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Article type : Original Research

Interspecific Variation and Elevated CO₂ Influence the Relationship Between Plant Chemical Resistance and Regrowth Tolerance

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Short Running Title: Elevated CO₂, Plant Resistance and Tolerance

ABSTRACT

To understand how comprehensive plant defense phenotypes will respond to global change, we investigated the legacy effects of elevated CO₂ on the relationships between chemical resistance (constitutive and induced via mechanical damage) and regrowth tolerance in four milkweed species (*Asclepias*). We quantified potential resistance and tolerance tradeoffs at the physiological level following simulated clipping/mowing, which are relevant to milkweed ecology and conservation. We examined the legacy effects of elevated CO₂ on four hypothesized tradeoffs between: 1) plant growth

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1002/ECE3.6284](https://doi.org/10.1002/ECE3.6284)

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31 rate and constitutive chemical resistance (foliar cardenolide concentrations), 2) plant growth rate and
32 mechanically induced chemical resistance, 3) constitutive resistance and regrowth tolerance, and 4)
33 regrowth tolerance and mechanically induced resistance. We observed support for one tradeoff
34 between plant regrowth tolerance and mechanically induced resistance traits that was, surprisingly,
35 independent of CO₂ exposure. Across milkweed species, mechanically induced resistance increased by
36 28% in those plants previously exposed to elevated CO₂. In contrast, constitutive resistance, and the
37 diversity of mechanically induced chemical resistance traits declined in response to elevated CO₂ in two
38 out of four milkweed species. Finally, previous exposure to elevated CO₂ uncoupled the positive
39 relationship between plant growth rate and regrowth tolerance following damage. Our data highlight
40 the complex and dynamic nature of plant defense phenotypes under environmental change and
41 question the generality of physiologically-based defense tradeoffs.

42

43 **Key Words**- *Asclepias*, Cardenolides, Global change ecology, Plant-herbivore interactions, Resistance to
44 herbivory.

45 INTRODUCTION

46 Plants employ a suite of defensive traits to deter and minimize the impacts of herbivory (Agrawal and
47 Fishbein 2006). As a result, critical factors that contribute to plant fitness in the context of damage may
48 be overlooked when defenses are studied in isolation (Baucom and De Roode 2011). Regrowth
49 tolerance and chemical resistance are two major strategies of defense that plants employ against
50 herbivory, and that jointly influence fitness (Strauss and Agrawal 1999; Stamp 2003; Núñez-Farfán et al.
51 2007; Agrawal 2011; Fornoni 2011; Zas et al. 2011). One major form of defense, resistance to herbivory,
52 occurs through physical and chemical traits such as trichomes, latex exudation, thorns, and toxic
53 secondary metabolites that together reduce herbivore performance (Rhoades 1985). Multiple resistance
54 traits can be both constitutively expressed before damage and induced following damage, however
55 chemical resistance is perhaps best known for this temporal strategy (Agrawal and Karban 1999; Karban
56 and Baldwin 2013). Because resistance traits are themselves metabolically costly (Gershenson 1994;
57 Strauss et al. 2002; Bekaert et al. 2012), it is thought that these costs manifest in the form of trade-offs
58 with other plant functions like growth rates (van der Meijden et al. 1988; Fineblum et al. 1995; Stamp
59 2003; Agrawal 2011). Plant tolerance to herbivory, or compensatory growth following damage,
60 minimizes fitness losses through simultaneous shifts in physiology and resource allocation (Rosenthal
61 and Kotanen 1994; Strauss and Agrawal 1999; Fornoni et al. 2003).

62

63 Numerous hypotheses have been developed to predict resource allocation to the competing plant
64 functions of chemical resistance and regrowth tolerance, often with variable generality and empirical
65 support (*reviewed in* Stamp 2003). On a macroevolutionary level, the resource availability hypothesis
66 (RAH) predicts that high resource environments select for fast-growing species that invest in regrowth
67 tolerance following damage rather than chemical defense production (Coley and Chapin 1985; Endara
68 and Coley 2011). But fewer hypotheses address the interplay of tolerance and chemical defense within
69 populations (Hahn and Maron 2016; Hahn et al. 2019) or physiologically within the lifetime of
70 individuals. At the cellular and tissue level, the growth-differentiation balance hypothesis (GDB) posits
71 that plants in high resource environments will not be limited by photosynthesis, and will allocate more
72 energy into regrowth rather than into cellular differentiation-related processes such as secondary
73 metabolism (Herms and Mattson 1992). At intermediate resource conditions, the GDB predicts that
74 plants will be limited in growth but not photosynthetic capacity and will produce more secondary
75 metabolites relatively cheaply. Due to the need to test at multiple resource levels, and measure not only
76 growth rate, but net assimilation and secondary metabolism, the GDB has proven difficult to test but
77 still provides a useful framework of plant defense at the physiological level (Stamp 2004). In general,
78 trade-offs between tolerance and chemical resistance arise as a result of plant allocation strategies
79 meant to optimize fitness in a variable environment (Züst and Agrawal 2017). Therefore, understanding
80 the environmental conditions under which trade-offs manifest is of critical importance.

81
82 The rapidly rising concentration of atmospheric carbon dioxide is well-known to influence chemical
83 resistance to herbivores and plant growth rates. Both the composition and concentration of
84 constitutively expressed and induced plant secondary metabolites change in response to elevated CO₂
85 depending on the class of compounds considered (Hunter 2001; Bidart-Bouzat et al. 2005; Ryan et al.
86 2010; Robinson et al. 2012; Zavala et al. 2013; Klaiber et al. 2013; Jia et al. 2016). Further, elevated CO₂
87 suppresses the synthesis of jasmonic acid and stimulates the production of salicylic acid, compromising
88 the plant's ability to mount an induced resistance response (Ode et al. 2014).

89
90 Changes in phytohormonal signaling pathways also mediate plant growth and regrowth tolerance
91 following damage under elevated CO₂ (Guo et al. 2012). In general, by increasing photosynthesis and
92 water use efficiency, elevated CO₂ positively affects plant growth rates (Drake et al. 1997; Ainsworth &
93 Long 2005; Robinson et al. 2012; Bazzaz et al. 2013). However, the direct effects of elevated CO₂ on
94 plant regrowth tolerance following damage can be negative (Wilsey 2001; Marshall et al. 2008; Lau and

95 Tiffin 2009; Guo et al. 2012) partially because of increased nutrient limitation under elevated CO₂ paired
96 with phytohormonal suppression. Studies that explore the integrated influence of elevated CO₂ on the
97 relationships between resistance and tolerance are sorely lacking.

98
99 Even less is known about the lingering effects of past CO₂ enrichment on plants. Though not ecologically
100 plausible, the modulation of exposure to environmental change drivers such as elevated CO₂ partially
101 reveals energetic allocation decisions made by plants under future conditions, and the persistence of
102 those responses. Extrapolations based on the substantial below-ground carbon sink and increased soil
103 microbial turnover that develops in response to elevated CO₂ predict mixed but lingering effects of
104 elevated CO₂ on plant regrowth tolerance (Hungate et al. 2006; Stiling et al. 2013). To our knowledge,
105 only two studies have examined plant responses to elevated CO₂ beyond the cessation of enrichment,
106 and found lasting effects on aspects of root morphology such as fine root hairs (Stiling et al. 2013) and
107 increases in regrowth tolerance following fire (Bain and Day 2019). These studies follow plant and
108 arthropod communities in the years following enrichment cessation, yet how plant physiological
109 properties will respond to abrupt changes in CO₂ enrichment over the course of a growing season
110 remains to be tested.

111
112 Here, we investigate the legacy effects of elevated CO₂ on the chemical resistance traits and regrowth
113 tolerance of four milkweed species (*Asclepias*). Specifically, we examined the effects of elevated CO₂ on
114 four hypothesized tradeoffs between: 1) initial growth rate and constitutive chemical resistance, 2)
115 initial growth rate and mechanically induced chemical resistance, 3) constitutive chemical resistance and
116 regrowth tolerance following damage, and 4) regrowth tolerance and mechanically induced chemical
117 resistance. To our knowledge, no theory exists to predict the interaction between resistance and
118 regrowth tolerance strategies under changing carbon supplementation. Nevertheless, we predicted that
119 elevated CO₂ would: a) induce higher growth rates and regrowth rates and depress constitutive
120 secondary metabolites following the GDB hypothesis; and b) mitigate, in part, any tradeoff between
121 chemical resistance traits and regrowth tolerance in milkweed. By analyzing changes in plant tolerance
122 and resistance chemistry, we aimed to improve our understanding of how future environmental
123 conditions may influence the defensive phenotype of plants, with implications for the herbivore
124 communities that damage them.

