

# Structural basis for C4 photosynthesis without Kranz anatomy in leaves of the submerged freshwater plant Ottelia alismoides

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Shijuan Han, Stephen C. Maberly, Brigitte Gontero, Zhenfei Xing, Wei Li, et al.. Structural basis for C4 photosynthesis without Kranz anatomy in leaves of the submerged freshwater plant Ottelia alismoides. Annals of Botany, 2020, 125 (6), pp.869-879. 10.1093/aob/mcaa005 . hal-02572396

# HAL Id: hal-02572396 https://amu.hal.science/hal-02572396

Submitted on 13 May 2020

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1	Original Article
2	Structural basis for C4 photosynthesis without Kranz
3	anatomy in leaves of the submerged freshwater plant Ottelia
4	alismoides
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#### ABSTRACT

Background and Aims Ottelia alismoides (Hydrocharitaceae) is a freshwater
macrophyte that, unusually, possesses three kinds of carbon dioxide-concentrating
mechanisms. Here we describe its leaf anatomy and chloroplast ultrastructure, how
they are altered by CO<sub>2</sub> concentration and may underlie C<sub>4</sub> photosynthesis.

Methods Light and transmission electron microscopy were used to study the
anatomy of mature leaves of *O. alismoides* grown at high and low CO<sub>2</sub> concentrations.
Diel acid change and the activity of PEP carboxylase were measured to confirm that
CAM activity and C<sub>4</sub> photosynthesis were present.

10 • Key Results When O. alismoides was grown at low CO<sub>2</sub> the leaves performed both 11 C4 and CAM photosynthesis whereas with high CO<sub>2</sub> leaves used C4 photosynthesis. 12 The leaf comprised an upper and lower layer of epidermal cells separated by a large 13 air space occupying about 22% of the leaf transverse-section area, and by mesophyll 14 cells connecting the two epidermal layers. Kranz anatomy was absent. At low CO<sub>2</sub>, 15 chloroplasts in the mesophyll cells were filled with starch even at the start of the 16 photoperiod, while epidermal chloroplasts had small starch grains. The number of 17 chloroplasts in the epidermis was greater than in the mesophyll cells. At high CO<sub>2</sub>, the 18 structure was unchanged but the thickness of the two epidermal layers, the air space, 19 mesophyll and the transverse-section area of cells and air space were greater.

Conclusions Leaves of *O. alismoides* have epidermal and mesophyll cells that
contain chloroplasts and large air spaces but lack Kranz anatomy. The high starch
content of mesophyll cells suggests they may benefit from an internal source of CO<sub>2</sub>,
for example via C4 metabolism, and are also sites of starch storage. The air spaces
may help in the recycling of decarboxylated or respired CO<sub>2</sub>. The structural similarity
of leaves from low and high CO<sub>2</sub> is consistent with the constitutive nature of

1	bicarbonate and C4 photosynthesis. There is sufficient structural diversity within the
2	leaf of O. alismoides to support dual-cell C4 photosynthesis even though Kranz
3	anatomy is absent.
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6	
7	Key words: aerenchyma, bicarbonate use, CAM, CO <sub>2</sub> acclimation, CO <sub>2</sub> -
8	concentrating mechanism (CCM), chloroplast ultrastructure, freshwater macrophyte,
9	Hydrocharitaceae

2

### **INTRODUCTION**

3 In their evolution from terrestrial ancestors, freshwater plants have traded-off 4 problems of water shortage for problems of carbon-shortage (Maberly and Gontero, 2018). Carbon-shortage is mainly caused by low rates of CO<sub>2</sub> diffusion across 5 6 boundary layers surrounding aquatic leaves (Black et al., 1981). Additionally, 7 especially in productive lakes, generation of CO<sub>2</sub> concentrations below air-8 equilibrium and, oxygen concentrations above air-equilibrium, can together stimulate 9 photorespiration (Maberly, 1996; Sand-Jensen et al., 2019). In response to the absence 10 of water shortage and the presence of carbon limitation, the leaves of submerged 11 freshwater plants have thin cuticles, lack stomata and sub-stomatal spaces and have 12 chloroplasts in epidermal cells (Sculthorpe, 1967). Laminar leaves are generally thin 13 with a high specific leaf area (Enríquez et al., 1996; Poorter et al., 2009) and the 14 lamina often comprises only two or three cell layers (Maberly and Gontero, 2018). 15 Aerenchyma is a common feature of aquatic plants (Sculthorpe, 1967) and is present 16 in the roots, leaves and stems of most aquatic species (Silveira et al., 2016).

17 In addition to these structural changes, freshwater macrophytes employ a 18 number of avoidance, exploitation and amelioration strategies to overcome carbon 19 limitation (Klavsen et al., 2011). Amelioration strategies involve active CO2-20 concentrating mechanisms (CCMs) that increase CO<sub>2</sub> around the primary carboxylase, 21 ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). There are three known 22 CCMs in freshwater macrophytes: of the species investigated, about half have the 23 ability to use HCO<sub>3</sub><sup>-</sup>, 8% have CAM and 4% have C<sub>4</sub> (Maberly and Gontero, 2017; 24 Iversen et al., 2019). Bicarbonate is the dominant form of inorganic carbon when pH

1 is between about 6.3 and 10.1 and even when CO<sub>2</sub> becomes depleted as pH increases, 2 bicarbonate can be present at appreciable concentrations. C4 photosynthesis and CAM 3 depend on temporary fixation of bicarbonate by phosphoenolpyruvate carboxylase 4 (PEPC) to form a C<sub>4</sub> compound that is subsequently decarboxylated, raising the CO<sub>2</sub> 5 concentration around the active site of Rubisco (Keeley, 1981; Bowes et al., 2002). 6 The carboxylation and decarboxylation processes are separated spatially in C4 7 photosynthesis and temporally in CAM. C4 photosynthesis reduces photorespiration 8 during the day in aquatic and terrestrial plants. In addition, CAM can reduce the loss 9 of carbon from dark respiration and extend the duration of carbon uptake to the night 10 (Maberly and Madsen, 2002; Pedersen et al., 2011).

11 Function and structure are often closely associated (Smith et al., 2012). In 12 freshwater macrophytes, use of bicarbonate can be associated with polar leaves with 13 high pH produced at the adaxial/upper surface and low pH at the abaxial/lower 14 surface (Steemann Nielsen, 1947; Prins and Elzenga, 1989). Terrestrial CAM plants 15 have large vacuoles to store the C<sub>4</sub> compound, often in the form of malate, 16 accumulated during the night (Nelson and Sage, 2008; Silvera et al., 2010). Terrestrial 17 C<sub>4</sub> plants, typically have a specialised 'Kranz anatomy', that comprises mesophyll 18 cells with C<sub>4</sub> photosynthesis surrounding bundle sheath cells where CO<sub>2</sub> is 19 concentrated and assimilated by the Calvin-Benson-Bassham cycle (Sage and Monson, 20 1999). In addition to different photosynthetic enzymes, these two types of cell also 21 differ in starch content and chloroplast ultrastructure (Edwards et al., 2004) so that the 22 initial carboxylation and the subsequent decarboxylation can be spatially separated in 23 these two distinctive types of photosynthetic cell (Raghavendra and Sage, 2011; Sage, 24 2016). However, a few terrestrial plants within the dicot Amaranthaceae family, e.g. 25 Bienertia cycloptera, Bienertia sinuspersici and Suaeda aralocaspica (formerly

Borszczowia aralocaspica), operate C4 photosynthesis through the spatial separation
 of dimorphic chloroplasts within a single cell (Voznesenskaya et al., 2001;
 Voznesenskaya et al., 2002; Voznesenskaya et al., 2003; Edwards et al., 2004; Akhani
 et al., 2005).

