



HAL
open science

Different CO₂ acclimation strategies in juvenile and mature leaves of *Ottelia alismoides*

Wen Min Huang, Hui Shao, Si Ning Zhou, Qin Zhou, Wen Long Fu, Ting Zhang, Hong Sheng Jiang, Wei Li, Brigitte Gontero, Stephen C. Maberly

► **To cite this version:**

Wen Min Huang, Hui Shao, Si Ning Zhou, Qin Zhou, Wen Long Fu, et al.. Different CO₂ acclimation strategies in juvenile and mature leaves of *Ottelia alismoides*. *Photosynthesis Research*, In press, 10.1007/s11120-018-0568-y . hal-01875659

HAL Id: hal-01875659

<https://amu.hal.science/hal-01875659v1>

Submitted on 9 Jan 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Article (refereed) - postprint

Huang, Wen Min; Shao, Hui; Zhou, Si Ning; Zhou, Qin; Fu, Wen Long; Zhang, Ting; Jiang, Hong Sheng; Li, Wei; Gontero, Brigitte; Maberly, Stephen C. 2018. **Different CO₂ acclimation strategies in juvenile and mature leaves of *Ottelia alismoides*.**

© Springer Nature B.V. 2018

This version available <http://nora.nerc.ac.uk/521020/>

NERC has developed NORA to enable users to access research outputs wholly or partially funded by NERC. Copyright and other rights for material on this site are retained by the rights owners. Users should read the terms and conditions of use of this material at <http://nora.nerc.ac.uk/policies.html#access>

This is a post-peer-review, pre-copyedit version of an article published in *Photosynthesis Research*, 138 (2). 219-232. The final authenticated version is available online at: <https://doi.org/10.1007/s11120-018-0568-y>

There may be differences between this version and the publisher's version. You are advised to consult the publisher's version if you wish to cite from this article.

Contact CEH NORA team at
noraceh@ceh.ac.uk

1 **Different CO₂ acclimation strategies in juvenile and mature leaves of *Ottelia alismoides***

2

3 Wen Min Huang^{1,2,*}, Hui Shao^{1,3,*}, Sining Zhou⁴, Qin Zhou⁵, Wenlong Fu¹, Ting Zhang⁶, Hong
4 Sheng Jiang^{1,2}, Wei Li^{1,2}, Brigitte Gontero^{3,†}, Stephen C. Maberly^{7, †}

5

6 1. Key Laboratory of Aquatic Botany and Watershed Ecology, Wuhan Botanical Garden,
7 Chinese Academy of Sciences, Wuhan 430074, China.

8 2. Hubei Key Laboratory of Wetland Evolution & Ecological Restoration, Wuhan Botanical
9 Garden, Chinese Academy of Sciences, Wuhan, 430074, China.

10 3. Aix Marseille Univ, CNRS, BIP, UMR 7281, 31 Chemin Joseph Aiguier, CS70071, 13402
11 Marseille Cedex 20, France.

12 4. Sino-Danish Center, The University of Chinese Academy of Sciences, Beijing 101400,
13 China.

14 5. School of Resources and Environmental Science, Hubei University, Wuhan 430074, China.

15 6. School of Resource and Environmental Engineering, East China University of Science and
16 Technology, Shanghai 200237, China.

17 7. Lake Ecosystems Group, Centre for Ecology & Hydrology, Lancaster Environment Centre,
18 Library Avenue, Bailrigg, Lancaster LA1 4AP, UK.

19 *These authors contributed equally to the work.

20

21 †Corresponding authors: Gontero B (bmeunier@imm.cnrs.fr) and Maberly, S.C
22 (scm@ceh.ac.uk)

23

24

25 **Keywords:** Bicarbonate use; C4 metabolism; carbon dioxide concentrating mechanism (CCM);
26 Crassulacean Acid Metabolism (CAM); freshwater macrophyte; leaf maturity

27 **Abbreviations:**

28 Alk: alkalinity; CAM: Crassulacean Acid Metabolism; CCM: carbon dioxide concentrating
29 mechanism; C_T: concentration of total inorganic carbon; FW: fresh weight; HC: high CO₂ LC:
30 low CO₂; NAD(P)-ME: NAD(P)-malic enzyme; PEP: phosphoenol pyruvate; PEPC: PEP
31 carboxylase; PPDK: pyruvate phosphate dikinase; Rubisco: ribulose 1,5-bisphosphate
32 carboxylase-oxygenase; SD: standard deviation.

33

34 **Abstract**

35 The freshwater macrophyte, *Ottelia alismoides*, is a bicarbonate user performing C₄
36 photosynthesis in the light, and Crassulacean Acid Metabolism (CAM) when acclimated to low
37 CO₂. The regulation of the three mechanisms by CO₂ concentration was studied in juvenile and
38 mature leaves. For mature leaves, the ratios of phosphoenolpyruvate carboxylase (PEPC) to
39 ribulose-bisphosphate carboxylase/oxygenase (Rubisco) are in the range of that of C₄ plants
40 regardless of CO₂ concentration (1.5 ~ 2.5 at low CO₂, 1.8 ~ 3.4 at high CO₂). In contrast, results
41 for juvenile leaves suggest that C₄ is facultative and only present under low CO₂. pH-drift
42 experiments showed that both juvenile and mature leaves can use bicarbonate irrespective of
43 CO₂ concentration, but mature leaves have a significantly greater carbon extracting ability than
44 juvenile leaves at low CO₂. At high CO₂, neither juvenile nor mature leaves perform CAM as
45 indicated by lack of diurnal acid fluctuation. However, CAM was present at low CO₂, though
46 the fluctuation of titratable acidity in juvenile leaves (15 to 17 μequiv g⁻¹ FW) was slightly but
47 significantly lower than in mature leaves (19 to 25 μequiv g⁻¹ FW), implying that the capacity
48 to perform CAM increases as leaves mature. The increased CAM activity is associated with
49 elevated PEPC activity and large diel changes in starch content. These results show that in *O.*
50 *alismoides*, CCMs are more effective in mature compared to juvenile leaves, and C₄ is
51 facultative in juvenile leaves but constitutive in mature leaves.

52

53 **Introduction**

54 The low rate of diffusion and frequently depleted concentration of CO₂ in water coupled with
55 the kinetic inefficiency and poor specificity for CO₂ of the primary carboxylating enzyme
56 ribulose 1,5-bisphosphate carboxylase-oxygenase (Rubisco) can restrict the productivity of
57 submerged macrophytes (Vadstrup and Madsen, 1995; Maberly & Gontero, 2017). However,
58 the presence of carbon dioxide-concentrating mechanisms (CCMs) in some freshwater plants
59 can reduce or overcome the problem of limited inorganic carbon supply. The most frequent
60 CCM in freshwater plants is based on the biophysical active uptake of bicarbonate, which is
61 found in ~45% of tested species (Maberly and Gontero 2017). In addition, Crassulacean Acid
62 Metabolism (CAM) and C₄ metabolism are also present in some freshwater plants (Keeley
63 1981; Bowes and Salvucci 1989; Bowes et al. 2002; Keeley and Rundel 2003). In terrestrial
64 plants adapted to xeric conditions, CAM can be widespread and plays a major role in the water-
65 use efficiency of the plant (Silvera et al. 2010). In aquatic plants, CAM occurs in about ~8% of
66 tested species (Maberly & Gontero 2017) and acts as a carbon-conserving and concentrating
67 mechanism that reduces the loss of respiratory carbon at night and exploits nocturnal CO₂
68 concentration that is often higher than during the day (Klavsen et al. 2011). Terrestrial C₄
69 carbon fixation is known in about 3% of species and involves spatial separation of
70 carboxylation and decarboxylation between different cells, mesophyll and bundle-sheath, or
71 occasionally within one type of cell (Sage 2016). In aquatic plants, C₄ photosynthesis is found
72 in about 4% of tested species, occurs within a single cell, and reduces the effects of
73 photorespiration especially when water column concentrations of oxygen are high and CO₂ are
74 low (Bowes et al. 2002).

75 In CAM and C₄ photosynthesis, the first carboxylating enzyme is usually
76 phosphoenolpyruvate carboxylase (PEPC) (Osmond 1978). In C₄ photosynthesis, PEPC is
77 active during the day but in CAM it is only active during the night (Hatch 1987). PEPC uses
78 phosphoenolpyruvate (PEP) and external or internal respiratory CO₂ to produce the C₄
79 compound oxaloacetate (OAA) that in turn is converted into malate. In C₄ photosynthesis, the
80 malate is decarboxylated close to Rubisco while in CAM, it is transported and stored in the
81 vacuole resulting in the well-known acidification observed during the night. In CAM, during

82 the day, the malic acid is decarboxylated into pyruvate, a C₃ compound and CO₂, that is then
83 fixed by Rubisco within the Calvin-Benson-Bassham cycle. Nocturnal CO₂ fixation via PEPC
84 requires an adequate pool of carbohydrates to generate PEP, in addition to that needed for other
85 metabolic processes including dark respiration, organic carbon export and growth (Nobel and
86 Hartsok 1983). In several CAM species, PEPC-mediated carboxylation is indeed closely linked
87 to the diel turnover of starch, since the amount of CO₂ taken up during the night depends on the
88 availability of C₃-carbon substrate (PEP) produced from the nocturnal degradation of starch
89 accumulated during the day (Ceusters et al. 2014). Thus, in these plants there is a large diel
90 change in transitory starch content as well as acidity (Neuhaus and Schulte 1996).

91 CAM metabolism is a plastic process in freshwater and terrestrial plants. In freshwater
92 plants, its expression can be regulated by a range of environmental parameters, e.g. irradiance,
93 CO₂ availability, temperature and nutrient availability (Keeley et al. 1983; Aulio 1985; Madsen
94 1987; Bowes and Salvucci 1989; Robe and Griffiths 1990; Hostrup and Wiegleb 1991; Klavsen
95 and Maberly 2010; Zhang et al. 2014; Shao et al. 2017). The activity of CAM may also be
96 dependent on leaf maturity and in many terrestrial species CAM is only fully expressed in
97 mature leaves (Jones 1975; Ting et al. 1996; Wen et al. 1997; Taybi et al. 2002). Whether
98 comparable ontogenetic change occurs in aquatic CAM plants has not been widely studied.
99 There is very little information on the influence of leaf maturity on aquatic CAM plants
100 (Klavsen and Madsen 2008) or on the interplay between developmental and environmental
101 factors. In the terrestrial plant, *Mesembryanthemum crystallinum*, salinity was originally
102 reported to induce CAM (Winter and von Willert 1972), but it was later shown that CAM was
103 a genetically controlled developmental programme that was just accelerated by the salinity
104 stress (Adams et al. 1998). C₄ photosynthesis is usually constitutive in terrestrial plants but is
105 facultative in the freshwater macrophytes *Hydrilla verticillata* (Bowes et al. 2002) and *Egeria*
106 *densa* (Casati et al. 2000). Use of bicarbonate can also be plastic and in *Elodea canadensis* and
107 *Ranunculus peltatus* is down-regulated at high CO₂ concentration (Sand-Jensen and Gordon
108 1986; Madsen et al. 1996).

109 It has been shown that in *Ottelia alismoides* from the Hydrocharitaceae, C₄ metabolism
110 and bicarbonate use are constitutive, while CAM metabolism is facultative and only induced at
111 low CO₂ (Zhang et al. 2014). *O. alismoides* operates CAM at night and C₄ metabolism during

112 the day. It is the only known aquatic species to operate CAM and C4 in the same tissue, and
113 one of only two known aquatic species to operate CAM and bicarbonate use (Shao et al. 2017)
114 although six species of *Portulaca* are also known to have constitutive C4 metabolism and
115 facultative CAM induced by water stress (Koch and Kennedy 1980; Guralnick et al. 2002;
116 Holtum et al. 2017).

