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RESEARCH ARTICLE

Herbivores disrupt clinal variation in plant responses to water limitation

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Abstract

1. Plasticity in plant traits, including secondary metabolites, is critical to plant survival and competitiveness under stressful conditions. The ability of a plant to respond effectively to combined stressors can be impacted by crosstalk in biochemical pathways, resource availability and evolutionary history, but such responses remain underexplored. In particular, we know little about intraspecific variation in response to combined stressors or whether such variation is associated with the stress history of a given population.
2. Here, we investigated the consequences of combined water and herbivory stress for plant traits, including relative growth rate, leaf morphology and various measures of phytochemistry, using a common garden of *Asclepias fascicularis* milkweeds. To examine how plant trait means and plasticities depend on the history of environmental stress, seeds for the experiment were collected from across a gradient of aridity in the Great Basin, United States. We then conducted a factorial experiment crossing water limitation with herbivory.
3. Plants responded to water limitation alone by increasing the evenness of UV-absorbent secondary metabolites and to herbivory alone by increasing the richness of metabolites. However, plants that experienced combined water and herbivory stress exhibited similar phytochemical diversity to well-watered control plants. This lack of plasticity in phytochemical diversity in plants experiencing combined stressors was associated with a reduction in relative growth rates.
4. Leaf chemistry means and plasticities exhibited clinal variation corresponding to seed source water deficits. The total concentration of UV-absorbent metabolites decreased with increasing water availability among seed sources, driven by higher concentrations of flavonol glycosides, which are hypothesized to act as antioxidants, among plants from drier sites. Plants sourced from drier sites exhibited higher plasticity in flavonol glycoside concentrations in response to water limitation, which increased phytochemical evenness, but simultaneous herbivory dampened plant responses to water limitation irrespective of seed source.
5. *Synthesis*. These results suggest that climatic history can affect intraspecific phytochemical plasticity, which may confer tolerance to water limitation, but that co-occurring herbivory disrupts such patterns. Global change is increasing the

frequency and intensity of stress combinations, such that understanding intraspecific responses to combined stressors is critical for predicting the persistence of plant populations.

KEYWORDS
chemical ecology, drought, herbivory, intraspecific variation, milkweed, multiple stressors, phytochemical diversity, plasticity

1 | INTRODUCTION

Organisms frequently respond to combined stressors non-additively (Smit et al., 2009; Zhang & Sonnewald, 2017), such that characterizing the impact of co-occurring stressors is necessary to predict the ecological impacts of global change (Beauchesne et al., 2021; Orr et al., 2020). Resource limitation exerts strong selection on organismal traits. For example, plants adapted to dry environments can use water more efficiently (Picotte et al., 2007). A trait that increases fitness in the context of one environmental stressor may also produce tolerance of a second stressor ('co-tolerance'), or such a trait may reduce performance when the organism is exposed to a second stressor (a 'trade-off,' e.g. Battaglia & Sharitz, 2006; Feckler et al., 2018; Lucas et al., 2013). Whether a given trait produces co-tolerance or a trade-off depends on its cost and on its potential to be multifunctional. For example, plant investment in roots may confer tolerance to both flooding and shade but make the plant more susceptible to competition in high-light environments (Lucas et al., 2013). Some studies, like those cited above, have addressed the continuum between co-tolerance and trade-offs using comparisons among different species, but we know less about whether there may be similar variation in responses to combined stressors among populations within species. Such understanding will contribute to our ability to predict whether populations will be differentially vulnerable to global change (DeMarche et al., 2019).

Plants use complex hormonal signalling to initiate plastic trait responses to their environments (Atkinson et al., 2015; Felton & Korth, 2000; Nguyen et al., 2016). Phenotypes that are induced upon exposure to multiple stressors, as well as their success at mitigating the stress, thus depend partly on the degree of integration of the biochemical pathways triggered by each stressor (Nguyen et al., 2016; Suzuki et al., 2014). For example, some biotic and abiotic stressors induce conflicting hormonal response mechanisms (Atkinson et al., 2015; Suzuki et al., 2014), which cause trade-offs that plants must manage to maximize fitness (Berens et al., 2019; Yin et al., 2023). It is not yet clear the extent to which such trade-offs can vary among plant populations within species, causing some populations to be more susceptible to combined stressors than others. Among plant populations spanning climatic gradients, genetic variation in phytochemical plasticity in response to climatic stress is apparently common and potentially adaptive (Diethelm et al., 2022; Matesanz & Ramírez-Valiente, 2019), but we do not know how such responses interact with the biotic environment.

Both water stress and herbivory are common in terrestrial plants. In response to each of these stressors acting alone, plants often mount an induced response to mitigate the stress. For example, plants experiencing water limitation may increase their concentrations of antioxidants, which can reduce the cellular damage caused by water stress (Kaminska-Rozek & Pukacki, 2004), whereas plants experiencing herbivory may increase their concentrations of defensive metabolites, deterring further attack (Baldwin, 1998). The few studies to date of combined drought and herbivory suggest that their co-occurrence creates a metabolic cost, or trade-off, which limits the plant's phenotypic response and thereby reduces fitness. For example, water limits the otherwise typical induction of higher nectar volume in *Nicotiana quadrivalvis* following herbivory, which may negatively impact seed set (Halpern et al., 2010). In addition, lower concentrations of plant secondary metabolites, such as antioxidants, are induced under combined drought and herbivory than under drought alone (Mundim & Pringle, 2018). These reduced reactions could negatively impact individual plant performance and even population persistence under global change (Caswell, 1983).

