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Crassulacean acid metabolism enhances underwater photosynthesis and diminishes photorespiration in the aquatic plant *Isoetes australis*

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Summary

• Underwater photosynthesis by aquatic plants is often limited by low availability of CO_2, and photorespiration can be high. Some aquatic plants utilize crassulacean acid metabolism (CAM) photosynthesis. The benefits of CAM for increased underwater photosynthesis and suppression of photorespiration were evaluated for *Isoetes australis*, a submerged plant that inhabits shallow temporary rock pools.

• Leaves high or low in malate were evaluated for underwater net photosynthesis and apparent photorespiration at a range of CO_2 and O_2 concentrations.

• CAM activity was indicated by 9.7-fold higher leaf malate at dawn, compared with at dusk, and also by changes in the titratable acidity (μmol H^+ equivalents) of leaves. Leaves high in malate showed not only higher underwater net photosynthesis at low external CO_2 concentrations but also lower apparent photorespiration. Suppression by CAM of apparent photorespiration was evident at a range of O_2 concentrations, including values below air equilibrium. At a high O_2 concentration of 2.2-fold the atmospheric equilibrium concentration, net photosynthesis was reduced substantially and, although it remained positive in leaves containing high malate concentrations, it became negative in those low in malate.

• CAM in aquatic plants enables higher rates of underwater net photosynthesis over large O_2 and CO_2 concentration ranges in floodwaters, via increased CO_2 fixation and suppression of photorespiration.

Introduction

Submerged aquatic plants display a suite of adaptations which facilitate inorganic carbon uptake, as carbon acquisition for underwater photosynthesis is greatly impeded by slow diffusion of gases in water compared with in air. Morphological adaptations include filamentous leaves to reduce the thickness of diffusive boundary layers and thin or absent cuticles to lower resistance to CO_2 uptake from the water (Maberly & Madsen, 2002). In addition, some plants can utilize alternative CO_2 sources; for example, floating leaves directly access atmospheric CO_2, and large permeable root systems access sediment CO_2 (Maberly & Madsen, 2002). Physiological adaptations to deal with low CO_2 availabilities in floodwaters include those that increase CO_2 at Rubisco, commonly referred to as carbon-concentrating mechanisms (CCMs) (Maberly & Madsen, 2002; Raven *et al.*, 2008); these are bicarbonate use (Prins & Elzenga, 1989), C_4 photosynthesis (Magnin *et al.*, 1997), C_3–C_4 intermediates (Keeley, 1999) and crassulacean acid metabolism (CAM) photosynthesis (Keeley, 1981; Madsen, 1985; Raven *et al.*, 1988).

In addition to enhancing photosynthesis under water, CCMs might also reduce photorespiration in submerged aquatic plants (Maberly & Madsen, 2002). Photorespiration results from the oxygenase activity of Rubisco and is promoted by a low CO_2 : O_2 ratio (Ogren, 1984; Sage, 2004). The low CO_2 availability in aquatic environments in itself lowers the CO_2 : O_2 ratio; moreover, as O_2 is 28.5-fold less soluble in water than is CO_2 (Baranenko *et al.*, 1990), O_2 tends to build up inside gas spaces in photosynthetic tissues during the daytime as escape to the

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surrounding solution can be slow (Bowes, 1993). Thus, with CCMs such as CAM, a more favourable CO_2 : O_2 ratio presumably can be maintained at Rubisco and thus diminish photorespiration. Inferences regarding reduced photorespiration in aquatic plants with CCMs are based on lower CO_2 compensation points than those found in species lacking CCMs (Salvucci & Bowes, 1983; Madsen, 1987); direct assessments of photorespiration in aquatic plants possessing CAM are lacking.

CAM in aquatic plants (Keeley, 1998a), as in terrestrial plants (Lüttge, 2002), involves night-time fixation of CO_2 into malate, followed by daytime decarboxylation of the malate so that CO_2 is supplied to the C_3 pathway without the need for concurrent inward diffusion of external CO_2. CAM in aquatic plants was first reported for *Isoetes howellii* (Keeley, 1981) and has subsequently been shown to occur in most species of *Isoetes*, and in some species within the genera *Crassula*, *Eleocharis*, *Littorella*, *Sagittaria* and *Scirpus* (Keeley, 1998a). CAM activity is indicated by large diurnal fluctuations in malate concentration, frequently measured as changes in the titratable acidity of tissue extracts (Keeley, 1998a). In *Isoetes* species, night-time fixation of CO_2 may contribute up to 50% of the inorganic carbon fixed, but huge variation is present both within and between species (Keeley, 1983a, 1985). When water recedes, most species of *Isoetes* form 'aerial leaves' that lack CAM and form stomata (Keeley *et al.*, 1983a,b), emphasizing the role of CAM as a CCM in submerged aquatic plants.

