

1 **Physiological adjustments of a Mediterranean shrub to long-term experimental**
2 **warming and drought treatments**

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28 **Abstract**

29 Warmer temperatures and extended drought in the Mediterranean Basin are becoming
30 increasingly important in determining plant physiological processes and affecting the
31 regional carbon budget. The responses of plant physiological variables such as shoot
32 water potential (Ψ), carbon-assimilation rates (A), stomatal conductance (g_s) and
33 intrinsic water-use efficiency ($iWUE$) to these climatic regimes, however, are not well
34 understood. We conducted long-term (16 years) field experiments with mild nocturnal
35 warming (+0.6 °C) and drought (-20% soil moisture) in a Mediterranean early-
36 successional shrubland. Warming treatment moderately influenced Ψ , A and g_s
37 throughout the sampling periods, whereas drought treatment strongly influenced these
38 variables, especially during the summer. The combination of a natural drought in
39 summer 2003 and the treatments significantly decreased A and $iWUE$. Foliar $\delta^{13}C$
40 increased in the treatments relative to control, but not significantly. The values of Ψ , A
41 and g_s were correlated negatively with vapor-pressure deficit (VPD) and positively with
42 soil moisture and tended to be more dependent on the availability of soil water. The
43 plant, however, also improved the acclimation to drier and hotter conditions by
44 physiological adjustments (g_s and $iWUE$). Understanding these physiological
45 processes in Mediterranean shrubs is crucial for assessing further climate change
46 impacts on ecosystemic functions and services.

47 **Keywords:** global warming, frequent droughts, physiological response; long-term
48 experiments; plant acclimation

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51 **1. Introduction**

52 Mediterranean-type shrubland ecosystems occupy large areas of the global
53 terrestrial surface and provide important ecosystemic services, such as carbon storage,
54 global biogeochemical cycles and the conservation of biodiversity [1–5]. The impacts
55 of anthropogenic climate change, however, are gradually representing a prominent
56 disturbance, affecting from individuals to ecosystems [3,5–7]. Numerous studies of
57 Mediterranean ecosystems have reported that global warming is advancing plant spring
58 phenology and extending growing periods [3,8–10]. In contrast, rapid changes in the
59 patterns of temperature and precipitation have also negatively affected plant growth and
60 survival by reducing water availability, ultimately leading to catastrophic carbon
61 starvation and widespread mortality [11–15]. Robust climatic models have projected a
62 continuous increase in warming and drought severity in the Mediterranean Basin for
63 the coming decades, which could severely impact carbon sinks in shrubland ecosystems
64 and alter regional carbon budgets [16,17].

65 Many studies over the latest several decades have reported physiological [18–23],
66 morphological [3,24,25] and genetic [3,26–28] changes in Mediterranean plants in
67 response to warming and drying conditions. Among these changes, physiological
68 adjustments are considered the most rapid and effective, because they can increase
69 photosynthetic rates or decrease water loss and improve intrinsic water-use efficiency
70 (iWUE) via stomatal conductance (g_s) [3,11,25,29–31]. Recent studies have also
71 demonstrated the resistance and resilience of terrestrial biomes to climatic change by

72 the modulation of the responses to inter-seasonal and inter-annual stresses over time
73 [25,32–34]. Changes in iWUE regulated by g_s have been widely studied by the
74 variation of foliar carbon isotope ($\delta^{13}\text{C}$) composition [35], so WUE can be evaluated
75 by changes in the amount of foliar $\delta^{13}\text{C}$ [36–39]. Our understanding of the effects of
76 future scenarios of warming and drought on these physiological processes in
77 Mediterranean shrubs, however, remains poor due to a lack of long-term data sets.

78 Climatic experiments have provided effective approaches for studying the potential
79 physiological and phenological changes in plants to future climatic regimes [40–44].
80 Numerous field experiments manipulating in Mediterranean ecosystems have helped to
81 identify the physiological [18–21,45], morphological [24,25] and structural [11,34,46]
82 adjustments of plants for coping with changes in climatic regimes. Long-term
83 precipitation-manipulation experiments in Mediterranean forests have highlighted
84 dampened effects on carbon assimilation [45], biomass accumulation [47,48] and
85 aboveground net primary production (ANPP) [48]. The physiological adjustments over
86 time in response to new climatic regimes, however, are still unclear because most
87 experimental studies have short terms (<5 years) [18–21,23]. Long-term experiments
88 are consequently desirable for interpreting the cumulative effect of certain climatic
89 regimes and detecting the patterns of plant physiological responses [42,45,48].

90 We carried out a long-term nocturnal warming and drought experiment in a
91 Mediterranean early-successional shrubland from 1999 to 2014 (16 years), which is one
92 of the longest climate-manipulation experiments ever conducted. Species in early-
93 successional stages are sensitive to rapid climate change but have received little

94 attention [3,18,20,49]. We studied the shrub *Erica multiflora*, which is widely
95 distributed in western and central Mediterranean Basin and is one of the dominant
96 species at the study site [18,20]. Previous experimental studies conducted in this
97 experimental site have observed that the rates of carbon assimilation in *E. multiflora*
98 were not affected by warming but were significantly decreased by drought throughout
99 the first two years of treatment [18]. Prieto et al [20] reported that warming tended to
100 increase the rate of carbon-assimilation of this species in cold seasons, but the response
101 to drought depended on the year and season. Our sampling periods were: 1999-2001
102 (short-term), 2003-2005 (medium-term) and 2014 (long-term). The specific objectives
103 of our study were to (i) verify if warming and drought exacerbate the loss of shoot water
104 in Mediterranean shrub species, (ii) investigate the effects of experimental warming and
105 drought on A_g , $iWUE$ ($A_g \text{ gs}^{-1}$) and foliar $\delta^{13}\text{C}$ levels at different timescales, and (iii)
106 determine the effect of long-term ecosystem exposure to warmer and drier conditions
107 on plant physiological adjustments. The results will be crucial for identifying the
108 potential physiological responses to climatic changes and will help us to understand
109 further the effects of climate on terrestrial ecosystemic functions and services.

