Combined impacts of prolonged drought and warming on plant size and foliar chemistry

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ORIGINAL ARTICLE

Combined impacts of prolonged drought and warming on plant size and foliar chemistry

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Running title: Consequences of prolonged drought and warming to *Plantago lanceolata* traits

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Abstract

• **Background and Aims** Future shifts in precipitation regimes and temperature are expected to dramatically affect plant traits. To date, many studies have explored the effects of acute stresses, but few have investigated the consequences of prolonged shifts in climatic conditions on plant growth and chemistry.

• **Methods** We assessed plant size and performed metabolite profiling of naturally occurring *Plantago lanceolata* plants growing under different precipitation (ambient, 50% less than ambient = drought) and temperature (ambient, +~0.8, +~2.4, +~4.0 ^{o}C above ambient) treatments at the Boston Area Climate Experiment (BACE, constructed in 2007).

• **Key Results** The analysis of several primary and secondary metabolites revealed striking effects of drought and lesser effects of warming on leaf chemistry. Compared to the ambient condition, plants in the drought plots had lower concentrations of foliar nitrogen, amino acids, and most sugars and higher concentrations of sorbitol, a common stress-induced metabolite. Moreover, drought-exposed plants showed lower leaf concentrations of catalpol, an iridoid glycoside well known to affect the performance of herbivores.

• **Conclusions** While the effects of warming were less pronounced, the temperature extremes (i.e., the highest temperatures) resulted in most distinct plant responses to drought. We discuss how these changes in chemistry might impact plant responses to abiotic and biotic stress.

Key words: amino acids; Boston Area Climate Experiment, climate warming; drought, foliar chemistry; iridoid glycosides; metabolite profiling; *Plantago lanceolata*; precipitation; sugars

INTRODUCTION

Global air temperatures are expected to increase by an average of 2-4 ^{o}C by the end of the century, especially at higher latitudes, and will likely be accompanied by changes in precipitation (IPCC, 2014). Warmer temperatures and drought are expected to dramatically affect ecosystem processes, and alter morphological and chemical plant traits that affect the performance of plants. To date, many studies have explored the effects of acute or short-term individual stresses on plant growth and chemistry, but relatively few have investigated the consequences of sustained long-term (> 5 years) shifts in certain abiotic conditions (Metz *et al.* 2014) and even fewer have manipulated multiple climatic factors simultaneously (Suseela *et al.* 2014, 2015; Van De Velde *et al.* 2015). This is a critical gap, since the effects of two or more climatic variables, such as temperature and drought, are typically non-additive and may shift with time (Dieleman *et al.* 2012; Gargallo-Garriga *et al.* 2015).

Short-term drought generally inhibits photosynthesis, suppresses shoot growth and triggers rapid changes in leaf chemistry, including increased concentrations of metabolites with osmoregulatory function, a loss of nutrients, changes in C/N/P/K stoichiometry, and decreased or enhanced concentrations of secondary metabolites (Selmar and Kleinwaechter 2013; Moradi 2016; Goufo *et al.* 2017). The short-term effects of warming are less pronounced but higher temperatures generally lead to increased growth, while temperature-induced changes in the concentrations of primary and secondary metabolites vary among genotypes and depending on the metabolite (Maenpaa *et al.* 2013; Virjamo *et al.* 2014).

The long-term effects of stress are often distinct and may function either via direct effects on the plant or indirectly via changes in the environment. Sustained drought stress causes shifts in traits that enhance avoidance and/or changes in the concentrations of stress-related metabolites,

such as osmoregulators and antioxidants, that increase tolerance to drought (Rodgers *et al.* 2012; Moradi 2016), or can influence plants via changes in ecosystem processes (Suseela *et al.* 2014). Mild warming typically increases plant growth and the concentrations of certain primary metabolites, i.e., sugars, amino acids, and other organic acids (Hu *et al.*, 2013; Zhang *et al.*, 2016). Importantly, the combination of drought and warming often has pronounced effects on growth and plant chemistry (Tharayil *et al.* 2011; Hoeppner and Dukes 2012; Gargallo-Garriga *et al.* 2015; Song *et al.* 2016). For example, drought and high temperatures can substantially reduce plant biomass (Song *et al.* 2016) and alter the foliar concentrations of primary as well as secondary metabolites (Tharayil *et al.* 2011; Suseela *et al.* 2015). In particular, foliar concentrations of metabolites that function as osmoregulators and antioxidants increase under the combination of drought and warming (Suseela *et al.* 2015). These changes help plants to mitigate the effects of climatic stresses (Suseela *et al.* 2015; Moradi *et al.* 2017). Furthermore, they may impact interactions between plants and their herbivores. To our knowledge no studies have examined the combined effects of long-term drought and warming on both primary and secondary metabolites important for plant stress tolerance and plant-herbivore interactions.

Plantago lanceolata L. (Plantaginaceae) is an excellent study system to explore the effects of prolonged drought and warming on plant size and leaf chemistry. It is an annual or facultative perennial cosmopolitan herbaceous weed with a wide tolerance to temperature and water limitation (Cavers *et al.* 1980; Prudic *et al.* 2005; Rodgers *et al.* 2012; Cranston *et al.* 2016; USDA, NRCS 2017). *Plantago* also has a well characterized chemistry (M. Deane Bowers *et al.* 1992; Janković *et al.* 2012; Schweiger, Baier, *et al.* 2014), including various osmoregulators such as proline (Patel and Vora 1985) and two iridoid glycosides, aucubin and catalpol. These metabolites of *P. lanceolata* are quite responsive to various environmental factors (Schweiger, Heise, *et al.* 2014; Pankoke *et al.* 2015) and differences in concentrations likely modify the their

stress tolerance (Backhaus *et al.* 2014) and their interactions with herbivores (M. Deane Bowers *et al.* 1992).

In this study we leveraged the long-running Boston Area Climate Experiment (BACE, constructed in 2007, Hoeppner and Dukes, 2012) to examine the consequences of prolonged shifts in climate on plant size and primary and secondary leaf metabolites. Specifically we quantified the effects of sustained precipitation reduction and warming on growth- and chemistry-related traits in naturally occurring *P. lanceolata* plants. We measured plant size, foliar N, foliar C, C/N ratio, and profiled diverse plant metabolites (including sugars, di- and tricarboxylic acids, the sugar alcohol sorbitol, the cyclic polyol *myo*-inositol, amino acids, and iridoid glycosides). We compared plants growing under ambient precipitation and drought and under the four temperatures. We expected that plants in plots with prolonged exposure to the combination of drought and warming would be distinct from plants in plots exposed to only one stress. Moreover, we expected that the effects of drought given its shallow rooting system (Tsialtas *et al.* 2001; Mommer *et al.* 2010). We expected the effects of warming to be less since it is common at different latitudes (USDA, NRCS 2017). Specifically, we predicted that plants would be smaller in drought plots, and would have lower leaf N due to limited water and nutrient uptake. At the metabolome level, we expected drought-induced decreases in the concentrations of N-containing metabolites and iridoid glycosides but increases in stress-responsive osmoregulatory metabolites. Our study provides new insights into the consequences of prolonged water deficits and warming to plant metabolism and its potential effects on plant-herbivore interactions.