CAM photosynthesis is particularly frequent among isoetids (Raven *et al.*, 1988; Keeley, 1998a). Isoetids are aquatic plants with short, stiff leaves arranged in a rosette (Hutchinson, 1975), often with a thick cuticle; they lack stomata when submerged, and have large lacunae providing a low resistance pathway for gas diffusion. The roots are normally unbranched and make up 50% or more of the total plant mass (Hutchinson, 1975). Isoetids generally inhabit oligotrophic softwater lakes (Smolders *et al.*, 2002), which are low in inorganic carbon, so an ability to use the sediment CO_2 pool is particularly advantageous (Maberly & Madsen, 2002). Isoetids may also occupy high-altitude wetlands (primarily the genus *Isoetes*; Keeley, 1998a) or seasonal wetlands such as dunes (*Littorella uniflora*; Pedersen *et al.*, 2006) and temporary pools (rain-fed rock pools; *Isoetes* spp.; Keeley, 1998a). In these habitats with high plant biomass and a relatively low volume of water, together resulting in large diurnal fluctuations in dissolved CO_2, CAM is the most common CCM (Keeley & Rundel, 2003; Raven *et al.*, 2008).

The present study tested two hypotheses, using the CAM isoetid species *Isoetes australis* (Keeley, 1983b). Firstly, the hypothesis was tested that leaves high in malate show enhanced underwater net photosynthesis, particularly at low external CO_2 concentrations, compared with leaves low in malate. Secondly, the long-standing hypothesis that CAM reduces photorespiration, based on low CO_2 compensation points (Salvucci & Bowes, 1983; Maberly & Madsen, 2002), was tested in experiments where underwater net photosynthesis and apparent photorespiration were measured while external CO_2 and O_2 concentrations were manipulated. The data show that tissues high in malate have enhanced underwater net photosynthesis at low external CO_2 concentrations and that apparent photorespiration is also diminished by CAM.

Materials and Methods

Plant material

Intact turfs (20 cm wide and 2 cm deep; this depth sampled almost all of the shallow sediment in the pools) of *Isoetes australis* Williams were collected in October 2009 from temporary shallow granite rock pools (Fig. 1; 118.2896°E, 30.7468°S) *c.* 400 km east of Perth, Western Australia. The