125
126

127 **MATERIALS AND METHODS**

128

129 *Study System:*

130 The four milkweed, *Asclepias*, species used in our study (*A. syriaca*, *A. speciosa*, *A. incarnata*, and *A.*
131 *curassavica*) originate from North and Central America (Woodson 1954) and support herbivores that
132 range from phloem feeding insects such as oleander aphids (*Aphis nerii*) to chewing insects capable of
133 removing large amounts of tissue, like monarch caterpillars (*Danaus plexippus*), and long horn beetles
134 (*Tetraopes* spp.). Most milkweed herbivores specialize within the genus because *Asclepias* produce a
135 well-characterized suite of defenses against herbivory.

136

137 To physically deter feeding by arthropod herbivores, milkweed plants exude latex, produce trichomes,
138 and increase leaf toughness (Hochwender et al. 2000; Zalucki et al. 2001; Agrawal and Fishbein 2006;
139 Agrawal and Konno 2009). However, milkweeds are best known for synthesizing a class of toxic steroids
140 known as cardenolides that disrupt Na⁺/K⁺-ATPase in the Na⁺/K⁺-channels of animal cells (Agrawal et al.
141 2012). The composition and concentration of cardenolides produced constitutively by milkweed plants
142 vary substantially within and among milkweed species (Rasmann and Agrawal 2011; Agrawal et al.
143 2012). Damage induces quick increases in cardenolide concentrations and changes in cardenolide
144 composition (Malcolm and Zalucki 1996). Regrowth following damage also plays a prominent role in the
145 defensive phenotype of milkweeds (Agrawal and Fishbein 2008; Tao et al. 2016). Despite a growing body
146 of work illustrating the effects of environmental change on milkweed chemistry and milkweed growth
147 (Vannette and Hunter 2011; Matiella 2012; Tao et al. 2014; Andrews 2015), no study to date has
148 explored the interplay between milkweed chemical resistance traits (both constitutive and induced) and
149 regrowth tolerance under future environmental conditions.

150

151 We grew four species of milkweed under ambient (400 ppm) and elevated (760 ppm) concentrations of
152 atmospheric CO₂ at the University of Michigan Biological Station (UMBS). To manipulate atmospheric
153 CO₂ concentrations, we used an outdoor array consisting of 40 open-top chambers, with 20 chambers
154 maintained at ambient CO₂ and 20 chambers maintained at elevated CO₂ from May through August of
155 2015. Chambers were 1 m high cubes with an octagonal top of diameter of 0.8 m composed of a PVC
156 frame and clear plastic walls following a modified design of Drake, Leadley, Arp, Nassiry, & Curtis (1989).

157

158 We chose *Asclepias* species that vary in foliar cardenolide concentrations. Specifically, we included *A.*
159 *incarnata* (low cardenolide), *A. speciosa*, *A. syriaca* (both medium cardenolide), and *A. curassavica* (high
160 cardenolide). Seeds of *A. speciosa* and *A. curassavica* were obtained from commercial sources (Prairie
161 Moon Nurseries, Winona, USA) and seeds of *A. incarnata* and *A. syriaca* were collected locally
162 (Cheboygan county, MI). We surface sterilized all seeds following a six-week cold stratification period
163 (for all but tropical *A. curassavica*), and germinated seeds on moist filter paper for 1 week. We planted
164 seedlings in 983 cm³ deepots™ (6.9 cm diameter by 35.6 cm height) containing Metromix 360 (SunGro
165 Horticulture, Vancouver, BC) and Osmocote controlled release fertilizer [N:P:K:16:16:16 ppm N (g/g)]
166 (ICL Specialty Fertilizers, Dublin, USA) on 5-May-15. Germinated seedlings were watered daily and grown
167 in the UMBS greenhouse for two weeks before they were moved to randomly assigned chambers in the
168 CO₂ array. Once in the array, potted plants were maintained under their CO₂ treatments for three
169 months. To minimize the entrance of herbivores into the chambers, we placed fine mesh coverings over
170 the openings of each chamber and physically removed any herbivores that we observed during daily
171 visual inspections.

172
173 Within each chamber, we grew as many as seven plants of each milkweed species. Low germination
174 success limited the number of *A. speciosa* and *A. syriaca* used in this study, and not all milkweed species
175 were represented in every chamber. Overall, our eight treatments (2 CO₂ treatments x 4 milkweed
176 species) combined for a total of 442 plants, with exact replicate numbers reported in Table 1.

177
178 Using a LI-COR 320 IRGA (LI-COR, Lincoln, USA), we monitored atmospheric CO₂ concentrations daily in
179 the 20 elevated CO₂ chambers and in one randomly selected ambient CO₂ chamber. Concentrations of
180 CO₂ were adjusted throughout the day to maintain the target of 760 ppm in each elevated chamber. The
181 ambient temperature inside each chamber was recorded every hour using a thermochron datalogger
182 (Thermochron, Baulkham Hills, Australia). Elevated CO₂ chambers averaged 21.03 (±0.034) °C, and
183 ambient CO₂ chambers averaged 21.24 (± 0.038) °C, roughly 2°C higher than the outside average
184 temperature of 18.93 (± 0.039) °C.

185
186 *Simulated Damage and Growth Measures:*

187 Three months following the initial transfer of plants into the array, we simulated clipping/mowing by
188 cutting all plants at the soil line. Many milkweed habitats important to the specialist herbivores
189 associated with milkweed are located near roadways and agricultural fields that are regularly mowed.

190 Properly timed mowing can improve reproduction and decrease predator abundance of certain
191 milkweed specialists, including the monarch butterfly (Haan and Landis 2019). Thus, our simulated
192 mowing represents an ecologically relevant stress regularly experienced by many milkweed plants.
193 Moreover, at our field site in northern Michigan, we have observed chipmunks (*Tamius striatus*),
194 milkweed stem weevils (*Rhyssomatus lineaticollis*) and porcupines (*Erethizon dorsatum*) all remove the
195 entire above ground tissues of milkweed plants. Other herbivores such as monarch caterpillars, and
196 milkweed tussock moths (*Euchaetes egle*), have also been observed to remove large amounts of foliage.
197 Thus, our clipping treatment also represents severe but not infrequent levels of herbivore damage
198 experienced by milkweed plants. We recognize that mechanical damage does not completely mimic
199 actual herbivory because oral secretions and regurgitant released from the herbivore at the time of
200 feeding can enter wounded plant tissue, inducing the release of jasmonic acid, a phytohormone critical
201 to the production of defensive secondary metabolites (McCloud and Baldwin 1997).

202
203 The aboveground biomass that we removed was dried at 60°C, weighed, and used to calculate growth
204 rate prior to damage (below). Cut plants were watered, moved to the UMBS greenhouse, and
205 maintained under identical (ambient CO₂) conditions for three weeks due to external limitations on use
206 of the chambers. However, by re-growing clipped plants under ambient CO₂ we are able to isolate the
207 legacy effects of altered carbon availability prior to damage on regrowth tolerance, and potential
208 tradeoffs between growth and resistance. Thus, we can examine the repercussions of previous energetic
209 allocation decisions made by plants under carbon enriched conditions in comparison to those under
210 ambient conditions. After a three-week period, the aboveground regrowth plant material was
211 harvested, dried at 60°C, and weighed as a measure of regrowth tolerance.

212
213 For a measure of growth rate prior to damage, we divided the aboveground dry biomass of the plant by
214 64 days (the number of days since the seedling had been transferred to soil) following Agrawal &
215 Fishbein (2008). Similarly, to calculate plant regrowth rate following mechanical damage, we divided the
216 mass of the regrowth material by 21 days (the length of time plants were allowed to regrow following
217 damage). Differences in regrowth rate following damage are important for the competitive success and
218 ultimate fitness of plants (Züst and Agrawal 2017).