5 C4 photosynthesis is also present in freshwater plants from the monocot 6 Hydrocharitaceae family: Egeria densa, Ottelia alismoides and Ottelia acuminata 7 (Casati et al., 2000; Lara et al., 2002; Zhang et al., 2014), as well as in the well-8 studied species Hydrilla verticillata, where it takes place within a single cell (Bowes 9 and Salvucci, 1989; Bowes, 2011). In E. densa and H. verticillata, C4 photosynthesis 10 is induced by carbon limitation whereas in O. alismoides it is present in mature leaves regardless of the CO<sub>2</sub> concentration (Zhang et al., 2014; Shao et al., 2017; Huang et 11 12 al., 2018). In contrast, the CAM activity of O. alismoides is facultative and is induced 13 at low CO<sub>2</sub> but absent at high CO<sub>2</sub> (Zhang et al., 2014). In addition, O. alismoides has 14 a constitutive ability to use bicarbonate and is the only plant known to have three 15 different CCMs (Zhang et al., 2014; Shao et al., 2017; Huang et al., 2018).

16 In terrestrial plants, elevated CO<sub>2</sub> concentrations can alter anatomical structure 17 (Pritchard et al., 1999; Uprety et al., 2001). Leaves of freshwater plants grown in air 18 and water can have very different morphologies and structure (Maberly and Gontero, 19 2018). However, the anatomical response of leaves of freshwater plants to different 20 CO<sub>2</sub> concentrations has not been studied. Since O. alismoides is the only known 21 species with three kinds of CCMs, we hypothesized that its leaf anatomy and 22 chloroplast ultrastructure might be peculiar and reflect the integration of these three 23 different processes and that they might be affected by CO<sub>2</sub> concentration.

#### MATERIALS AND METHODS

## 2 Plant material

3 On 22 March 2018, seeds of O. alismoides were sown in plastic pots (11 cm in 4 diameter and 7 cm deep) containing sterile soil from nearby Donghu Lake and covered with 2 cm of sterile tap water. The chemical composition of the tap water was 5 analyzed. The concentrations of total nitrogen (TN) and total phosphorus (TP) were 6 7 determined spectrophotometrically after digestion with K<sub>2</sub>S<sub>2</sub>O<sub>8</sub> (Huang et al., 1999). The concentrations of  $Na^+$ ,  $K^+$ ,  $Ca^{2+}$  and  $Mg^{2+}$  were determined using optical emission 8 9 spectrometry (ICP-OES) Optima 8000DV (Perkin Elmer, MA, USA) after addition of 200  $\mu$ L HNO<sub>3</sub> to 10 mL tap water. The concentrations of Cl<sup>-</sup>, SO<sub>4</sub><sup>2-</sup> and NO<sub>3</sub><sup>-</sup> were 10 measured using Dionex ICS-5000<sup>+</sup> HPIC system (Thermo Fisher Scientific, MA, 11 12 USA). The composition is shown in Supplementary Data Table S1.

The pots were placed in a plant growth chamber at 28 °C, 12/12 hours 13 photoperiod (150  $\mu$ mol photon m<sup>-2</sup> s<sup>-1</sup>, photosynthetically active radiation). The water 14 15 level was increased as the seedlings grew to keep them submerged. When the 16 seedlings were about 4 cm tall, the pots were placed in 1 L glass beakers in the growth 17 chamber. After 40 days, three to five seedlings (~8 cm tall) were transplanted into 18 another plastic pot (15 cm diameter, 12 cm deep) containing the sterile soil. These 19 containers were placed in a 400 L tank (64 cm deep) located in a glasshouse receiving 20 natural daylight. The tap water in the tank was changed weekly and snails were 21 removed daily.

22

# 23 Response of mature *O. alismoides* leaves to different CO<sub>2</sub> concentrations

1 In August 2018, when the plants had produced many broad, oval-shaped mature 2 leaves, one pot was placed in each of eight white plastic buckets ( $25 \times 25 \times 35$  cm). These were filled with tap water and placed in the rooftop tanks, surrounded by water 3 4 to keep the water temperature consistent among all the buckets, but the solution in 5 each bucket was independent from the others. High and low CO<sub>2</sub> concentrations were 6 produced using the method described in Shao et al. (2017) with four replicates of each 7 treatment. The water in the buckets was changed twice during the 40-days acclimation. 8 Low CO<sub>2</sub> (LC) was produced by allowing plant photosynthesis to deplete inorganic 9 carbon and increase pH while high CO<sub>2</sub> (HC) was produced by adding a CO<sub>2</sub> solution 10 to produce a set pH twice each day. In the morning (between 08:00 and 09:00) and 11 afternoon (between 17:30 to 19:00), the water was gently stirred to thoroughly mix it and pH was measured with a pH electrode and temperature was measured with a 12 13 thermometer. Alkalinity was measured every two days by Gran titration (Shao et al., 14 2017). On each sampling occasion, CO<sub>2</sub>-saturated tap water was added to the HC 15 treatment, to bring the pH to 6.8. Concentrations of CO<sub>2</sub> were calculated from pH, 16 alkalinity and temperature (Maberly, 1996). In the LC treatment, the pH increased from 8.0 to over 9.8 and the CO<sub>2</sub> concentration varied between 0.1 and 13 µmol L<sup>-1</sup> 17 with a mean of 2.4  $\mu$ mol L<sup>-1</sup>. In the HC treatment, the pH varied between 6.6 and 6.9, 18 producing CO<sub>2</sub> concentrations between 481  $\mu$ mol L<sup>-1</sup> and 1110  $\mu$ mol L<sup>-1</sup> with a mean 19 of 720 µmol L<sup>-1</sup>. Water temperature ranged between 25 and 35 °C with a mean of 30 20 21 °C. As a reference, the CO<sub>2</sub> concentration in equilibrium with 400 ppm atmospheric CO<sub>2</sub> will be about 14 µmol L<sup>-1</sup> at 30 °C. Changes in CO<sub>2</sub> concentration in the two 22 23 treatments are shown in Supplementary Data Fig. S1. After 40-days acclimation to LC 24 and HC treatments, fully expanded mature leaves that had been produced during the

experiment and appeared to be of similar age were sampled for structure and
 physiology studies.

3

## 4 Light Microscopy and Transmission Electron Microscopy

5 Leaf segments (3 mm  $\times$  3 mm) sampled at 0500 and 1800 were fixed overnight in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C and post-fixed in 1% 6 7 OsO<sub>4</sub> at 4°C for 2.5 h (Farnese et al., 2017). They were dehydrated using a stepwise ethanol series (30, 50, 70, 90 and 100%) and processed with a mixed solution of 8 9 ethanol and acetone, then infiltrated in a mixture of acetone and epoxy resin (1: 1 for 1 h, and then 1: 2 for 8 h) and finally embedded using SPI-PON 812 at 60°C for 48 h. 10 11 Semi-thin sections (1.5 µm) and ultrathin sections (72 nm) were obtained on a Leica 12 EM UC7 ultramicrotome. Semi-thin sections were stained with methylene blue and 13 observed using a Motic BA310 digital light microscope. Quantitative characteristics 14 of leaf structures were measured using the Motic Images Plus 2.0 ML software. For 15 the ultrathin sections, after staining with uranyl acetate and lead citrate, they were 16 observed and photographed with a HT7700 transmission electron microscope (Hitachi 17 High-Tech, Japan). Leaf chloroplasts and starch grains in electron micrograph were measured using Image J software. The distribution of chloroplasts in cells was 18 19 assessed by measuring the number of chloroplasts per unit length of cell wall at 20 different locations from the semi-thin sections under the light microscope, including 21 the upper epidermis next to water, the upper epidermis next to air space, the upper 22 mesophyll cells, the lower mesophyll cells, the lower epidermis next to air space and 23 the lower epidermis next to water.

# 2 Enzyme activity measurement

Mature leaves were collected at 0500 and 1800 using the extraction and assay
protocols for Rubisco and PEPC activities as described previously (Zhang et al., 2014;
Shao et al., 2017; Huang et al., 2018). Enzyme activities were calculated from the rate
of disappearance of NADH at 340 nm and 25°C measured with a microplate reader
(Tecan M200 PRO, Austria).

8

# 9 CAM activity measurement

10 CAM activity was assessed by calculating the daily change in titratable acidity, in
11 leaves harvested at 0500 and 1800 as previously described (Zhang et al., 2014).
12 Briefly, to measure leaf acid content, 10 mL CO<sub>2</sub>-free deionized water were added to
13 the leaf samples of known fresh weight (0.2 ~ 0.5 g) and then boiled for 30 min. The
14 acidity was titrated to pH 8.3 using 0.01 N NaOH.