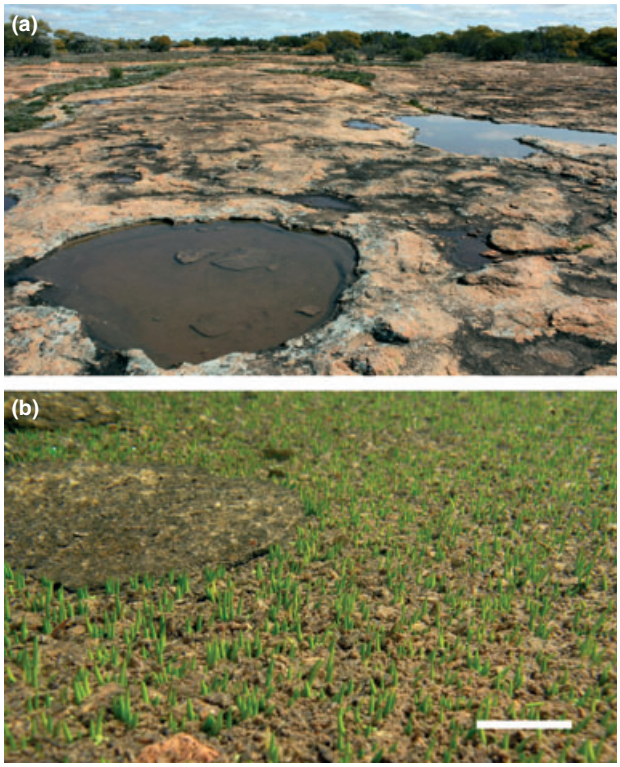


Fig. 1 Temporary granite rock pools in south-western Australia (a) and typical underwater vegetation (b). The temporary rock pools are rainwater-fed each winter and persist for 3–5 months. The water is shallow (5–10 cm) and low in conductivity (< 10–80 μS cm^{-1}); the sediment consists of weathered granite, is low in organic matter (2.3 ± 0.6% DW; n = 5) and often has a depth of < 2 cm. Nevertheless, dense populations of *Isoetes australis* often develop (b) and can coexist with other plants (species of *Glossostigma*, *Crassula*, *Marselia* and *Eleocharis*) typical of temporary granite rock pools (Tuckett *et al.*, 2010). Bar, 5 cm.

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turfs were transported air-exposed under plastic, in 4.5-l white plastic vessels, to a phytotron (15°C night, 20°C day) in Perth.

Most experiments used plants maintained in submerged cultures, 3–5 cm below the surface of 'artificial floodwater' (defined below) that was topped up with deionized water following evaporation. In one experiment, previously fully submerged plants were de-submerged and maintained in waterlogged sediment, with shoots in air, for 6 wk. Leaves formed in air were compared with those of plants in a continuously submerged treatment. The artificial floodwater was a solution with a final electrical conductivity of 85 μS cm^{-1} and consisting of deionized water containing (in mol m^{-3}): 0.15 Ca^{2+}, 0.10 Mg^{2+}, 0.10 K^+, 0.30 Cl^-, 0.10 SO_4^{2-} and 0.10 HCO_3^-. This low-EC solution was designed to simulate the water in the temporary rock pools from which the specimens were collected. In the phytotron, *I. australis* continued to grow with a leaf turnover rate of *c.* 1 leaf wk^{-1}. Seedlings of the genera *Glossostigma* and *Crassula* were removed weekly in order to maintain a monoculture of *I. australis*. Leaf tissues were used for up to 6 wk following turf collection.

Underwater net photosynthesis – CO_2 response

Underwater net photosynthesis by leaves was measured using the method described in Colmer & Pedersen (2008). Leaf tips (5–10 mm), without the achlorophyllous white base (hereafter referred to as 'leaves'), were excised and placed in glass vials (10 ml) with stoppers. Each vial contained two leaves in incubation medium, and two glass beads for mixing as the vials rotated on a wheel within an illuminated water bath (20°C). Photosynthetically active radiation (PAR) inside the submerged glass vials was 350 μmol m^{-2} s^{-1} (measured using a 4π US-SQS/L; Walz, Effeltrich, Germany).

The incubation medium had the same composition as the artificial floodwater (described in the previous section) but various amounts of $KHCO_3$ (described further on in this paragraph) and the solution also contained 2-(N-morpholino) ethanesulfonic acid (MES) at 2.5 mol m^{-3}, with pH adjusted to 6.00 using KOH. The dissolved O_2 concentration in the incubation medium was set at 50% of air equilibrium, by bubbling in 1 : 1 v/v of N_2 and air (before adjustment of dissolved CO_2); this avoided build-up of high O_2 during the measurements. As vials were incubated in the light immediately after adding the leaves, and as these produce O_2 when in the light, there was no risk of tissue hypoxia. Dissolved CO_2 treatments were imposed by adding specific concentrations of $KHCO_3$ to the incubation medium with pH always adjusted to pH 6.00 using various amounts of KOH depending on $KHCO_3$ added, to provide a range of CO_2 concentrations from 30 to 2000 mmol m^{-3} (Stumm & Morgan, 1996). K_2SO_4 was added as required in the various treatments so that K^+ concentration was equal across treatments. For each CO_2 concentration, vials without leaves served as blanks.

Following incubations of known durations (60–75 min), dissolved O_2 concentrations in the vials were measured using a Clark-type O_2 microelectrode (Revsbech, 1989; OX-25; Unisense A/S, Aarhus, Denmark), connected to a pico ampere meter (PA2000; Unisense A/S). The electrode was calibrated immediately before use.