117 *O. alismoides* leaves differ in size and shape and develop from a basal rosette (Cook and
118 Urmi-König 1984; Yu and Yu 2009). The first juvenile leaves are only 2 to 4 cm wide and 18
119 to 20 cm long, and are linear or lanceolate and either sessile or attenuate (Fig. 1). Under summer
120 conditions, juvenile leaves can develop into mature leaves that have a petiole that can be up to
121 50 cm long and are ovate to cordate, 18 to 20 cm long and up to 20 cm wide. In addition to
122 these morphological characteristics, the fresh weight and leaf area of mature leaves is four to
123 five greater than juvenile leaves but the specific leaf area is 1.15-times greater in juvenile than
124 in mature leaves, while the content of chlorophyll *a* and *b* on a fresh weight basis is similar
125 (Table S1). Previously, we studied fully expanded mature leaves of *O. alismoides*, but we
126 observed that there were significant differences in the diurnal change of acidity between mature
127 and juvenile leaves (unpublished observations). Little is known about differential expression of
128 CCMs in different types of leaf in freshwater plants although Maberly and Spence (1983)
129 reported that linear leaves of *Potamogeton x zizii* were restricted to CO₂ while broad leaves
130 could also use bicarbonate.

131 In the present study, we tested the hypothesis that CAM activity, C4 metabolism and
132 bicarbonate use, will differ between mature and juvenile leaves. We further hypothesised that
133 there will be an interplay between leaf development and acclimation to CO₂ concentration.

134

135 **Materials and methods**

136 Plant material and leaf sampling

137 Seeds of *O. alismoides* were germinated on 20 March 2017 in a one liter glass beaker filled
138 with sterile tap water with an alkalinity of about 1.9 mequiv L⁻¹ and a low nutrient concentration
139 (TP = 0.05 mg L⁻¹; TN = 1.35 mg L⁻¹). The beaker was placed in a growth chamber at 25 °C
140 and the water was replaced daily. Six weeks after germination, two or three seedlings (about 8
141 cm tall) comprising two to four juvenile leaves were transplanted into a plant pot (15 cm

142 diameter, 12 cm high) containing sterile soil from nearby Donghu Lake. The pots were placed
143 in a tank (64 cm deep) that was located in a glasshouse on the flat roof of the laboratory,
144 containing about 400 L of tap water. The water level was increased gradually as the plants grew
145 in order to ensure that the whole plant was fully submerged in the water. Snails and moribund
146 leaves were removed daily and the tap water in the tank was replaced every 2 days.

147

148 Acclimation to CO₂

149 After seven to eight weeks, in mid-July 2017, the plants were 25-30 cm tall with mature and
150 newly produced juvenile leaves. Four pots containing *O. alismoides* plants of similar height
151 from the glasshouse, were placed into each of two white plastic tanks (65 × 45 × 35 cm)
152 containing tap water that was renewed twice a week. The tanks were placed in a constant
153 temperature room at 25 ± 2°C and were illuminated with FSL T5/865 28 W fluorescence tubes,
154 producing 140-150 μmol photon m⁻² s⁻¹ (photosynthetically available radiation; Li-Cor
155 underwater sensor, UWQ, connected to a Li-Cor LI-1400 data logger) at the water surface with
156 a 14 hour light (08:00 to 22:00) and 10 hour dark photoperiod. In each tank, pH was measured
157 with a combination pH electrode (model IP-600-9 Jenco Instruments, USA) centrally located
158 15 cm below the water surface, connected to a microcomputer pH controller (model 6311, Jenco
159 Instruments, USA). The plants were grown at two CO₂ concentrations. In the high CO₂
160 treatment (HC), the target pH of 7.0 was maintained between 6.90 and 7.18 by bubbling the
161 growth medium with pure CO₂ from four tubes, one in each corner of the tank, under the control
162 of the computer. On approximately eight occasions during the daytime, the water was
163 thoroughly mixed and the pH was recorded. In the low CO₂ treatment (LC) the natural
164 photosynthetic activity of the plants depleted the inorganic carbon concentration of the water.
165 The water was thoroughly mixed and the pH measured as described above. The pH ranged from
166 8.65 to over 9.64. Alkalinity was measured every two days (see method below). Over the whole
167 period of acclimation, the CO₂ concentration calculated from pH, alkalinity and temperature
168 using the equations in Maberly (1996), was between 302 and 604 μmol L⁻¹ for the HC treatment,
169 and 0.1 μmol L⁻¹ and 5 μmol L⁻¹, for the LC treatment. These are equivalent to between 22 and
170 44 times and between 1 and 36% times the concentration at equilibrium with 400 ppm for the
171 HC treatment and the LC treatment, respectively. After 14 days, juvenile leaves (newly

172 produced, long and narrow) and mature leaves (oval) from the plants treated with HC and LC
173 were sampled at 07:30 (towards the end of the dark period, hereafter referred to as ‘dawn’) and
174 21:30 (towards the end of the photoperiod, hereafter referred to as ‘dusk’), respectively. Leaves
175 grown at LC tended to have a layer of marl (calcite) on the upper surface which was gently
176 removed by rubbing. After cleaning, the leaves were immediately placed on aluminium foil on
177 top of ice and kept in the dark to reduce metabolic changes. Measurements were made either
178 on one large mature leaf or on two to three juvenile leaves. Each leaf was photographed, blotted
179 dry and weighed. Half of the material was used to determine enzyme activity and pigment
180 content, the other half to determine acidity and starch content. Samples were taken in triplicate
181 from different plants and stored in liquid nitrogen before measurement. Chlorophyll
182 fluorescence and rates of oxygen exchange were measured on fresh leaves in the morning.

183

184 Overnight changes in gas exchange, enzyme activity and chemical composition in detached
185 leaves

186 In order to analyse the capture of respired CO₂ by different types of leaf, one mature leaf or
187 three juvenile leaves (about 1.0 g fresh weight in both cases), detached from the plants grown
188 under HC and LC at dusk were placed in 580 mL gas-tight glass bottles, containing the tap
189 water enriched with CO₂ to a final concentration of 560 μmol L⁻¹, and a dissolved oxygen
190 concentration of 7.7 mg L⁻¹. All the bottles were incubated in the dark, unstirred, at 25°C for 11
191 hours. In addition, at the start of the overnight incubation experiment, one mature or three
192 juvenile leaves were collected from the plants grown either at high or low CO₂, wrapped in foil
193 envelopes and frozen in liquid nitrogen for later determination of acidity, starch and enzyme
194 activity. After overnight incubation, the alkalinity, pH and O₂ concentration of the incubation
195 medium were measured as described below. Meanwhile, the detached leaves in the bottles were
196 removed and acidity, starch and enzyme activity were measured. Measurements were made in
197 triplicate.

198

199 pH-drift

200 About 0.2–0.3 g fresh weight of juvenile and mature leaves grown at HC and LC were washed
201 in tap water and then incubated in 65 mL glass tubes with ground glass stoppers. The glass

202 tubes contained 60 mL of test solution (equimolar concentrations of NaHCO₃ and KHCO₃ at
203 an overall concentration of 1 mM) and about 5 mL air. The glass tubes were incubated, unstirred,
204 in a growth room at 25°C and 120–135 μmol photon m⁻²s⁻¹ PAR. After 24 h continuous
205 irradiance, the final pH was measured with the pH electrode (model IP-600-9 Jenco Instruments,
206 USA) and the final alkalinity in the solution was measured by Gran titration (see below).

207

208 Measurement of CAM activity

209 CAM activity was measured as a change in titratable acidity between minimal acidity levels at
210 dusk and maximal levels at dawn. To measure acidity, 10 mL CO₂-free milliQ water was added
211 to the leaf samples (0.2-0.5 g fresh weight) that had been stored in liquid nitrogen and then
212 boiled for 30 minutes. After cooling to room temperature, the samples were titrated to an
213 endpoint of pH 8.3 with 0.01 N NaOH (Madsen 1987; Zhang et al. 2014). Other methods, e.g.
214 those used by Keeley and co-workers and others titrate to pH 6.4 and Keeley et al. (1983) report
215 that this produced 8% lower estimates of acidity compared to titrating to pH 8.3.

216

217 Measurement of enzyme activity

218 The extraction and assay of PEPC, Rubisco, PPDK, NAD-ME and NADP-ME was based on
219 the methods described by Zhang et al. (2014) and Shao et al. (2017). Enzyme activity was
220 assessed from the rate of appearance or disappearance of NADPH or NADH at 340 nm at 25°C,
221 using a microplate reader (Tecan M200 PRO, Austria). A calibration curve for both cofactors
222 was produced to convert absorbance to concentration.

223

224 Measurement of starch, alkalinity and pH

225 Starch content was determined by the amyloglucosidase assay according to Smith and Zeeman
226 (2006) and Shao et al. (2017). Alkalinity was measured by Gran titration with a standardized
227 solution of HCl (Shao et al. 2017). pH was measured with the pH electrode (model IP-600-9
228 Jenco Instruments, USA) connected to the microcomputer pH controller.

229

230 Measurement of leaf area, chlorophyll content and chlorophyll fluorescence

231 Projected (1-sided) leaf area was estimated from photographs analysed using AreaAna software

232 (Huazhong University of Sciences and Technology, China). Chlorophyll *a* and *b* were extracted
233 overnight in 90% ethanol from samples collected in the morning, and concentrations calculated
234 according to the method of Brain and Solomon (2007). Parameters of chlorophyll variable
235 fluorescence were determined with a pulse modulated fluorometer PAM 2100 (Walz, Germany).
236 Prior to measurements the leaves were kept in the dark for 15 minutes to minimize fluorescence
237 quenching. The parameter F_v/F_m was used as an indicator of the maximal quantum yield of
238 photosystem II (PSII) (Kitajima and Butler 1975). The effective quantum yield of PSII was also
239 obtained using the Win Control software (Walz, Germany).

240

241 Oxygen exchange

242 Oxygen exchange was measured with an optical oxygen electrode system (Unisense OX-13298
243 and a Unisense microsensor multimeter Version 2.01) in a glass and Perspex chamber (62 mL)
244 according to the method of Shao et al. (2017). The chamber was placed in a constant
245 temperature water bath at 25°C and illuminated from the side by fluorescent tubes (36 W, 6500
246 K colour temperature) producing a photon irradiance of 115 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The oxygen electrode
247 was calibrated in the chamber with tap water (alkalinity $\sim 1.9 \text{ mequiv L}^{-1}$) that had been
248 vigorously bubbled either with air from outside the laboratory (100% saturation) or with
249 nitrogen (0% saturation). About 0.4 g fresh weight of leaf was placed in the chamber filled with
250 water from the tank in which the plants were growing and hence measurements were made at
251 the CO_2 concentration at which they were grown. Changes of oxygen concentration were
252 recorded for 5 to 10 minutes on a computer connected to the Unisense meter. After measuring
253 photosynthesis, the chamber was placed in the dark and the decline in oxygen concentration
254 was recorded for 15 minutes.

255

256 Statistical analysis

257 The data were analyzed using SPSS 16.0 (SPSS Inc., Chicago, USA). The significance of CO_2
258 treatment and leaf maturity were determined with one- and two-way ANOVA (with Duncan's
259 and Tukey's post hoc tests). Comparisons of physiological parameters between attached and
260 detached leaves were determined with *t*-tests. Pearson correlation was used to test the
261 correlation between acidity and other parameters (e.g. PEPC activity, starch) and the

262 significance level was set at 5%.

263

264 **Results**

265 Comparison of mature and juvenile leaves at different CO₂ concentration

266 At high CO₂ at dawn, the activities of PEPC and PPDK were significantly lower in juvenile
267 leaves than in mature leaves on a fresh weight basis. However, compared to mature leaves, the
268 activity of Rubisco was markedly higher in juvenile leaves. NAD-ME and NADP-ME were not
269 significantly different between the two types of leaf. The only significant difference in the
270 PEPC to Rubisco ratio was at dusk when mature leaves had a significantly higher ratio than
271 juvenile leaves (Fig. 2, Table 1).

