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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 CO_2 acclimation strategies in juvenile and mature leaves of *Ottelia alismoides***.

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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

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Different CO_2 acclimation strategies in juvenile and mature leaves of *Ottelia alismoides*

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

Abbreviations:

Alk: alkalinity; CAM: Crassulacean Acid Metabolism; CCM: carbon dioxide concentrating mechanism; C_T: concentration of total inorganic carbon; FW: fresh weight; HC: high CO_2 LC: low CO_2; NAD(P)-ME: NAD(P)-malic enzyme; PEP: phosphoenol pyruvate; PEPC: PEP carboxylase; PPDK: pyruvate phosphate dikinase; Rubisco: ribulose 1,5-bisphosphate carboxylase-oxygenase; SD: standard deviation.

Abstract

The freshwater macrophyte, *Ottelia alismoides*, is a bicarbonate user performing C4 photosynthesis in the light, and Crassulacean Acid Metabolism (CAM) when acclimated to low CO_2. The regulation of the three mechanisms by CO_2 concentration was studied in juvenile and mature leaves. For mature leaves, the ratios of phosphoenolpyruvate carboxylase (PEPC) to ribulose-bisphosphate carboxylase/oxygenase (Rubisco) are in the range of that of C4 plants regardless of CO_2 concentration (1.5 ~ 2.5 at low CO_2, 1.8 ~ 3.4 at high CO_2). In contrast, results for juvenile leaves suggest that C4 is facultative and only present under low CO_2. pH-drift experiments showed that both juvenile and mature leaves can use bicarbonate irrespective of CO_2 concentration, but mature leaves have a significantly greater carbon extracting ability than juvenile leaves at low CO_2. At high CO_2, neither juvenile nor mature leaves perform CAM as indicated by lack of diurnal acid fluctuation. However, CAM was present at low CO_2, though the fluctuation of titratable acidity in juvenile leaves (15 to 17 μequiv g^{-1} FW) was slightly but significantly lower than in mature leaves (19 to 25 μequiv g^{-1} FW), implying that the capacity to perform CAM increases as leaves mature. The increased CAM activity is associated with elevated PEPC activity and large diel changes in starch content. These results show that in *O. alismoides*, CCMs are more effective in mature compared to juvenile leaves, and C4 is facultative in juvenile leaves but constitutive in mature leaves.

Introduction

The low rate of diffusion and frequently depleted concentration of CO_2 in water coupled with the kinetic inefficiency and poor specificity for CO_2 of the primary carboxylating enzyme ribulose 1,5-bisphosphate carboxylase-oxygenase (Rubisco) can restrict the productivity of submerged macrophytes (Vadstrup and Madsen, 1995; Maberly & Gontero, 2017). However, the presence of carbon dioxide-concentrating mechanisms (CCMs) in some freshwater plants can reduce or overcome the problem of limited inorganic carbon supply. The most frequent CCM in freshwater plants is based on the biophysical active uptake of bicarbonate, which is found in ~45% of tested species (Maberly and Gontero 2017). In addition, Crassulacean Acid Metabolism (CAM) and C4 metabolism are also present in some freshwater plants (Keeley 1981; Bowes and Salvucci 1989; Bowes et al. 2002; Keeley and Rundel 2003). In terrestrial plants adapted to xeric conditions, CAM can be widespread and plays a major role in the water-use efficiency of the plant (Silvera et al. 2010). In aquatic plants, CAM occurs in about _8% of tested species (Maberly & Gontero 2017) and acts as a carbon-conserving and concentrating mechanism that reduces the loss of respiratory carbon at night and exploits nocturnal CO_2 concentration that is often higher than during the day (Klavsen et al. 2011). Terrestrial C4 carbon fixation is known in about 3% of species and involves spatial separation of carboxylation and decarboxylation between different cells, mesophyll and bundle-sheath, or occasionally within one type of cell (Sage 2016). In aquatic plants, C4 photosynthesis is found in about 4% of tested species, occurs within a single cell, and reduces the effects of photorespiration especially when water column concentrations of oxygen are high and CO_2 are low (Bowes et al. 2002).

In CAM and C4 photosynthesis, the first carboxylating enzyme is usually phosphoenolpyruvate carboxylase (PEPC) (Osmond 1978). In C4 photosynthesis, PEPC is active during the day but in CAM it is only active during the night (Hatch 1987). PEPC uses phosphoenolpyruvate (PEP) and external or internal respiratory CO_2 to produce the C4 compound oxaloacetate (OAA) that in turn is converted into malate. In C4 photosynthesis, the malate is decarboxylated close to Rubisco while in CAM, it is transported and stored in the vacuole resulting in the well-known acidification observed during the night. In CAM, during

the day, the malic acid is decarboxylated into pyruvate, a C3 compound and CO_2, that is then fixed by Rubisco within the Calvin-Benson-Bassham cycle. Nocturnal CO_2 fixation via PEPC requires an adequate pool of carbohydrates to generate PEP, in addition to that needed for other metabolic processes including dark respiration, organic carbon export and growth (Nobel and Hartsok 1983). In several CAM species, PEPC-mediated carboxylation is indeed closely linked to the diel turnover of starch, since the amount of CO_2 taken up during the night depends on the availability of C3-carbon substrate (PEP) produced from the nocturnal degradation of starch accumulated during the day (Ceusters et al. 2014). Thus, in these plants there is a large diel change in transitory starch content as well as acidity (Neuhaus and Schulte 1996).

CAM metabolism is a plastic process in freshwater and terrestrial plants. In freshwater plants, its expression can be regulated by a range of environmental parameters, e.g. irradiance, CO_2 availability, temperature and nutrient availability (Keeley et al. 1983; Aulio 1985; Madsen 1987; Bowes and Salvucci 1989; Robe and Griffiths 1990; Hostrup and Wiegleb 1991; Klavsen and Maberly 2010; Zhang et al. 2014; Shao et al. 2017). The activity of CAM may also be dependent on leaf maturity and in many terrestrial species CAM is only fully expressed in mature leaves (Jones 1975; Ting et al. 1996; Wen et al. 1997; Taybi et al. 2002). Whether comparable ontogenetic change occurs in aquatic CAM plants has not been widely studied. There is very little information on the influence of leaf maturity on aquatic CAM plants (Klavsen and Madsen 2008) or on the interplay between developmental and environmental factors. In the terrestrial plant, *Mesembryanthemum crystallinum*, salinity was originally reported to induce CAM (Winter and von Willert 1972), but it was later shown that CAM was a genetically controlled developmental programme that was just accelerated by the salinity stress (Adams et al. 1998). C4 photosynthesis is usually constitutive in terrestrial plants but is facultative in the freshwater macrophytes *Hydrilla verticillata* (Bowes et al. 2002) and *Egeria densa* (Casati et al. 2000). Use of bicarbonate can also be plastic and in *Elodea canadensis* and *Ranunculus peltatus* is down-regulated at high CO_2 concentration (Sand-Jensen and Gordon 1986; Madsen et al. 1996).

