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From plants to predators: investigating the phytochemical landscape from the herbivore's perspective

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Abstract

The phytochemical landscape—the diverse and spatially heterogeneous distribution of plant chemical compounds—plays a crucial role in shaping tritrophic interactions. Focused on the perspective of herbivores, this dissertation investigates variations in plant chemistry along an environmental gradient, the responses of herbivore predators to climatic conditions, and the resulting impacts on trophic dynamics. Specifically, it presents an overview of how environmental stress, predator-exposure, and food plant identity interact to influence the larval performance of a specialist Lepidopteran, the monarch butterfly (*Danaus plexippus* L.), which is exclusively reliant on *Asclepias* (milkweed) species during its larval stage. Focusing on two prevalent species of western milkweed (*A. fascicularis* and *A. speciosa*), this dissertation provides invaluable insights into the relationships between herbivores, their natural enemies, and the ever-changing environment that they inhabit.

Chapter 1 delves into the effects of climate at the seed source on plant chemical plasticity in response to water stress. By employing common gardens of *A. fascicularis* and *A. speciosa* sourced from sites across an aridity gradient, the study uncovers patterns of constitutive and induced expression of antioxidant compounds in the chemical class flavonols. Flavonols were found in higher constitutive concentrations in plants sourced from drier sites, and both species exhibited increased leaf flavonol concentrations in response to water stress. Interestingly, *A. fascicularis* plants from wetter sites displayed higher flavonol plasticity, while *A. speciosa* demonstrated weaker patterns. Such opposing patterns of constitutive and induced flavonol expression led to a reduction in

the variation between populations under water stress, suggesting the influence of local adaptation in shaping phytochemical strategies for water limitation.

Chapter 2 investigated the consequences of combined water and herbivory stress on plant traits and phytochemical diversity using *A. fascicularis* milkweeds. With this study, I found that water limitation alone increased the evenness of UV-absorbent secondary metabolites (plant defensive phytochemicals), whereas herbivory alone increased the richness of metabolites. However, plants experiencing combined water and herbivory stress displayed similar phytochemical diversity to control plants, associated with a reduction in relative growth rates. Leaf chemistry average constitutive levels and plasticities exhibited clinal variation corresponding to seed-source water deficits, suggesting climatic history can influence phytochemical plasticity, while co-occurring herbivory disrupted these patterns.

Chapter 3 builds upon the previous studies to explore the tritrophic perspective, with a focus on the non-consumptive impacts of predators on monarch larval performance. Here, I investigated how exposure to predators affected larval development and survival, and whether those effects could be modulated by food plant identity, using the two species of milkweed from Chapter 1. I found that monarchs developed more slowly when exposed to predators, particularly when feeding on *A. speciosa*. Overall, the results suggest that larvae developing on architecturally simple *A. speciosa* plants spent more time avoiding predators and less time eating compared to larvae on bushier, more branching *A. fascicularis* plants. The findings underscore the role of predation risk in monarch larval performance and highlight the importance of larval food plant species in mediating non-consumptive predator effects.

Lastly, Chapter 4 examines the interplay between predator exposure, abiotic conditions, and food plant identity in shaping monarch larval performance across a water-availability gradient. Exposure to predators significantly reduced larval survival, and had sub-lethal effects where increasing diversity for predator communities lead to greater delays in larvae reaching adulthood. Moreover, extreme climatic conditions influenced the developmental timing and adult size of monarch larvae, further highlighting the complexity of interactions between biotic and abiotic stressors impacting larval success.

In conclusion, this dissertation underscores the vital role of the phytochemical landscape in shaping tritrophic interactions. Specifically, it sheds light on how environmental stress, predator-exposure, and food plant identity interact to influence the larval performance of a specialist herbivore, the monarch butterfly.

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Chapter 4

Background

A central goal of chemical ecology is to understand how plant chemistry mediates community dynamics across different spatial and temporal scales (Raguso et al. 2015, Dyer et al. 2018). Plant chemistry is itself dynamic, varying within and among species and across environmental gradients (Gershenzon 1984, Kergunteuil et al. 2019). In turn, this variation influences trophic interactions as it affects herbivores, which are themselves a critical food source for various predators (Hunter 2016, Kessler and Kalske 2018).

Plants can respond to herbivory with various defenses, including an increase in the concentration of defensive metabolites to discourage further attacks (Baldwin 1998). Alternatively, plants may attract the natural enemies of herbivores using chemical signals (Schoonhoven et al. 2005). An increase in chemical concentrations or diversity, including the number and relative abundance of phytochemical compounds produced, can deter herbivores (Glassmire et al. 2016, Richards et al. 2016). Plants can also alter their growth patterns to increase defensive structures such as thorns or leaf hairs (Schuman and Baldwin 2016, War et al. 2012). Additionally, plants may employ compensatory growth, a process where they accelerate their growth rate to recover from herbivore damage and offset the impact of herbivory on their overall fitness (Strauss and Agrawal 1999). Over time, populations of plants can adapt to be more herbivore resistant, creating intraspecific variation (Hahn and Maron 2016).

Plants also display remarkable adaptability in responding to abiotic stress, adapting to minimize its adverse effects on their growth and survival. For example, plants can implement various strategies to mitigate the impact of water stress, through changes to their primary and secondary metabolites. Primary metabolites in plants are essential

compounds like carbohydrates, proteins, and nucleic acids, involved in basic growth and development. Secondary metabolites, such as alkaloids, phenolics, and terpenoids, are compounds serving a diverse suite of ecological roles, including defense. For example, plants experiencing water scarcity often elevate the synthesis of antioxidants such as phenolics, which help combat cellular damage induced by drought stress (Treutter 2006, Mundim and Pringle 2018). Plants can also alter their levels of carbohydrates, such as sucrose and glucose, to conserve energy and support crucial metabolic processes during water scarcity (Chaves et al. 2003). These chemical alterations play a crucial role in bolstering the plant's ability to withstand drought and, ultimately, enhance its resilience in challenging environmental conditions (Chaves et al. 2002). Over time, natural selection may favor plants with traits like improved water-use efficiency, deeper root systems, or increased production of compounds that mitigate oxidative stress, leading to the emergence of drought-tolerant plant populations (Westerband et al. 2021).

With global climate change, plants are increasingly being exposed to multiple stressors (Surówka et al. 2020). When stressors co-occur, they can create non-additive metabolic costs, potentially limiting phenotypic responses (Suzuki et al. 2014, Atkinson et al. 2015). Certain biotic and abiotic stressors trigger opposing response mechanisms (Atkinson et al. 2015), and the consequences of such conflicting signaling on plant phenotypes remain understudied (Suzuki et al. 2014). Furthermore, how changes in plant phenotypes in response to combined stressors impact herbivore development and survival is not well understood (Couture et al. 2015, Lin et al. 2023).

Plants in the genus *Asclepias* (Apocynanceae; milkweeds) are an excellent system for studying the effect of phytochemistry on plant-herbivore-predator interactions.

Milkweeds are known for their defensive chemistry, particularly cardenolides, toxic steroidal glycosides that disrupt the osmotic balance in cells (Malcolm 1994, Malcolm and Zalucki 1996). Furthermore, milkweeds show variation both among and within species in their production of defensive compounds (Agrawal et al. 2012). Cardenolides are constitutive, but their production can also be up-regulated following herbivore damage (Rasmann et al. 2009, Agrawal and Hastings 2019). Milkweed also produces other secondary metabolites, including pregnane glycosides and phenolics, although the ecological roles of these other compounds are less understood (Agrawal et al. 2009, Si et al. 2022). Pregnane glycosides regulate appetite in some vertebrates (Komarnytsky et al. 2013a, 2013b), and flavonoids such as quercetin glycoside act as oviposition stimulants for females of the monarch butterfly (*Danaus plexippus* L.), a milkweed-specialist herbivore (Haribal and Renwick 1996, Baur et al. 1998). Milkweed also accumulates latex in pressurized cells, which acts as both a physical defense and a chemical defense because latex contains cardenolides and possibly other toxic metabolites (Malcolm and Zalucki 1996, Zalucki et al. 2001).

Milkweed is the primary larval food plant for the monarch butterfly, which can sequester plant metabolites for their own defense (Malcolm 1994). Despite the monarch's tolerance of the milkweed's cardiac glycosides, these toxins still affect both larval development and adult fitness (Zalucki et al. 2001, Agrawal et al. 2021). Monarchs are known for their transcontinental, multigenerational migration (Urquhart and Urquhart 1978, Agrawal 2017). There are two major, semi-independent migratory subpopulations of monarchs in North America (Brower and Boyce 1991; Brower 1995). The western population of monarchs generally overwinter on the coast of California before expanding

across the West during the spring and summer to reproduce, followed by a fall migration back to California (Urquhart and Urquhart 1977, Dingle et al. 2005). This population is far less studied than the eastern monarchs, which are known for overwintering in Mexico before expanding back into Canada (Urquhart and Urquhart 1977, Dingle et al. 2005, Dyer and Forister 2016). Although both populations are declining, the western population is declining at a faster rate (Espeset et al. 2016, Schultz et al. 2017, Pelton et al. 2019) In the last 40 years, the overwintering population of Western monarch butterflies has plummeted by ~95% (Schultz et al. 2017, Pelton et al. 2019). Several factors have been suggested to explain the precipitous drop in numbers, including pesticide-use and habitat loss (Pelton et al. 2019, Halsch et al. 2020). Shifting climate, principally the increase of drought in the West, is also implicated in the decline of monarchs (Stevens and Frey 2010, Crone et al. 2019). Water deficits—an environmental stress that is predicted to increase in frequency as the climate changes—can drive variation in the phytochemical landscape by reducing plant growth and altering plant nutritional and defensive chemistry (Huberty and Denno 2004, Osakabe et al. 2014, Mundim and Pringle 2018). Specialist herbivores, which consume only a limited number of closely related plant species, are influenced by variation in plant chemistry both among and within species. These chemical differences significantly impact the population dynamics of specialist herbivores by affecting larval development and susceptibility to natural enemies (Awmack and Leather 2002, Rayor 2004, Kichenin et al. 2013).

This dissertation investigates the phytochemical landscape from an herbivore's perspective, seeking to understand how plant chemistry varies across a gradient of water availability, how herbivore predators vary with climatic conditions, and how climatic

conditions themselves influence trophic dynamics. The investigation of tri-trophic relationships amidst changing climatic conditions further deepens our comprehension of the factors influencing herbivore vulnerability in the context of continuous global environmental transformations. The studies comprising this dissertation provide valuable insights into the dynamic and delicate interplay between herbivores, their resources, and the ever-changing climate they inhabit.

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Chapter 1

Climatic history, constraints, and the plasticity of phytochemical traits under water stress

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Abstract

Environmental stress can induce changes in organismal traits and in resulting intraspecific variation. The nature of such effects will depend on the plasticity of trait expression and on any ecological constraints to such expression. Plants can mitigate abiotic stress, like drought, by changing their chemistry, but the ability to induce costly metabolites may be under strong local selection and ecologically constrained. Here we asked whether climate at the seed source predicts plant chemical plasticity in response to water stress and what the consequences are for intraspecific variation in phytochemical traits. To this end, we used common gardens of two widespread species of western milkweed (*Asclepias fascicularis* and *Asclepias speciosa*) that had been collected from sites across an aridity gradient. Both species produce high concentrations of leaf flavonols, which are hypothesized to mitigate water stress by functioning as antioxidants. These compounds were found in higher constitutive concentrations in plants sourced from drier sites, and both species responded to water stress in the common garden by increasing leaf flavonol concentrations. Interestingly, flavonol plasticity was higher in plants sourced from wetter sites in *A. fascicularis*, with similar, but weaker, patterns in *A. speciosa*. These opposing patterns in constitutive and induced flavonol expression reduced the variation between populations in leaf flavonol concentrations under water stress. These results suggest that local adaptation in plants can shape phytochemical strategies for water limitation but that the cost of metabolite production may ultimately limit the range of phytochemical variation.

Keywords co-gradient plasticity, constraints, drought, intraspecific variation, milkweed, phytochemistry, resource availability

1 | Introduction

Intraspecific variation may increase diversity and resilience in ecological communities (Bolnick et al. 2011), but it remains unclear what environmental factors favor such variation (Kuppler et al. 2020). For example, theory can alternatively predict stressful environmental conditions to increase or to decrease intraspecific variation (Hoffmann and Merilä 1999). Empirically, stress appears to reduce genetically determined phenotypic variance (*i.e.*, heritability) within populations, but this effect is stronger in some traits than in others (Charmantier and Garant 2005). Stress can also influence the magnitude of phenotypic variation among populations, but if there is a directional link, it is not yet clear (Matesanz and Ramírez-Valiente 2019). Given that global change is exerting increasing stress on organisms worldwide (Orr et al. 2020), a better understanding of how such conditions will affect phenotypic variation within species is necessary.

Phenotypic variation in the same environment may be driven either by variation in fixed genetic traits or by genetic variation in plasticity (*i.e.*, gene-by-environment interactions, G × E). Within a set of genotypes, then, any changes in phenotypic variation with environmental stress will be the result of G × E. The mechanisms that favor more plastic genotypes may depend on spatial scale: differential selection and drift are more likely among populations than within them. Among populations, for example, higher plasticity could be adaptive in populations that have experienced more, and more

predictable, climatic variation (Pratt and Mooney 2013, Leung et al. 2020). On the other hand, resource availability may consistently constrain plasticity and associated trait expression among individuals regardless of their evolutionary history (Valladares et al. 2007, Van Buskirk and Steiner 2009). If so, then phenotypic variation may be lower among populations under stress compared to variation among populations in less stressful conditions, similar to an apparent trend of reduced heritability under stress within populations (Charmantier and Garant 2005). The consequences of such reduced variation could include both reduced survival of individuals and reduced resilience of the ecosystem under rapid environmental change (Fox et al. 2019, Wilcox et al. 2020).

A recent meta-analysis reported that among-population variation in trait plasticity (*i.e.*, P × E) is common in terrestrial plants (occurring in 77% of 151 case studies) (Matesanz and Ramírez-Valiente 2019). Among the 20 studies that had assessed whether such P × E effects led to more or less variation among populations in stressful environments, however, there was no clear pattern (Matesanz and Ramírez-Valiente 2019). Population differentiation in stressful environments was found to be higher, lower, or equivalent to differentiation in less-stressful environments in roughly equal proportions. These results should be considered in the light of at least two contextual variables: (i) how do we define stress?, and (ii) which traits?

Stress can be defined most broadly as any time an organism experiences conditions outside its fundamental niche (Steinberg 2012), but such a broad definition can conflate novelty with unfavorable conditions (Schlichting 2008). Within populations, unfavorable environmental conditions appear to reduce trait variation, whereas novel conditions do the opposite (Charmantier and Garant 2005). This distinction may be

similarly important among populations. Here, we define stress as severe resource limitation, with the potential for trade-offs in allocation of the resource or its products among biosynthetic pathways (Auld et al. 2010). This definition lends itself, in turn, to a specific consideration of the biochemical traits of individuals, which may provide direct insight into such patterns of allocation (Bradshaw 1965).

Plants provide particularly interesting study systems for investigating biochemical traits due to their extraordinary chemical diversity, which can drive associated variation in terrestrial food webs and biogeochemical cycles (Hunter 2016). Phytochemical plasticity can mitigate harm to plants from both abiotic and biotic stresses (Agrawal 1998, Chaves et al. 2003). Both the production of stress-mitigating secondary metabolites (*i.e.,* trait expression) and the mechanisms allowing for induction of such metabolites (*i.e.*, plasticity) can be costly (Baldwin 1998, Agrawal 2001). The magnitude of induced phytochemical responses to an abiotic stress, such as drought, is thus likely to depend both on past selection by the local environment and on present resource availability. Yet few studies have investigated how stress impacts the magnitude of intraspecific variation in phytochemical trait expression.

Severe and unprecedented droughts are predicted by climate models for the western United States (Cook et al. 2015), where water availability is already a key selective agent in natural plant populations. The Great Basin Desert, a 540,000 km^2 watershed in the western U.S., receives ~80% of its annual precipitation between October and March. Water deficits for plants are thus both variable and predictable within years: low in spring when water is plentiful and temperatures are cooler, and high in late summer when there has been little precipitation for several months and temperatures are

high. Indeed, this marked seasonality causes plants at relatively wetter sites to experience higher intra-annual variation in water deficits. The Great Basin also contains hundreds of ranges and drainages, which create heterogenous water availability on the landscape that favors strong local adaptation in plants (Svejcar et al. 2017, Baughman et al. 2019). Plasticity is likely to be important to plant survival in this heterogeneous environment, and climate change may only amplify its importance (Hendry 2016).

Here we asked whether two widely distributed Great Basin plant species exhibit population-specific phytochemical trait expression and plasticity in response to acute water stress. We also asked how these patterns affect intraspecific variation in phytochemical traits among populations. To answer these questions, we collected seeds of the two most widespread species of western milkweed (*Asclepias speciosa* and *Asclepias fascicularis*) from six sites spanning a 500-mm range in climatic water deficits (*i.e.*, the evaporative demand of vegetation not met by available water, Stephenson 1998). We define "constitutive" chemistry as the concentrations of metabolites produced under well watered conditions and "induced" chemistry as that produced under water stress. We hypothesized that climate at the seed-source location would shape constitutive phytochemistry, as well as the degree of phytochemical plasticity in response to acute water stress. With the understanding that producing high concentrations of secondary metabolites can be costly, we predicted that plants from wetter sites would produce lower constitutive concentrations of water-stress-mitigating metabolites. However, because wetter sites can exhibit higher variation than drier sites in water deficits within years, we predicted that plants sourced from wetter sites would also exhibit higher plasticity in the production of such metabolites. Finally, we predicted that higher metabolic costs under

water stress would constrain variation in induced phytochemical trait expression relative to that under non-stressful conditions.

2 | Methods

2.1 | Study system

Asclepias speciosa (showy milkweed) and *A. fascicularis* (narrowleaf milkweed) are widespread in the western United States (Woodson 1954, Dilts et al. 2019). The two species exhibit distinct morphologies—*A. fascicularis* has narrow, glabrous leaves, whereas *A. speciosa* has wide, pubescent leaves (Agrawal et al. 2009a)—but both can be found across a wide range of water availabilities, including in very dry locations (down to at least 100 mm of annual precipitation). Neither species produces many of the toxic cardenolides for which milkweeds are best known (Rasmann and Agrawal 2011), but both species produce a broad range of other UV-absorbent secondary metabolites, including flavonol glycosides, small phenolics, and pregnane glycosides (Mundim and Pringle 2020). We predicted that flavonol glycosides would be particularly likely to respond to drought because flavonols can act as antioxidants, mitigating water stress by scavenging reactive oxygen species (Kaminska-Rozek and Pukacki 2004). The biological roles of pregnane glycosides in milkweeds are unknown, although they could act as herbivore deterrents, especially in plants with low cardenolide content (Zehnder and Hunter 2007).

2.2 | Seed sources

To seed the experiments, we collected seeds from six sites spanning 385 km of the Great Basin Desert, USA, in fall 2016 (Fig. 1). To estimate the typical drought stress at each of the sites, we calculated the cumulative annual climatic water deficit (CWD) (Fig. 1; Appendix S1: Methods S1). Climatic water deficit (CWD) explicitly accounts for how temperature and precipitation interact to affect plant water balance, with temperature driving water demand through increased potential evapotranspiration and precipitation driving the amount of water available in the system (Stephenson 1990, 1998). CWD is the single most important climate variable for predicting the distribution of many plants in the Great Basin (Dilts et al. 2015). 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) (Fig. 1). From highest to lowest CWD (mm), our seed-source sites were: Fallon, NV (FN; 988.98 mm), Auburn, CA (CA; 985.68 mm), Pyramid Lake, NV (PL; 919.62 mm), Battle Mountain, NV (BM; 847.74 mm), Reno, NV (RN; 587.19 mm), and Verdi, NV (VE; 460.3 mm) (Appendix S1: Table S1).

Prior to using mean annual CWD as our main predictor, we explored its relationship to other bioclimatic and water-balance variables (Appendix S1: Fig. S1). CWD was positively related to mean annual temperature (Pearson's $r = 0.77$, df = 4, $p < 0.08$) and mean August temperature ($r = 0.95$, df = 4, $p < 0.004$). In contrast, CWD was not strongly related to precipitation ($r = 0.10$, df = 4, $p = 0.8$), suggesting that water deficits can still be high at wetter sites when temperatures are high. Wetter sites had higher variation than drier sites in water deficits within years ($r = -0.84$, df = 4, $p < 0.04$) but all sites showed similar variation in CWD and precipitation between years ($r = -0.39$, df = 4, $p = 0.4$ and $r = -0.06$, df = 4, $p = 0.9$, respectively).

2.3 | Experimental design

To determine whether intraspecific trait variation could be predicted by seed-source CWD and how such variation is affected by water limitation, we conducted a drought experiment with *A. fascicularis* and *A. speciosa* in a glasshouse. We germinated 12 *A. fascicularis* seeds from each of three maternal families from all six sites ($N = 216$), and 12 *A. speciosa* seeds from each of three maternal families from four of the sites (FN, PL, BM, and RN; $N = 144$). We randomly assigned six plants from each maternal family to the control (well watered) treatment and the other six plants to the dry treatment ($n = 36$ per seed-source site). Prior to beginning the dry treatments, mortality was higher for *A. speciosa* than for *A. fascicularis*, such that the final average n was ~33 for *A. fascicularis* but ~17 for *A. speciosa*.

We used a gravimetric dry-down treatment to expose plants to drought stress for four weeks in March–April 2017. Seeds were germinated in Petri dishes under lights (L18:D6) at 25° C in November 2016. Plants were grown in 4x9.5-in treepots containing 1500 g of a mixture of 2:1:1 parts sand:peat moss:composted bark. Pots were completely randomized on tables in the glasshouse and fertilized weekly with 24:8:16 N:P:K fertilizer. We calculated gravimetric soil water content using 13 treepots with 1500 g of the same soil. Saturated mass was measured 2 h after fully saturating the pots; dry mass was measured after oven drying for 48 h at 90° C. 100% soil saturation was estimated as: saturated mass – dry mass. We allowed control plants to dry to 70% soil saturation and dry plants to 10% soil saturation. These treatments are, respectively, what these milkweeds might experience at the weedy edge of an irrigated agricultural field in

Nevada (Irmak et al. 2007) and the plants' wilting point. In the fourth and last week of the experiment, we increased the dry treatment to 30% soil saturation to maintain plant survival.

2.4 | Plant traits

To verify the efficacy of our drought treatment and to explore differences in physiological responses to drought, which may mediate the plant's metabolic allocation, we measured the following plant traits: change in plant height; whole-plant dry biomass of roots and shoots; root:shoot ratio; leaf mass per area (LMA); and stomatal conductance. Plant height was recorded prior to beginning the dry treatment and again prior to harvesting the plants. Roots, stems, and leaves were harvested separately, washed, and dried at 60 °C for 72 h before weighing. Tissues were weighed in microcentrifuge tubes, and the weight of the tube was subtracted. Prior to weighing, 41 microcentrifuge tubes that had contained stem tissue were accidentally discarded; we thus present results on both root:shoot ratios and root:leaf ratios. A leaf in position three of the phylotaxis was collected for LMA, which was estimated by dividing the dry weight of the leaf in mg by the estimated leaf area in mm^2 (length x width). Stomatal conductance was measured between 1200–1400 h using an SC1 Porometer (Decagon).

To determine how water stress affected phytochemical trait expression, we measured plant UV-absorbent secondary chemistry. Prior to harvest, leaves and fine roots were collected and stored at -80° C. These tissues were later freeze-dried, ground, and extracted in 100% methanol with a cardenolide internal standard (digitoxin). UV-absorbent peaks were measured on a high-performance liquid chromatography (HPLC)

system with a diode array detector recording peaks that absorbed between 200–330 nm (see additional methods in Methods S2). We retained peaks for our analysis that could be consistently identified from mass fragments using low-resolution mass spectrometry and/or that were present in a majority of the samples of a given species (*A. fascicularis* or *A. speciosa*) and respective tissue (leaf or root).

2.5 | Statistical analysis

All analyses were conducted in R version 4.0.5 (R Core Team 2021).

To determine whether trait plasticity under acute water stress depended on climatic history at the seed source, we used generalized linear mixed models (GLMMs) from the 'glmmTMB' R package (Brooks et al. 2017). Preliminary analyses showed strong trait differences and a lack of correspondence in phytochemical compounds between species, such that we ran separate models for each species. Each saturated model started with the fixed effects of water treatment, seed-source CWD, the water × CWD interaction, and the random intercept effects of plant maternal family nested within the seed-source site. To compare effect sizes among response variables, we normalized responses and CWD values using the 'BBmisc' package (Bischl et al. 2017), and we report beta coefficients (β) 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.

To understand how the dry treatment affected growth, we used GLMMs with log-transformed plant height as a tweedie-distributed variable, log-transformed biomass as a Gaussian-distributed variable, and log-transformed root:shoot and root:leaf ratios as

Gaussian-distributed variables. To understand how the dry treatment affected physiology, we used GLMMs with stomatal conductance at the beginning and end of the experiment as Gaussian-distributed variables and log-transformed leaf-mass-per-area (LMA) as a Gaussian-distributed variable. To understand how the dry treatment affected phytochemistry, we first explored variation among unique compounds and their relationship to total concentrations using graphical data exploration. We then analyzed the responses of total concentration, the concentration of water-responsive flavonol glycosides, and the concentration of pregnane glycosides using GLMMs with Gaussian-distributed variables. We also assessed constitutive patterns in the dominant leaf flavonol in each species using GLMMs with control-plant flavonol concentrations as the response and seed-source CWD as the sole fixed predictor.

The best model for each response was selected based on the lowest sample-size corrected Akaike Information Criterion (AICc), and any marginal predictors (≤ 2 Δ AICc) were evaluated using log-likelihood ratio tests (LRT) in the 'lmtest' R package (Zeileis and Hothorn 2002). Marginal and conditional R^2 values were calculated for each best model in the 'MuMIn' package. Marginal means were calculated in the effects package (Fox 2003, Fox and Weisberg 2019).

To test whether the dry treatment affected the magnitude of intraspecific variation in phytochemistry, we first calculated coefficients of variation among populations in the control and dry treatment using the 'cvcqv' package (Beigy 2019) and Mahmoudvand-Hassani confidence intervals (Mahmoudvand and Hassani 2009). We then also compared among-population coefficients of variation between the dry and control treatments using

modified signed-likelihood ratio tests (Krishnamoorthy and Lee 2014) in the 'cvequality' package (Marwick and Krishnamoorthy 2019).

Finally, to explore the potential value of flavonol plasticity to plant performance, we investigated the relationship between plasticity in leaf flavonols and the maintenance of plant biomass in the dry treatment. Plasticity was estimated as the change in mean trait value per maternal family between the dry and control treatments. We then applied a simple linear model with change in biomass as a function of change in flavonol concentration to data from both species. Plant species and average seed mass were examined as potential covariates but neither improved model fit (not shown).

3 | Results

3.1 | Growth and physiological responses to water stress

The dry treatment produced evidence of water limitation for both species. Water limitation tended to reduce plant growth and biomass (Tables S2-S3). The dry treatment reduced *A. fascicularis* plant biomass by ~17%, but this effect was stronger in plants whose seeds originated from drier sites (Fig. S2a; $\beta_{water \times CWD}$ = -0.33 ± 0.14, z = -2.44, P < 0.02). In *A. speciosa,* the dry treatment reduced plant biomass by 20% on average (Fig. S3); this effect was not significant (LRT water: χ^2 = 0.50, df = 1, P = 0.5) but was most pronounced in plants originating from the driest site (Fig. S2b).

Seed-source CWD had a stronger effect on root:shoot ratios in *A. speciosa* than in *A. fascicularis*, but both species increased their root:leaf ratios in the dry treatment (Appendix S1: Tables S2-S3). *Asclepias speciosa* produced higher root:shoot ratios than

A. fascicularis (Welch's t = -5.36, $p < 0.0001$). The root:shoot ratio of *A. speciosa* plants was higher in plants sourced from drier sites ($\beta_{CWD} = 0.36 \pm 0.15$, z = -2.40, $P < 0.02$) and the root:leaf ratio was marginally higher in the dry treatment (Appendix S1: Fig. S2d; LRT water: $\chi^2 = 2.18$, df = 1, $P = 0.1$). *Asclepias fascicularis* plants also increased their root:leaf ratio in the dry treatment (Appendix S1: Fig. S2c; $\beta_{water} = 0.28 \pm 0.13$, $z = 2.21$, $P < 0.03$), but this effect was not apparent in the root:shoot ratio (LRT water: $\chi^2 = 0.00$, df = 1, $P = 0.9$), and did not depend on seed-source CWD (LRT CWD: $\chi^2 = 0.14$, df = 1, $P = 0.7$).

Plasticity in the physiological traits of stomatal conductance and LMA was stronger in *A. fascicularis* than in *A. speciosa* (Tables S2-S3). Stomatal conductance at the end of the experiment was lower in dry *A. fascicularis* plants than in controls (β_{water} = -0.70 ± 0.21, z = -3.39, $P < 0.0008$) but was not different between control and dry *A. speciosa* plants (LRT water: $\chi^2 = 0.28$, df = 1, $P = 0.6$). Water limitation reduced LMA in both species, but the uncertainty in LMA response was higher in *A. speciosa* (*A. fascicularis*: $\beta_{water} = -0.28 \pm 0.14$, z = -1.94, $P < 0.06$; *A. speciosa*: $\beta_{water} = -0.28 \pm 0.17$, z = -1.62, $P = 0.1$). In *A. fascicularis*, control plants originating from wetter sites also had higher LMA than control plants originating from drier sites ($\beta_{CWD} = -0.24 \pm 0.12$, z = -1.96, $P < 0.06$), such that the direction of plasticity in LMA paralleled the constitutive decline in LMA with seed-source CWD in this species.

3.2 | Phytochemical responses to water stress

Flavonol glycosides present in the leaves of both species dominated the total concentration of UV-absorbent secondary metabolites. In total, we retained nine UV-absorbent secondary metabolites from *A. fascicularis* leaves, twelve from *A. fascicularis* roots, six from *A. speciosa* leaves, and ten from *A. speciosa* roots for our analysis (Appendix S2). Fifteen, or ~40%, of these 37 unique compounds were putatively identified as pregnane glycosides present in *A. fascicularis* leaves and roots and *A. speciosa* roots. We also identified one putative cardenolide in *A. speciosa* roots, one putative saponin in *A. fascicularis* leaves, and two putative small phenolics in *A. fascicularis* roots. Flavonol glycosides (henceforth, flavonols) were present only in the leaves of both species. Moreover, the leaves of each species contained a single dominant flavonol that comprised ~77% of the total UV-absorbent metabolite concentration: a quercetin-glucoside-rhamnoside (QGR) in *A. fascicularis* leaves and a quercetin-glucoside (QG) in *A. speciosa* leaves (Appendix S1: Fig. S4).

The dominant flavonol in each species tended to be found in higher constitutive concentrations in plants sourced from drier sites (Fig. 2). In particular, well watered plants originating from drier sites had higher concentrations of QGR in the leaves of *A. fascicularis* than plants originating from wetter sites ($\beta_{CWD} = 0.33 \pm 0.14$, $z = -2.38$, $P < 0.02$). There was no statistical relationship between QG concentrations and the CWD of the seed source in well watered *A. speciosa* leaves (LRT CWD: $\chi^2 = 0.60$, df = 1, $P = 0.4$), but this lack of pattern was driven mostly by plants sourced from Reno, NV, which contained relatively high concentrations of the flavonol despite their relatively wet source location compared to plants sourced from the other three sites (Fig. 2).