15

### 16 Statistical analysis

17 SPSS 20 (SPSS Inc., Chicago, IL, USA) was used to analyze the data. An 18 independent sample t-test was used for two groups of data with normal distribution, 19 the Mann-Whitney U-test was used for two groups of data where the distribution was 20 not normal and the Kruskal-Wallis test was used for three or more groups of data 21 where the distribution was not normal. The significance level of the statistics was 22 accepted at P < 0.05.

# 2

# RESULTS

# **3** C<sub>4</sub> photosynthesis and CAM activity

4 The enzyme activities of PEPC and Rubisco in mature leaves under HC and LC 5 treatments were measured to confirm that C4 photosynthesis was present. There was no significant difference in PEPC activities between leaves collected at dusk and 6 7 dawn regardless of the treatments, but the PEPC activities were significantly higher in 8 leaves grown at a low versus a high concentration of CO<sub>2</sub> (Fig. 1A). Rubisco activity 9 was significantly lower in leaves collected at dawn versus dusk, but was not affected 10 by CO<sub>2</sub> concentration (Fig. 1B). The ratio of PEPC to Rubisco was between 1.6 and 11 4.2 (Fig. 1C) within a range typical of terrestrial C<sub>4</sub> plants (Zhang et al., 2014). This 12 ratio was significantly higher at low than at high CO<sub>2</sub> both at dawn and dusk. At dawn, 13 the ratio was about 2.6-fold higher in low compared to high CO<sub>2</sub> leaves suggesting 14 that leaves from low CO<sub>2</sub> had more active C<sub>4</sub> photosynthesis than those from high 15  $CO_2$ . Leaves at low  $CO_2$  had a marked diel change in acidity that was significantly higher (58  $\mu$ equiv g<sup>-1</sup> FW) than at high CO<sub>2</sub> concentrations (19  $\mu$ equiv g<sup>-1</sup> FW, Fig. 16 2). These results confirm that these mature O. alismoides leaves perform both C4 17 18 photosynthesis and CAM when acclimated to low CO<sub>2</sub> and C<sub>4</sub> photosynthesis at high 19 CO<sub>2</sub>.

20

# 21 Effects of CO<sub>2</sub> concentration on leaf anatomy

Transverse sections showed that there were no major differences in basic structureand types of cell in leaves grown at high and low concentrations of CO<sub>2</sub> (Fig. 3).

Leaves from both treatments comprised an upper and lower layer of epidermal cells separated by a large air space and between one and three but most often two stacked mesophyll cells connecting the two epidermal layers. In transverse section, the epidermal cells varied in shape between rectangular and elliptical while the mesophyll cells were less elongated (Fig. 3B, E). Parenchymal cells containing some chloroplasts occurred around the vascular bundle, the bundle sheath, but this was not surrounded by mesophyll cells and thus Kranz anatomy was absent (Fig. 3C, F).

8 Leaf width was significantly greater at high (8.56 (mean) cm  $\pm$  0.50 (SD)) 9 compared to low CO<sub>2</sub> concentration (5.44 cm  $\pm$  0.18), but leaf length was unaltered at 10  $\sim$ 12 cm (Table 1). Consequently, the length to width ratio was greater at low CO<sub>2</sub>. 11 The leaves of *O. alismoides* were thicker at high than at low CO<sub>2</sub> concentration (Fig. 12 3, Table 1). The ratio of air space area to leaf area in transverse section was 0.22 to 13 0.23 but was not affected significantly by CO<sub>2</sub> concentration (Table 1). The air spaces 14 were bounded top and bottom by the epidermal cells and surrounded at the sides by a 15 network of mesophyll cells and so were not connected to one another (see also 16 Supplementary Data Fig. S2).

17 The epidermis and mesophyll of plants grown at high CO<sub>2</sub> concentration were 18 significantly thicker and of greater cell area than in plants grown at low CO<sub>2</sub> 19 concentration (Fig. 4). Furthermore, the upper epidermis was thicker than the lower 20 epidermis, both at low and high CO<sub>2</sub> (P < 0.001), but the area was not different. The 21 thickness and area of an air space were correspondingly greater at high CO<sub>2</sub> 22 concentration.

23

## 24 Effects of CO<sub>2</sub> concentration on chloroplasts

1 Chloroplasts were present in epidermal and mesophyll cells (Fig. 3). Since 2 chloroplasts can differ in cells carrying out different processes, such as in C4 3 photosynthesis, we characterized chloroplast size, shape and ultrastructure in leaves 4 grown at high and low CO<sub>2</sub>. In leaves grown at high CO<sub>2</sub>, the chloroplasts in 5 epidermis and mesophyll cells were all nearly spherical with large starch grains (Fig. 6 5A, C, D, E), which occupied ~65 and ~80% of the chloroplast area both at dusk and 7 dawn (Fig. 6E & F). In contrast, in leaves grown at low CO<sub>2</sub>, epidermal chloroplasts 8 were spindle-shaped (Fig. 5F), while the mesophyll chloroplasts were spherical (Fig. 9 5I) and consequently, their minor axis length was shorter (Fig. 6B). In the leaves collected at dusk, starch occupied 40% and 64% of the chloroplast area in the 10 11 epidermal and the mesophyll cells, respectively (Fig. 6E). The same trend was also 12 observed in the leaves sampled at dawn (Fig. 6F), small and large starch grains were 13 distributed in the chloroplasts of the epidermal and mesophyll cells, respectively (Fig. 14 5H & J). Grana with many thylakoids were clearly visible in epidermal chloroplasts 15 (Fig. 5B & G) whereas in mesophyll chloroplasts, the grana thylakoids were indistinct 16 because of the high starch content.

The average area of a chloroplast was greater in leaves grown at high versus low CO<sub>2</sub> in both types of cell (Fig. 6C). The area of a chloroplast was greater in mesophyll than in epidermal cells for both CO<sub>2</sub> treatments. At high CO<sub>2</sub>, the number of mitochondria within 1 μm around a chloroplast in mesophyll and epidermal cells was not significantly different (Fig. 6D). However, at low CO<sub>2</sub>, the number of mitochondria around chloroplasts in mesophyll cells was significantly higher than in the epidermal cells.

The distribution of chloroplasts in epidermal and mesophyll cells was 2 measured under different CO<sub>2</sub> concentrations (Fig. 7). The frequency of chloroplasts 3 in the upper and lower epidermis was significantly greater than in the mesophyll cells. 4 The chloroplast frequency at the cell walls next to air spaces in the lower epidermis 5 was less than the equivalent location in the upper epidermis, at high and low CO<sub>2</sub>. The 6 frequency of chloroplasts at the upper epidermis next to an air space was significantly 7 lower in leaves acclimated to low compared to high CO<sub>2</sub>.

- 8
- 9

### **DISCUSSION**

#### 10 Adaptation to photosynthesis underwater

11 The presence of chloroplasts in epidermal cells is a common feature in submerged angiosperms, but rare in their terrestrial ancestors (Sculthorpe, 1967; Rascio, 2002; 12 13 Maberly and Gontero, 2018). Mature leaves of O. alismoides have two types of 14 photosynthetic cell, epidermal and mesophyll. In contrast, leaves from three other 15 species of Hydrocharitaceae, H. verticillata, E. densa and Elodea callitrichoides, only 16 have two layers of epidermal cells and no mesophyll cells (Falk and Sitte, 1963; 17 Pendland, 1979; Hara et al., 2015). As a consequence, the leaf thickness of O. 18 alismoides, especially in the leaves grown at high CO<sub>2</sub>, at 196 µm, is much greater 19 than the median (95 µm) for submerged leaves from a range of freshwater 20 macrophytes (Maberly and Gontero, 2018) and greater than E. callitrichoides and E. 21 densa estimated from published images to be about 65 µm. While Black et al. (1981) 22 estimated that the internal resistance to diffusion of CO<sub>2</sub> within the relatively thin 23 leaves of four *Potamogeton* species (48 to 63 µm) was only 3 to 4% of the total, the 24 internal resistance in the thicker leaves of O. alismoides could be greater but could be

1 offset by the high porosity (ratio of air space to transverse sectional leaf area, 0.22 to 2 0.23). In O. alismoides, the sizes of the upper and lower epidermal cells are similar 3 while in *H. verticillata*, the upper epidermal cells are noticeably larger than the lower 4 cells (Pendland, 1979). In E. callitrichoides the transverse sectional area of the upper 5 epidermal cells is about four-times that of the lower epidermis (Falk and Sitte, 1963), 6 assuming the orientation in Fig. 1 in Falk and Sitte (1963) is correct since the adaxial 7 and abaxial layers are not specifically labelled. Similarly, in E. densa, the transverse 8 sectional area of the upper epidermal cells is about five-times larger than that of the 9 lower epidermal cells (Hara et al., 2015). The functional significance of these 10 differences is currently unknown but they could be linked to the use of bicarbonate 11 involving different processes in upper and lower cell layers (Steemann Nielsen, 1947; 12 Prins and Elzenga, 1989).