It has been shown that in *Ottelia alismoides* from the Hydrocharitaceae, C4 metabolism and bicarbonate use are constitutive, while CAM metabolism is facultative and only induced at low CO_2 (Zhang et al. 2014). *O. alismoides* operates CAM at night and C4 metabolism during

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

O. alismoides leaves differ in size and shape and develop from a basal rosette (Cook and Urmi-König 1984: Yu and Yu 2009). The first juvenile leaves are only 2 to 4 cm wide and 18 to 20 cm long, and are linear or lanceolate and either sessile or attenuate (Fig. 1). Under summer conditions, juvenile leaves can develop into mature leaves that have a petiole that can be up to 50 cm long and are ovate to cordate, 18 to 20 cm long and up to 20 cm wide. In addition to these morphological characteristics, the fresh weight and leaf area of mature leaves is four to five greater than juvenile leaves but the specific leaf area is 1.15-times greater in juvenile than in mature leaves, while the content of chlorophyll *a* and *b* on a fresh weight basis is similar (Table S1). Previously, we studied fully expanded mature leaves of *O. alismoides*, but we observed that there were significant differences in the diurnal change of acidity between mature and juvenile leaves (unpublished observations). Little is known about differential expression of CCMs in different types of leaf in freshwater plants although Maberly and Spence (1983) reported that linear leaves of *Potamogeton x zizii* were restricted to CO_2 while broad leaves could also use bicarbonate.

In the present study, we tested the hypothesis that CAM activity, C4 metabolism and bicarbonate use, will differ between mature and juvenile leaves. We further hypothesised that there will be an interplay between leaf development and acclimation to CO_2 concentration.

Materials and methods

Plant material and leaf sampling

Seeds of *O. alismoides* were germinated on 20 March 2017 in a one liter glass beaker filled with sterile tap water with an alkalinity of about 1.9 mequiv L^{-1} and a low nutrient concentration (TP = 0.05 mg L^{-1}; TN = 1.35 mg L^{-1}). The beaker was placed in a growth chamber at 25 °C and the water was replaced daily. Six weeks after germination, two or three seedlings (about 8 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 CO_2
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 CO_2 concentrations. In the high CO_2
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 CO_2 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 CO_2 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 CO_2 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

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

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

In order to analyse the capture of respired CO_2 by different types of leaf, one mature leaf or three juvenile leaves (about 1.0 g fresh weight in both cases), detached from the plants grown under HC and LC at dusk were placed in 580 mL gas-tight glass bottles, containing the tap water enriched with CO_2 to a final concentration of 560 μmol L^{-1}, and a dissolved oxygen concentration of 7.7 mg L^{-1}. All the bottles were incubated in the dark, unstirred, at 25℃ for 11 hours. In addition, at the start of the overnight incubation experiment, one mature or three juvenile leaves were collected from the plants grown either at high or low CO_2, wrapped in foil envelopes and frozen in liquid nitrogen for later determination of acidity, starch and enzyme activity. After overnight incubation, the alkalinity, pH and O_2 concentration of the incubation medium were measured as described below. Meanwhile, the detached leaves in the bottles were removed and acidity, starch and enzyme activity were measured. Measurements were made in triplicate.

pH-drift

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

tubes contained 60 mL of test solution (equimolar concentrations of $NaHCO_3$ and $KHCO_3$ at an overall concentration of 1 mM) and about 5 mL air. The glass tubes were incubated, unstirred, in a growth room at 25℃ and 120–135 µmol photon $m^{-2}s^{-1}$ PAR. After 24 h continuous irradiance, the final pH was measured with the pH electrode (model IP-600-9 Jenco Instruments, USA) and the final alkalinity in the solution was measured by Gran titration (see below).

Measurement of CAM activity

CAM activity was measured as a change in titratable acidity between minimal acidity levels at dusk and maximal levels at dawn. To measure acidity, 10 mL CO_2-free milliQ water was added to the leaf samples (0.2-0.5 g fresh weight) that had been stored in liquid nitrogen and then boiled for 30 minutes. After cooling to room temperature, the samples were titrated to an endpoint of pH 8.3 with 0.01 N NaOH (Madsen 1987; Zhang et al. 2014). Other methods, e.g. those used by Keeley and co-workers and others titrate to pH 6.4 and Keeley et al. (1983) report that this produced 8% lower estimates of acidity compared to titrating to pH 8.3.

Measurement of enzyme activity

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

Measurement of starch, alkalinity and pH

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

Measurement of leaf area, chlorophyll content and chlorophyll fluorescence

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

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

Oxygen exchange

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

Statistical analysis

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

significance level was set at 5%.

Results

Comparison of mature and juvenile leaves at different CO_2 concentration

At high CO_2 at dawn, the activities of PEPC and PPDK were significantly lower in juvenile leaves than in mature leaves on a fresh weigth basis. However, compared to mature leaves, the activity of Rubisco was markedly higher in juvenile leaves. NAD-ME and NADP-ME were not significantly different between the two types of leaf. The only significant difference in the PEPC to Rubisco ratio was at dusk when mature leaves had a significantly higher ratio than juvenile leaves (Fig. 2, Table 1).

At low CO_2, the juvenile leaves had significantly lower PEPC (1.64-fold, SD = 1.72) and Rubisco (1.42-fold, SD = 0.64) activity than mature leaves on a fresh weight basis (Fig. 2, Table 1) and also on an area leaf basis (data not shown). In contrast, the activities of PPDK, NAD-ME and NADP-ME were not significantly affected by leaf maturity (Fig. 2, Table 1). The PEPC to Rubisco ratios in juvenile and mature leaves at dawn and dusk, were not significantly different (Fig. 2). At low and high CO_2, the content of chlorophyll *a*, chlorophyll *b* and total chlorophyll per unit fresh weight was not significantly different in juvenile and mature leaves (Fig. 3A) according to the one-way ANOVA analysis. However, a two-way ANOVA, showed that leaf maturity has a significant effect on the content of chlorophyll *a* and total chlorophyll because of an interaction between leaf maturity and CO_2 (Table 2).

