside (PPG) production (mg PPG g^{-1} plant dry weight) across populations in young leaves of *Mimulus guttatus*. Two PPGs (PPG 6 and 7) were present in quantities too low (relative to the other PPGs) to appear in the figure. Patterns of PPG levels in leaves of this age are largely representative of those in the other leaf ages evaluated. Error bars represent ± 1 standard error (SE) from the mean



the among-family level in total PPGs and in 4 of the 7 individual PPGs (Table 1). Heritability estimates for each PPG, calculated for the oldest and youngest leaf pairs only, ranged from <0.01 to 0.62 (Table 2). The range of within-population variation for total PPG concentration approached that of among-population variation for some populations (e.g., 19–166 mg g^{-1} dry weight in oldest leaves of Population 12),

but not others (e.g., 22–48 mg g^{-1} dry weight in the oldest leaves of Population 4).

Finally, when we grouped populations according to life history strategy (annual vs. perennial), we discovered substantial effects of life history on the multivariate PPG phenotype ($F_{7, 276}=14.74$, $P<0.001$). Likewise, annual and perennial populations differed or were marginally different in most but not all of their individual PPG concentrations (Table 1, Fig. 4), with perennial populations generally containing higher concentrations.

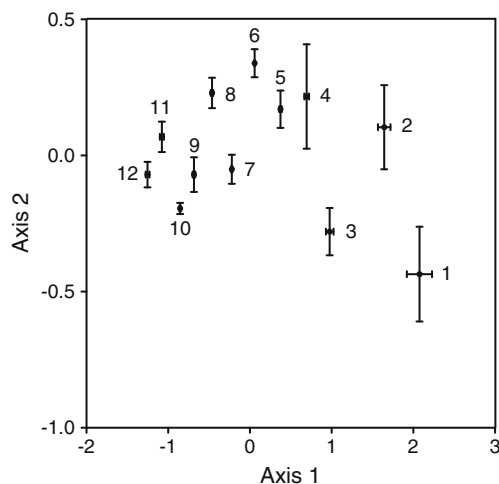


Fig. 3 Nonmetric multidimensional scaling (NMDS) ordination plot comparing the multivariate PPG phenotype in the oldest leaf pair for twelve *Mimulus guttatus* populations. Each point represents the mean of the NMDS scores for multiple individuals sampled for a population. Population identification numbers correspond with those in Fig. 2. Standard error bars (± 1 standard error (SE) from the mean) represent the variance of the NMDS scores in the x and y directions within a particular population. Data points near each other in the NMDS plots are more similar than points further apart. NMDS axes do not have associated quantitative units

Patterns of Variation in M. guttatus Induced Phytochemistry Populations responded to 2nd leaf damage differently, in terms of their multivariate PPG phenotypes, when oldest and youngest leaves were considered (significant population * damage interactions; oldest leaves, *Wilks* $\lambda=0.829$, $F_{49, 755.7}=1.41$, $P=0.038$; youngest leaves, *Wilks* $\lambda=0.654$, $F_{49, 842.1}=1.50$, $P=0.017$). Damage did not have an overall (across population) effect on the multivariate PPG phenotype in leaves of either age (oldest leaves, $F_{7, 148}=1.82$, $P=0.088$; youngest leaves, $F_{7, 165}=1.44$, $P=0.191$), although the overall effect might be considered marginally significant in the oldest leaves.

Individual PPGs exhibited patterns of induction similar to those in the multivariate PPG analysis. Individual PPGs did not increase or decrease in concentration overall (across populations in response to damage), but there was among-population genetic variation for this response in many of the PPGs (significant population * damage interactions; Table 1). In response to damage, several populations exhibited increases in individual PPG levels (relative to controls), some produced lower levels of PPGs, and some showed no

Table 2 Heritabilities (across populations) for each individual phenylpropanoid glycoside (PPG), in the youngest and oldest leaves. Heritability estimates were calculated using covariances among full-siblings

Trait	h ² (Youngest leaf age)	h ² (Oldest leaf age)
Conandroside	0.09	<0.01
Verbascoside	0.62	0.22
PPG 3	0.28	0.29
PPG 4	0.16	0.07
PPG 5	0.26	0.21
PPG 6	0.43	0.06
PPG 7	0.04	0.07
Total PPGs	0.05	0.10

significant changes in PPG levels (Fig. 5). The degree of response to damage varied with population and PPG, but generally was an increase or decrease in concentration of less than 50 % of the constitutive concentration.