13 Well-developed aerenchyma is a common feature of aquatic plants (Jung et al., 14 2008). O. alismoides contains large air spaces between the epidermal layers while in 15 H. verticillata and E. callitrichoides, there are numerous small intercellular spaces 16 between the upper and lower epidermal cells that comprise a small proportion of the 17 leaf volume (Falk and Sitte, 1963; Pendland, 1979). In many isoetids, the aerenchyma 18 is a large proportion of the leaf volume and is continuous from the roots to the leaves, 19 allowing sedimentary CO<sub>2</sub> to be taken up and fixed (Madsen et al., 2002) and oxygen 20 to be supplied to the roots (Sand-Jensen and Prahl, 1982). Since in O. alismoides the 21 air spaces are discrete (Supplementary Data Fig. S2), and so not connected to the 22 roots, these two processes are unlikely to occur. Air spaces comprise about 22 to 23% 23 of the transverse section leaf area in O. alismoides, which is within the broad range 24 recorded for terrestrial leaves (3 - 73%, Slaton and Smith, 2002; Earles et al., 2018). 25 In terrestrial leaves, the intercellular air spaces are connected to the atmosphere via

1	stomata and help to maximize the mesophyll surface area in contact with atmospheric
2	CO2. Aquatic plants generally lack functional stomata, so the air spaces within a leaf
3	are not connected to the exterior. The air spaces provide buoyancy, allowing the
4	leaves to float towards the surface where light is higher, but could also act as a
5	reservoir of respiratory CO2 (Wetzel et al., 1984) or photorespiratory CO2
6	(Søndergaard and Wetzel, 1980) as can also occur in terrestrial C3 plants (Busch et al.,
7	2013). However, a calculation for low CO <sub>2</sub> leaves based on a one-sided specific leaf
8	area of 100.5 cm <sup>2</sup> g <sup>-1</sup> FW and net rates of photosynthesis at around air-equilibrium
9	CO2 of 2 µmol g <sup>-1</sup> FW h <sup>-1</sup> (Zhang et al., 2014), air space area as a proportion of leaf
10	area of 0.23 (this study) and maximal CO <sub>2</sub> partial pressure in the air space of 10,000
11	ppm (Madsen, 1987) suggests that air-space CO2 could only support net
12	photosynthesis for about 5 minutes. Nevertheless, if C4 decarboxylation occurs in the
13	mesophyll cells, as seems possible given the relatively high chloroplast density in
14	these cells in relation to their distance from external inorganic carbon sources, then
15	the air spaces may serve to trap and recycle CO2 produced by C4 decarboxylation.
16	This could be efficient as loss of CO <sub>2</sub> from the air spaces to the outside will be limited
17	by the chloroplasts in the epidermis and the low rate of CO <sub>2</sub> exchange between the
18	leaf and the bulk water as a result of transport limitation across the boundary layer. At
19	25 °C the air-equilibrium molar ratio of oxygen to CO <sub>2</sub> in water is about 28-fold less
20	than in air, so the air spaces may also provide a means to reduce the concentration of
21	photosynthetically produced oxygen within mesophyll and epidermal cells.

# 23 C<sub>4</sub> photosynthesis and absence of Kranz anatomy

1 In the Hydrocharitaceae, four species are known to have constitutive or facultative C4 2 photosynthesis, H. verticillata, E. densa, O. acuminata and O. alismoides (Bowes et al., 2002; Lara et al., 2002; Zhang et al., 2014; Yin et al., 2017) and lack Kranz 3 4 anatomy. There is evidence that H. verticillata has single cell C4 photosynthesis 5 (Bowes et al., 2002). In this respect, it is similar to single-cell terrestrial C<sub>4</sub> plants 6 (Voznesenskaya et al., 2001; Voznesenskaya et al., 2002) such as B. cycloptera and S. 7 aralocaspica where C4 production and decarboxylation occur in different parts of a 8 cell (Edwards et al., 2004; Edwards and Voznesenskaya, 2011; Sharpe and Offermann, 9 2014; von Caemmerer et al., 2014). In these terrestrial plants, C4 decarboxylation 10 occurs in mitochondria using NAD malic enzyme (NAD-ME). In O. alismoides, 11 Zhang et al. (2014) suggested, on the basis of enzyme activity measurements, that 12 NAD-ME is also the decarboxylating enzyme, unlike in H. verticillata where NADP-13 ME is believed to be involved (Bowes, 2011). The location of primary CO<sub>2</sub> fixation 14 and decarboxylation in O. alismoides, and whether they take place in a single cell, is 15 unknown. Here we show that the mesophyll cells of O. alismoides have a high starch 16 content which is consistent with these cells either being the site of high rates of 17 photosynthesis resulting from decarboxylation, producing CO<sub>2</sub> locally or being sites 18 of starch storage. However, the high frequency of mitochondria around mesophyll 19 chloroplasts in leaves acclimated to low CO<sub>2</sub> supports the possibility that these cells 20 are the site of decarboxylation by the mitochondrial NAD-ME, as does the presence 21 of chloroplasts in mesophyll cells despite their distance from external sources of 22 inorganic carbon. In this dual-cell model, the O. alismoides epidermal cells would 23 then perform the function of the terrestrial C<sub>4</sub> mesophyll cells by producing a C<sub>4</sub> 24 product and the O. alismoides mesophyll cells would perform the function of the

terrestrial C<sub>4</sub> bundle sheath cells in decarboxylating the C<sub>4</sub> product. However, it is
 also possible that both processes could be occurring within the mesophyll cells.

Some C<sub>4</sub> terrestrial plants have dimorphic chloroplasts in a single cell 3 4 (Voznesenskaya et al., 2001; Voznesenskaya et al., 2002; Voznesenskaya et al., 2003; Akhani et al., 2005; Sharpe and Offermann, 2014). The different forms of chloroplasts 5 6 in the Kranz anatomy cells are linked to different energy requirements and fixation of 7 carbon (Edwards et al., 2004). Dimorphic chloroplasts differing in size and 8 ultrastructure also occur in leaves of freshwater plants. In Cabomba caroliniana 9 (Cabombaceae), the chloroplasts in mesophyll cells have larger starch grains, more 10 thylakoids per granum, and are larger than epidermal chloroplasts (Galati et al., 2015; 11 Table 2). Species within the aquatic angiosperm family Podostemaceae also have two 12 types of chloroplasts. Small chloroplasts with a normal grana ultrastructure and very 13 small starch grains occur at the upper tangential walls of epidermal cells, while large 14 chloroplasts with more thylakoids per granum and many well-developed starch grains 15 occur at the lower tangential walls of these cells and also in mesophyll cells (Fujinami 16 et al., 2011; Table 2). However, there is no evidence for CCMs in C. caroliniana (Yin 17 et al., 2017). In the Podostemaceae, although carbon isotope values range widely 18 between -12.8 and -38.6‰ (Ziegler and Hertel, 2007) at different locations suggesting 19 that there could be differences in discrimination resulting from C<sub>4</sub> photosynthesis or 20 bicarbonate use, differences in carbon isotope signature caused by the source 21 inorganic carbon cannot be excluded so the presence or absence of CCMs in these 22 plants is currently unknown.