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117 **2. Materials and methods**

118 2.1 Study site

119 We carried out a field experiment on a south-facing hill (13% slope) in Garraf Natural
120 Park near Barcelona (northeastern Spain) (41°18' N, 1°49' E; 210 m a.s.l.). The climate
121 at the experimental site is typically Mediterranean, with mild winters, dry summers and
122 rainy springs and autumns. The mean annual air temperature during the study period
123 was 15.8 °C, with the maximum mean summer temperature (June-August) of 23.5 °C.
124 The mean annual precipitation was 537.3 mm, as much as 70% of which falls in spring
125 and autumn. The soil is calcareous and composed of marls and limestone, with depths
126 of 10-40 cm. The site suffered two large fires in summer 1982 and spring 1994, which
127 degraded the vegetation to early-successional shrubland. Most of the current vegetation
128 has sprouted from underground organs after the two fires. The vegetation is co-
129 dominated by *E. multiflora*, a species widely distributed in the Mediterranean Basin. *E.*
130 *multiflora* grows mainly in the spring but also in the autumn; flowering begins in late
131 summer and ends the following spring.

132 2.2 Experimental manipulations

133 Nine plots (5 × 4 m²) were randomly organized in three blocks, with each block
134 having one warming, one drought and one control plot. Each treatment thus had three
135 replicates. The experiment was maintained from 1999 to 2014.

136 The warming treatment consisted of a passive nocturnal warming by covering the
137 plots with aluminum curtains. This covering system reduces the amount of long-wave

138 infrared radiation reflected back to the atmosphere at night [40]. A light scaffold was
139 installed in each warming plot for supporting the covering. The curtains were
140 automatically unfolded at night and retracted during the day and were controlled by
141 light sensors (below and above 200 lux, respectively). This passive nocturnal warming
142 is realistic and effective, because the effects of global warming are predicted to be
143 higher at night than during the day [40,49]. The curtains were automatically retracted
144 during rain to avoid influencing the hydrological cycle.

145 The drought treatment extended the summer drought to the following spring and
146 autumn by preventing rainwater from entering the plots using transparent waterproof
147 roofs [40]. Scaffolds were also installed in the drought plots, but the curtain material
148 was transparent plastic. Rain sensors activated the curtains to cover the plots whenever
149 it rained during the treatment period and retracted them when the rain stopped. Rain
150 was sensed by a sensitive (>5 mm rainfall) tipping-bucket rain gauge. The rainwater
151 blocked by the waterproof plastic was drained outside the plots. The curtains were also
152 automatically retracted to avoid damage during winds exceeding $10 \text{ m}\cdot\text{s}^{-1}$. The drought
153 plots were treated the same as the control plots during the rest of the year. The control
154 plots had similar scaffolds as the warming and drought plots but without curtains.

155 2.3 Environmental conditions

156 Air temperature and precipitation have been recorded at the study site since 1998.
157 Soil temperature at -5 cm depth was recorded by temperature sensors distributed in each
158 plot. Precipitation was recorded by a tipping-bucket rain gauge 1.5 m above the ground.
159 Relative humidity was recorded every 30 minutes at a nearby meteorological station.

160 Vapor-pressure deficit (VPD) was calculated every 30 minutes from the relative
161 humidity and air temperature. Soil moisture in the top 15 cm of soil was measured
162 weekly by Time Domain Reflectometry (TDR) using three probes in each plot.

163 2.4 Measurements of shoot water potential and gas exchange

164 We monitored the shoot water potential (ψ), foliar carbon-assimilation rates (A)
165 and gas exchange (gs) for 3-6 consecutive days per season during the sampling periods
166 (spring 1999 to winter 2001, winter 2003 to summer 2005 and winter 2014 to autumn
167 2014). Five current-year shoots of *E. multiflora* with similar growth performance were
168 collected in each plot to measure the changes of ψ at midday (11:00-13:00, solar time)
169 using a Scholander-type pressure chamber (PMS Instruments, Corvallis, USA).
170 Measured Erica plants were always the same ones, permanently marked with labels at
171 the beginning of experiments. Foliar A and gs were measured on three consecutive days
172 in each season with a portable gas-exchange system (an ADC4 system configured with
173 a chamber model PLC4B (Hoddesdon, Hertfordshire, UK) from 1999 to 2001, a
174 CIRAS2 system (Hitchin, Hertfordshire, UK) from 2003 to 2005 and an LI-6400XT
175 system (LI-COR Inc., Lincoln, USA) from 2013 to 2014). Two to six sunny and
176 current-year shoots with similar growth status were collected from each plot, and gas
177 exchange was measured in triplicate. Leaf area was measured using ImageJ 1.46r (NIH,
178 Maryland, USA) from a photocopy of all leaves on the twigs. Intrinsic water-use
179 efficiency (iWUE) was calculated from the gas-exchange measurements as the ratio A
180 gs^{-1} . The medium- (2003-2005) and long-term (2014) periods focused on gs and iWUE.

181 2.5 Isotopic and elemental analyses

182 Foliar $\delta^{13}\text{C}$ content was measured for current-year leaves in August 2000, 2004
183 and 2014. Leaves from three *E. multiflora* plants were collected in each plot and
184 analyzed in an EA1108 elemental analyzer (Carlo Erba, Milano, Italy) coupled to a
185 Delta C isotope ratio mass spectrometer with a CONFLO II interface (Thermo Finnigan
186 MAT, Bremen, Germany). The results were calibrated with interspersed international
187 isotopic carbon standards (IAEA, Vienna, Austria). Values are expressed relative to
188 Pee Dee Belemnite (PDB) standard for $\delta^{13}\text{C}$ as:

$$189 \quad \delta^{13}\text{C} = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000 (\text{‰})$$

190 where ^{13}C is the heavier carbon isotope and R is the ratio of the heavier to the
191 lighter isotope ($^{13}\text{C}:^{12}\text{C}$) for the sample and standard. The accuracy of the measurements
192 was $\pm 0.15\text{‰}$ for $\delta^{13}\text{C}$.