Although induction of higher concentrations of hormones and secondary metabolites has been shown to mitigate plant stress from drought and herbivory, there are also other, less studied dimensions of plant metabolic responses to stress. In particular, plant metabolic profiles can vary in diversity, including variation in both the number of unique compounds produced (i.e. richness) and the relative abundance of compound production (i.e. evenness; Wetzel & Whitehead, 2020). Phytochemical diversity appears to have strong impacts on herbivores (Glassmire et al., 2016; Richards et al., 2015; Whitehead et al., 2021) and may thus influence how much herbivory individual plants receive (Glassmire et al., 2019). Plastic changes in phytochemical diversity can be triggered by both the plant's abiotic and biotic environments. Herbivory is usually predicted to increase phytochemical diversity, but such a response could result either from adaptive induced defence or from chemical degradation caused by the mechanical damage (Philbin et al., 2022). Resource limitation associated with abiotic stress might be expected to constrain phytochemical diversity by causing the plant to focus investment on higher concentrations of critical compounds (Volf et al., 2022), although little support has been found for this prediction to date.

We have previously shown that plants from populations of a widespread milkweed (Apocynaceae: *Asclepias fascicularis*) in the western United States respond to acute water stress by plastically adjusting their phytochemistry in directions consistent with local adaptation to seed-source water deficits (Diethelm et al., 2022). Here, we hypothesized that these apparently adaptive responses to water limitation would be influenced by co-occurring herbivory. Using *A. fascicularis* seeds collected from five populations across a 500-mm gradient of climatic water deficits, we conducted a factorial experiment crossing water limitation with herbivory in an outdoor common garden. We examined plasticity in trait responses to acute stress among seed source populations. We predicted that combined water and herbivory stress would produce interactive, antagonistic responses relative to the responses to each stressor in isolation. Specifically, we predicted that plants from wetter sites, which also experience greater variation in water deficits within years (Diethelm et al., 2022), would exhibit greater plasticity in their responses to water stress, but that this plasticity would be inhibited when herbivory co-occurred. We also predicted that herbivory would increase phytochemical diversity and that water stress would reduce phytochemical diversity, but that the metabolic cost of combined stressors would constrain phytochemical diversity most severely.

2 | MATERIALS AND METHODS

2.1 | Study system

Asclepias fascicularis (narrowleaf milkweed) is one of the most widely distributed milkweed species in the western United States and an important food-plant species for the monarch butterfly (*Danaus plexippus*), among other specialist herbivores (Dilts et al., 2019; Woodson, 1954). Narrowleaf milkweed is found across a wide range of water availabilities, including in very dry locations (down to at least 100 mm of annual precipitation; Woodson, 1954). Milkweeds are well known for their chemical defences, which are sequestered by monarch butterflies. Unlike many milkweeds, *A. fascicularis* contains few cardiac glycosides, but the leaves contain high concentrations of flavonols and both leaves and roots contain diverse pregnane glycosides (Diethelm et al., 2022; Mundim & Pringle, 2020; Rasmann & Agrawal, 2011). Flavonols are hypothesized to play a role in the mitigation of plant water stress by functioning as antioxidants, and the biological role of the pregnane glycosides remains unknown (Diethelm et al., 2022; Kaminska-Rozek & Pukacki, 2004; Zehnder & Hunter, 2007).

To explore the role of climatic history in plant trait responses to combined stressors, we collected seeds from five sites spanning a west-to-east transect of 385 km of the Great Basin Desert, United States, in fall 2016 (Figure S1). Annual precipitation at these five sites encompasses the 5th–95th percentiles of annual precipitation among 453 occurrence records for *A. fascicularis* in the western United States (Dilts et al., 2019). To estimate the typical drought stress at each of these sites, we calculated the cumulative annual climatic water deficit (CWD) using monthly precipitation and temperature data from the PRISM Data Explorer tool (PRISM Climate Group, 2019) for the years 2004–2016 (for more details, see Diethelm et al., 2022). The seed source sites with high CWD (hereafter, dry) experience more water limitation on an annual basis than the sites with low CWD (hereafter, wet). From dry to wet, these sites were Fallon, NV (FN; 989.0 ± 19.7 mm); Georgetown, CA (CA; 985.7 ± 39.4 mm); Battle Mountain, NV (BM; 847.7 ± 29.5 mm); Reno, NV (RN; 587.2 ± 20.0); and Verdi, NV (VE; 460.3 ± 16.7; Figure S1 and Table S1). In a previous study conducted in a glasshouse, plants from drier source populations had higher constitutive concentrations of leaf flavonols, whereas plants from wetter source populations exhibited higher induction of leaf flavonols under acute water stress (Diethelm et al., 2022).

2.2 | Water limitation × herbivory experiment

To investigate how exposure to combined stressors affects plant traits, we conducted a factorial common-garden experiment manipulating water and herbivory. This experiment was conducted from May to October 2017 in a 30 × 10-m outdoor plot at the University of Nevada, Reno, Valley Road Agricultural Station. *Asclepias fascicularis* seeds from each of the five seed source sites, representing four maternal families per site and 16 plants per family ($N = 320$), were germinated in May 2017 and transferred to 0.5-L pots of Full Circle® Soar potting soil to grow for 2 months. In July 2017, the 236 plants that had successfully germinated were transplanted into the plot in a randomized block design (Figure S2). Some of these plants failed to establish in the plot, reducing the total number of plants to 147 (see Table 1 for n per treatment). All plants were fertilized once per month with 1:1:1 Triple Pro® NPK fertilizer pellets.