Water limitation drove increases in the total concentration of UV-absorbent secondary metabolites in the leaves of both milkweed species, and this induction was driven by increases in flavonols (Fig. 3; Appendix S1: Table S4; Appendix S2). In *A. fascicularis*, the magnitude of the flavonol response depended on seed-source CWD, such that only plants originating from wetter sites increased expression of flavonols under water limitation (Fig. 3a; $\beta_{water \times CWD} = -0.26 \pm 0.13$, $z = -1.99$, $P < 0.05$; Appendix S1: Fig. S5, Table S4). In *A. speciosa*, flavonols increased in the dry treatment ($\beta_{water} = 0.70 \pm 0.25$, $z = 2.77$, $P < 0.006$), but this response did not depend statistically on seed-source CWD (LRT CWD: $\chi^2 = 0.94$, df = 1, $P = 0.3$; Appendix S1: Table S4). Nevertheless, the *A. speciosa* leaves of plants sourced from wetter sites also tended to show larger increases in flavonol expression under water stress (Fig. 3b; Appendix S1: Fig. S5), except for plants from Reno, NV, which contained higher constitutive flavonols. Because plants from drier sites tended to contain higher constitutive flavonols, whereas plants from wetter sites tended to increase flavonols more under drought, among-population intraspecific variation in flavonol concentrations was higher in well watered plants than in water-stressed plants in both species (Fig. 3c,d). This change was marginally significant by a modified signed-likelihood ratio test for *A. fascicularis* ($F = 3.38$, df = 5, $P < 0.07$) but not for *A. speciosa* ($F = 1.11$, df = 3, $P = 0.3$). Finally, higher flavonol plasticity was weakly associated with better biomass maintenance in the dry treatment across both species (Fig. 4; $\beta_{\text{flavonol response}} = 0.35 \pm 0.19$, $t = 1.79$, $P < 0.09$).

Roots, unlike leaves, did not contain flavonols, and the total concentration of UV-absorbent compounds in roots did not respond to water limitation in either species (Appendix S1: Table S4). Likewise, the diverse pregnane glycosides did not respond to

water limitation, although they were generally found in higher concentrations in plants from wetter sites (Fig. S6; Table S4).

4 | Discussion

To predict the ecological consequences of drought and other stressors, we must understand how acute stress to organisms interacts with past selection by the local environment to affect trait expression and resulting variation. Here we have shown that two widespread species of western milkweeds show phytochemical trait expression consistent with local adaptation to seed-source water deficits. In particular, the same chemical compounds that were induced upon acute water stress exhibited higher constitutive concentrations in plants sourced from drier sites (*i.e.,* plasticity occurred in a "co-gradient" direction, Lusk et al. 2008). Interestingly, however, plants sourced from wetter sites tended to increase the concentrations of these putatively stress-mitigating secondary metabolites more than plants sourced from drier sites in response to acute water stress. Acute water stress thus reduced intraspecific variation in the concentrations of these dominant metabolites. We also found a weak but positive correlation between phytochemical plasticity and biomass maintenance under reduced water. Together these results suggest that despite the potential for induced chemical responses to mitigate plant water stress, such responses may be constrained by the cost of metabolite production. The consequence may be reduced phytochemical variation among populations as environmental stress becomes more severe.

Our predictions for trait expression and plasticity were based on a definition of stress as severe resource limitation, and plant growth and physiological traits suggested

that our dry treatment successfully produced such limitation in both species. Nevertheless, the two species appeared to regulate their physiology differently. In particular, *A. fascicularis* appears to be relatively more isohydric than *A. speciosa*. *Asclepias fascicularis* leaves were unique in their regulation of stomatal conductance in the dry treatment, and we have also found that *A. fascicularis* plants maintain a more constant leaf water potential than *A. speciosa* (Pringle, unpublished data). In contrast, *A. speciosa* produced higher root:shoot ratios than *A. fascicularis*, and constitutive *A. speciosa* root:shoot ratios also varied positively with water deficits at the seed-source site. Higher allocation to roots may allow *A. speciosa* to better tolerate drought, whereas isohydricity in *A. fascicularis* may indicate more drought-avoidance (McDowell et al. 2008). Changes in the respective traits of each species in the dry treatment, however, suggested the potential for allocation trade-offs that could affect phytochemical trait expression.

In this study, phytochemical trait expression and plasticity were associated with climatic history at the seed-source site as characterized by the annual cumulative climatic water deficit (CWD). Importantly for regions with highly seasonal precipitation, this statistic was correlated not with precipitation, but with late-summer temperatures. Moreover, CWD at our source sites is driven by low late-summer water supply in combination with high temperatures. Predicted higher summer temperatures under climate change (Hegewisch, et al. 2021) may thus be sufficient—irrespective of changes in precipitation—to increase the duration and frequency of future drought stress for plants in our study region. Late-summer may also be a critical time for the *A. fascicularis* and *A. speciosa* milkweeds examined here because it is when these plants begin their

reproductive investment in fruit. Summer temperature and its effects on plant water balance may thus exert strong selection on drought-mitigating phytochemistry.

The patterns in phytochemical trait expression that we observed are consistent with local adaptation to CWD because the direction of plasticity paralleled constitutive differences in phytochemical traits, with clinal variation along the water-deficit gradient. Although a true test of whether plasticity is adaptive requires an association between plasticity and a reliable measure of fitness (van Kleunen and Fischer 2005), patterns resulting from adaptive plasticity would (i) produce reaction norms driving traits closer to the values favored by selection in the new environment (Ghalambor et al. 2007) and (ii) vary clinally along environmental gradients (Muir and Angert 2017). Given how little we know about phytochemical responses to drought in most species (Mundim and Pringle 2018), including the two milkweeds examined here, we took an untargeted approach to the measurement of UV-absorbent secondary metabolites. This discovery-oriented approach led to the clear identification of flavonols as phenolic compounds that both dominated the overall concentration of UV-absorbent metabolites and responded most strongly to the dry treatment. This finding is consistent with an important role for flavonols in reducing oxidative stress under water deficits, preventing damage to cell membranes from reactive oxygen species (Kaminska-Rozek and Pukacki 2004). Moreover, the positive relationship between flavonol plasticity and biomass maintenance is preliminary evidence of a fitness-related benefit of these compounds. The conclusion that flavonols may represent important, adaptive phytochemicals in these milkweeds is also consistent with phylogenetic conservatism in these compounds across the broader milkweed phylogeny (Agrawal et al. 2009b) and with the higher concentration of these

compounds among milkweed species whose leaf traits are adapted to more arid environments (Agrawal et al. 2009a).

Focusing on flavonols, then, as putative water-stress-mitigating metabolites, our results generally supported our predictions: plants sourced from drier sites contained higher constitutive concentrations of these compounds, whereas plants sourced from wetter sites exhibited higher plasticity. These results are thus also consistent with the hypotheses underlying these predictions, namely: (i) there is a cost to flavonol production; and (ii) given this cost, plasticity is favored in predictably variable environments. In particular, constitutively higher production of flavonols is avoided by plants sourced from locations with plentiful early-season water, but these plants also experience larger annual variation in water deficits due to the bigger differential between wet springs and dry summers. These conclusions fit the patterns observed in *A. fascicularis*, but they require some allowances in *A. speciosa*. Indeed, neither the constitutive expression nor the plasticity of flavonols was statistically dependent on seed-source CWD in *A. speciosa*. Nevertheless, we suspect that flavonols play fundamentally similar roles in the two species. *P* values are context dependent (Hartig and Barraquand 2022), and we had lower statistical power to assess the effect of seed-source CWD in *A. speciosa*, due to fewer source sites and an ~50% lower sample size per site than in *A. fascicularis*. In many respects, *A. speciosa* plants sourced from the wettest site for this species (Reno, NV) behaved more like plants from much drier sites, whereas the patterns among plants sourced from the other three sites were more similar to those in *A. fascicularis*. We can only speculate that perhaps the microenvironment of the seasonal ditch along which *A. speciosa* seeds were collected in Reno, NV, was distinct from the

site's broader climatic patterns (McLaughlin et al. 2017), or that plants in this relatively urban environment were once planted from other sources (Auffret et al. 2014). The possibility that the observed patterns in water-stress-mitigating phytochemicals, which were statistically supported in *A. fascicularis*, are common to other widespread Great Basin species thus merits further study.

Ultimately, we are interested in how commonly such population-dependent patterns in constitutive and drought-induced phytochemicals will reduce intraspecific phytochemical variation if our study area continues to aridify. Again suggesting a cost to flavonol production, plants that were sourced from the driest sites and produced the highest constitutive concentrations of leaf flavonols also produced little, if any, additional flavonols in the dry treatment. On average, these plants also lost more biomass in the dry treatment, a consequence that did not depend on the maternal effect of seed size, suggesting that resource availability constrained flavonol production across populations under acute water stress. Such ecological limits on trait expression and/or plasticity may be commonplace (Valladares et al. 2007, Auld et al. 2010). We know of only three other studies that have examined the effect of resource stress on among-population phytochemical variation. Two of these studies examined primary metabolites: nutrient stress reduced variation in leaf nutrient concentrations (Andivia et al. 2012), whereas drought stress reduced variation in glucose:sucrose ratios but increased variation in the concentration of soluble carbohydrates (Lázaro-Nogal et al. 2016). A third study examined between-population expression of secondary metabolites under drought and found a pattern similar to ours: in a perennial grass, in which isoprenes are proposed to act as critical antioxidants, drought stress reduced the between-population difference in

leaf isoprene concentrations (Ahrar et al. 2017). The generality of these results deserves further study, especially considering the possible consequences of changes in phytochemical variation for plant populations and ecological communities.

To the extent that phytochemistry mediates plant responses to stress, changes in phytochemistry may also provide early indicators of ecosystem responses to global change. Phytochemical diversity is hypothesized to regulate the structure and diversity of food webs and the dynamics of biogeochemical cycles (Hunter 2016, Wetzel and Whitehead 2020). Determining on what scales global change stressors tend to change phytochemical variation—among species, among populations, and/or among individuals—and whether the scale of phytochemical variation, or lack thereof, impacts ecological dynamics will thus also be important. For example, lower diversity in plant communities has been shown to reduce functional resilience to disturbance (Wilcox et al. 2020) and variation in soil mineralization processes (Rewcastle et al. 2022). Future work should examine whether such patterns can be traced back to phytochemistry, and whether the processes that drive phytochemical variation at the community level parallel, or not, the intraspecific patterns that we have begun to document here.

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Figure Legends

Fig. 1 The six study sites described by water-balance variables, based on 1981 to 2010 climate normal. Colors on the map show the climatic water deficit (CWD) gradient with green representing less arid areas and brown representing more arid areas. The isolines show actual evapotranspiration (AET—simultaneous availability of water and energy) in millimeters. Sites are (bottom-left to top-right) California (CA), Verdi (VE), Reno (RE), Pyramid Lake (PL), Fallon (FA), and Battle Mountain (BM).

Fig. 2 The leaf concentration (mg/g) of the dominant flavonol in control (well watered) *Asclepias fascicularis* and *Asclepias speciosa* plants. Points show the mean concentration of quercetin-glucoside-rhamnoside (QGR) in *A. fascicularis* and of quercetin glucoside (QG) in *A. speciosa*; bars show SE. SE for climatic water deficits was calculated interannually from 2004–2016.

Fig. 3 Interaction plots showing the responses of leaf C_{15} flavonol glycosides to water treatment × climatic water deficit (CWD) at the seed source. (**a**) Responses in *A Asclepias fascicularis* leaves and (**b**) responses in *Asclepias speciosa* leaves. Interaction plots show reaction norms separately by source location, colored by their location along a gradient of annual cumulative climatic water deficit from dry to wet. Points represent means and bars represent standard errors. The best general linear models for each response retained the fixed predictors: (**a**) water treatment × CWD; (**b**) water treatment. Estimated among-population coefficients of variation (CV) with confidence intervals for flavonol concentrations in (**c**) *A. fascicularis* and (**d**) *A. speciosa* plants in the control and dry treatments.

Fig. 4 Relationship between the change in dry biomass of experimental plants and the change in C_{15} flavonol glycoside concentrations. Most plants at the end of the experiment were smaller in the dry treatment than in the control, so change in biomass is referred to in the text as biomass maintenance. Points represent maternal families. Circles represent *A Asclepias fascicularis* plants, and diamonds represent *Asclepias speciosa* plants. The

regression line represents the marginal ($P < 0.09$) relationship across species described in the text.

Fig. 1

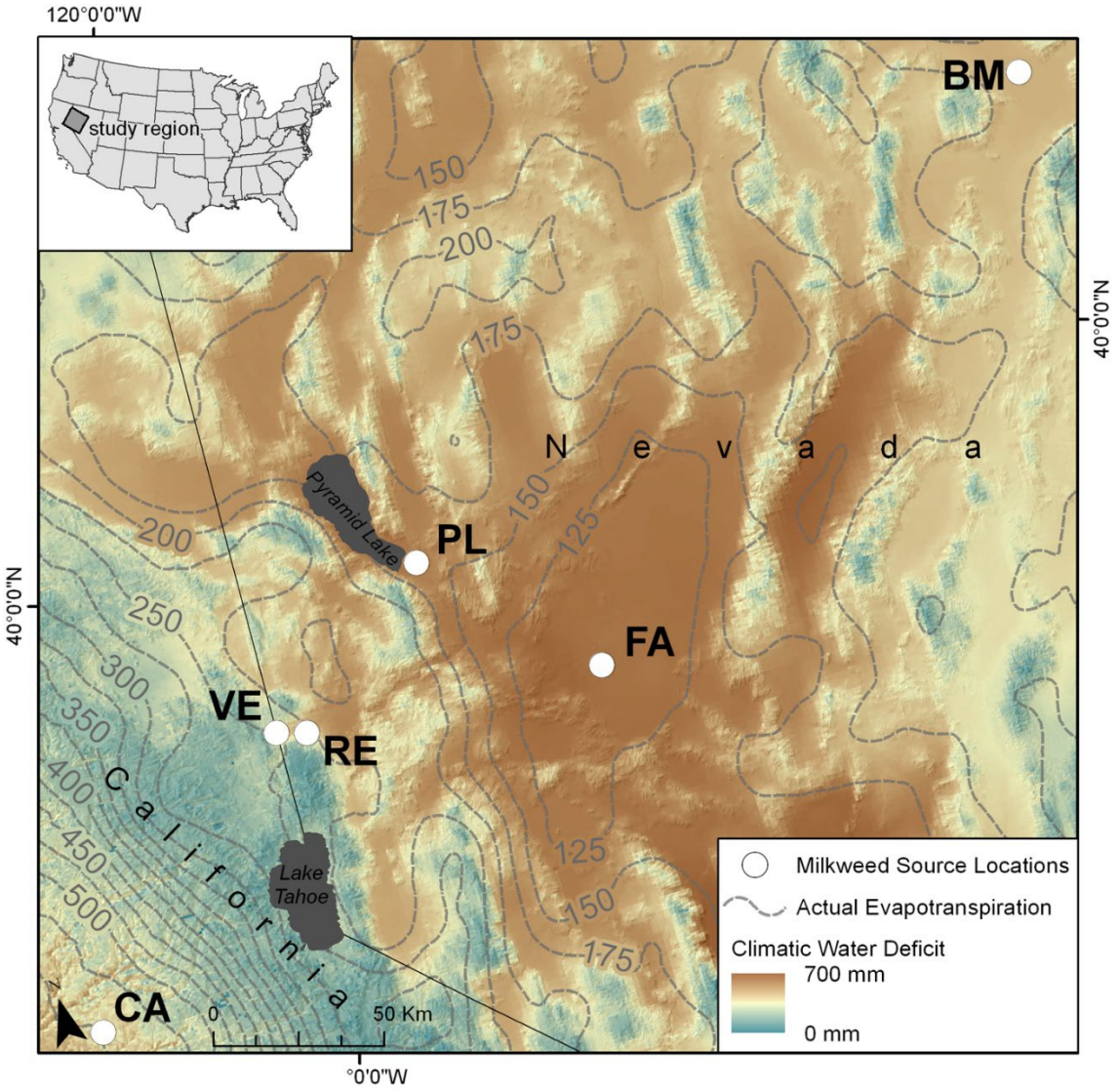

120°0'0"W
study region
BM
150
175
200
175
40°0'0"N
N e v a d a
Pyramid Lake
PL
150
125
200
250
FA
40°0'0"N
300
VE
350
RE
400
C a l i f o r n i a
450
Lake Tahoe
500
125
150
175
CA
0
50 Km
°0'0"W
Milkweed Source Locations
Actual Evapotranspiration
Climatic Water Deficit
700 mm
0 mm

Fig. 2

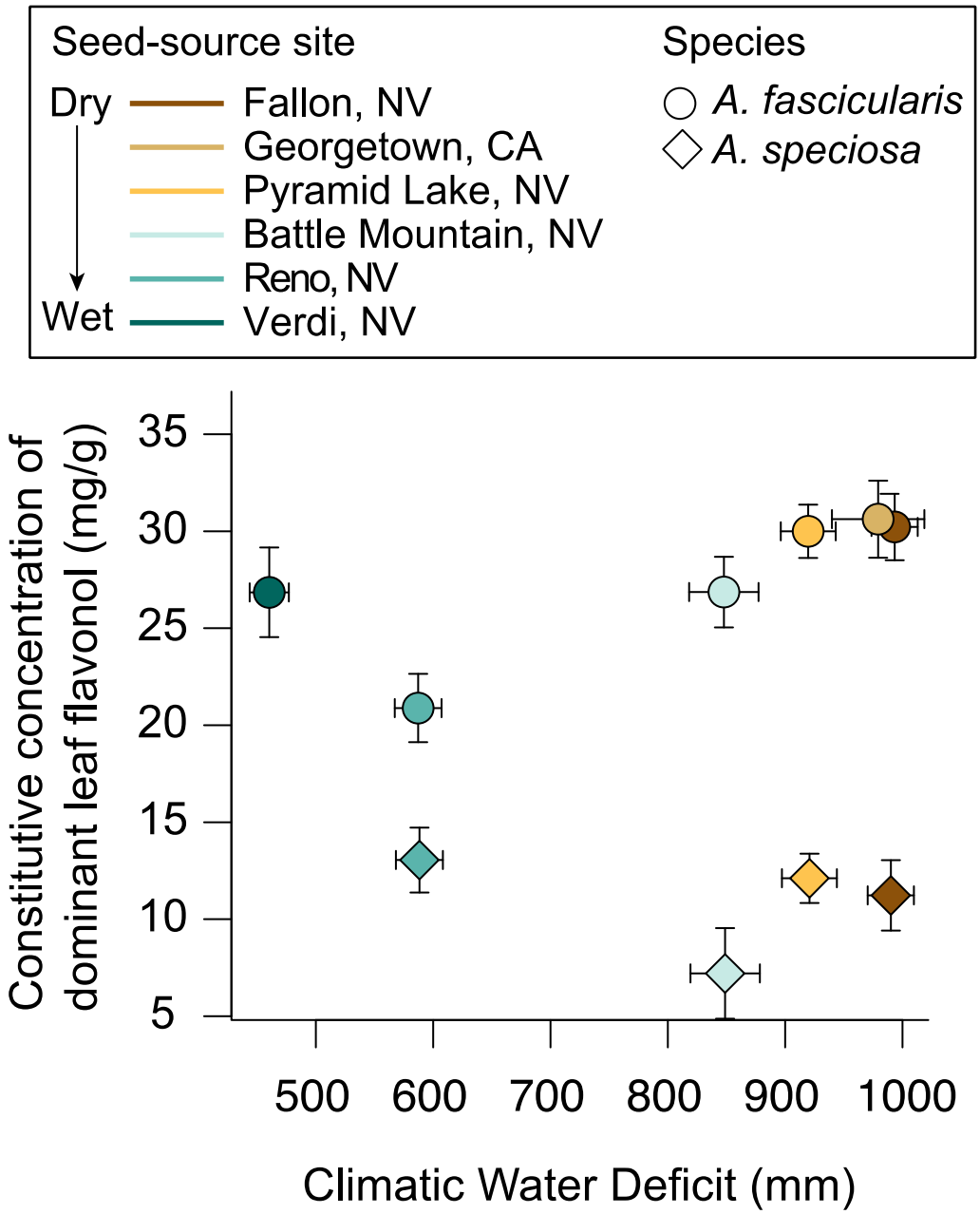

Seed-source site
Dry
Wet
Fallon, NV
Georgetown, CA
Pyramid Lake, NV
Battle Mountain, NV
Reno, NV
Verdi, NV
Species
A. fascicularis
A. speciosa
Constitutive concentration of dominant leaf flavonol (mg/g)
35
30
25
20
15
10
5
500
600
700
800
900
1000
Climatic Water Deficit (mm)

Fig. 3

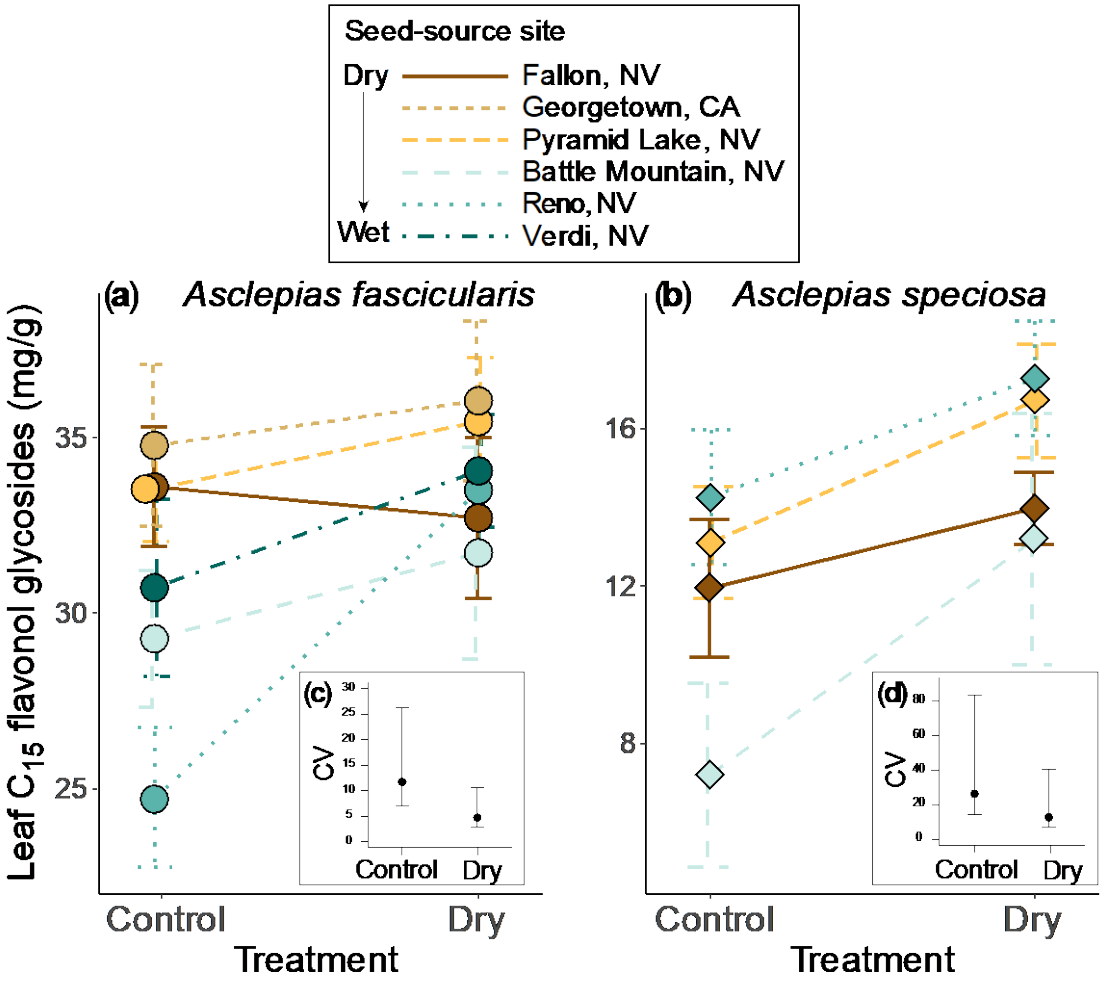
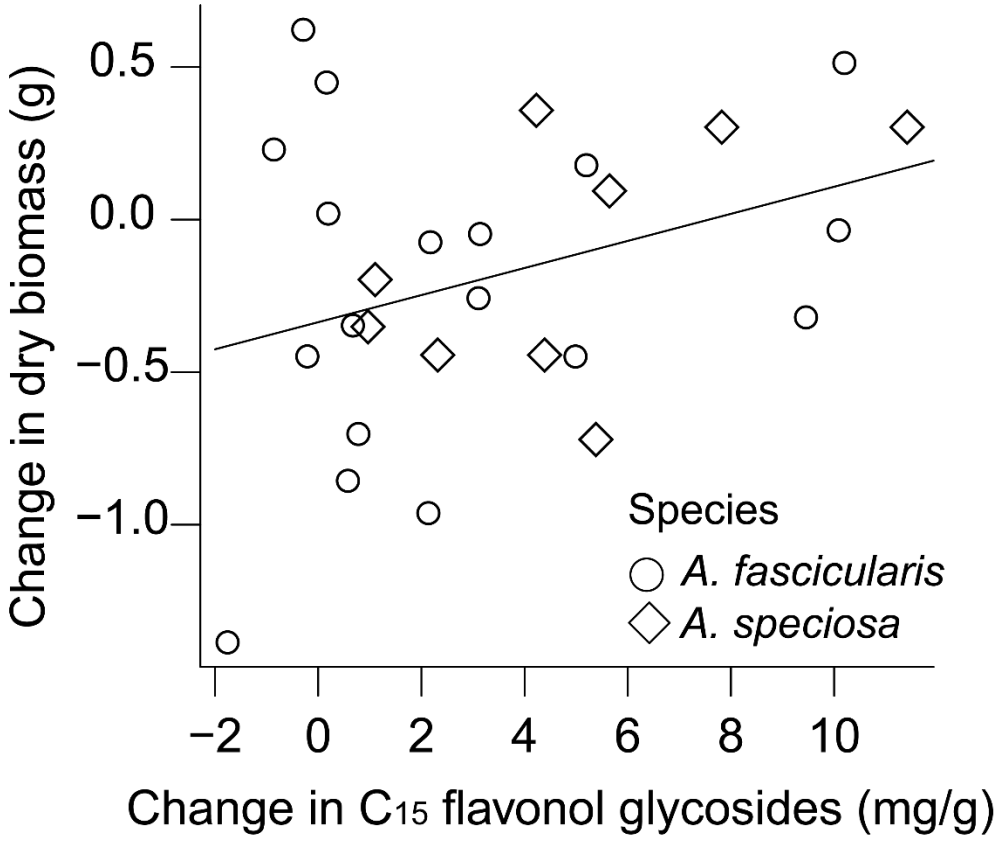

Seed-source site
Dry
Wet
Fallon, NV
Georgetown, CA
Pyramid Lake, NV
Battle Mountain, NV
Reno, NV
Verdi, NV
(a) Asclepias fascicularis
(b) Asclepias speciosa
Leaf C15 flavonol glycosides (mg/g)
35
30
25
16
12
8
(c)
(d)
CV
Control
Dry
Treatment

Fig. 4


0.5
0.0
−0.5
−1.0
Change in dry biomass (g)
−2
0
2
4
6
8
10
Change in C15 flavonol glycosides (mg/g)
Species
A. fascicularis
A. speciosa

Supplemental Tables and Figures

Fig. S1 Pair plots showing correlations among water-balance and climatic variables for the six seed-provenance sites. Plots above the diagonal show raw points; numbers below the diagonal show corresponding Pearson's correlation coefficients. CWD = mean annual climatic water deficit; Mean Temp = mean annual temperature; Mean Aug Temp = mean August temperature; PPT = mean annual precipitation; AET = mean annual actual evapotranspiration; PET = mean annual potential evapotranspiration; CWD_winyr_cv = coefficient of variation in climatic water deficit among months within years; TMean_winyr_cv = coefficient of variation in mean temperature among months within years; PPT_winyr_cv = coefficient of variation in precipitation among months within years; CWD_btyr_cv = coefficient of variation in climatic water deficit between years; TMean_btyr_cv = coefficient of variation in mean temperature between years; PPT_btyr_cv = coefficient of variation in precipitation between years. All summary variables based on climate normals from 2004–2016.

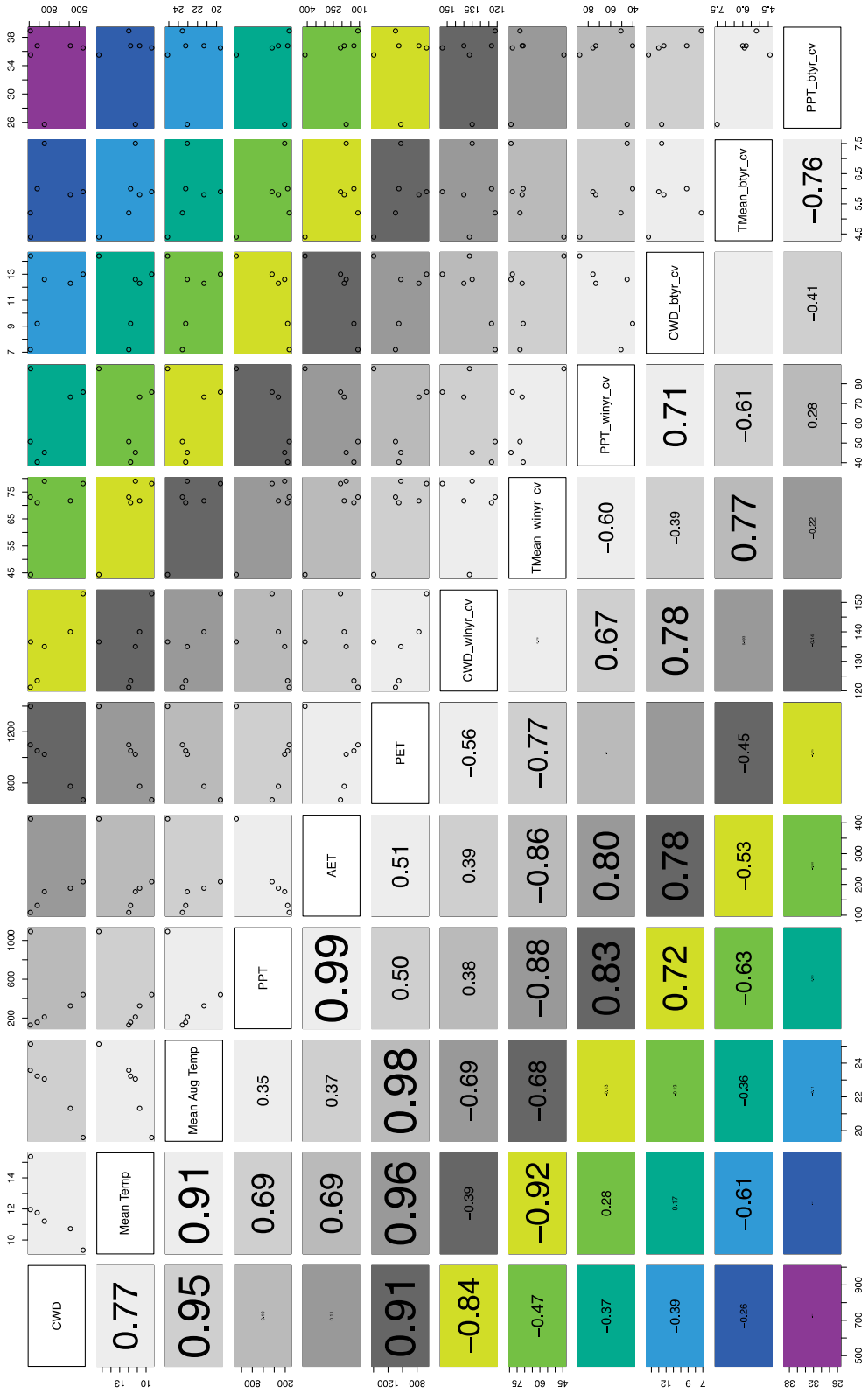

CWD
Mean Temp
Mean Aug Temp
PPT
AET
PET
CWD_winyr_cv
TMean_winyr_cv
PPT_winyr_cv
CWD_btyr_cv
TMean_btyr_cv
PPT_btyr_cv
0.77
0.95
0.91
0.69
0.69
0.96
−0.39
−0.92
0.28
0.17
−0.61
0.99
0.50
0.38
−0.88
0.83
0.72
−0.63
0.51
0.39
−0.86
0.80
0.78
−0.53
0.98
−0.69
−0.68
−0.36
−0.56
−0.77
0.67
0.78
−0.45
−0.60
−0.39
0.77
0.71
−0.61
−0.41
−0.84
−0.47
−0.37
−0.39
−0.26
−0.76

Fig. S2 Interaction plots showing plant growth responses to water treatment × climatic water deficit (CWD) at the seed source. (**a**,**b**) Dry biomass of roots and shoots (*i.e.*, whole-plant) and (**c**,**d**) root:leaf ratios in both species, (**a**,**c**) *Asclepias fascicularis* and (**b**,**d**) *Asclepias speciosa*. Points represent means and bars represent SE. The best general linear mixed models for each response retained the fixed predictors: (**a**) water treatment × CWD; (**b**) intercept only; (**c**) water treatment; and (**d**) intercept only.