272 At low CO₂, the juvenile leaves had significantly lower PEPC (1.64-fold, SD = 1.72) and
273 Rubisco (1.42-fold, SD = 0.64) activity than mature leaves on a fresh weight basis (Fig. 2, Table
274 1) and also on an area leaf basis (data not shown). In contrast, the activities of PPDK, NAD-
275 ME and NADP-ME were not significantly affected by leaf maturity (Fig. 2, Table 1). The PEPC
276 to Rubisco ratios in juvenile and mature leaves at dawn and dusk, were not significantly
277 different (Fig. 2). At low and high CO₂, the content of chlorophyll *a*, chlorophyll *b* and total
278 chlorophyll per unit fresh weight was not significantly different in juvenile and mature leaves
279 (Fig. 3A) according to the one-way ANOVA analysis. However, a two-way ANOVA, showed
280 that leaf maturity has a significant effect on the content of chlorophyll *a* and total chlorophyll
281 because of an interaction between leaf maturity and CO₂ (Table 2).

282 At low CO₂, the maximal photochemical efficiency of PSII (Fv/Fm) and the actual
283 photochemical efficiency of PSII, or yield, were slightly but significantly higher in the mature,
284 compared to the juvenile leaves (ratio of 1.1, SD = 0.03 for Fv/Fm and 1.1, SD = 0.01 for yield).
285 In contrast, at high CO₂, the maximal photochemical efficiency of PSII was slightly but
286 significantly higher in the juvenile leaves (ratio of 1.05, SD = 0.03), while the yield was similar
287 in both types of leaf (Fig. 3B, Table 2). At both low and high CO₂, the rate of net photosynthesis
288 and the rate of respiration were not significantly different for juvenile and mature leaves (Fig.
289 3C, Table 2).

290 At low CO₂, for both types of leaf, there were marked diel changes in acidity. The change
291 was significantly lower in juvenile leaves (15 µequiv g⁻¹ FW) than in mature leaves (19 µequiv

292 g^{-1} FW) (Fig. 4A). In contrast, at high CO_2 , there was no significant difference in the diel change
293 in acidity between juvenile and mature leaves and the average diel acidity change was only 8.2
294 $\mu\text{equiv g}^{-1}$ FW (Fig. 4A). At dusk and dawn, at low CO_2 , starch content was similar in juvenile
295 and mature leaves while at high CO_2 , it was significantly lower in juvenile leaves compared to
296 mature leaves (Fig. 4B).

297

298 Responses of juvenile and mature leaves to variable CO_2

299 In mature leaves, the Rubisco activity was significantly higher at low, compared to high CO_2 in
300 both dusk (2.45-fold, SD = 0.64) and dawn (1.74-fold, SD = 0.89). However, in juvenile leaves,
301 the Rubisco activity was significantly lower at low, compared to high CO_2 (1.41-fold, SD =
302 0.62) at dusk (Fig. 2A). The PEPC activity at low CO_2 was significantly higher than at high
303 CO_2 in mature leaves both at dawn and dusk (1.83-fold, SD = 1.72; 1.43-fold, SD = 1.30). The
304 same tendency was found in juvenile leaves (1.76-fold, SD = 1.16), but only at dawn (Fig. 2B).
305 The PPDK activity at low CO_2 was significantly higher than at high CO_2 in mature leaves at
306 dusk (1.35-fold, SD = 1.87), the same tendency was found in juvenile leaves at dawn (1.59-
307 fold, SD = 1.35) (Fig. 2D). There was no significant effect of CO_2 concentration on NAD-ME
308 and NADP-ME activities from mature and juvenile leaves at either dusk or dawn (Fig. 2E, 2F).

309 In mature leaves, Fv/Fm was slightly but significantly higher (1.1-fold, SD = 0.03) at low,
310 compared to high CO_2 but in juvenile leaves, the opposite, significant, response (0.93-fold, SD
311 = 0.02) was found. The yield was not significantly affected by CO_2 concentration in the mature
312 leaves, however it was slightly but significantly lower (1.2-fold, SD = 0.01) in the juvenile
313 leaves at low compared to high CO_2 (Fig. 3B, Table 2). The rate of net photosynthesis at high
314 CO_2 was significantly higher than at low CO_2 in mature (1.4-fold, SD = 0.7) and juvenile leaves
315 (1.3-fold, SD = 0.6). The respiration rate was significantly lower in juvenile leaves (1.5-fold,
316 SD = 0.4) at low CO_2 but in mature leaves, this rate was not affected by CO_2 concentration (Fig.
317 3C, Table 2). There was no significant effect of CO_2 concentration on chlorophyll content from
318 mature and juvenile leaves at dusk or dawn (Fig. 3A, Table 2).

319 The diel change in acidity was significantly greater at low than at high CO_2 for both
320 juvenile (1.65-fold, SD = 0.61) and mature leaves (2.34-fold, SD = 0.79) (Fig. 4A, Table 2). At
321 dusk, the amount of starch was significantly greater at high than at low CO_2 in both juvenile

322 (1.63-fold, SD = 2.06) and mature leaves (1.75-fold, SD = 2.42) (Fig. 4B, Table 2). The amount
323 of starch left at the beginning of the next day in juvenile and mature leaves was both
324 significantly lower at low, compared to high CO₂. Compared to dusk, the amount of starch
325 present at dawn was reduced by 75 and 67 % for mature and juvenile leaves respectively at low
326 CO₂, but at high CO₂ this reduction was only 31 and 42 % for mature and juvenile leaves
327 respectively (Fig. 4B).

328

329 Overnight changes in leaves detached from plants grown at high and low CO₂

330 The overnight incubation experiments and acclimation experiments were performed on the
331 same plants material collected at dusk. The measurements, the next morning, were made on
332 leaves attached to the plant in the acclimation experiments or detached from the plant in the
333 overnight incubation experiment. This provided an opportunity to compare responses of
334 attached and detached juvenile or mature leaves from plants grown at low or high CO₂.

335

336 *Enzyme activity*

337 The pattern of enzyme activity in detached leaves was very similar to that found in attached
338 leaves. Irrespective of the leaf type, the activities of Rubisco, PEPC and PPDK in leaves
339 detached from plants grown at low CO₂ were significantly higher than those in leaves detached
340 from plants grown at high CO₂, at either the start or the end of the overnight incubation. In
341 contrast, the activities of NAD-ME and NADP-ME were the same in both types of leaf
342 irrespective of CO₂ concentration (Fig. S1). In detached mature leaves from plants grown at
343 low CO₂ at dawn, the activity of PPDK was significantly higher in detached than in attached
344 leaves, but there were no other significant differences for any of the other enzyme activities
345 (Table 3).

346

347 *Rates of gas exchange in the dark*

348 Rates of respiratory O₂ consumption were similar in detached juvenile and mature leaves from
349 high CO₂ or low CO₂ treated plants (Fig. 5A). For the mature leaves detached from plants grown
350 at high CO₂, there was a net release of CO₂ and the molar ratio of CO₂ released to O₂ consumed

351 was 0.13 (SD = 0.02). For the juvenile leaves detached from plants grown at high CO₂, there
352 was a net uptake of CO₂ and the ratio of CO₂ consumed to O₂ consumed was 0.83 (SD = 0.13).
353 In contrast, at low CO₂, there was a net uptake of CO₂ for both types of detached leaves. The
354 CO₂ uptake was 120 % (SD = 11) and 280 % (SD = 20) of O₂ uptake for mature and juvenile
355 leaves, respectively (Fig. 5A).

356

357 *Acidity and starch*

358 The pattern of acidity changes in attached and detached leaves was not significantly different
359 (Fig. 4A and Fig. 5B; Table 3). Like in the previous experiment with attached leaves (see Fig.
360 4A), the diel change in acidity was not significantly different in mature leaves detached from
361 plants grown at high CO₂. However, for plants grown at low CO₂ the change in acidity in
362 detached juvenile and mature leaves was significantly different at 17 and 25 µequiv g⁻¹ FW,
363 respectively (Fig. 5B).

364 There were no significant differences for starch variation between attached and detached
365 leaves at dusk (Table 3). Like in the acclimation experiments using attached leaves, the amount
366 of starch decreased after overnight incubation in all detached leaves from plants grown at either
367 low or high CO₂ (Fig. 5C). Moreover, after the overnight incubation, when compared to the
368 corresponding attached leaves, the starch content was significantly higher in juvenile leaves
369 detached from plants grown at high CO₂ and mature leaves detached from plants grown at low
370 CO₂ (Table 3).

371

372 *Capture of respired CO₂ by malic acid*

373 Using the assumptions that 2 moles of H⁺ are equivalent to 1 mole of malic acid and that the
374 respiratory quotient (CO₂/O₂) is 1, the percent of dark respiration that could be trapped as malic
375 acid was 6.3% for juvenile leaves and 4.9% for mature leaves detached from plants grown at
376 high CO₂. The equivalent value for leaves detached from plants grown at low CO₂, was 8.4%
377 and 6.6% for juvenile and mature leaves, respectively.

378

379 Relationships between acidity, starch and enzyme activities for plants grown at different
380 concentrations of CO₂

381 We combined data from juvenile and mature leaves to analyze the relationships between
382 enzyme activities and change in acidity. For plants grown at low CO₂, there were no significant
383 relationships between acidity and PEPC activity for attached leaves grown under low CO₂
384 neither at dusk ($r=0.688$, $P=0.131$) nor at dawn ($r=0.719$, $P=0.107$). However, there was a
385 significant positive relationship between the change in acidity and PEPC activity (with
386 combined data from dawn and dusk) for attached leaves grown at low CO₂ ($r=0.624$, $P=0.03$,
387 Fig. 6A). In contrast, the activities of Rubisco, PPDK, NAD-ME and NADP-ME were not
388 correlated to change in acidity ($P>0.05$). Combining data from mature leaves grown at both
389 CO₂ concentrations, a positive correlation between change in acidity and consumption of starch
390 was observed ($r= 0.885$, $P=0.019$, Fig. 6B); this relationship was absent in juvenile leaves.

391

392 pH-drift

393 The pH-drift experiments provided clear evidence for bicarbonate use in both types of leaf
394 grown at different CO₂ concentrations since final CO₂ concentrations were substantially less
395 than 1 $\mu\text{mol L}^{-1}$. Both the mature and juvenile leaves grown at high CO₂ were able to raise the
396 pH to over 9.20, at which point the [CO₂] and [HCO₃⁻] were 0.113 $\mu\text{mol L}^{-1}$ and 0.11 mmol L^{-1}
397 for mature leaves, 0.282 $\mu\text{mol L}^{-1}$ and 0.373 mmol L^{-1} for juvenile leaves, respectively (Table
398 4). Both the mature and juvenile leaves grown at low CO₂ were able to raise the pH to over
399 10.0, at which point the [CO₂] and [HCO₃⁻] were 0.023 $\mu\text{mol L}^{-1}$ and 0.18 mmol L^{-1} for mature
400 leaves and 0.093 $\mu\text{mol L}^{-1}$ and 0.387 mmol L^{-1} for juvenile leaves, respectively (Table 4). The
401 alkalinity in the HCML treatment was substantially reduced at the end of the drift probably
402 because of carbonate precipitation or release of organic acid or both. The carbon uptake ability
403 estimated using the quotient of final [C_T] to alkalinity (C_T/Alk) is shown in Table 4. The C_T/Alk
404 for mature and juvenile leaves grown at high CO₂ were both significantly higher than at low
405 CO₂. Moreover, the ratio for mature leaves grown at low CO₂ was statistically different and
406 lower than for juvenile leaves at low CO₂ (Table 4).