193 2.6 Data analyses

194 For all physiological parameters (Ψ , A, gs, iWUE and $\delta^{13}\text{C}$), we calculated one
195 value per plot as the mean of all plants measured per plot. The warming and drought
196 treatments were always separately compared with the controls. The differences between
197 the treatments and controls were analyzed by analyses of variance using R version 3.2.3
198 (R Core Development Team, 2015). The significance level was $p < 0.05$ for the statistical
199 tests within year and season.

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205 **3. Results**

206 Climatic parameters

207 The climatic parameters (rainfall, temperature and VPD) varied greatly throughout
208 the sampling periods (Fig. 1). The highest temperatures were recorded in summer
209 (mean of June, July and August), with the highest summer temperature of 26.4 °C in
210 2003. The rainy seasons were usually spring and autumn. VPD was correlated with
211 accumulated precipitation and temperature, with extremely severe deficits in summer
212 2003. The mean annual soil temperature at -5 cm depth was on average 0.6 °C higher
213 in the warming than in the control plots. There were no significant differences between
214 warming and control plots in 2014 (Fig. 2a). Soil moisture in the 0-15 cm layer
215 fluctuated with season and year during the sampling periods, with wet springs and
216 autumns and dry summers (Fig. 2b). Soil moisture averaged 20% lower in the drought
217 treatment than the control plots throughout the sampling periods ($p<0.01$).

218 Shoot water potential (ψ)

219 Shoot water potential (ψ) of *E. multiflora* varied with VPD and was lowest in
220 summer (Fig. 3). The warming treatment did not significantly affect ψ compared to the
221 control, but ψ was significantly lower in the drought treatment throughout the sampling
222 periods ($p<0.01$).

223 Foliar gas exchange

224 Carbon-assimilation rates (A) was highly variable in the control plants inter-
225 seasonally and inter-annually throughout the sampling periods. Photosynthesis was

226 generally highest during the wet seasons (spring and autumn) and lowest during the hot
227 and dry summers (Fig. 4). Neither warming nor drought treatment significantly
228 decreased the values of A when compared to control, and warming treatment even had
229 some positive effects. The values of A, however, decreased significantly under both
230 warming and drought in the extremely hot and dry summer of 2003 ($p<0.01$ and $p<0.05$,
231 respectively). The values of g_s fluctuated greatly with season and year and was lowest
232 in winters and highest in the wet springs and autumns (Fig. 5), but g_s in the warming
233 and drought treatments was similar to that in the controls. The iWUE in control was
234 generally highest in summer, except for the extreme summer of 2003 (Fig. 6). The
235 (iWUE) was generally higher in the warming and drought treatments but decreased
236 significantly in summer 2003 due to the combination of the extremely hot summer and
237 the experimental warming ($p<0.05$) and drought ($p<0.05$).

238 Shoot water potential (ψ), Carbon-assimilation rates (A) and stomatal conductance
239 (g_s) were correlated negatively with VPD and positively with soil moisture, indicating
240 a strong dependence on soil moisture. The (iWUE), however, was not affected when
241 soil-water potential and VPD were high (Fig. 7).

242 Variability of isotopic values (^{13}C)

243 The foliar $\delta^{13}\text{C}$ values in *E. multiflora* varied greatly during the sampling periods
244 (Fig. 8). The $\delta^{13}\text{C}$ values after one year of both the warming and drought treatments
245 were similar to those in the controls. After five years of treatment, however, were
246 significantly higher in the warming treatment than the controls. Neither the warming
247 nor the drought treatments had significant effects on $\delta^{13}\text{C}$ after 15 years of treatment.

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249 **4. Discussion**

250 Shoot water potential (ψ) in *E. multiflora* was highly variable inter-seasonally and
251 was lowest in hot and dry summers (Fig. 3). The lower ψ in the experimental treatments
252 was likely induced mainly by increased evapotranspiration in the warming treatment
253 and by the decrease in soil moisture due to the exclusion of rainwater in the drought
254 treatment [18,20]. The effects on ψ differed greatly between warming and drought
255 treatment, consistent with the previous studies showing that the impacts of drought
256 treatment on plant water potential were stronger than those of warming treatment
257 [18,20]. Our results indicated that hot and dry summers accelerated the loss of water
258 from plant shoots, which can cause large-scale crown die-off, mortality and vegetation
259 shifts in Mediterranean ecosystems [11,14,15]. Long periods of warmer and drier
260 climate combined with extreme conditions in the future would likely cause more severe
261 shoot-water deficits in Mediterranean shrubland ecosystems [15,17].

262 Foliar photosynthesis is an important physiological process of plant life that is
263 strongly affected by conditions of temperature and water across various types of
264 ecosystems [23,31,34]. The moderate warming and prolonged drought treatments
265 decreased photosynthetic rates, although the experimental warming had some positive
266 effects in wet seasons (Fig. 4). Photosynthesis in *E. multiflora* was strongly correlated
267 with soil moisture, and it was significantly lower in both the warming and drought
268 treatments during the extreme summer in 2003, probably due to the lower water
269 availability [14,17].

270 Stomatal adjustment is the primary regulator of foliar hydrological changes, which
271 control the response of WUE to climatic stresses [23,31]. Photosynthesis is highly
272 correlated with g_s , although the mechanisms by which water stress limits
273 photosynthesis by stomatal closure or metabolic impairment are controversial
274 [23,30,31]. Most studies accept that stomatal closure is the main determinant of
275 photosynthesis under moderate drought stress [20,23,45,50]. Foliar photosynthetic rates
276 are also well correlated with g_s in some studies of Mediterranean species [18–20,45].
277 Plant photosynthesis, however, may not be limited by stomatal closure but by metabolic
278 impairment [3,20,23,50,51]. Our study indicated that foliar photosynthetic rates were
279 significantly lower in both the warming and drought treatments in the extreme 2003
280 summer, but this effect was probably associated with the impairment of photosynthetic
281 enzymes, because the treatments did not significantly affect g_s [23,31,51].