219

220 *Chemical Analyses & Resistance Classifications:*

221 We collected samples of the original aboveground foliage, and the regrowth foliage of each plant for
222 cardenolide analysis using established methods (Zehnder and Hunter 2009; Vannette and Hunter 2011;
223 Tao and Hunter 2012). Roughly 20 mg of dried plant material was ground in a ball mill, deposited in 1 mL
224 methanol, and stored at -10°C prior to analysis. Cardenolides were extracted, separated and quantified
225 with a 0.15mg/mL digitoxin internal standard (Sigma Chemical Company, St. Louis, Missouri, USA), by
226 reverse-phase high-performance liquid chromatography (HPLC) on a Waters Acquity UPLC with PDA
227 detector (Waters Corporation, Milford, MA, USA). Peaks with symmetrical absorbance between 217-222
228 nm were identified as cardenolides. Cardenolide concentrations were calculated as the sums of all
229 separated peak areas, corrected by the concentration of the internal digitoxin standard and sample dry
230 mass. We used digitoxin as an internal standard because it is absent from *Asclepias* and because purified
231 standards remain unavailable for a majority of milkweed cardenolides. We recognize that cardenolides
232 may differ in their concentration-area relationships, and our estimates of cardenolide concentration
233 should be considered as measured in digitoxin-equivalents. Because milkweed plants were grown in
234 field mesocosms which excluded herbivores all season, the foliar cardenolides measured from plants
235 prior to simulated damage represent natural levels of constitutive resistance. Conversely, the foliar
236 cardenolide concentrations of regrown tissue following clipping, represent mechanically induced
237 resistance.

238

239 *Statistical Analyses:*

240 In all analyses that follow, we used either linear mixed models (LMMs; Lme4 package) or generalized
241 linear mixed models (GLMMs; Lme4 package). To account for variation among chambers and the non-
242 independence of plants grown within the same individual chamber, we included chamber identity as a
243 random effect in all of our models described below. This design allows us to test our hypotheses at the
244 level of plant individuals to capture relevant variation in our analyses, while accounting for multiple
245 plants within chambers. We performed all statistical tests in R version 3.6.0 (R Development Core Team,
246 2018), and selected models using likelihood ratio tests (Burnham and Anderson 2002). Variables were
247 transformed to best achieve normality of error as tested by the Shapiro-Wilk normality test.
248 Homogeneity of variance and distribution of residuals were inspected using quantile-quantile and
249 residuals fitted-value plots to check for conformation to model assumptions (Crawley 2012).

250

251 *Testing for Tradeoffs Among Milkweed Growth, Regrowth Tolerance, and Resistance Chemistry*

252 1) *Plant growth rate and chemical resistance before damage*: We used an LMM with log-transformed
253 initial foliar cardenolide concentrations as the dependent variable and square-root-transformed growth
254 rate prior to clipping, CO₂ treatment, and milkweed species as fixed effects. An interaction between
255 growth rate prior to clipping and CO₂ indicates a difference between the CO₂ treatments in the extent to
256 which growth rate correlates with the production of cardenolides.

257
258 2) *Plant growth rate before damage and mechanically induced resistance of regrowth tissues*: We used
259 an LMM with log-transformed foliar cardenolide concentrations of the regrowth foliage as the
260 dependent variable and square-root-transformed growth rate prior to clipping, CO₂ treatment, and
261 milkweed species as fixed effects. An interaction between initial growth rate and CO₂ indicates a
262 difference between CO₂ treatments in the potential tradeoff between plant growth rate before damage
263 and chemical resistance after damage.

264
265 3) *Chemical resistance before damage and regrowth tolerance*: Likewise, we ran an LMM with square-
266 root-transformed regrowth rate as the response variable and log-transformed initial foliar cardenolide
267 concentrations, CO₂ treatment, and milkweed species as fixed effects. An interaction between initial
268 foliar cardenolide concentration and CO₂ indicates a difference between atmospheres in the relationship
269 between initial plant chemical resistance and regrowth.

270
271 4) *Regrowth tolerance and the mechanically induced resistance of regrowth tissues*: Lastly, we ran an
272 LMM with log-transformed regrowth foliar cardenolide concentrations as the response variable and
273 square-root-transformed regrowth rate, CO₂ treatment, and milkweed species as fixed effects. A
274 significant interaction between CO₂ treatment and regrowth rate would signify a difference between the
275 two atmospheres in any correlation between the two defense traits.

276
277 *Elevated CO₂, Milkweed Species, and Plant Growth and Resistance Profiles*:

278 While the tradeoff model framework described above provided some information on how growth rates
279 and chemical resistance responded to our treatments, we also performed the following additional
280 analyses to ask further questions about defense phenotypes. To determine the effects of our treatments
281 on plant growth rate prior to damage and regrowth rate after damage, we used CO₂ treatment, the
282 probability of regrowth, and milkweed species as fixed effects and square-root transformed growth
283 rates (mg/day) as response variables. Not all milkweed individuals regrew following damage. We

284 therefore used generalized linear mixed models with binomial error distributions and logit link functions
285 to assess the effects of plant species and CO₂ treatment on the proportion of milkweed plants that
286 regrew following damage.

287

288 We then examined how CO₂ treatment and species influenced the relationship between growth rate
289 prior to damage and regrowth rate following damage, using an LMM with square-root transformed
290 regrowth rate as the response variable and square-root transformed initial growth rate, CO₂ treatment
291 and species as fixed effects.

292

293 Plant chemical defense encompasses not only the total concentration of defense compounds but also
294 the diversity of chemical species produced. We therefore examined the relationships between
295 cardenolide community diversity and growth rates. We calculated cardenolide diversity using the
296 Shannon diversity index borrowed from the biodiversity literature: $H = -\sum(P_i \log[P_i])$ where P_i is the
297 relative amount of a cardenolide peak compared to the total amount of cardenolides in an individual
298 plant (Rasmann & Agrawal 2011). Similar to above, we selected simplified models from two starting
299 LMMs: 1) with constitutive foliar cardenolide concentrations as the dependent variable and square-root-
300 transformed growth rate prior to clipping, CO₂ treatment, and milkweed species as fixed effects; and 2)
301 with mechanically induced foliar cardenolide concentrations as the dependent variable and square-root-
302 transformed regrowth rate, CO₂ treatment, and milkweed species as fixed effects.

303

304 To compare the effects of CO₂ treatment, and milkweed species on the community of cardenolide
305 compounds produced in the plants before and after damage, we used permutational multivariate
306 analysis of variance (PerMANOVA; Anderson, 2001). The model included CO₂ treatment, milkweed
307 species, tissue type, and their interactions as fixed effects, and Bray-Curtis distance of percentage
308 weight of each foliar cardenolide peak as dependent variables. To visualize these differences, we used
309 non-metric multidimensional scaling (NMDS) with 999 permutations per model run and a maximum of
310 500 runs per dimension (model stress = 0.200). PerMANOVA and NMDS scaling were performed using
311 the VEGAN package in R (Oksanen et al. 2017).

312

313 **RESULTS**

314

315 **Only Regrowth Tolerance and Induced Resistance Traded Off Among Individuals Following Mechanical**
316 **Damage**

317 1) *Plant growth rate and constitutive resistance*: Milkweed growth rate prior to damage was unrelated
318 to foliar constitutive cardenolide concentrations prior to damage (initial growth rate: $F_{1,195} = 2.72$, $p =$
319 0.100 , Fig. 1a, Table 2). Elevated CO_2 had no effect on this nonsignificant relationship (CO_2 *initial growth
320 rate: $F_{1,195} = 0.46$, $p = 0.499$).

321
322 2) *Plant growth rate before damage and mechanically induced resistance*: Instead of a tradeoff between
323 growth rate prior to damage and the mechanically induced chemical resistance of regrown tissues
324 following damage, we found a positive relationship that weakened (became less steep) under elevated
325 CO_2 (CO_2 *initial growth rate: $F_{1,215} = 5.33$, $p = 0.022$, Fig. 1b, Table 2).

326
327 3) *Constitutive resistance before damage and regrowth tolerance after damage*: Similarly, we observed a
328 weak positive relationship between constitutive chemical resistance and regrowth tolerance
329 (constitutive resistance: $F_{1,208} = 3.66$, $p = 0.057$, Fig. 1c, Table 2). Model selection eliminated models
330 containing the influence of CO_2 on this relationship.