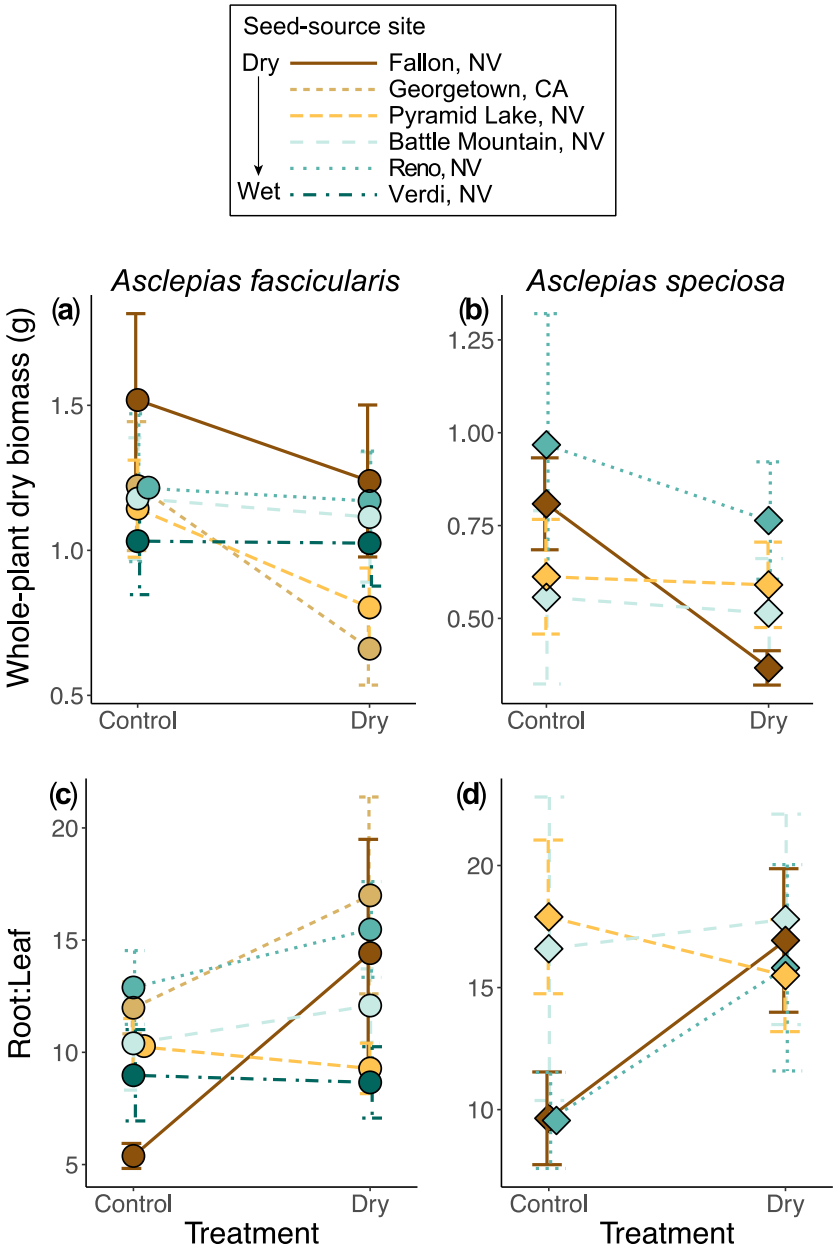


Fig. S3 Effect of the dry treatment on whole-plant dry biomass contingent on the mean fixed effect of seed-source climatic water deficit (mm) and random effect of plant genotype.

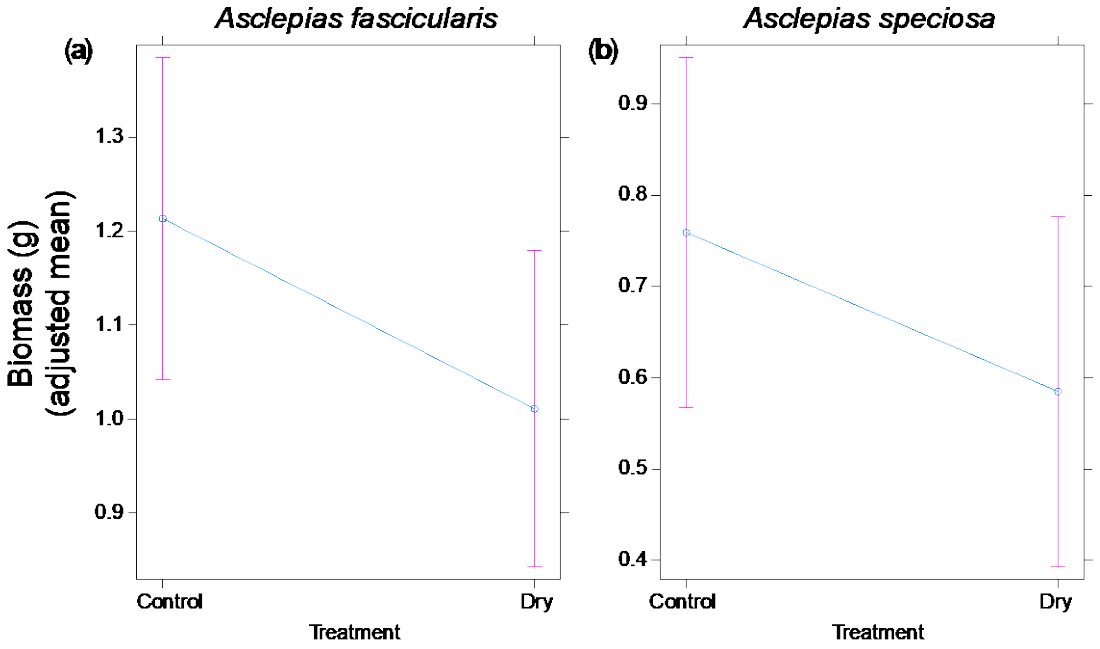


Fig. S4 Correlations between the total concentration of consistently identified UV absorbent metabolites and the concentration of C_{15} flavonol glycosides (QGR and KGR in *A. fascicularis*; QGR and QG in *A. speciosa*). These C_{15} flavonol glycosides were also the only four compounds in leaves that were induced upon water limitation.

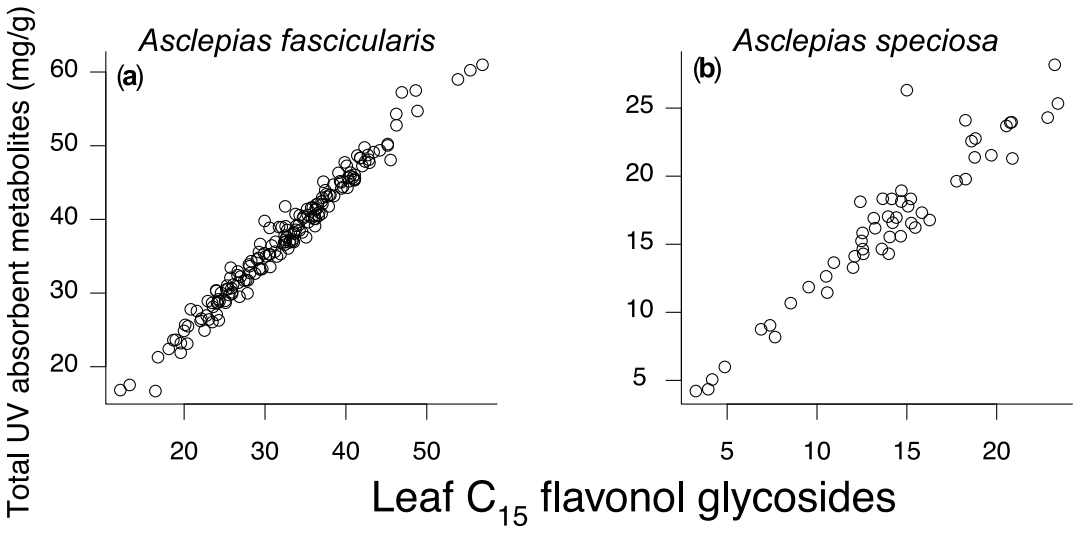


Fig. S5 Flavonol plasticity in response to acute water stress among seeds sourced from along a climatic water deficit gradient. Points show maternal-family mean differences between dry treatment and well watered concentrations and SE. SE error bars for climatic water deficits were calculated interannually from 2004–2016.

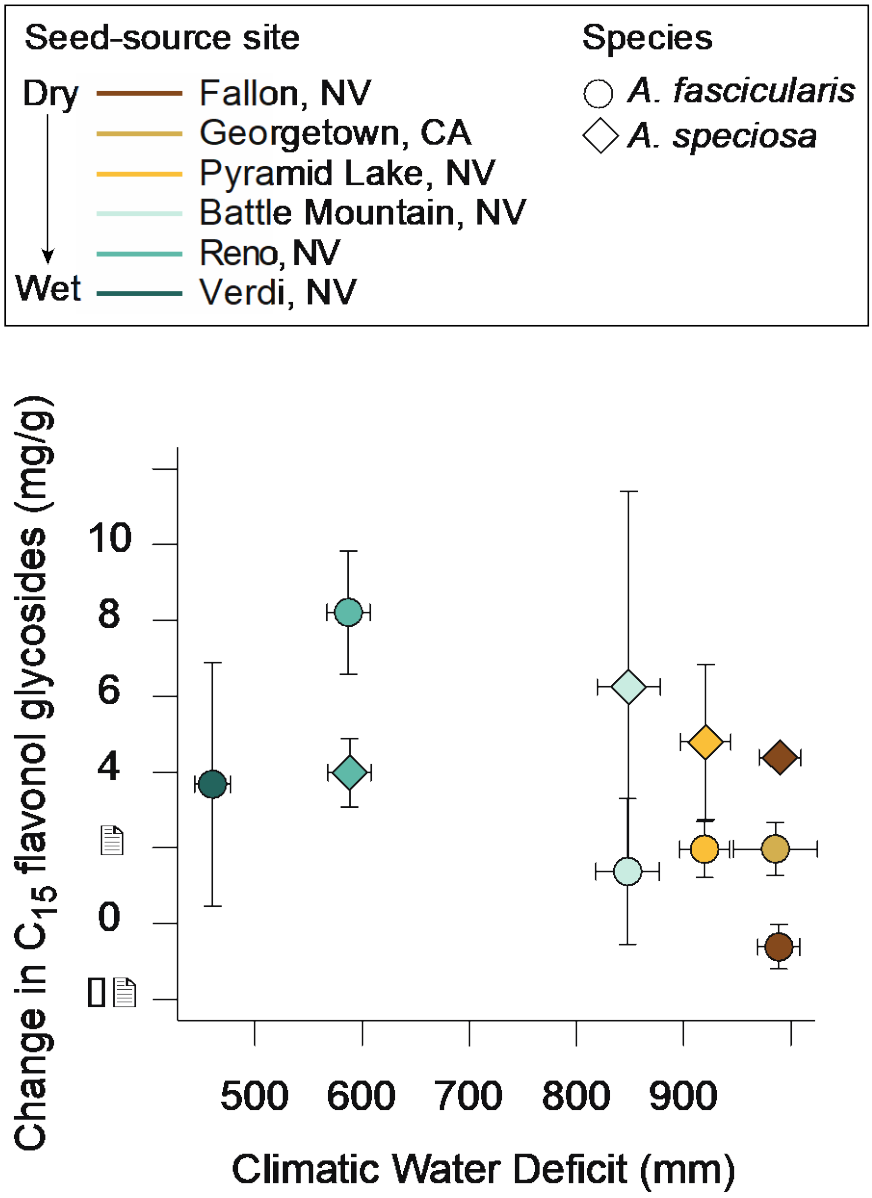


Fig. S6 Constitutive concentrations of pregnane glycoside compounds in the leaves and roots of control plants (*i.e.,* well watered). Points show the mean concentration among all plants sourced from each site and bars show SE. SE for climatic water deficits was calculated interannually from 2004–2016. Note the differences in scale on the y-axis. Insets show representations of leaves and roots of each species; no pregnane glycosides were identified in the leaves of *A. speciosa.*

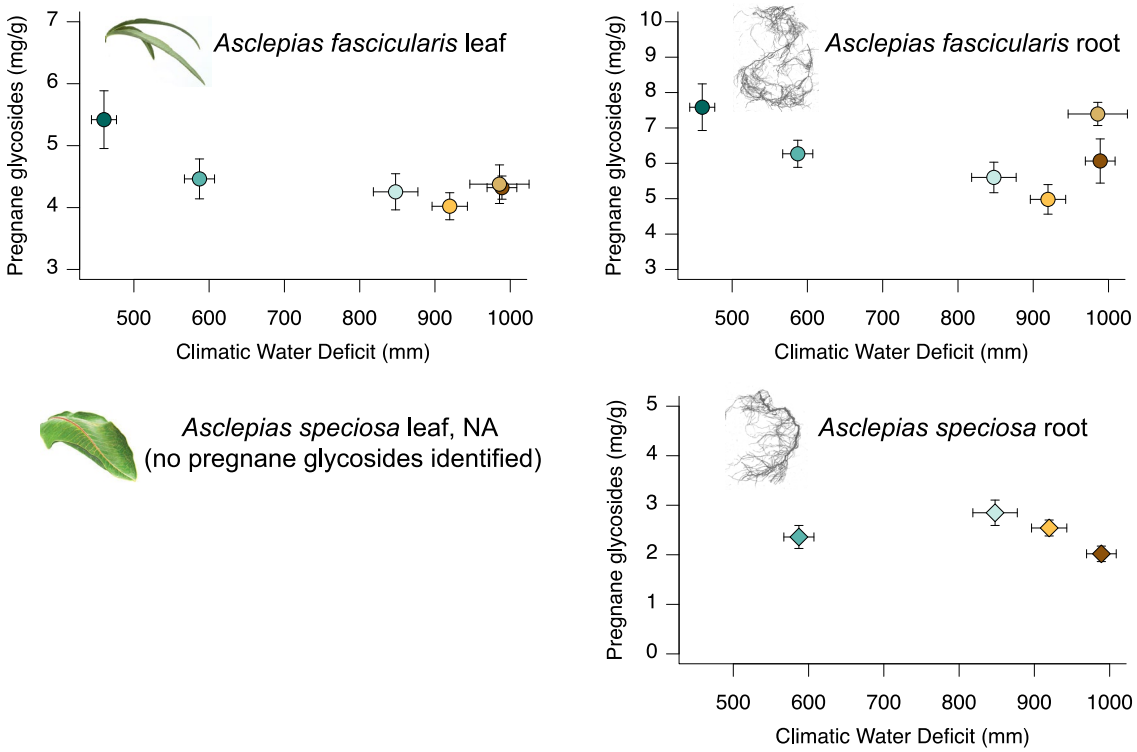


Table S1. Seed-provenance site locations for *Asclepias fascicularis* and *Asclepias speciosa* plants in the common garden experiments.

Site	Latitude	Longitude
California	38.87178726	-120.8187756
Verdi	39.52318534	-119.9985313
Reno	39.50261419	-119.8986715
Pyramid Lake	39.86179074	-119.3868993
Fallon	39.47482708	-118.6575129
Battle Mountain	40.662576	-116.9320299

Table S2 Plant physical and physiological responses in the glasshouse drought experiment.

Response	Plant part	Treatment (mean ± SE (*N*))		Best model
		Control	Dry	
Asclepias fascicularis				
Height change (cm)	*Shoot*	0.8 ± 0.2 (*97*)	0.3 ± 0.2 (*103*)	Treatment
Biomass (g)	*Root*	0.90 ± 0.06 (*92*)	0.76 ± 0.05 (*96*)	Treatment x CWD
Biomass (g)	*Shoot*	0.31 ± 0.03 (*92*)	0.25 ± 0.03 (*96*)	Treatment x CWD
Root:shoot biomass ratio		4.11 ± 0.25 (*92*)	4.02 ± 0.25 (*96*)	Intercept
Root:leaf biomass ratio		10.0 ± 0.7 (*92*)	12.8 ± 1.2 (*96*)	Treatment
Stomatal conductance (mmol/m2/s)	*Leaf*	232.5 ± 17.2 (*44*)	168.4 ± 10.2 (*51*)	Treatment
LMA (mg/mm2)	*Leaf*	0.05 ± 0.004 (*95*)	0.04 ± 0.002 (*94*)	Treatment + CWD
Asclepias speciosa				
Height change (cm)	*Shoot*	0.5 ± 0.4 (*34*)	0.4 ± 0.5 (*33*)	Intercept
Biomass (g)	*Root*	0.61 ± 0.09 (*32*)	0.49 ± 0.06 (*32*)	Intercept
Biomass (g)	*Shoot*	0.14 ± 0.04 (*32*)	0.11 ± 0.03 (*32*)	CWD
Root:shoot biomass ratio		6.88 ± 0.67 (*32*)	8.19 ± 1.05 (*32*)	CWD
Root:leaf biomass ratio		13.5 ± 1.7 *(32*)	16.3 ± 1.9 (*32*)	Intercept
Stomatal conductance (mmol/m2/s)	*Leaf*	180.6 ± 17.2 (*21*)	190.8 ± 17.4 (*19*)	Intercept
LMA (mg/mm2)	*Leaf*	0.04 ± 0.002 (*33*)	0.03 ± 0.002 (*29*)	Treatment

Table S3. Model selection results for growth and physiological traits from the global GLMM. Parameters in the model (K), degrees of freedom error (df), Aikaike's Information Criterion for small sample sizes (AICc), the difference in AIC (dAIC), and variance of the random intercept terms are shown. All models include a random effect of plant genotype nested within seed source site. Only models with dAICc < 2 are shown. Marginal (fixed effects only; R^2_M) and conditional (fixed + random effects; R^2_C) R^2 values are also shown.

							Random			
Species	Model	Fixed effects	K	df (N)	AICc	dAIC	Family	Site	R^2_M	R^2_C
Asclepias fascicularis	Height change (cm) ~	Treatment	6	194	130	0	0.378	0.072	NA	NA
	Total dry biomass (g) ~	Treatment * CWD	7	181	536.9	0.0	0.000	0.000	0.05	0.05
		Treatment	**5**	**183**	**538.5**	**1.6**				
	Root:shoot ratio ~	Intercept	4	184	454.8	0.0	0.087	0.103	0	0.26
		CWD	**5**	**183**	**456.7**	**1.9**				
	Root:leaf ratio ~	Treatment	5	183	514.1	0.0	0.100	0.080	0.02	0.21
		Treatment + CWD	**6**	**182**	**516.1**	**2.0**				
	Stomatal conductance ~	Treatment	5	90	280.9	0.0	0.021	0.000	0.11	0.13
	(mmol m^{-2} s^{-1})	**Treatment + CWD**	**6**	**89**	**282.0**	**1.1**				
	Leaf mass per area ~	Treatment + CWD	6	183	552.4	0.0	0.009	0.066	0.07	0.14
	(mg mm^{-2})	**Treatment * CWD**	**7**	**182**	**552.6**	**0.2**				
		Treatment	**5**	**184**	**553.3**	**0.8**				
		CWD	**5**	**184**	**554.0**	**1.6**				
Asclepias speciosa	Height change (cm) ~	Intercept only	5	62	82.1	0	0.153	0.234	NA	NA
		CWD	**6**	**61**	**82.8**	**0.7**				
	Total dry biomass (g) ~	Intercept only	4	60	174.5	0.0	0.000	0.000	0	0
		CWD	**5**	**59**	**176.0**	**1.5**				
		Treatment	**5**	**59**	**176.3**	**1.9**				
	Root:shoot ratio ~	CWD	5	59	195.9	0.0	0.000	0.000	0.08	0.08
		Treatment + CWD	**6**	**58**	**197.2**	**1.3**				
	Root:leaf ratio ~	Intercept only	4	60	188.3	0.0	0.000	0.025	0	0.03
		Treatment	**5**	**59**	**188.5**	**0.2**				
		CWD	**5**	**59**	**188.8**	**0.5**				
		Treatment + CWD	**6**	**58**	**188.8**	**0.5**				
	Stomatal conductance ~	Intercept only	4	36	104.1	0	0.133	0.000	0	0.20
	(mmol m^{-2} s^{-1})									
	Leaf mass per area ~	Treatment	5	57	144.3	0.0	0.059	0.016	0.04	0.17
	(mg mm^{-2})	**Intercept only**	**4**	**58**	**144.5**	**0.2**				
		Treatment + CWD	**6**	**56**	**145.2**	**0.8**				
		CWD	**5**	**57**	**145.4**	**1.1**				

Table S4. Model selection results for chemical traits from the global GLMM. Parameters in the model (K), degrees of freedom error (df), Aikaike's Information Criterion for small sample sizes (AICc), the difference in AIC (dAIC), and variance of the random intercept terms are shown. All models include a random effect of plant genotype nested within seed source site. Only models with dAICc < 2 are shown. Marginal (fixed effects only; R^2_M) and conditional (fixed + random effects; R^2_C) R^2 values are also shown.

							Random			
Species	Model	Fixed effects	K	df (N)	AICc	dAIC	Family	Site	R^2_M	R^2_C
Asclepias fascicularis	Total UV absorbent conc (mg/g) ~ Leaves	Treatment * CWD	7	168	479.3	0.0	0.227	0.000	0.05	0.28
		Treatment	5	170	479.7	0.4				
		Treatment + CWD	6	169	480.9	1.6				
	Total UV absorbent conc (mg/g) ~ Roots	Intercept only	4	193	552.9	0.0	0.077	0.006	0	0.08
		CWD	5	192	553.4	0.6				
		Treatment	5	192	554.1	1.3				
		Treatment + CWD	6	191	554.7	1.9				
	QGR + KGR (flavonoids) (mg/g) ~ Leaves ONLY	Treatment * CWD	7	168	481.4	0.0	0.192	0.000	0.07	0.26
		Treatment	5	170	482.9	1.5				
		Treatment + CWD	6	169	483.1	1.8				
	Pregnane glycosides (mg/g) Leaves	CWD	5	170	455.4	0	0.250	0.000	0.08	0.34
		Intercept only	**4**	**171**	**457**	**1.6**				
		Treatment + CWD	**6**	**169**	**457.1**	**1.7**				
	Pregnane glycosides (mg/g) Roots	Intercept only	4	193	550.2	0	0.040	0.106	0	0.15
		Treatment	**5**	**192**	**551.3**	**1.1**				
		CWD	**5**	**192**	**551.7**	**1.5**				
Asclepias speciosa	Total UV absorbent conc (mg/g) ~ Leaves	Treatment + CWD	6	47	151.4	0.0	0.179	0.000	0.21	0.37
		Treatment	**5**	**48**	**152.0**	**0.6**				
		CWD	**5**	**48**	**153.3**	**1.9**				
	Total UV absorbent conc (mg/g) ~ Roots	CWD	5	61	189.1	0.0	0.139	0.000	0.09	0.23
		Intercept only	**4**	**62**	**189.5**	**0.4**				
	QGR + QG (flavonoids) (mg/g) ~ Leaves ONLY	Treatment	5	48	153.1	0.0	0.186	0.000	0.13	0.31
		Treatment + CWD	**6**	**47**	**154.4**	**1.2**				
	Pregnane glycosides (mg/g) Roots ONLY	Intercept	4	62	192.4	0.0	0.109	0.000	0	0.11
		Treatment	**5**	**61**	**193.1**	**0.6**				
		CWD	**5**	**61**	**193.1**	**0.7**				
		Treatment + CWD	**6**	**60**	**194**	**1.6**				

Methods S1 Calculation of cumulative annual climatic water deficit (CWD)

To calculate monthly climatic water deficit (CWD) for each seed-provenance site, we acquired: (i) total monthly precipitation and mean monthly temperature using the PRISM Data Explorer tool for the years 2004–2016 (PRISM Climate Group 2019); (ii) elevation from the 10-m National Elevation Dataset (USGS 2019), which was used to derive slope and aspect; and (iii) the predicted soil water holding capacity from USDA SSURGO (USDA 2018), following Dilts (2014). These monthly data were then summed annually and averaged over the period 2004–2016 for each site.

Literature Cited

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Methods S2 Analytical Chemistry Supplementary Methods

HPLC-UV

Analysis of *Asclepias fascicularis* and *Asclepias speciosa* leaves and roots from the glasshouse drought experiment used samples extracted in methanol with a 0.075 mg/mL digitoxin internal standard.

Analysis was performed using a HPLC-UV system (Agilent 1100 Series instrument) equipped with a reversed phase column (Nucleodur Sphinx RP, 250 x 4.6 mm, 5 µm particle size; Macherey-Nagel, Düren, Germany). Mobile phases were water (A) and acetonitrile (B), starting with 20% B, followed by a gradient to 68% B in 24 min at a constant flow of 1 ml/min, followed by a washing and reequilibration cycle. The eluent was monitored by a photodiode array detector at 219 nm. All peaks were quantified as digitoxin equivalents based on the internal standard digitoxin applying a relative molar response factor of 1.0.

LC-IonTrap-MS (low resolution mass spectrometer)

Analysis was done by LC-MS using a Bruker Esquire 6000 ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) operated in alternating ionization mode in the range m/z 60–1,400 (capillary exit voltage, +110/-110 eV; capillary voltage, +4,000/-4,000V; nebulizer pressure, 35 psi; drying gas, 11 l min-1; gas temperature, 330°C) coupled to an Agilent 1100 series HPLC (Agilent Technologies, Waldbronn, Germany). Elution was accomplished using a Nucleodur Sphinx RP column (250 x 4.6 mm, 5 µm; Macherey- Nagel, Düren, Germany). Mobile phases were 0.2% formic acid (v:v) (A) and acetonitrile (B), starting with 20% , followed by a gradient to 68% B in 24 min followed by a washing and reequilibration cycle. MS2 spectra were recorded in positive and negative ionization mode in AutoMS modus.

LC-Q-ToF-MS (high resolution mass spectrometer)

To determine the exact mass of metabolites, ultra-high-performance liquid chromatography–electrospray ionization– high resolution mass spectrometry (UHPLC–ESI–HRMS) was performed with a Dionex Ultimate 3000 series UHPLC (Thermo Scientific) and a Bruker timsToF mass spectrometer (Bruker Daltonik, Bremen, Germany). UHPLC was used applying a reversed-phase Zorbax Eclipse XDB-C18 column (100 mm × 2.1 mm, 1.8 µm, Agilent Technologies, Waldbronn, Germany) with a solvent system of 0.1% formic acid (A) and acetonitrile (B) at a flow rate of 0.3 ml/min. The elution profile was the following: 0 to 0.5 min, 5% B; 0.5 to 11.0 min, 5% to 60% B in A; 11.0 to 11.1 min, 60% to100% B, 11.1 to 12.0 min, 100% B and 12.1 to 15.0 min 5% B. Electrospray ionization (ESI) in negative/positive ionization mode was used for the coupling of LC to MS. The mass spectrometer parameters were set as follows: capillary voltage 4.5 KV/3.5KV, end plate offset of 500V, nebulizer pressure 2.8 bar, nitrogen at 280°C at a flow rate of 8L/min as drying gas. Acquisition was achieved at 12 Hz with a mass range from m/z 50 to 1500. At the beginning of each chromatographic analysis 10uL of a sodium formate-isopropanol solution (10 mM solution of NaOH in 50/50 (v/v%) isopropanol water containing 0.2% formic acid) was injected into the dead volume of the sample injection for re-calibration of the mass spectrometer using the expected cluster ion m/z values.

Appendix S2. Analytical information for the UV-absorbent chemistry of plants in the glasshouse drought experiment.

AppendixS2. Analytical information for the UV-absorbent chemistry of plants in the glasshouse drought experiment.