At low CO_2, the maximal photochemical efficiency of PSII (Fv/Fm) and the actual photochemical efficiency of PSII, or yield, were slightly but significantly higher in the mature, compared to the juvenile leaves (ratio of 1.1, SD = 0.03 for Fv/Fm and 1.1, SD = 0.01 for yield). In contrast, at high CO_2, the maximal photochemical efficiency of PSII was slightly but significantly higher in the juvenile leaves (ratio of 1.05, SD = 0.03), while the yield was similar in both types of leaf (Fig. 3B, Table 2). At both low and high CO_2, the rate of net photosynthesis and the rate of respiration were not significantly different for juvenile and mature leaves (Fig. 3C, Table 2).

At low CO_2, for both types of leaf, there were marked diel changes in acidity. The change 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 µequiv 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

(1.63-fold, SD = 2.06) and mature leaves (1.75-fold, SD = 2.42) (Fig. 4B, Table 2). The amount of starch left at the beginning of the next day in juvenile and mature leaves was both significantly lower at low, compared to high CO_2. Compared to dusk, the amount of starch present at dawn was reduced by 75 and 67 % for mature and juvenile leaves respectively at low CO_2, but at high CO_2 this reduction was only 31 and 42 % for mature and juvenile leaves respectively (Fig. 4B).

Overnight changes in leaves detached from plants grown at high and low CO_2

The overnight incubation experiments and acclimation experiments were performed on the same plants material collected at dusk. The measurements, the next morning, were made on leaves attached to the plant in the acclimation experiments or detached from the plant in the overnight incubation experiment. This provided an opportunity to compare responses of attached and detached juvenile or mature leaves from plants grown at low or high CO_2.

Enzyme activity

The pattern of enzyme activity in detached leaves was very similar to that found in attached leaves. Irrespective of the leaf type, the activities of Rubisco, PEPC and PPDK in leaves detached from plants grown at low CO_2 were significantly higher than those in leaves detached from plants grown at high CO_2, at either the start or the end of the overnight incubation. In contrast, the activities of NAD-ME and NADP-ME were the same in both types of leaf irrespective of CO_2 concentration (Fig. S1). In detached mature leaves from plants grown at low CO_2 at dawn, the activity of PPDK was significantly higher in detached than in attached leaves, but there were no other significant differences for any of the other enzyme activities (Table 3).

Rates of gas exchange in the dark

Rates of respiratory O_2 consumption were similar in detached juvenile and mature leaves from high CO_2 or low CO_2 treated plants (Fig. 5A). For the mature leaves detached from plants grown at high CO_2, there was a net release of CO_2 and the molar ratio of CO_2 released to O_2 consumed

was 0.13 (SD = 0.02). For the juvenile leaves detached from plants grown at high CO_2, there was a net uptake of CO_2 and the ratio of CO_2 consumed to O_2 consumed was 0.83 (SD = 0.13). In contrast, at low CO_2, there was a net uptake of CO_2 for both types of detached leaves. The CO_2 uptake was 120 % (SD = 11) and 280 % (SD = 20) of O_2 uptake for mature and juvenile leaves, respectively (Fig. 5A).

Acidity and starch

The pattern of acidity changes in attached and detached leaves was not significantly different (Fig. 4A and Fig. 5B; Table 3). Like in the previous experiment with attached leaves (see Fig. 4A), the diel change in acidity was not significantly different in mature leaves detached from plants grown at high CO_2. However, for plants grown at low CO_2 the change in acidity in detached juvenile and mature leaves was significantly different at 17 and 25 μequiv g^{-1} FW, respectively (Fig. 5B).

There were no significant differences for starch variation between attached and detached leaves at dusk (Table 3). Like in the acclimation experiments using attached leaves, the amount of starch decreased after overnight incubation in all detached leaves from plants grown at either low or high CO_2 (Fig. 5C). Moreover, after the overnight incubation, when compared to the corresponding attached leaves, the starch content was significantly higher in juvenile leaves detached from plants grown at high CO_2 and mature leaves detached from plants grown at low CO_2 (Table 3).

Capture of respired CO_2 by malic acid

Using the assumptions that 2 moles of H^+ are equivalent to 1 mole of malic acid and that the respiratory quotient (CO_2/O_2) is 1, the percent of dark respiration that could be trapped as malic acid was 6.3% for juvenile leaves and 4.9% for mature leaves detached from plants grown at high CO_2. The equivalent value for leaves detached from plants grown at low CO_2, was 8.4% and 6.6% for juvenile and mature leaves, respectively.

Relationships between acidity, starch and enzyme activities for plants grown at different concentrations of CO_2

We combined data from juvenile and mature leaves to analyze the relationships between enzyme activities and change in acidity. For plants grown at low CO_2, there were no significant relationships between acidity and PEPC activity for attached leaves grown under low CO_2 neither at dusk (r=0.688, *P*=0.131) nor at dawn (r=0.719, *P*=0.107). However, there was a significant positive relationship between the change in acidity and PEPC activity (with combined data from dawn and dusk) for attached leaves grown at low CO_2 (r=0.624, *P*=0.03, Fig. 6A). In contrast, the activities of Rubisco, PPDK, NAD-ME and NADP-ME were not correlated to change in acidity (*P*>0.05). Combining data from mature leaves grown at both CO_2 concentrations, a positive correlation between change in acidity and consumption of starch was observed (r= 0.885, *P*=0.019, Fig. 6B); this relationship was absent in juvenile leaves.

pH-drift

The pH-drift experiments provided clear evidence for bicarbonate use in both types of leaf grown at different CO_2 concentrations since final CO_2 concentrations were substantially less than 1 μmol L^{-1}. Both the mature and juvenile leaves grown at high CO_2 were able to raise the pH to over 9.20, at which point the $[CO_2]$ and $[HCO_3^-]$ were 0.113 μmol L^{-1} and 0.11 mmol L^{-1} for mature leaves, 0.282 μmol L^{-1} and 0.373 mmol L^{-1} for juvenile leaves, respectively (Table 4). Both the mature and juvenile leaves grown at low CO_2 were able to raise the pH to over 10.0, at which point the $[CO_2]$ and $[HCO_3^-]$ were 0.023 μmol L^{-1} and 0.18 mmol L^{-1} for mature leaves and 0.093 μmol L^{-1} and 0.387 mmol L^{-1} for juvenile leaves, respectively (Table 4). The alkalinity in the HCML treatment was substantially reduced at the end of the drift probably because of carbonate precipitation or release of organic acid or both. The carbon uptake ability estimated using the quotient of final $[C_T]$ to alkalinity (C_T/Alk) is shown in Table 4. The C_T/Alk for mature and juvenile leaves grown at high CO_2 were both significantly higher than at low CO_2. Moreover, the ratio for mature leaves grown at low CO_2 was statistically different and lower than for juvenile leaves at low CO_2 (Table 4).