331
332 4) *Regrowth tolerance and mechanically induced resistance of regrown tissues*: In contrast to the first
333 three potential tradeoffs, we observed a significant tradeoff between regrowth tolerance and the
334 mechanically induced chemical resistance of regrown foliage (Regrowth rate*milkweed species: $F_{1,215} =$
335 7.18 , $p = 0.0001$, Fig. 1d, Table 2). The tradeoff was determined by two of the four milkweed species (*A.*
336 *incarnata* and *A. speciosa*). As above, our selection process eliminated models containing the influence
337 of CO_2 on this relationship.

338
339 **Elevated CO_2 eliminated the positive relationship between initial growth rate and regrowth tolerance**
340 **following damage.**

341 Across all milkweed species, elevated CO_2 induced an average 24% increase in growth rate (CO_2 :
342 $F_{1,151} = 9.71$, $p = 0.002$, Fig. 2a) illustrating the classic effect of CO_2 fertilization on plant growth (Kimball
343 1983; Leadley et al. 1999). Initial growth rates of milkweed increased most strongly in *A. syriaca* (43%)
344 followed by *A. incarnata* (31%), *A. curassavica* (12%) and *A. speciosa* (7%) (species* CO_2 : $F_{3,409} = 3.24$,
345 $p = 0.022$, Fig. 2a). Surprisingly, previous CO_2 exposure had no effect on regrowth tolerance across
346 milkweed species (CO_2 : $F_{1,61} = 0.09$, $p = 0.77$, Fig. 2b, Table 2), nor was there an interaction between

347 species and CO₂ treatment on milkweed regrowth tolerance (species*CO₂: F_{3,207} = 0.83, p = 0.477, Table
348 2). This result contradicted our original prediction that increased carbon availability and reduced water
349 loss under elevated CO₂ would favor faster rates of regrowth in damaged plants. Milkweed regrowth
350 rate following damage was highest in *A. curassavica* (10.05 ± 0.45 mg/day) and lowest in *A. syriaca* (2.12
351 ± 0.34 mg/day) (species: F_{3,208} = 24.27, p < 0.0001, Fig. 2b, Table 2).

352
353 Intriguingly, elevated CO₂ weakened the positive relationship between initial plant growth rate and
354 regrowth rate following damage (Regrowth rate* CO₂: F_{1,263} = 5.99, p = 0.015, Fig. 3, Table 3). In other
355 words, future atmospheric concentrations of CO₂ uncoupled the relationship between regrowth
356 tolerance following damage and initial growth rate before damage. Following mechanical damage, only
357 278 of the 442 plants (63%) regrew aboveground tissue. Despite previous carbon supplementation,
358 elevated CO₂ did not affect the probability of regrowth ($\chi^2 = 0.16$, p = 0.6875, Fig. 4), nor was there an
359 interaction between milkweed species and CO₂ treatment on regrowth probability ($\chi^2 = 1.47$, p = 0.689,
360 Fig. 4).

361

362 ***Elevated CO₂ altered the magnitude and diversity of chemical resistance.***

363 Elevated CO₂ reduced constitutive resistance in *A. incarnata* by 37%, in *A. syriaca* by 10%, slightly in *A.*
364 *curassavica* by 5%, and increased constitutive resistance in *A. speciosa* by 22% (species*CO₂: F_{3,207} = 3.84,
365 p = 0.010, Fig. 5a, Table 2). Milkweed species was by far the most important determinant of constitutive
366 cardenolide concentration (species: F_{3,207} = 189.32, p < 0.0001, Fig. 5a, Table 2). In those plants that did
367 regrow following damage, mechanically induced resistance varied substantially by milkweed species
368 (species: F_{3,215} = 8.59, p < 0.0001, Fig. 5b, Table 2). *A. curassavica* again produced the highest
369 concentrations of foliar cardenolides, followed by *A. speciosa*, *A. syriaca* and *A. incarnata*. Across all four
370 species, mechanically induced resistance increased by 28% in those plants previously exposed to
371 elevated CO₂ (CO₂: F_{1,213} = 4.90 p = 0.028, Fig. 5b, Table 2).

372

373 The diversity of cardenolides produced constitutively among milkweed species increased by 24% under
374 elevated CO₂ (CO₂: F_{1,68} = 4.08, p = 0.047, Fig. 5c, Table 4). Despite a species-specific effect of elevated CO₂
375 on the total concentration of constitutive resistance, there was no such effect on the diversity of
376 cardenolides produced constitutively (species*CO₂: F_{3,206} = 2.04, p = 0.109, Fig. 5c, Table 4). Conversely,
377 the diversity of cardenolides produced in the mechanically induced resistance profiles of both *A.*

378 *incarnata*, and *A. speciosa* declined by 70% and 11% after previous exposure to elevated CO₂
379 (species*CO₂: F_{3,20}= 2.67, p = 0.048, Fig. 5d, Table 4).

380
381 When comparing the composition of cardenolide communities among individuals before and after
382 damage, the difference between constitutive and mechanically induced foliar tissue was the strongest
383 driver of community dissimilarity as determined by PerMANOVA (resistance type: F_{1,410}= 55.38, p =
384 0.001, R²=0.15, Fig. 6, Table 5). There were slight differences between these two resistance profiles
385 among milkweed species driven by elevated CO₂ (resistance type*species*CO₂: F_{2,410} = 2.39, p = 0.001,
386 R²=0.013, Fig. 6, Table 5), and these slight differences likely represent the changes in cardenolide
387 diversity detected above.

388

389 **DISCUSSION**

390

391 Our study reveals the limitations of a tradeoff framework at the physiological level when considering
392 how complex defense phenotypes respond to environmental change. Of the four hypothesized tradeoffs
393 among aspects of plant growth and resistance framing the study, we found support for only one
394 between regrowth tolerance and mechanically induced chemical resistance (foliar cardenolide
395 concentration following mechanical damage). The strength of this tradeoff was unaffected by previous
396 exposure to elevated CO₂ but varied substantially among milkweed species, presumably reflecting
397 species-specific allocation patterns to defense following damage. In contrast to expected tradeoffs, we
398 found positive relationships among some growth and resistance traits. However, the positive
399 relationship between growth rate prior to damage and mechanically induced chemical resistance was
400 weaker under previous exposure to elevated CO₂. Our data add to a growing body of work that
401 demonstrates the complex nature of plant growth and resistance relationships and highlights the need
402 to test allocation strategies of plants in the context of rapidly changing environmental resources on
403 ecological time scales as well as across evolutionary contexts.

404

405 Multiple mechanisms may govern the direction and magnitude of growth and resistance relationships in
406 plants. These mechanisms include nutrient limitation, allocation costs, genetic linkage of defense traits,
407 and ecological costs (Simms and Rausher 1987; Strauss et al. 1999; Fine et al. 2006; Boege et al. 2007;
408 Wise and Abrahamson 2007; Tucker and Avila-Sakar 2010; Tao et al. 2016; Züst and Agrawal 2017).
409 Among plants that regrew following damage, we found evidence of a tradeoff between mechanically

410 induced cardenolide concentrations and regrowth tolerance in three of four milkweed species (Fig. 1d).
411 This finding supports previous studies that have reported negative relationships between milkweed
412 growth and cardenolide production (Hochwender et al. 2000; Züst et al. 2015; Tao et al. 2016).
413 However, ours is the first study within the milkweed system to show interspecific differences in
414 regrowth tolerance and mechanically induced resistance relationships following damage. Interestingly,
415 previous exposure to elevated CO₂ had no effect on the strength of this tradeoff, indicating that the
416 legacy of carbon supplementation in isolation may not be a critical driver of plant induced defense
417 syndromes. Only the tropical *A. curassavica*, native to central America, failed to display a tradeoff
418 between mechanically induced resistance and regrowth tolerance. Higher herbivore pressure at
419 southern latitudes may select for higher levels of both defense traits in this species as compared to the
420 other three perennials native to N. America (Rasmann and Agrawal 2011). The positive relationship
421 between innate plant growth and mechanically induced resistance could also reflect selection for
422 vigorous plants capable of mounting a strong response to herbivory (Hahn *et al.*, 2019, Fig. 1c).
423 Interestingly, with faster pre-damage growth rates under elevated CO₂, plants produced lower levels of
424 induced resistance likely as a result of suppressed phytohormonal signaling pathways (Guo et al. 2012;
425 Ode et al. 2014).
426
427 Despite finding no influence of elevated CO₂ on three of the four relationships between growth and
428 resistance in our study, elevated CO₂ altered aspects of both milkweed growth and resistance
429 independently. Notably, elevated CO₂ uncoupled the positive relationship between initial plant growth
430 rate and regrowth tolerance following damage (Fig. 3). Often plants with high innate growth rates can
431 regrow faster following damage (Rosenthal and Kotanen 1994). However, in our study, those plants that
432 were fast growing under elevated CO₂ did not maintain a proportionately high level of regrowth under
433 ambient CO₂ following damage. Because the regrowth period took place in a greenhouse under ambient
434 CO₂ with homogenous soil nutrients and water availability, these data potentially indicate the legacy of
435 elevated CO₂ in altering phytohormonal signaling pathways responsible for regrowth tolerance (Guo et
436 al. 2012). The constitutive resistance of both *A. incarnata* and *A. syriaca* declined under elevated CO₂
437 and increased in *A. speciosa*. Despite these effects of elevated CO₂ on constitutive defense, no legacy of
438 this treatment was detected in the mechanically induced resistance response of the milkweed species.
439 Such conserved induction responses despite previous exposure to elevated CO₂ suggests that changes in
440 chemical resistance due to elevated CO₂ detected by this and other studies (Zavala et al. 2013, 2017;
441 Ode et al. 2014), rely on continuous carbon supplementation and simultaneous manipulation of