Relationship Between Physical and Chemical Defenses We found no significant correlation between constitutive trichome production and constitutive total PPG concentration in the youngest leaves across populations (Pearson correlation coefficient=0.042; $P=0.616$). Rather, the relationship between constitutive phytochemistry and trichome production varied among populations (Fig. 6; significant effect of

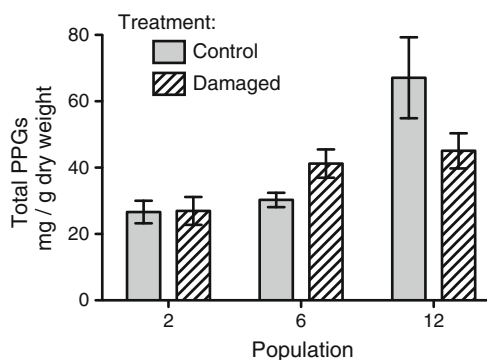


Fig. 5 Mean values (+/- 1 SE) for total phenylpropanoid glycoside (PPG) concentrations in the oldest leaves of control and damaged *Mimulus guttatus* from three populations. This figure demonstrates all three patterns of damage response found across populations: no change in total PPG concentration (Population 2), increase in total PPG concentration (Population 6), and decrease in total PPG concentration (Population 12). Patterns are similar across leaves of different ages (no significant damage × leaf age interactions)

population × total PPG (covariate) interaction on trichome density: $F_{11, 100}=2.42, P=0.010$). Populations varied in their trichome induction response to damage (significant population × damage interaction; $F_{10, 220}=2.23, P=0.017$). However, we found no clear relationship between induction of physical and chemical defenses (total PPGs), either within populations or across populations. Pearson correlation coefficients for within population comparisons ranged from