407

408 Discussion

409 The results presented here are concordant with earlier work showing that mature leaves of *O.*
410 *alismoides* have a constitutive use of bicarbonate and C4 metabolism, and operate CAM

411 facultatively at low CO₂ (Zhang et al. 2014; Yin et al. 2017; Shao et al. 2017) and confirmed
412 our hypotheses that: i) juvenile leaves behave differently to mature leaves, and ii) that
413 developmental stage and CO₂ concentration both have an effect on CCM activity. Although the
414 conclusion that *O. alismoides* possesses C4 metabolism relies mainly on enzyme activity, it is
415 consistent with studies performed with other species such as *Hydrilla* where interpretation of
416 enzyme activities have been confirmed by other approaches such as radiocarbon pulse-chase
417 experiments (Salvucci and Bowes Bowes et al. 2002). Although CAM was induced at low CO₂
418 in both types of leaf, the extent of CAM was lower in juvenile leaves. Even in mature leaves,
419 the magnitude of titratable acid change (maximum of 34 µequiv g⁻¹ fresh weight; Zhang et al.
420 2014), is substantially lower than in other freshwater plants such as *Isoetes* (Pedersen et al.
421 2011). The smaller diel change in acidity measured in *O. alismoides* is not the result of the
422 different pH end-point used here (pH 8.3) and in Pedersen et al. (2011) (pH 6.4), since Keeley
423 et al. (1983) showed that the difference between acidity measured at these two pH end-points
424 was only 8 %. However, similar low CAM activity, has been found in two species of the
425 terrestrial plant *Portulaca* that operate C4 photosynthesis and CAM when droughted. Values of
426 up to 9 and 54 µequiv g⁻¹ fresh weight in *P. cyclophylla* and *P. digyna* (Holtum et al. 2017) are
427 in the same range as for *O. alismoides*. The low CAM activity in plants with an additional C4
428 metabolism may therefore result from an anatomical and/or biochemical limitation.

429 Leaf maturity seems to be a factor determining the magnitude of induced CAM activity in
430 *O. alismoides*. A similar developmental effect on CAM activity can occur in terrestrial plants.
431 Gas exchange measurements showed that under drought conditions CAM was only present in
432 mature leaves of *Clusia rosea* (Winter et al. 2008). PEPC is a highly regulated enzyme (Winter
433 1980) that plays a key role in C4 and CAM. In *O. alismoides*, the activity of PEPC during day
434 and night, increased with leaf maturity, especially at low CO₂, concurrent with an increase in
435 CAM activity. Similarly, in terrestrial plants, PEPC activity was higher in mature than in young
436 leaves in *Mesembryanthemum crystallinum* (von Willert et al. 1976), *Bryophyllum calycinum*
437 (Nishida 1978) and in *Peperomia camptotricha* (Ting et al. 1993). Therefore, the possibility
438 that environmental signals, in concert with developmental processes, affects an accelerated shift
439 towards CAM is certainly plausible in *O. alismoides*.

440 During the day, CO₂ is fixed and partly converted into transitory starch that is degraded

441 during the following night (Huber 1983). The diurnal turnover of transitory starch is an
442 important characteristic of plant metabolism and its alteration by environmental conditions may
443 affect both plant growth and plant habit (Schulze et al. 1990). In several CAM-induced plants,
444 the metabolic products of nocturnal starch degradation are mainly converted into the primary
445 CO₂ acceptor PEP (Smith and Bryce 1992). For example, in *M. crystallinum*, the induction of
446 CAM by exposure to salinity was accompanied by increased activities of a range of starch-
447 degrading enzymes (Paul et al. 1993). Therefore, the large increase in starch turnover when
448 CAM was induced in both juvenile and mature leaves of *O. alismoides* is consistent with the
449 important role of starch metabolism in CAM plants. Especially in mature leaves, there was a
450 positive correlation between change in starch and CAM activity. The higher starch content at
451 the end of the night in detached leaves compared to attached leaves implies that there are
452 alternative fates for starch in attached leaves including export for growth of young leaves and
453 the plant as a whole as also found in *M. crystallinum* by Borland and Dodd (2002).

454 In the present study, at high and low CO₂ the PEPC to Rubisco ratio in mature leaves is in
455 a range typical of terrestrial C₄ plants and consistent with previous reports showing that C₄
456 metabolism is constitutive in *O. alismoides* (Zhang et al. 2014; Shao et al. 2017; Yin et al. 2017).
457 Similarly, in juvenile leaves grown at low CO₂, the PEPC to Rubisco ratio was between 1.4 ~
458 2.2, that is also consistent with C₄ metabolism. In contrast, in juvenile leaves grown at high
459 CO₂, the PEPC to Rubisco ratio (0.9 ~ 1.1), was significantly lower than that of mature leaves
460 and implies that C₄ metabolism is absent. Although juvenile leaves are produced at the base of
461 the plants, the low light is unlikely to be responsible for the lack of C₄ metabolism since Shao
462 et al. (2017) showed that the PEPC to Rubisco ratio of mature leaves at high CO₂ and low light
463 was between 2 and 3. This down-regulation of C₄ metabolism at high CO₂ is the pattern also
464 found in other C₄ freshwater plants such as *Hydrilla verticillata* (Bowes et al. 2002) and *Egeria*
465 *densa* (Casati et al. 2000). Further studies are ongoing to characterise C₄ photosynthesis in
466 leaves of *O. alismoides* in the light.

467 Our data showed that photosynthetic carbon uptake was able to drive the concentration of
468 CO₂ in the solution well below 1 μM (i.e. about 7% of air-equilibrium) with both types of leaf
469 irrespective of CO₂. This indicates that carbon uptake was not relying solely on passive
470 diffusion of CO₂, since freshwater macrophytes restricted to CO₂ typically have CO₂

471 compensation point ranging from 2 to 6 μM (Maberly and Spence 1983). Our results suggest
472 that *O. alismoides* can use bicarbonate like many other macrophytes (Maberly and Gontero
473 2017). The markedly higher value of C_T/Alk in juvenile leaves compared with mature leaves
474 when grown at low CO_2 indicate that the mature leaves have a greater ability for carbon
475 extracting from the solution, especially at low CO_2 .

476 Hitherto, *O. alismoides*, is the only known species with three CCMs: bicarbonate use, C4
477 metabolism and CAM. These CCMs are differentially regulated in response to high and low
478 CO_2 and in the two types of leaf. In juvenile leaves, C4 metabolism was not induced at high
479 CO_2 , CAM activity was 1.7-fold lower, while bicarbonate use was the least down-regulated by
480 1.3-fold. In mature leaves, CAM was the most strongly down-regulated by 2.3-fold at high CO_2 ,
481 bicarbonate use was down-regulated by 1.6-fold while C4 metabolism was not affected.
482 Moreover, CAM in *O. alismoides* is induced by a combination of factors including low CO_2
483 and leaf aging. High light and temperature are also likely to promote CAM in this species
484 because Zhang et al. (2014) found greater changes in acidity (34 compared to 19 $\mu\text{equiv g}^{-1}$
485 FW) in mature leaves grown at natural light and temperatures up to 31°C. These conditions also
486 produced a greater PEPC to Rubisco ratio of about 6.0 (compared to 3.4 here) and a lower
487 C_T/Alk of 0.27 (compared to 0.47 here). Therefore the regulation of CCMs in *O. alismoides* is
488 controlled by a combination of external environmental factors and internal ontogeny. More
489 work is required to understand better the molecular mechanisms underlying the regulation of
490 these CCMs.

491

492 **Conclusions and outlook**

493 Mature leaves of *O. alismoides* possess bicarbonate use and C4 metabolism constitutively and
494 operate low-level CAM at low CO_2 , while juvenile leaves only have bicarbonate use as a
495 constitutive CCM and operate CAM and C4 facultatively. The magnitude of CAM activity in
496 juvenile leaves is lower than in mature leaves but both are in a similar range to two species of
497 terrestrial C4-CAM from the genus *Portulaca*. The costs and benefits of operating multiple
498 CCMs in parallel in aquatic and terrestrial plants and the mechanisms involved in regulating
499 them require further study. There might be value in research that combines studies on aquatic
500 and terrestrial plants since the environmental stresses (CO_2 or water) differ while the responses

501 are similar.

502

503 This manuscript contains supplementary information.

504

505 **Acknowledgements**

506 This research was supported by the Strategic Priority Research Program of the Chinese
507 Academy of Sciences (XDPB0201), the Chinese Academy of Sciences President's International
508 Fellowship Initiative to SCM and BG (2015VBA023, 2016VBA006) and the National Key
509 Research and Development Program of China (2016YFA0601000). We thank the two reviewers
510 for their helpful comments.

511

512 **Compliance with ethical standards**

513 Not applicable

514

515 **References**

- 516 Adams P, Nelson DE, Yamada S, Chmara W, Jensen RG, Bohnert HJ, Griffiths H (1998)
517 Growth and development of *Mesembryanthemum crystallinum* (Aizoaceae). *New Phytol*
518 138:171–190
- 519 Aulio K (1985) Differential expression of diel acid metabolism in two life forms of *Littorella*
520 *uniflora* (L.) Aschers. *New Phytol* 100:533–536
- 521 Borland AM, Dodd AN (2002) Carbohydrate partitioning in crassulacean acid metabolism
522 plants: reconciling potential conflicts of interest. *Funct Plant Biol* 29:707–716
- 523 Bowes G, Salvucci ME (1989) Plasticity in the photosynthetic carbon metabolism of submerged
524 aquatic macrophytes. *Aquat Bot* 34:233–266
- 525 Bowes G, Rao SK, Estavillo GM, Reiskind JB (2002) C4 mechanisms in aquatic angiosperms:
526 comparisons with terrestrial C4 systems. *Funct Plant Biol* 29:379–392
- 527 Brain RA, Solomon KR (2007) A protocol for conducting 7-day daily renewal tests with *Lemna*
528 *gibba*. *Nat Protoc* 2:979–987

529 Casati P, Lara MV, Andreo CS (2000) Induction of a C₄-like mechanism of CO₂ fixation in
530 *Egeria densa*, a submersed aquatic species. *Plant Physiol* 123:1611-1621

531 Ceusters J, Borland AM, Taybi T, Frans M, Godts C, De Proft MP (2014) Light quality
532 modulates metabolic synchronization over the diel phases of crassulacean acid metabolism.
533 *J Exp Bot* 65:3705–3714

534 Cook CDK, Urmi-König K (1984) A revision of the genus *Ottelia* (Hydrocharitaceae). 2. The
535 species of Eurasia, Australasia and America. *Aquat Bot* 20:131-177

536 Guralnick LJ, Edwards GE, Ku MSB, Hockema B, Franceschi VR (2002) Photosynthetic and
537 anatomical characteristics in the C₄-crassulacean acid metabolism-cycling plant,
538 *Portulaca grandiflora*. *Funct Plant Biol* 29: 763–773

539 Hatch MD (1987) C₄ photosynthesis: a unique blend of modified biochemistry, anatomy and
540 ultrastructure. *Biochim Biophys Acta* 895:81-106

541 Holtum JAM, Hancock LP, Edwards EJ, Winter K (2017) Optional use of CAM photosynthesis
542 in two C₄ species, *Portulaca cyclophylla* and *Portulaca digyna*. *J Plant Physiol* 214:91–
543 96

544 Hostrup O, Wiegleb G (1991) The influence of different CO₂ concentrations in the light and the
545 dark on diurnal malate rhythm and phosphoenolpyruvate carboxylase activities in leaves
546 of *Littorella uniflora* (L.) Aschers. *Aquat Bot* 40:91–100

547 Huber SC (1983). Relation between photosynthetic starch formation and dry-weight
548 partitioning between the shoot and root. *Can J Bot* 61:2709–2716

549 Jones MB (1975) The effect of leaf age on leaf resistance and CO₂ exchange of the CAM plant
550 *Bryophyllum fedtschenkoi*. *Planta* 123:91–96

551 Keeley JE (1981) *Isoetes howelli*—a submerged aquatic CAM plant. *Am J Bot* 68:420–424

552 Keeley JE, Walker CM, Mathews RP (1983) Crassulacean acid metabolism in *Isoetes bolanderi*
553 in high elevation oligotrophic lakes. *Oecologia* 58:63–69

554 Keeley JE, Rundel PW (2003) Evolution of CAM and C₄ carbon concentrating mechanisms.
555 *Int J Plant Sci* 164:S55–S77

556 Kitajima M, Butler WL (1975) Quenching of chlorophyll fluorescence and primary
557 photochemistry in chloroplasts by dibromothymoquinone. *Biochim Biophys Acta*