442 phytohormonal signaling pathways rather than previous allocation decisions made by the plant before
443 damage.

444

445 Monarch caterpillars are iconic milkweed herbivores undergoing significant declines, due, in part, to
446 changing environmental conditions in both overwintering and summer breeding grounds (Stephen
447 Malcolm, 2017; Stenoien et al., 2016). Roadside milkweed patches are important habitat for monarchs
448 and regularly experience mowing events (Mueller and Baum 2014; Kasten et al. 2016). Appropriately
449 timed mowing treatments can increase monarch fecundity within milkweed patches by increasing the
450 availability of high quality foliage and releasing monarchs from the presence of enemies (Borkin 1982;
451 Fischer et al. 2015; Knight et al. 2019; Haan and Landis 2019). Our study reveals that elevated CO₂
452 changes the composition and reduces the diversity of cardenolides produced after simulated mowing in
453 both *A. incarnata*, and *A. speciosa*, two milkweed species commonly found in the N. American summer
454 breeding grounds (Woodson 1954). Critically, the composition of cardenolide communities produced by
455 milkweed can alter monarch interactions with natural enemies, such as a prevalent protozoan pathogen
456 (Sternberg et al. 2012; Decker et al. 2018, 2019). Given the conservation importance of roadside
457 milkweed patches that are regularly mowed throughout N. America, changes in regrowth tissue
458 chemical quality could have implications for monarch populations. Yet, attempts to predict how
459 migratory monarchs that depend on roadside milkweed corridors will perform under global
460 environmental change remain challenging (Zipkin et al. 2012).

461

462 Our study, though comprehensive in its investigation of growth and chemical resistance before and after
463 damage, does not incorporate the entire suite of defenses expressed by milkweeds. Additional direct
464 and indirect defenses include trichomes, latex, leaf toughness, and volatile emissions that attract natural
465 enemies (Hochwender et al. 2000; Zalucki et al. 2001; Agrawal and Fishbein 2006; Agrawal and Konno
466 2009; Meier and Hunter 2019). This suite of defense strategies may also generate resource-based
467 tradeoffs and alter plant-herbivore interactions (Züst et al. 2015; Züst and Agrawal 2017). Thus, further
468 studies exploring the fitness costs of regrowth tolerance and multiple defenses under future
469 environmental conditions, and the responses of herbivore populations to these changes, are greatly
470 needed.

471

472 On an evolutionary timescale, the influence of resource clines has illustrated the existence of tradeoffs
473 between growth and resistance, lending broad support to the RAH (Coley and Chapin 1985; Strauss and

474 Agrawal 1999; Endara and Coley 2011). Currently, no well-established theory makes predictions about
475 how tradeoffs among defense traits will respond to rapid environmental change within one generation.
476 In our study, the identity of the milkweed species determined our ability to detect a tradeoff between
477 regrowth tolerance and resistance following mechanical damage, and previous exposure to elevated CO₂
478 weakened a positive relationship between innate growth rate and constitutive defense. Given the rapid
479 rate of environmental change predicted globally (IPCC 2013), studies measuring the rate of plant
480 resistance and growth evolution as well as which environmental change drivers are crucial determinants
481 of plant fitness will be vital to predicting plant-insect interactions. This knowledge can be used to inform
482 policy decisions which reduce the use of pesticides (Strauss and Murch 2004) and improve weed control
483 programs (Williams et al. 2004).

484

485 **Acknowledgements:** Many thanks to H., Streit, R. Ricart, C. Chappell, R. Peterson, J. Kristofik, B. Shilling,
486 K. Moriarty, A. Meier, K. Crocker, K. Sanchez, J. Den Uyl, A. Potts. This work was supported by National
487 Science Foundation grants DEB-1256115 and DEB-1257160.

488

489 **Author Contributions:** LED and MDH designed the experiment, collected and analyzed the data. LED
490 wrote the manuscript, the MDH contributed significantly to drafts and approved the final version.

491

492 **Competing Interest Statement:** Authors have no sources of conflict of interest.

493

494 **Data Accessibility:** Data are available in the Dryad Digital Repository:

495 <https://doi.org/10.5061/dryad.v6wwpzgs3>

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716 **Figure Legends**

717

718 **Figure 1.** Support for only one of the four hypothetical tradeoffs relating the growth rate of milkweeds
719 before and after damage to their constitutive and mechanically induced foliar cardenolide
720 concentrations before and after damage. Milkweeds were grown under either elevated (760 ppm) or
721 ambient (400ppm) atmospheric concentrations of CO₂. a) Nonsignificant effects of CO₂ treatment and
722 pre-damage growth rate on milkweed constitutive cardenolide concentrations (mg/g dry mass) before
723 damage. b) Positive effects of CO₂ treatment and pre-damage growth rate on mechanically induced
724 cardenolide concentrations in regrown leaves (mg/g dry mass) after damage. c) Slight effects of
725 milkweed constitutive cardenolide concentrations before damage on regrowth rate after damage
726 contingent upon milkweed species. d) Significant tradeoff between mechanically induced cardenolide
727 concentrations (mg/g dry mass) in regrowth leaves and the regrowth rate (mg/day) of milkweeds.
728 Regressions are represented with 95% confidence intervals and milkweed species codes are as follows:
729 CUR = *A. curassavica* (diamond), INC= *A. incarnata* (square), SPE = *A. speciosa* (triangle), and SYR = *A.*
730 *syriaca* (circle). In figures (a, b, & c), light gray shapes represent plants grown under ambient CO₂ and
731 dark gray shapes are those grown under elevated CO₂. In figure (d) shading corresponds to milkweed
732 species.

733

734 **Figure 2.** Elevated CO₂ increased initial milkweed growth rate but had no lasting effects on regrowth rate
735 following damage. The effects of CO₂ treatment and milkweed species on a) initial growth rate prior to
736 damage (mg dry mass of above ground tissue/ 64 days), and b) nonsignificant effects of elevated CO₂
737 and milkweed species on regrowth rate following damage (mg dry mass of above ground tissue/21
738 days). In boxplots, dark lines represent the median, box boundaries represent first and third quartiles,
739 and whiskers extend to the most extreme data point less than 1.5 times the interquartile range from the
740 box. Milkweed species codes are the same as above. Data are grouped by species and CO₂ treatment for
741 ease of interpretation; however, the interaction term was not retained in our models of regrown plants.

742

743 **Figure 3.** Elevated CO₂ uncoupled the positive relationship between initial plant growth rate and
744 regrowth rate following damage. Light gray circles and lines represent plants grown under ambient CO₂
745 and dark gray triangles and lines are those grown under elevated CO₂. Regressions are represented with
746 95% confidence intervals.

747

748 **Figure 4.** Variation among milkweed species in the probability of regrowth after mechanical damage.
749 Light gray bars represent plants grown under ambient CO₂ and dark gray bars are those grown under
750 elevated CO₂. Data are grouped by species and CO₂ treatment for ease of interpretation, however the
751 interaction term was not significant in the models. Milkweed species codes are the same as above.