To evaluate plant responses to single versus combined stressors, plants were randomly assigned to one of four possible treatments: well-watered control (well-watered + no herbivory); dry (dry + no herbivory); herbivory (well-watered + herbivory); or dry + herbivory. Well-watered and dry irrigation lines alternated from east to west through the plot. To enhance establishment, all plants were watered daily to soil saturation using drip irrigation for the first 8 weeks. For 38 days beginning 6 September 2017, well-watered plants received 84 mL of water to an ~157 cm^2 soil area per day while the dry lines received no irrigation. The well-watered treatment was designed to simulate precipitation at the wettest seed source site (VE), whereas the drought treatment was designed to bring plants to their wilting point. After 1 month, plants in the dry treatment were beginning to show severe wilt, so we supplemented them with 84 mL over 2 days in early October.

Herbivory treatments were implemented over 3 weeks, starting in mid-September. Leaves were mechanically damaged to produce ~15% tissue loss, using a rolling leather punch to simulate herbivory (Baldwin, 1990). To test if this mechanical damage effectively simulated damage by herbivores, a randomly selected subset of plants ($n = 30$) received ~5% leaf removal by monarch (*Danaus plexippus*) caterpillars in addition to ~10% mechanical damage. Caterpillars were obtained from a laboratory colony at the University of California, Davis, and were maintained on *A. fascicularis* plants in a glasshouse. In the common garden, larvae were restrained to ~5%

TABLE 1 *Asclepias fascicularis* plant physical and physiological responses from a factorial manipulation of water availability (well-watered, dry) and herbivory. 'Best model' indicates the top model determined by AIC_c.

	Treatment (mean ± SE [*N*])				
Response	**Well-watered**	**Dry**	**Well-watered + Herbivory**	**Dry + herbivory**	**Best model**
Relative growth rate	0.017 ± 0.0022 (*44*)	0.017 ± 0.0025 (*32*)	0.018 ± 0.0017 (*36*)	0.0095 ± 0.0018 (*34*)	CWD + Water*Herbivory
Leaf width (mm)	3.21 ± 0.13 (*44*)	3.03 ± 0.12 (*32*)	3.57 ± 0.14 (*37*)	3.22 ± 0.17 (*34*)	Water
Water-use efficiency (δ13C)	−28.08 ± 0.15 (*42*)	−27.86 ± 0.12 (*32*)	−28.24 ± 0.11 (*37*)	−28.07 ± 0.12 (*34*)	Water + Herbivory
Foliar water content	0.30 ± 0.009 (*44*)	0.29 ± 0.011 (*32*)	0.28 ± 0.008 (*37*)	0.27 ± 0.011 (*34*)	Herbivory
Leaf mass per area (mg/mm^2)	0.092 ± 0.004 (*44*)	0.09 ± 0.004 (*32*)	0.09 ± 0.003 (*37*)	0.09 ± 0.004 (*34*)	Intercept
Trichome density	3.64 ± 0.29 (*44*)	3.25 ± 0.31 (*32*)	3.76 ± 0.36 (*37*)	3.42 ± 0.45 (*34*)	Intercept
Carbon to nitrogen ratio (C:N)	13.4 ± 0.28 (*42*)	13.21 ± 0.24 (*32*)	13.31 ± 0.25 (*37*)	13.61 ± 0.26 (*34*)	Intercept

of above-ground plant tissue using netted bags. We checked plants periodically and removed caterpillars once all of the leaves in the netted section had been consumed. The use of caterpillars in this study was not subject to animal care approval.

Plant responses to the treatments were quantified as follows. To calculate changes in above-ground relative growth rate (RGR), we measured: (i) plant height (cm) and (ii) the number of >4-cm-long stems branching directly from the main plant axis at the beginning and end of treatments. We then multiplied (i) by (ii) to estimate plant size. Relative growth rate was calculated per day using the natural logarithms of the change in size, divided by the 38 days of the experiment. At harvest, six leaves from the second whorl of the main axis of each plant were separated to assess the following traits: leaf width, leaf mass per area (LMA), foliar water content, trichome density and secondary metabolites. We measured leaf width (mm) at the widest point of three leaves and averaged those measurements per plant. To determine LMA (mg dry mass/mm^2), we cut a rectangle of 1-cm length of the fourth leaf, measured the width and dried it at 60°C for 48 h before reweighing. Foliar water content was calculated as the difference between the wet and dry masses divided by the area of the same rectangle. To determine trichome density, we counted the number of trichomes on a randomly selected 2-mm wide leaf edge. To assess plant water-use efficiency, we analysed ∂13-carbon isotopes (Farquhar et al., 1989) from homogenously ground leaf and stem material at the UC Davis Stable Isotope Facility. These same samples were also analysed for percent carbon (C) and percent nitrogen (N), which we used to calculate the C:N ratio.