Discussion

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

411 facultatively at low CO_2 (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 CO_2 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 CO_2
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 CO_2, 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, CO_2 is fixed and partly converted into transitory starch that is degraded

during the following night (Huber 1983). The diurnal turnover of transitory starch is an important characteristic of plant metabolism and its alteration by environmental conditions may affect both plant growth and plant habit (Schulze et al. 1990). In several CAM-induced plants, the metabolic products of nocturnal starch degradation are mainly converted into the primary CO_2 acceptor PEP (Smith and Bryce 1992). For example, in *M. crystallinum*, the induction of CAM by exposure to salinity was accompanied by increased activities of a range of starch-degrading enzymes (Paul et al. 1993). Therefore, the large increase in starch turnover when CAM was induced in both juvenile and mature leaves of *O. alismoides* is consistent with the important role of starch metabolism in CAM plants. Especially in mature leaves, there was a positive correlation between change in starch and CAM activity. The higher starch content at the end of the night in detached leaves compared to attached leaves implies that there are alternative fates for starch in attached leaves including export for growth of young leaves and the plant as a whole as also found in *M. crystallinum* by Borland and Dodd (2002).

In the present study, at high and low CO_2 the PEPC to Rubisco ratio in mature leaves is in a range typical of terrestrial C4 plants and consistent with previous reports showing that C4 metabolism is constitutive in *O. alismoides* (Zhang et al. 2014; Shao et al. 2017; Yin et al. 2017). Similarly, in juvenile leaves grown at low CO_2, the PEPC to Rubisco ratio was between 1.4 ~ 2.2, that is also consistent with C4 metabolism. In contrast, in juvenile leaves grown at high CO_2, the PEPC to Rubisco ratio (0.9 ~ 1.1), was significantly lower than that of mature leaves and implies that C4 metabolism is absent. Although juvenile leaves are produced at the base of the plants, the low light is unlikely to be responsible for the lack of C4 metabolism since Shao et al. (2017) showed that the PEPC to Rubisco ratio of mature leaves at high CO_2 and low light was between 2 and 3. This down-regulation of C4 metabolism at high CO_2 is the pattern also found in other C4 freshwater plants such as *Hydrilla verticillata* (Bowes et al. 2002) and *Egeria densa* (Casati et al. 2000). Further studies are ongoing to characterise C4 photosynthesis in leaves of *O. alismoides* in the light.

Our data showed that photosynthetic carbon uptake was able to drive the concentration of CO_2 in the solution well below 1 μM (i.e. about 7% of air-equilibrium) with both types of leaf irrespective of CO_2. This indicates that carbon uptake was not relying solely on passive diffusion of CO_2, since freshwater macrophytes restricted to CO_2 typically have CO_2

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

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

Conclusions and outlook

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

are similar.

This manuscript contains supplementary information.

Acknowledgements

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

Compliance with ethical standards

Not applicable

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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 CO_2 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) PPDK, (E) NAD-ME and (F) NADP-ME. There are four treatments: mature leaves grown
659 at high CO_2 (HC, 302 to 604 μmol L^{-1}); juvenile leaves grown at high CO_2 (HC, 302 to 604
660 μmol L^{-1}); mature leaves grown at low CO_2 (LC, 0.1 to 5 μmol L^{-1}); juvenile leaves grown at
661 low CO_2 (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 CO_2 concentration on pigment content for juvenile and mature leaves (A),
666 chlorophyll fluorescence (B) and rate of O_2 exchange (C) of *O. alismoides*. There are four
667 treatments: mature leaves grown at high CO_2 (HC, 302 to 604 μmol L^{-1}); juvenile leaves grown
668 at high CO_2 (HC, 302 to 604 μmol L^{-1}); mature leaves grown at low CO_2 (LC, 0.1 to 5 μmol L^{-1});
669 juvenile leaves grown at low CO_2 (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 CO_2 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 CO_2 (HC, 302 to 604 μmol L^{-1}); juvenile leaves grown at high CO_2 (HC, 302 to
677 604 μmol L^{-1}); mature leaves grown at low CO_2 (LC, 0.1 to 5 μmol L^{-1}); juvenile leaves grown
678 at low CO_2 (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

(P<0.05); NS, not significant.

Fig. 5 Responses of detached leaves from *O. alismoides* during an overnight incubation in the dark at high CO_2. (A) gas exchange rate, (B) acidity and (C) starch content. There are four treatments: detached mature leaves grown at high CO_2 (HC, 302 to 604 µmol L^{-1}); detached juvenile leaves grown at high CO_2 (HC, 302 to 604 µmol L^{-1}); detached mature leaves grown at low CO_2 (LC, 0.1 to 5 µmol L^{-1}); detached juvenile leaves grown at low CO_2 (LC, 0.1 to 5 µmol L^{-1}). Mean values with their SD (n=3) are presented and differences among means were tested using one-way ANOVA with Duncan's and Tukey's post hoc tests. Data with different letters are significantly different within the four treatments (P<0.05); NS, not significant.

Fig. 6 Relationship between PEPC activity or starch content and CAM activity. (A) Relationship between PEPC and CAM activity for mature and juvenile leaves of *O. alismoides* grown at low CO_2 (0.1 to 5 µmol L^{-1}); Pearson correlation for PEPC activity (y) *vs* CAM activity (x): y= 1.06x + 14.27, P<0.05, R^2= 0.39. (B) Relationship between change in starch content and CAM activity for mature leaves of *O. alismoides* grown at low and high CO_2 concentration (low CO_2, 0.1 to 5 µmol L^{-1}; high CO_2, 302 to 604 µmol L^{-1}); Pearson correlation for change in starch (y) *vs* CAM activity (x): y=0.59x+15.26, P<0.05, R^2= 0.78.

Table 1 Effects of CO_2, leaf maturity and time of day on enzyme activities in *O. alismoides*. The plants were grown at high CO_2 (302 to 604 µmol L^{-1}) or low CO_2 (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 represents mature *vs* juvenile leaves. Results of a two-way ANOVA are presented, with significant effects shown in bold. $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$.