282 Intrinsic water-use efficiency (iWUE) is an important indicator of plant
283 physiological response to environmental changes [23,31,39]. In our study, the plants
284 increased iWUE to reduce water loss in summer, except in the extreme 2003 summer
285 (Fig. 6), consistent with the theory that the balance between carbon assimilation and
286 the control of water loss is optimized in hot and dry summers [29,30,39]. Climatic
287 extremes such as during the 2003 summer, however, can cause severe plant-water
288 deficits that can irreversibly damage plant hydraulic systems [11,12,14,15,17]. Changes
289 in foliar $\delta^{13}\text{C}$ during warmer and drier conditions can increase plant WUE via
290 photosynthetic or metabolic processes [35–37,39,52]. Our results, however, showed
291 that $\delta^{13}\text{C}$ did not change significantly in the warming and drought treatments throughout

292 the study period, except in the warming treatment in 2004 when $\delta^{13}\text{C}$ was significantly
293 higher, which was probably due to the higher iWUE after the extremely hot summer in
294 2003 (Fig. 8). Various studies have reported that ^{13}C discrimination decreases with the
295 concentration of intercellular carbon dioxide due to stomatal closure, and ultimately
296 WUE [3,38,39,52]. Foliar $\delta^{13}\text{C}$ was therefore higher under the warming and drought
297 conditions, consistent with the findings of other experiments in terrestrial ecosystems
298 where plants regulate the relationship between WUE and climate change [32,37,39].

299 The available information on global change indicates that long periods of warming
300 have been impacting terrestrial ecosystems since the mid-20th century [5,16]. We have
301 also concluded that the severe decrease in water availability had substantial effects on
302 physiological processes, such as Ψ , A and g_s , whereas the effects of the mild warming
303 treatment were very mild (nearly no influences). These differential treatment effects
304 indicate that low water availability may have a more direct impact on plants [23,31].
305 These physiological adjustments, however, are essential for adapting to changes in
306 climatic regimes. Our study shows that the physiological responses (A, g_s , iWUE and
307 $\delta^{13}\text{C}$) to long-term experimental warming and drought decrease over time, indicating
308 acclimation to the climatic stresses [21,23,45,46]. Robust models, however, predict that
309 global warming will continue to increase, accompanied by other stresses such as
310 extreme droughts and heat waves [5,16,17]. Mediterranean species have shown some
311 acclimation responses and strategies, but these could be potentially overwhelmed by
312 future climate, leading to increased risks of xylem embolism, crown die-off and
313 mortality, which would affect future ecosystemic carbon budgets.

314

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535 **Figures**

536 Fig. 1 Seasonal variation of mean air temperature, precipitation and vapor-pressure
537 deficit (VPD) during the sampling periods 1999-2001, 2003-2005 and 2014. Wi, winter;
538 Sp, spring; Su, summer; Au, autumn.

539 Fig. 2 (a) Changes of seasonal soil temperature in the control and nocturnal warming
540 treatment during the sampling periods. (b) Changes of seasonal soil moisture in the
541 control and drought treatment during the sampling periods. Significant differences
542 between the control and treatment plots are indicated by asterisks (**, $p < 0.01$). Each
543 treatment had three replicates ($n=3$ plots). Wi, winter; Sp, spring; Su, summer; Au,
544 autumn.

545 Fig. 3 Changes in shoot water potential in *Erica multiflora* during the sampling periods
546 in the control and warming treatment and drought treatment. Significant differences
547 between the control and treatment plots are indicated by asterisks (*, $p < 0.05$; **, $p < 0.01$). Each treatment had three replicates ($n=3$ plots). Wi, winter; Sp, spring; Su,
548 summer; Au, autumn.

550 Fig. 4 Seasonal variation of net photosynthetic rates in *Erica multiflora* in the control
551 and treatment plots during the experimental periods. Error bars indicate the standard
552 errors of the means ($n=3$ plots). Significant differences between the control and
553 treatment plots are indicated by asterisks (*, $p < 0.05$; **, $p < 0.01$). Wi, winter; Sp, spring;
554 Su, summer; Au, autumn.

555 Fig. 5 Seasonal stomatal conductance in *Erica multiflora* in the control and the warming

556 and drought treatments during the medium- and long-term study periods. Error bars
557 indicate the standard errors of the means (n=3 plots). Wi, winter; Sp, spring; Su,
558 summer; Au, autumn.

559 Fig. 6 Intrinsic water-use efficiency (iWUE, A g^{-1}) in *E. multiflora* leaves in the control
560 and the warming and drought treatments during the medium- and long-term sampling
561 periods. Error bars indicate the standard errors of the means (n=3 plots). Significant
562 differences are indicated by asterisks (*, $p < 0.05$). Wi, winter; Sp, spring; Su, summer;
563 Au, autumn.

564 Fig. 7 The relationships of shoot water potential (SWP), net photosynthetic rate,
565 stomatal conductance and intrinsic water-use efficiency (iWUE) with vapor-pressure
566 deficit (VPD) and soil moisture during the sampling periods.

567 Fig. 8 Foliar $\delta^{13}\text{C}$ in *E. multiflora* in summer 2000, 2004 and 2014. Error bars indicate
568 the standard errors of the means (n=3 plots). Significant differences with control are
569 indicated by asterisks (*, $p < 0.05$).