MATERIALS AND METHODS

Study Site

The Boston-Area Climate Experiment (BACE) was constructed in 2007 in an old-field ecosystem in Waltham, Massachusetts (42°23.1′N, 71°12.9′W). Mean annual precipitation and temperature in nearby Boston are ~1000 mm and ~10 ^{o}C, respectively. The study site has a loam topsoil (Mesic Typic Dystrudept; Haven series) with 45% sand, 46% silt and 9% clay (gravel content: 7%) and a gravelly sandy loam subsoil (Auyeung *et al.* 2013; Suseela *et al.* 2014). Prior to construction the site was maintained by periodic mowing. In 2016, the most common plant species in the experimental plots, in addition to *P. lanceolata*, were *Achillea millefolium*, *Asclepias syriaca*, *Chenopodium album*, *Lepidium virginicum*, *Linaria vulgaris*, *Poa trivialis*, *Solidago canadensis*, *Verbascum thapsus*, and *Veronica arvensis.*

BACE consists of three replicate blocks with three levels of precipitation and four levels of temperature that are manipulated in a full-factorial, split-plot design (for a total of 36 experimental plots). The soil around each 2 x 2 m plot had been trenched to 60 cm depth and plots were lined with polyethylene sheets to prevent the movement of water and nutrients between plots. The three precipitation regimes per block were ambient, -50% (hereafter "drought"), and +50%, and were achieved using rainout shelters and a sprinkler system, respectively. Clear, corrugated polycarbonate slats (Rooflite®, Rimol Greenhouse Systems) removed 50% of incoming precipitation in the drought plots. Such rainout shelters are widely used to study plant responses to water deficits (Kreyling *et al.* 2017). In this study, plants subjected to the ambient and drought treatments were analyzed.

Within each precipitation treatment group, there were four temperature treatment levels, unwarmed (ambient), and low (+~0.8 ^{o}C), medium (+~2.4 ^{o}C), and high (+~4 ^{o}C) warming (Suseela *et al.* 2015). Each temperature treatment was applied to a 2 m x 2 m plot and all four treatments were repeated in each precipitation regime. Infrared heaters of different wattages were installed 1 m above the ground at each plot corner of the low (200 W), medium (600 W), and

high (1000 W) warming treatments and faced towards the center of the plots at a 45° downward angle to provide relatively uniform warming. Infrared radiometers measured canopy temperatures in the unwarmed and high warming plots, and a control system (LabView National Instruments, Austin, Texas, USA) provided active feedback control to maintain the target temperatures of the other warming treatment plots. Further details of the treatments and their consequences can be found in Suseela *et al.* (2012), Hoeppner and Dukes (2012), and Auyeung *et al.* (2013).

Plant species

Plantago lanceolata was introduced into North America over 200 years ago and is now common in lawns and gardens, in agricultural fields, and in hayfields. Although exotic, it is fed upon by several herbivore species native to North America (Bowers 1983; Thomas *et al.* 1987). *Plantago lanceolata* grows naturally at BACE and in the surrounding landscape and the population includes both seedlings and older vegetative clones. We only sampled from vegetative clones to avoid confounding the effects of plant ontogeny. Aucubin and catalpol, the characteristic secondary metabolites in *P. lanceolata* (Bowers and Stamp 1992, 1993), typically deter generalist herbivores, but attract specialist herbivores and even aid in their defense against predators upon sequestration (Bowers 1983; Theodoratus and Bowers 1999; Dobler *et al.* 2011).

Water availability

Soil moisture was determined volumetrically (v/v) using time domain reflectrometry (TDR) sensors placed in the upper 10 cm of soil (see Auyeung *et al.*, 2013). TDR sensors were permanently installed (at both 10 and 30 cm soil depth) to provide integrated measures of volumetric soil moisture. Measurements were taken weekly during the growing season using a

portable TDR-100 (Campbell Scientific, Logan, UT, USA). We report soil moisture at 10 cm 2 days before harvest.

Plant harvest and determination of morphological and chemical plant traits

Plants were sampled on 1 June 2016. [This date was chosen so that these data would inform our future experiments examining the consequences of any chemical changes to the performance of the Baltimore checkerspot butterfly (BCB; *Euphydryas phaeton*; Lepidoptera: Nymphalidae), which feed on *P. lanceolata* in Massachusetts.] It is important to note that all sampled plants were free of herbivores. We also selected plants that had no close plant neighbors. This was done to ensure that any potential effects of shading would be minimized, and to ensure that clonal individuals were only sampled once. With the exception of the ambient precipitation by low warming treatment (n=4), there were six plants per treatment. The lower sample size in the ambient by warming treatment was due to the limited number of plants in that treatment.

Plant size and leaf harvest. For each plant, we measured the total number of leaves and the length of the longest leaf. To determine effects of the long-term drought and warming treatments on leaf chemistry, we collected leaves from each plant between 08:30 and 09:30. We sampled the three youngest fully expanded leaves of each plant, to control for the effects of leaf ontogeny on chemistry (Quintero and Bowers 2012) and to ensure sufficient material for chemical analyses. The three leaves were placed into Falcon tubes and immediately placed in a cooler filled with dry ice to stop any enzymatic degradation. Samples were then lyophilized and ground in a KLECO ball mill (Garcia Machine, Visalia, CA, USA). Dried samples were stored in sealed Eppendorf tubes in dessicated chambers until chemical analyses.

182 *Carbon and nitrogen.* We determined foliar %C and %N by dry combustion with a CHN
183 analyzer at Tufts University. In brief, 5 ± 0.5 mg leaf powder per sample were analyzed using a
184 vario MICRO cube (Elementar Americas, Mt. Laurel, NJ, USA).
185 *Metabolite profiling of primary and secondary leaf metabolites.* Polar leaf metabolites were
186 analyzed by targeted metabolite profiling using two analytical platforms. On both platforms,
187 blanks (without biological material) as well as several reference standards (from Sigma-Aldrich,
188 Steinheim, Germany; AppliChem, Darmstadt, Germany; Merck, Darmstadt, Germany; Roth,
189 Karlsruhe, Germany; Macherey-Nagel, Düren, Germany; Agilent Technologies, Waldbronn,
190 Germany; Phytoplan Diehm & Neuberger, Heidelberg, Germany) were analyzed.
191 Concentrations of sugars, di- and tricarboxylic acids, the sugar alcohol sorbitol, the cyclic
192 polyol *myo*-inositol, and iridoid glycosides were determined using a gas chromatograph coupled
193 to a flame ionization detector (GC-FID). GC analysis of derivatized compounds is commonly
194 applied both for primary metabolites (Pankoke and Müller 2013; Schweiger, Baier, *et al.* 2014)
195 as well as for iridoid glycosides (Bowers and Stamp 1992, 1993; Quintero and Bowers 2012;
196 Pankoke and Müller 2013). Leaf powder (4 mg) was extracted and derivatization performed
197 using a modified protocol after Schweiger *et al.* (2014). Samples were extracted at room
198 temperature (RT) with a 1:2.5:1 (v/v/v) chloroform:methanol:Millipore-H_2O mixture (360 µL;
199 chloroform: HPLC grade, AppliChem; methanol: LC-MS grade, Fisher Scientific,
200 Loughborough, UK) containing ribitol (99%, Sigma-Aldrich) as internal standard by vortexing
201 and centrifugation. Phase separation was induced by addition of 140 µL Millipore-H_2O, followed
202 by vortexing and centrifugation. Aliquots of the methanol-water phases were dried under
203 nitrogen. Samples were derivatized at 37 °C with O-methylhydroxylamine hydrochloride (≥
204 98%, Sigma-Aldrich; 20 mg mL^{-1} in pyridine) and N-methyl-N-trimethylsilyltrifluoracetamide
205 (≥ 95%, Macherey-Nagel) for 90 and 30 min, respectively. Metabolite concentrations were