Factors	Parameters									
	Rubisco		PEPC		PPDK		NAD-ME		NADP-ME	
	P	Sum of squares	*P*	Sum of squares	*P*	Sum of squares	*P*	Sum of squares	*P*	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

Table 2 Effects of CO_2 and leaf maturity on physiological parameters in *O. alismoides*. The plants were grown at high CO_2 (302 to 604 µmol L^{-1}) or low CO_2 (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 end of the photoperiod). Pigment content, chlorophyll fluorescence and rates of oxygen exchange were measured on leaves collected in the morning.Maturity represents mature *vs* juvenile leaves. Results of a two-way ANOVA are presented, with significant effects shown in bold. $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$.

Parameters	Factors					
	CO_2		Maturity		CO_2×Maturity	
	P	Sum of squares	*P*	Sum of squares	*P*	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 *a*	0.868	9.10×10^{-5}	**0.030***	0.02	0.527	0.001
Chlorophyll *b*	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

Table 3 Comparison of physiological parameters in attached and detached leaves. The attached leaves were collected from the plants treated with either high CO_2 (302 to 604 µmol L^{-1}) or low CO_2 (0.1 to 5 µmol L^{-1}) at dawn and dusk. The detached leaves grown at high or low CO_2 were collected at dusk and incubated overnight in tap water enriched with CO_2 (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