In terrestrial C<sub>4</sub> plants, cells or parts of cells where C<sub>4</sub> fixation occurs have
 smaller chloroplasts with less starch than chloroplasts in cells or parts of cells where

1 decarboxylation occurs (Table 2). Chloroplasts from the epidermal cells of O. 2 alismoides are similar to those involved in C4 fixation while chloroplasts from 3 mesophyll cells are similar to those involved in C<sub>4</sub> decarboxylation, suggesting that 4 C<sub>4</sub> photosynthesis in this species may involve both cell types. However, although the 5 biochemical evidence suggests that O. alismoides belongs to the NAD-ME sub-type 6 of C<sub>4</sub> (Zhang et al., 2014), its pattern of thylakoids per granum in epidermal compared 7 to mesophyll cells is closer to that of terrestrial NADP-ME C<sub>4</sub> plants, suggesting that 8 there is not a direct 'read-across' between C<sub>4</sub> photosynthesis in aquatic and terrestrial 9 plants.

# 10 **Responses to CO<sub>2</sub>**

11 To maximise plant productivity there is an intricate relationship between leaf structure 12 and function (Oguchi et al., 2018). In terrestrial plants, where water can be limiting, 13 there is an evolutionary pressure to maximise the ratio of carbon gain to water loss. 14 Cell size and leaf thickness are dependent on environmental conditions (Zeiger, 1983; 15 Jones, 1985; Radoglou and Jarvis, 1990). In Liquidambar styraciflua, Pinus taeda, 16 and Brassica juncea, high CO<sub>2</sub> also has a structural effect, increasing the thickness of 17 the upper and lower epidermis and mesophyll cell of leaves (Rogers et al., 1983; 18 Uprety et al., 2001). Elevated CO<sub>2</sub> generally increases the size of terrestrial plants 19 (Pritchard et al., 1999). We found the same here for the freshwater plant O. alismoides, 20 suggesting that the increase of this resource has a universal effect on plant size.

21

# 22 Conclusions

1 O. alismoides has three CCMs that requires structural and functional coordination to 2 operate efficiently. Unlike terrestrial plants, the anatomy of *O. alismoides* is relatively 3 simple, and spongy and palisade tissues are absent, as they are in submerged leaves of 4 all aquatic plants (Maberly and Gontero, 2018). The leaf comprises two types of 5 photosynthetic cell, epidermal and mesophyll. The conceptual overview summarizing 6 the structure of O. alismoides leaves acclimated to high CO2 and low CO2 7 concentrations is shown in Supplementary Data Fig. S3. Epidermal cells, containing 8 chloroplasts, maximise uptake of external CO<sub>2</sub>, aided by bicarbonate use, while the mesophyll cells may be sites where CO2 is concentrated by decarboxylation. 9 10 Abundant discrete air spaces provide buoyancy but may also trap (photo)-respiratory 11 CO<sub>2</sub>, or CO<sub>2</sub> produced by decarboxylation, permitting its refixation. Overall, there is 12 sufficient structural diversity within the leaf of O. alismoides to support dual-cell C4 13 photosynthesis even though Kranz anatomy is absent. However, further studies are 14 needed to conclude definitively if O. alismoides has dual-cell C4 with the mesophyll 15 cells representing the site of decarboxylation. Work is underway to test this by 16 locating key photosynthesis enzymes in the epidermal and mesophyll cells.

- 17
- 18

# SUPPLEMENTARY DATA

Supplementary data consist of the following. Figure S1: Fluctuations of CO<sub>2</sub> concentration in high CO<sub>2</sub> and low CO<sub>2</sub> treatments during the 40-days acclimation.
Figure S2: Photographs of the surface of a mature *O. alismoides* leaf from the low CO<sub>2</sub> treatment using a laser scanning confocal microscope. Figure S3: Conceptual overview summarizing the structure of *O. alismoides* leaves acclimated to high CO<sub>2</sub> and low CO<sub>2</sub> concentrations. Table S1: The chemical composition of the tap water used in the growth experiments.

# 

# ACKNOWLEDGEMENTS

3	We thank Yuan Xiao for providing the TEM service and Jun Men for assistance in the
4	chemical analysis of water (Analysis and Testing Center, Institute of Hydrobiology,
5	Chinese Academy of Sciences). This work was supported by the Strategic Priority
6	Research Program of the Chinese Academy of Sciences (XDB31000000), Chinese
7	Academy of Sciences President's International Fellowship Initiative to SCM and BG
8	(2015VBA023, 2016VBA006), and the National Scientific Foundation of China
9	(31860101, 31970368).
10	
10	
11	
12	Ι ΙΤΕΡΑΤΙΙΡΕ ΔΊΤΕΝ
12	LITERATURE CITED
13	Akhani H, Barroca J, Koteeva N, et al. 2005. Bienertia sinuspersici (Chenopodiaceae): a
14	new species from Southwest Asia and discovery of a third terrestrial C4 plant without
15	Kranz anatomy. Systematic Botany <b>30</b> : 290-301.
16	
	Black MA, Maberly SC, Spence DHN. 1981. Resistances to carbon dioxide fixation in four
17	Black MA, Maberly SC, Spence DHN. 1981. Resistances to carbon dioxide fixation in four submerged freshwater macrophytes. <i>New Phytologist</i> 89: 557-568.
17 18	<ul> <li>Black MA, Maberly SC, Spence DHN. 1981. Resistances to carbon dioxide fixation in four submerged freshwater macrophytes. <i>New Phytologist</i> 89: 557-568.</li> <li>Bowes G. 2011. Single-cell C<sub>4</sub> photosynthesis in aquatic plants. In: Raghavendra AS, Sage</li> </ul>
17 18 19	<ul> <li>Black MA, Maberly SC, Spence DHN. 1981. Resistances to carbon dioxide fixation in four submerged freshwater macrophytes. New Phytologist 89: 557-568.</li> <li>Bowes G. 2011. Single-cell C<sub>4</sub> photosynthesis in aquatic plants. In: Raghavendra AS, Sage RF, eds. C<sub>4</sub> photosynthesis and related CO<sub>2</sub> concentrating mechanisms. Dordrecht:</li> </ul>
17 18 19 20	<ul> <li>Black MA, Maberly SC, Spence DHN. 1981. Resistances to carbon dioxide fixation in four submerged freshwater macrophytes. <i>New Phytologist</i> 89: 557-568.</li> <li>Bowes G. 2011. Single-cell C<sub>4</sub> photosynthesis in aquatic plants. In: Raghavendra AS, Sage RF, eds. <i>C<sub>4</sub> photosynthesis and related CO<sub>2</sub> concentrating mechanisms</i>. Dordrecht: Springer, 63-80.</li> </ul>
17 18 19 20 21	<ul> <li>Black MA, Maberly SC, Spence DHN. 1981. Resistances to carbon dioxide fixation in four submerged freshwater macrophytes. New Phytologist 89: 557-568.</li> <li>Bowes G. 2011. Single-cell C<sub>4</sub> photosynthesis in aquatic plants. In: Raghavendra AS, Sage RF, eds. C<sub>4</sub> photosynthesis and related CO<sub>2</sub> concentrating mechanisms. Dordrecht: Springer, 63-80.</li> <li>Bowes G, Rao SK, Estavillo GM, Reiskind JB. 2002. C<sub>4</sub> mechanisms in aquatic</li> </ul>
17 18 19 20 21 22	<ul> <li>Black MA, Maberly SC, Spence DHN. 1981. Resistances to carbon dioxide fixation in four submerged freshwater macrophytes. New Phytologist 89: 557-568.</li> <li>Bowes G. 2011. Single-cell C<sub>4</sub> photosynthesis in aquatic plants. In: Raghavendra AS, Sage RF, eds. C<sub>4</sub> photosynthesis and related CO<sub>2</sub> concentrating mechanisms. Dordrecht: Springer, 63-80.</li> <li>Bowes G, Rao SK, Estavillo GM, Reiskind JB. 2002. C<sub>4</sub> mechanisms in aquatic angiosperms: comparisons with terrestrial C<sub>4</sub> systems. Functional Plant Biology 29:</li> </ul>