Projected areas of leaf samples were measured using a leaf area meter (Li-3000; Li-Cor, Lincoln, NE, USA). Projected areas were converted into actual surface areas using an empirical relationship obtained from measurements of areas based on leaf diameters and lengths of the cylindrical leaves and conical tips. The ratio of actual area : projected was 3.633 (n = 10). Fresh and dry masses (after freeze-drying) of leaf samples were also determined. The ratio of actual leaf area : dry mass was 17.54 g m^{-2} (n = 4).

Underwater net photosynthesis – O_2 response

Underwater net photosynthesis by the leaves was measured using a system identical to the one already described for the CO_2 response; however, initial O_2 concentration was manipulated rather than CO_2. The initial O_2 concentration was adjusted by bubbling containers with N_2 or O_2 and then mixing these as required to obtain the desired O_2 concentration. After mixing, $KHCO_3$ was injected to obtain 30 mmol CO_2 m^{-3} at pH 6.00 in the incubation medium in all vials. Increasing apparent photorespiration relative to photosynthesis was evident as a decline in net O_2 production rate, below the maximal rate when O_2 was optimal. In some samples (i.e. tissues low in malate), net O_2 consumption occurred (i.e. O_2 production from photosynthesis became less than O_2 consumption), as photorespiration exceeded photosynthesis. 'Dark respiration' was also measured for comparative purposes (see below). The rates of net photosynthesis are presented in Fig. 5 for the mean O_2 concentration in the vial during the incubation, as measured with an O_2 microelectrode.

Underwater dark respiration (R_d)

Respiration by leaves when under water in darkness was measured, using a system identical to that already described for the CO_2 response, but with the lights off. The initial O_2 concentration of the medium was adjusted by bubbling containers with N_2 or O_2 and then mixing these as required to obtain the desired O_2 concentrations of half air equilibrium, air equilibrium and double air equilibrium. After mixing, $KHCO_3$ was injected to obtain 30 mmol CO_2 m^{-3} at pH 6.00. Four leaf tips, rather than two as used in the underwater net photosynthesis experiments, were used in order to keep incubation times at *c.* 2 h. Net O_2 uptake in the vials

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was measured in darkness and the projected area of the tissue and the dry mass were recorded.

Tissue organic acids

Leaves sampled at dawn or dusk were frozen in liquid N_2, freeze-dried, ground in a ball mill, and then extracted in ice-cold 5% (v/v) perchloric acid (Fan *et al.*, 1993). The extract was mixed using a vortex, centrifuged at 12096 ***g*** for 30 min, and the supernatant was collected. The pellet was extracted a second time in ice-cold 5% (v/v) perchloric acid. The combined supernatant from the two extractions was pH-adjusted to 3.0–3.5 using K_2CO_3 to precipitate the perchlorate. The sample was again centrifuged and the supernatant collected, and the volume was measured. The extract was filtered (0.22 μm) before high-performance liquid chromatography (HPLC) analyses.

Organic acids in tissue extracts were analysed by HPLC (600E pump, 717plus autoinjector, 996 Photodiode array detector; Waters, Milford, MA, USA) by slightly adapting the method described by Cawthray (2003). In brief, separation was achieved at 22 ± 0.5°C on a Prevail C-18 column (250 × 4.6 mm internal diameter with 5-μm packing; Alltech Associates, Deerfield, IL, USA) using a mobile phase consisting of 25 mM KH_2PO_4 at pH 2.50 and 1 ml min^{-1}. A gradient elution programme with 60% methanol was used every fifth sample to flush the column of the more hydrophobic compounds and negate carryover. Detection and quantification were at 210 nm, with Photo Diode Array (PDA) acquisition from 195 to 400 nm to enable positive identification of organic acids by comparison of the retention time and PDA peak spectral analyses, including peak purity, of standards with those of the unknowns. Calibration curves for each organic acid were generated from peak area vs the mass of standard organic acid injected. Data acquisition and processing were with EMPOWER® chromatography software (Waters). Retention times and PDA spectral data for organic acid standards, including malic, *iso*-citric, malonic, shikimic, lactic, acetic, maleic, citric, succinic, fumaric, *cis*-aconitic, and *trans*-aconitic acids, were used to identify organic acids in tissue extracts. Samples were at pH < 4.0 before HPLC and typical sample injection volumes ranged from 20 to 100 μl. The method was verified by spiking malate and citrate into some additional *I. australis* leaf samples immediately before extraction; the recovery for malate was 101 ± 6 and for citrate 98 ± 6 (mean ± SE; n = 4). Data presented were not adjusted.