Species	Tissue	Retention time (min)-LCUV	RT-LCMS	m/z neg*	m/z pos*	MW	sum formula	UV spectra	fragments neg m/z	fragments pos m/z	**Tentative** ID	References
Asclepias fascicularis	leaf	6.82	7.3	***609.1462***[a]	***611.1609***[c]	610	C27H30O16			303 465	Quercetin-glucoside-rhamnoside	Haribal, M. & J. A. A. Renwick. 1996. Oviposition stimulants for the monarch butterfly: flavonol glycosides from *Asclepias curassavica*. Phytochemistry 41: 139-144. compound #3
Asclepias fascicularis	leaf	7.958	8.2	***593.1514***[a]	***595.166***[c]	594	C27H30O15			387 449	Kaempferol-glucoside-rhamnoside	El-Askary, H. 2003. Pregnene glycoside and monoterpene derivative from *Solenostemma argel* Hayne. Bull Fac Pharm Cairo University. 41: 131-137. compound #3
Asclepias fascicularis	leaf	17.366	17.8	***962.4746***[b]	***918.4838***[c]	917	C48H71N1O16				pregnane glycoside	Warashina, T., & N. Tadataka. 1996. Steroidal glycosides from roots of *Cynanchum caudatum*. III. Chemical & Pharmaceutical Bulletin 44: 358-363. compound #3
Asclepias fascicularis	leaf	20.354										
Asclepias fascicularis	leaf	21.461	22.5	***931.4667***[a] ***/ 977.4763***[b]	***950.5091***[d]	932	C49H72O17		809	309 327 345	benzoylated pregnane glycoside	Oberai, K., M. P. Khare, A. Khare. 1985. A pregnane ester triglycoside from *Sarcostemma brevistigma*. Phytochemistry. 24: 3011-3013. compound #1
Asclepias fascicularis	leaf	21.947	22.9	***931.4673***[a] ***/ 977.4735***[b]	***950.5103***[d]	932	C49H72O17		809		benzoylated pregnane glycoside	Oberai, K., M. P. Khare, A. Khare. 1985. A pregnane ester triglycoside from *Sarcostemma brevistigma*. Phytochemistry. 24: 3011-3013. compound #1
Asclepias fascicularis	leaf	22.237	23.3	915.6[a]	939.5[e]	916	C49H72O16		731 749	311 329	benzoylated pregnane glycoside	Vleggar, R., F. R. Van Heerden, L. A. P. Anderson, G. L. Erasmus. 1993. Toxic constituents of the Asclepiadaceae. Structure elucidation of sarcovimiside A-C, pregnane glycosides of *Sarcostemma viminale*. Journal of the Chemical Society, Perkin Transactions 1: Organic and Bio-Organic Chemistry (1972-1999). 483-487. compound #A6
Asclepias fascicularis	leaf	24.009	25.1	1075.6[a]	1099.6[e]	1076	C63H80O15		953	309 327 345	benzoylated pregnane glycoside	
Asclepias fascicularis	leaf	24.823	25.9	1059.7[a]	1083.6[e]	1060	C63H80O14		937	311	saponin	Sylla, B., S. Levoie, J. Legault, C. Gauthier, A. Pichette. 2019. Synthesis, cytotoxicity and anti-inflammatory activity of rhamnose-containing ursolic and betulinic acid saponins. RSC Advances 9: 39743-39757.compound #18
Asclepias fascicularis	root	6.351		***565.1924***[b]	***538.2288***[d] ***; 543.1845***[e] ***; 559.1578***[f]	520	C26H32O11				phenolic	
Asclepias fascicularis	root	7.307		***581.2236***[a]	***600.2655***[d] ***; 543.1845***[e] ***; 559.1578***[f]	582	C28H38O13				phenolic	
Asclepias fascicularis	root	11.32										
Asclepias fascicularis	root	11.527										
Asclepias fascicularis	root	15.991	16.6	841.5[b]	819.5[e]	796	C42H68O14				pregnane glycoside	Warashina, T. & T. Noro. 2000. Cardenolide and oxypregnane glycosides from the root of *Asclepias incarnata* L. Chemical & Pharmaceutical Bulletin. 48: 516-524. compound #3
Asclepias fascicularis	root	17.554	18.3	939.6[a]	963.5[e]	940	C49H80O17				pregnane glycoside	Fumiko, A. & T. Yamauchi. 2000. Pregnane glycosides from the roots of *Asclepias tuberosa.* Chemical & Pharmaceutical Bulletin. 48: 1017-1022. compound #13
Asclepias fascicularis	root	19.17	19.5	939.6[a]	963.5[e]	940	C49H80O17				pregnane glycoside	Fumiko, A. & T. Yamauchi. 2000. Pregnane glycosides from the roots of Asclepias tuberosa. Chemical & Pharmaceutical Bulletin. 48: 1017-1022. compound #13
Asclepias fascicularis	root	20.354										
Asclepias fascicularis	root	21.461		***931.4667***[a] ***/ 977.4763***[b]	***950.5091***[c]	932	C49H72O17				benzoylated pregnane glycoside	Oberai, K., M. P. Khare, A. Khare. 1985. A pregnane ester triglycoside from *Sarcostemma brevistigma*. Phytochemistry. 24: 3011-3013. compound #1
Asclepias fascicularis	root	21.947		***931.4673***[a] ***/ 977.4735***[b]	***950.5103***[c]	932	C49H72O17				benzoylated pregnane glycoside	Oberai, K., M. P. Khare, A. Khare. 1985. A pregnane ester triglycoside from *Sarcostemma brevistigma*. Phytochemistry. 24: 3011-3013. compound #1
Asclepias fascicularis	root	22.197									benzoylated pregnane glycoside	
Asclepias fascicularis	root	22.237		915.6[a]	939.5[e]	916	C49H72O16				benzoylated pregnane glycoside	Vleggar, R., F. R. Van Heerden, L. A. P. Anderson, G. L. Erasmus. 1993. Toxic constituents of the Asclepiadaceae. Structure elucidation of sarcovimiside A-C, pregnane glycosides of *Sarcostemma viminale*. Journal of the Chemical Society, Perkin Transactions 1: Organic and Bio-Organic Chemistry (1972-1999). 483-487. compound #A6
Asclepias speciosa	leaf	6.82	7.3	***609.1462***[a]	***611.1609***[c]	610	C27H30O16				Quercetin-glucoside-rhamnoside	Haribal, M. & J. A. A. Renwick. 1996. Oviposition stimulants for the monarch butterfly: flavonol glycosides from *Asclepias curassavica*. Phytochemistry 41: 139-144. compound #3
Asclepias speciosa	leaf	7.774	8.3	463.0[a]	465.0[c]	464	C21H20O12			303	Quercetin-glucoside	
Asclepias speciosa	leaf	8.766	9.4	477.1[a]	479.1[c]	478	C22H22O12			317	Isorhamnetin-glucoside	
Asclepias speciosa	leaf	17.366	18.3	807.5[b]	785.5[e]	762			613			
Asclepias speciosa	leaf	17.841										
Asclepias speciosa	leaf	20.354	21.4	675.4[a]	699.3[e]	676	C33H56O14		397 415			
Asclepias speciosa	root	7.307										
Asclepias speciosa	root	7.572	8.1	***581.2238***[a]	***600.2651***[d]	582	C28H38O13		401			
Asclepias speciosa	root	7.774	8.4	***551.2132***[a]	***570.2554***[d]	552	C27H36O12					
Asclepias speciosa	root	11.527	12	953.3[a]	977.3[e]	954			757			
Asclepias speciosa	root	13.93	14.5	843.5[b]	821.5[e]	798	C42H70O14					
Asclepias speciosa	root	15.991	16.5	***616.2216***[a]		617	C31H39N1O10S1		491	311	labriformin (cardenolide)	Cardenolides in the latex and leaves of seven Asclepias species and Calotropis procera: Seiber, James N.; Nelson, Carolyn J.; Lee, S. Mark; Phytochemistry,Volume21, Issue9, Pages2343-8, 1982. compound #10
Asclepias speciosa	root	17.554	18.1	939.6[a]	963.5[e]	940	C49H80O17				pregnane glycoside	Fumiko, A. & T. Yamauchi. 2000. Pregnane glycosides from the roots of *Asclepias tuberosa.* Chemical & Pharmaceutical Bulletin. 48: 1017-1022. compound #13
Asclepias speciosa	root	18.532	19.5	939.6[a]	963.5[e]	940	C49H80O17			617	cinnamoylated pregnane glycoside	Fumiko, A. & T. Yamauchi. 2000. Pregnane glycosides from the roots of *Asclepias tuberosa.* Chemical & Pharmaceutical Bulletin. 48: 1017-1022. compound #13
Asclepias speciosa	root	19.77	20.8	1265.7[a]	1289.7[e]	1266					cinnamoylated pregnane glycoside	
Asclepias speciosa	root	20.354	21.4	947.5[b]	925.5[e]	902			679			

**Values for m/z of molecular ion and fragment ions from low resolution mass spectrometry (LC-ESI-IonTrap-MS); values in bold and italics from high-resolution mass spectrometry (LC-ESI-Q-ToF-MS)*

[a] $[M-H]^-$
[b] $[M+HCOOH-H]^-$
[c] $[M+H]^+$
[d] $[M+NH4]^+$
[e] $[M+Na]^+$
[f] $[M+K]^+$

Chapter 2

Herbivores disrupt clinal variation in plant responses to water limitation

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Abstract

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. 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 from within the Great Basin, USA. We then conducted a factorial experiment crossing water limitation with herbivory. 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 control plants. This lack of plasticity in phytochemical diversity in plants experiencing combined stressors was associated with a reduction in relative growth rates. 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. These results suggest that climatic history can affect within-species patterns in 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 *Asclepias*, drought, herbivory, intraspecific variation, milkweed, multiple stressors, phytochemistry, phytochemical diversity, plasticity

1 | Introduction

Organisms frequently respond to combined stressors non-additively (Smit et al. 2009, Zhang and Sonnewald 2017), such that characterizing the impact of co-occurring stressors is necessary to predict the ecological impacts of global change (Orr et al. 2020, Beauchesne et al. 2021). 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 and Sharitz 2006, Lucas et al. 2013, Feckler et al. 2018). 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 signaling to initiate plastic trait responses to their environments (Felton and Korth 2000, Atkinson et al. 2015, 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 (Suzuki et al. 2014, Nguyen et al. 2016). For example, some biotic and abiotic stressors induce conflicting hormonal response mechanisms (Suzuki et al. 2014, Atkinson et al. 2015), 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 (Matesanz and Ramírez-Valiente 2019, Diethelm et al. 2022), 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 and 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 and 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 distribution or relative abundance of compound production (*i.e.*, evenness) (Wetzel and Whitehead 2020). Phytochemical diversity appears to have strong impacts on herbivores (Richards et al. 2015, Glassmire et al. 2016, 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 defense 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 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 | 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, among other specialist herbivores (Woodson 1954, Dilts et al. 2019). 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 defenses, which are sequestered by monarch butterflies (*Danaus plexippus*). 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 (Rasmann and Agrawal 2011, Mundim and Pringle 2020, Diethelm et al. 2022). 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 (Kaminska-Rozek and Pukacki 2004, Zehnder and Hunter 2007, Diethelm et al. 2022).

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 across the Great Basin Desert, USA, in fall 2016 (Fig. S1). To estimate the typical drought stress at each of these sites, we calculated the cumulative annual climatic water deficit (CWD) using data from the years 2004–2016 following 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) (Fig. 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–October 2017 in a 30x10-m outdoor plot at the University of Nevada, Reno, Valley Road Agricultural Station. Seeds were germinated in May 2017 and transferred to 0.5-L pots of Full Circle® Soar potting soil to grow for two months. In July 2017, *A. fascicularis* plants from the five seed-source sites, representing four maternal families per site and 16 plants per family, were transplanted into the plot in random order ($N = 320$). Some plants failed to establish, reducing the total number of plants to 147. All plants were fertilized once per month with 1:1:1 NPK fertilizer pellets.

To evaluate plant responses to single versus combined stressors, plants were randomly assigned to one of four possible treatments: control (control water + no herbivory); dry (dry + no herbivory); herbivory (control water + herbivory); or dry + herbivory. Control 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, control plants received 84 mL of water to an ~157 cm^2 soil area per day while the dry lines received no irrigation. The control 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 one month, plants in the dry treatment were beginning to show severe wilt, so we supplemented them with 84 mL over two 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% of aboveground plant tissue using netted bags. We checked plants periodically and removed caterpillars once all the leaves in the netted section had been consumed.

Plant responses to the treatments were quantified as follows. To calculate changes in aboveground 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 physical 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 analyzed ∂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 analyzed 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 8,000 absorbance units (AU)/min and a minimum height of 5,000 AU/min. The chemical identities of key peaks were verified by by LC-Q-ToF-MS at the Max Planck Institute for Chemical Ecology (Appendix S3).

2.3 | Statistical analysis

To analyze 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 2021). To compare effect sizes, we normalized 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 S1-S2 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. The total concentration of UV-absorbent plant metabolites was not changed by the addition of monarch herbivory to mechanical damage ($t = 0.26$, df = 57, $P = 0.8$), 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 marginal predictors (Δ AIC ≤ 2) were evaluated using log-likelihood ratio tests (LRT) between models in the 'lmtest' package (Zeileis and Hothorn 2002). Marginal and conditional R^2 values were calculated for selected models in the 'MuMIn' package.

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 as flavonol glycosides (hereafter flavonols) and 16 as benzoylated pregnane glycosides (hereafter pregnane glycosides) (Appendix S2–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 from wetter to drier seed sources (β_{CWD} = 0.23 ± 0.08, z = 2.61, P < 0.01; Fig. S2b,e), a pattern driven by an increase in the concentration of flavonols from wetter to drier seed sources (β_{CWD} = 0.27 ± 0.08, z = 3.38, P < 0.0008, Fig. 1a; Table S2). The dominant flavonol, QGR, in particular was found in higher concentrations in plants from drier sites (β_{CWD} = 0.26 ± 0.10, z = 2.62, P < 0.009; Table S2). Leaf chemical diversity (H) was higher in plants sourced from wetter sites (Fig. 1b), partly because evenness was higher among plants with lower concentrations of the dominant flavonols (Fig. S2). Plants from wetter sites also had higher metabolite richness (β_{CWD} = -0.17 ± 0.08, z = -2.1, P < 0.04, Fig. S2), due to the higher richness of pregnane glycosides in plants from wetter sites (β_{CWD} = -0.24 ± 0.08, z = -3.00, P < 0.003; Table S2). Relative growth rate was marginally higher among plants from relatively wetter seed sources (β_{CWD} = -0.14 ± 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-S3). Water limitation reduced leaf width: water-limited plants produced leaves ~6% narrower than well watered plants (β_{water} = -0.28 ± 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 (β_{water} = 0.28 ± 0.15, z = 2.0, P = 0F.05; Table 1). Herbivory, meanwhile, marginally increased the richness (S) of pregnane glycosides in the leaves ($\beta_{herbivory}$ = 0.29 ± 0.16, z = 1.83, P = 0.07) and reduced foliar water content ($\beta_{herbivory}$ = -0.35 ± 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 1and S3).

3.4 | Plant responses contingent on multiple stressors

Co-occurring herbivory and water limitation resulted in slower growing plants (Fig. 2) and dampened phytochemical responses to stress (Fig. 3). Relative growth rates of the plants experiencing either water limitation or herbivory alone were not different from those of control plants, but plants experiencing both stressors grew 45% more slowly ($\beta_{water:herbivory}$ = -0.57 ± 0.32, z = -1.78, P < 0.08; Fig. 2). Water limitation increased leaf chemical diversity (H) only in plants without herbivory ($\beta_{water:herbivory}$ = 0.38 ± 0.14, z = 2.67, P < 0.008; Fig. 3a; Table S2). This increase in chemical diversity corresponded partly to a reduction in the QGR concentrations in the leaves of dry plants, which increased phytochemical evenness ($\beta_{water:herbivory}$ = 0.56 ± 0.14, z = 3.94, P < 0.0009; correlation between QGR and evenness: Pearson's r = -0.50, df = 141, P < 0.0001; Fig 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 (Fig. 3c).

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 ($\beta_{water:CWD} = 0.38 \pm 0.14$, z = 2.67, P < 0.008; Fig. 4a), and this clinal variation in responses to dry conditions was disrupted by co-occurring herbivory (Fig. 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 (Fig. S2).

4 | Discussion

Plants routinely experience multiple stressors. To predict the ecological consequences of multiple stressors, we must understand whether co-occurring stressors interact to produce non-additive effects on 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 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 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, Whitehead et al. 2021, Volf et al. 2022), 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 operate by means of 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, control plants in this outdoor experiment contained 62% higher concentrations of flavonols than control plants in our previous experiment, which was conducted in a glasshouse (50.5 mg/g versus 31.2 mg/g). Because flavonols can also act as antioxidants against UV radiation (Fischbach et al. 1999, Dixon et al. 2001), 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 then 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 aboveground, which suggests that disruption of plasticity by combined stressors produces a fitness cost. Nevertheless, we note that aboveground growth rates may be an insufficient proxy for plant fitness if plants are instead allocating energy to other functions necessary for survival, such as belowground growth or carbohydrate storage (Peng et al. 2020, Huang et al. 2021).

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 defense, the ecological role of phytochemical evenness *per se* is less studied (Wetzel and Whitehead 2020) but may also be protective (Whitehead et al. 2021, Salgado et al. 2023). 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 showed that 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 and 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 defense 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 defense remains unclear, although the presence of pregnane glycosides often trades off with that of the better known cardiac glycosides, suggesting a potential defensive role (Zehnder and Hunter 2007, Diethelm et al. 2022). It is thus possible that the higher richness of pregnane glycosides following herbivory acts as an induced defense. 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 plastic responses to abiotic stress, which is consistent with local adaptation, 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. Indeed, the 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 stresses in the study of the consequences of intraspecific variation for community dynamics.

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Tables

Table 1. *Asclepias fascicularis* physical and physiological responses from a factorial manipulation of water-availability (control, dry) and herbivory. "Best model" indicates the top model determined by AIC_c.

Response	Treatment (mean ± SE (*N*))				Best model
	Control	*Dry*	*Herbivory*	*Dry + Herbivory*	
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/mm2)	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

Figure Legends

Figure 1

Plant phytochemistry in *Asclepias fascicularis* leaves in (**a**) phytochemical diversity ($P = 0.09$) and (**b**) total UV-absorbent concentration (mg/g; $P < 0.01$) across the climatic water deficit (CWD) of the seed provenance location. Points show the mean value ($n = 143$) and are colored by their location along a gradient of water availability from dry to wet. Horizontal bars represent SE of the mean trait value and vertical bars represent the SE of the CWD value. SE for climatic water deficits was calculated interannually from 2004–2016.

Figure 2

The response of relative growth rate to reduced water and herbivory alone and in combination. Interaction lines show reaction norms separated 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

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, blue dashed lines represent treatments with herbivory. In all plots, points represent means and bars represent SE. Changes in 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 generalized linear models for the interaction effects of water-limitation and herbivory as follows: ** $P < 0.01$, * $P < 0.05$.

Figure 4

Phytochemical diversity in *A. fascicularis* in the outdoor common garden in response to (**a**) the dry treatment alone, and (**b**) the dry + herbivory treatment. Interaction plots in

(**a,b**) show reaction norms separately by seed-provenance ($P < 0.009$), colored by their location along a gradient of climatic water deficit from dry to wet.

Figures

Fig. 1

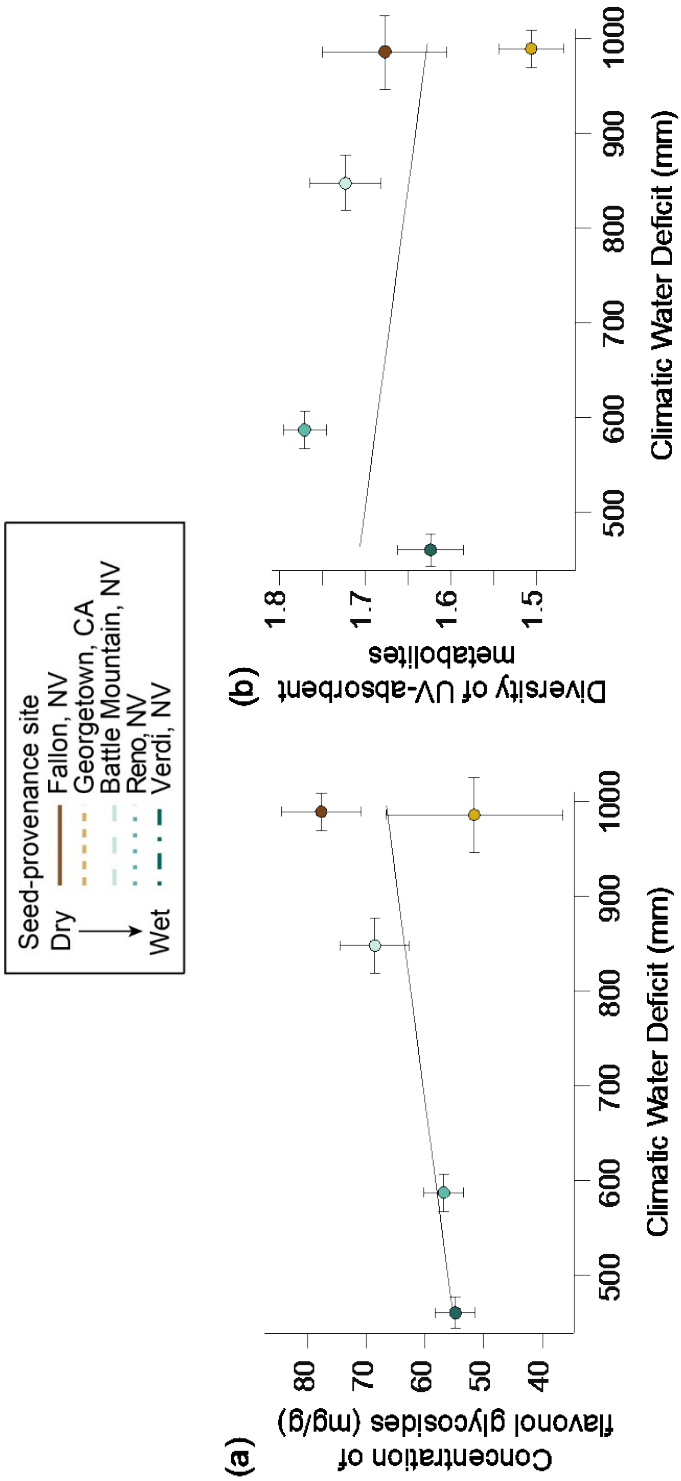


Fig. 2

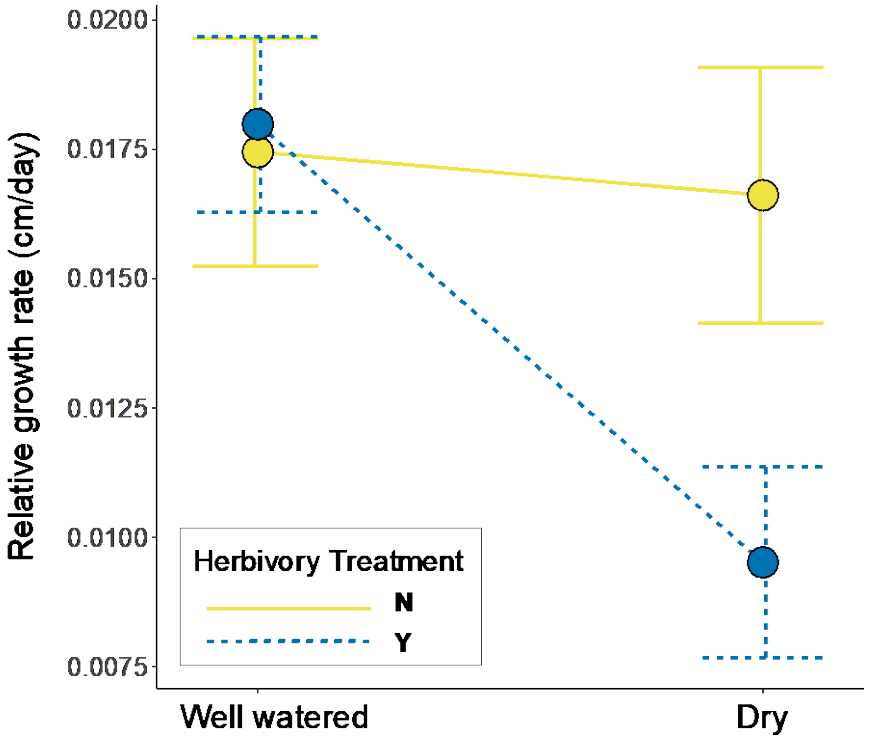

0.0200
0.0175
0.0150
0.0125
0.0100
0.0075
Relative growth rate (cm/day)
Herbivory Treatment
N
Y
Well watered
Dry

Fig. 3

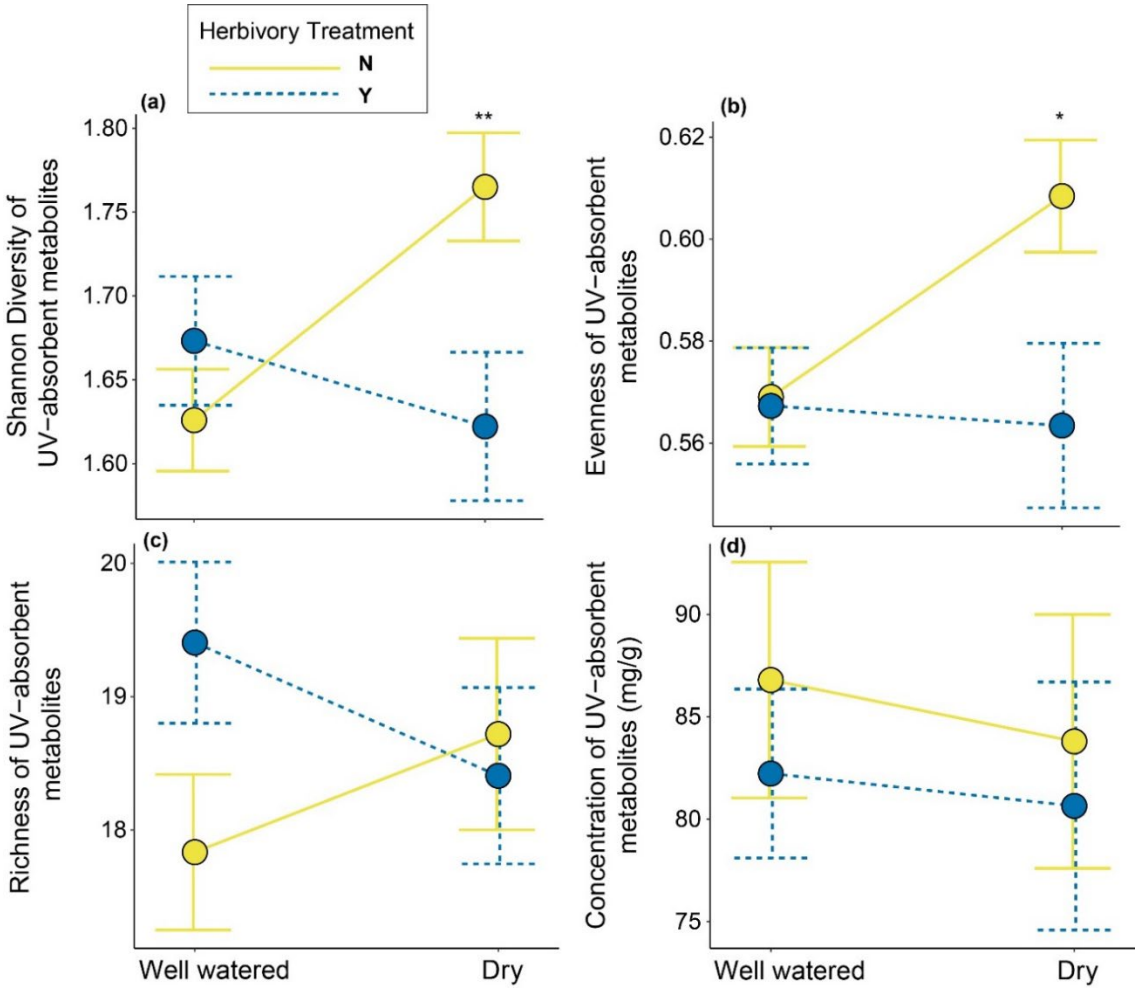
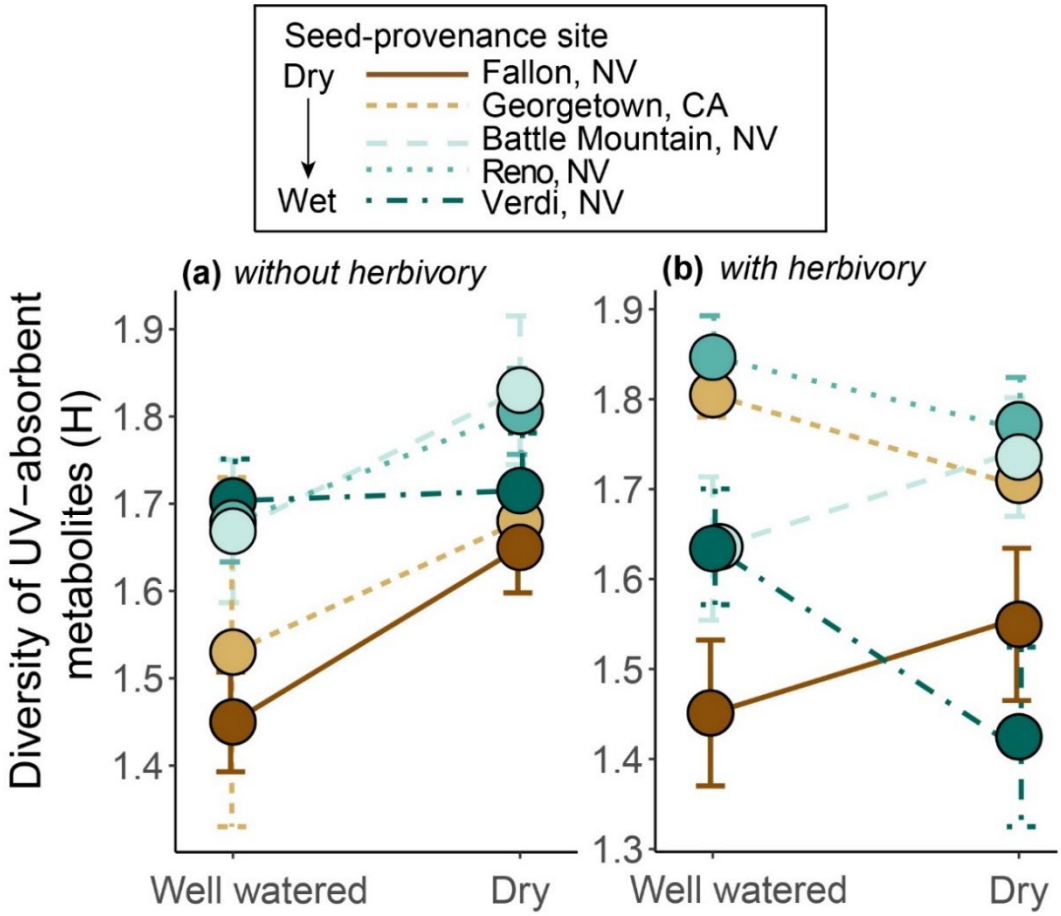

Herbivory Treatment
N
Y
(a)
(b)
(c)
(d)
Shannon Diversity of UV−absorbent metabolites
Evenness of UV−absorbent metabolites
Richness of UV−absorbent metabolites
Concentration of UV−absorbent metabolites (mg/g)
1.80
1.75
1.70
1.65
1.60
0.62
0.60
0.58
0.56
20
19
18
90
85
80
75
**
*
Well watered
Dry
Well watered
Dry

Fig. 4


Seed-provenance site
Dry
Wet
Fallon, NV
Georgetown, CA
Battle Mountain, NV
Reno, NV
Verdi, NV
(a) without herbivory
(b) with herbivory
Diversity of UV−absorbent metabolites (H)
1.9
1.8
1.7
1.6
1.5
1.4
1.3
Well watered
Dry

Supplemental Tables and Figures

Figure S1. Seed collection sites from across a climatic water deficit gradient, based on 1981 to 2010 climate normal. The isolines distinguish actual evapotranspiration (i.e., simultaneous availability of water and energy) in millimeters. Site names are as follows: California (CA), Verdi (VE), Reno (RE), Fallon (FA), and Battle Mountain (BM). Adapted with permission from "Climatic history, constraints, and the plasticity of phytochemical traits under water stress," by Diethelm *et al*., 2022, *Ecosphere* 13; e4167.

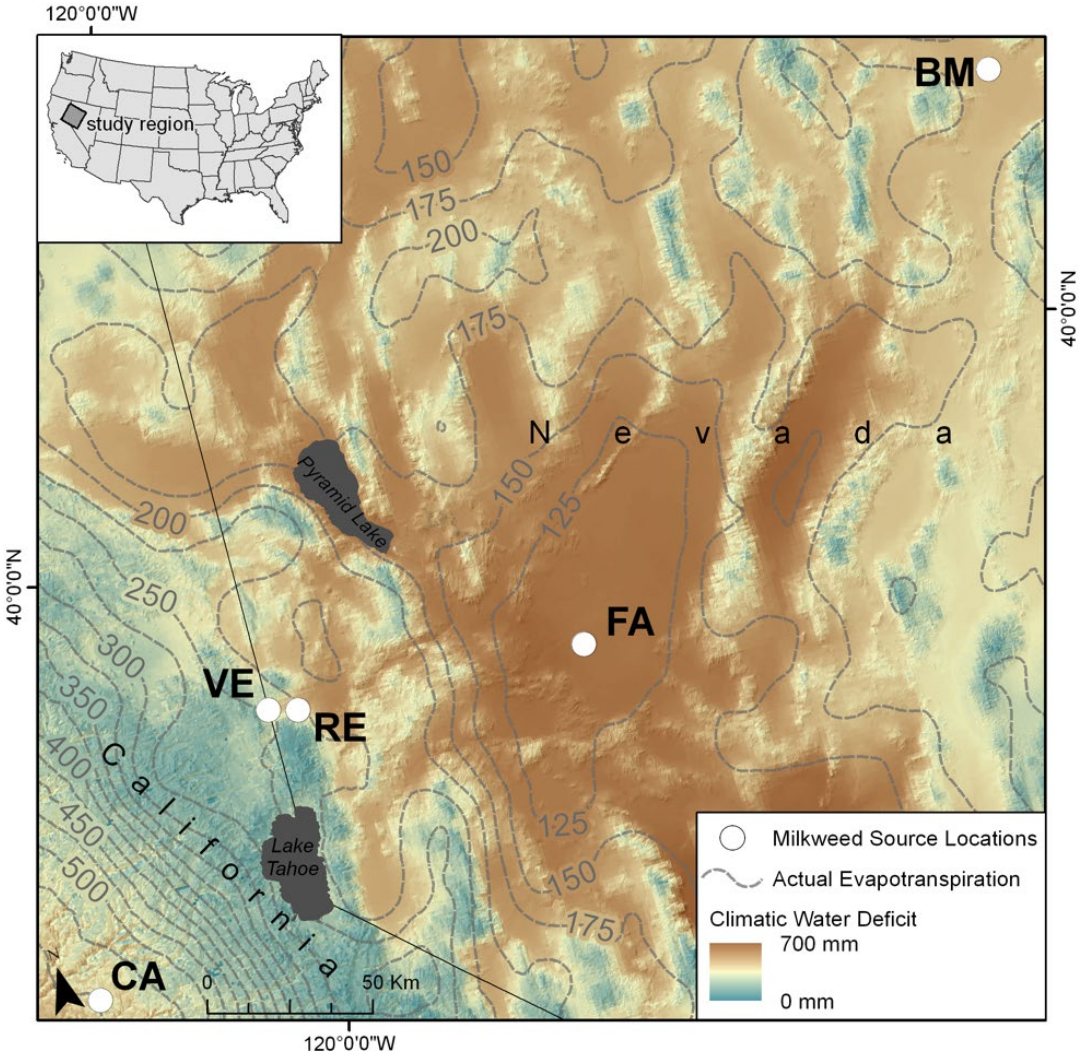


Figure S2. Phytochemistry in metabolites in *Asclepias fascicularis* in a common garden in response to (**a,b,c**) the dry treatment alone, and (**d,e,f**) the dry + herbivory treatment as separately by seed-provenance. Interaction plots show reaction norms for **(a,d)** richness, **(b,e)** total concentration, and **(c,f)** evenness per seed-source with locations colored in a gradient of dry to wet climatic water deficit values.