To investigate how our treatments affected plant secondary metabolites, we performed an untargeted analysis of the UV-absorbent metabolites in the remaining two leaves. Leaves were stored in a −80°C freezer until they were freeze-dried, ground and extracted in methanol with a digitoxin internal standard. Extracts were then run on an UPLC-UV system; for detailed methods, see Appendix S1. UV absorbance spectra were recorded from 200 to 330 nm, and peak areas were quantified at 219 nm. Compounds were differentiated based on retention time and the UV spectrum (Appendix S2) and concentrations were estimated as digitoxin equivalents. We also quantified the richness (S), evenness (J) and the Shannon diversity index (H) of all of the UV-absorbent peaks for each sample with a minimum area of 8000 absorbance units (AU) and a minimum height of 5000 AU. The chemical identities of key peaks were verified by LC-Q-ToF-MS at the Max Planck Institute for Chemical Ecology (Appendices S1 and S3).

2.3 | Statistical analysis

To analyse plant responses to experimental treatments, we used generalized linear mixed models (GLMMs) from the *glmmTMB* package (Brooks et al., 2017) in R version 3.6.1 (R Core Team, 2022). To compare effect sizes, we z-transformed all continuous variables before analysis using the *BBmisc* package (Bischl et al., 2017), and we report beta coefficients (β) from the models with standard errors. We assessed the residuals of each fitted model, and we square-root or log-transformed response variables when these transformations provided a better fit to the gaussian distribution as needed. All plant-trait models used plant maternal family nested within the seed source site as random intercept effects to account for non-independence within families or sites.

To determine the predictors of plant traits, we used backward selection (Zuur et al., 2009) in the *MuMIn* package (Barton, 2019); see Tables S2 and S3 for model selection details. To determine whether interactive effects between stressors depended on seed source, each saturated model began with a three-way interaction among water treatment, herbivory treatment and seed source CWD. Neither the concentration nor the richness of UV-absorbent plant metabolites was changed by the addition of monarch herbivory to mechanical damage (respectively, Welch's $t = -0.12$, $\text{df} = 56.1$, $p = 0.9$; Welch's $t = 0.53$, $\text{df} = 65.8$, $p = 0.6$; Figure S3), so all herbivory treatments were pooled in subsequent analyses.

Models were selected based on the lowest sample size-corrected Akaike information criterion (AICc), and any marginally significant predictors were evaluated using log-likelihood ratio tests (LRT) between models in the *lmtest* package (Zeileis & Hothorn, 2002). Marginal and conditional R^2 values were calculated for selected models in the *MuMIn* package. Data are archived in Dryad (Diethelm et al., 2023).

3 | RESULTS

3.1 | Identification of plant secondary metabolites

Overall, we quantified 56 unique UV-absorbent metabolites in *A. fascicularis* leaves, including five compounds that were putatively identified, using the UV spectra and exact masses, as flavonol glycosides (hereafter flavonols) and 16 as benzoylated pregnane glycosides (hereafter pregnane glycosides) (Appendices S2 and S3). Three dominant flavonols were present in all plants, with a single flavonol—quercetin–glucoside–rhamnoside (QGR)—comprising ~75% of the total concentration of UV-absorbent metabolites. Isorhamnetin–glucoside–rhamnoside (IGR) was the second most abundant flavonol, followed by isorhamnetin–glucoside (IG). Unlike the flavonols, the pregnane glycosides were highly variable in their presence among individual plants, with 0–13 unique pregnane glycosides per plant.

3.2 | Seed source climate and plant trait means

Seed source CWD was an important predictor of plant trait means, particularly for phytochemistry. The total concentration of UV-absorbent metabolites (mg/g) in the leaves increased ~10% from wetter to drier seed sources ($\beta_{\mathrm{CWD}} = 0.23 \pm 0.08$, $z = 2.61$, $p < 0.01$; Figure S4b,e), a pattern driven by an ~14% increase in the concentration of flavonols from wetter to drier seed sources ($\beta_{\mathrm{CWD}} = 0.27 \pm 0.08$, $z = 3.38$, $p < 0.0008$, Figure 1a; Table S2). The dominant flavonol, QGR, in particular was found in ~16% higher concentrations in plants from drier sites ($\beta_{\mathrm{CWD}} = 0.26 \pm 0.10$, $z = 2.62$, $p < 0.009$; Table S2). Leaf chemical diversity (H) was higher in plants sourced from wetter sites (Figure 1b), partly because evenness was higher among plants with lower concentrations of the dominant flavonols (Figure S4). Plants from wetter sites also had higher metabolite richness ($\beta_{\mathrm{CWD}} = -0.17 \pm 0.08$, $z = -2.1$, $p < 0.04$, Figure S4), due to a higher richness of pregnane glycosides ($\beta_{\mathrm{CWD}} = -0.24 \pm 0.08$, $z = -3.00$, $p < 0.003$; Table S2). Relative growth rate was marginally higher among plants from relatively wetter seed sources ($\beta_{\mathrm{CWD}} = -0.14 \pm 0.08$, $z = -1.77$, $p < 0.08$; Table S3).