206 determined *via* GC-FID (GC-2010 Plus equipped with AOC-20s auto sampler and AOC-20i auto
207 injector, Shimadzu, Kyoto, Japan) using a VF-5 ms column (30 m x 0.25 mm i.d., 10 m guard
208 column, Varian, Palo Alto, CA, USA) with 225 °C inlet temperature and 1.12 mL min^{-1} carrier
209 gas (H_2) column flow rate. The oven temperature was 80 °C (hold for 3 min) and then ramped (5
210 °C min^{-1}) to 325 °C. For the peaks that were absent in the blanks, Kováts retention indices (RIs;
211 Kováts, 1958) were determined based on measurements of n-alkanes (C8-C40, Sigma-Aldrich)
212 and used for peak identifications *via* comparison with RIs of reference standards. Peaks were
213 integrated after file conversion using Xcalibur (1.4.SR1, Thermo Electron, Rodano, Italy).
214 Thereby, peak areas of analytes belonging to the same metabolite were added together.
215 Amino acids were analyzed by ultra-high performance liquid chromatography coupled to
216 fluorescence detection (UHPLC-FLD) modified after Jakobs & Müller (2018). Leaf powder (4
217 mg) was extracted threefold with 80% methanol (LC-MS grade, Fisher Scientific) containing
218 norvaline and sarcosine (Agilent Technologies) as internal standards by vortexing and
219 centrifugation at RT. Supernatants were pooled, filtered (0.2 µm polytetrafluorethylene filters,
220 Phenomenex, Torrance, CA, USA), and analyzed *via* UHPLC-FLD (1290 Infinity UHPLC with
221 1260 Infinity FLD, Agilent Technologies, Santa Clara, CA, USA). Samples were mixed with
222 borate buffer and pre-column derivatized by addition of ortho-phthaldialdehyde (OPA) reagent
223 (10 mg mL^{-1} in 0.4 M borate buffer and 3-mercaptoproprionic acid, Agilent Technologies) and
224 subsequently 9-fluorenyl-methyl chloroformate (FMOC) reagent (2.5 mg mL^{-1} in acetonitrile,
225 Agilent Technologies). Amino acids were separated at 40 °C on a ZORBAX Eclipse Plus C18
226 column (250 mm x 4.6 mm, 5 µm particle size, with guard column, Agilent Technologies) using
227 a gradient of mobile phase A [1.4 g Na_2HPO_4 (> 99.5%, AppliChem), 3.8 g $Na_2B_4O_7$ x 10 H_2O
228 (≥ 99.5%, Sigma-Aldrich), and 32 mg NaN_3 (≥ 98%, Roth) in 1 L Millipore-H_2O, pH = 8.2] to
229 mobile phase B [4.5:4.5:1 (v/v/v) mixture of methanol (LC-MS grade, Fisher Scientific),

acetonitrile (LC-MS grade, VWR International, Fontenay-sous-Bois, France), and Millipore-H_2O] with a flow rate of 1.5 mL min^{-1}. The gradient was ramped from 2 to 57% B within 43.4 min, followed by column equilibration. The FLD excitation and emission wavelengths were set to 340 and 450 nm, respectively, for the OPA-derivatized primary amino acids and to 260 and 325 nm, respectively, for the FMOC-derivatized secondary amino acids. Those metabolites that were absent in the blanks were identified *via* comparison of retention times with those of reference standards. Peaks were integrated in OpenLab ChemStation (C.01.06, Agilent Technologies).

Statistical analyses

Statistical analyses were done using JMP version 12 (SAS Institute Inc.). A Shapiro-Wilk goodness-of-fit test was used to ensure normality. Because some TDR sensors were broken we could not test the effects of temperature on volumetric water availability. We tested the effects of precipitation by pooling across temperature treatment levels (n=4 for drought and n=7 for ambient) using a one-tailed t-test. For plant traits, precipitation and temperature treatments were fixed effects. To determine the effects of these treatments on the number of leaves per plant, the length of the longest leaf, foliar %carbon, %nitrogen, C/N, aucubin concentration, and catalpol concentration, two-way ANOVAs were used.

The peak areas of the metabolites were related to those of the internal standards (GC-FID: ribitol; UHPLC-FLD: norvaline for primary, sarcosine for secondary amino acids) and the dry weights (dw) of the samples (leaf powder), yielding relative concentrations. For the iridoid glycosides, absolute concentrations were additionally calculated to be able to compare these values with those in the literature. For that, response factors between ribitol and the iridoid glycosides were determined using the same ribitol concentration as in the samples (see above)

and four concentrations of the iridoid glycosides in the linear range. Response factors were (averaged over concentrations and technical duplicates) 1.5 and 1.4 for aucubin and catalpol, respectively. Only those metabolites that occurred in more than 50% of the replicates of at least one treatment (precipitation x temperature) group were retained. A principal component analysis (PCA) was performed in R (R Core Team 2016) after replacement of zero values by small random numbers (10^{-13}-10^{-12}) and autoscaling (i.e., mean-centering and scaling to unit variance). Fold changes (mean metabolite concentrations in treatment groups divided by the mean metabolite concentrations in the common control group, i.e., ambient precipitation and ambient temperature) were calculated for metabolites that occurred in > 50% of the replicates of all treatment groups and $\log_2$-transformed for scale symmetry. Metabolite pool sizes were considered to be decreased by the treatment (compared to the common control group) if fold changes were < 0.5 (< - 1 on $\log_2$ scale) and considerably increased if fold changes were > 2 (> 1 on $\log_2$ scale).

Clustering of treatment groups and metabolites was performed based on mean fold changes (see above) using the average linkage hierarchical clustering method based on Pearson correlations in Cluster 3.0 (de Hoon *et al.* 2004). The heatmap was constructed with Java TreeView 1.1.6r4 (Saldanha 2004). Heatmap stripes were mapped on a metabolic pathway map that was modified after Schweiger *et al.* (2014) and relies on the KEGG PATHWAY database (Kaneshia and Goto, 2000; http://www.genome.jp/kegg/).

RESULTS

Soil moisture

At the time of harvest the volumetric water (θv) availability in the top 10 cm of soil was different between the two precipitation treatments (t = 2.35, p = 0.03). Specifically, the volumetric water

availability was nearly four times higher in the ambient (0.211 ± 0.03 θv, mean ± se, n = 7) than in the drought (0.047 ± 0.01 θv, n = 4) plots. Although statistical analyses could not be done, water availability was similar across the temperature treatment levels.

Plant size

The size of *P. lanceolata* plants was quite similar across all treatments (Table 1). For the number of leaves there was a marginal effect of precipitation ($F_{1,38}$ = 4.01, p = 0.052) but no effect of temperature ($F_{3,38}$ = 2.32, p = 0.09) or their interaction ($F_{3,38}$ = 2.05, p = 0.12). Surprisingly, plants growing in the drought plots tended to have more leaves. For the length of the longest leaf there were no effects of precipitation ($F_{1,38}$ = 1.46, p = 0.23), temperature ($F_{3,38}$ = 1.98, p = 0.13), or their interaction ($F_{3,38}$ = 0.74, p = 0.53).

Carbon and nitrogen

Percent nitrogen and C/N ratio were significantly influenced by precipitation (N: $F_{1,38}$ = 15.81, p < 0.01; C/N: $F_{1,38}$ = 20.24, p < 0.001) but not by temperature (N: $F_{3,38}$ = 0.65, p = 0.59; C/N: $F_{3,38}$ = 0.95, p = 0.42) or their interaction (N: $F_{3,38}$ = 1.99, p = 0.13; C/N: $F_{3,38}$ = 0.98, p = 0.41) (Figure 1). Since there were no effects of precipitation ($F_{1,38}$ = 0.41, p = 0.52), temperature ($F_{3,38}$ = 0.05, p = 0.98), or their interaction ($F_{3,38}$ = 1.56, p = 0.21) on percent carbon (data not shown), this indicates that shifts in nitrogen were driving the difference in C/N. Overall, percent nitrogen was about 40% higher in the ambient (2.1 ± 0.1%; mean ± se across all temperature levels) compared to the drought treatment (1.5 ± 0.1%).

Metabolite responses

Principle component analyses revealed strong effects especially of the precipitation treatment on the leaf metabolite profiles of *P. lanceolata* (Figure 2). Across all temperatures, the metabolite profiles of plants grown under drought clustered separate from samples taken from plants grown under ambient precipitation mainly along the first principal components (Figure 2a,c). The effects of warming on leaf chemistry were less pronounced. Drought effects on metabolite profiles were, however, strongest in plants grown under the two highest temperature levels, indicating that warming reinforced the drought effects (Figure 2a,c).