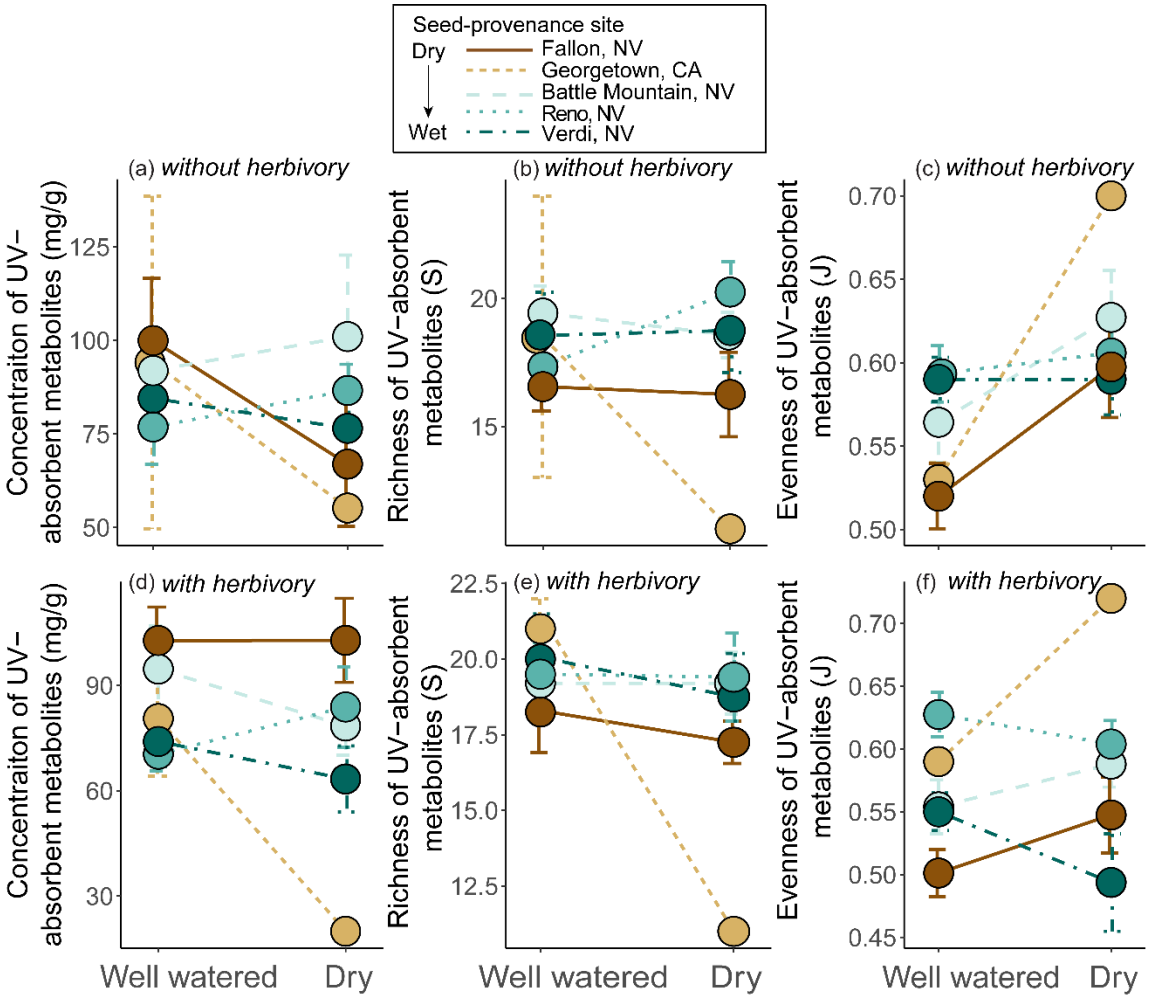


Table S1. Seed-provenance site in decimal degrees from low to high climatic water deficit for *Asclepias fascicularis* plants used in common garden experiment.

Site	Latitude	Longitude
Fallon (FN)	39.47482708	-118.6575129
California (CA)	38.87178726	-120.8187756
Battle Mountain (BM)	40.662576	-116.9320299
Reno (RN)	39.50261419	-119.8986715
Verdi (VE)	39.52318534	-119.9985313

Table S2. Model selection results for *Asclepias fascicularis* chemical leaf traits in a common garden experiment from the global GLMM. Parameters in the model (K), degrees of freedom error (df), Aikaike's Information Criterion for small sample sizes (AICc), the difference in AIC (dAIC), and variance of the random intercept terms are shown. All models include a random effect of plant genotype nested within seed provenance site. Only models with dAICc < 2 are shown. Marginal (fixed effects only; R^2_M) and conditional (fixed + random effects; R^2_C) R^2 values are also shown.

						Random effect			
Model	Fixed effects	K	df (N)	AICc	dAIC	Family	Site	R^2_M	R^2_C
UV-absorbent metabolite diversity (Shannon index; H) ~	CWD * Water + Herbivory * Water	9	134	388.2	0.0	0.034	0.108	0.11	0.26
	CWD * Water + CWD * Herbivory + Water * Herbivory	**10**	**133**	**390.0**	**1.8**				
UV-absorbent total richness (S) ~	CWD	5	138	410.9	0.0	0.000	0.000	0.03	0.03
	CWD + Herbivory	**6**	**137**	**411.7**	**0.8**				
	Intercept only	**4**	**139**	**412.9**	**1.9**				
UV-absorbent metabolite evenness (J) ~	CWD * Water + Herbivory * Water	9	134	388.2	0.0	0.059	0.091	0.13	0.28
	CWD * Water + Herbivory	**8**	**135**	**389.9**	**1.7**				
Total UV-absorbent metabolites (mg/g)~	CWD	5	138	408.6	0.0	0.000	0.000	0.05	0.05
	CWD + Herbivory	**6**	**137**	**410.3**	**1.7**				
	CWD + Water	**6**	**137**	**410.5**	**1.9**				
Total flavonol glycosides (mg/g)~	CWD	5	138	404.3	0.0	0.000	0.000	0.07	0.07
	CWD + Herbivory	**4**	**139**	**406.0**	**1.7**				
	CWD + Water	**6**	**137**	**406.0**	**1.7**				
QGR (mg/g)~	CWD	5	138	404.0	0.0	0.000	0.000	0.07	0.08
	Intercept only	**4**	**139**	**404.7**	**0.7**				
	CWD + Water	**6**	**137**	**405.0**	**1.0**				
	CWD * Water	**7**	**136**	**405.2**	**1.2**				
	Water	**5**	**138**	**405.6**	**1.6**				
IGR (mg/g)~	Intercept only	4	139	403.3	0.0	0.149	0.000	0.00	0.15
	CWD	**5**	**138**	**404.4**	**1.1**				
	CWD * Herbivory	**7**	**136**	**404.7**	**1.4**				
	Water	**5**	**138**	**404.9**	**1.6**				
Benzoylated pregnane glycosides (mg/g) ~	Intercept only	4	139	407.8	0.0	0.046	0.079	0.00	0.12
	CWD	**5**	**138**	**408.8**	**1.0**				
	Water	**5**	**138**	**409.4**	**1.6**				
Benzoylated pregnane glycoside richness ~	CWD + Herbivory	6	137	405.8	0.0	0.000	0.000	0.08	0.08
	CWD	**5**	**138**	**406.9**	**1.1**				
	CWD * Herbivory	**7**	**136**	**407.7**	**1.9**				

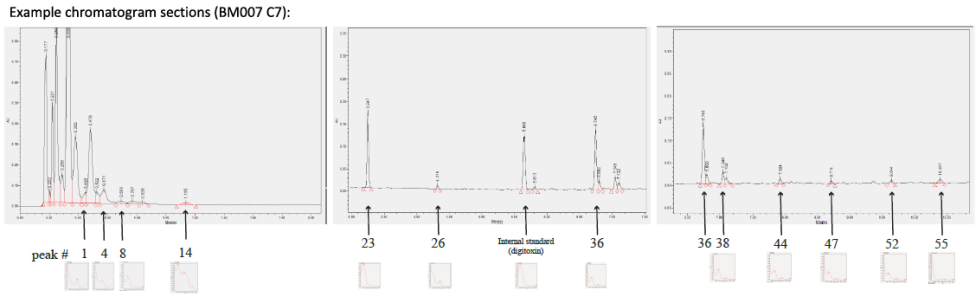
Table S3. Model selection results for *Asclepias fascicularis* physical leaf traits in a common garden experiment from the global GLMM. Parameters in the model (K), degrees of freedom error (df), Aikaike's Information Criterion for small sample sizes (AICc), the difference in AIC (dAIC), and variance of the random intercept terms are shown. All models include a random effect of plant genotype nested within seed provenance site. Only models with dAICc < 2 are shown. Marginal (fixed effects only; R^2_M) and conditional (fixed + random effects; R^2_C) R^2 values are also shown.

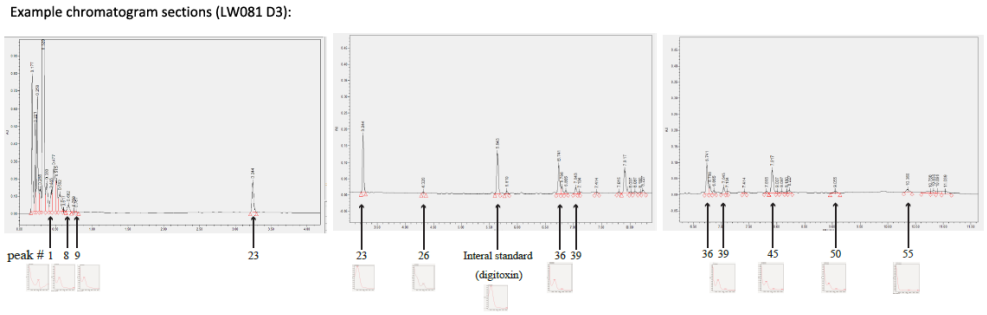
						Random			
Model	Fixed effects	K	df (N)	AICc	dAIC	Family	Site	R^2_M	R^2_C
Relative growth rate ~	CWD + Water * Herbivory	8	138	417.0	0.0	0.000	0.000	0.09	0.09
$(LN(size_2)-LN(size_1))/d$	CWD + Water	6	140	417.6	0.6				
	Water * Herbivory	7	139	417.9	0.9				
	CWD + Water + Herbivory	7	139	417.9	0.9				
Leaf width (mm) ~	Water + Herbivory	6	141	410.9	0.0	0.218	0.000	0.03	0.25
	Water	5	142	411.8	0.9				
	Water * Herbivory	7	140	412.3	1.4				
	Herbivory	5	142	412.4	1.5				
	CWD + Water + Herbivory	7	140	412.7	1.8				
	Intercept only	4	143	412.8	1.9				
Water-use efficiency ($\delta 13C$) ~	Water + Herbivory	6	139	401.5	0.0	0.281	0.000	0.03	0.30
	Water	5	140	401.6	0.1				
	Intercept only	4	141	402.7	1.2				
	Herbivory	5	140	403.0	1.5				
Foliar water content ~	Herbivory	5	142	415.5	0.0	0.074	0.046	0.03	0.15
(mg/mm^2)	Water + Herbivory	6	141	416.2	0.7				
	CWD + Herbivory	6	141	417.4	1.9				
Leaf mass per area ~	Intercept only	4	143	419.5	0.0	0.120	0.000	0.00	0.12
(mg/mm^2)	Herbivory	5	142	420.8	1.3				
	CWD	5	142	421.4	1.9				
Trichome density ~	Intercept only	4	143	416.2	0.0	0.124	0.000	0.00	0.13
	Water	5	142	416.7	0.5				
Carbon to Nitrogen Ratio ~	Intercept only	4	141	418.3	0.0	0.021	0.006	0.00	0.03
(C:N)	CWD	5	140	418.8	0.5				
	Herbivory	5	140	420.2	1.9				

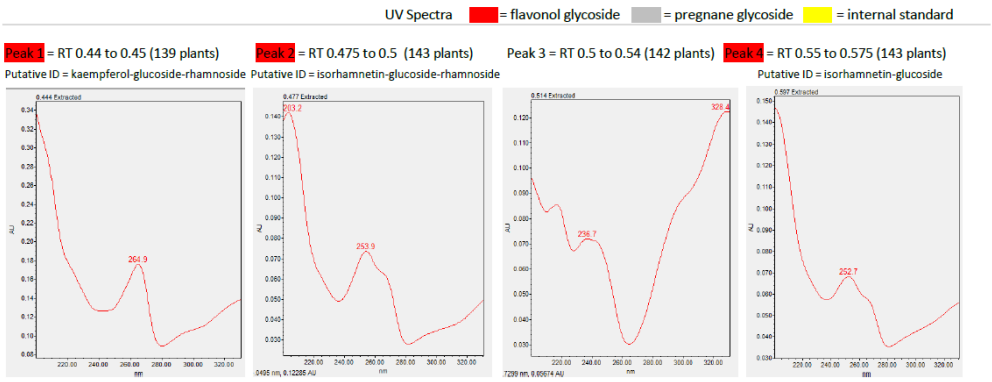
Appendix S1. UPLC-UV Instrument Method

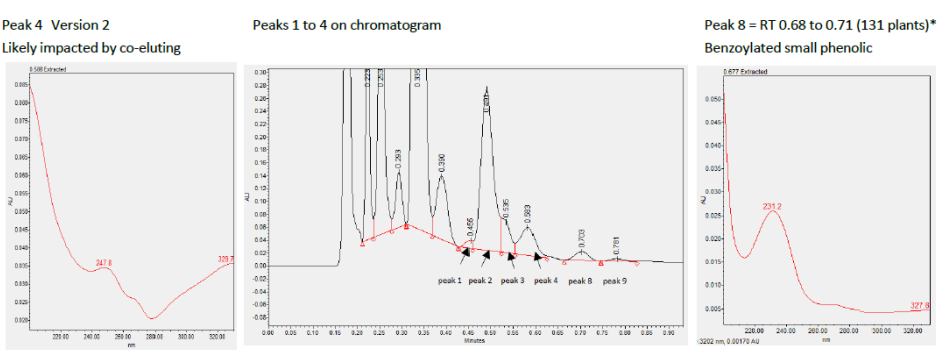
Analysis of *Asclepias fascicularis* foliar phytochemistry used samples extracted in methanol with a 0.15 mg/mL digitoxin internal standard. Analysis was performed using a UPLC-UV system (Waters Inc., Milford, MA, USA) and an ACQUITY UPLC BEH C18 (2.1 x 50 mm, 1.7µm) reversed phase column. Mobile phases were 0.2% phosphoric acid (v:v) (A) and acetonitrile (B), starting with 20% B, followed by a gradient to 68% B in 10.6 min at a constant flow of 0.7 mL/min, followed by a washing and reequilibration cycle. All peaks were quantified as digitoxin equivalents based on the internal standard digitoxin applying a relative molar response factor of 1.0. We exclude any peaks with an area < 8,000 AU/min and a height < 5,000 AU/min.

Appendix S2 Peak identity based on retention times and UV-spectra for the UV-absorbent chemistry of *Asclepias fascicularis* plants in the outdoor water x herbivory experiment.

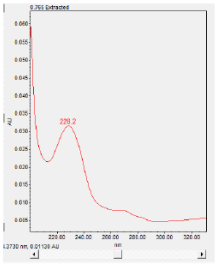




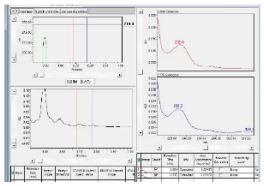




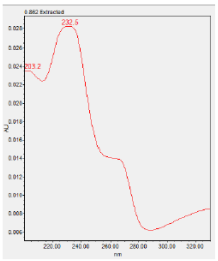
Peak 9 = RT 0.754 to 0.79 (95 plants)*
Benzoylated small phenolic



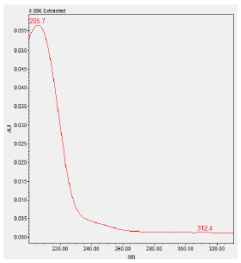
*Similar shape and RT time but diff. molec
Also diff. maxima w/ Peak 8 ~231 & Peak 9 ~228



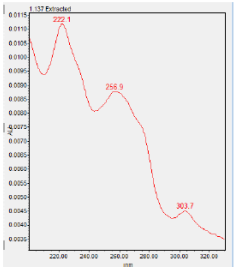
Peak 11 = RT 0.866



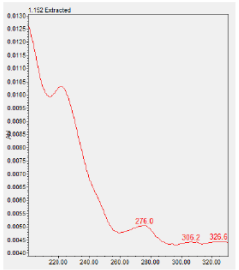
Peak 12 = RT 0.88 to 1.32 (16 plants)



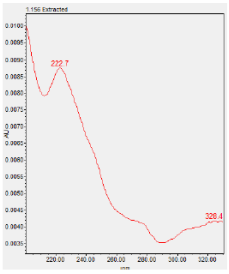
Peak 14 = RT 1.134 to 1.14 (3 plants)



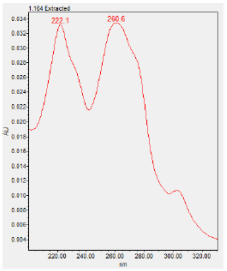
Peak 15 = RT 1.13 to 1.5 (2 plants)



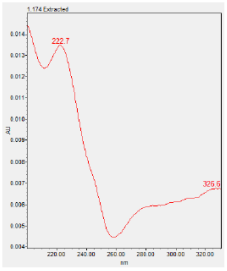
Peak 17= RT 1.15 (1 plant)



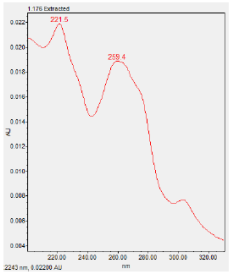
Peak 18 = RT 1.17 (1 plant)



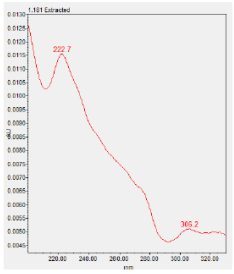
Peak 19 = RT 1.23 (1 plant)



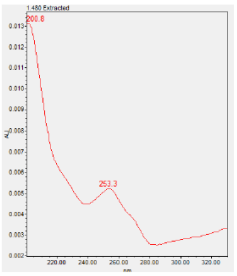
Peak 20= RT 1.175 (1 plant)



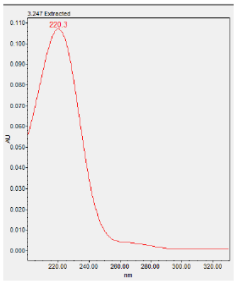
Peak 21= RT 1.174 to 1.79 (2 plants)



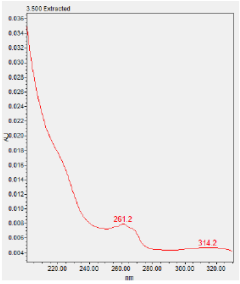
Peak 22 = RT 1.19 to 1.55
(24 plants)



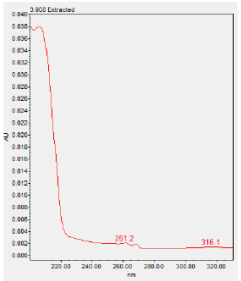
Peak 23 = RT 3.2 (control, all plants)
likely digitoxin disassociate



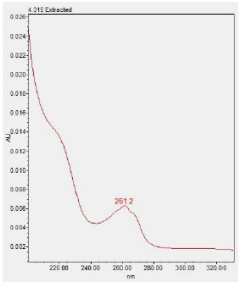
Peak 24 = RT 3.4 (1 plant)



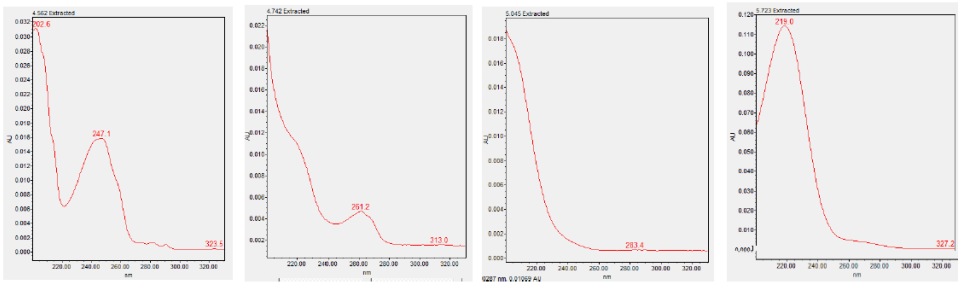
Peak 25 = RT 4.0 (4 plants)



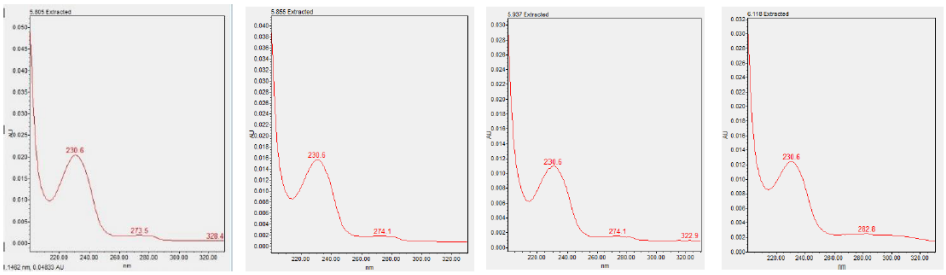
Peak 26 = RT 4.32 to 4.74 (13 plants)



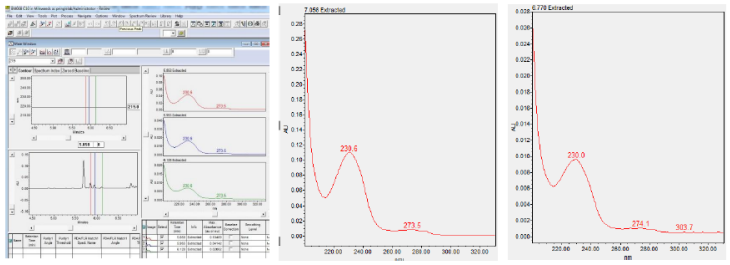
Peak 27 = RT 4.54 to RT 4.57 (3 plants) Peak 28 = RT 4.7 (3 plants) Peak 29 = RT 5.0 LW082 D6 only Peak 30 = Digitoxin RT 5.6 (143 plants)



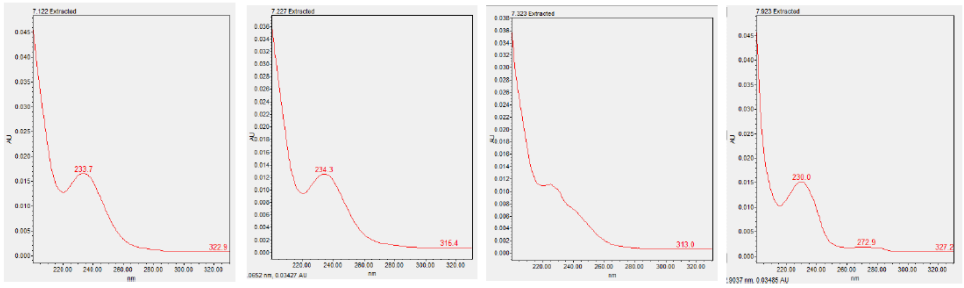
Peak 31 = RT 5.8 to 5.851 (45 plants)** Peak 32 = RT 5.852 to 5.93 (31 plants)** Peak 33 = RT 5.94 to 6.102 (21 plants)** Peak 34 = RT 6.11 to 6.4 (11 plants)**



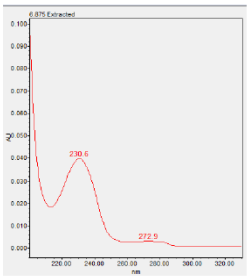
Similar shape and RT time but diff. molec Peak 35 = RT 6.5 to RT 6.7 (8 plants) * Peak 36 = RT 6.7 to RT 6.8 (125 plants) ***



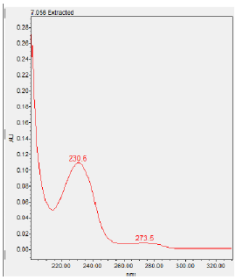
Peak 40 = RT 7.125 to 7.18 (33 plants) Peak 42 = RT 7.225 to 7.32 (10 plants) Peak 43 = RT 7.322 to 7.5 (14 plants) Peak 44 = RT 7.9 to 8.006**** (101 plants)



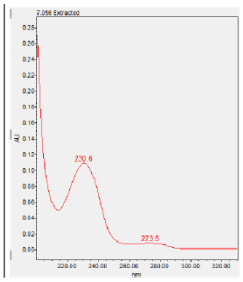
Peak 37 = RT 6.8 to 6.9 (109 plants)***



Peak 38 = RT 7.0 to 7.1 (101 plants)***



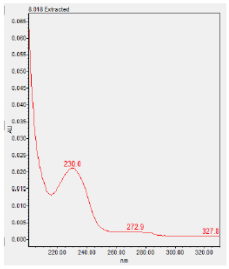
Peak 39 = RT 7.0 to RT 7.15 (27 plants)***

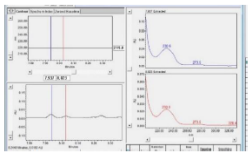


***similar shape and RT but diff. molec

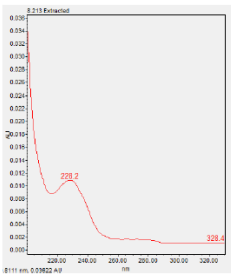
Peak 45 = RT 8.022 To 8.18**** (31 plants)

****similar shape and RT but diff. molec

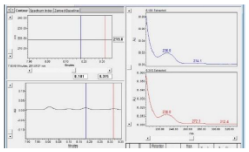




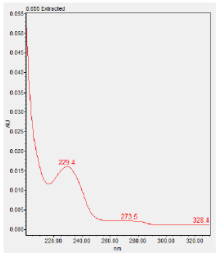
Peak 46 = RT 8.21 to RT 8.32 (21 plants)



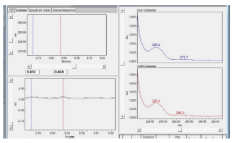
Division between 8.18 and 8.21 based on below:



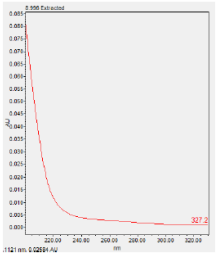
Peak 47 = RT 8.65 to 8.8 (29 plants)



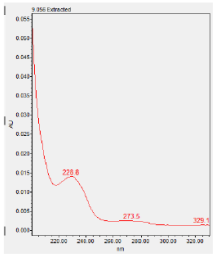
Peak 48 = RT 8.996 (1 plant)



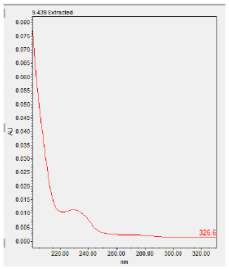
Peak 49 = RT 8.996 to 9.045 (93 plants)



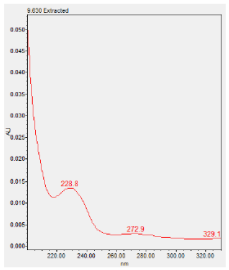
Peak 50 = RT 9.054 to 9.40 (8 plants)



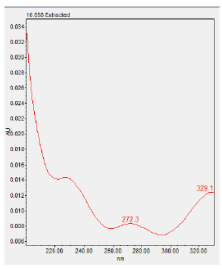
Peak 51 =RT 9.43 to 9.624 (4 plants)



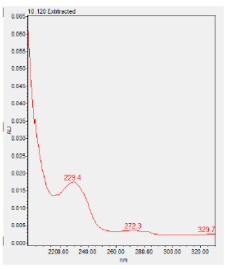
P52 RT 9.628 to 9.74 (18 plants)



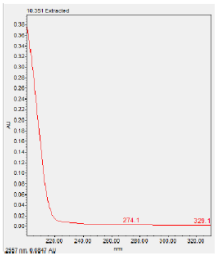
Peak 53 = RT 9.9 to 10.05 (44 plants)



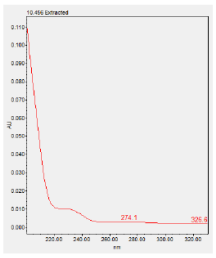
Peak 54 = RT 10.08 to 10.2 (18 plants)



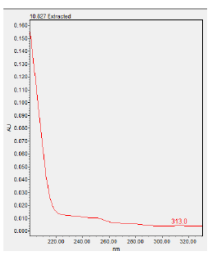
Peak 55 =RT 10.4 to 10.45 (96 plants)



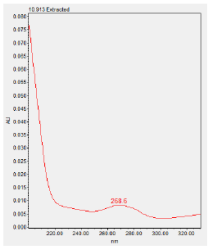
Peak 56 = RT 10.46 to 10.78 (9 plants)



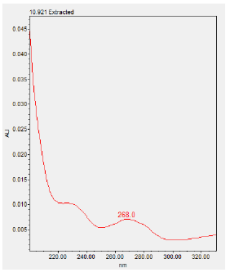
Peak 57= RT 10.83 to 10.91 (8 plants)



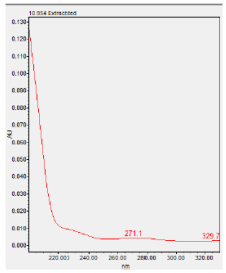
Peak 58 = RT 10.914 (2 plants)



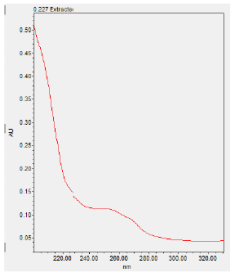
Peak 59 RT 10.92 to 10.93 (3 plants)



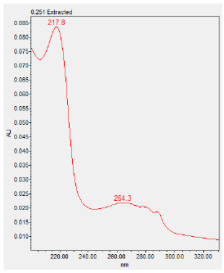
Peak 60 = RT 10.938 to 10.984 (21 plants)



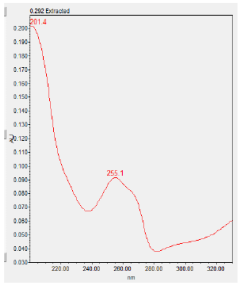
Peak 61 RT 0.218 to 0.224 (143 plants)



Peak 62 RT 0.248 to 0.254 (143 plants)

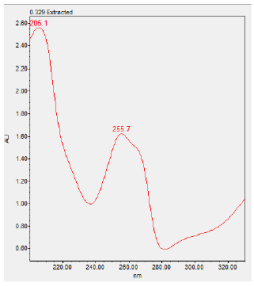


Peak 63 RT 0.287 To RT 0.296 (143 plants)



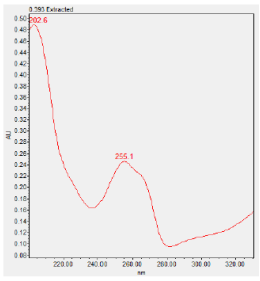
Peak 64 RT 0.33 to RT 0.35 (143 plants)

Putative ID = quercetin-glucoside-rhamnoside



Peak 65 RT 0.37 to RT 0.4 (131 plants)