752
753 **Figure 5.** Elevated CO₂ altered the total concentration of milkweed constitutive defense and the
754 diversity of mechanically induced defense following damage. (a) The effects of elevated atmospheric
755 concentrations of CO₂ on constitutive cardenolide concentrations of milkweed (mg/g dry mass), (b) the
756 mechanically induced cardenolide concentrations of milkweeds (mg/g dry mass), (c) the diversity of
757 cardenolides produced constitutively, and the d) the diversity of cardenolides produced in the
758 mechanically induced resistance response following damage. Data are grouped by species and CO₂
759 treatment for ease of interpretation; however, the interaction term was not retained in our models for b
760 and c. Dark gray points represent plants grown under elevated CO₂ and light gray points and lines are
761 those grown under ambient CO₂. Milkweed species codes are the same as above.

762
763 **Figure 6.** Previous exposure to elevated CO₂ caused slight changes in both the constitutive and
764 mechanically induced cardenolide communities of milkweed. Dark gray points represent the constitutive
765 cardenolide communities produced by plants before damage, and light gray points are the cardenolide
766 communities detected in the mechanically induced response of milkweed following damage. Those
767 plants grown under ambient CO₂ are in the upper panel and those grown under elevated CO₂ are in the
768 lower panel. Milkweed species codes are the same as above.

Tables

Table 1. Sample sizes of 442 milkweed plants grown under either ambient (400 ppm) or elevated (760 ppm) CO₂ grouped (a) by species and (b) by their distribution in 40 open-top chambers. Species codes are: CUR = *A. curassavica*, SYR = *A. syriaca*, SPE = *A. speciosa*, INC= *A. incarnata*.

(a)

CO ₂ Treatment	species	N
ambient	<i>A. curassavica</i>	84
	<i>A. incarnata</i>	105
	<i>A. speciosa</i>	22
	<i>A. syriaca</i>	25
elevated	<i>A. curassavica</i>	81
	<i>A. incarnata</i>	91
	<i>A. speciosa</i>	23
	<i>A. syriaca</i>	11

(b)

CO ₂ Treatment	chamber	CUR				SYR				
		INC	SPE	SYR	chamber	CUR	INC	SPE	SYR	
elevated	1	4	3	0	2	21	4	4	2	1
ambient	2	6	6	1	2	22	5	6	2	0
elevated	3	6	7	1	0	23	2	5	3	0
ambient	4	3	6	2	3	24	5	5	0	1
elevated	5	4	4	3	1	25	6	6	2	1
ambient	6	4	4	0	1	26	4	6	1	0
elevated	7	5	6	2	0	27	2	4	0	1
ambient	8	4	6	1	1	28	4	6	0	1
elevated	9	1	6	0	0	29	5	5	2	0
ambient	10	4	5	3	1	30	3	5	0	2
elevated	11	4	5	1	0	31	5	2	0	1
ambient	12	4	6	2	1	32	4	3	0	0

elevated	13	3	1	1	0	33	4	6	3	0
ambient	14	2	5	2	3	34	4	5	0	1
elevated	15	6	3	1	1	35	2	3	1	2
ambient	16	5	5	0	3	36	6	6	3	2
elevated	17	3	4	1	0	37	4	6	1	1
ambient	18	5	4	0	0	38	5	6	2	1
elevated	19	5	6	0	0	39	6	5	0	0
ambient	20	5	6	2	1	40	2	4	0	1

769 **Table 2.** ANOVA tables of linear mixed effects models used to investigate the four putative trade-offs proposed in this study. Model selection
 770 was performed using maximum likelihood. Tables were produced with the R package LmerTest, using Type III sums of squares with Satterthwaite
 771 approximation for degrees of freedom, random effects estimates ± 1 standard deviation, and fixed effects parameter estimates ± 1 standard
 772 deviation.

773

Tradeoff 1: constitutive resistance ~ species + CO₂ + sqrt(growth rate) + CO₂*sqrt(growth rate) + random = chamber

	<i>species</i>	CO ₂	sqrt(growth rate)	<i>species</i> *CO ₂	atm*sqrt(growth rate)	Random Effect \pm SD
<i>F</i>	F_{3,207} = 189.32	F _{1,193} = 0.29	F _{1,195} = 2.72	F_{3,207} = 3.84	F _{1,195} = 0.46	chamber
<i>p</i>	< 0.0001	0.59346	0.10044	0.01047	0.49931	0.06885 \pm 0.2624

774

775

Tradeoff 2: induced resistance ~ sqrt(growth rate) + species + CO₂ + CO₂*sqrt(growth rate) + random = chamber

	sqrt(growth rate)	<i>species</i>	CO ₂	<i>species</i> *sqrt(growth rate)	CO ₂ *sqrt(growth rate)	Random Effect \pm SD
<i>F</i>	F _{1,214} = 0.58	F_{3,215} = 8.59	F_{1,213} = 4.90	F _{3,215} = 1.73	F_{1,215} = 5.33	chamber
<i>p</i>	0.44782	< 0.0001	0.028	0.16224	0.02188	0.0003061 \pm 0.0175

776

777

Tradeoff 3: sqrt(regrowth rate) ~ log(constitutive) + species + CO₂ + species*CO₂ + random = chamber

	log(constitutive)	<i>species</i>	CO ₂	<i>species</i> *CO ₂	Random Effect \pm SD
<i>F</i>	F _{1,208} = 3.66	F_{3,208} = 24.27	F _{1,61} = 0.09	F _{3,207} = 0.83	chamber
<i>p</i>	0.05716	< 0.0001	0.77115	0.47673	0.02114 \pm 0.1454

778

779

Tradeoff 4: induced resistance \sim sqrt(regrowth rate) + species + CO₂ + species*sqrt(regrowth rate) + random = chamber

	<i>sqrt(regrowth rate)</i>	<i>species</i>	CO ₂	<i>species*sqrt(regrowth rate)</i>	Random Effect \pm SD
F	F_{1,216}= 21.11	F_{3,216}= 14.37	F _{1,45} = 0.16	F_{3,215}= 7.18	chamber
p	< 0.0001	< 0.0001	0.6879328	0.00013	0.007618 \pm 0.08728

780

781

782 **Table 3.** ANOVA table of a linear mixed effects model describing the effects of elevated CO₂ on the relationship between initial plant growth rate
 783 and regrowth rate following damage. As above, model selection was performed using maximum likelihood. Tables were produced with the R
 784 package LmerTest, using Type III sums of squares with Satterthwaite approximation for degrees of freedom, random effects estimates \pm 1
 785 standard deviation, and fixed effects parameter estimates \pm 1 standard deviation.

786

Model: *sqrt(regrowth rate) \sim sqrt(growth rate) + CO₂ + species + species* sqrt(growth rate) + CO₂* sqrt(growth rate) + random= chamber*

	<i>sqrt(growth rate)</i>	<i>species</i>	CO ₂	<i>species*sqrt(growth rate)</i>	<i>species* CO₂</i>	<i>CO₂*sqrt(growth rate)</i>	Random Effect \pm SD
F	F _{1,261} = 0.01	F_{3,257}= 2.88	F_{1,260}= 5.95	F _{3,257} = 2.14	F _{3,260} = 1.17	F_{1,263}= 5.99	chamber
p	0.90362	0.03633	0.01543	0.09542	0.32088	0.01505	0.07448 \pm 0.2729

787

788

789 **Table 4.** ANOVA tables of linear mixed effects models describing the relationships between the diversity of constitutive and induced
 790 cardenolides and growth rates dependent on milkweed species and elevated CO₂. As above, model selection was performed using maximum
 791 likelihood. Tables were produced with the R package LmerTest, using Type III sums of squares with Satterthwaite approximation for degrees of
 792 freedom, random effects estimates \pm 1 standard deviation, and fixed effects parameter estimates \pm 1 standard deviation.

793

Model: constitutive diversity ~ sqrt(growth rate) + species + CO₂ + species*CO₂ + random = chamber

	sqrt(growth rate)	species	CO ₂	species* CO ₂	Random Effect ±SD
<i>F</i>	F _{1,201} = 0.76	F_{3,207}= 260.56	F_{1,68}= 4.077	F _{3,206} = 2.04	chamber
<i>p</i>	0.38452	< 0.0001	0.04741	0.10937	0.003±0.054

Model: induced diversity ~ sqrt(regrowth rate) + species + CO₂ + sqrt(regrowth rate)*species + species*CO₂ + random = chamber

	sqrt(regrowth rate)	species	CO ₂	sqrt(regrowth rate)*species	species* CO ₂	Random Effect ±SD
<i>F</i>	F _{1,211} =1.94	F_{3,205}=29.67	F _{1,72} =1.95	F_{3,206}=4.62	F_{3,203}=2.67	chamber
<i>p</i>	0.1646	<0.0001	0.16661	0.003752	0.04841	0.003±0.057

Table 5. PerMANOVA describing the effects of elevated CO₂ on the composition of constitutive and induced cardenolide communities.