Compared to plants grown under ambient precipitation, plants grown in drought plots had lower leaf concentrations of most primary metabolites including proline and most other amino acids, but higher concentrations of malate, citrate, and sorbitol (Figure 2b,d). The concentrations of the two iridoid glycosides aucubin and catalpol ranged from 0.9 to 6.5% dw and 0.4 to 6.6% dw, respectively, and these two metabolites responded differently to the treatments (Figure 2b, Figure 3, Table 2). Whereas the aucubin concentrations were influenced both by precipitation ($F_{1,38} = 4.31$, $p = 0.04$) and temperature ($F_{3,38} = 4.22$, $p = 0.01$) but not their interaction ($F_{3,38} = 0.81$, $p = 0.50$), the concentrations of catalpol were only influenced by precipitation ($F_{1,38} = 8.59$, $p < 0.01$; temperature: $F_{3,38} = 0.65$, $p = 0.59$; interaction: $F_{3,38} = 1.20$, $p = 0.32$). Aucubin concentrations were generally higher under drought and decreased with temperature (Figure 3). In contrast, catalpol had about 40% lower concentrations in plants subjected to drought compared to plants grown under ambient precipitation (averaged across all temperature levels).

Cluster analysis confirmed that the effects on the foliar metabolite profiles were stronger for the drought than for the warming treatment but that warming reinforced the effects of drought, as seen in the fold changes and clustering of treatment groups predominantly according to the precipitation treatment (Figure 4). Moreover, two distinct clusters of treatment-responsive metabolites were found, confirming that most metabolites were reduced (Cluster III) and only

some (Cluster I; malate, citrate, sorbitol, to a lesser extent aucubin) were increased in concentrations under drought. The drought-responsive metabolites were spread across the major plant primary and secondary metabolic pathways (Figure 5). Metabolites specifically increased under drought were related to sugar and sugar alcohol metabolism (sorbitol) and part of the citric acid cycle (malate, citrate), while those decreased under drought were related to amino acid metabolism and to a lesser degree terpenoid biosynthesis.

DISCUSSION

Striking differences in the foliar leaf metabolite profiles were observed, despite no differences in plant size at the time of sampling. As expected, the effects of the drought treatment were strong and most pronounced under warming. In general, the effects of warming were much weaker. At this time of year water is generally less limiting and temperatures are lower, but later in the season, when it is both drier and hotter, phenotypic differences are more pronounced (Rodgers *et al.* 2012). Thus, the chemical changes reported in the current study provide a conservative estimate of the effects of prolonged precipitation deficits on plant traits.

Effects of drought and warming on the leaf metabolome

There were large differences in the foliar concentrations of primary and secondary metabolites in response to reduced water availability, despite the absence of differences in leaf number or size. While the effects of drought were stronger than the effects of warming, drought effects on metabolite profiles were strongest in plants grown under the two highest temperature levels, indicating that warming reinforced the drought effects.

Foliar N, amino acids, and the iridoid glycoside catalpol were reduced under drought, while sorbitol, two acids of the citric acid cycle (malate and citrate), and aucubin were increased. We

had expected drought-induced increases of sorbitol, malate, citrate, and proline since they are all stress-responsive osmoregulatory metabolites (Venekamp 1989; Rai 2002; Reddy *et al.* 2004; Singh *et al.* 2015). We observed partial support for this expectation. Sorbitol, malate, and citrate all increased, but proline concentrations were lower in plants grown under drought.

The response of proline to drought was quite unexpected given that many studies indicate that proline is associated with plant tolerance to drought and extreme temperatures (Wang *et al.* 2003; Reddy *et al.* 2004; Verbruggen and Hermans 2008; Moradi 2016). While other free amino acids may also play a major role in a plant's osmotic adjustment capacity (Rai 2002; Hu *et al.* 2015), none of the amino acids were higher in this condition. Rather most amino acids were lower or unchanged in concentrations in the plants grown in the drought treatment. Several factors may have contributed to the reduced leaf amino acid concentrations under drought. First, foliar N levels were ca. 40% higher in the ambient precipitation regime, suggesting that plants in the drought plots may have been N-limited which could have limited the biosynthesis of amino acids. The capacity to take up N-containing nutrients from the soil is typically lower under water limitation because drought-induced stomatal closure reduces the transpiration stream and nutrient mobility (da Silva *et al.* 2011). In this way, warming could result in more rapid desiccation and thus enhance the effects of drought. In contrast to N-containing osmolytes, the production of non N-containing compounds with osmoregulatory function such as sorbitol, citrate, and malate may be less constrained by N nutrition.

Second, proline may be a less important osmolyte for *P. lanceolata*. In the closely related species *Plantago major*, sorbitol but not proline was increased in response to salt stress (Hassan *et al.* 2016), and in general sorbitol concentrations are strongly increased by drought and salt stress in many *Plantago* species (Pommerrenig *et al.* 2007; Hassan *et al.* 2016). Sorbitol may be especially important when N is limiting. Thus we suggest that under N limitation there may be a

373 shift from accumulation of N-containing osmolytes, like proline, to non-N-containing osmolytes
374 like sorbitol.

375 The effects of drought on secondary metabolism can be variable (Chaves *et al.* 2003; Selmar
376 and Kleinwaechter 2013; Metz *et al.* 2014). We had predicted that as a result of resource
377 limitation in the drought treatment, an increase in compounds involved in osmoregulation would
378 be associated with decreased iridoid glycoside concentrations. Indeed, catalpol concentrations
379 were ca. 40% lower in leaves of plants grown in the drought plots. In contrast, aucubin
380 concentrations were higher in these plants. As aucubin is the biosynthetic precursor of catalpol
381 (Damtoft 1994), it is possible that the biosynthetic conversion of aucubin to catalpol was
382 impaired in drought-exposed plants. Alternatively, catalpol may be more prone to degradation.
383 Whatever the mechanism, similar to the current study, catalpol concentrations have been shown
384 to be more responsive to low mineral N availability or to interspecific competition (Pankoke *et*
385 *al.* 2015) and to arbuscular mycorrhiza (Schweiger, Baier, *et al.* 2014) than those of aucubin.
386 Consistent with the current study, aucubin and catalpol were reported to make up high
387 proportions (> 2-4% for each iridoid glycoside) of the leaf dry matter of *P. lanceolata* (Bowers
388 and Stamp 1992, 1993; M. Deane Bowers *et al.* 1992; Quintero and Bowers 2012; Pankoke and
389 Müller 2013).

390 Cluster analysis revealed that plants in the drought treatment were clustered together and that
391 warming magnified the effects of drought (Figure 4 top). The chemistry of the plants also
392 clustered in intriguing ways (Figure 4 side). While most chemicals responded similarly (Cluster
393 III) with lower concentrations under drought, those in Cluster I (especially malate, citrate, and
394 sorbitol) were higher. These results imply that metabolic shifts are correlated and are
395 corroborated in Figure 5. From Figure 5 it is apparent that metabolites related to sugar and sugar
396 alcohol metabolism (sorbitol) or a part of the citric acid cycle (malate, citrate) increased under

drought. In contrast, amino acid metabolism and to a lesser degree terpenoid biosynthesis decreased under drought.

Overall, our results indicate that shifts in environmental conditions, especially to changes in soil water availability will impact *P. lanceolata* chemistry in predictable ways. The underlying mechanisms for this shift deserve further study. These shifts could reflect plasticity to changes in the environment or may be a result of genetic differentiation after years of prolonged exposure. Also, given that these treatments can lead to secondary oxidative stress, the response of other metabolites such as flavonoids and phenylpropanoid glycosides (Janković *et al.* 2012), and enzymes, which function as antioxidants, should be considered in future studies.

Besides chemical adjustments to drought in individual plants, other external factors probably modify the severity of drought effects on plant traits in natural communities. Our results suggest that periods of high temperature will exacerbate the effects of drought. In addition, the composition of the surrounding plant community may affect water availability in the soil, depending on root architectures and water uptake efficiencies. Moreover, plant associations with mycorrhizal fungi may improve the plant's drought tolerances (Ruiz-Lozano *et al.* 2012) and affect plant chemistry (Asensio *et al.* 2012). Thus, to understand long-term effects of drought on plant traits it is important to study drought effects under multi-factorial field conditions.