3.3 | Plant responses dominated by single stressors

Water limitation and herbivory each caused changes in some plant traits irrespective of the other stressor (Tables S2 and S3). Water limitation reduced leaf width: water-limited plants produced leaves ~6% narrower than well-watered plants ($\beta_{\mathrm{water}} = -0.28 \pm 0.15$, $z = -1.92$, $p = 0.055$; Table 1). Water-limited plants also had higher leaf δ13C values than well-watered plants, indicating higher leaf water use efficiency ($\beta_{\mathrm{water}} = 0.28 \pm 0.15$, $z = 2.0$, $p = 0.05$; Table 1). Herbivory, meanwhile, marginally increased the richness (S) of pregnane glycosides in the leaves ($\beta_{\mathrm{herbivory}} = 0.29 \pm 0.16$, $z = 1.83$, $p = 0.07$) and reduced foliar water content ($\beta_{\mathrm{herbivory}} = -0.35 \pm 0.15$, $z = -2.28$, $p < 0.03$, Table 1). Trichome density, LMA and the C:N ratio were not affected by either of the treatments (Table 1 and Table S3).

3.4 | Plant responses contingent on multiple stressors

Co-occurring herbivory and water limitation resulted in slower growing plants (Figure 2) and dampened phytochemical responses to stress (Figure 3). Relative growth rates of the plants experiencing either water limitation or herbivory alone were not different from those of well-watered control plants, but plants experiencing both stressors grew 45% more slowly ($\beta_{\mathrm{water:herbivory}} = -0.57 \pm 0.32$, $z = -1.78$, $p < 0.08$; Figure 2). Water limitation increased leaf chemical diversity (H) only in plants without herbivory ($\beta_{\mathrm{water:herbivory}} = 0.38 \pm 0.14$, $z = 2.67$, $p < 0.008$; Figure 3a; Table S2). This increase in

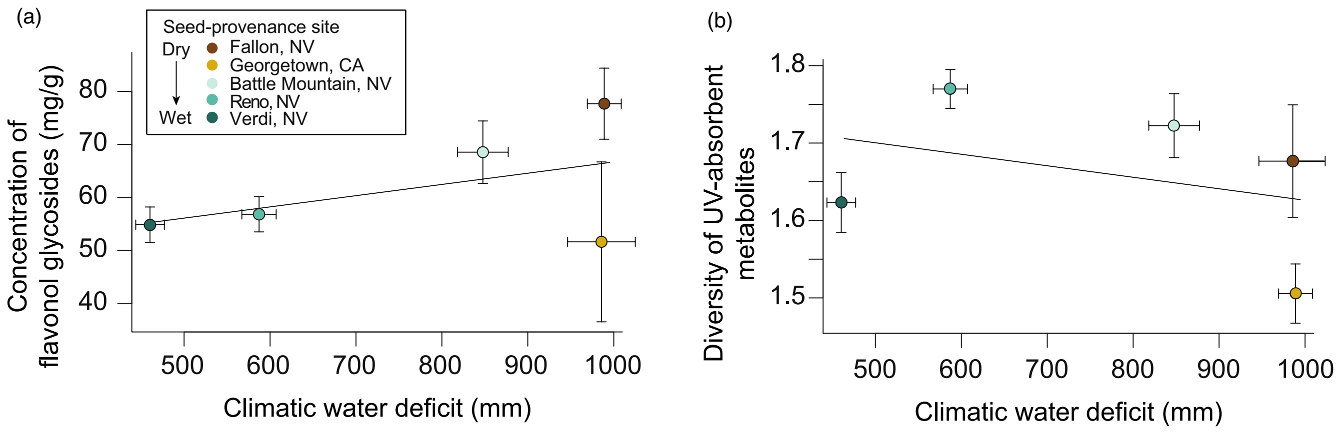

FIGURE 1 Phytochemistry in *A. fascicularis* leaves measured as (a) total flavonol–glycoside concentration (mg/g; $p < 0.001$) and (b) phytochemical diversity (Shannon index; $p = 0.09$) across the climatic water deficit (CWD) of the seed source location. Points show the mean value of all plants from each seed source ($n = 143$) and are coloured by their location along the gradient of water availability from wet to dry. Vertical bars represent SE of the mean trait value and horizontal bars represent the SE of the CWD value, calculated interannually from 2004 to 2016.

chemical diversity corresponded partly to a reduction in the QGR concentrations in the leaves of dry plants, which increased phytochemical evenness ($\beta_{\text{water:herbivory}}=0.56\pm0.14$, $z=3.94$, $p<0.0009$; correlation between QGR and evenness: Pearson's $r=-0.50$, df = 141, $p<0.0001$; Figure 3b,d). Herbivory induced more unique compounds (i.e. higher phytochemical richness) than water limitation (Table S2), but induction of phytochemical richness was also dampened when plants experienced simultaneous stressors (Figure 3c).

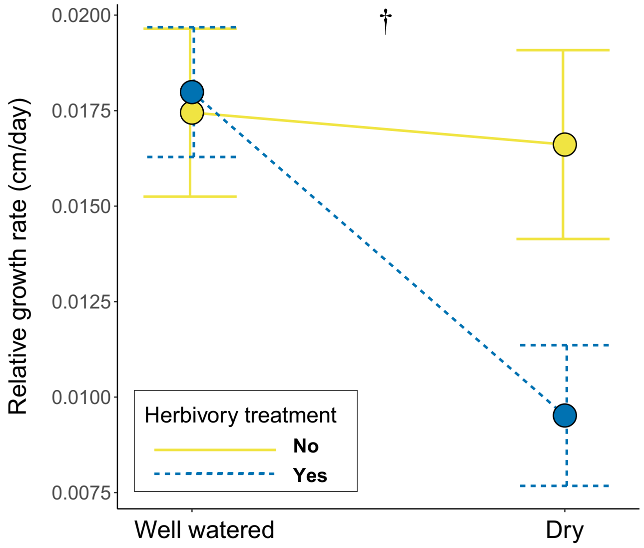

FIGURE 2 Responses of relative growth rate to water availability and herbivory. Interaction lines show reaction norms separately by water × herbivory treatments. Yellow solid lines represent treatments with no herbivory, and blue-dashed lines represent treatments with herbivory. Points represent means and bars represent SE. The cross (†) represents $p<0.08$, as determined by the best-fit general linear mixed model for the interaction effect between water limitation and herbivory.