<i>PerMANOVA</i>			
	<i>F</i>	<i>R</i> ²	<i>P</i>
species	F _{3,410} = 12.12	0.10	0.001
CO ₂	F _{1,410} = 1.06	0.003	0.404
resistance type	F_{1,410}= 55.38	0.15	0.001
resistance type*species	F_{3,410}= 4.74	0.04	0.001
resistance type*CO ₂	F _{1,410} = 1.04	0.003	0.391
species*CO₂	F_{2,410}= 2.26	0.011	0.003
resistance type*species*CO₂	F_{2,410}= 2.39	0.013	0.001

Figure 1.

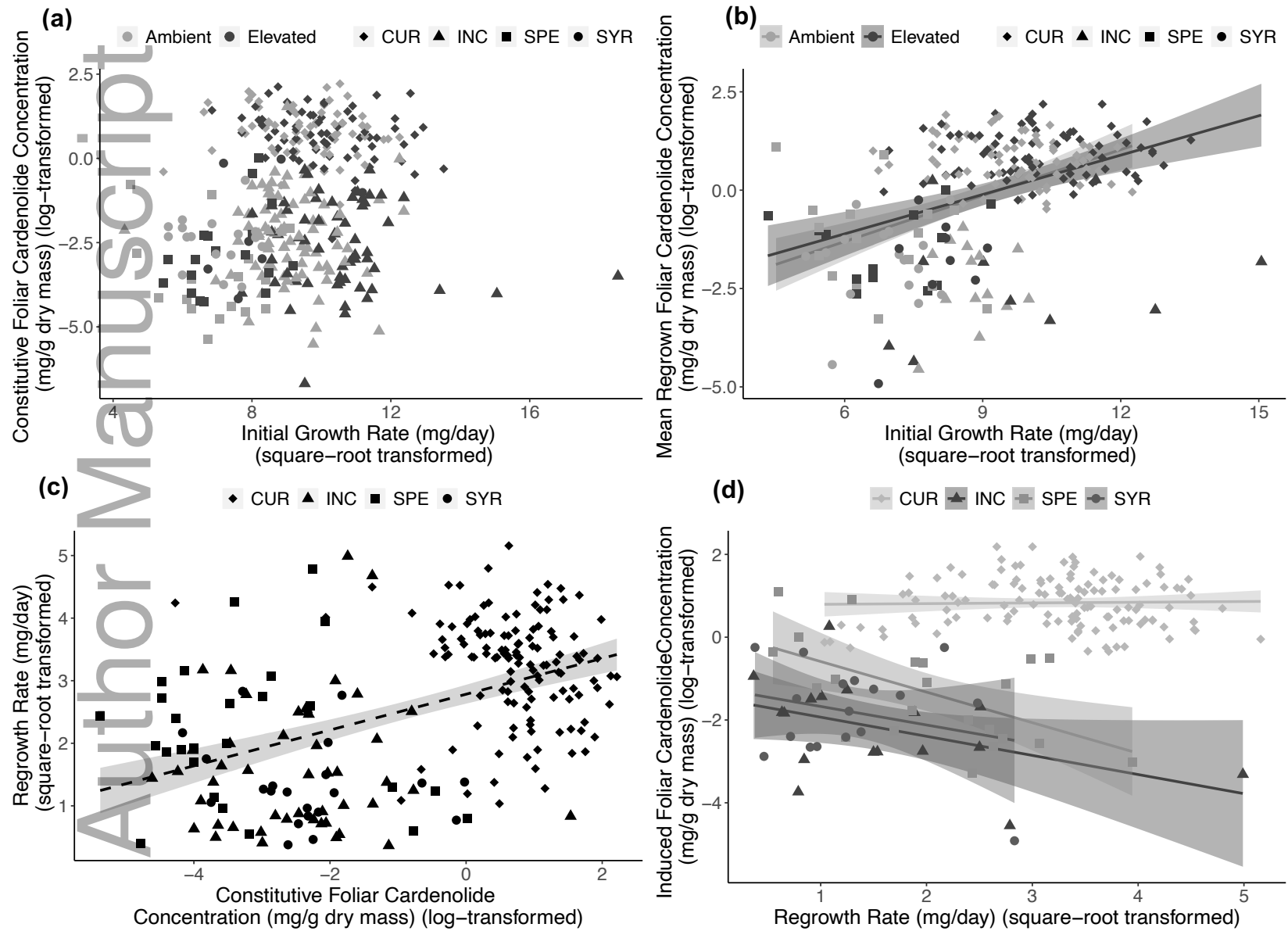
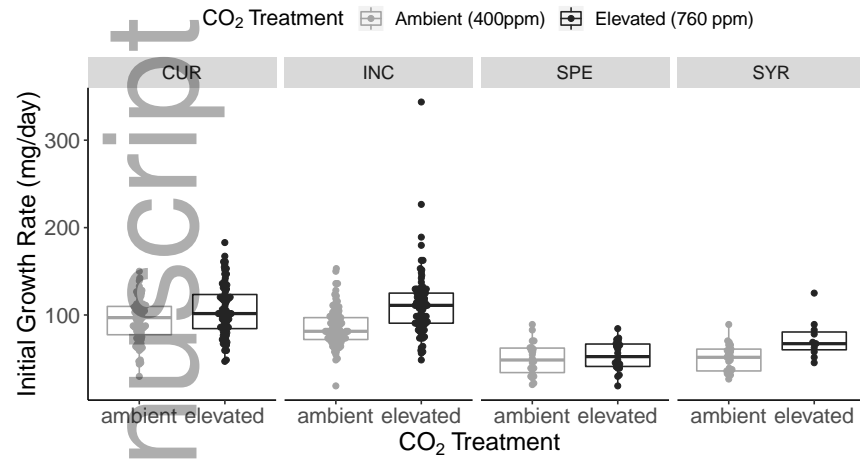


Figure 2.

(a)



(b)