Implications for plant-herbivore interactions

Most herbivores are nitrogen (N)-limited (Mattson 1980), so changes in nutritional profiles of leaves can impact herbivore feeding, growth, reproduction, and survival. Likewise, changes in plant secondary metabolites may have similar direct effects on herbivores and indirectly affect their susceptibility to predators (Bowers 1983; Theodoratus and Bowers 1999; Tomczak and Müller 2017). While plant size and thus the quantity of leaf material available to herbivores was

similar across treatments in early June, the lower foliar concentrations of N, amino acids, and catalpol in drought-stressed *P. lanceolata* plants probably have important consequences for herbivores. The decreased concentrations of N and amino acids under drought are expected to negatively affect herbivores (Mattson 1980). It is, however, the balance between nutritional and defense compounds that determines how well herbivores survive, develop, and reproduce on the plants. We suggest that the effects of the reduced catalpol concentrations in the drought treatment would depend on the dietary breadth of the herbivore. June coincides with the occurrence of the last two larval instars of *Euphyrdryas phaeton* prior to adult emergence in late June and early July (M.D. Bowers *et al.* 1992). *Euphydryas phaeton* and other North American specialist herbivores species evolved on native plant species that produce iridoid glycosides and subsequently have incorporated *P. lanceolata* into their diet (M.D. Bowers *et al.* 1992). The native host of *E. phaeton* is *Chelone glabra* (Plantaginaceae) which produces high concentrations of catalpol and very little aucubin. Since catalpol may act as oviposition stimulant as shown for another specialist butterfly species (Pereyra and Bowers 1988), reductions in catalpol might hamper the ability of adult female butterflies to detect *P. lanceolata* in drought-prone habitats. Moreover, since iridoid glycosides are sequestered as a defense against predators and parasitoids by some specialist herbivores (Bowers 1980; Theodoratus and Bowers 1999; Dobler *et al.* 2011), lower concentrations of catalpol may make the larvae and emerging adults in drought-prone habitats more susceptible to their enemies. In contrast, generalist herbivores are predicted to perform better on drought-stressed plants with lower concentrations of catalpol. Future studies are needed to examine the consequences of the chemical responses to stress observed in *P. lanceolata* plants and should also consider effects of precipitation and warming on both iridoid glycoside concentrations and β-glucosidase activity, since both traits form a dual defense system (Pankoke *et al.* 2013).

CONCLUSION

Climate projections indicate more variable precipitation and higher air temperatures. Compared to warming, we found that that the reduction of precipitation exerts a stronger influence on the polar leaf metabolome of *P. lanceolata* that might influence plant tolerance to further stress and suitability for associated herbivores, pathogens, and members of higher trophic levels. While warming had less of an effect, the magnitude of drought effects was reinforced by warming. These effects may, in part, reflect the direct and indirect impacts of prolonged exposure to drought and temperature. They likely also reflect rapid shifts (plasticity) in chemistry as environmental conditions change. If so, then as the severity of drought and warming increase over the season the consequences are likely to be even more pronounced. Clearly, more frequent drought periods and warming will have profound impacts on the metabolism of plants and will likely alter the behavior and performance of herbivores. This will probably feed back to affect the performance of the plants, since damage to resource-limited plants is likely to have a greater effect on their long-term performance. How these shifts in plant traits will affect the ecological outcome of plant-herbivore-predator/parasitoid and plant-pathogen interactions remains unknown.

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TABLES

TABLE 1. Effects of precipitation and temperature treatments on the number of leaves and length of the longest leaf of *Plantago lanceolata* plants; mean ± se of n = 4-6. There were no significant effect of precipitation, temperature and their interaction on these plant traits but a trend for more leaves in the drought treatment (see results for statistics).

Precipitation	**Temperature**	**# Leaves**	**Leaf length (cm)**
ambient	*ambient*	5.7 ± 0.5	20.2 ± 1.6
	+~0.8 ^{o}C	6.0 ± 1.7	19.6 ± 0.3
	+~2.4 ^{o}C	6.7 ± 0.9	18.2 ± 1.5
	+~4 ^{o}C	5.7 ± 1.1	17.3 ± 0.9
drought	*ambient*	10.3 ± 2.2	20.4 ± 1.3
	+~0.8 ^{o}C	5.5 ± 0.8	15.2 ± 1.6
	+~2.4 ^{o}C	8.8 ± 0.5	18.1 ± 2.3
	+~4 ^{o}C	5.8 ± 0.5	16.0 ± 1.9

TABLE 2. Metabolites, grouped according to their chemical class, in *Plantago lanceolata* leaf tissue. The analytical platform (GC-FID, gas chromatography coupled to flame ionization detection; UHPLC-FLD, ultra-high performance liquid chromatography coupled to fluorescence detection) as well as chromatographic retention parameters (RI, Kováts retention index; RT, retention time) are given. If one metabolite produced more than one analyte (GC-FID), retention parameters are given for all analytes. Names of organic acids are given both as anions and in protonated form. Abbreviations of metabolites are given in brackets. Metabolites were identified *via* comparison of retention parameters to those of reference standards. Note that cystine is a dimer of cysteine.

Metabolite	**Analytical platform**	**Retention parameter**	
Sugars			
fructose [FRC]	GC-FID	RI	1858/1868
glucose [GLC]	GC-FID	RI	1883/1901
sucrose [SUC]	GC-FID	RI	2613
Di- and tricarboxylic acids			
malonate / malonic acid [MALO]	GC-FID	RI	1201
succinate / succinic acid [SUCC]	GC-FID	RI	1311
fumarate / fumaric acid [FUM]	GC-FID	RI	1348
malate / malic acid [MAL]	GC-FID	RI	1482
citrate / citric acid [CIT]	GC-FID	RI	1809
Sugar alcohols and cyclic polyols			
sorbitol [SOR]	GC-FID	RI	1920

myo-inositol [INO]	GC-FID	RI	2075
Amino acids			
aspartate / aspartic acid [ASP]	UHPLC-FLD	RT	2.8 min
glutamate / glutamic acid [GLU]	UHPLC-FLD	RT	4.6 min
asparagine [ASN]	UHPLC-FLD	RT	8.7 min
serine [SER]	UHPLC-FLD	RT	9.4 min
glutamine [GLN]	UHPLC-FLD	RT	10.9 min
glycine [GLY]	UHPLC-FLD	RT	12.3 min
threonine [THR]	UHPLC-FLD	RT	12.7 min
citrulline [CITR]	UHPLC-FLD	RT	13.6 min
arginine [ARG]	UHPLC-FLD	RT	15.0 min
alanine [ALA]	UHPLC-FLD	RT	15.7 min
γ-aminobutyrate / γ-aminobutyric acid [GABA]	UHPLC-FLD	RT	16.4 min
tyrosine [TYR]	UHPLC-FLD	RT	18.9 min
cystine [CYS-CYS]	UHPLC-FLD	RT	21.7 min
valine [VAL]	UHPLC-FLD	RT	23.6 min
methionine [MET]	UHPLC-FLD	RT	24.2 min
tryptophan [TRP]	UHPLC-FLD	RT	26.3 min
phenylalanine [PHE]	UHPLC-FLD	RT	27.3 min
isoleucine [ILE]	UHPLC-FLD	RT	27.7 min
leucine [LEU]	UHPLC-FLD	RT	29.3 min
lysine [LYS]	UHPLC-FLD	RT	30.3 min
proline [PRO]	UHPLC-FLD	RT	39.0 min

Iridoid glycosides

aucubin [AUC]	GC-FID	RI	2767
catalpol [CAT]	GC-FID	RI	2857

651

652

FIGURE LEGENDS

FIG. 1. Effects of precipitation and temperature treatments on A) percent nitrogen, B) carbon/nitrogen ratios, C) aucubin concentrations, and D) catalpol concentrations in *Plantago lanceolata* plants; mean ± se of *n* = 4-6. dw: dry weight

FIG. 2. Principal component analysis of leaf metabolites of *Plantago lanceolata* subjected to different precipitation and temperature treatments. Concentrations of sugars, di- and tricarboxylic acids, the sugar alcohol sorbitol, the cyclic polyol *myo*-inositol, and iridoid glycosides (**a,b**) and amino acids (**c,d**)**.** Score plots (**a,c**) with the percent total variance explained by the first two principal components (PCs) in brackets, median scores of each group shown as larger open symbols and convex hulls surrounding each treatment group. Loadings plots (**b,d**) with loading axes on the top and right, loadings depicted as arrows and metabolites abbreviated as in Table 3. *n* = 4-6. Note: Zero values were replaced by random small numbers and data autoscaled.