558 376:105–115

559 Klavsen SK, Madsen TV (2008) Effect of leaf age on CAM activity in *Littorella uniflora*. *Aquat*

560 *Bot* 89:50–56

561 Klavsen SK, Maberly SC (2010) Effect of light and CO₂ on inorganic carbon uptake in the

562 invasive aquatic CAM plant *Crassula helmsii*. *Funct Plant Biol* 37:737–747

563 Klavsen SK, Madsen TV, Maberly SC (2011) Crassulacean acid metabolism in the context of

564 other carbon-concentrating mechanisms in freshwater plants: a review. *Photosynth Res*

565 109:269–279

566 Koch K, Kennedy RA (1980) Characteristics of crassulacean acid metabolism in the succulent

567 C₄ dicot, *Portulaca oleracea* L. *Plant Physiol* 65:193–197

568 Maberly SC, Spence DHN (1983) Photosynthetic inorganic carbon use by freshwater plants. *J*

569 *Ecol* 71:705–724

570 Maberly SC (1996) Diel, episodic and seasonal changes in pH and concentrations of inorganic

571 carbon in a productive lake. *Freshwat Biol* 35:579–598

572 Maberly SC, Madsen TV (2002) Use of bicarbonate ions as a source of carbon in photosynthesis

573 by *Callitriche hermaphroditica* L. *Aquat Bot* 73:1-7

574 Maberly SC, Gontero B (2017) Ecological imperatives for aquatic CO₂-concentrating

575 mechanisms. *J Exp Bot* 68:3797-3814

576 Madsen TV (1987) The effect of different growth conditions on dark and light carbon

577 assimilation in *Littorella uniflora*. *Physiol Plantarum* 70:183–188

578 Madsen TV, Maberly SC, Bowes G (1996) Photosynthetic acclimation of submersed

579 angiosperms to CO₂ and HCO₃⁻. *Aquat Bot* 53:15-30

580 Neuhaus HE, Schulte N (1996) Starch degradation in chloroplasts isolated from C₃ or CAM

581 (crassulacean acid metabolism)-induced *Mesembryanthemum crystallinum* L. *Biochem J*

582 318:945–953

583 Nishida K (1978) Effect of leaf age on light and dark ¹⁴CO₂ fixation in a CAM plant,

584 *Bryophyllum calycinum*. *Plant & Cell Physiol* 19:935–941

585 Nobel PS, Hartsok TL (1983) Relationships between photosynthetically active radiation,

586 nocturnal acid accumulation, and CO₂ uptake for a Crassulacean Acid Metabolism plant,

587 *Opuntia ficus-indica*. *Plant Phys* 71:71-75

588 Osmond CB (1978) Crassulacean Acid Metabolism: a curiosity in context. *Ann Rev Plant*
589 *Physiol* 29:379-414

590 Paul MJ, Loos K, Stitt M, Ziegler P (1993) Starch-degrading enzymes during the induction of
591 CAM in *Mesembryanthemum crystallinum*. *Plant Cell Environ* 16:531–538

592 Pedersen O, Rich SM, Pulido C, Cawthray GR, Colmer TD (2011) Crassulacean acid
593 metabolism enhances underwater photosynthesis and diminishes photorespiration in the
594 aquatic plant *Isoetes australis*. *New Phytol* 190:332–339

595 Robe WE, Griffiths H (1990) Photosynthesis of *Littorella uniflora* grown under two PAR
596 regimes: C₃ and CAM gas exchange and the regulation of internal CO₂ and O₂
597 concentrations. *Oecologia* 85:128–136

598 Sage RF (2016) A portrait of the C₄ photosynthetic family on the 50th anniversary of its
599 discovery: species number, evolutionary lineages, and Hall of Fame. *J Exp Bot* 67:4039–
600 4056

601 Salvucci ME and Bowes G (1983) Two photosynthetic mechanisms mediating the low
602 photorespiratory state in submersed aquatic angiosperms. *Plant Physiol* 73:488-496

603 Sand-Jensen K, Gordon DM. (1986). Variable HCO₃⁻ affinity of *Elodea canadensis* Michaux
604 in response to different HCO₃⁻ and CO₂ concentrations during growth. *Oecologia* 70:426–
605 432

606 Schulze W, Stitt M, Schulze ED, Neuhaus HE, Fichtner K (1990) A quantification of the
607 significance of assimilatory starch for growth of *Arabidopsis thaliana* L. Heynh. *Plant*
608 *Physiol* 95:890–895

609 Shao H, Gontero B, Maberly SC, Jiang HS, Cao Y, Li W, Huang WM (2017) Responses of
610 *Ottelia alismoides*, an aquatic plant with three CCMs, to variable CO₂ and light. *J Exp Bot*
611 68:3985–3995

612 Silvera K, Neubig KM, Whitten WM, Williams NH, Winter K, Cushman JC (2010) Evolution
613 along the crassulacean acid metabolism continuum. *Funct Plant Biol* 37:995–1010

614 Smith AM, Zeeman SC (2006) Quantification of starch in plant tissues. *Nat Protoc* 1:1342–
615 1345

616 Smith JAC, Bryce JH (1992) Metabolite compartmentation and transport in CAM plants. In:
617 Tobin AK (ed) *Plant organelles*. Cambridge University Press, Cambridge, U.K. pp 141–

618 167

619 Taybi T, Cushman JC, Borland AM (2002) Environmental, hormonal and circadian regulation
620 of crassulacean acid metabolism expression. *Funct Plant Biol* 29:669–678

621 Ting IP, Hann J, Sipes D, Patel A, Walling LL (1993) Expression of P-enolpyruvate carboxylase
622 and other aspects of CAM during the development of *Peperomia camptotricha* leaves. *Bot*
623 *Acta* 106:313–319

624 Ting IP, Patel A, Kaur S, Hann J, Walling L (1996) Ontogenetic development of crassulacean
625 acid metabolism as modified by water stress in *Peperomia*. In: Winter K, Smith JAC (eds)
626 Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution. Springer,
627 Heidelberg, pp 204–215

628 Vadstrup M, Madsen TV (1995) Growth limitation of submerged aquatic macrophytes by
629 inorganic carbon. *Freshwat Biol* 34:411–419

630 von Willert DJ, Kirst GO, Treichel S, von Willert K (1976) The effect of leaf age and salt stress
631 on malate accumulation and phosphoenolpyruvate carboxylase activity in
632 *Mesembryanthemum crystallinum*. *Plant Sci Lett* 7:341–346

633 Wen H, Wagner J, Larcher W (1997) Growth and nocturnal acid accumulation during early
634 ontogeny of *Agave attenuate* grown in nutrient solution and in vitro culture. *Biol Plant*
635 39:1–11

636 Winter K, von Willert DJ (1972) NaCl-induzierter Crassulaceen säurestoffwechsel bei
637 *Mesembryanthemum crystallinum*. *Zeitschrift für Pflanzenphysiologie* 67:166–170

638 Winter K (1980) Day/night changes in the sensitivity of phosphoenolpyruvate carboxylase to
639 malate during Crassulacean acid metabolism. *Plant Physiol* 65:792–796

640 Winter K, Garcia M, Holtum JAM (2008) On the nature of facultative and constitutive CAM:
641 environmental and developmental control of CAM expression during early growth of
642 *Clusia*, *Kalanchoë*, and *Opuntia*. *J Exp Bot* 59:1829–1840

643 Yin LY, Li W, Madsen TV, Maberly SC, Bowes G (2017) Photosynthetic inorganic carbon
644 acquisition in 30 freshwater macrophytes. *Aquat Bot* 140:48–54

645 Yu LF, Yu D (2009) Responses of the threatened aquatic plant *Ottelia alismoides* to water level
646 fluctuations. *Fund Appl Limnol* 175:295–300

647 Zhang YZ, Yin LY, Jiang HS, Li W, Gontero B, Maberly SC (2014) Biochemical and

648 biophysical CO₂ concentrating mechanisms in two species of freshwater macrophyte
649 within the genus *Ottelia* (Hydrocharitaceae). *Photosynth Res* 121:285–297
650

651 **Figure legends**

652

653 **Fig. 1** Photographs of mature (A) and juvenile (B) leaves of *O. alismoides*. The scale bar equals
654 5 cm.

655

656 **Fig. 2** Influence of CO₂ concentration on activities of enzymes from *O. alismoides* collected at
657 dawn and dusk for juvenile and mature leaves. (A) Rubisco, (B) PEPC, (C) PEPC:Rubisco ratio,
658 (D) PPK, (E) NAD-ME and (F) NADP-ME. There are four treatments: mature leaves grown
659 at high CO₂ (HC, 302 to 604 μmol L⁻¹); juvenile leaves grown at high CO₂ (HC, 302 to 604
660 μmol L⁻¹); mature leaves grown at low CO₂ (LC, 0.1 to 5 μmol L⁻¹); juvenile leaves grown at
661 low CO₂ (LC, 0.1 to 5 μmol L⁻¹). Mean values with their SD (n=3) are presented and differences
662 among means were tested using one-way ANOVA with Duncan's and Tukey's post hoc tests.
663 Data with different letters are significantly different within the four treatments (*P*<0.05).

664

665 **Fig. 3** Influence of CO₂ concentration on pigment content for juvenile and mature leaves (A),
666 chlorophyll fluorescence (B) and rate of O₂ exchange (C) of *O. alismoides*. There are four
667 treatments: mature leaves grown at high CO₂ (HC, 302 to 604 μmol L⁻¹); juvenile leaves grown
668 at high CO₂ (HC, 302 to 604 μmol L⁻¹); mature leaves grown at low CO₂ (LC, 0.1 to 5 μmol L⁻¹);
669 juvenile leaves grown at low CO₂ (LC, 0.1 to 5 μmol L⁻¹). Mean values with their SD (n=3)
670 are presented and differences among means were tested using one-way ANOVA with Duncan's
671 and Tukey's post hoc tests. Data with different letters and symbols are significantly different
672 within the four treatments (*P*<0.05).

673

674 **Fig. 4** Influence of CO₂ concentration on acidity (A) and starch content (B) of *O. alismoides*
675 from dawn and dusk for juvenile and mature leaves. There are four treatments: mature leaves
676 grown at high CO₂ (HC, 302 to 604 μmol L⁻¹); juvenile leaves grown at high CO₂ (HC, 302 to
677 604 μmol L⁻¹); mature leaves grown at low CO₂ (LC, 0.1 to 5 μmol L⁻¹); juvenile leaves grown
678 at low CO₂ (LC, 0.1 to 5 μmol L⁻¹). Mean values with their SD (n=3) are presented and
679 differences among means were tested using one-way ANOVA with Duncan's and Tukey's post
680 hoc tests. Data with different letters are significantly different within the four treatments

681 ($P<0.05$); NS, not significant.

682

683 **Fig. 5** Responses of detached leaves from *O. alismoides* during an overnight incubation in the
684 dark at high CO₂. (A) gas exchange rate, (B) acidity and (C) starch content. There are four
685 treatments: detached mature leaves grown at high CO₂ (HC, 302 to 604 μmol L⁻¹); detached
686 juvenile leaves grown at high CO₂ (HC, 302 to 604 μmol L⁻¹); detached mature leaves grown
687 at low CO₂ (LC, 0.1 to 5 μmol L⁻¹); detached juvenile leaves grown at low CO₂ (LC, 0.1 to 5
688 μmol L⁻¹). Mean values with their SD (n=3) are presented and differences among means were
689 tested using one-way ANOVA with Duncan's and Tukey's post hoc tests. Data with different
690 letters are significantly different within the four treatments ($P<0.05$); NS, not significant.