1	Bowes G, Salvucci ME. 1989. Plasticity in the photosynthetic carbon metabolism of					
2	submersed aquatic macrophytes. Aquatic Botany 34: 233-266.					
3	Busch FA, Sage TL, Cousins AB, Sage RF. 2013. C3 plants enhance rates of photosynthesis					
4	by reassimilating photorespired and respired CO <sub>2</sub> . Plant, Cell & Environment 36:					
5	200-212.					
6	Casati P, Lara MV, Andreo CS. 2000. Induction of a C <sub>4</sub> -like mechanism of CO <sub>2</sub> fixation in					
7	Egeria densa, a submersed aquatic species. Plant Physiology 123: 1611-1621.					
8	Earles JM, Théroux-Rancourt G, Roddy AB, Gilbert ME, McElrone AJ, Brodersen CR.					
9	2018. Beyond porosity: 3D leaf intercellular airspace traits that impact mesophyll					
10	conductance. Plant physiology 178: 148-162.					
11	Edwards GE, Franceschi VR, Voznesenskaya EV. 2004. Single-cell C4 photosynthesis					
12	versus the dual-cell (Kranz) paradigm. Annual Review of Plant Biology 55: 173-196.					
13	Edwards GE, Voznesenskaya EV. 2011. C4 Photosynthesis: Kranz forms and single-cell C4					
14	in terrestrial plants. In: Raghavendra AS, Sage RF, eds. C4 photosynthesis and related					
15	CO <sub>2</sub> concentrating mechanisms. Dordrecht: Springer, 29-61.					
16	Enríquez S, Duarte CM, Sand-Jensen K, Nielsen SL. 1996. Broad-scale comparison of					
17	photosynthetic rates across phototrophic organisms. Oecologia 108: 197-206.					
18	Falk H, Sitte P. 1963. Zellfeinbau bei Plasmolyse. Protoplasma 57: 290-303.					
19	Farnese FS, Oliveira JA, Paiva EAS, et al. 2017. The involvement of nitric oxide in					
20	integration of plant physiological and ultrastructural adjustments in response to					
21	arsenic. Frontiers in Plant Science 8: 516.					
22	Fujinami R, Yoshihama I, Imaichi R. 2011. Dimorphic chloroplasts in the epidermis of					
23	Podostemoideae, a subfamily of the unique aquatic angiosperm family					
24	Podostemaceae. Journal of Plant Research 124: 601-605.					
25	Galati BG, Gotelli MM, Rosenfeldt S, Lattar EC, Tourn GM. 2015. Chloroplast					
26	dimorphism in leaves of Cabomba caroliniana (Cabombaceae). Aquatic Botany 121:					
27	46-51.					

1	Hara T, Kobayashi E, Ohtsubo K, et al. 2015. Organ-level analysis of idioblast patterning				
2	in Egeria densa Planch. leaves. Plos One 10: e0118965.				
3	Hodge AJ, McLean JD, Mercer FV. 1955. Ultrastructure of the lamellae and grana in the				
4	chloroplasts of Zea mays L. The Journal of Biophysical and Biochemical Cytology 1:				
5	605-614.				
6	Huang WM, Shao H, Zhou SN, et al. 2018. Different CO <sub>2</sub> acclimation strategies in juvenile				
7	and mature leaves of Ottelia alismoides. Photosynthesis Research 138: 219-232.				
8	Huang XF, Chen WM, Cai QM. 1999. Survey, observation and analysis of lake ecology.				
9	Beijing: Standards Press of China (in Chinese).				
10	Iversen LL, Winkel A, Baastrup-Spohr L, et al. 2019. Catchment properties and the				
11	photosynthetic trait composition of freshwater plant communities. Science 366: 878-				
12	881.				
13	Jones HG. 1985. Adaptive significance of leaf development and structural responses to				
14	environment. In: Baker NR, Davies WJ, Ong CK, eds. Control of leaf growth.				
15	Cambridge: Cambridge University Press, 155-173.				
16	Jung J, Lee SC, Choi H-K. 2008. Anatomical patterns of aerenchyma in aquatic and wetland				
17	plants. Journal of Plant Biology 51: 428-439.				
18	Keeley JE. 1981. Isoetes howellii: a submerged aquatic CAM plant? American Journal of				
19	Botany 68: 420-424.				
20	Klavsen SK, Madsen TV, Maberly SC. 2011. Crassulacean acid metabolism in the context				
21	of other carbon-concentrating mechanisms in freshwater plants: a review.				
22	Photosynthesis Research 109: 269-279.				
23	Laetsch WM. 1968. Chloroplast specialization in dicotyledons possessing the C4-				
24	dicarboxylic acid pathway of photosynthetic CO2 fixation. American Journal of				
25	Botany 55: 875-883.				
26	Laetsch WM, Price I. 1969. Development of the dimorphic chloroplasts of sugar cane.				
27	American Journal of Botany 56: 77-87.				

1	Lara MV, Casati P, Andreo CS. 2002. CO <sub>2</sub> -concentrating mechanisms in <i>Egeria densa</i> , a				
2	submersed aquatic plant. Physiologia Plantarum 115: 487-495.				
3	Maberly SC. 1996. Diel, episodic and seasonal changes in pH and concentrations of				
4	inorganic carbon in a productive lake. Freshwater Biology 35: 579-598.				
5	Maberly SC, Gontero B. 2017. Ecological imperatives for aquatic CO <sub>2</sub> -concentrating				
6	mechanisms. Journal of Experimental Botany 68: 3797-3814.				
7	Maberly SC, Gontero B. 2018. Trade-offs and synergies in the structural and functional				
8	characteristics of leaves photosynthesizing in aquatic environments. In: Adams III				
9	WW, Terashima I, eds. The leaf: a platform for performing photosynthesis. Cham:				
10	Springer International Publishing, 307-343.				
11	Maberly SC, Madsen TV. 2002. Freshwater angiosperm carbon concentrating mechanisms:				
12	processes and patterns. Functional Plant Biology 29: 393-405.				
13	Madsen TV. 1987. Interactions between internal and external CO <sub>2</sub> pools in the				
14	photosynthesis of the aquatic CAM plants Littorella uniflora (L.) Aschers and Isoetes				
15	lacustris L. New Phytologist 106: 35-50.				
16	Madsen TV, Olesen B, Bagger J. 2002. Carbon acquisition and carbon dynamics by aquatic				
17	isoetids. Aquatic Botany 73: 351-371.				
18	Nelson EA, Sage RF. 2008. Functional constraints of CAM leaf anatomy: tight cell packing				
19	is associated with increased CAM function across a gradient of CAM expression.				
20	Journal of Experimental Botany <b>59</b> : 1841-1850.				
21	Oguchi R, Onoda Y, Terashima I, Tholen D. 2018. Leaf anatomy and function. In: Adams				
22	III WW, Terashima I, eds. The leaf: a platform for performing photosynthesis. Cham:				
23	Springer International Publishing, 97-139.				
24	Pedersen O, Rich SM, Pulido C, Cawthray GR, Colmer TD. 2011. Crassulacean acid				
25	metabolism enhances underwater photosynthesis and diminishes photorespiration in				
26	the aquatic plant Isoetes australis. New Phytologist 190: 332-339.				

1	Pendland J. 1979. Ultrastructural characteristics of Hydrilla leaf tissue. Tissue & Cell 11: 79-				
2	88.				
3	Poorter H, Niinemets Ü, Poorter L, Wright IJ, Villar R. 2009. Causes and consequences				
4	of variation in leaf mass per area (LMA): a meta-analysis. New phytologist 182: 565-				
5	588.				
6	Prins HBA, Elzenga JTM. 1989. Bicarbonate utilization: function and mechanism. Aquatic				
7	<i>Botany</i> <b>34</b> : 59-83.				
8	Pritchard SG, Rogers HH, Prior SA, Peterson CM. 1999. Elevated CO <sub>2</sub> and plant structure:				
9	a review. Global Change Biology 5: 807-837.				
10	P'yankov VI, Voznesenskaya EV, Kondratschuk AV, Black Jr CC. 1997. A comparative				
11	anatomical and biochemical analysis in Salsola (Chenopodiaceae) species with and				
12	without a Kranz type leaf anatomy: a possible reversion of $C_4$ to $C_3$ photosynthesis.				
13	American Journal of Botany 84: 597-606.				
14	Radoglou KM, Jarvis PG. 1990. Effects of CO <sub>2</sub> enrichment on four poplar clones. I. Growth				
15	and leaf anatomy. Annals of Botany 65: 617-626.				
16	Raghavendra AS, Sage RF. 2011. Introduction. In: Raghavendra AS, Sage RF, eds. C <sub>4</sub>				
17	photosynthesis and related CO <sub>2</sub> concentrating mechanisms. Dordrecht: Springer, 17-				
18	25.				
19	Rascio N. 2002. The underwater life of secondarily aquatic plants: some problems and				
20	solutions. Critical Reviews in Plant Sciences 21: 401-427.				
21	Rogers HH, Thomas JF, Bingham GE. 1983. Response of agronomic and forest species to				
22	elevated atmospheric carbon dioxide. Science 220: 428-429.				
23	Sage RF. 2016. A portrait of the C4 photosynthetic family on the 50th anniversary of its				
24	discovery: species number, evolutionary lineages, and hall of fame. Journal of				
25	Experimental Botany 67: 4039-4056.				
26	Sage RF, Monson RK. 1999. C4 plant biology. San Diego: Academic Press.				