FIG. 3. Effects of different precipitation and temperature treatments on the concentrations of the iridoid glycosides (a) aucubin and (b) catalpol in *Plantago lanceolata* leaves; mean ± se of n = 4-6. dw: dry weight.

FIG. 4. Cluster heatmap based on fold changes of leaf metabolite concentrations of *Plantago lanceolata* subjected to different precipitation and temperature treatments. Clustering was performed based on mean fold changes (i.e., mean metabolite concentrations in treatment groups divided by those in the common control group; means of *n* = 4-6). Both treatment groups and

metabolites were clustered using the average linkage hierarchical clustering method based on Pearson correlations. Only those 29 metabolites were included that occurred in > 50% of the replicates of all treatment groups. The color code for $\log_2$-scaled fold changes is given at the top. On this color bar, fold change thresholds (orig., untransformed) of < 0.5 (considerable decrease in metabolite pool sizes) and > 2 (considerable increase in metabolite pool size), respectively, are indicated. Abbreviations of metabolites as in Table 2.

FIG. 5. Metabolic pathway map showing fold changes of leaf metabolite concentrations of *Plantago lanceolata* subjected to different precipitation and temperature treatments. The map shows a part of the primary metabolism as well as the biosynthesis of iridoid glycosides. Only some major pathway intermediates are shown; dashed arrows mean that intermediates were omitted. The names of the metabolites that were found in *P. lanceolata* leaves in this study are written in black, whereas others are given in grey. The heatmap stripes (mean $\log_2$-scaled fold changes compared to the common control group) were derived from the cluster heatmap (Figure 2); the corresponding color bar ($\log_2$ scale and original scale) and order of treatments (same order as derived by clustering, see Figure 2) are given in the keys at the top. Full names of metabolites are given in the lower key and in Table 2.

Figure 1

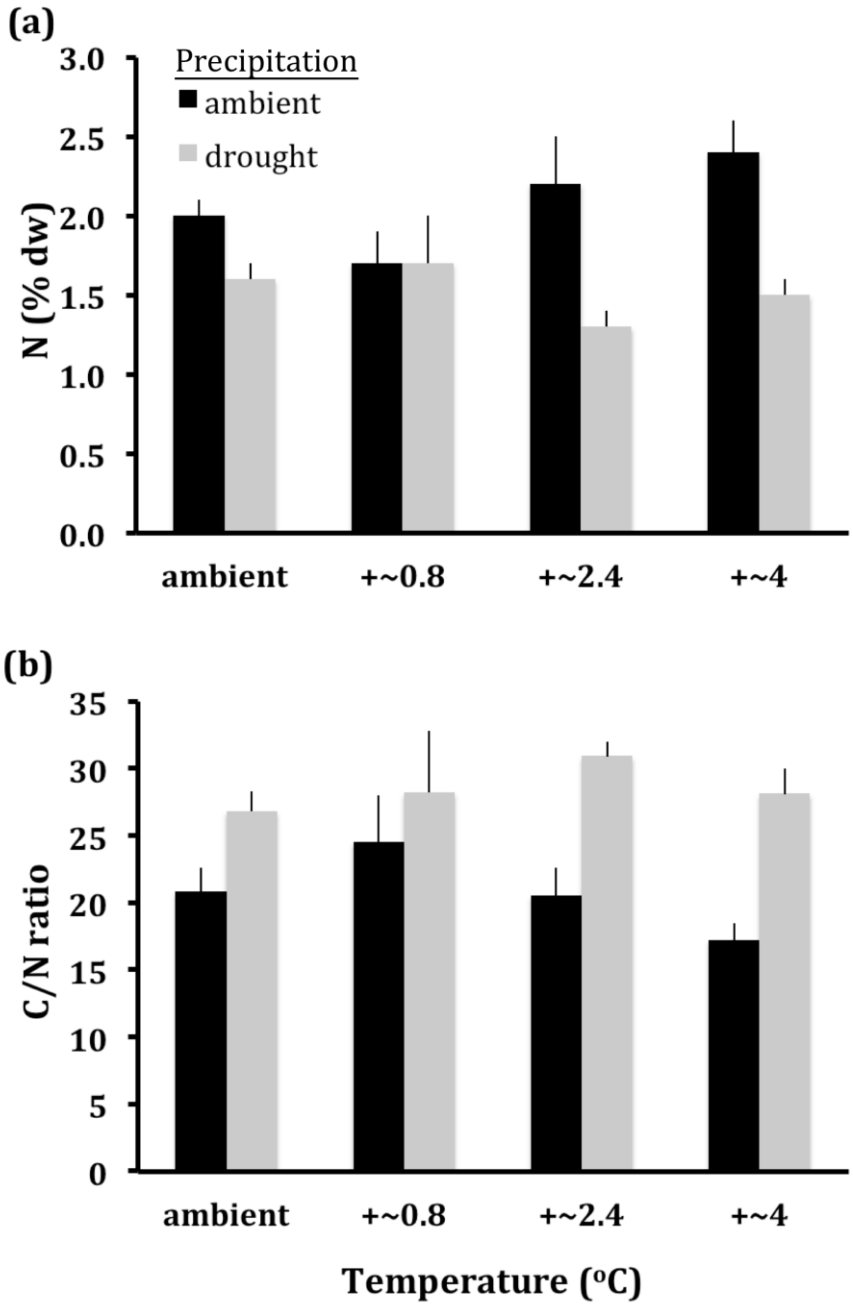

(a)
Precipitation
ambient
drought
N (% dw)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ambient
+~0.8
+~2.4
+~4
(b)
C/N ratio
35
30
25
20
15
10
5
0
ambient
+~0.8
+~2.4
+~4
Temperature (°C)