Putative ID = quercetin-glucoside



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

Species	Tissue	Retention time (min)-LCUV Reno	RT-QToF Jena	m/z neg*	m/z pos*	MW	sum formula	UV spectra	**Tentative** ID	AD_PeakNo.[+]	Reference
Asclepias fascicularis	leaf	0.33	0.4	609.1463[a]	611.161[c]	610	C27H30O16		Quercetin-glucoside-rhamnoside	Peak 64	Haribal, M. & J. A. A. Renwick. 1996. Oviposition stimulants for the monarch butterfly: flavonol glycosides from *Asclepias curassavica* . Phytochemistry 41: 139-144. compound #3
Asclepias fascicularis	leaf	0.37	0.5	463.0876[a]	465.1039[c]	464	C21H20O12		Quercetin-glucoside	Peak 65	
Asclepias fascicularis	leaf	0.44	0.5	593.1514[a]	595.166[c]	594	C27H30O15		Kaempferol-glucoside-rhamnoside	Peak 1	El-Askary, H. 2003. Pregnene glycoside and monoterpene derivative from *Solenostemma argel* Hayne. Bull Fac Pharm Cairo University. 41: 131-137. compound #3 **Could not find journal-formatted reference; not sure if peer-reviewed.
Asclepias fascicularis	leaf	0.5	0.6	623.1616[a]	625.1768[c]	624	C28H32O16		Isorhamnetin-glucoside-rhamnoside	Peak 2	Bouratoua, A., Khalfallah, A., Kabouche, A., Semza, Z., & Kabouche, Z. (2013). Total phenolic quantification, antioxidant, antibacterial activities and flavonoids of Algerian Calotropis procera (Asclepiadaceae). *Der Pharmacia Lettre* , *5* (4), 204-207. compound #1
Asclepias fascicularis	leaf	0.55	0.7	477.1035[a]	479.1189[c]	478	C22H22O12		Isorhamnetin-glucoside	Peak 4	
Asclepias fascicularis	leaf	0.76	0.8	237.0404[a]		238	C11H10O6		Benzoylated small phenolic	Peak 9	
Asclepias fascicularis	leaf	6.7	6.7	931.4673[a] / 977.4735[b]	950.5103[d]	932	C49H72O17		Benzoylated pregnane glycoside	Peak 36	Oberai, K., M. P. Khare, A. Khare. 1985. A pregnane ester triglycoside from *Sarcostemma brevistigma* . Phytochemistry. 24: 3011-3013. compound #1
Asclepias fascicularis	leaf	7	7	915.4756[a] / 961.4779[b]	934.5155 [d]	916	C49H72O16		Benzoylated pregnane glycoside	Peak 38	Vleggar, R., F. R. Van Heerden, L. A. P. Anderson, G. L. Erasmus. 1993. Toxic constituents of the Asclepiadaceae. Structure elucidation of sarcovimiside A-C, pregnane glycosides of *Sarcostemma viminale* . Journal of the Chemical Society, Perkin Transactions 1: Organic and Bio-Organic Chemistry (1972-1999). 483-487. compound #A6
Asclepias fascicularis	leaf	6.8	7.1	933.4818[a] / 979.4881[b]	952.5242[d]	934	C49H74O17		Benzoylated pregnane glycoside	Peak 37	El Sayed, K. A., A. F. Halim, A. M. Zaghloul, J. D. McChesney, M. P. Stone, M. Voehler, K. Hayashi. 1995. Pregnane glycosides from *Stapelia variegata* . Phytochemistry 39: 395-403. compound #5
Asclepias fascicularis	leaf	7.9	7.9		1094.5890[d]	1076	C63H80O15		Benzoylated pregnane glycoside	Peak 44	
Asclepias fascicularis	leaf	8	8.1	915.4753[a] / 961.4782[b]	934.5153[d]	916	C49H72O16		Benzoylated pregnane glycoside	Peak 45	Vleggar, R., F. R. Van Heerden, L. A. P. Anderson, G. L. Erasmus. 1993. Toxic constituents of the Asclepiadaceae. Structure elucidation of sarcovimiside A-C, pregnane glycosides of *Sarcostemma viminale* . Journal of the Chemical Society, Perkin Transactions 1: Organic and Bio-Organic Chemistry (1972-1999). 483-487. compound #A6
Asclepias fascicularis	leaf	8.7	8.7	1059.5517[a] / 1105.5558[b]	1078.5917[d]	1060	C63H80O14		Saponin	Peak 47	Sylla, B., S. Levoie, J. Legault, C. Gauthier, A. Pichette. 2019. Synthesis, cytotoxicity and anti-inflammatory activity of rhamnose-containing ursolic and betulinic acid saponins. RSC Advances 9: 39743-39757.compound #18
Asclepias fascicularis	leaf	9.4	9.4	1203.6272[a] / 1249.6286[b]	1222.6695[d]	1204	C63H96O22		Benzoylated pregnane glycoside	Peak 50	Kitagawa, I., R. Zhang, J. D. Park, N. I. Baek, Y. Takeda, M. Yoshikawa, H. Shibuya. 1992. Indonesian medicinal plants. I. Chemical structures of calotroposides A and B, two new oxypregnane-oligoglycosides from the root of Calotropis gigantea (Asclepiadaceae). Chemical & Pharmaceutical Bulletin. 40: 2007-2013. compound #2
Asclepias fascicularis	leaf	9.628	9.7	1203.6197[a] / 1249.6330[b]	1222.6742[d], 1227.6240[e]	1204	C70H92O17		Benzoylated pregnane glycoside	Peak 52	
Asclepias fascicularis	leaf	10.1	10.1	1187.6295[a] / 1233.6374[b]	1206.6765[d]	1188	C70H92O16		Benzoylated pregnane glycoside	Peak 54	

**Values for m/z of molecular ion and fragment ions from high-resolution mass spectrometry (LC-ESI-Q-ToF-MS)*

[a] $[M-H]^-$

[b] $[M+HCOOH-H]^-$

[c] $[M+H]^+$

[d] $[M+NH4]^+$

[e] $[M+Na]^+$

[+]see 2017_Af_outdoorCG_chemistry_UVspectra_Diethelm.pdf at associated Dryad URL

Chapter 3

Feeding amidst fear: Food plant species, predators, and larval performance of the monarch butterfly (*Danaus plexippus*)

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Abstract

Predation risk influences behavior in organisms, shaping their growth and physiology, and ultimately impacts their survival and reproductive success. Although predation is known to cause significant mortality for eggs and neonate larvae of the monarch butterfly (*Danaus plexippus*), non-consumptive impacts of predators, such as behavioral changes, may also influence larval growth and adult fitness, perhaps contributing to the population decline of this flagship species. In this study, we investigated how predation risk affects monarch larval performance, as well as whether these effects are modulated by food plant identity. In particular, we compared larval responses to predation risk when feeding on two common species of western milkweed, *Asclepias speciosa* and *Asclepias fascicularis*, which differ in various traits, including architectural complexity. We found that predator impacts differed between the two years of our study. In the first year of our study, monarchs developed from larvae to adults more slowly when exposed to predators, but only when eating one of the two larval food plants (*A. speciosa*). In both years, larvae eating *A. speciosa* had slower rates of weight gain compared to larvae eating *A. fascicularis,* but only when exposed to predators. Our results suggest that larvae feeding on more architecturally simple *A. speciosa* plants spend more time avoiding predators and less time eating than larvae feeding on co-occurring *A. fascicularis*. Our findings highlight the role of predation risk in monarch larval performance and the role that larval food plant species plays in the magnitude of non-consumptive predator effects .

Keywords caterpillar, landscape of fear, milkweed, plant architecture, predation risk

1 | Introduction

Recent declines in North American monarch butterfly (*Danaus plexippus*) populations have sparked investigations into the role of predators and parasites in monarch survival (Prysby 2004, Pelton et al. 2019, Hermann et al. 2019), yet the impact of non-consumptive effects arising from exposure to natural enemies has received less attention (Sheriff et al. 2020, but see Lee et al. 2021). As larvae, monarchs are specialist herbivores that feed on milkweed plants in the genus *Asclepias*, sequestering toxic cardiac glycosides (cardenolides) from these plants for their own protection (Agrawal 2017). Despite their sequestration of these compounds, monarch larvae survival rates in the wild average ~10 percent, with strong top-down effects of predators and parasitoids on larvae (Prysby and Oberhauser 2004, De Anda and Oberhauser 2015, Nail et al. 2015).

At least ten orders of generalist predatory arthropods consume monarchs (Hermann et al. 2019) by either tolerating or behaviorally avoiding monarch toxicity (Rayor 2004, De Anda and Oberhauser 2015). In particular, predators in the families Coccinellidae, Formicidae, Mantidae, Pentatomidae, Reduviidae, Salticidae, Thomisidae, and Vespidae exert strong top-down pressure by consuming monarch larvae (Oberhauser et al. 2015). Two particularly important natural enemies are the tachinid fly *Lespesia archippivora* and the protozoan parasite *Ophryocystis elektroscirrha* (OE). Parasitism by tachinid flies varies among years and locations but has been estimated to affect ~20% of late instar monarchs in the Eastern United States (Oberhauser et al. 2017). Infection rates of OE also vary both seasonally and annually, but typically 8-30% of the population is

heavily infected (Altizer et al. 2000, Majewska et al. 2022). Infection by OE reduces monarch survival but can also have sub-lethal impacts, including smaller adult size, decreased male reproductive success, reduced flight capabilities, and diminished likelihood of successful migration (Altizer and Oberhauser 1999, Bradley and Altizer 2005).

Monarch larvae respond to predation risk by increasing defensive behaviors, which potentially increase their chance of escaping predation but negatively impact their development. Exposure to parasites and predators may divert larvae from eating, potentially reducing or prolonging their growth (Rypstra et al. 2007, Sheriff et al. 2020). Monarch larvae use sensilla hairs to listen to their surroundings and will respond to the sounds of predators by increasing defensive behavioral responses (Taylor and Yack 2019). Sound frequencies in the range of those produced by flying predators and parasitoids can elicit larval behaviors such as freezing, contracting from the anterior and posterior ends, and aggressively headbanging by vertical flicking of the thorax (Taylor and Yack 2019). In a laboratory setting, the buzzing of wasps, but not mosquitoes, reduced monarch survival and pupal sizes even in the absence of a physical predator (Lee et al. 2021). Monarchs also respond physiologically to intermittent stressors: for example, acute periods of increased ambient noises caused larvae to increase their heart rates in the lab (Davis et al. 2018), although it is not clear whether this physiological response results in slower development. It is also uncertain how monarch performance is affected in an outdoor setting where there are inevitably multiple sources of cues and repeated physical exposure to predators.

The identity of the larval food plant could also potentially interact with predation risk to influence monarch larval performance. Milkweeds contain a suite of defensive traits, including cardenolides, latex, and trichomes, which vary in expression among species (Malcom 1991). Despite being milkweed specialists, monarch larvae are affected by these defenses, and larvae feeding on highly defended milkweeds experience longer development times (Ladner and Altizer 2005, Yang et al. 2020). Time that larvae spend cleaning latex from their mouthparts and head, grazing down trichomes, and processing high levels of cardenolides slows down their consumption of plant tissue, which increases both development time and exposure to predators (Yang et al. 2020). In addition, the concentration of food plant cardenolides can influence the morphology of adult wings, which in turn determines the success of migration to annual overwintering locations (Decker et al. 2019, Soule et al. 2020). Changes in larval food intake and resulting allocation to adult tissue caused by predation risk may similarly influence wing morphometrics, although to our knowledge this has not been studied.

Differences in plant architecture between different milkweed food plant species may be another important but overlooked factor that contributes to monarch larval performance. Plant structure and leaf morphology can affect predation risk by altering the search efficiency of predators, either by limiting the mobility of the predator or by increasing the number of refuges that conceal herbivores (Clark and Messina 1998, Feng et al. 2015). For example, several studies have shown that parasitoids find hosts more successfully on architecturally simple plants (e.g., little to no branching) compared to more complex plants (Andow and Prokrym 1990, Geitzenauer and Bernays 1996, Lukianchuk and Smith 1997). Furthermore, larvae reared on simple, non-branching

plants may have to spend more time hiding from predators instead of consuming plant material, potentially slowing their development. Conversely, increased plant branching may enhance predator foraging success, particularly if the predator is cursorial or relies on web-building to trap prey (Reynolds and Cuddington 2012, Nell and Mooney 2019). Leaf size and the degree of overlap among leaves can also affect predator search efficiency (Kareiva and Perry 1989).

The two most widespread western *Asclepias* species, *A. fascicularis* and *A. speciosa,* vary considerably in their morphology and other traits (Woodson 1954, Dilts et al. 2019). *Asclepias speciosa* generally produces a large, single stem with large, non-overlapping leaves compared to the multiple-stemmed, bushy *A. fascicularis,* which has many small but often overlapping leaves. The impact of these structural differences on predator exposure remains unexplored for western monarchs. Previous studies have focused on the impact of varying plant defense expression on monarch larvae. *Asclepias speciosa* produces more latex, trichomes and cardenolides than *A. fascicularis* (Yang et al. 2020). Larvae reared on the better defended *A. speciosa* have slower growth rates and reach smaller maximum sizes than larvae on *A. fascicularis* (Ladner and Altizer 2005, Yang et al. 2020). No studies to our knowledge have compared adult wing morphology for larvae reared on these two milkweed species.

To investigate how exposure to predators impacts larval development and survival for a specialist herbivore, as well as whether these impacts are modulated by larval food plant species, we conducted a two-year experiment using common gardens. Using *A. speciosa* and *A. fascicularis* and predator-exclusion cages, we conducted a 2x2 factorial experiment, crossing predator exclusion with larval food plant. We then posed the

following questions: 1) Do predators pose a serious threat to the survival of monarch larvae? 2) Are there also important non-consumptive effects of predators on monarch development? 3) Do consumptive and/or non-consumptive effects of predators depend on larval food plant species? 4) Do predation risk and larval food identity plant affect adult morphometrics? We predicted that predators would reduce survival and that monarchs exposed to predators would take longer to develop and emerge as smaller adults because they spend more time hiding and less time eating compared to monarchs protected from predators. We expected this effect to be more pronounced on larvae consuming *A. speciosa*, either because larvae on this food plant have fewer and less effective places to hide and/or because larvae have to spend more time exposed to predators to effectively consume this more defended species. Finally, we predicted that these effects on larval development would extend to adult wing morphometrics, allowing butterflies that developed on *A. fascicularis* to develop larger wing area.

2 | Methods

2.1 | Study system

The western monarch butterfly breeds primarily west of the Rocky Mountains in North America and overwinters along the California coast (Jepsen and Black 2015). This western population of monarchs has significantly declined in the last 40 years, with pressures from habitat loss, pesticides, and climate change (Espeset et al. 2016, Pelton et al. 2019). *Asclepias fascicularis* and *A. speciosa* are the two most widespread western milkweed species and the most used in habitat restoration in the West (Woodson 1954,

Landis and Savoie 2018, Dilts et al. 2019). These species exhibit distinct morphologies and differ in their expression of plant defensive traits (Zalucki et al. 2012). *Asclepias fascicularis* has narrow, glabrous leaves and extensive branching, with less latex, trichomes, or cardiac glycosides than *A. speciosa*, which also has more wide, pubescent leaves (Woodson 1954, Agrawal et al. 2009a).

2.2 | Experimental design

To investigate the effects of larval food plant species and exposure to predators on monarch development and survival, we conducted factorial common-garden experiments during two breeding seasons (2018: n = 318; 2019: n = 174) at the University of Nevada, Reno, Agricultural Research Station in Reno, NV. We used seeds collected from across a 370-km environmental gradient in northern Nevada and California, with 4-5 natal regions per species per garden, to capture some of the breadth of intraspecific variation in phenotype (Diethelm et al. 2022). In 2019, we also included seeds for both milkweed species grown commercially in California's Central Valley (Hedgerow Farms in Winters, CA), which is considered high-quality breeding habitat for monarchs (Dilts et al. 2019). The milkweed plants used in each year were germinated ~4-6 months prior to the start of that year's experiment. The experiments began in September and ended in October.

All seeds were first rinsed in a 5% bleach solution and then scarified with a small nick in the seed coat. The seeds were germinated on moist tissues in Petri dishes that were positioned beneath a 20-h light source from April – May in 2018 and 2019. When germinating seeds had ≥ 2-mm root growth, they were transplanted into a 1:4 ratio of Full Circle (Full Circle Soils & Compost, Gardenerville, NV) SOAR potting soil to sand in

475 cm^3 pots. Plants were maintained a 25 ± 2 °C, 16L:8D light cycle and watered daily. Once seedlings developed two sets of adult leaves, they were fertilized weekly with 3 mL of 24-8-16 NPK (MiracleGro®). Plants were transplanted to the outdoor garden in mid-June in 2018 and early-July in 2019. In both years, plants were watered using drip irrigation three times per wk at 63 mL/min for 16 min from June-August and for 10 min from September-October.

We established a colony of monarchs from gravid females caught near Reno, NV. Females were inspected for infection by the parasite *Ophryocystis elektroscirrha* (Phylum Apicomplexa) by applying clear tape to each side of the abdomen and checking for spores following Altizer et al. (2000). Only uninfected females were used. Mated F2 females oviposited onto milkweed plants in the greenhouse, and larvae remained on natal stalks until their second instar. To account for differences in starting weights among larvae, all second-instar larvae were weighed prior to being randomly assigned, where possible, to a plant of the same species as their natal stalk in the common garden.

To account for expected uneven survival between control plants and predator-exclusion plants, ~25% of the caterpillars were assigned to predator-exclusion caged plants and ~75% to control plants (yr1: $N_{A.\ fascicularis}$: cage = 38, mock-cage = 123; $N_{A.\ speciosa}$: cage = 37, mock-cage = 119; yr2: : $N_{A.\ fascicularis}$: cage = 16, mock-cage = 76; $N_{A.\ speciosa}$: cage = 17, mock-cage = 60). Predator-exclusion cages consisted of a wire frame with a fitted fine-weave, sheer-voile fabric cover, which was buried into the soil to enclose the plant. The control treatment consisted of an open-sided cage, or mock-cage, placed over the plant to mimic the shading that occurred in the full predator-exclusion treatment. To track differences in larval developmental timing and survival between

plants, plants in the control treatment were checked daily for larvae, whereas individuals in the predator-exclusion treatment were checked weekly.

To standardize search time, each plant was checked for 2 min and any known predators on the plant were noted. Any larvae missing for 4 days in a row were considered predated following De Anda and Oberhauser (2015), except for plants with over 20 stems, which were checked for 5 days in a row to account for the possibility that larvae had been obscured within the plant's dense architecture. To prevent the movement of 5th-instar caterpillars, which are known to disperse from natal plant, all individuals were caged at this stage of development. Surviving larvae pupated in the field and were given one day to fully harden before being brought into the greenhouse to finish development. Time to emergence was calculated as the number of days from placement into the common garden to eclosure.

We accounted for larval movement prior to the 5^{th} instar using two approaches. First, six individuals from the control treatment were randomly selected to have a circular barrier around their plant, coated with an insect barrier adhesive ("Tree Tanglefoot"; the Scotts Company®), which allowed us to catch early instar larvae moving between plants and evaluate the frequency of such movements. Second, we briefly scanned neighboring plants for larvae as we moved through the garden. No individuals were caught in the tanglefoot barrier between plants, suggesting that movement between plants was limited and disappearances were indicative of predation events. We occasionally found more than one larva on a plant. In these cases, we were usually able to identify which plant they belonged to by their developmental stage, otherwise they were excluded from the survival analyses.

2.3 | Monarch performance

Monarch performance was assessed using metrics of developmental timing and weight. To determine how larval growth differed between milkweed species and predation treatments, we measured total larval weight gain (g/d) as the late-fifth-instar (pre-pupal) weight minus the starting weight and then standardized by number of days in the common-garden environment. We used pre-pupal caterpillars for these measurements to minimize concerns about differences in gut contents, as the gut is fully emptied before pupation. Adults that emerged successfully were checked for OE infection using an infection scale from 0-5, where 0 represents no infection and 5 is heavily infected (> 1000 spore per cm^2 of abdomen; Altizer et al. 2000). To measure the dry weight of adults, individuals were euthanized within 24 hours of eclosing and oven-dried at 55 °C for 72 hrs. Adult weights included the wings.

2.4 | Morphometrics

To investigate the impact of larval food plant species and predation risk on wing morphometrics, we measured morphological variables that are suggested to influence the flight performance of butterflies and other insects (Altizer and Davis 2010, Soule et al. 2020). Specifically, we measured wing area (mm^2), wing loading (body mass in g/wing area in mm^2), and wing roundness ($4 \times [\text{area}/(\pi \times \text{length})^2$). The right forewing was utilized for morphometric measurements, unless extensively damaged, in which case we used the left forewing following Altizer and Davis (2010). Wings were first scanned at

300 DPI with a clear ruler and then measured for wing area and length using ImageJ software (U.S. National Institutes of Health, Bethesda, MD).

2.5 | Statistical analysis

All analyses were conducted in R version 4.2.3 (R Core Team 2022). Individuals that suffered mortality from accidental deaths and any individuals in the predator-exclusion treatment where predators had been observed were omitted from our analyses.

To determine if the impact of predator exposure on larval performance varied between milkweed species, we compared the fit of models with and without a predation x food plant interaction for the following performance variables (Table 1): survival, time to reach last instar (d), time to emerge as adult, rate of larval weight gain (g/d), and adult dry wet (g). We used log-likelihood ratio tests (LRT) from the 'lmtest' R package to evaluate differences in model fit (Hothorn et al. 2022). All models were run as linear regression models (Gaussian distribution), except for survival which was run as generalized linear models with binomial distributions. To account for potential differences in abiotic conditions and measured differences in biotic pressures between years, the experimental results for 2018 and 2019 were modeled separately. Preliminary analyses also showed that the inclusion of the degree of OE infection did not improve model fit, so this variable was discarded. We report effect sizes as beta coefficients (β) and standard errors.

To investigate potential causal relationships between exposure to predators, larval food plant species, and adult wing morphometrics, we used a Structural Equation Model implemented with the 'Lavaan' R package (Rosseel 2012). All measured variables were

first standardized, and no latent variables were utilized in any models. We again conducted separate analyses for each year of the experiment. We excluded wing loading from these models because of its correlation to both larval weight gain and wing area.

3 | Results

There were two key differences in biotic pressures between the first and second year of the experiment. First, in 2018, the presence of OE was widespread, with an average parasite load of >900 spores/cm^2 on the abdomen of surviving adults (n = 45), suggesting that most adults were heavily infested (Altizer et al. 2000). In 2019, by contrast, we identified no infections in eclosing adults (n = 31). Second, although we found no tachinid parasitism in 2018, 32% of all pupating individuals were parasitized by the tachinid fly *Lespesia archippivora* in 2019. None of the parasitized monarch pupae survived.

Exposure to predators generally reduced monarch survival, but the effects of predation pressure varied between years. In 2018, exposure to predators reduced survival by ~50% on *A. speciosa* compared to individuals protected from predation, whereas exposure to predators had minimal effects on the survival of larvae reared on *A. fascicularis* ($\beta_{\text{caging:milkweed}}$ = -1.23 ± 0.82, z = -1.50, P = 0.13; Fig. 1a,b). In 2019, exposure to predators greatly reduced survival for monarchs on both larval food plant species, with only 10% of exposed individuals surviving compared to 67% when protected (β_{caging} = -2.91 ± 0.47, z = -6.19, P < 0.001; Fig. 1c,d). For individuals protected from predators, 18% of larvae developing on *A. fascicularis* and 12% of larvae on *A.*

speciosa survived to eclose in 2018. In 2019, the survival rates among larvae protected from predators improved to 69% on *A. fascicularis* and 65% on *A. speciosa*.

In addition to affecting survival, larval food plant identity influenced the effect of predator exposure on larval developmental timing, albeit only in one year of the experiment. In 2018, larvae reared on *A. speciosa* reached the fifth instar ~4 days more slowly when exposed to predators, but exposure to predators did not affect development time on *A. fascicularis* ($\beta_{caging:milkweed} = 4.39 \pm 2.33$, $z = 1.89$, $P = 0.06$; Fig. 2a). This effect was even more pronounced in time to adult emergence, with those individuals feeding on *A. speciosa* reaching maturity ~7 days more slowly when exposed to predators ($\beta_{caging:milkweed} = 9.06 \pm 3.46$, $z = 2.61$, $P < 0.02$; Fig. 3a). We did not observe this effect in 2019, when neither exposure to predators ($\beta_{caging} = 1.35 \pm 1.12$, $z = 1.22$, $P = 0.2$), nor milkweed identity significantly impacted the time needed to reach the last instar ($\beta_{milkweed} = -1.32 \pm 1.12$, $z = -1.18$, $P = 0.25$; Fig. 2b). Likewise, we found no difference in time to adult emergence based on predator treatment or food plant species in 2019 (Fig. 3b).

Despite the inconsistency between years in developmental timing, larvae feeding on *A. speciosa* exhibited slower weight gain compared to monarchs on *A. fascicularis* in both 2018 and 2019 (2018: $\beta_{milkweed} = -0.0085 \pm 0.0048$, $z = -1.77$, $P = 0.08$; 2019: $\beta_{milkweed} = -0.01 \pm 0.005$, $z = -1.94$, $P = 0.06$). In both years, the effect was driven by predator-exposed monarchs on *A. speciosa* (Fig. 2c,d). In 2018, larvae on *A. speciosa* gained an average of 0.051 g per day (n = 5, SD = 0.016) when protected from predators but only an average of 0.034 g per day (n = 7, SD = 0.016) when exposed to predators. Similarly, in 2019, larvae on *A. speciosa* gained an average of 0.056 g per day (n = 13, SD = 0.015) when protected from predators but only 0.039 g per day (n = 7, SD = 0.023)

when exposed. In 2018, slower weight gain produced marginally smaller adults on *A. speciosa* ($\beta_{milkweed}$ = -0.021 ± 0.012, z = -1.76, P = 0.09; Fig. 3c). In 2019, despite a slower rate of larval weight gain on *A. speciosa*, adult weight was not different between milkweed species ($\beta_{milkweed}$ = 1.01 ± 0.84, z = 1.91, P = 0.2; Fig. 3d).

Predator exposure had a negative indirect effect on wing area in 2019, whereas OE infection had a negative indirect effect on wing roundness in 2018 (Fig. 4). Food plant identity also had a direct effect on wing morphometrics. Larvae eating *A. speciosa* emerged with smaller, rounder wings, although these effects were less pronounced in 2019 (Fig. 4).

4 | Discussion

In this study, exposing monarch larvae to their predators had a dual impact: not only did predators reduce survival, but they also triggered non-consumptive effects that negatively impacted monarch development. In 2018, exposure to predators reduced survival and prolonged larval development only for larvae on *A. speciosa*, with minimal effects on larvae on *A. fascicularis*. In both experimental years, larvae eating *A. speciosa* had slower rates of weight gain than those eating *A. fascicularis* but only when larvae were exposed to predators. Interestingly, larvae protected from predators performed similarly on both milkweed species, despite the presumed stronger chemical and physical defenses produced by *A. speciosa* (Agrawal et al. 2009b). This suggests that monarch larvae developing on *A. speciosa* allocated relatively more time to predator avoidance and less time to feeding than those developing on *A. fascicularis*.

Taken together, our results indicate that monarchs developing on *A. speciosa* have reduced larval performance in the presence of predators. Yet we do not have evidence that this reduction was influenced by the higher levels of plant defense found in *A. speciosa* because larvae performed similarly on the two plants in the absence of predators. One possible explanation for these observed differences in larval performance is instead the differences in morphology between the two plant species. Larvae may be able to hide and continue eating more easily on *A. fascicularis* plants with bushy architecture, or predator movement could be restricted by the overlap in branches on *A. fascicularis*, compared to *A. speciosa*, which is sparsely branched with leaves that rarely overlap. Despite the larger leaves of *A. speciosa*, which potentially offer bigger hiding places, the thin leaves of *A. fascicularis* could act as refuge due to the presence of numerous branching stems. In addition, when plants were flowering, the compact, clustered umbels of *A. fascicularis* could provide greater shelter for larval concealment than the looser and larger umbels of *A. speciosa*.

Wing shape was also directly impacted by larval food plant species and indirectly impacted by natural enemies in both years of the study, but the impact of predator exposure varied between experimental years. In 2018, larval food plant species had the greatest impact on wing characteristics, whereas in 2019, larval weight gain had a stronger direct effect. These findings align with prior research, demonstrating the influence of larval food plant identity on the shape of forewings in monarch butterflies (Freedman and Dingle 2018, Decker et al. 2019, Soule et al. 2020). Here, we demonstrate that there is also an indirect, non-consumptive effect of exposure to predators that influences wing shape, which can be affected by food plant identity. Resulting wing size

and shape can have implications for successful long-distance dispersal of the breeding generations of monarchs and for body condition upon returning to the overwintering grounds for the late-fall generation. A rounder wing shape is associated with shorter, less elongated wings, which are helpful for agile maneuvers like evading predators but less suitable for the long-distance gliding flight needed for migration or long-distance dispersal (Satterfield and Davis 2015, Freedman and Dingle 2018, but see Li et al. 2016).

Because natural enemies have previously been shown to exert strong effects on the survival of monarch larvae (Prysby 2004), we expected to see higher survival when larvae were protected from predators than when they were exposed. We did indeed observe this in 2019, but, in 2018, exposure to predators reduced survival only for larvae on *A*. *speciosa*. Over the two study years, we also observed a fourfold difference in survivorship for monarchs protected from predators, with the lower rates of survival in 2018 potentially associated with the higher levels of OE infection that year. Although we could not directly test larval infection rates, the high prevalence of OE among adults suggests widespread larval infection. OE generally weakens the immune system and reduces survival (de Roode et al. 2009). As for the substantial increase in tachinid parasitism in year 2019, this aligns with previous work, which has shown that parasitism is widely variable year-to-year, and even patch-to-patch (Oberhauser et al. 2007).

Our findings differ from those of Lee et al. (2021), who observed faster developmental times for monarchs exposed to predator auditory cues in a controlled laboratory environment. It is possible that larvae exhibit increased developmental speed in the presence of a single stressful cue but that multiple threats slow down development, particularly if these threats collectively indicate elevated levels of danger. This

discrepancy could be attributed to the fact that, in natural common gardens, monarchs are likely subjected to a multitude of auditory cues, encompassing various predator sounds as well as ambient noises such as road traffic. Furthermore, larvae in our study consumed intact, living plants, whereas Lee et al. (2021) used leaf clippings of *A. syriaca*. Species-specific variations, including plant defenses and the disarming of latex (Zalucki et al. 2001), could also contribute to the differences in our results.

Our study underscores the potential interaction between predation risk and larval food plant species, and the importance of considering larval food plant identity in understanding monarch larval performance. Importantly, our findings carry implications for monarch conservation efforts, which often focus on planting milkweeds for monarchs (Pleasants 2017, Thogmartin et al. 2017). This highlights the need for further investigation into how the consumptive and non-consumptive impacts of predators may depend on which milkweed species are available.

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Tables

Table 1: Model selection for interaction or additive effects between the larval host-plant species (milkweed) and exposure to natural enemies (caging) treatments using log-likelihood ratio tests (LRT). Second-order Akaike Information Criterion values (AICc) are bolded for each best fit model.

Year	Response Variable	Relationship between milkweed and caging treatments	LRT p-value	AICc
2018	Survival	*	P = 0.14	**250.3**
		+		250.4
	Larval developmental	*	P = 0.06	**382.5**
	timing to last instar (d)	+		383.8
	Total larval weight gain	*	P = 0.07	-291.3
	(g/d)	+		**-291.7**
	Adult developmental	*	P = 0.04	**328.8**
	timing (d)	+		330.9
	Adult dry weight (g)	*	P = 0.9	-161.6
		+		**-164.2**
2019	Survival	*	P = 0.5	135.0
		+		**133.3**
	Larval developmental	*	P = 0.4	209.1
	timing to last instar (d)	+		**207.2**
	Total larval weight gain	*	P = 0.1	-198.9
	(g/d)	+		**-199.0**
	Adult developmental	*	P = 0.5	168.1
	timing (d)	+		**165.7**
	Adult dry weight (g)	*	P = 0.5	**-93.2**
		+		-95.6

Figure Legends

Fig. 1 Impact of milkweed (*Asclepias)* larval-host plant species and exposure to natural enemies on monarch (*Danaus plexippus*) survival in 2018 (**a,b**) and 2019 (**c,d**) in common garden environments in Reno, NV. Larvae were either to exposed to natural enemies (mock-cage) or protected in full-cage enclosures (cage).

Fig. 2 Impact of milkweed (*Asclepias)* larval food plant species on monarch (*D. plexippus*) larval developmental timing to reach the last instar (**a,c**) and total larval weight gain as standardized by day (**b,d**) across years ($n_{2018} = 40$, $n_{2019} = 44$) when reared in a common garden in Reno, NV. Larvae were either protected from natural enemies by a full-cage enclosure or exposed in a mock-cage. Center points represent means and bars represent SE. Model p-values are represented by line length: a long line indicates the interaction effect between milkweed species and exposure to predators, while short lines represent the effect of milkweed species alone.

Fig. 3 Impact of milkweed (*Asclepias)* larval-host plant species on monarch (*Danaus plexippus*) adult developmental timing (**a,c**) and weight gain (**b,d**) across years ($n_{2018} = 38$, $n_{2019} = 35$) when reared in a common garden in Reno, NV. Larvae were either protected from natural enemies by a full-cage enclosure or exposed in a mock-cage. Center points represent means and bars represent SE. Model p-values are represented by line length: a long line indicates the interaction effect between milkweed species and exposure to predators, while short lines represent the effect of milkweed species alone.

Fig. 4 Path models testing the hypothesized causal relationships between milkweed (*Asclepias*) larval-host plant species, exposure to natural enemies, monarch larval weight gain (g/d), and adult monarch wing morphometrics for 2018 (a; $c2 = 1.58$, $df = 4$, $P = 0.81$) and 2019 (b; $c2 = 4.1$, $df = 2$, $P = 0.13$). The values on the arrows are path coefficients (β), representing magnitude of effects and the signs (+/-) indicate the

direction of the effect. Coefficients in bold are from paths where $P < 0.05$. Solid lines represent direct effects, while dashed lines are indirect effects.