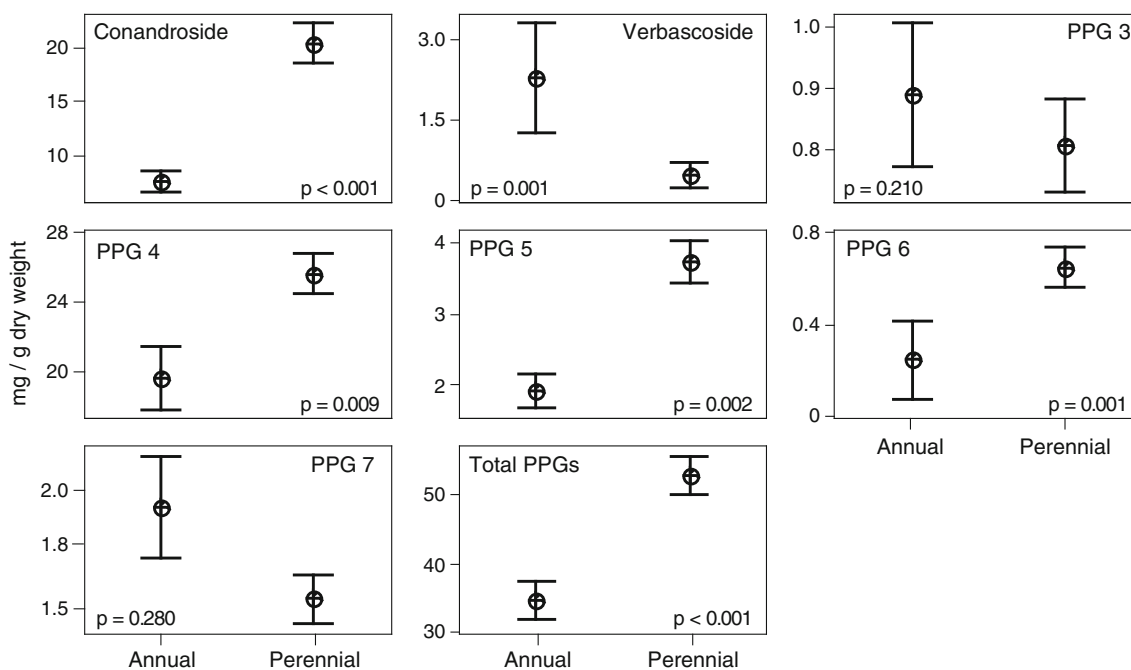


Fig. 4 Mean values (across populations) for annual or perennial life history strategies and individual phenylpropanoid glycoside (PPG) concentrations for *Mimulus guttatus*. Values are provided only for older leaves, as patterns did not differ significantly across leaves of

different ages. P -values are based on the effects of life history strategy upon a particular PPG when the effects of population nested within life history strategy were taken into account. Error bars represent +/- 1 SE from the mean. Note the differences in scale among y-axes

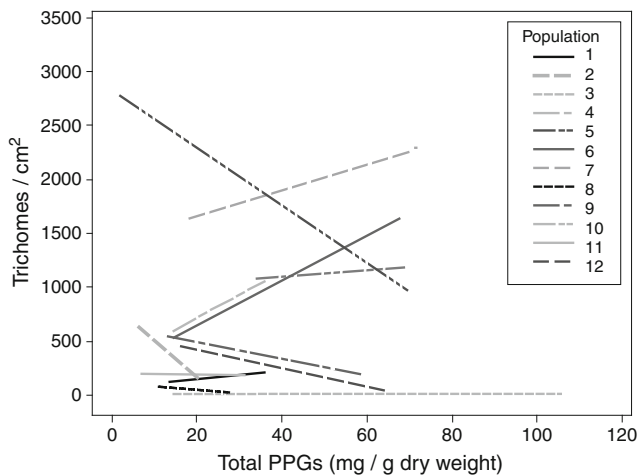


Fig. 6 The relationship between constitutive levels of total phenylpropanoid glycosides (PPGs) and constitutive trichome density in the youngest leaf age for *Mimulus guttatus* across populations. Lines represent the trend lines from linear regression; each population is represented by a unique line

−0.976 to 0.539 with associated *P*-values from 0.139 to 0.951, and the Pearson correlation coefficient for the across population comparison=0.073; *P*=0.686.

Feeding Trials with *Junonia coenia* Buckeye larval weight was significantly affected by diet ($F_{5, 54}=7.56, P<0.001$) after 12 days of feeding. Verbascoside serves as a kairomone to buckeye larvae; larval weight was four times higher on diets with verbascoside or *P. lanceolata* leaves than on controls (Fig. 7). There was no evidence of a dose-dependent response to amount of verbascoside, as larval weights did not differ among the five verbascoside-containing diets (Fig. 7). Survival of larvae was similarly high on all diets (90 % or higher).

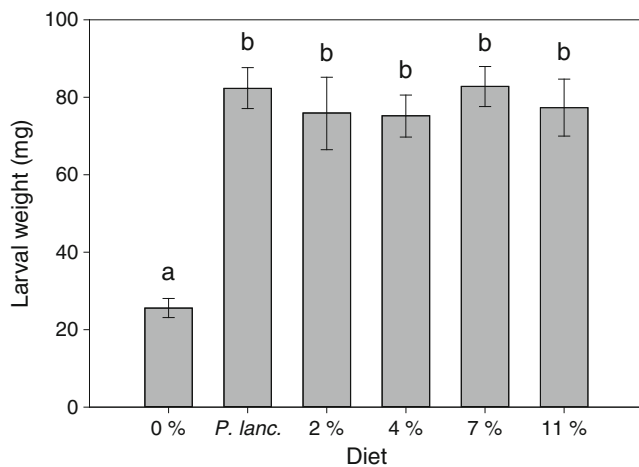


Fig. 7 Weight of *Junonia coenia* larvae after 12 days of feeding on artificial diets. Control = 0 %, no plant material or verbascoside added; *P. lanc.* = powdered *Plantago lanceolata* leaves added as 5 % dry weight of the diet; 2, 4, 7, 11 % = % dry weight verbascoside. Error bars represent +/- 1 SE from the mean. Letters above bars indicate significant differences at *P*<0.001 from Tukey *post-hoc* tests