Figure 2

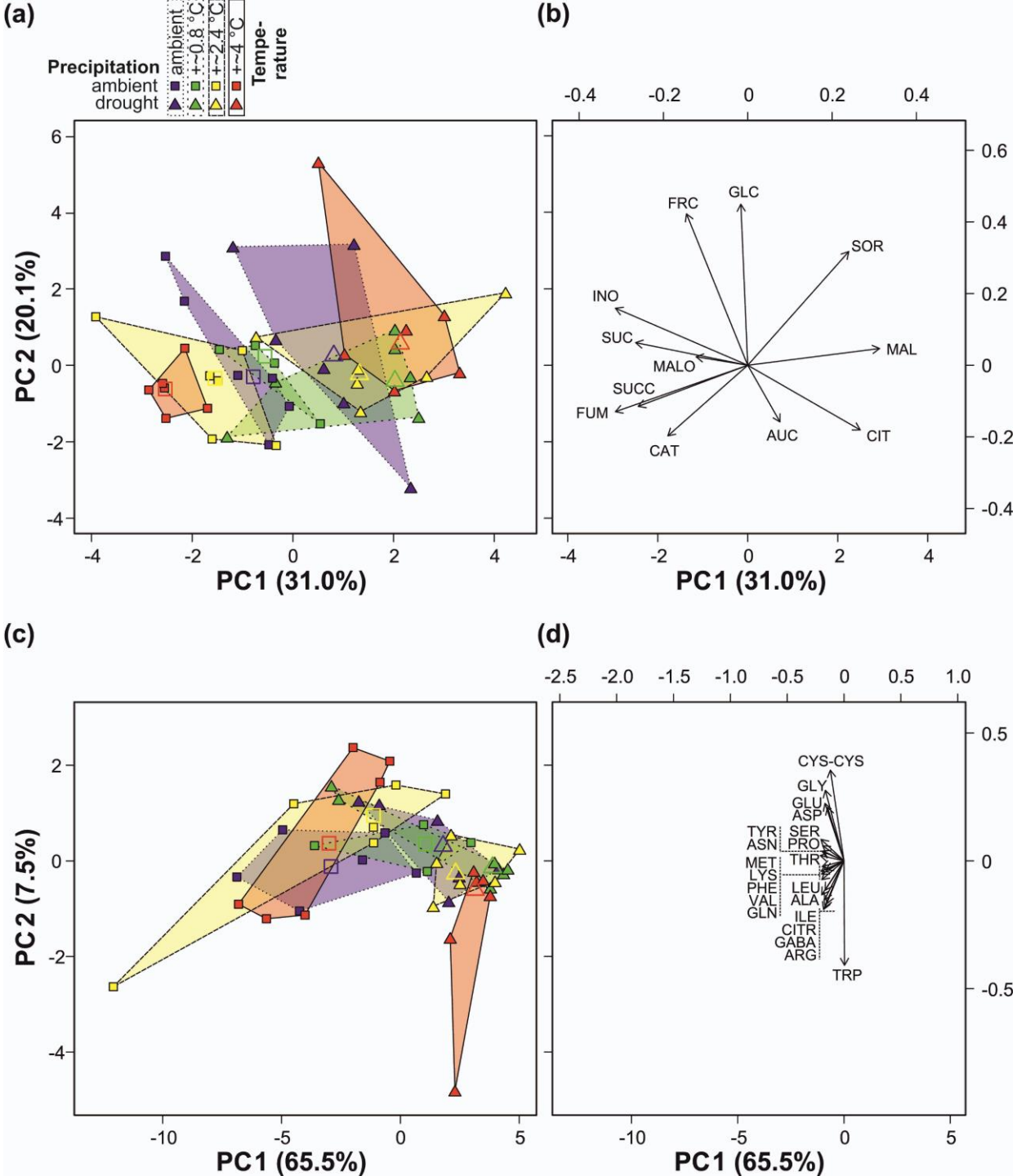

(a)
(b)
(c)
(d)
Precipitation
ambient
drought
Tempe-rature
ambient
+~0.8 °C
+~2.4 °C
+~4 °C
PC2 (20.1%)
PC1 (31.0%)
FRC
GLC
SOR
INO
SUC
MAL
MALO
SUCC
FUM
CAT
AUC
CIT
PC2 (7.5%)
PC1 (65.5%)
CYS-CYS
GLY
GLU
ASP
TYR
SER
ASN
PRO
MET
THR
LYS
PHE
LEU
VAL
ALA
GLN
ILE
CITR
GABA
ARG
TRP

Figure 3

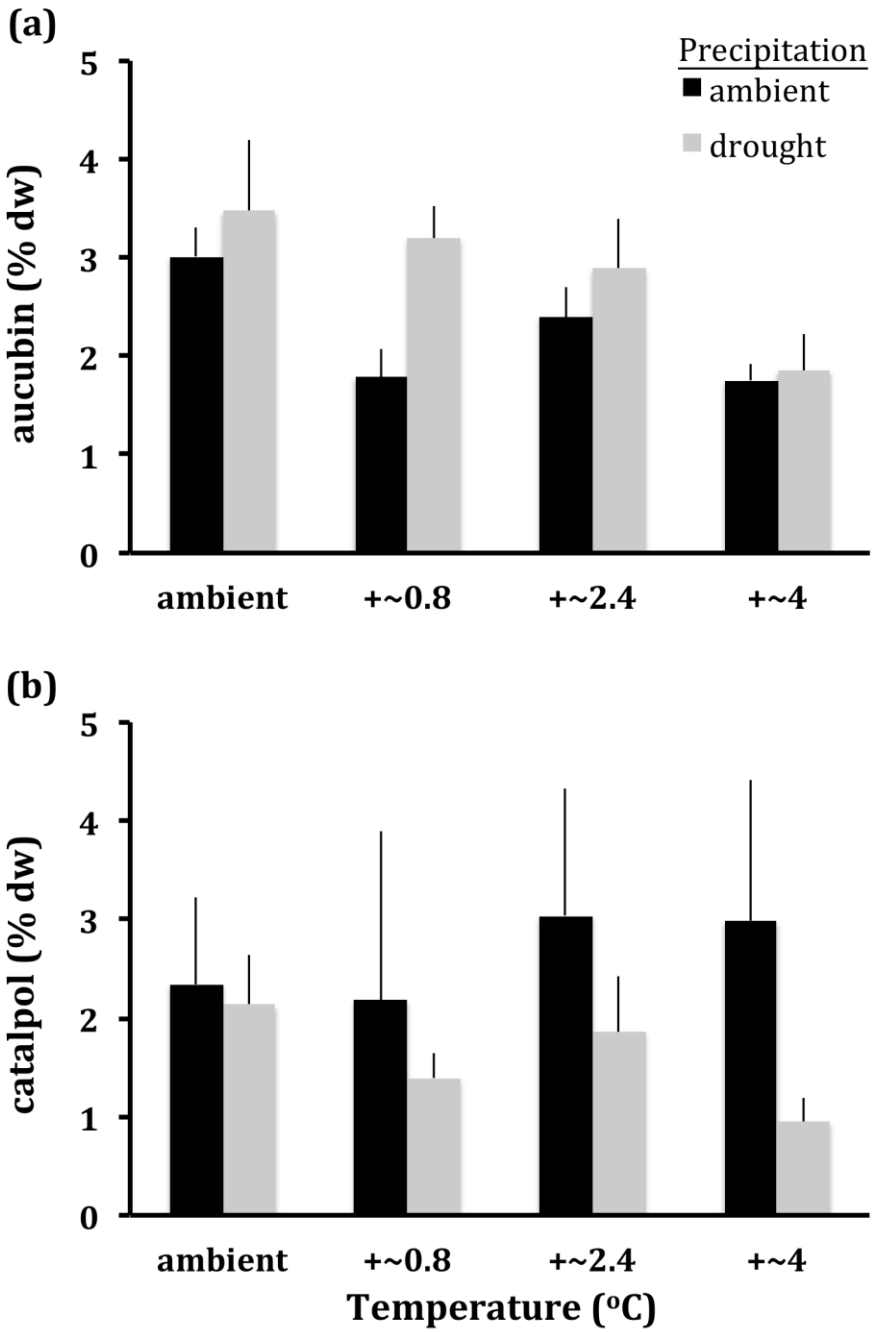

(a)
Precipitation
ambient
drought
aucubin (% dw)
0
1
2
3
4
5
ambient
+~0.8
+~2.4
+~4
(b)
catalpol (% dw)
0
1
2
3
4
5
ambient
+~0.8
+~2.4
+~4
Temperature (°C)