Figures

Fig. 1

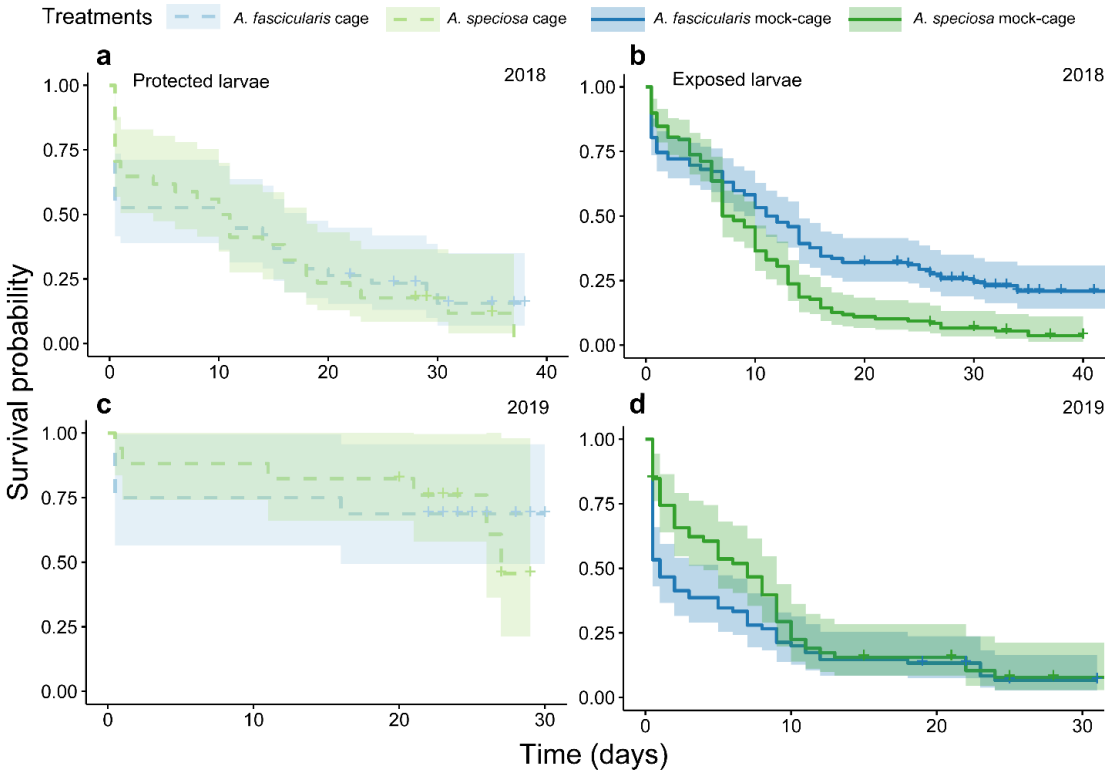


Fig. 2

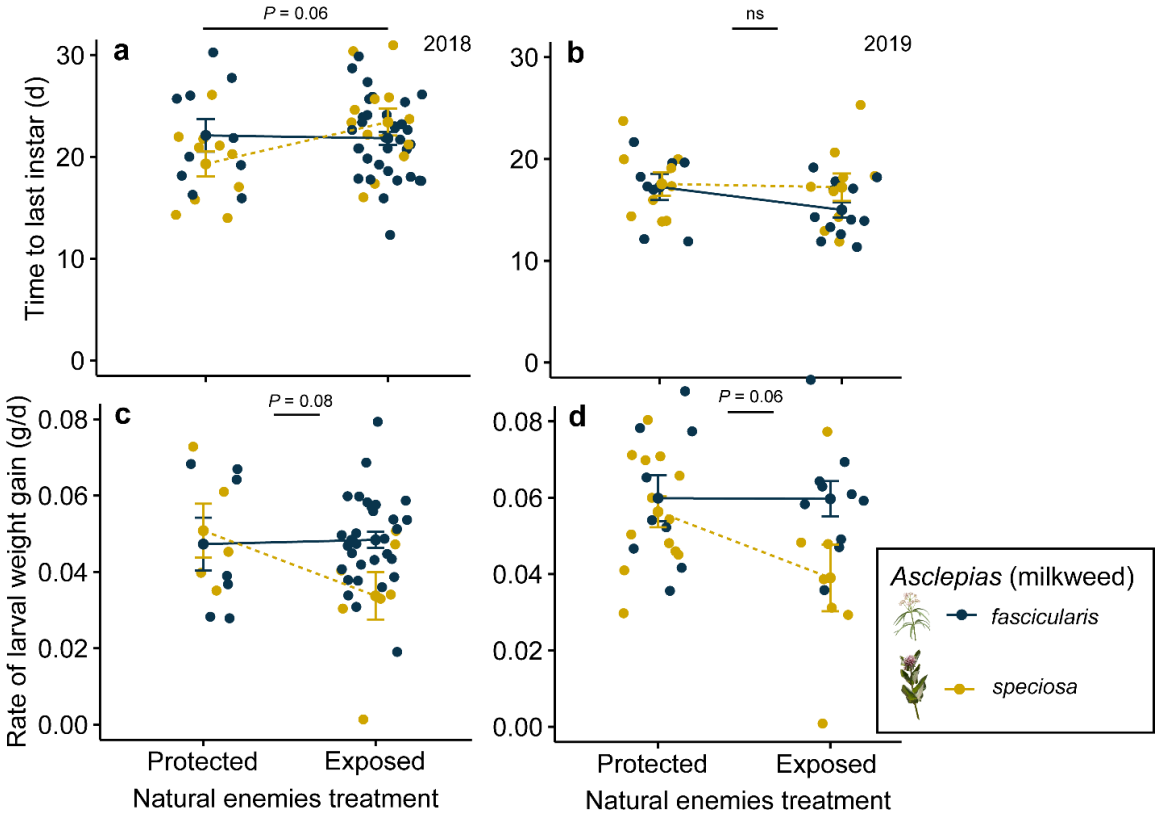

P = 0.06
ns
a
b
2018
2019
Time to last instar (d)
30
20
10
0
P = 0.08
P = 0.06
c
d
Rate of larval weight gain (g/d)
0.08
0.06
0.04
0.02
0.00
Protected
Exposed
Natural enemies treatment
Asclepias (milkweed)
fascicularis
speciosa

Fig. 3

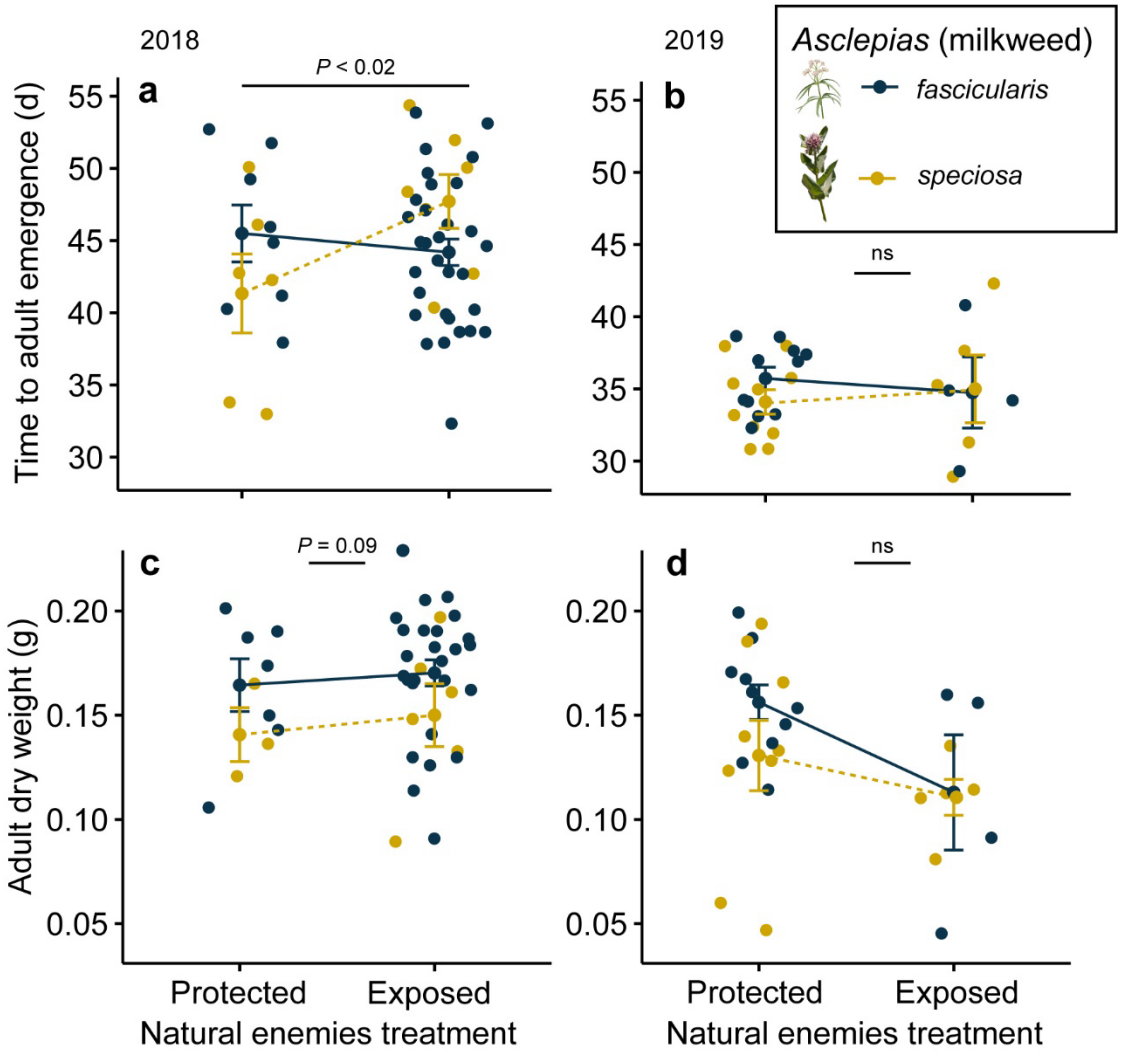
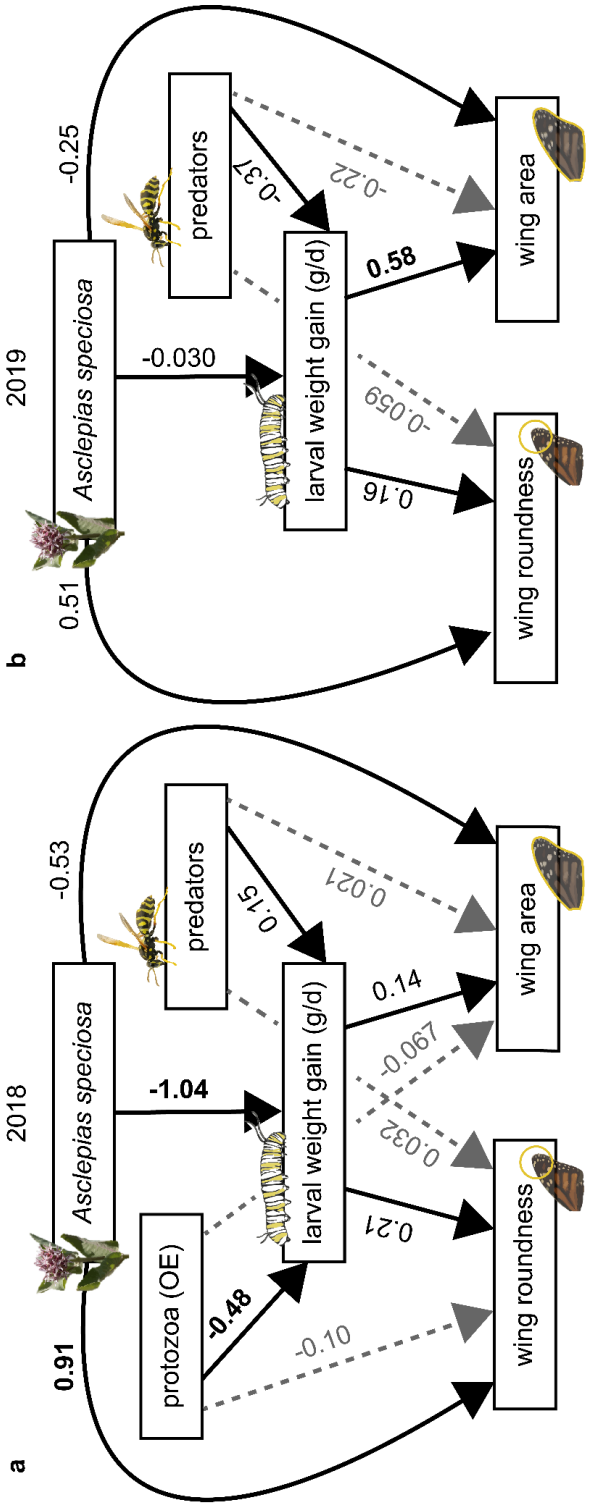

2018
2019
a
b
c
d
P < 0.02
P = 0.09
ns
ns
Asclepias (milkweed)
fascicularis
speciosa
Time to adult emergence (d)
Adult dry weight (g)
55
50
45
40
35
30
0.20
0.15
0.10
0.05
Protected
Exposed
Natural enemies treatment

Fig. 4


a
2018
Asclepias speciosa
0.91
-0.53
predators
protozoa (OE)
-0.48
-1.04
larval weight gain (g/d)
0.15
0.021
0.14
-0.067
0.032
0.21
-0.10
wing area
wing roundness
b
2019
Asclepias speciosa
0.51
-0.25
predators
-0.37
-0.22
-0.030
larval weight gain (g/d)
0.58
-0.059
0.16
wing area
wing roundness

Chapter 4

Larval performance of specialist butterfly from a tritrophic perspective under varying climatic conditions

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Abstract

Biotic and abiotic stressors significantly influence herbivore population dynamics, yet their relative contributions to larval performance have not been thoroughly examined from a tritrophic perspective. Abiotic conditions both directly impact herbivore development and indirectly affect larvae by altering larval food plant chemistry. Additionally, abiotic conditions shape predator community composition and behaviors, leading to changes in hunting strategies, activity patterns, and foraging efficiency. With this study, we investigated the interplay between predator exposure, abiotic conditions, and food plant identity in shaping the larval performance of the monarch butterfly (*Danaus plexippus* L.). Using four common gardens across a 513-km water-availability gradient in northern Nevada and California, we found that exposure to predators significantly reduced larval survival. Predator communities also had sub-lethal effects, as an increase in the diversity of predators delayed larvae from reaching adulthood. Extreme climatic conditions, including elevated temperatures and air pollution from smoke, influenced the developmental timing of monarch larvae, resulting in longer time to adult eclosion and, in certain cases, smaller adult sizes. These findings highlight the complexity of interactions between biotic and abiotic stressors impacting monarch larval success. This study reveals broader insights into how predator communities and abiotic conditions shape larval performance in herbivorous insects, emphasizing the importance of understanding these ecological dynamics in addressing environmental challenges.

Keywords multitrophic interactions, monarch butterfly, *Danaus plexippus*, climate change

1 | Introduction

Predation during the larval stage exerts a significant influence on arthropod populations (Vidal and Murphy 2018). However, the impact of predation is not consistent across environments and habitats (Hunter 2016, Kergunteuil et al. 2019). The abundance, distribution, and diversity of predators can vary along environmental gradients (Schmitz 2008, Smit et al. 2009), which suggests that predation risk can be modulated by abiotic conditions which influence predator behavior and availability (Menge and Sutherland 1987, Han et al. 2019). As a result, understanding the variation of predators across arthropod communities is essential for understanding the factors shaping population dynamics and predicting ecological community responses to changing environmental conditions.

Abiotic stressors can exert both direct and indirect effects on herbivore survival through altering larval developmental timing and survival (Zalucki 1982, Nail et al. 2015), or through impacts on larval food plant quality (Pellissier et al. 2012, Couture et al. 2015). Temperature plays a pivotal role in determining insect larval fate (Régnière et al. 2012), with lethal effects often contingent on the duration of exposure to temperatures over thermal maximums (Dixon et al. 2009). Elevated temperatures can also cause sub-lethal effects through changes to herbivore feeding behavior. For example, high temperatures can trigger compensatory feeding behavior for larvae, where larvae consume more low-quality host plant tissue to meet their nutritional needs, delaying development and reducing adult size (Hamann et al. 2021). Likewise, larvae may spend more time resting during periods of intense heat, resulting in reduced adult size (Hagstrum and Subramanyam 2010). Air quality is also of concern, particularly in

regions affected by pollution and wildfires, as it poses both lethal and sub-lethal risks for larvae (Tan et al. 2018). Prolonged exposure to abiotic stressors can impede larval development and overall survival, exacerbating the pressures faced by populations of herbivorous insects.

Here, we investigate the relative contributions of biotic and abiotic stressors on the larval performance of an herbivore species in decline: the monarch butterfly (*Danaus plexippus*). The western monarch butterfly, which breeds primarily west of the Rocky Mountains in North America and overwinters along the California coast (Urquhart and Urquhart 1977, Freedman et al. 2021), has severely declined in the last 40 years as a result of habitat loss, pesticides, and climate change (Schultz et al. 2017, Pelton et al. 2019). Yet, the combined effects of changing abiotic conditions, altered larval food plant quality and defensive traits (*Asclepias*; milkweeds), and shifts in predator-prey interactions have not been thoroughly investigated (Dyer and Forister 2016).

By conducting a series of common garden experiments, we sought to capture natural environmental variation within western breeding grounds for monarchs, accounting for a range of biotic and abiotic stressors. Specifically, we asked: How do biotic and abiotic stressors interact to influence monarch butterfly larval development and survival? We predicted the following: 1) Predator communities will vary with environmental conditions, with wetter areas harboring a higher diversity of predators, 2) Larval survival will vary among sites, with the lowest survival rates observed at sites with higher predator diversity and poorer air quality, 3) Larvae will develop more slowly at sites with higher predator diversity and lower air quality, potentially leading to smaller adult individuals, and 4) These effects will be mediated by larval food plant identity.

Understanding the dynamic interplay between biotic and abiotic stressors influencing larval survival in the face of climate change is essential for predicting and managing potential shifts in insect populations and for developing conservation strategies amidst a changing environment.

2 | Methods

2.1 | Study system

The monarch butterfly relies on milkweeds (*Asclepias* spp.) as the sole larval food plants, which enables monarchs to sequester toxic compounds produced by the plants (cardiac glycosides; cardenolides) that in turn affect their predation risk (Malcolm 1994, Agrawal et al. 2021). Monarch larval development and survival are affected by ambient temperatures, with a thermal tolerance range of 42 to -20 °C (Nail et al. 2015). Extended exposure to temperatures of ≥33 °C can cause significant larval mortality (Zalucki 1982). Monarch larvae dealing with thermal stress rely on crucial periods of relief, such as cooler overnight temperatures and days in between with less intense heat compared to uninterrupted daily highs, to effectively cope with the challenging conditions (Nail et al. 2015, Couture et al. 2015).

2.2 | Experimental design

To understand how biotic and abiotic stressors affect monarch larval performance in northern Nevada and California, we investigated larval developmental timing and survival in four separate common-gardens across a 513-km gradient of water-availability

(Table 1). Because monarchs expand eastward from the coast of California during the summer breeding season, each survival experiment was implemented sequentially from west to east in the following order: (1) Davis, CA, at the University of California, Davis, Plant Sciences Field Station (38.51878, -121.76897); (2) Reno, NV, at the University of Nevada, Reno (UNR) Valley Road Field Lab (39.539966, -119.804368); (3) Fallon, NV, at the UNR Fallon Research Center (39.45759, -118.77941); and (4) near Austin, NV, at the UNR Gund Ranch Research and Training Facility Research (39.90138, -116.58536). To assess plant water availability among sites, we calculated the annual climatic water deficit (CWD) for each site following Diethelm et al. (2022). Higher CWD values represent drier sites. From highest to lowest CWD, our common-garden sites were as follows: Davis (851.6 mm); Reno (711.2 mm); Fallon (703.3 mm), and Austin (556.6 mm).

To capture the range of phenotypic variation within species (Diethelm et al. 2022), we collected seeds of *Asclepias fascicularis* and *Asclepias speciosa* from 4–5 natal regions across a 610-km environmental gradient between 2017–2019. For all experimental plants, we first cold-stratified seeds at ~5 °C for 3–4 weeks. Seeds were then soaked in 5% bleach water for 15 minutes, manually stratified, and placed in 475 cm^3 pots with a 1:4 ratio of potting soil (Full Circle Soils & Compost, Gardnerville, NV) to sand. To induce germination, pots were saturated with 100 mg L^{-1} of ethephon (2-chloroethylphosphonic acid; Bayer CropScience AG) and then incubated at 23 °C for 3–5 days following Yerka et al. (2013). For *Asclepias eriocarpa*, plants were started from seed collection sites within California's Central Valley and were allowed to germinate without stratification. Plants were then kept under a 25 ± 2 °C, 16L:8D light cycle and

watered 3x per week. Once seedlings reached ~10 cm in height, they were fertilized weekly with 3 mL of a 24–8–16 NPK (MiracleGro®) solution. Plants for the three Nevada common-garden sites were transplanted in June–July of 2019, after 3–5 months of growth, and plants for the Davis, CA, site were transplanted in April of 2020, after 5–9 months of growth. Watering practices were determined by the infrastructure at each site but were consistent for all plants within each site. In Austin, NV, we used flood irrigation every other week from June–September, whereas in Davis, CA, Reno, NV, and Fallon, NV, we used drip irrigation three times per week from May–August.

All larvae used in the Davis, CA, site were the offspring of two wild-caught females (State of California, Department of Fish and Wildlife, Specific Use Permit ID S-183180002-18318-001). We then mated 25 individuals from the F1 generation of those initial females and used those larvae (F2) for the Reno and Fallon, NV, common-garden sites. Adults emerging in those sites were mated and their offspring (F3) were used in Austin, NV. Adults were examined for *Ophryocystis elektroscirrha* (OE) infection following Altizer et al. (2000) and infected individuals were euthanized. Gravid females oviposited onto milkweed plants within a glasshouse, where larvae remained on natal stalks until reaching the 2nd instar, when they were moved to experimental plants in the outdoor garden.

To determine whether larval food plant identity modulates the impacts of predators or abiotic conditions, larvae were placed onto the same species of milkweed as their natal stalk wherever possible. Each plant hosted a single larva for the duration of its survival, however, plants that were not damaged by the original larva were sequentially reused. Following the methods described in Chapter IV, we allocated 1/4 of the

caterpillars to predator-exclusion caged plants and the remaining 3/4 to open-sided mock-cages. The cages, consisting of a wire frame with a fine-weave-voile fabric cover, were buried into the soil to fully enclose the plant. The mock-cage replicated shading effects of the predator-exclusion treatment.

The experiment ran from June–August of 2021. For larvae in the control (mock-cage) treatment, which were exposed to predators, we conducted daily assessments to monitor survival and larval developmental timing, whereas the predator-exclusion treatment was checked every 2–3 days. To control the search effort, we conducted 2-minute checks of each plant, during which we noted the instar value of surviving larvae and counted any predators present. Larvae missing for four consecutive days were considered predated according to De Anda and Oberhauser (2015), except for plants with over 20 stems, which were checked for five consecutive days to account for potential concealment within the dense architecture. To limit the movement of 5^{th} instar larvae, any surviving larvae on plants in the control treatment were caged. All individuals pupated in the field. Pupae were weighed 1–2 days later before being transferred to a greenhouse to complete their development. Time to emergence was calculated as the number of experimental days, starting with placement of the larva onto a plant in the garden and ending when the adult eclosed.

To assess potential differences in predator assemblages among sites, we employed visual surveys on individual plants and pitfall traps located in each garden (Table 1). The four pitfall traps were randomly placed in the experimental garden, avoiding a two-meter distance from the garden edges to prevent capturing arthropods from outside the experimental area. Traps were buried to be level with the ground and held ~80 mL of 1:8

white vinegar to DI water with a few drops un-scented dish soap (ECOS by Earth Friendly Products), and were changed every 3–4 days during the initial 14-day experimental period in the corresponding garden. Contents were then rinsed, sorted by taxonomic order using a dissection microscope, and then placed into 80% ethanol for storage.

2.3 | Statistical analysis

All analyses were conducted in R version 4.3.0 (R Core Team 2022).

To examine the impact of abiotic and biotic conditions on monarch survival (defined as successful emergence), we used the R-package 'survival' to generate Kaplan-Meier survival curves as a function of time (Therneau and Grambsch 2000). To assess the impact of predator exposure on survival, we employed generalized linear regressions with binomial distributions. Food plant species, which can modify survival outcomes (Zalucki et al. 2001), was also included as a factor. To account for site-level variation in biotic and abiotic conditions, we included the common-garden site as a factor.

To evaluate the relative importance of various abiotic stressors on survival across instars, we calculated the following variables per instar-period for each individual larva: number of days over critical maximum thermal threshold (> 33 °C; Zalucki 1982), average daily maximum temperature, average daily minimum temperature, average air quality index (AQI), and number of days with > 200 $PM_{2.5}$ (criteria of "very unhealthy"; Environmental Protection Agency). We used random forest analysis from the 'randomForest' package in R to select which biotic and abiotic variables were best suited to model survival in each instar (Wiener and Liaw 2002). After identifying the most

influential variables, we then modeled survival at each instar value with the selected predictors using generalized linear regressions with binomial distributions. To investigate sublethal effects of biotic and abiotic stressors, we also modeled larval developmental timing (days to reach adulthood) using a linear model with the variables selected by random forest as predictors as well as the covariate of common garden location (site).

We scaled all continuous variables to compare effect sizes across response variables. To perform comparisons among all levels of a factor variable, we conducted posthoc comparisons using the 'multcomp' R package and reported the corresponding adjusted p-values (Hothorn et al. 2023). For all models that evaluated survival, we excluded accidental deaths and larvae from any predator-exclusion cages where predators were observed. For evaluating sublethal effects on monarch larval performance, we also excluded individuals in predator-exclusion cages where predators were observed. We calculated diversity of predators per site from pitfall trap contents, using the exponential term of the Shannon index (q = 1; Chao et al. 2014)

3 | Results

Exposure to predators strongly reduced larval survival (n = 710; β_{caging} = -6.20 ± 1.02, z = -6.06, P < 0.0001; Fig. 1). Among individuals protected from predators, survival varied across sites, ranging from 27.3–61.8% (n = 198), whereas larvae exposed to predators suffered low survival rates in all gardens, with survival ranging from 0–0.9% (n = 502). Contrary to our prediction, larval food plant species had no effect on adult survival ($\beta_{Af\text{-}Ae}$ = -1.53 ± 1.05, z = -1.47, P = 0.3; $\beta_{As\text{-}Ae}$ = -1.3 8± 1.05, z = -1.32, P = 0.4; $\beta_{As\text{-}Af}$ = 0.15 ± 0.31, z = 0.48, P = 0.9).

Larvae at different sites faced variable biotic and abiotic conditions, including differences in predator assemblages among sites and among individual plants, as well as fluctuating levels of air pollution and temperature extremes (Table 1). Pitfall trap contents suggested that larvae at the Davis, CA, site experienced predatory arthropods in similar abundance to what larvae experienced at the Reno, NV, site. However, almost 30% more predators were visually observed on milkweed plants during survival checks in Reno as compared to Davis. The diversity of predators in pitfall traps was also lowest in the Davis garden (Fig. S1). In contrast, larvae in Fallon, NV, contended with a relatively lower abundance of predators, but with a moderate predator diversity. Within a site, flowering plants attracted more predators ($\beta_{nectar} = 5.48 \pm 0.73$, $z = 7.53$, $P < 0.0001$; Fig. S2), such that we also observed higher densities of predators at sites with a higher proportion of nectar availability (Fig. S2). Regarding climatic conditions, larvae at the Davis site encountered the highest maximum daily high temperatures (up to 42 °C), while larvae at the Fallon site had the lowest daily maximum temperatures (up to 28 °C). Air quality also varied considerably among the sites. Larvae in Davis, CA, experienced zero days of poor air quality ($PM_{2.5} > 100$ AQI), larvae in Austin, NV, experienced only three such days, whereas larvae in Fallon, NV, and Reno, NV, experienced more 3–4 weeks of these conditions (Table 1).

Given these differences in abiotic conditions among sites, survival varied considerably among sites even for monarch larvae protected from predators. Larvae protected from predators survived best in the Davis, CA, and Austin, NV, sites, with no significant difference between them ($\beta_{DA\text{-}AU} = -0.22 \pm 0.49$, $z = -0.53$, $P = 0.9$; Fig. 1a,d). Larvae in the Reno, NV, garden had the lowest survival rate, with significantly less

survivorship than in Davis, CA ($\beta_{RN\text{-}DA}$ = -1.18 ± 0.45, z = -2.60, P = 0.46) and Austin, NV ($\beta_{RN\text{-}AU}$ = -1.438 ± 0.39, z = -3.64, P < 0.002; Fig. 1a,d). Larvae in Fallon, NV, fared worse than those in Austin ($\beta_{FN\text{-}AU}$ = -1.21 ± 0.46, z = -2.61, P < 0.05), but not differently than those in Davis ($\beta_{FN\text{-}DA}$ = -0.95 ± 0.52, z = -1.85, P = 0.2) or Reno ($\beta_{RN\text{-}FN}$ = -0.22 ± 0.43, z = -0.52, P = 0.9).

Random-forest variable selection indicated that exposure to predators explained the most variance in survival among early-instar monarchs, with climatic variables making smaller, though still important, contributions (Fig. 2a,b,c). After running generalized linear models based on the random-forest selected predictors, we found predator exposure had the greatest influence on survival for 2nd to 4th instars (instar2: n = 710; β_{caging} = -1.57 ± 0.21, z = -7.42, P < 0.0001; instar3: n = 199; β_{caging} = -2.77 ± 0.51, z = -5.34, P < 0.0001; instar4: n = 141; β_{caging} = -3.79 ± 0.94, z = -4.02, P < 0.0001; Fig. 2a,b,c). At the 2nd-instar stage, number of days > 33 °C increased larval survival ($\beta_{33°d}$ = 0.77 ± 0.14, z = 5.54, P < 0.0001), although increasing maximum daily high temperatures marginally decreased survival (β_{Tmax} = -0.20 ± 0.14, z = -1.49, P = 0.1). Air quality also decreased survival at this stage (β_{AQI} = -0.31 ± 0.12, z = -2.69, P < 0.008). At the 3rd-instar stage the pattern was similar with increasing survival as the number of days over 33 °C increased (instar3: $\beta_{33°d}$ = 1.93 ± 0.48, z = 3.98, P < 0.0001), yet increasing daily high temperatures decreased survival (β_{Tmax} = -1.61 ± 0.51, z = -3.15, P < 0.002). Larval food plant species also affected survival at the 2nd and 3rd instar stages. For both instars, larvae consuming *A. speciosa* had lower survival than larvae on *A. eriocarpa* (2nd instar: $\beta_{As\text{-}Ae}$ = -1.75 ± 0.58, z = -3.04, P < 0.0006; 3rd instar: $\beta_{As\text{-}Ae}$ = -2.35 ± 1.14, z = -2.08, P = 0.09). Second instar larvae also experienced reduced survival on *A. fascicularis* compared

to *A. eriocarpa* ($\beta_{Af\text{-}Ae}$ = -1.77 ± 0.57, z = -3.11, P < 0.005). At the 4th-instar stage, none of the climatic conditions suggested by Random-Forest variable selection had any significant impacts on survival, based on the model output. For 5th instar monarchs, which were all caged and therefore protected from predators, air quality had the highest impact on survival (β_{AQI} = -0.75 ± 0.26, z = -2.90, P < 0.004; Fig 2.d). This was similar for pupae, where increased wildfire smoke decreased survival (β_{AQI} = -0.74 ± 0.26, z = -2.85, P < 0.005; Fig 2.e).