691

692 **Fig. 6** Relationship between PEPC activity or starch content and CAM activity. (A)
693 Relationship between PEPC and CAM activity for mature and juvenile leaves of *O. alismoides*
694 grown at low CO₂ (0.1 to 5 μmol L⁻¹); Pearson correlation for PEPC activity (y) vs CAM activity
695 (x): $y= 1.06x + 14.27$, $P<0.05$, $R^2= 0.39$. (B) Relationship between change in starch content
696 and CAM activity for mature leaves of *O. alismoides* grown at low and high CO₂ concentration
697 (low CO₂, 0.1 to 5 μmol L⁻¹; high CO₂, 302 to 604 μmol L⁻¹); Pearson correlation for change in
698 starch (y) vs CAM activity (x): $y=0.59x+15.26$, $P<0.05$, $R^2= 0.78$.

699

700

701 **Table 1** Effects of CO₂, leaf maturity and time of day on enzyme activities in *O. alismoides*. The plants were grown at high CO₂ (302 to 604 μmol L⁻¹) or low
702 CO₂ (0.1 to 5 μmol L⁻¹). The leaves were collected at 07:30 (towards the end of the dark period) and 21:30 (towards the end of the photoperiod). Maturity
703 represents mature vs juvenile leaves. Results of a two-way ANOVA are presented, with significant effects shown in bold. ****P*<0.001, ***P*<0.01, **P*<0.05.
704

Factors	Parameters									
	Rubisco		PEPC		PPDK		NAD-ME		NADP-ME	
	<i>P</i>	Sum of squares	<i>P</i>	Sum of squares	<i>P</i>	Sum of squares	<i>P</i>	Sum of squares	<i>P</i>	Sum of squares
Time	0.000 ***	210.21	0.727	2.91	0.000 ***	705.25	0.935	0.04	0.001 **	9.35
CO ₂	0.001 **	108.76	0.000 ***	879.62	0.002 **	176.58	0.766	0.54	0.117	1.60
Maturity	0.097	19.13	0.001 **	363.21	0.034 *	72.45	0.134	14.83	0.146	1.36
Time×CO ₂	0.098	19.02	0.710	3.30	0.289	15.84	0.444	3.66	0.361	0.52
Time×Maturity	0.557	2.22	0.229	36.11	0.043 *	64.35	0.568	2.02	0.858	0.02
CO ₂ ×Maturity	0.000 ***	177.34	0.296	26.90	0.293	15.84	0.302	6.75	0.208	1.00
Time×CO ₂ ×Maturity	0.101	18.60	0.093	73.64	0.000 ***	316.10	0.513	2.65	0.578	0.19

705

706

707 **Table 2** Effects of CO₂ and leaf maturity on physiological parameters in *O. alismoides*. The plants were grown at high CO₂ (302 to 604 μmol L⁻¹) or low CO₂
708 (0.1 to 5 μmol L⁻¹). The leaves used for determination of acidity and starch were collected at 07:30 (towards the end of the dark period) and 21:30 (towards the
709 end of the photoperiod). Pigment content, chlorophyll fluorescence and rates of oxygen exchange were measured on leaves collected in the morning. Maturity
710 represents mature vs juvenile leaves. Results of a two-way ANOVA are presented, with significant effects shown in bold. ****P*<0.001, ***P*<0.01, **P*<0.05.
711

Parameters	Factors					
	CO ₂		Maturity		CO ₂ ×Maturity	
	<i>P</i>	Sum of squares	<i>P</i>	Sum of squares	<i>P</i>	Sum of squares
Diurnal change in acidity	0.000 **	633.76	0.153	27.76	0.043 *	57.77
Diurnal change in starch	0.013 **	102.82	0.315	15.60	0.006 **	132.08
F _v /F _m	0.382	5.21×10 ⁻⁵	0.014 *	0.001	0.000 ***	0.01
Yield	0.017 **	0.002	0.296	0.000	0.039 *	0.001
Respiratory rate	0.007 **	25.85	0.263	2.86	0.397	1.58
Photosynthetic rate	0.005 **	49.18	0.061	16.24	0.383	2.92
Chlorophyll <i>a</i>	0.868	9.10×10 ⁻⁵	0.030 *	0.02	0.527	0.001
Chlorophyll <i>b</i>	0.633	0.00	0.087	0.01	0.287	0.002
Total chlorophyll	0.761	0.001	0.039 *	0.05	0.398	0.01

712

713

714

Table 3 Comparison of physiological parameters in attached and detached leaves. The attached leaves were collected from the plants treated with either high CO₂ (302 to 604 μmol L⁻¹) or low CO₂ (0.1 to 5 μmol L⁻¹) at dawn and dusk. The detached leaves grown at high or low CO₂ were collected at dusk and incubated overnight in tap water enriched with CO₂ (560 μmol L⁻¹) and analysed at dawn. P values are presented for the differences between attached and detached leaves. Significant differences are shown in bold (***P*<0.001, **P*<0.01, **P*<0.05; *t*-test).

Time of day	Parameters	Attached vs detached mature leaves grown at high CO ₂	Attached vs detached juvenile leaves grown at high CO ₂	Attached vs detached mature leaves grown at low CO ₂	Attached vs detached juvenile leaves grown at low CO ₂
Dawn	Acidity	0.46	0.39	0.13	0.20
	Starch	0.82	0.0005***	0.007**	0.25
	PEPC	0.26	0.83	0.06	0.49
	Rubisco	0.08	0.11	0.88	0.53
	PPDK	0.24	0.19	0.0001**	0.26
	NAD-ME	0.43	0.06	0.21	0.39
	NADP-ME	0.63	0.33	0.09	0.61
Dusk	Acidity	0.86	0.89	0.72	0.34
	Starch	0.61	0.29	0.74	0.82
	PEPC	0.21	0.63	0.89	0.18
	Rubisco	0.08	0.11	0.51	0.06
	PPDK	0.12	0.27	0.11	0.14
	NAD-ME	0.24	0.19	0.11	0.45
	NADP-ME	0.78	0.73	0.26	0.93

Table 4 Conditions and calculated carbon concentrations at the end of pH-drift experiments for *O. alismoides*. Means with standard deviation (n=3) in parenthesis are presented. Data for C_T/Alkalinity with different letters are significantly different ($P<0.05$; one-way ANOVA with Duncan's and Tukey's post hoc tests). C_T stands for total inorganic carbon.

Type of leaf	Alkalinity (meq L ⁻¹)	Final pH	[C _T] (mmol L ⁻¹)	[CO ₂] (μmol L ⁻¹)	[HCO ₃ ⁻] (mmol L ⁻¹)	C _T /Alkalinity
Mature leaves grown at high CO ₂	0.17 (0.09)	9.24 (0.26)	0.13 (0.05)	0.11 (0.02)	0.11 (0.04)	0.75 (0.05) ^a
Juvenile leaves grown at high CO ₂	0.60 (0.19)	9.45 (0.19)	0.47 (0.17)	0.28 (0.23)	0.38 (0.16)	0.77 (0.07) ^a
Mature leaves grown at low CO ₂	0.94 (0.13)	10.18 (0.14)	0.44 (0.03)	0.02 (0.01)	0.18 (0.04)	0.47 (0.06) ^b
Juvenile leaves grown at low CO ₂	1.13 (0.04)	9.93 (0.17)	0.69 (0.11)	0.09 (0.07)	0.39 (0.13)	0.61 (0.08) ^c

Fig.1

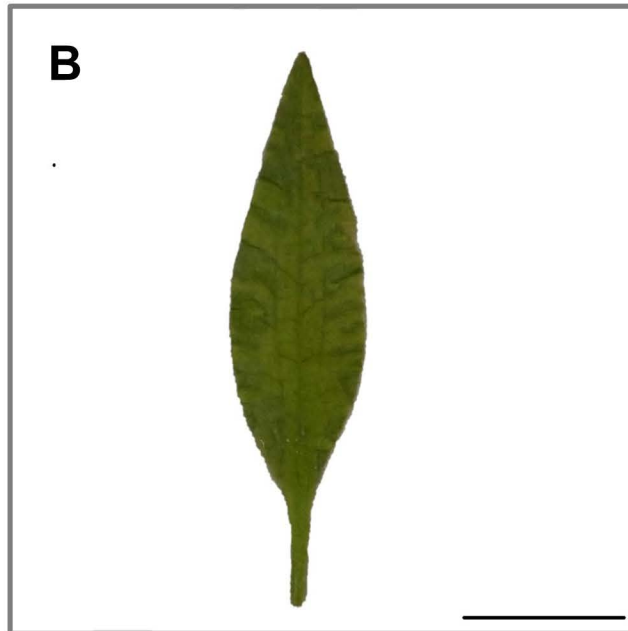
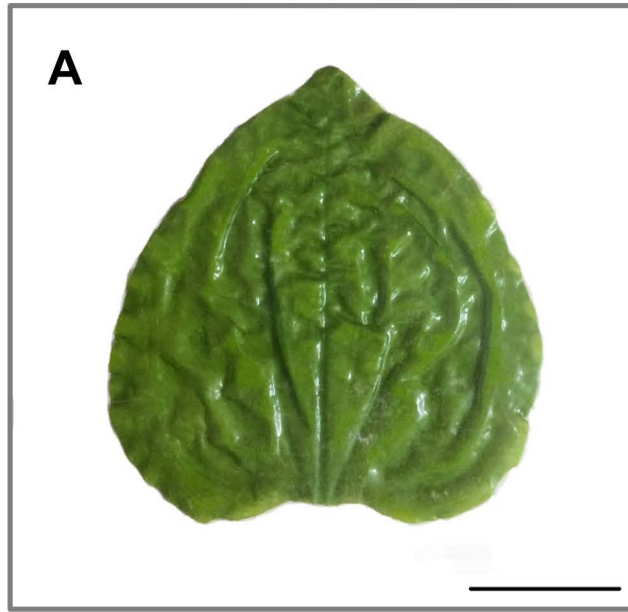
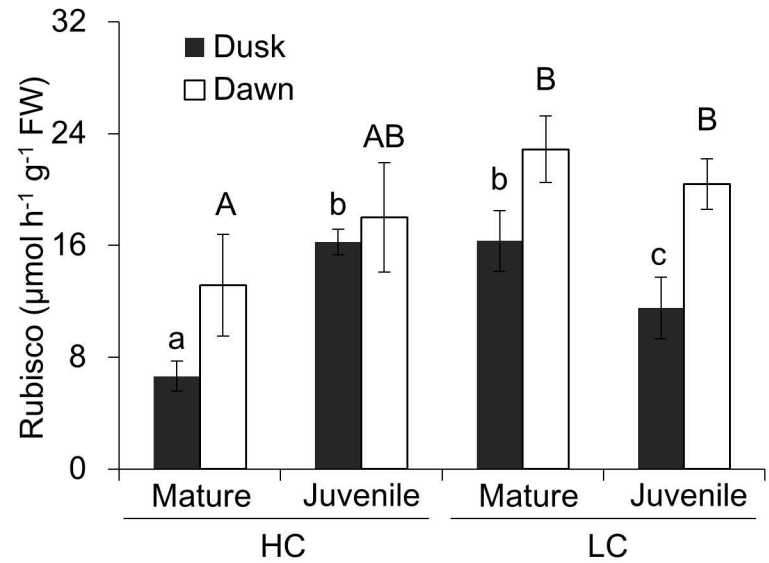
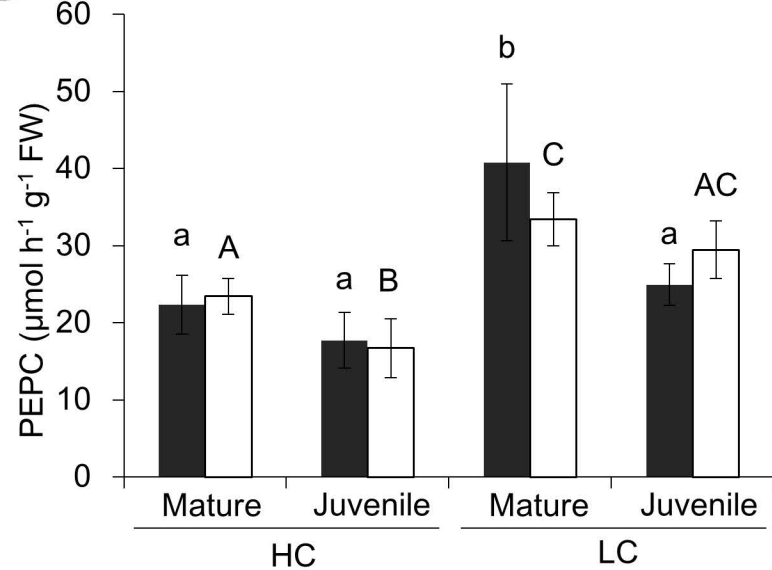