Figure 4

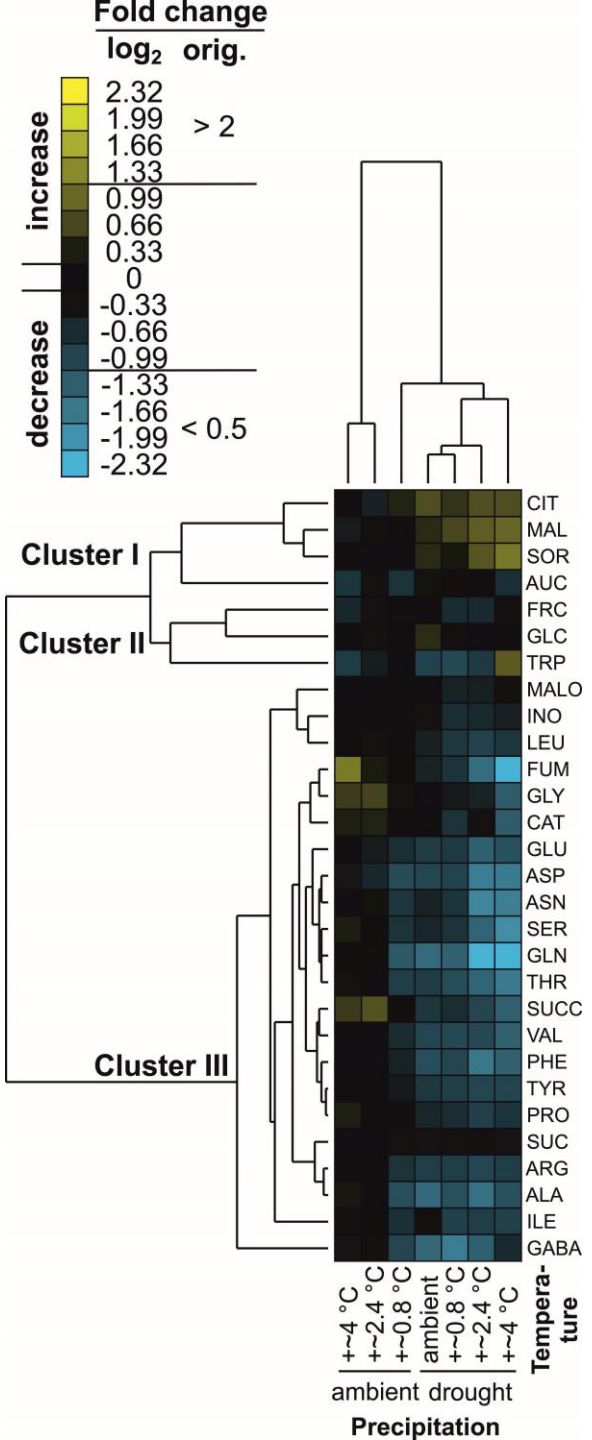
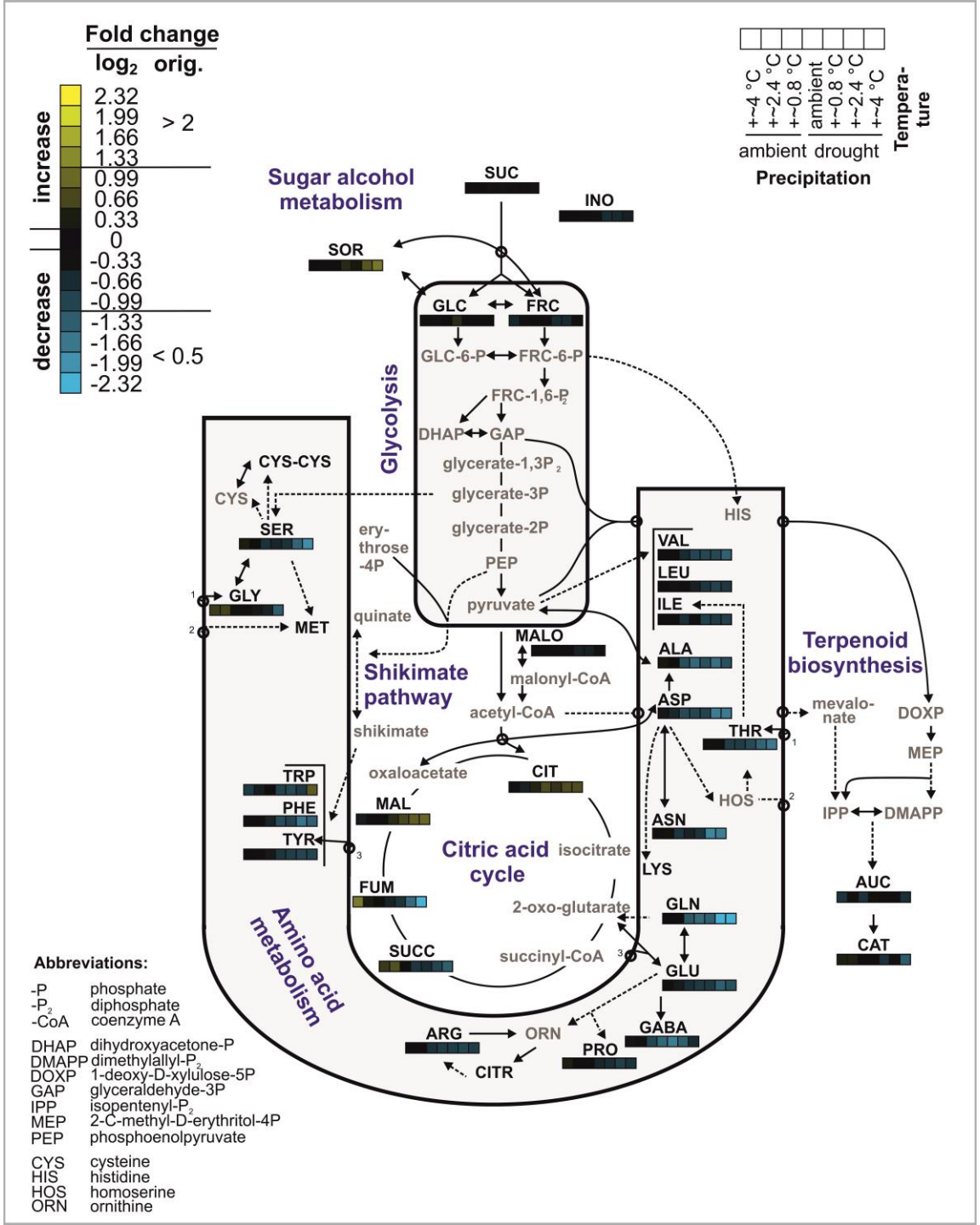

Fold change
log2 orig.
2.32
1.99
1.66
1.33
0.99
0.66
0.33
0
-0.33
-0.66
-0.99
-1.33
-1.66
-1.99
-2.32
> 2
< 0.5
increase
decrease
Cluster I
Cluster II
Cluster III
CIT
MAL
SOR
AUC
FRC
GLC
TRP
MALO
INO
LEU
FUM
GLY
CAT
GLU
ASP
ASN
SER
GLN
THR
SUCC
VAL
PHE
TYR
PRO
SUC
ARG
ALA
ILE
GABA
+~4 °C
+~2.4 °C
+~0.8 °C
ambient
+~0.8 °C
+~2.4 °C
+~4 °C
Tempera-ture
ambient drought
Precipitation

Figure 5


Fold change
log2 orig.
increase
decrease
2.32
1.99
1.66
1.33
0.99
0.66
0.33
0
-0.33
-0.66
-0.99
-1.33
-1.66
-1.99
-2.32
> 2
< 0.5
Precipitation
ambient
drought
+~4 °C
+~2.4 °C
+~0.8 °C
ambient
+~0.8 °C
+~2.4 °C
+~4 °C
Temperature
Sugar alcohol metabolism
SUC
INO
SOR
GLC
FRC
GLC-6-P
FRC-6-P
FRC-1,6-P2
DHAP
GAP
glycerate-1,3P2
glycerate-3P
glycerate-2P
PEP
pyruvate
Glycolysis
ery-throse -4P
quinate
Shikimate pathway
shikimate
CYS-CYS
CYS
SER
GLY
MET
MALO
malonyl-CoA
acetyl-CoA
oxaloacetate
CIT
MAL
FUM
SUCC
isocitrate
2-oxo-glutarate
succinyl-CoA
Citric acid cycle
TRP
PHE
TYR
Amino acid metabolism
HIS
VAL
LEU
ILE
ALA
ASP
THR
HOS
ASN
LYS
GLN
GLU
ARG
ORN
CITR
PRO
GABA
Terpenoid biosynthesis
mevalo-nate
DOXP
MEP
IPP
DMAPP
AUC
CAT
Abbreviations:
-P phosphate
-P2 diphosphate
-CoA coenzyme A
DHAP dihydroxyacetone-P
DMAPP dimethylallyl-P2
DOXP 1-deoxy-D-xylulose-5P
GAP glyceraldehyde-3P
IPP isopentenyl-P2
MEP 2-C-methyl-D-erythritol-4P
PEP phosphoenolpyruvate
CYS cysteine
HIS histidine
HOS homoserine
ORN ornithine