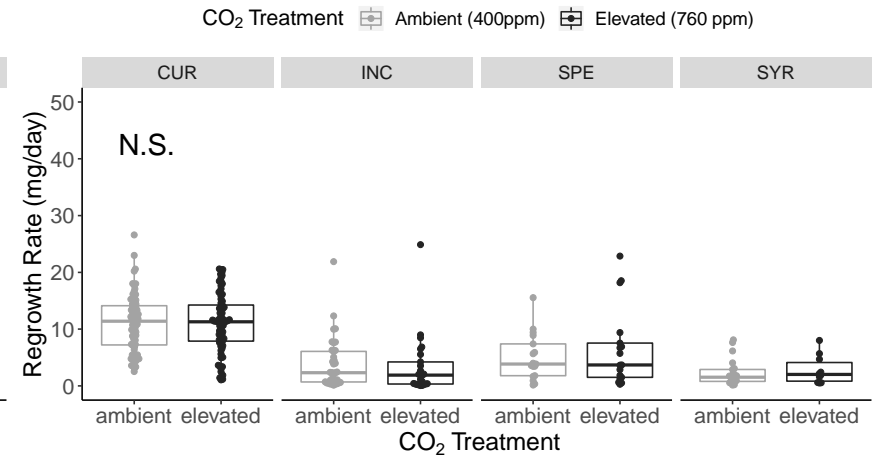


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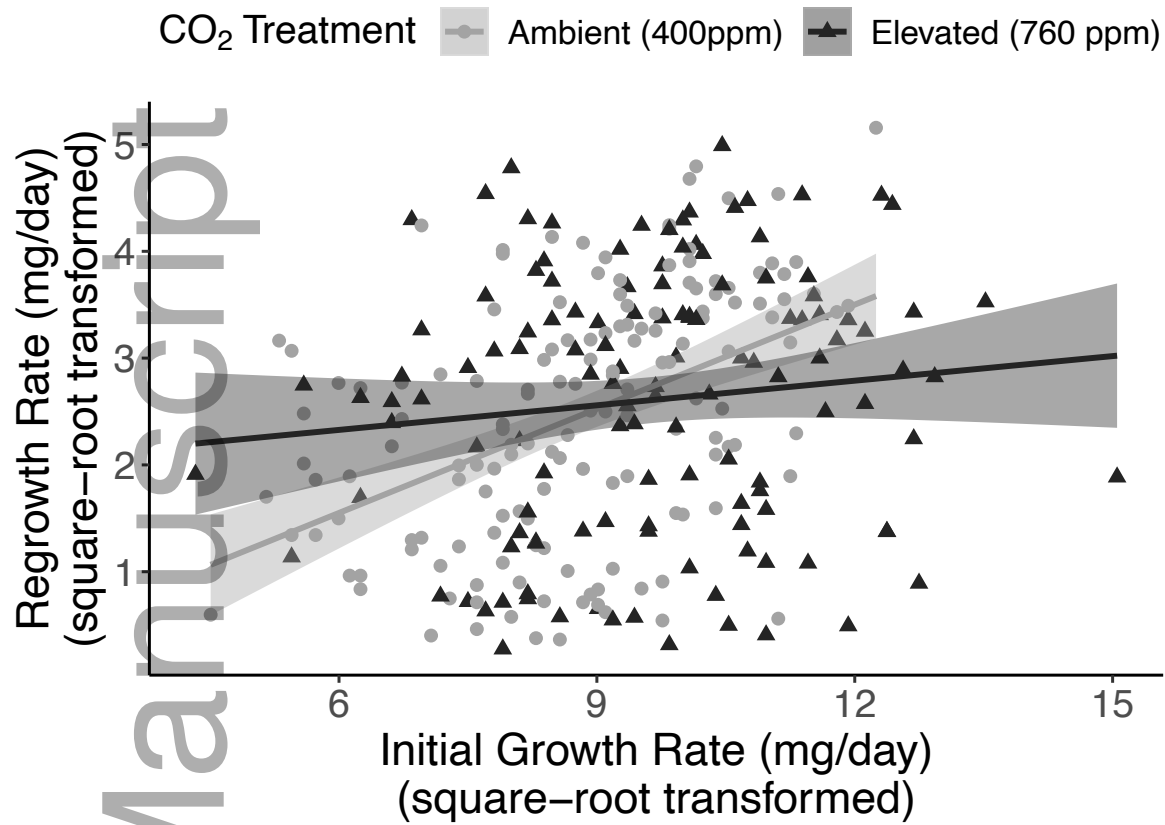


Figure 4.

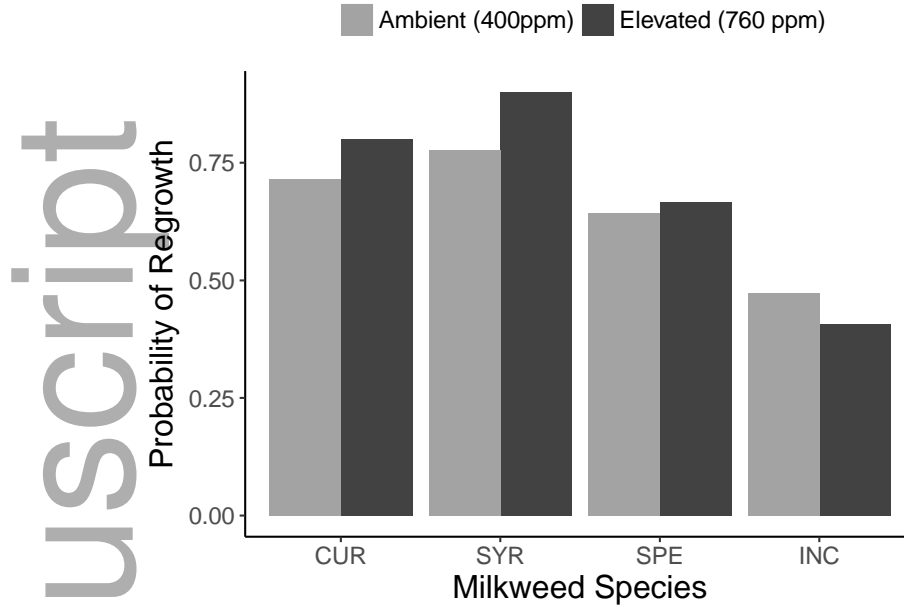


Figure 5.

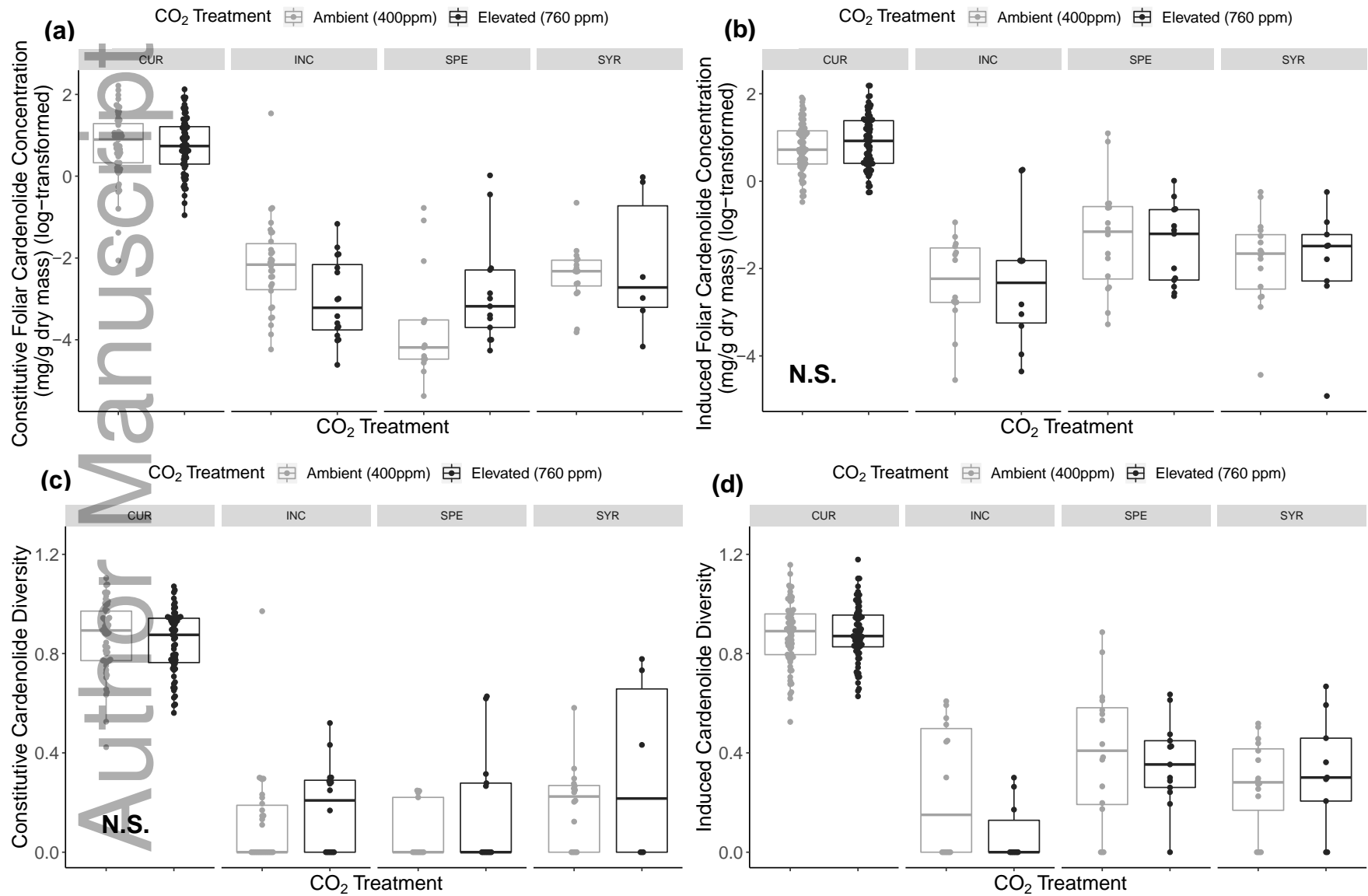


Figure 6.