Discussion

Substantial genetic variation exists both within and across populations of *M. guttatus* for constitutive levels of a suite of predominant foliar secondary compounds, the PPGs (Table 1, Figs. 2, 3). When populations were grouped according to life history strategy (annual vs. perennial), we observed significant differences in constitutive phytochemistry between the two strategies, with perennial populations generally producing higher concentrations of PPGs (Table 1, Fig. 4). In addition to among-plant variation, we found substantial within-plant variation in phytochemistry: constitutive phytochemical levels tended to be lower in younger leaves relative to older leaves (Table 1, Fig. 1). We found that PPGs generally are plastic in response to artificial damage, with a response that varies among populations (Table 1, Fig. 5). For most but not all of these aspects of constitutive and induced phytochemistry, similar patterns of variation were present regardless of whether individual PPGs, total PPGs, or the multivariate PPG phenotype was the dependent variable. Finally, while PPGs (including verbascoside) have been shown to act as allomones against generalist herbivores (Mølgaard, 1986; Holeski et al., unpublished data), we demonstrate that verbascoside functions as a feeding stimulant (kairomone) for a specialist herbivore, *Junonia co.*

The presence of heritable genetic variation for constitutive, individual PPGs indicates that they have the potential to evolve in response to selection. Traits that are the most tightly tied to fitness often may have the lowest heritabilities, as they are closely associated with the outcome of natural selection (Mousseau and Roff, 1987; but see Geber and Griffen, 2003). Thus, one explanation for our observations of low heritability and high concentrations of conandroside and other PPGs across populations is that they have recently been under selection in the natural populations from which our experimental populations were derived. Heritability estimates in the literature for resistance traits (including those in *M. guttatus*) have a wide range (Carroll et al., 2000; Ivey et al., 2009; Hakes and Cronin, 2011).

Substantial variation exists between constitutive, individual PPG concentrations in plants with annual vs. perennial life history strategies (Table 1, Fig. 4), and also between the two life history strategies for the multivariate PPG phenotype. One possible explanation for the lower levels of PPGs in annual populations is that these populations of *M. guttatus* may more frequently employ “behavioral” defenses, such as escape in space or time, than phytochemical defenses (Kursar and Coley, 2003; Carmona et al., 2011). Natural annual populations of *M. guttatus* have a much shorter generation time than do their perennial counterparts; often changing from vegetative to reproductive growth after producing only one or two true leaf pairs (Hall

and Willis, 2006). Annual plants could, thus, avoid herbivory by their shortened vegetative growth period alone, or by a short growth period that is asynchronous with the life cycle of many herbivores (Van der Meijden et al., 1988; Van der Putten, 2003). Few other studies have made direct comparisons between annual and perennial populations of the same or closely related species. In one study, Clauss et al. (2006) found that annual *Arabidopsis thaliana* plants from a single accession had lower concentrations of glucosinolates than those derived from a perennial *Arabidopsis lyrata* population.

Leaves of different ages differ greatly in their multivariate constitutive PPG phenotype, as well as in their concentrations of each individual PPG, with the youngest leaves generally containing the lowest concentrations (Table 1, Fig. 1). Ontogenetic patterns of secondary compound allocation have shown that compound concentrations can increase or decrease with leaf age, depending on plant species (Barton and Koricheva, 2010; Diezel et al., 2011). In our study, the differences in PPG concentrations could arise from two scenarios: 1) developmentally pre-programmed PPG concentrations for each leaf pair result in PPG concentrations that remain constant within a particular leaf as it ages and expands, or 2) physiological or allocation differences that lead to changes in concentrations of secondary compounds within particular leaves as they age and expand. Under scenario 1 but not scenario 2, we predict consistent PPG concentrations for a particular leaf regardless of its age or expansion state when sampled (Koricheva, 1999). Our experiment was not designed to differentiate between these two possibilities; we harvested all the leaf pairs of a particular plant at a defined developmental stage, allowing a direct comparison of leaves of the same developmental stage across plants, but not within plants.