In addition to affecting survival, predators also had sublethal effects that hindered the development of monarch larvae. At all sites, the presence of predators increased the number of days that are required for larvae to reach adulthood ($\beta_{p/a\ predator}$ = 5.71 ± 2.26, z = 2.53, P < 0.04, Fig. S3). Furthermore, sites with a higher diversity (q=1) of predators were associated with increased number of days to eclose as adults ($\beta_{predatorH'}$ = 1.62 ± 0., z = 2.60, P < 0.02; Fig. 3). Larvae in Fallon, NV, which had the highest diversity of predators (Table 1), required ~10–15 days longer to eclose as adults than larvae developing at the other three sites ($\beta_{FN\text{-}DA}$ = -15.2 ± 1.17, z = 12.99, P < 0.001; $\beta_{RN\text{-}FN}$ = -12.55 ± 1.16, z = -10.81, < 0.001; $\beta_{FN\text{-}AU}$ = 10.49 ± 1.13, z = 9.28, P < 0.001). Monarchs from Fallon also emerged as smaller adults (adult weight: $\beta_{FN\text{-}DA}$ = -0.074 ± 0.031, z = -2.37, P = 0.09; $\beta_{RN\text{-}FA}$ = 0.12 ± 0.030, z = 4.14, P , 0.001; $\beta_{FN\text{-}AU}$ = -0.14 ± 0.028, z = -5.11, P < 0.001), ranging from to 0.078–0.14 grams lighter in weight.

Likewise, climatic conditions that reduced survival had sub-lethal effects on larval development. As daily maximum temperatures increased, so did the time to reach adulthood (β_{Tmax} = 1.45 ± 0.56, z = 2.60, P < 0.02, Fig. S3). Poor air quality also delayed

maturation, with declining air quality resulting in more days spent in the 5th instar (β_{AQI} = 0.043 ± 0.012, z = 3.62, P < 0.0005, Fig. S3).

4 | Discussion

The present study sought to investigate the interplay of biotic and abiotic stressors on the larval performance of the monarch butterfly in its northern California and Nevada breeding grounds. Predator exposure emerged as a crucial determinant of monarch larval survival. However, abiotic conditions, especially air quality and temperature, also contributed substantially to survival. Our findings reveal a novel aspect of the impact of climate change: the potential detrimental effect of increasing levels of forest fire smoke on herbivore populations, in addition to the exposure to climatic extremes (Hagmann et al. 2021, Harvey et al. 2023).

In addition to lethal effects, biotic and abiotic stressors had sub-lethal effects of larval development by prolonging the time required for larvae to reach adulthood. Sites with higher predator diversity were associated with increased developmental time, suggesting that the risk of predation may influence the developmental pace of monarch larvae, which aligns with our findings from Chapter III. As daily maximum temperatures increased, the time taken for larvae to reach adulthood also increased. This indicates that temperature extremes might alter the developmental timing of monarch butterflies, while further increasing their risk of predation. Poor air quality conditions also delayed larval maturation by increasing the time that larvae spent in the 5th instar. These findings are in line with those of Tan et al. (2018), who found that smoke decreased survival, increased the time larvae took to develop, and decreased adult size in *Bicyclus anynana* butterfly

larvae. Adult size has been shown to influence fecundity in herbivorous insects (Honěk 1993), including lepidopterans, suggesting that the sub-lethal effects observed in larval development in this experiment may have broader implications for population dynamics and reproductive success. Our results also demonstrated the importance of considering the combined effects of biotic and abiotic stressors on monarch butterfly populations. Monarch larvae in Fallon, NV, faced a combination of factors, including higher predator diversity and poor air quality, resulting in prolonged larval development and smaller adult sizes. This cumulative impact suggests that addressing single stressors in isolation may not be sufficient for effective conservation.

Contrary to our initial prediction, the larval food plant species did not significantly influence survival to adulthood, although it did affect larval survival at the 2nd and 3rd instars. While larval food plant species identity can influence monarch larval development and survival due to variation in toxic compounds (Zalucki et al. 2001), our results suggest that other factors may override or interfere with the effects of milkweed species on survival across the entire larval developmental period. It is possible that the overall stress levels experienced by later-stage larvae, such as those induced by predators and abiotic conditions, could have masked the subtle differences in milkweed defenses between species. Extreme abiotic conditions may also have caused plant traits to respond similarly, thereby leading to similar expression of defense traits among species.

Increasing daily maximum temperatures did reduce survival, particularly at the 3rd instar stage. These findings align with those Nail et al. (2015), who found that 3rd instars were most susceptible to both cold and heat stress. Contrary to our predictions, exposure to temperatures over 33 °C positively impacted survival for 2nd and 3rd instar

monarchs. Further studies need to define how many days of exposure are beneficial compared to when increased temperatures become detrimental, and to tease apart any potential correlation between atmospheric smoke and lower ambient air temperatures. Indeed, follow-up controlled experiments could better explore each factor individually to understand the relationship between each stressor and monarch performance. Additionally, abiotic and biotic factors may interact differently in varying habitats and regions across the full expanse of the breeding range for western monarch butterflies.

The decline of the monarch butterfly is a pressing concern due to its cultural significance and potential conservation role as an umbrella species (Agrawal 2017, Schultz et al. 2017). Our study emphasizes the importance of considering biotic factors, particularly the negative impact of predators on monarch larval survival, in conservation efforts. We also highlight the delicate balance between survival and developmental timing under changing climatic conditions, stressing the need for a comprehensive understanding of these interactions in the face of climate change. Global climate change has led to higher maximum temperatures and increased air pollution (Hagmann et al. 2021, Intergovernmental Panel on Climate Chang 2023), which pose challenges to monarch populations. Rising temperatures and more frequent wildfires may further stress monarch populations by reducing survival and altering larval developmental timing. Understanding the cumulative effects of these factors is crucial for predicting the long-term viability of monarch butterfly populations in the fire-prone western North America.

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Tables

Table 1 Metrics of biotic and abiotic conditions across four common garden sites in northern California and Davis. Predators were collected in pitfall traps and diversity of predators per site was calculated as the exponential term of the Shannon index (q = 1). We calculated the average annual cumulative annual climatic water deficit (CWD; mm) per site. Higher CWD values represent drier sites.

Site	Experi-mental dates	Max. daily high temp. °C	Avg. daily temp. °C	Days over 33 °C	Days over $PM_{2.5}$ 100 AQI	Richness of predators in pitfall traps	Diversity (q=1) of predators in pitfall traps	Counts of predators observed on plants	CWD
Davis, CA	6/13/21-7/8/21	41.7	22	19	0	163	1.70	627	851.6
Fallon, NV	7/18/21-8/26/21	27.8	22	28	28	80	2.14	163	711.2
Reno, NV	7/26/21-8/25/21	32.8	24	23	25	164	2.27	874	703.3
Austin, NV	8/14/21-9/4/21	36.1	21	6	3	126	2.55	324	556.6

Figure Legends

Fig. 1 Impact of milkweed (*Asclepias*) larval-host plant species and exposure to natural enemies on monarch (*Danaus plexippus*) survival in common garden environments in (a) Davis, CA, (b) Reno, NV, (c) Fallon, NV, and (d) near Austin, NV. Larvae were either protected in full-cage enclosures (cage) or exposed to predators (mock-cage). Highlighted areas represent the 95 % confidence intervals

Fig. 2 Results of a Random Forest analysis evaluating the percent increase of mean square error (MSE) from omitting each selected predictor variable as it relates to monarch (*Danaus plexippus*) survival at each larval instar value using individuals experiencing different biotic and abiotic variables at four common-garden sites in northern California and Nevada.

Fig. 3 Impact of site-specific diversity of monarch butterfly (*Danaus plexippus*) larval predators (the exponential Shannon entropy; q = 1) on monarch larval developmental time to reach adulthood (days) in common garden areas across northern California and Nevada. Letters above individual boxplots are based on posthoc pairwise comparisons, denoting differences between the sites.

Fig. 1

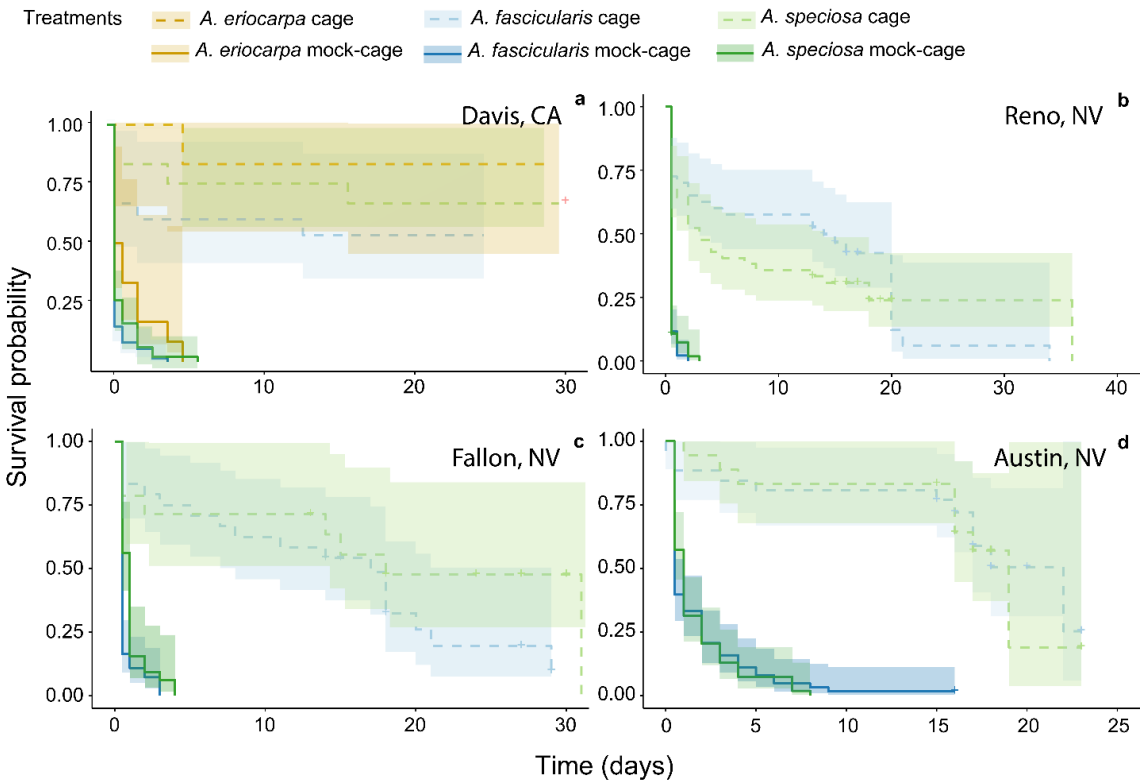

Treatments
A. eriocarpa cage
A. eriocarpa mock-cage
A. fascicularis cage
A. fascicularis mock-cage
A. speciosa cage
A. speciosa mock-cage
Davis, CA
a
Reno, NV
b
Fallon, NV
c
Austin, NV
d
Survival probability
Time (days)

Fig. 2

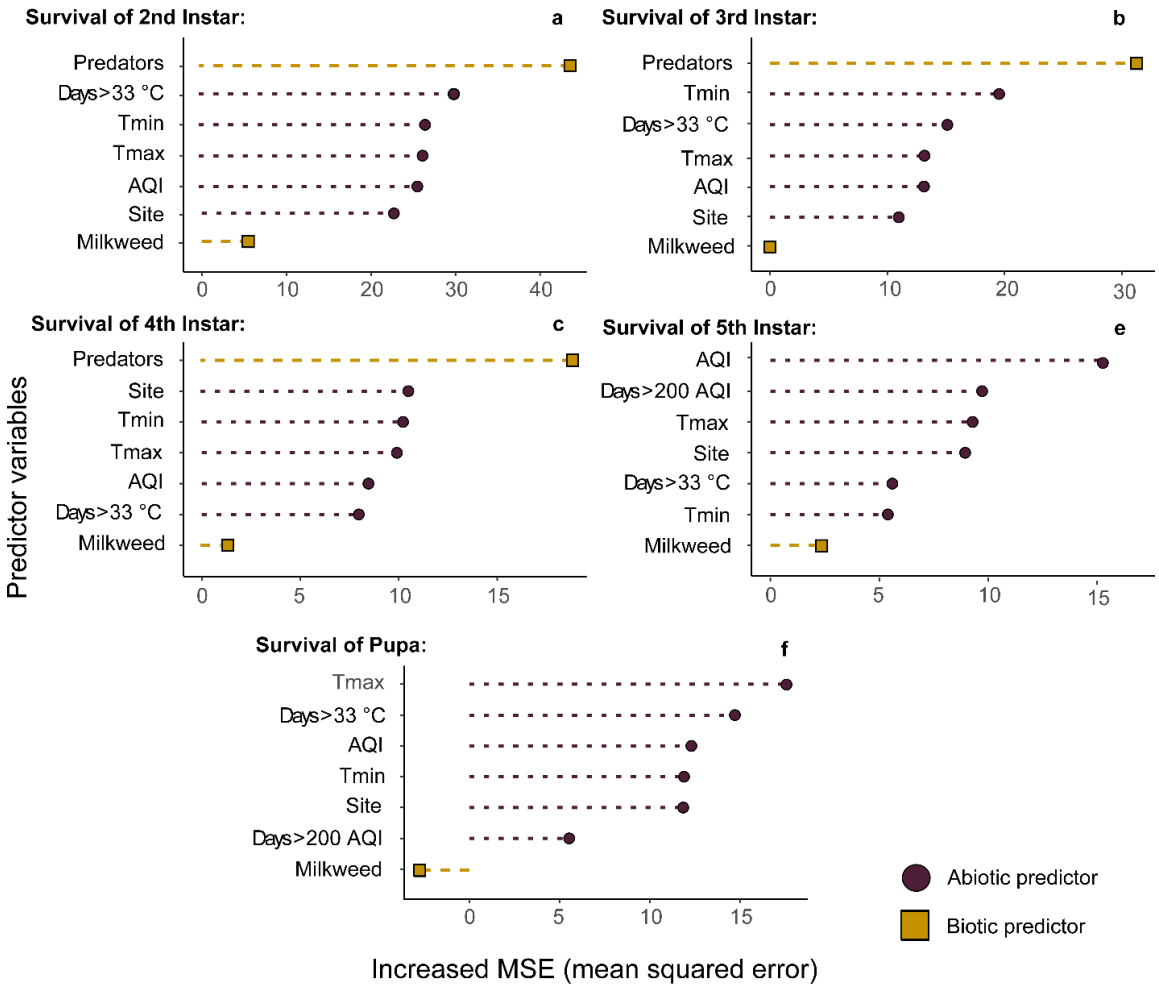
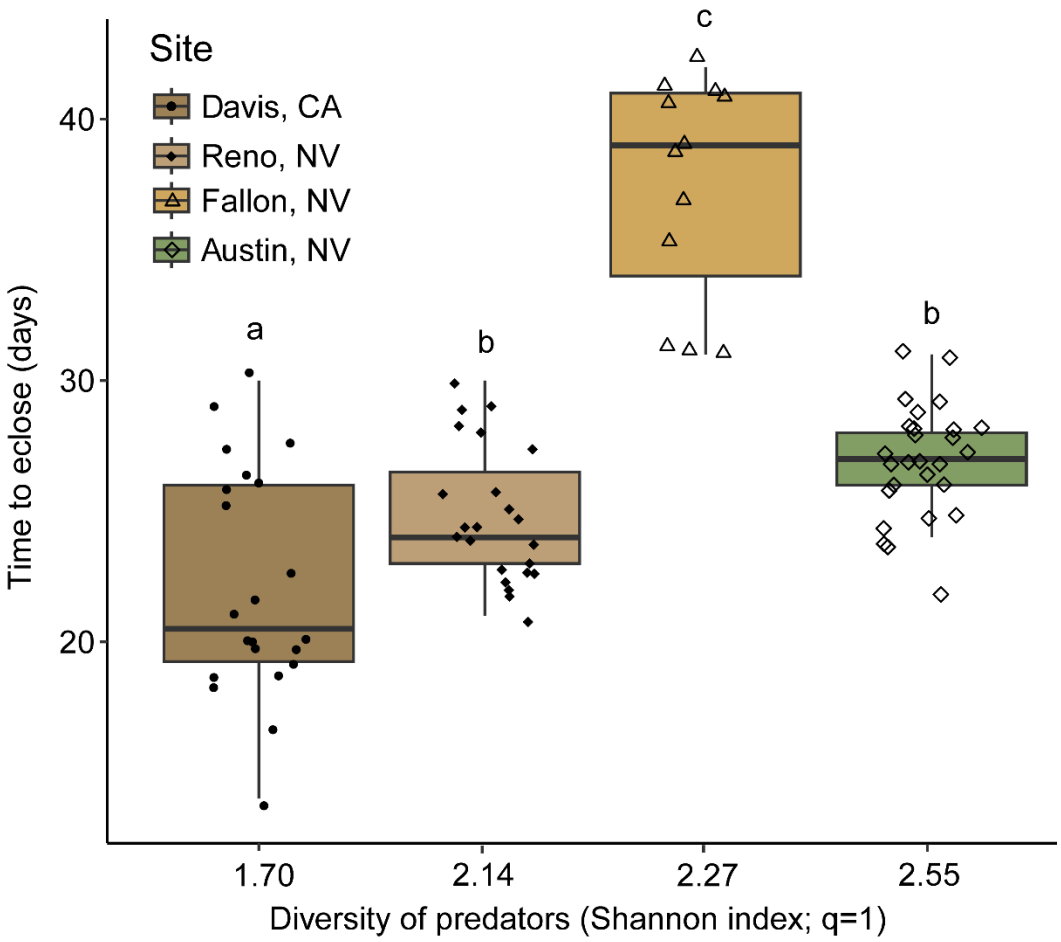

Survival of 2nd Instar:
a
Predators
Days>33 °C
Tmin
Tmax
AQI
Site
Milkweed
0
10
20
30
40
Survival of 3rd Instar:
b
Predators
Tmin
Days>33 °C
Tmax
AQI
Site
Milkweed
0
10
20
30
Survival of 4th Instar:
c
Predators
Site
Tmin
Tmax
AQI
Days>33 °C
Milkweed
0
5
10
15
Survival of 5th Instar:
e
AQI
Days>200 AQI
Tmax
Site
Days>33 °C
Tmin
Milkweed
0
5
10
15
Survival of Pupa:
f
Tmax
Days>33 °C
AQI
Tmin
Site
Days>200 AQI
Milkweed
0
5
10
15
Predictor variables
Increased MSE (mean squared error)
Abiotic predictor
Biotic predictor

Fig. 3


Site
Davis, CA
Reno, NV
Fallon, NV
Austin, NV
Time to eclose (days)
40
30
20
a
b
c
b
1.70
2.14
2.27
2.55
Diversity of predators (Shannon index; q=1)

Supplemental Tables and Figures

Table S1 Abundance of monarch butterfly (*Danaus plexippus*) larval predators as grouped by taxonomic orders, where Hymenoptera "other" represents both predatory and parasitic wasps. Predators were collected in pitfall traps across four common garden sites across northern California and Nevada.

Site	Trap ID	Hymenoptera Formicidae	Hymenoptera Other	Aranea	Coleoptera	Hemiptera	Total
Davis,	DA_1	54	1	1	12	1	69
CA	DA_2	15	0	4	2	0	21
	DA_3	29	0	10	2	0	41
	DA_4	21	1	6	4	0	32
Reno,	RN_1	10	0	2	0	0	12
NV	RN_2	18	0	10	2	0	30
	RN_3	3	0	2	0	0	5
	RN_4	19	1	12	0	1	33
Fallon,	FN_1	16	9	1	4	0	30
NV	FN_2	26	18	3	2	2	51
	FN_3	19	5	1	1	1	27
	FN_4	49	4	2	0	1	56
Austin,	AU_1	9	10	3	0	0	22
NV	AU_2	15	2	6	0	0	23
	AU_3	9	12	2	1	0	24
	AU_4	37	17	3	0	0	57

Fig. S1 Diversity (Shannon entropy; q =1) of monarch butterfly (*Danaus plexippus*) larval predators at four common garden sites from along a Climatic Water Deficit gradient, using monthly climate and soil content data from 2004–2016 that were summed annually and averaged over the time period. Predators were collected using pitfall traps.

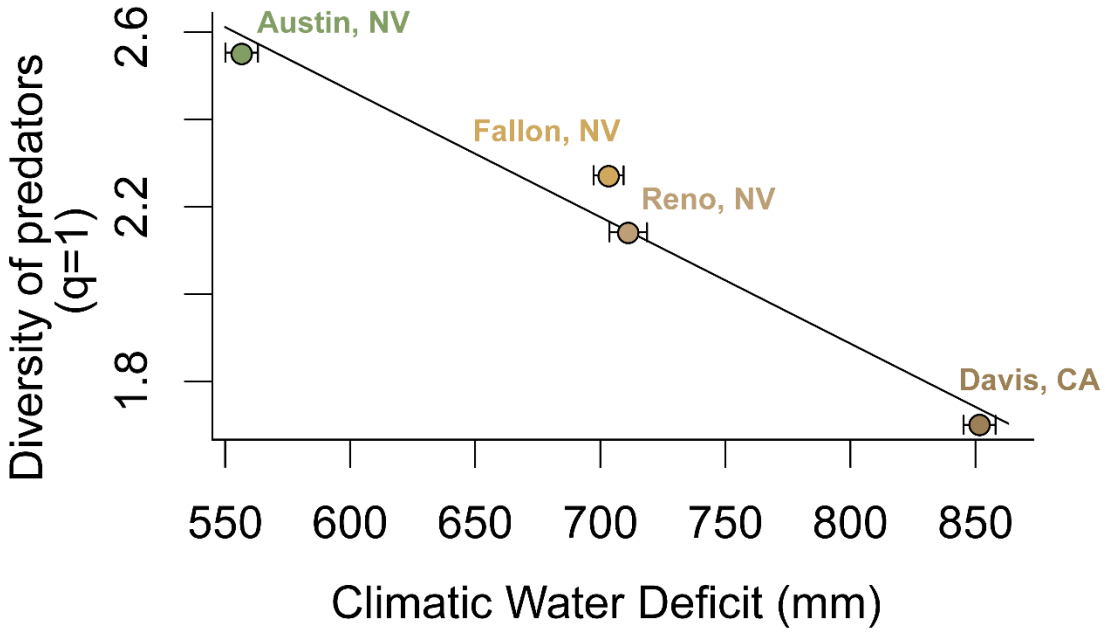


Fig. S2 Influence of nectar availability from milkweed plants (*Asclepias*) on the abundance of known monarch butterfly (*Danaus plexippus*) larval predators across four common garden areas across northern California and Nevada. Within each group, the boxes span from the 25th to the 75th percentile of the value distribution. Thick horizontal black lines denote median values. Vertical lines extending from the boxes indicate distribution of values, specifically the outermost values within 1.5 times the interquartile range of the 25th and 75th percentiles for each group. Jittered points represent the individual predators as collected using pitfall traps.

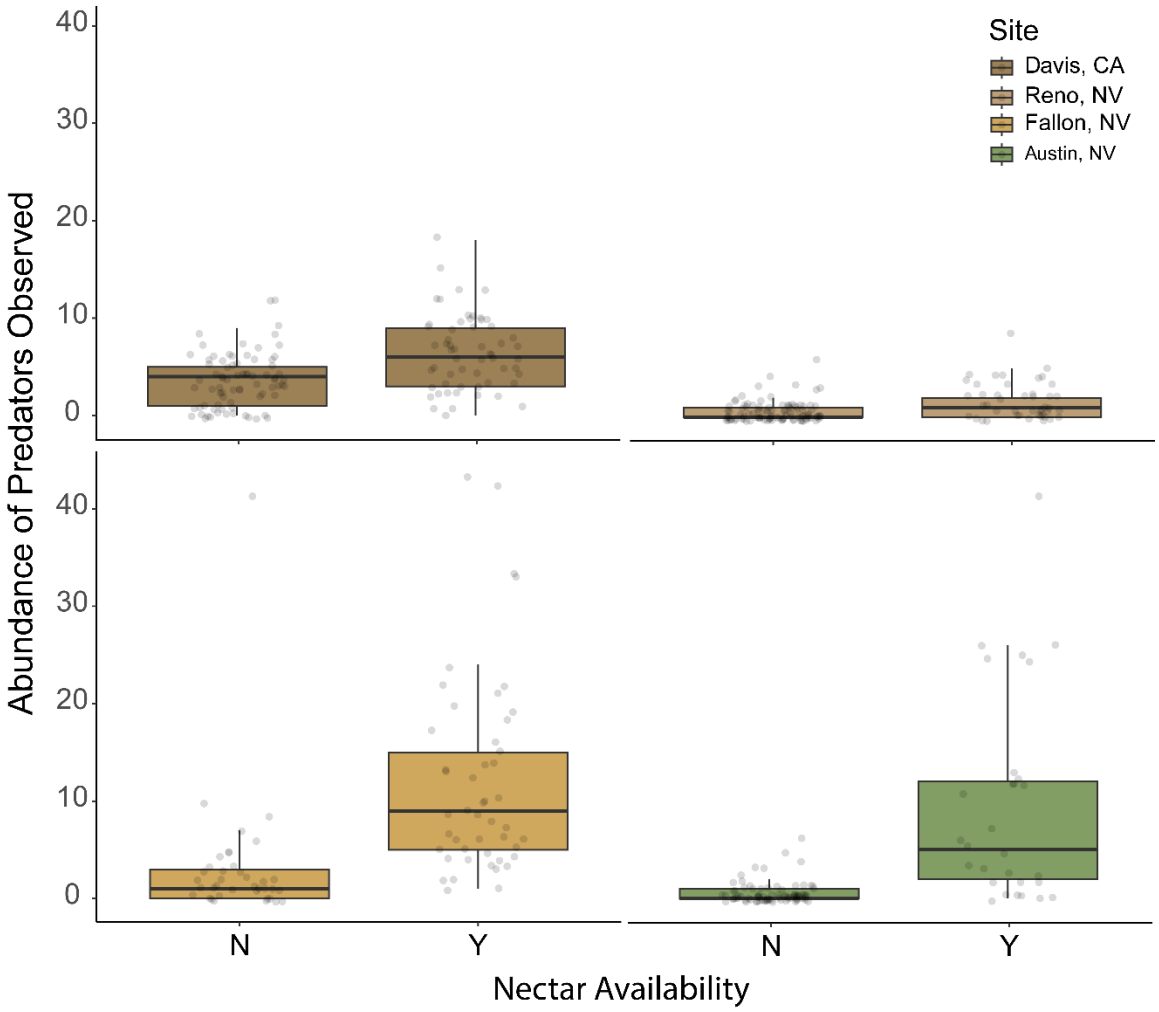
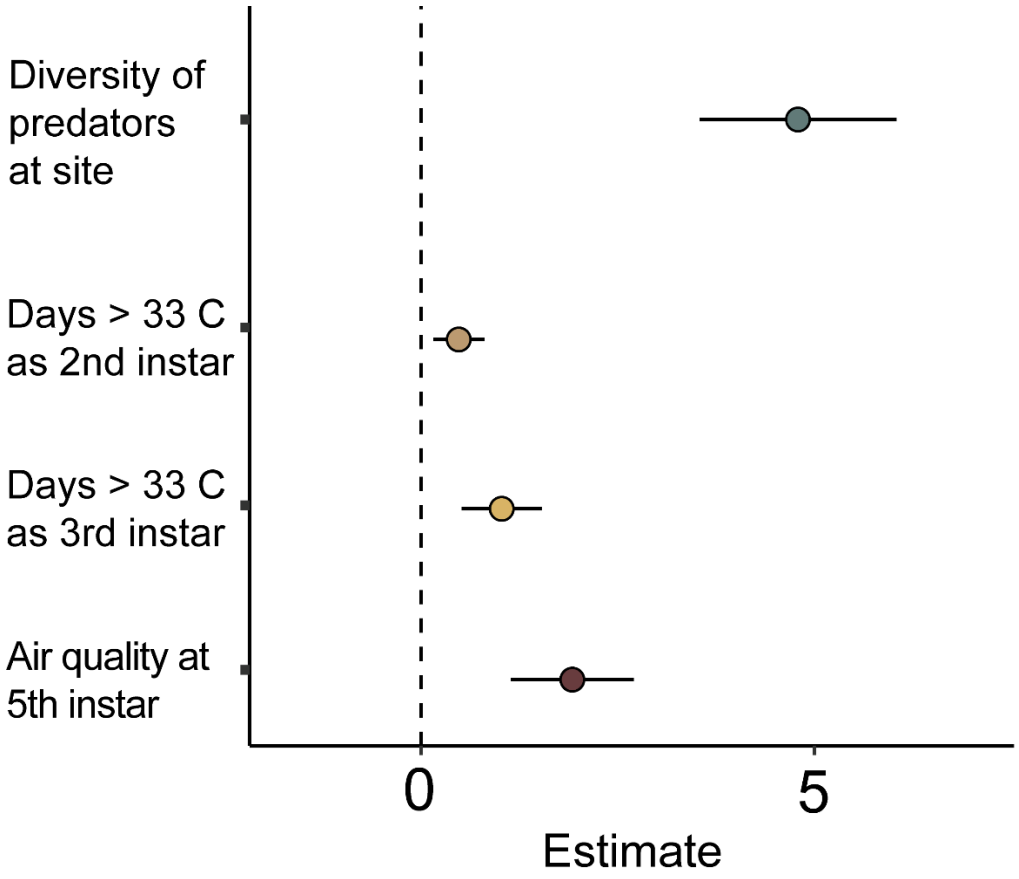


Fig. S4 Impact of relevant biotic and abiotic conditions monarch butterfly (*Danaus plexippus*) larval developmental timing (days to reach adulthood) from four common garden areas across a climatic gradient in northern California and Nevada. Predators were collected using pitfall traps and diversity was measured using the exponential term of the Shannon index. Each single point is the beta-co-efficient from a linear model evaluating the impact of all the predictors, with the horizonal line around the point representing the standard error.



Conclusion

Examining the influence of environmental conditions on multi-trophic interactions is critical to understanding herbivore vulnerability and resilience amidst global climate change (Hunter and Price 1992; Rosenblatt and Schmitz 2016). To investigate the impact of changing conditions, this dissertation research investigated the plant-herbivore-predator interactions across a gradient in abiotic conditions, with a central focus on water-availability.

Initially, I examined the impact of abiotic factors on plant trait responses within a controlled greenhouse setting. Those results suggested that local adaptation across an arid landscape can shape plant trait responses to water limitation at a population-level (Chapter I). Building upon these findings, I used plants with the same natal seed sites within an outdoor common garden setting to introduce a secondary, biotic stressor. This approach unveiled potential metabolic constraints underlying plant responses to co-occurring stressors (Chapter II). Collectively, these findings provide valuable insights into the variability of larval food plant quality across a landscape marked by diverse histories of stress exposure. A critical next step involves evaluating herbivore performance in relation to the plant trait responses observed in Chapter II to determine if there are biologically relevant effects for plant-herbivore interactions (Massad and Dyer 2010), and potential cascading trophic effects within the broader ecosystem (Mooney et al. 2010; Mohl et al. 2016).

To shift the perspective towards predators, I then examined the non-consumptive impacts of natural enemies on herbivore performance, and how these effects can be modulated by the identity of larval food plants. In investigating this less-explored aspect

of tritrophic interactions (Hermann and Landis 2017), my findings indicate that non-consumptive effects are indeed a significant factor in plant-herbivore-predator dynamics (Chapters III and IV). By assessing fear-induced impacts alongside consumptive effects, my findings suggest that non-consumptive effects may influence population dynamics even in the presence of consumptive pressures (Peers et al. 2018), although the extent of demographic implications and the potential of larval food plant identity to modulate those impacts warrants further investigation. Moreover, upon investigating the biotic and abiotic influences on larval survival, I found that predators exert significant top-down pressures, even under extreme abiotic conditions (Chapters IV). My results also indicate that a relatively unexplored facet of global climate change has the potential to disrupt herbivore populations: the detrimental effects of wildfire smoke on larval performance (Chapter IV; but see Tan et al. 2018).

The results presented in Chapters I-IV highlight how abiotic conditions contribute to variations in both bottom-up effects from host plants and top-down effects from predators on herbivore abundance and distribution. While the field-based studies of Chapters II-IV provide valuable insights into the complexities of natural settings, follow-up experiments in controlled environments are essential to disentangle the impacts of abiotic and biotic stressors on herbivore performance.

Collectively, this dissertation advances our understanding of plant-insect-predator interactions under varying abiotic conditions. Furthermore, it provides a roadmap for directing future studies that aim to untangle the complexities of tritrophic interactions in a rapidly changing world. This understanding of the intricate dynamics among herbivores, their larval-food resources, and their predators under varying abiotic conditions offers a

valuable framework for addressing current and future challenges in invertebrate conservation within a time of rapid insect declines (Wagner et al. 2021).

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