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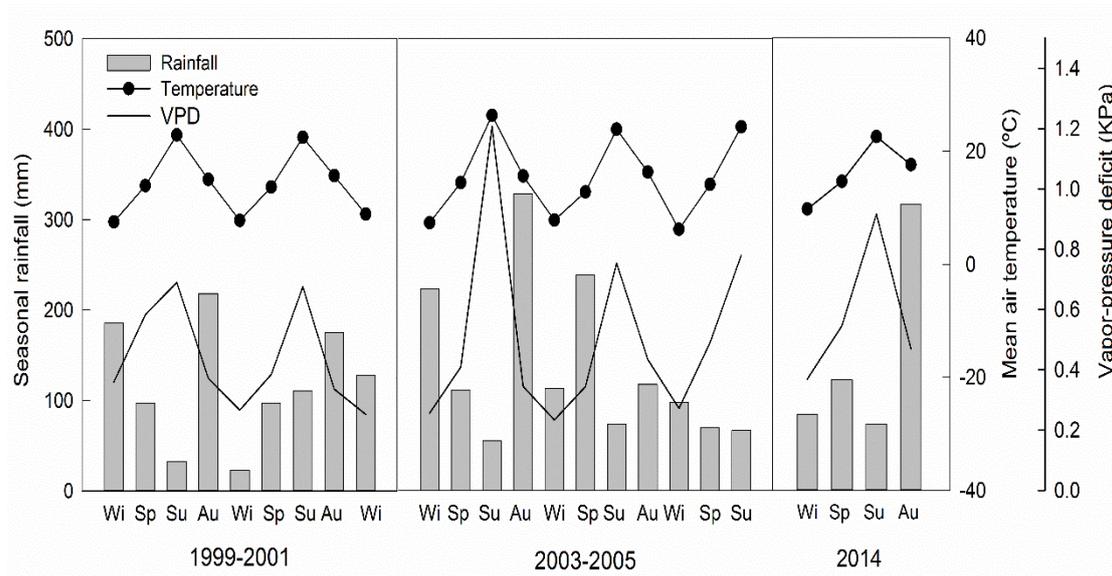
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Fig. 1

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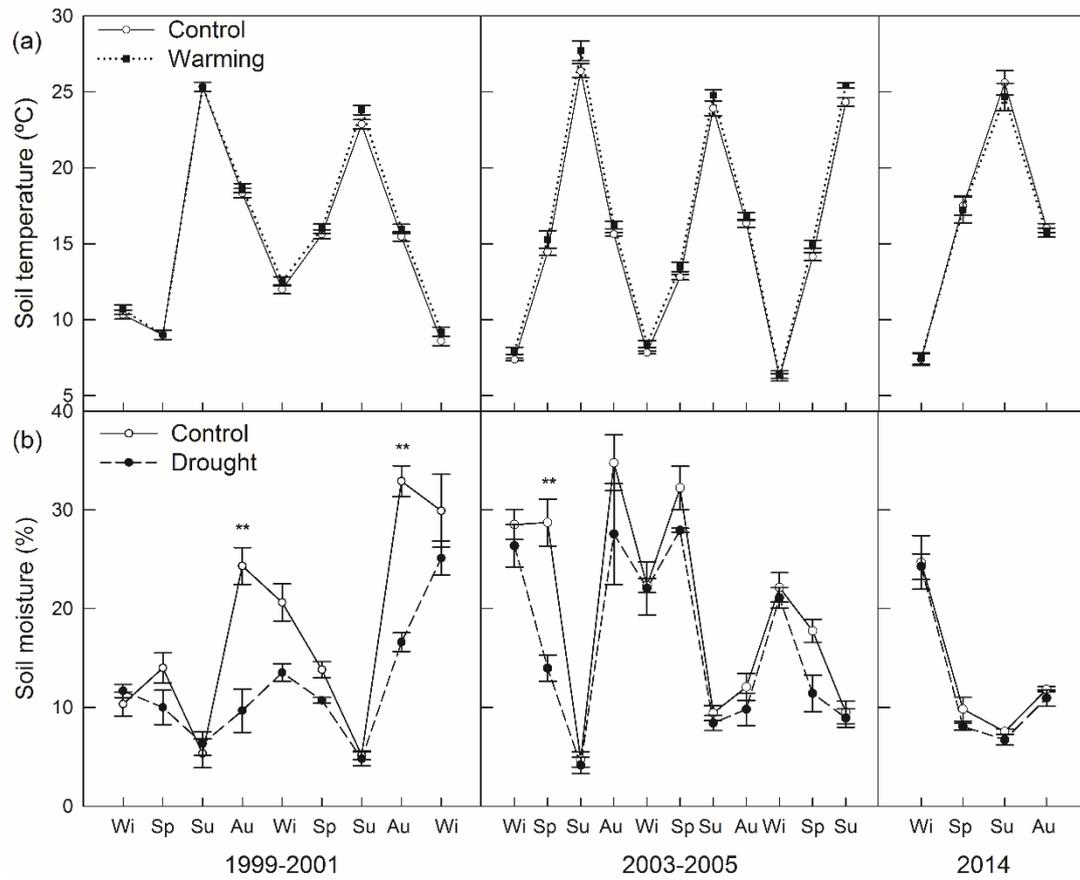
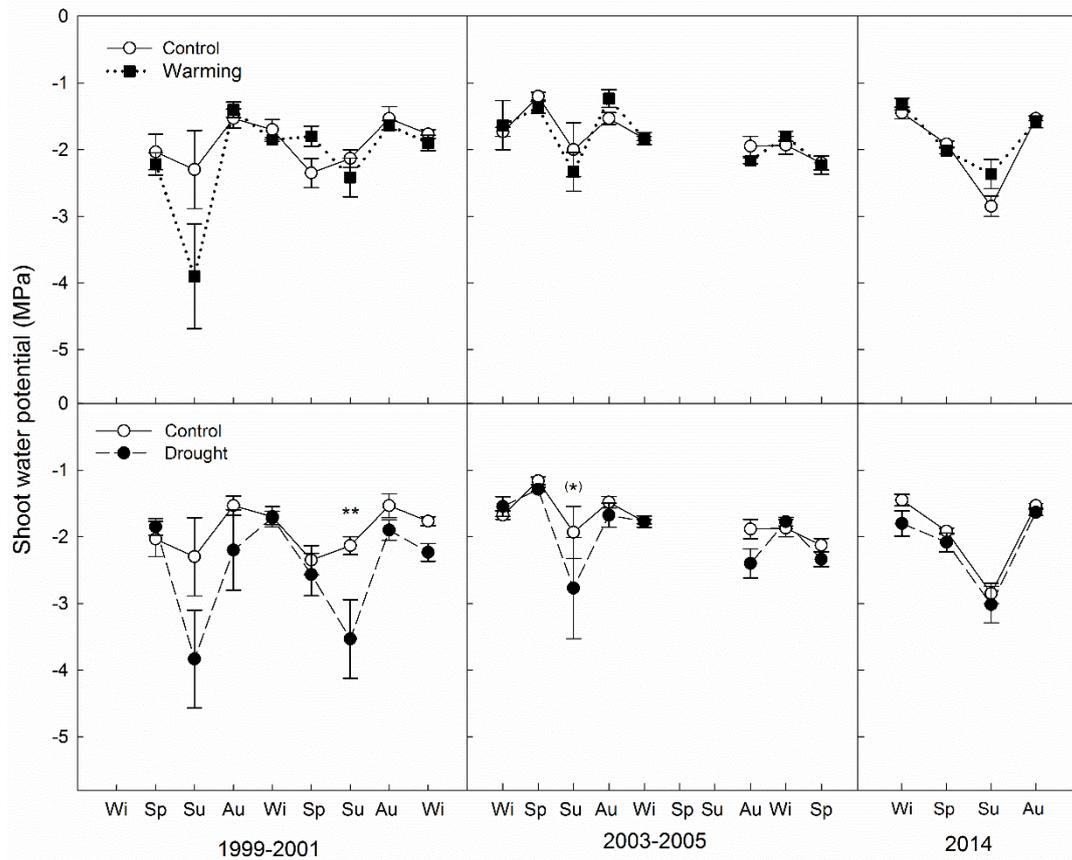