In our experiment, we used artificial herbivory, which is not intended to mimic herbivory by any specific herbivore species. It is well-established that a number of insect-derived cues can influence plant induced responses to particular herbivores, in addition to the general effects of tissue removal (e.g., Tian et al., 2012). Thus, just as individual PPGs may differentially affect particular herbivore species, their plastic response to herbivory also is likely to differ by herbivore species. By using artificial herbivory, we are able to track plant generalized responses to tissue loss, rather than specific response to any cue by a particular generalist or specialist herbivore (Hjältén, 2004).

Concentrations of some individual PPGs and the multivariate PPG phenotype are plastic in response to artificial herbivory; we found genetic variation at the population level in response to damage (i.e., no induction, induced increase in PPG level, or induced decrease; Table 1; Fig. 5). This result is similar to previous studies of trichome induction in *M. guttatus* (Holeski, 2007; Holeski et al., 2010). The

degree of response to damage in individual PPGs or the multivariate PPG phenotype does not vary significantly among leaves of different ages, suggesting either an immediate induction response that is sustained as new leaves develop, or a delayed response with systemic effects. As with our observed patterns of constitutive trichome production, variation among populations in their individual and multivariate PPG induction responses could be a result of differential selection pressures on the parental populations. Plasticity of defenses may be a means of minimizing allocational or ecological costs of defense production when herbivores are absent (Stamp, 2003).

The relationship between constitutive trichome production and constitutive total PPG concentration in the youngest leaf age ranged from positive to negative across populations (Fig. 6). This among-population variation supports our hypothesis that selection pressures by herbivores differed across the ancestral natural populations from which our populations were derived. If trichomes and PPGs act in defense against the same herbivores, and patterns of herbivory are the same across populations and over time, we would expect to see a strong positive correlation between the traits across all populations if both traits were necessary for defense. A negative correlation between the traits across all populations would be noted if production of both defenses is redundant, and defense production is costly. Our observed patterns indicate that the relationship between physical and chemical traits, and likely the types and patterns of herbivory across populations and over time, is not straightforward.

The PPG verbascoside acts as a kairomone for the specialist buckeye caterpillar, *J. coenia*. Buckeye larvae fed artificial diets with purified verbascoside grew at a rate equivalent to those fed artificial diet with dried *P. lanceolata*, one of their host plants (Fig. 7). There did not appear to be a dose-dependent response to verbascoside over the concentration range used in our feeding trial. While generalist herbivores are often deterred by secondary compounds, specialist herbivores can exhibit adaptations to specific phytochemical defenses in their hosts (Bowers and Puttick, 1988; Ali and Agrawal, 2012), even subverting the compounds for use against their own natural enemies. Buckeyes, for example, sequester iridoid glycosides (Bowers, 1984) that are used for their own defense. These compounds are commonly found in close taxonomic relatives to *M. guttatus*. However, gas chromatographic analysis of buckeye caterpillars from the artificial diet experiment showed that verbascoside is not sequestered by these larvae (D. Bowers, unpublished data). The number of PPGs that we detected, in addition to the variation in the levels of each produced, suggest that they may have evolved to function individually, additively, or synergistically against particular herbivores via evolutionary or coevolutionary processes such as defense escalation in response to multiple herbivore species (Agrawal et al., 2009; Becerra et al., 2009).

In summary, the populations of *M. guttatus* that we examined demonstrated substantial genetic variation among and/or within populations in each aspect of physical or chemical defense examined. Our identification of genetic variation in bioactive secondary compounds in the species opens new possibilities for the use of *M. guttatus* as a model study system for plant-insect interactions. We currently are mapping the genetic architecture of PPG production, which will allow us to evaluate the genetic basis for PPG production, as well as the relationship to trichome architecture (Holeski et al., 2010). Future work should include additional feeding trials to evaluate resistance against specialist and generalist herbivores, common garden studies to clarify the roles of genetic, environmental, and genetic x environmental effects on resistance traits, and reciprocal transplants to evaluate selection in natural populations.

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