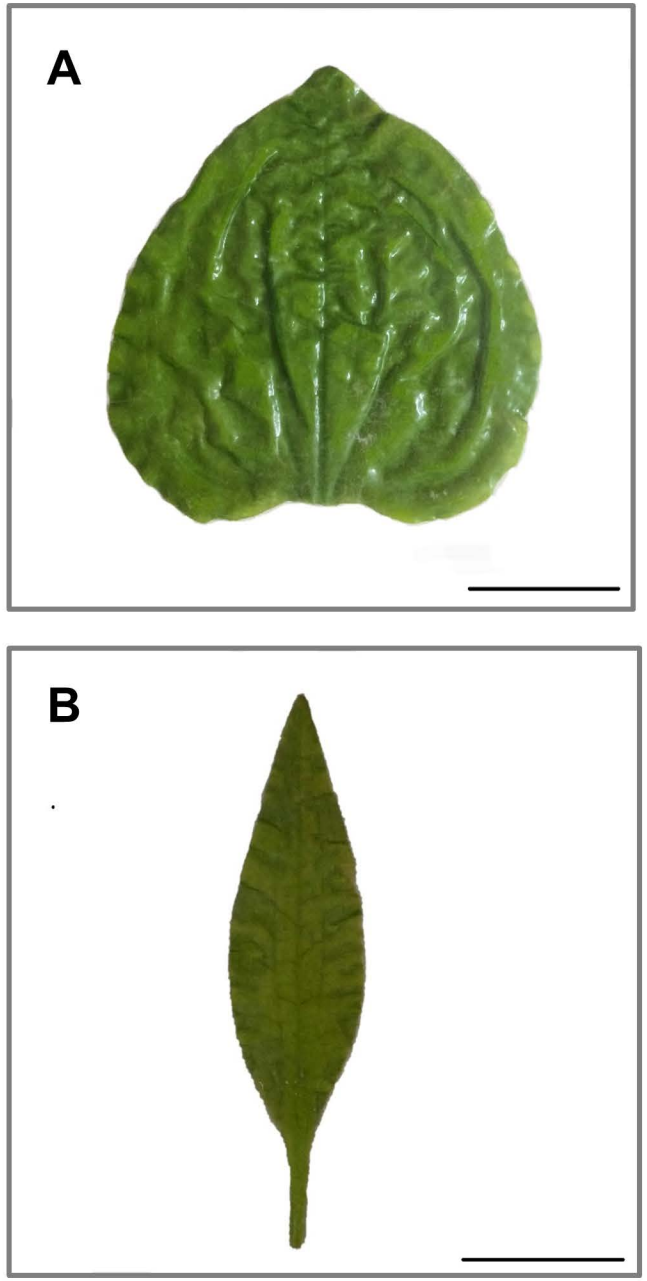


Fig.2

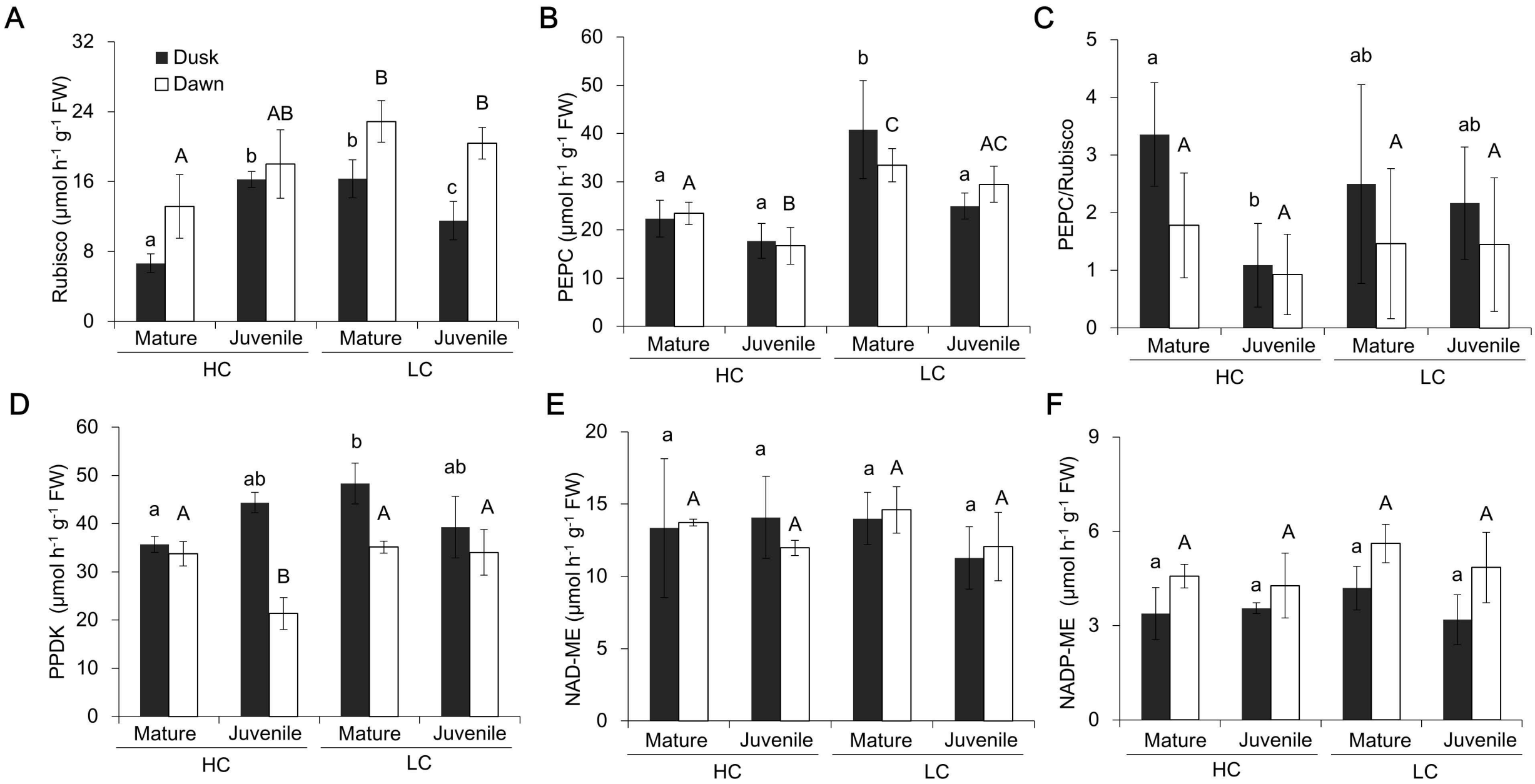

A
Rubisco (μmol h-1 g-1 FW)
Dusk
Dawn
Mature
Juvenile
Mature
Juvenile
HC
LC
B
PEPC (μmol h-1 g-1 FW)
C
PEPC/Rubisco
D
PPDK (μmol h-1 g-1 FW)
E
NAD-ME (μmol h-1 g-1 FW)
F
NADP-ME (μmol h-1 g-1 FW)

Fig.3

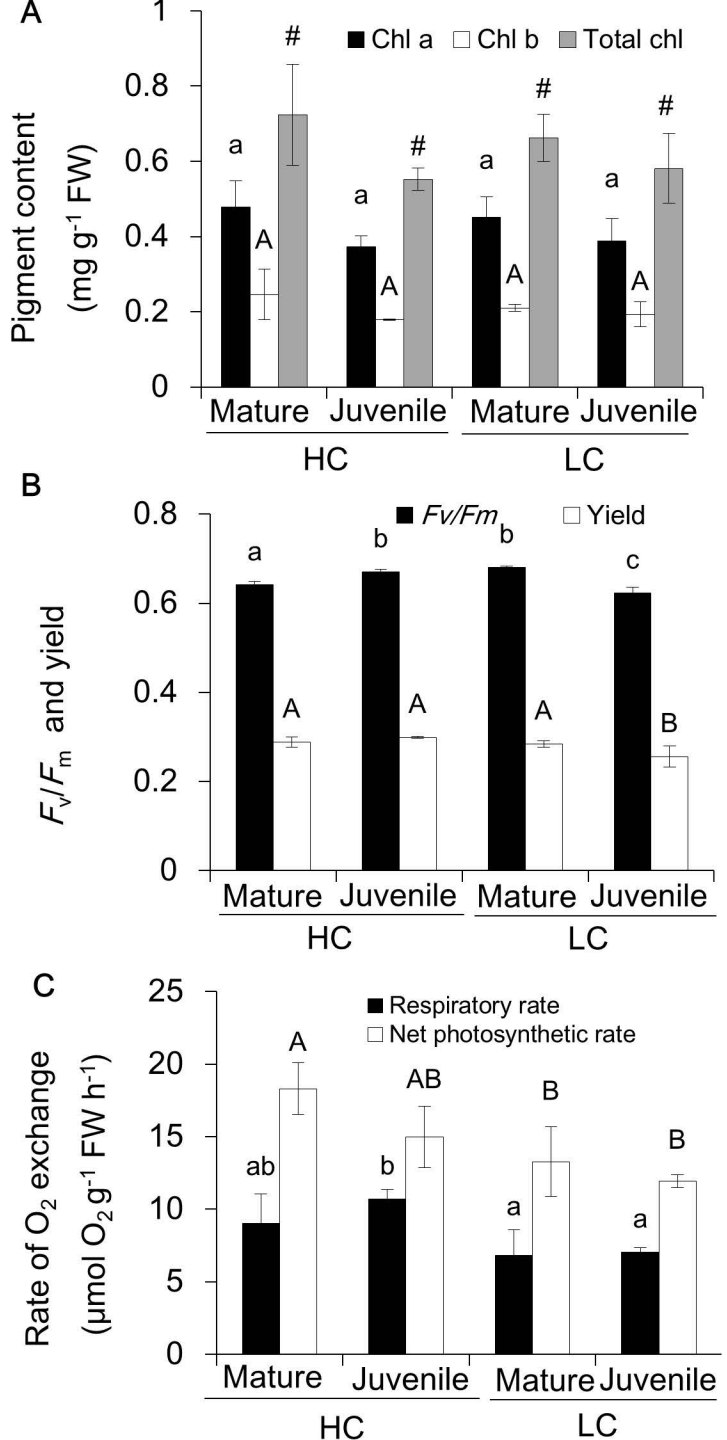

A
Chl a
Chl b
Total chl
Pigment content (mg g-1 FW)
Mature
Juvenile
Mature
Juvenile
HC
LC
B
Fv/Fm
Yield
Fv/Fm and yield
C
Respiratory rate
Net photosynthetic rate
Rate of O2 exchange (μmol O2 g-1 FW h-1)