Fig. 2

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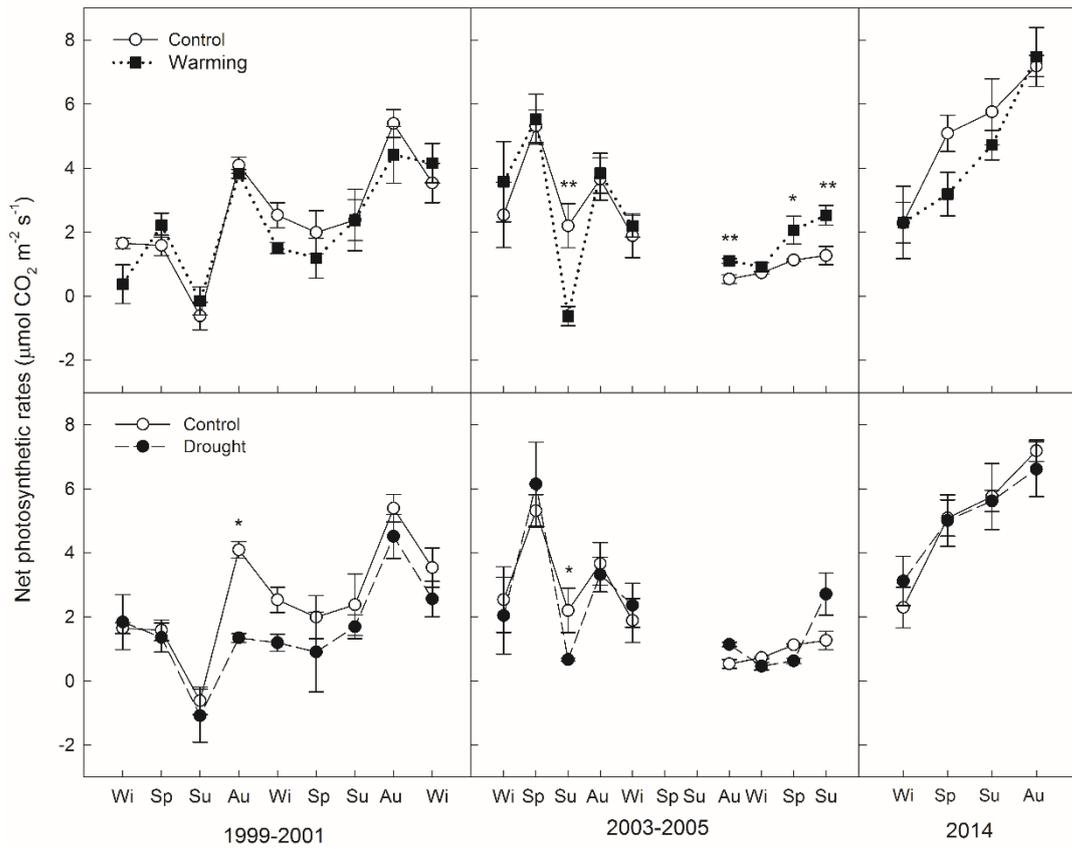
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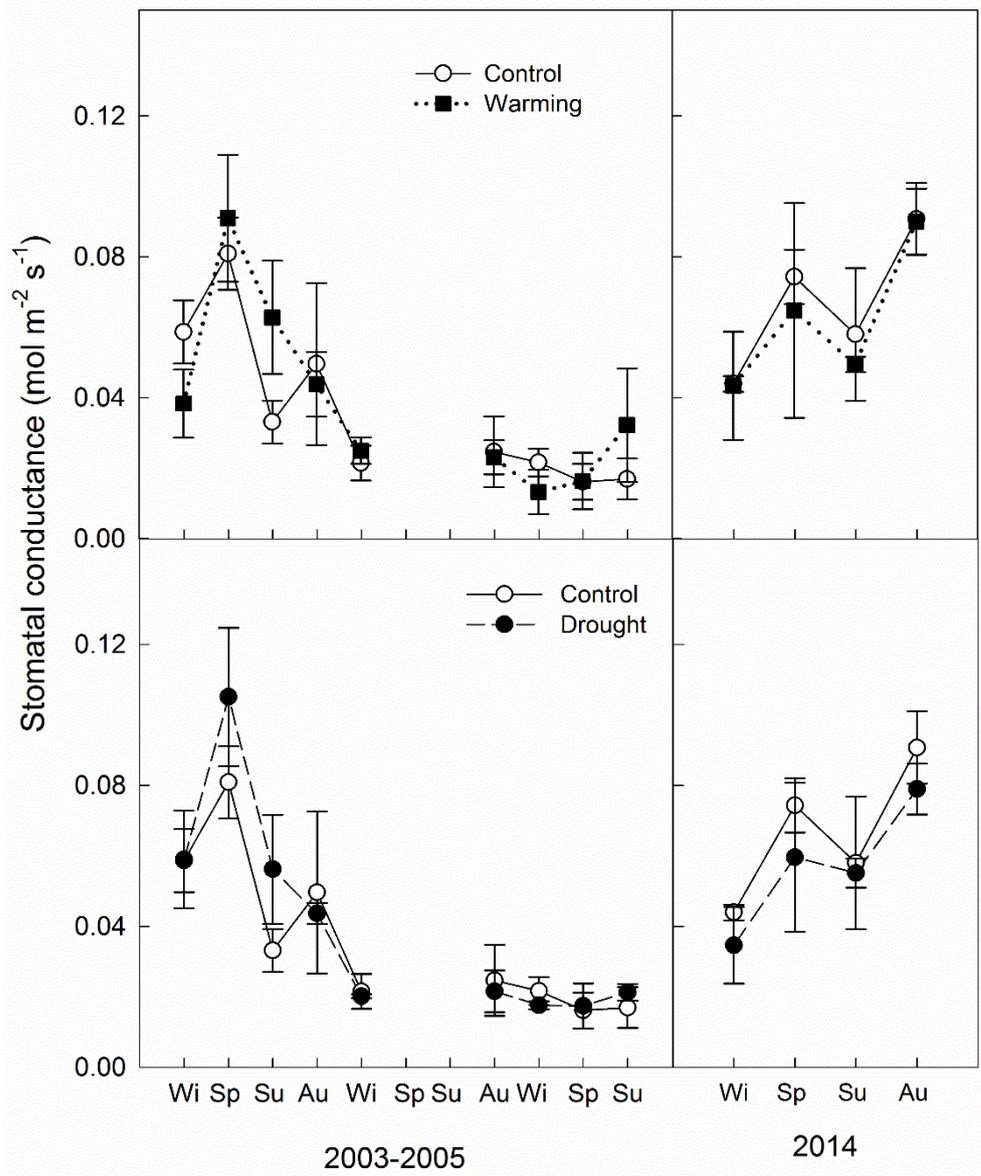
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Fig. 5