3.5 | Impacts of seed source on plant response to combined stressors

Climatic water deficit at the seed source did not modulate the antagonistic effect of combined stressors on relative growth rate (water × herbivory × CWD: LRT: $\chi^2=0.52$, df = 3, $p=0.9$) or phytochemical diversity (water × herbivory × CWD: LRT: $\chi^2=0.94$, df = 2, $p=0.6$). Nevertheless, seed source climate did affect plasticity in phytochemical diversity (Table S2), though not in the direction that we had predicted. Plants from drier sites showed larger increases in phytochemical diversity than plants from wetter sites when experiencing water limitation in isolation ($\beta_{\text{water:CWD}}=0.38\pm0.14$, $z=2.67$, $p<0.008$; Figure 4a), and this clinal variation in responses to dry conditions was disrupted by co-occurring herbivory (Figure 4b). In particular, co-occurring herbivory disrupted clinal variation in the reductions of leaf flavonol concentrations, which had increased evenness in plants experiencing only water limitation (Figure S4).

4 | DISCUSSION

Plants routinely experience multiple stressors. To predict the ecological consequences of this, we must understand whether co-occurring stressors interact to produce non-additive effects on

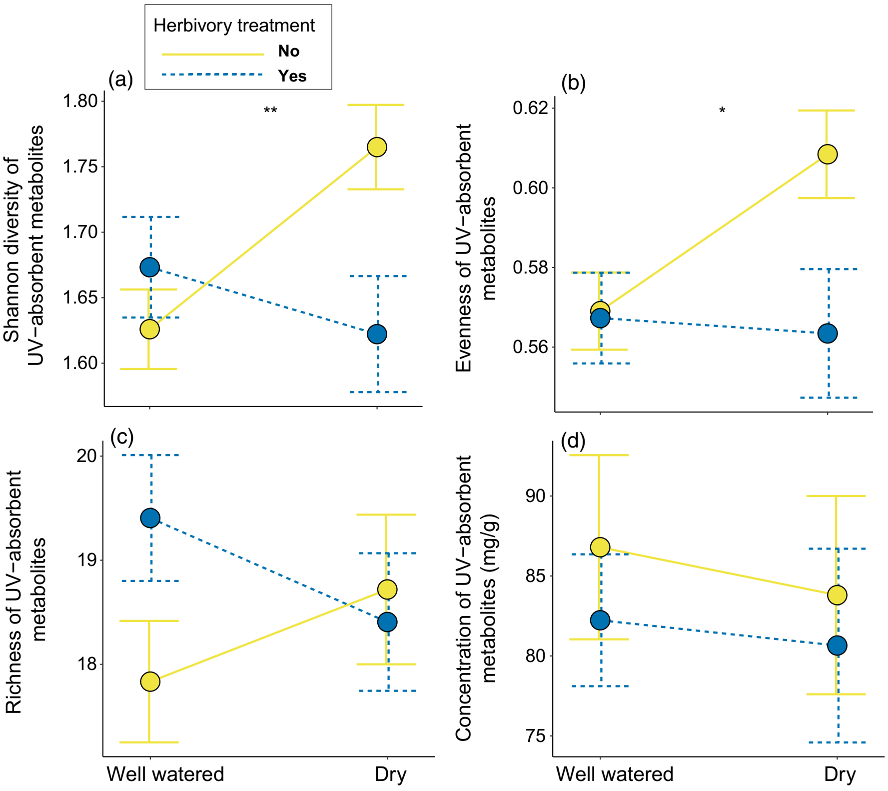

FIGURE 3 Responses of phytochemical (a) diversity, (b) evenness, (c) richness and (d) total concentration (mg/g) to water availability and herbivory. Interaction lines show reaction norms separately by water × herbivory treatments. Yellow solid lines represent treatments with no herbivory, and blue-dashed lines represent treatments with herbivory. In all plots, points represent means and bars represent SE. Changes in (a) phytochemical diversity in the factorial water × herbivory experiment were driven by changes in both (b) metabolite evenness and (c) metabolite richness. Significance values are from the best-fit general linear mixed models for the interaction effects of water limitation and herbivory as follows: **$p<0.01$, *$p<0.05$.