Fig.4

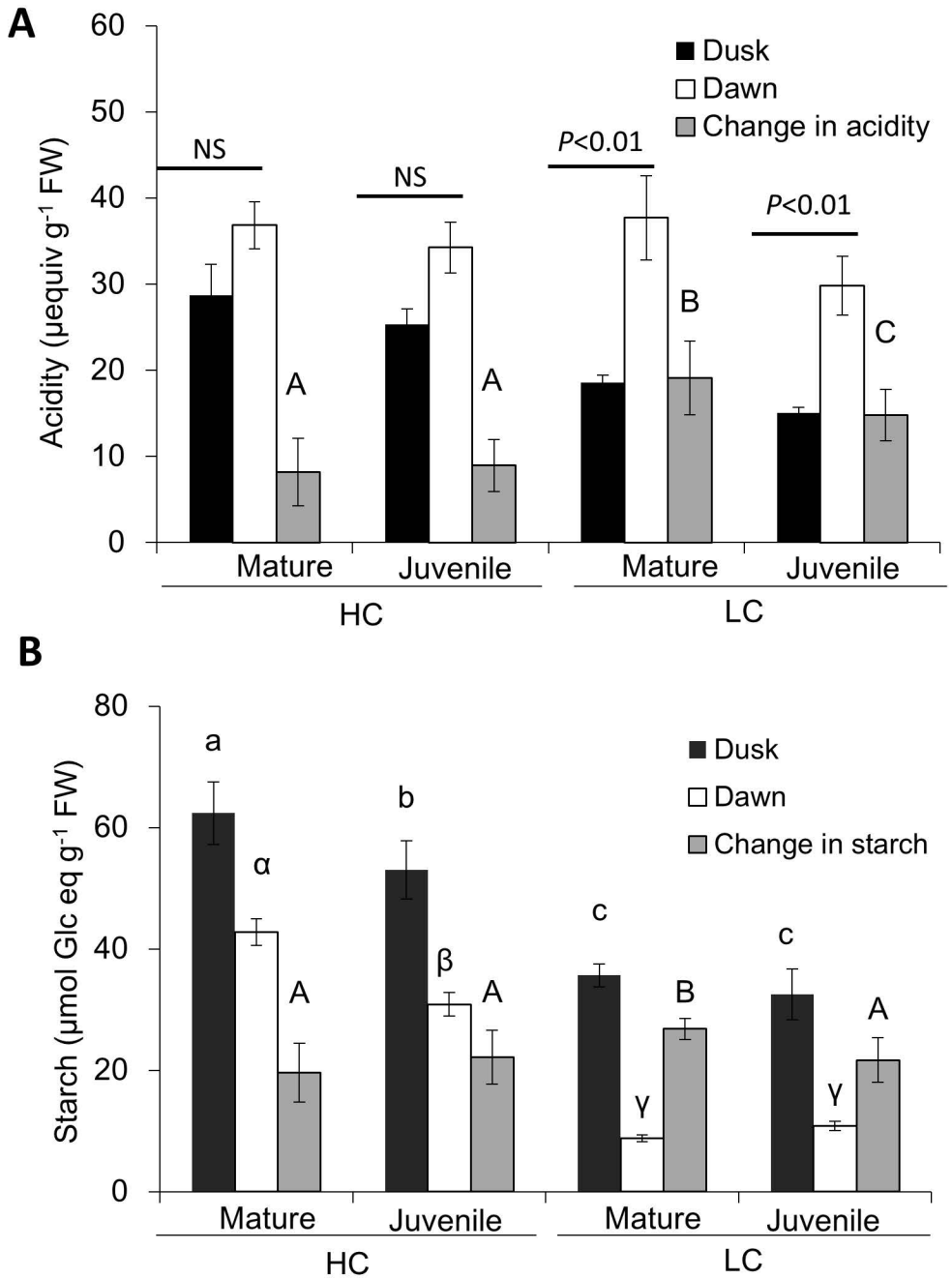

A
Acidity (μequiv g-1 FW)
60
50
40
30
20
10
0
Dusk
Dawn
Change in acidity
NS
NS
P<0.01
P<0.01
A
A
B
C
Mature
Juvenile
Mature
Juvenile
HC
LC
B
Starch (μmol Glc eq g-1 FW)
80
60
40
20
0
Dusk
Dawn
Change in starch
a
α
A
b
β
A
c
γ
B
c
γ
A
Mature
Juvenile
Mature
Juvenile
HC
LC

Fig.5

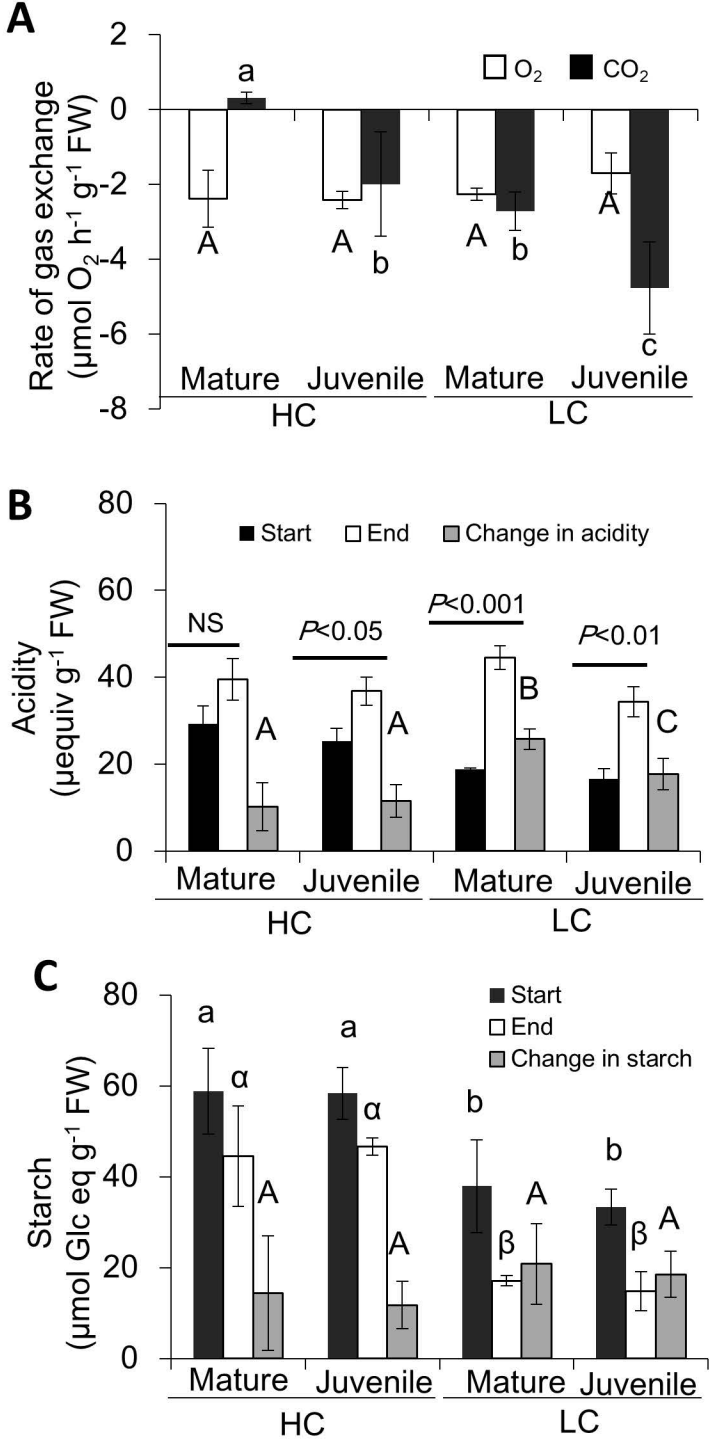
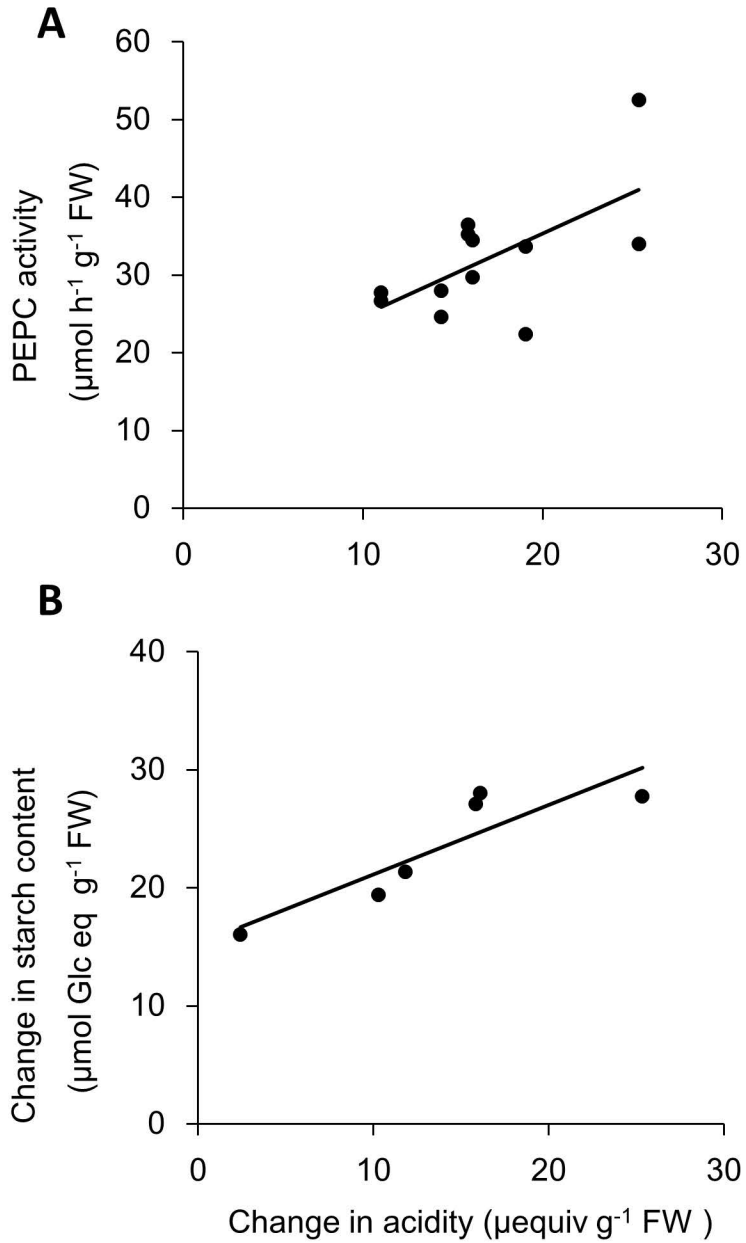