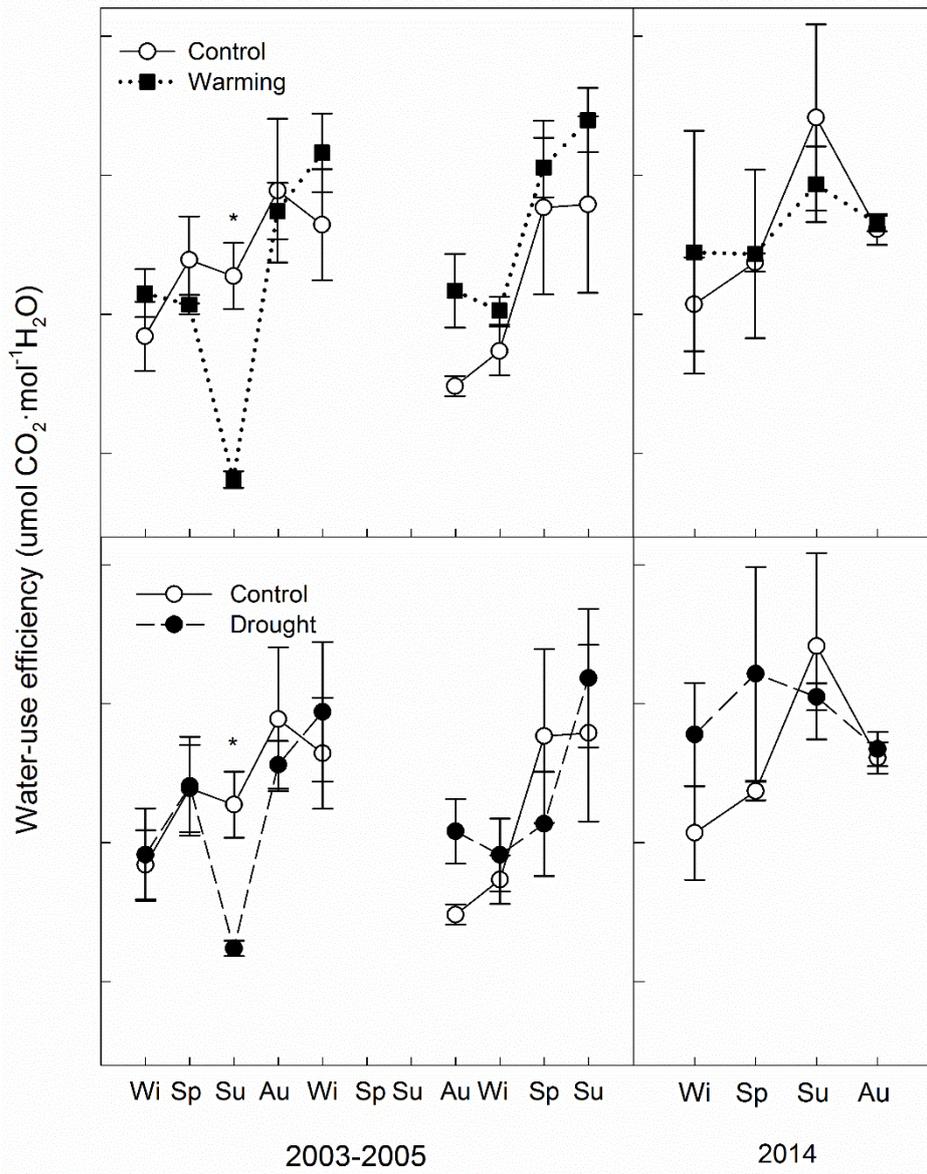
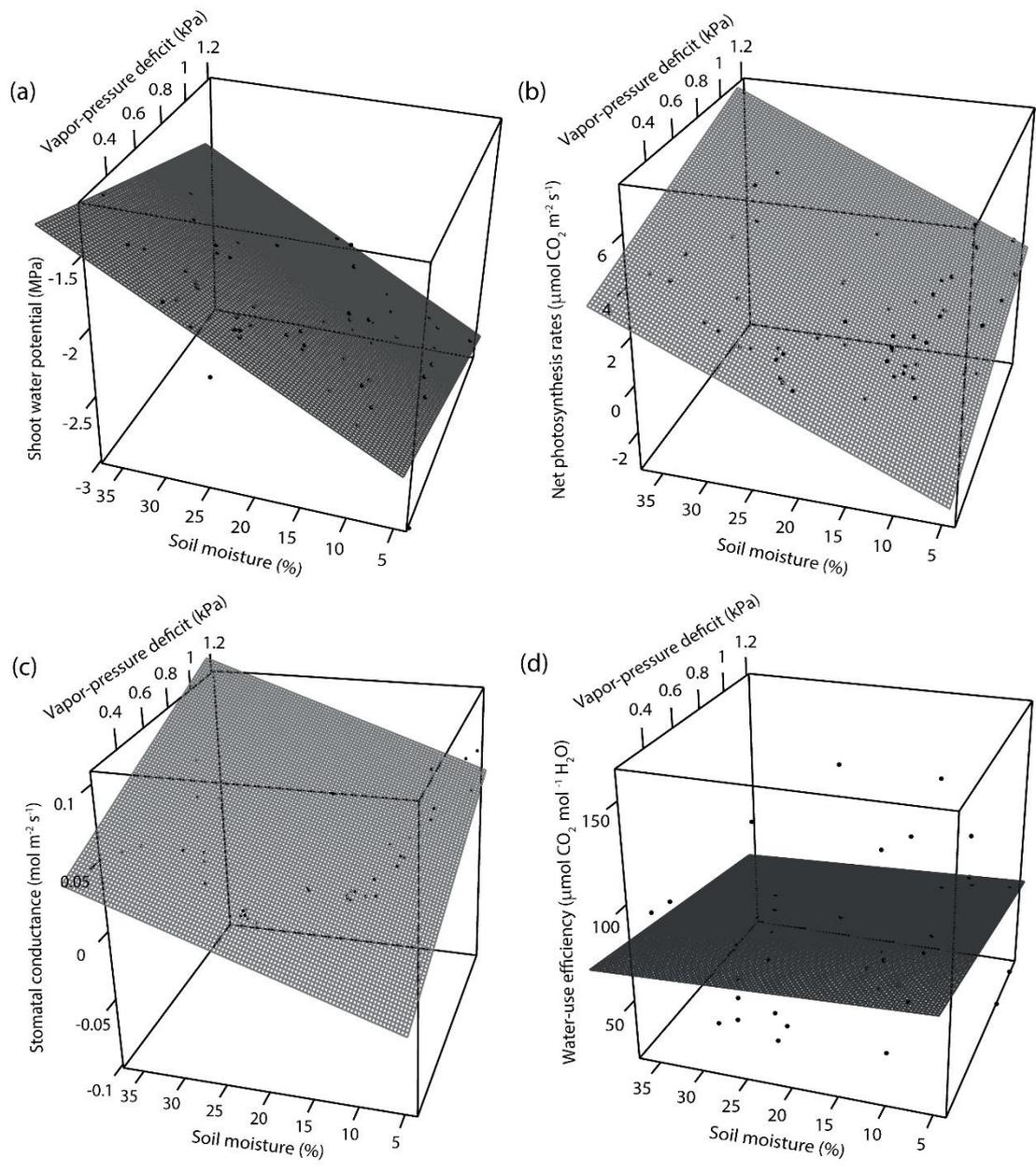