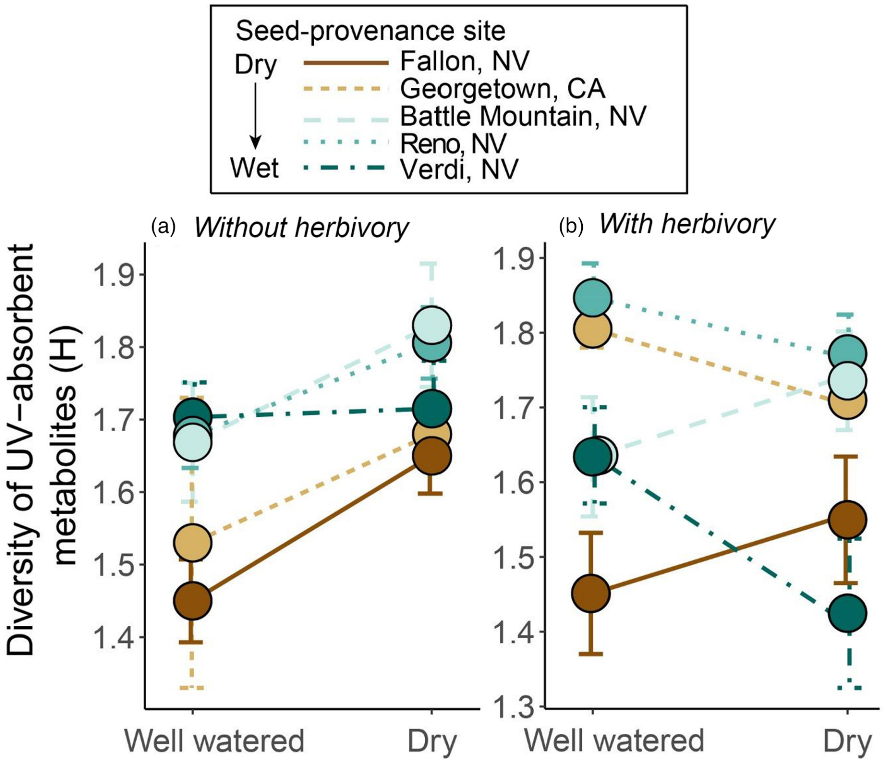

FIGURE 4 Among-population variation in the responses of phytochemical diversity (Shannon index) to the dry treatment in a common garden, (a) without and (b) with combined herbivory. The interaction plots show reaction norms separately by seed source ($p < 0.008$) and are coloured by their location along a gradient of climatic water deficits from dry to wet.

phenotypes, as well as whether these consequences depend on variable population histories of exposure to stress. Here, we have shown that *A. fascicularis* milkweeds sourced from a water-deficit gradient show clinal increases in phytochemical diversity in response to water limitation by itself, but that co-occurring herbivory disrupts this pattern. Moreover, although we observed that the diversity of secondary metabolites increased in response to both water limitation and herbivory in isolation—corresponding to an increase in evenness following water limitation and an increase in richness following herbivory—plants that experienced combined water and herbivory stress had similar phytochemical diversity to well-watered control plants. We speculate that simultaneous water limitation and herbivory exert antagonistic effects on phytochemical diversity because there are trade-offs between the biochemical networks required to respond to each stressor (Demmig-Adams et al., 2013). To the extent that phytochemical diversity or its components can improve the performance of individual plants (Glassmire et al., 2019; Volf et al., 2022; Whitehead et al., 2021), these results suggest that plants in natural communities may be less able to mitigate stress than would be suggested by experiments that manipulate only one stressor at a time.

Our results thus support our general predictions that combined water and herbivory stress produce interactive, antagonistic responses, and that the metabolic cost of combined stressors can constrain phytochemical diversity. Yet our results do not support our specific prediction that these effects would result from herbivory constraining leaf-flavonol induction under water stress in plants sourced from wetter sites. As in our prior work (Diethelm et al., 2022), plants from drier sites contained higher constitutive concentrations of flavonols in the leaves, which is consistent with a role for these compounds in mitigating water stress, and these compounds dominated the plants' plastic responses to water limitation. In this study, however, in contrast to our previous one, plants from drier sites actually exhibited higher plasticity in flavonol concentrations than plants from wetter sites. Surprisingly, in this study, plants from drier sites reduced their concentrations of leaf flavonols under water limitation as opposed to increasing them. Interestingly, well-watered control plants in this outdoor experiment contained 62% higher concentrations of flavonols than well-watered control plants in our previous experiment, which was conducted in a glasshouse (50.5 mg/g vs. 31.2 mg/g). Because flavonols can also act as antioxidants against UV radiation (Dixon et al., 2001; Fischbach et al., 1999), plants in this outdoor garden may have had more flavonols because they were not beneath UV-filtering glass. The reduction in leaf flavonols under water stress in plants sourced from dry sites could thus have been triggered by the metabolic cost of flavonols: such high flavonol concentrations may be beneficial only under higher water availability, where their cost is more easily maintained (Scheiner et al., 2020).

Co-occurring herbivory disrupted this pattern in flavonol plasticity, most notably by causing plants from our driest site—Fallon, NV—to still produce some of the highest concentrations of leaf flavonols among our experimental plants, even under dry conditions. We have previously shown that higher flavonol plasticity is associated with the maintenance of total biomass under water stress in these milkweeds (Diethelm et al., 2022). Likewise, in this study, reduced plasticity was associated with lower relative growth rates above-ground, which suggests that disruption of plasticity by combined stressors produces a fitness cost. Nevertheless, we note that above-ground growth rates may be an insufficient proxy for plant fitness if plants are instead allocating energy to other functions necessary for survival, such as below-ground growth or carbohydrate storage (Huang et al., 2021; Peng et al., 2020).