Fig.2

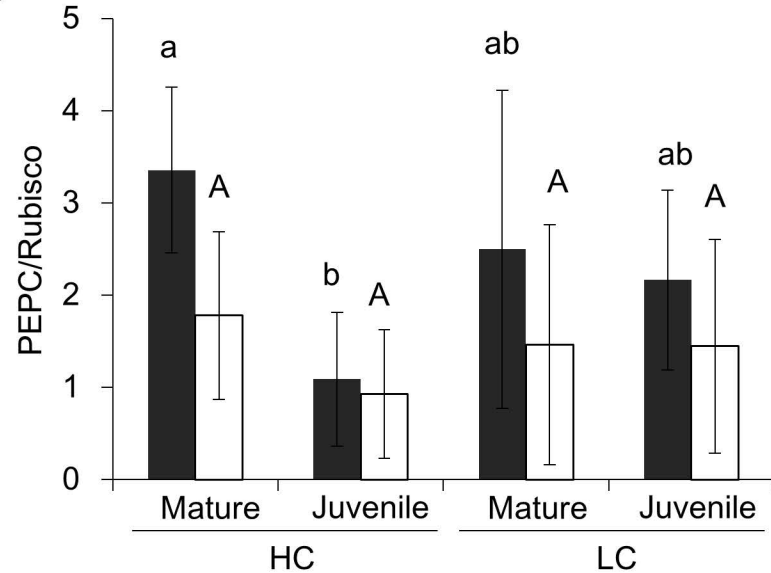
A



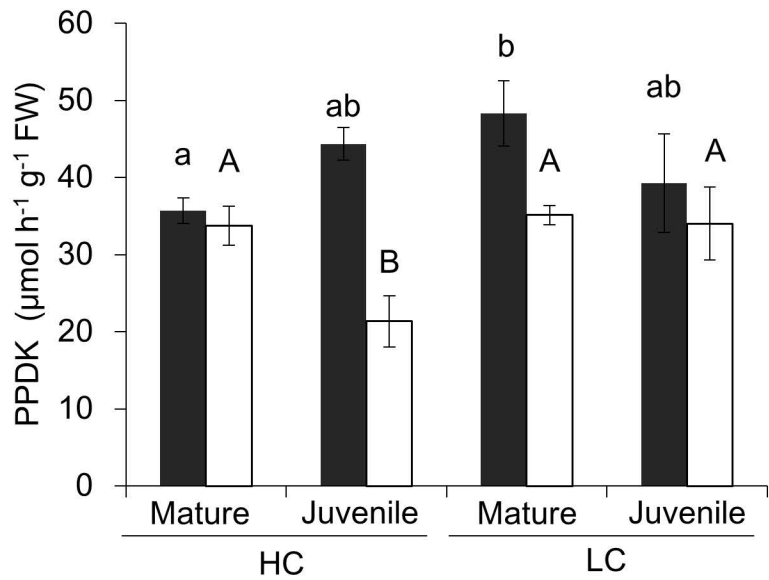
B



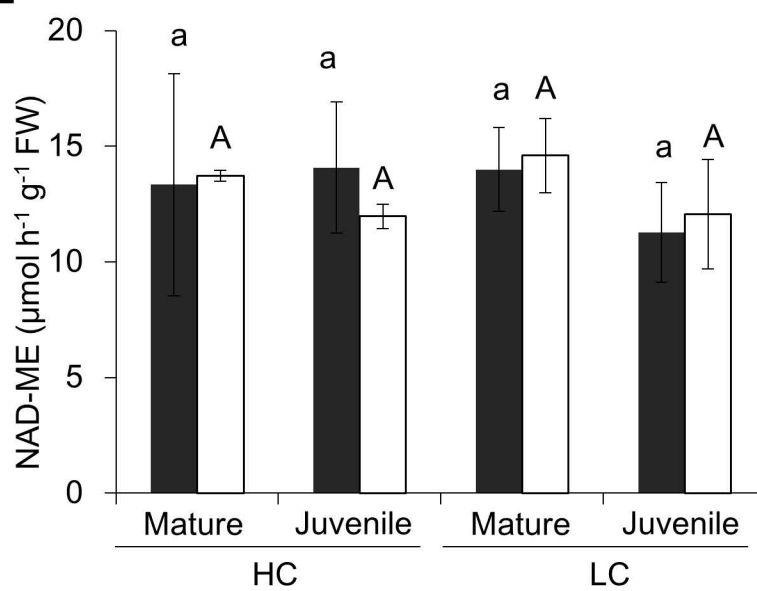
C



D



E



F

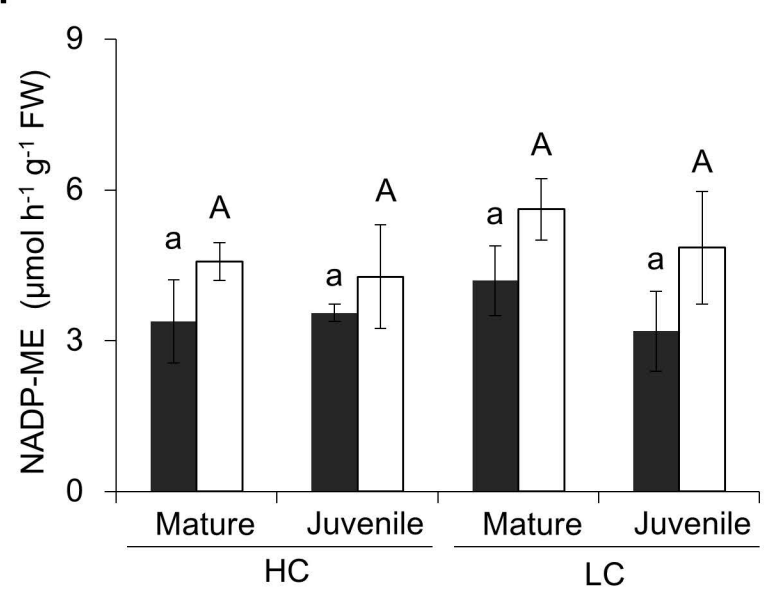


Fig.3

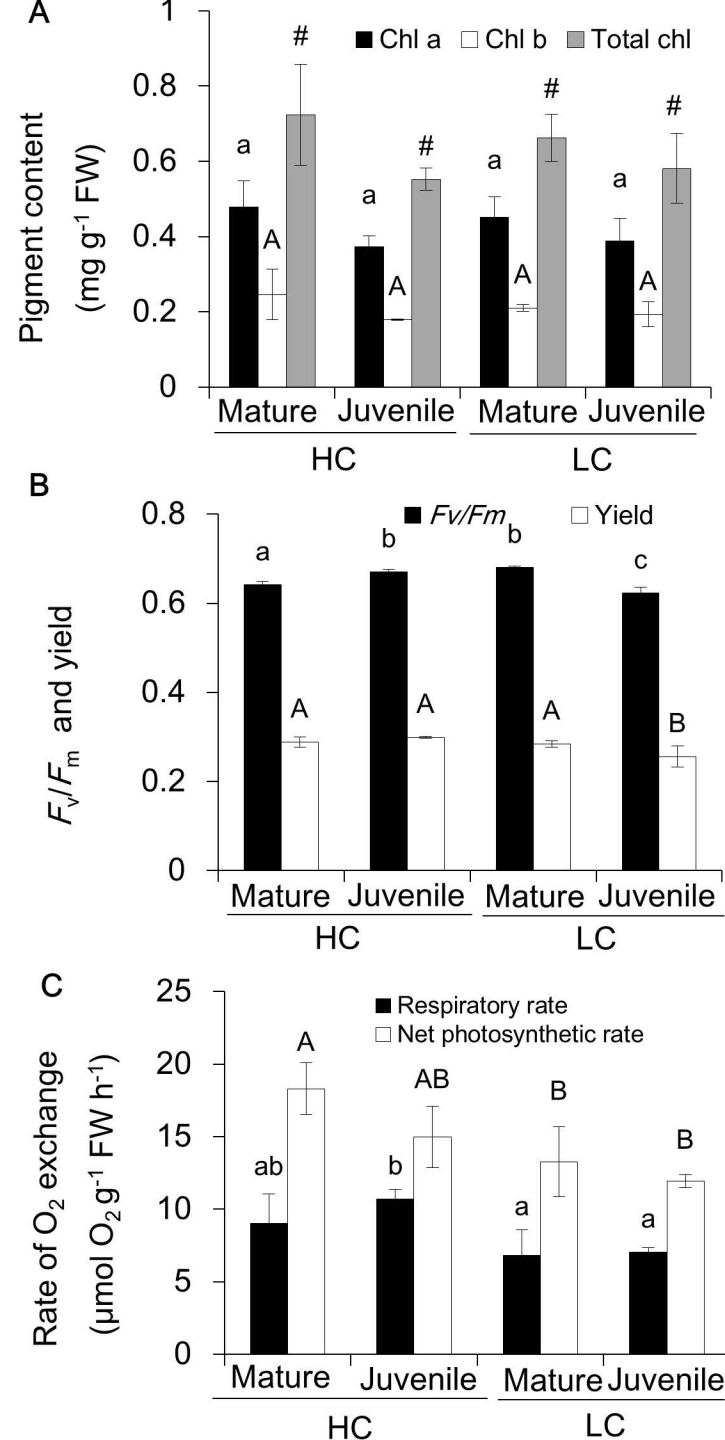


Fig.4

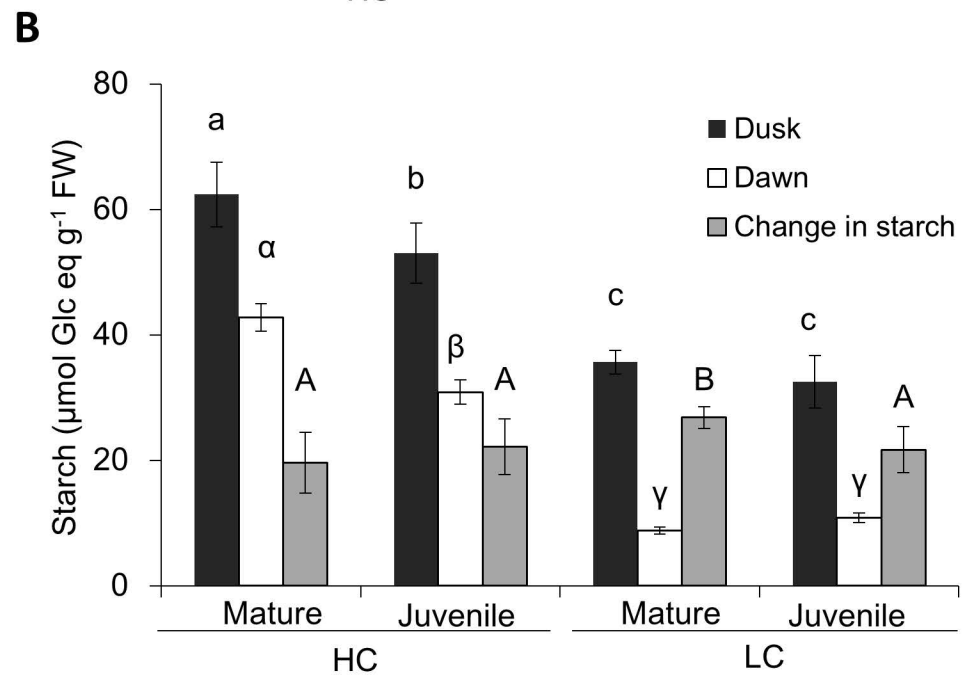
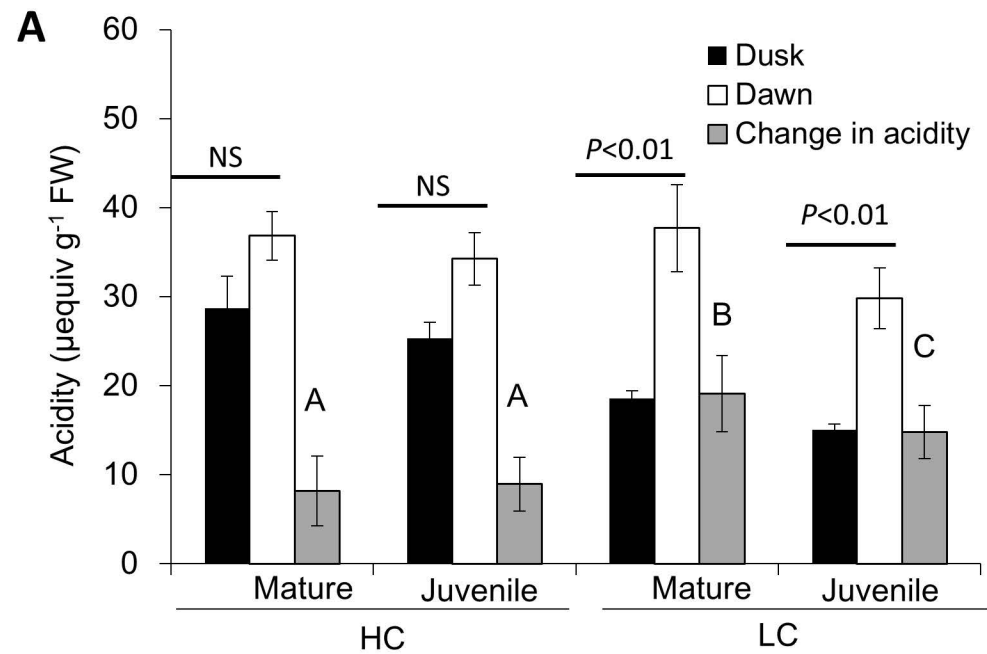