1	Sand-Jensen K, Andersen MR, Martinsen KT, Borum J, Kristensen E, Kragh T. 2019.			
2	Shallow plant-dominated lakes-extreme environmental variability, carbon cycling and			
3	ecological species challenges. Annals of Botany 124: 355-366.			
4	Sand-Jensen K, Prahl C. 1982. Oxygen exchange with the lacunae and across leaves and			
5	roots of the submerged vascular macrophyte, Lobelia dortmanna L. New Phytologist			
6	<b>91</b> : 103-120.			
7	Sculthorpe CD. 1967. The biology of aquatic vascular plants. London: Edward Arnold.			
8	Shao H, Gontero B, Maberly SC, et al. 2017. Responses of Ottelia alismoides, an aquatic			
9	plant with three CCMs, to variable CO2 and light. Journal of Experimental Botany 68			
10	3985-3995.			
11	Sharpe RM, Offermann S. 2014. One decade after the discovery of single-cell C <sub>4</sub> species in			
12	terrestrial plants: what did we learn about the minimal requirements of C <sub>4</sub>			
13	photosynthesis? Photosynthesis Research 119: 169-180.			
14	Silveira MJ, Harthman VC, Michelan TS, Souza LA. 2016. Anatomical development of			
15	roots of native and non-native submerged aquatic macrophytes in different sediment			
16	types. Aquatic Botany 133: 24-27.			
17	Silvera K, Neubig KM, Whitten WM, Williams NH, Winter K, Cushman JC. 2010.			
18	Evolution along the crassulacean acid metabolism continuum. Functional Plant			
19	<i>Biology</i> <b>37</b> : 995-1010.			
20	Slaton MR, Smith WK. 2002. Mesophyll architecture and cell exposure to intercellular air			
21	space in alpine, desert, and forest species. International Journal of Plant Sciences 163			
22	937-948.			
23	Smith RA, Lewis JD, Ghannoum O, Tissue DT. 2012. Leaf structural responses to pre-			
24	industrial, current and elevated atmospheric [CO2] and temperature affect leaf			
25	function in Eucalyptus sideroxylon. Functional Plant Biology 39: 285-296.			

1	Søndergaard M, Wetzel RG. 1980. Photorespiration and internal recycling of CO <sub>2</sub> in the				
2	submersed angiosperm Scirpus subterminalis. Canadian Journal of Botany 58: 591-				
3	598.				
4	Steemann Nielsen E. 1947. Photosynthesis of aquatic plants with special reference to the				
5	carbon-sources. Dansk Botanisk Arkiv 12: 1-71.				
6	Uprety DC, Dwivedi N, Mohan R, Paswan G. 2001. Effect of elevated CO <sub>2</sub> concentration				
7	on leaf structure of Brassica Juncea under water stress. Biologia Plantarum 44: 149-				
8	152.				
9	von Caemmerer S, Edwards GE, Koteyeva N, Cousins AB. 2014. Single cell C <sub>4</sub>				
10	photosynthesis in aquatic and terrestrial plants: a gas exchange perspective. Aquatic				
11	<i>Botany</i> <b>118</b> : 71-80.				
12	Voznesenskaya EV, Edwards GE, Kiirats O, Artyusheva EG, Franceschi VR. 2003.				
13	Development of biochemical specialization and organelle partitioning in the single-				
14	cell C4 system in leaves of Borszczowia aralocaspica (Chenopodiaceae). American				
15	Journal of Botany <b>90</b> : 1669-1680.				
16	Voznesenskaya EV, Franceschi VR, Kiirats O, Artyusheva EG, Freitag H, Edwards GE.				
17	2002. Proof of C <sub>4</sub> photosynthesis without Kranz anatomy in Bienertia cycloptera				
18	(Chenopodiaceae). The Plant Journal 31: 649-662.				
19	Voznesenskaya EV, Franceschi VR, Kiirats O, Freitag H, Edwards GE. 2001. Kranz				
20	anatomy is not essential for terrestrial C <sub>4</sub> plant photosynthesis. <i>Nature</i> <b>414</b> : 543-546.				
21	Wetzel RG, Brammer ES, Forsberg C. 1984. Photosynthesis of submersed macrophytes in				
22	acidified lakes. I. Carbon fluxes and recycling of CO2 in Juncus bulbosus L. Aquatic				
23	Botany 19: 329-342.				
24	Woo KC, Anderson JM, Boardman NK, Downton WJS, Osmond CB, Thorne SW. 1970.				
25	Deficient photosystem II in agranal bundle sheath chloroplasts of C <sub>4</sub> plants.				
26	Proceedings of the National Academy of Sciences 67: 18-25.				

1	Yin L, Li W, Madsen TV, Maberly SC, Bowes G. 2017. Photosynthetic inorganic carbon
2	acquisition in 30 freshwater macrophytes. Aquatic Botany 140: 48-54.
3	Zeiger E. 1983. The biology of stomatal guard cells. Annual Review of Plant Physiology 34:
4	441-475.
5	Zhang Y, Yin L, Jiang H-S, Li W, Gontero B, Maberly SC. 2014. Biochemical and
6	biophysical CO <sub>2</sub> concentrating mechanisms in two species of freshwater macrophyte
7	within the genus Ottelia (Hydrocharitaceae). Photosynthesis Research 121: 285-297.
8	Ziegler H, Hertel H. 2007. Carbon isotope fractionation in species of the torrenticolous
9	families Podostemaceae and Hydrostachyaceae. Flora 202: 647-652.
10	
11	

# 1 Legends

FIG. 1. Influence of CO<sub>2</sub> concentration on activities of enzymes from O. alismoides 2 3 leaves collected at dusk (1800) and dawn (0500). (A) PEPC activity. (B) Rubisco activity. (C) Ratio of PEPC to Rubisco activity. The mean values (n = 3-4), with their 4 SD are shown. The statistical differences between means were tested using 5 6 independent sample t-tests in panel A and B and independent sample t-tests and the 7 Mann-Whitney U test in panel C. The statistic above the horizontal line compares 8 leaves exposed to high CO<sub>2</sub> (black bars) and low CO<sub>2</sub> (white bars) collected at the same time (NS not significant, \*P < 0.05, \*\*\*P < 0.001); data with different small and 9 large letters are significantly different between dusk and dawn, under high and low 10 11 CO<sub>2</sub> treatments respectively (P < 0.05).

12

FIG. 2. Influence of high CO<sub>2</sub> (HC) and low CO<sub>2</sub> (LC) on acidity of *O. alismoides* leaves from dusk (1800) and dawn (0500). The mean values (n = 3-4), with their SD are shown. The statistical differences between means were tested using independent sample t-tests and the Mann-Whitney U test. The statistic above the horizontal line compares acidity in dusk and dawn (*NS* not significant, <sup>\*\*</sup>P < 0.01) within the same CO<sub>2</sub> treatment; changes in acidity with different letters are significantly different between HC and LC treatments (P < 0.05).

20

FIG. 3. Transverse sections of *O. alismoides* leaves under high CO<sub>2</sub> concentration (AC) and low CO<sub>2</sub> concentration (D-F) at dusk (1800). a, air space; le, lower epidermis;
m, mesophyll cell; ue, upper epidermis; v, vascular bundle. The arrowhead indicates
the chloroplasts. Scale bar = 100 μm.