A
Rate of gas exchange (μmol O_2 h^{-1} g^{-1} FW)
2
0
-2
-4
-6
-8
O_2
CO_2
a
A
A
b
A
b
A
c
Mature
Juvenile
Mature
Juvenile
HC
LC
B
Acidity (μequiv g^{-1} FW)
80
60
40
20
0
Start
End
Change in acidity
NS
P<0.05
P<0.001
P<0.01
A
A
B
C
Mature
Juvenile
Mature
Juvenile
HC
LC
C
Starch (μmol Glc eq g^{-1} FW)
80
60
40
20
0
Start
End
Change in starch
a
α
A
a
α
A
b
β
A
b
β
A
Mature
Juvenile
Mature
Juvenile
HC
LC

Fig.6


A
PEPC activity
(μmol h-1 g-1 FW)
60
50
40
30
20
10
0
0
10
20
30
B
Change in starch content
(μmol Glc eq g-1 FW)
40
30
20
10
0
0
10
20
30
Change in acidity (μequiv g-1 FW)

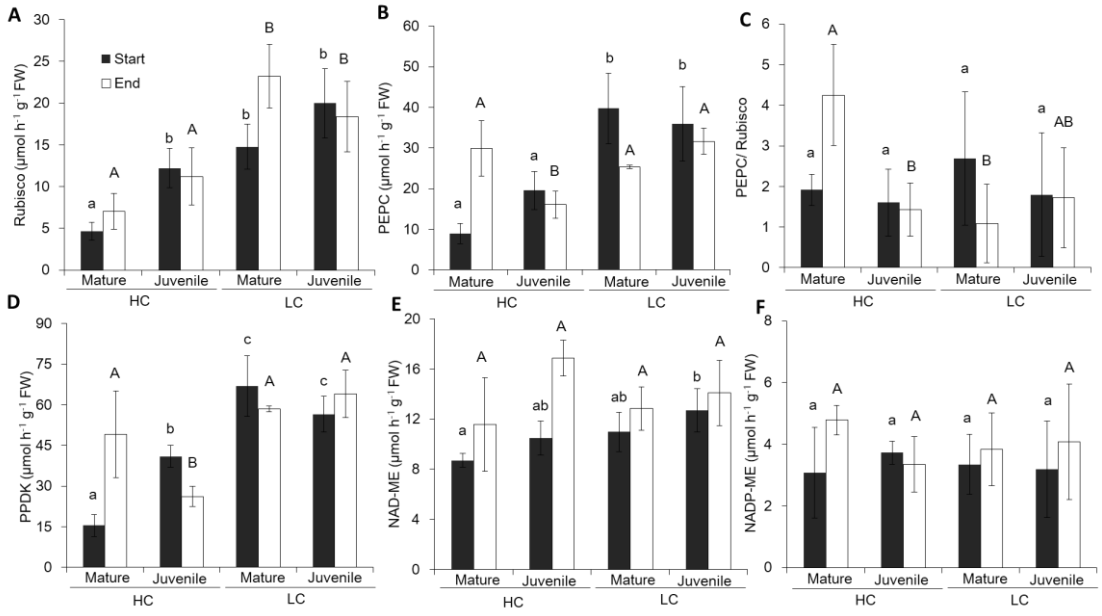


Figure S1: Changes in enzyme activities in detached mature or juvenile leaves from plants grown at high or low CO_2 after overnight incubation. (A) Rubisco activity, (B) PEPC activity, (C) the PEPC:Rubisco ratio, (D) PPDK activity, (E) NAD-ME activity and (F) NADP-ME activity. Mean ± SD is presented. 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 *a* (mg g^{-1})	0.53 (0.04)[a]	0.47 (0.03)[a]
Chl a/b	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]