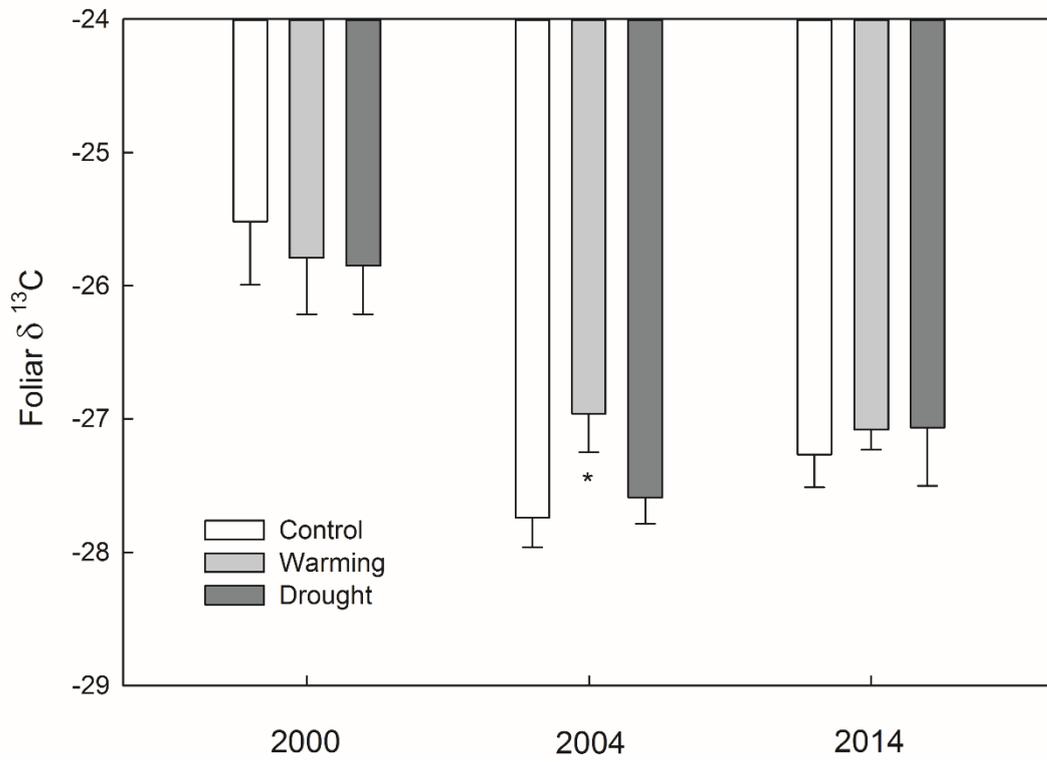
Fig. 6

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