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FIG. 4. Effects of CO<sub>2</sub> concentration on anatomical characteristics of *O. alismoides* leaves. (A) The thickness of upper epidermis, lower epidermis, mesophyll and air space in transverse section. (B) The area of upper epidermal cell, lower epidermal cell, mesophyll cell and air space in transverse section. The mean values ( $n \ge 30$ ), with their SD are shown. The statistical differences between means were tested using independent sample t-tests and the Mann-Whitney U test. The statistic above the 1 horizontal line compares leaves under high CO<sub>2</sub> (black bars) and low CO<sub>2</sub> (white bars)

2 concentration (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001).

3

FIG. 5. Ultrastructure of chloroplasts in *O. alismoides* leaves under different CO<sub>2</sub>
concentrations and times of day. The upper row cells are from high and the lower row
cells from low CO<sub>2</sub> concentration. A, B, F and G are epidermal cells at dusk (1800). C
and H are epidermal cells at dawn (0500). D and I are mesophyll cells from dusk
(1800) and E and J are mesophyll cells from dawn (0500). cw, cell wall; g, grana; m,
mitochondria; p, plastoglobuli; s, starch grain. Scale bar = 1 μm.

10

11 FIG. 6. Effects of CO<sub>2</sub> concentration on characteristics of chloroplasts located in 12 epidermal and mesophyll cells of O. alismoides leaves. (A & B) Chloroplast major 13 and minor axis length at dusk (1800). (C) Area of chloroplast at dusk (1800). (D) 14 Number of mitochondria within 1 µm of a chloroplast at dusk (1800). (E & F) Area 15 ratio of starch to chloroplast in O. alismoides leaves collected at dusk (1800) and dawn (0500). The mean values ( $n \ge 20$ ), with their SD are shown. The statistical 16 17 differences between means were tested using independent sample t-tests in panels A 18 and C and independent sample t-tests and the Mann-Whitney U test in panels B, D, E 19 and F. The statistic above the horizontal line compares high CO<sub>2</sub> (black bars) with low CO<sub>2</sub> (white bars) treatments (NS not significant, \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.0120 21 0.001); data with different small and large letters are significantly different between 22 chloroplasts in the two cell types, under high and low CO<sub>2</sub> treatments respectively (P 23 < 0.05).

24

FIG. 7. The number of chloroplasts per unit length of cell wall under different CO<sub>2</sub> concentrations at dusk (1800). UEW, Upper epidermis, wall next to water; UEA, Upper epidermis, wall next to air space; UM, Upper mesophyll cell; LM, Lower mesophyll cell; LEA, Lower epidermis, wall next to air space; LEW, Lower epidermis, wall next to water. Error bars represent SD ( $n \ge 20$ ). The statistic above the horizontal line compares high CO<sub>2</sub> (black bars) and low CO<sub>2</sub> (white bars) treatments based on independent sample t-tests and the Mann-Whitney U-test (*NS* not significant,

- 1 <sup>\*\*</sup>P < 0.01); data with different small and large letters are significantly different 2 among different locations based on the Kruskal-Wallis test (P < 0.05) under high and
- 3 low  $CO_2$  treatments.

# 4 SUPPLEMENTARY INFORMATION

Supplementary Data Fig. S1. Fluctuations of CO<sub>2</sub> concentration in high CO<sub>2</sub>
and low CO<sub>2</sub> treatments during the 40-days acclimation.

Supplementary Data Fig. S2. Photographs of the surface of a mature O. *alismoides* leaf from the low CO<sub>2</sub> treatment using a laser scanning confocal
microscope.

Supplementary Data Fig. S3. Conceptual overview summarizing the structure
 of *O. alismoides* leaves acclimated to high CO<sub>2</sub> and low CO<sub>2</sub> concentrations.

Supplementary Data Table S1. The chemical composition of the tap waterused in the growth experiments.

14

- 1 Table 1. Influence of CO<sub>2</sub> concentration on characteristics of *O. alismoides* leaves.
- 2 The mean values are given with SD in parenthesis. Significant differences between
- 3 leaves treated with different  $CO_2$  concentration are shown based on independent
- 4 sample t-test (NS not significant, \*P < 0.05, \*\*\*P < 0.001).

Characteristics	High CO <sub>2</sub>	Low CO <sub>2</sub>	Significance	n
Leaf length (cm)	12.08 (0.36)	11.58 (0.68)	NS	5
Leaf width (cm)	8.56 (0.50)	5.44 (0.18)	***	5
Length-width ratio	1.41 (0.06)	2.13 (0.12)	***	5
Leaf thickness (µm)	196 (17)	161 (18)	*	4
The ratio of air space to leaf area in transverse section	0.22 (0.03)	0.23 (0.02)	NS	4

6

1 Table 2. Characteristics of dimorphic chloroplasts in aquatic and terrestrial plants.

2

Environment /type/ species	Location	Area (µm²)	Thylakoids per granum	Starch	References
Aquatic C <sub>3</sub>					
Hydrobryum khaoyaiense	E F	$3.7 \pm 1.8$ 23.7 + 11	$3 \sim 4$ $4 \sim 5$	+ + + +	Fujinami et al. (2011)
Cabomba caroliniana	E	$\sim 3.8^{a}$	~9	+	Galati et al. (2015)
Aquatic NAD-MF C	M	~33.2*	~29	++	
Ottelia alismoides <sup>b</sup>	E	$9.0 \pm 2.3$	++	++	This study
Terrestrial NADP-ME C4	IVI	$13.8 \pm 3.3$	Ŧ	+ + +	
Zea mays	М	Small	+++	++	Hodge et al. (1955); Laetsch (1968)
	BS	Large	+	+ + +	()
Saccharum officinarum	М	Small	+ + +	+	Laetsch and Price (1969)
	BS	Large	_	+ + +	
Sorghum bicolor	М	nd	+ + +	nd	Woo et al. (1970)
	BS	nd	_	nd	
Salsola australis	M	6.67~8.2 <sup>a</sup>	6~12	+	P'yankov et al. (1997)
	BS	14.6~16.1ª	2~5	+++	
Amananthus adulis	п	See all		1	$L_{actach}$ (1069)
Amaraninus eaulis	r BS	Jarge	++	+++	Laeisen (1908)
Atriplex lentiformis	P	nd	2~3	+	Laetsch (1968)
in prese terriger mus	BS	nd	++	+ + +	
Atriplex spongiosa	M	nd	+	nd	Woo et al. (1970)
1 1 0	BS	nd	+ + +	nd	
Terrestrial Single-cell NAD-ME C4					
Bienertia cycloptera	$C^{c}$	nd	$\sim 2$	_	Voznesenskaya et al.
- <u>-</u>	$\mathrm{C}^{\mathrm{d}}$	nd	3~5	++	(2002)
Suaeda aralocaspica	$C^{c}$	3.48~4.0 <sup>a</sup>	+	+	Voznesenskaya et al. (2003)
	$\mathbf{C}^{d}$	$7.18 \sim 8.0^{a}$	+ + +	+ + +	× /

3 <sup>a</sup>Calculated according to axis length in reference

4 <sup>b</sup> Plants treated with low CO<sub>2</sub> concentrations

5 <sup>c</sup> Chloroplasts have structural characteristics like those in mesophyll

<sup>d</sup> Chloroplasts have structural characteristics like those in bundle sheath cell

7 – Lack grana/starch; + Rudimentary grana/starch; + + Contain grana/starch; + + Well-

8 developed grana/starch; BS = Bundle sheath cell; C = Chlorenchyma cell; E = Epidermal cell;

9 M = Mesophyll cell; P = Palisade cell; nd = not determined.

11

12

<sup>10</sup> 







Fig. 4









Fig. S1 1200 HC treatment
 LC treatment 1000 CO<sub>2</sub> concentration (µmol L<sup>-1</sup>) 0 800 600 15 10 5 **9 9** 2018/9/15 2018/9/7 2018/8/14 2018/8/22 2018/8/30 Date









1 Table S1

Component	Mean (SD)
Alkalinity	2200 (10)
ТР	1.61 (0.32)
TN	100 (5)
$Na^+$	635 (15)
$\mathbf{K}^+$	130 (19)
$Ca^{2+}$	2255 (21)
$Mg^{2+}$	789 (6)
Cl-	590 (2)
SO4 <sup>2-</sup>	670 (3)
NO <sub>3</sub> -	86 (2)