These changes in flavonol plasticity under single and combined stressors were a major driver of our patterns in phytochemical evenness. Although higher phytochemical diversity and richness are generally associated with better anti-herbivore defence, the ecological role of phytochemical evenness per se is less studied (Wetzel & Whitehead, 2020) but may also be protective (Salgado et al., 2023; Whitehead et al., 2021). A recent experimental study of phytochemical evenness in phenolics, including flavonols, found that mixtures with higher evenness were defended against more species of natural enemies (Whitehead et al., 2021). Another study found a negative correlation between phytochemical evenness and herbivory among lineages within a wetland grass species (Salgado et al., 2023). Extrapolating these findings to our system suggests that milkweeds experiencing combined drought and herbivory will be poorly defended against further herbivory. Indeed, in a study of the effects of water and herbivory stress in *A. syriaca* milkweeds on the development and survival of the specialized monarch butterfly (*Danaus plexxipus*), monarch caterpillars gained significantly more mass on plants that had experienced both low-water and herbivory than on plants that had experienced low-water alone (Hahn & Maron, 2018). Although much remains to be learned about the modes of action of phytochemical evenness in different plant species, phytochemical evenness could prove to be an important functional trait, particularly within species, given how relatively straightforward it may be for plants to regulate the relative abundance of their secondary metabolites from established biochemical pathways (Salgado et al., 2023).

Our measure of phytochemical diversity in this study, which accounted for the richness and abundance of individual molecules with chromophores as measured by a chromatographic method, is a measure of compositional diversity, rather than one of metabolic or structural complexity (Philbin et al., 2022). Hypotheses surrounding the potential importance of phytochemical richness to plant defence often emphasize the potential for synergisms in phytochemical mixtures (Gershenzon et al., 2012; Richards et al., 2016). Such synergisms may be better predicted from an understanding of structural complexity, to the extent that structure predicts function (Philbin et al., 2022). Our result that phytochemical richness increased following herbivory was driven by changes in the presence of ~13 pregnane glycosides, many of which may be structurally related to one another and which are highly variable among individual *A. fascicularis* plants. Whether pregnane glycosides play a role in milkweed defence remains unclear, although the presence of pregnane glycosides often trades off with that of the better known cardiac glycosides, suggesting a potential defensive role (Diethelm et al., 2022; Zehnder & Hunter, 2007). It is thus possible that the higher richness of pregnane glycosides following herbivory acts as an induced defence. Yet it is also possible that this higher richness followed simply from the degradation of one of the more common pregnane glycosides following herbivory. Future work should address these alternative possibilities.

Our results are consistent with a growing body of work indicating that there are trade-offs in plant responses to simultaneous abiotic and biotic stress (Atkinson et al., 2015; Raderschall et al., 2021; Yin et al., 2023). Our study builds on this prior work by showing that clinal variation in plants' plastic responses to abiotic stress, which is consistent with local adaptation or adaptive transgenerational plasticity (sensu Herman & Sultan, 2011), can be disrupted by co-occurring biotic stress. Interestingly, this disruption was uncovered most clearly in the patterns of phytochemical diversity, a relatively underexplored functional trait, and in relative growth rate, a frequent proxy for plant performance. Indeed, the other physical and ecophysiological traits that we examined did not show patterns that were as clearly influenced by seed source environment or as indicative of trade-offs, such that ecological studies of combined stressors in plants may benefit from explicitly including phytochemical traits. The results here suggest that the plasticity of plant traits in response to water stress may be locally adapted, but that the capacity of plant populations to persist under drought will depend on more than tolerance to water stress alone. Our findings support the need for including adaptive plasticity as well as realistic combinations of ecological stressors in the study of the consequences of intraspecific variation for population dynamics.

AUTHOR CONTRIBUTIONS

Aramee C. Diethelm and Elizabeth G. Pringle designed the study; Aramee C. Deithelm, Michael Reichelt and Elizabeth G. Pringle performed the experiments; Aramee C. Deithelm, Michael Reichelt and Elizabeth G. Pringle analysed the data; Aramee C. Diethelm and Elizabeth G. Pringle wrote the paper, with editorial suggestions from Michael Reichelt.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/1365-2745.14237.

DATA AVAILABILITY STATEMENT

All data and corresponding R code are archived in Dryad: https://doi.org/10.5061/dryad.k6djh9wd5 (Diethelm et al., 2023).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Seed collection sites from across a climatic water deficit gradient, based on 1981 to 2010 climate normal.

Figure S2. Experimental planting design in the common garden.

Figure S3. Comparison of the effects of different herbivory treatments on (a) the concentration of UV-absorbent metabolites and (b) the richness of UV-absorbent metabolites.

Figure S4. Phytochemistry in metabolites in *A. fascicularis* in a common garden in response to (a–c) the dry treatment alone, and (d–f) the dry + herbivory treatment as separately by seed-source. Interaction plots show reaction norms for (a, d) richness, (b, e) total concentration, and (c,f) evenness per seed-source with locations colored in a gradient of dry to wet climatic water deficit values.

Table S1. Seed-source site in decimal degrees from high to low climatic water deficit.

Table S2. Model selection results for *Asclepias fascicularis* chemical leaf traits in a common garden experiment from the global GLMM.

Table S3. Model selection results for *Asclepias fascicularis* physical leaf traits in a common garden experiment from the global GLMM.

Appendix S1. Instrument methods for plant chemical analyses.

Appendix S2. Example chromatograms and catalog of UV-spectra for all UV-absorbent metabolites.

Appendix S3. Analytical information for the UV-absorbent chemistry of plants in the outdoor water × herbivory experiment.

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