Fig.5

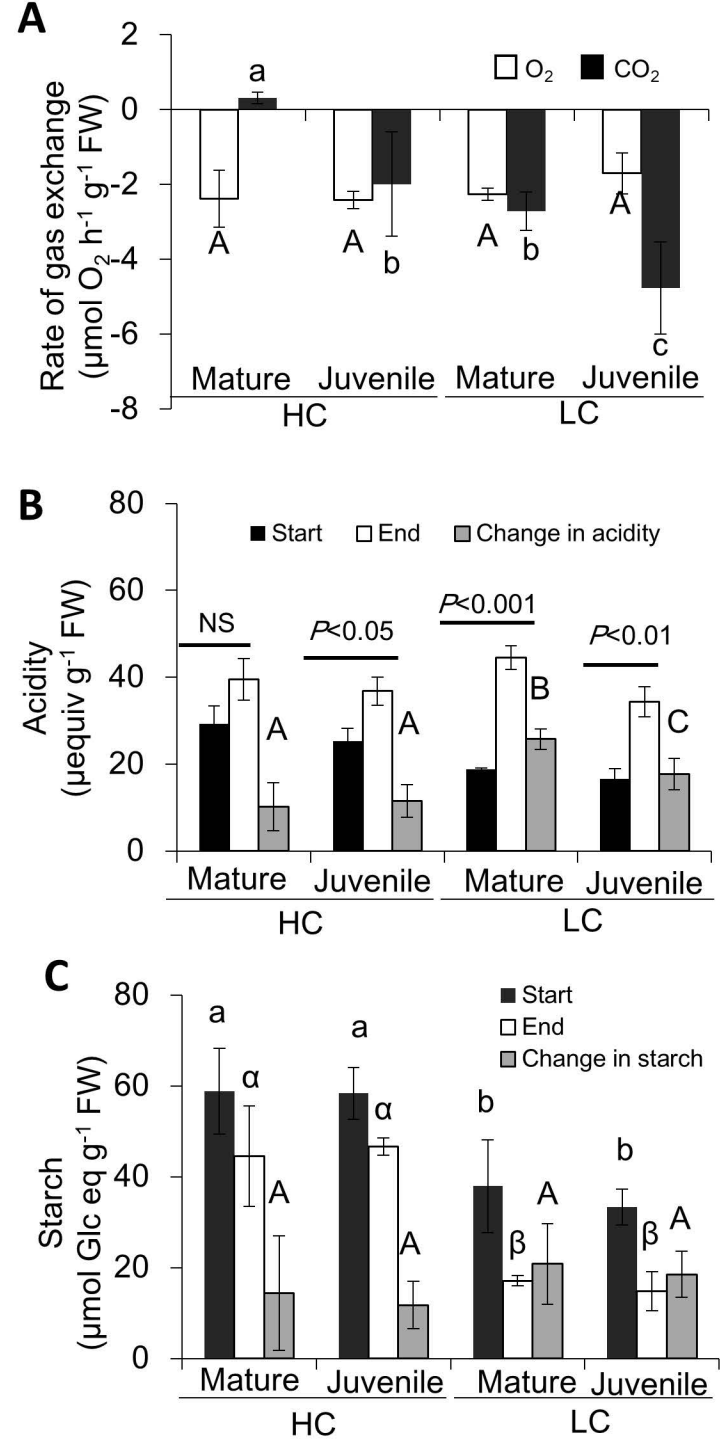
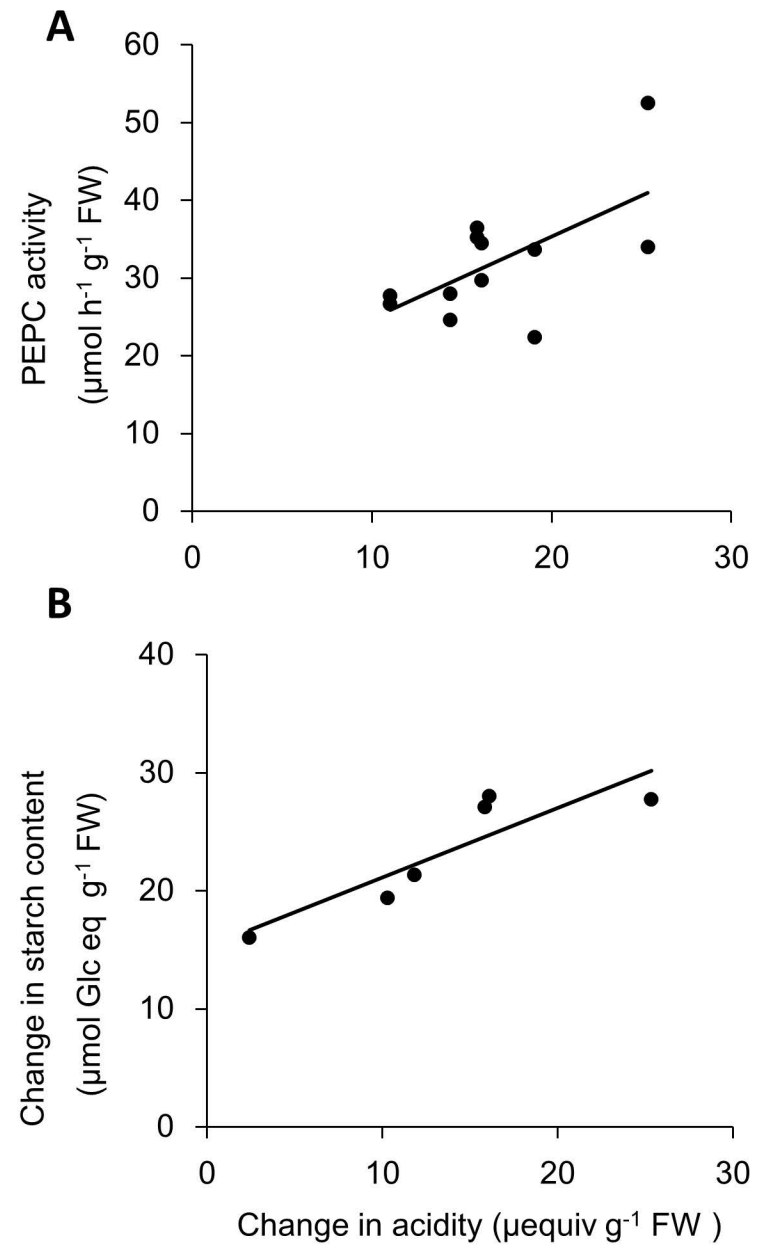


Fig.6



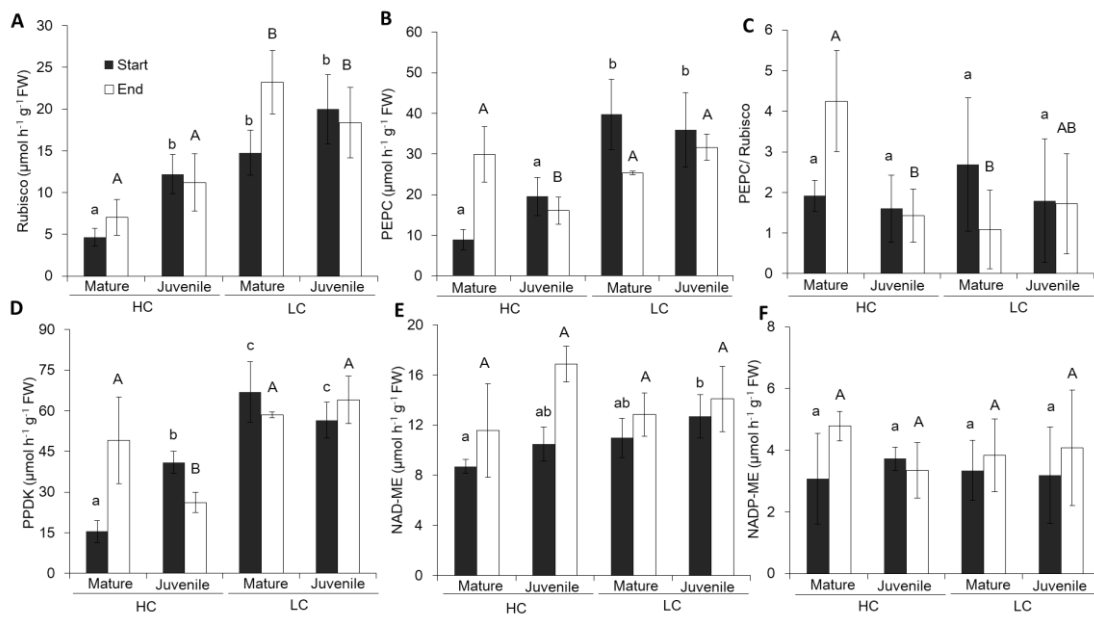


Figure S1: Changes in enzyme activities in detached mature or juvenile leaves from plants grown at high or low CO₂ after overnight incubation. (A) Rubisco activity, (B) PEPC activity, (C) the PEPC:Rubisco ratio, (D) PPDK activity, (E) NAD-ME activity and (F) NADP-ME activity. Mean \pm SD is presented. Data with different letters are significantly different within the four treatments ($P < 0.05$, one-way ANOVA).

Table S1: Inter-relationships between fresh weight, area, chlorophyll *a* and chlorophyll *b* for juvenile and mature leaves of *O. alismoides*. Data with different letters are significantly different.

Parameters	Mature leaf	Juvenile leaf
Fresh weight per leaf (g)	1.55 (0.33) ^a	0.34 (0.06) ^b
Leaf area (1-side, cm ²)	132.25 (16.79) ^a	33.73 (8.71) ^b
Chl <i>a</i> (mg g ⁻¹)	0.53 (0.04) ^a	0.47 (0.03) ^a
Chl <i>a/b</i>	2.15 (0.22) ^a	2.60 (0.40) ^a
Specific leaf area (1-sided cm ² g ⁻¹ FW)	86.17 (6.16) ^a	99.24 (9.18) ^b