Titratable tissue acidity

Diurnal changes in titratable tissue acidity (i.e. titrations of tissue homogenates) have been used as a proxy for diurnal changes in malate caused by night-time fixation of CO_2 to produce malate and daytime decarboxylation of malate (Lüttge, 2004). Thus, in addition to tissue organic acids, we also measured titratable tissue acidity to facilitate comparison with previous studies of CAM activity in species of *Isoetes*.

Leaves sampled at dawn or at dusk were immediately frozen (−18°C) and analysed within 1 h of sampling. Frozen tissue was homogenized using a mortar and pestle on ice, and following addition of CO_2-free deionized water (0.1 g tissue FW in 5 ml of water) the mixture was transferred to a glass vial and titrated to pH 6.40 with 0.01 N NaOH following the procedure of Keeley & Sandquist (1991).

Data analyses

GRAPHPAD PRISM 5.0 (GraphPad Software Inc., San Diego, CA, USA) was used for one- or two-way ANOVA (with Tukey or Bonferoni *post hoc* tests) and Student's *t*-tests to compare means. GRAPHPAD PRISM was also used to fit the data set to predictive models (Gaussian and Michaelis–Menten) in experiments with underwater net photosynthesis vs external CO_2 or O_2 concentrations.

Results

Leaf organic acids and titratable tissue acidity at dawn and dusk

A comparison of dawn and dusk malate or titratable tissue acidity is a diagnostic test for CAM activity (Lüttge, 2004), and we therefore measured these parameters in the chlorophyllous portion of leaves (i.e. the green leaf tips) of *I. australis*. CAM activity was indicated by the 9.7-fold higher malate concentration at dawn compared with dusk (Fig. 2). The citrate concentration was also higher at dawn, although the diurnal amplitude was much lower (1.9-fold)

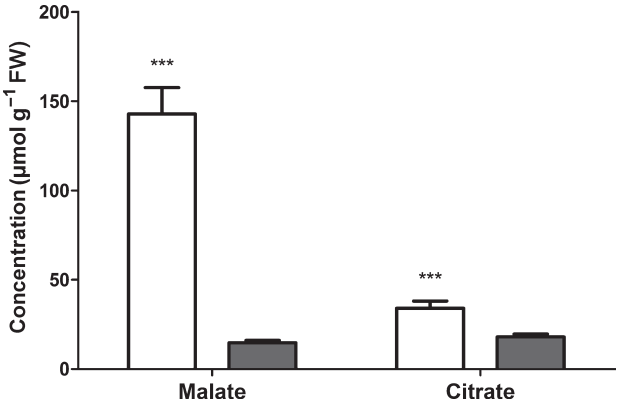


Fig. 2 Organic acids (malate and citrate) in leaves of submerged *Isoetes australis*. Tissue was sampled at dawn (open columns) following 16 h of preceding darkness or at dusk (closed columns) after 8 h in the light. Bars represent the mean ± SE (n = 4). ***Dawn and dusk values of both malate and citrate were significantly different (P < 0.001; Student's *t*-test).

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and the absolute concentration at dawn was only 24% that of malate (Fig. 2). The large change in the titratable acidity of leaf homogenates from 250 at dawn to 4 μmol H^+ equivalents g^{-1} FW at dusk (Fig. 3) supported the conclusion that there was substantial CAM activity. Furthermore, the ratio of Δ malate : Δ acidity was 0.46, close to the expected value of 0.50 when the titration starts at or below the first pKa of malate. Thus, malate was the major organic acid used to store CO_2 fixed during the night (Fig. 2).

Underwater net photosynthesis in response to dissolved CO_2 for leaves of contrasting malate status

The presence of CAM has been hypothesized to enable maintenance of net photosynthesis when external CO_2 is low in submerged plants (Madsen, 1987). We tested the potential benefits of CAM activity by comparing CO_2 response curves for leaves either high (dawn) or low (dusk) in malate. The CO_2-saturated rate of underwater net photosynthesis did not differ between high- and low-malate leaves, being 2.3 μmol O_2 m^{-2} s^{-1} in both cases (Fig. 4). The response curves of high- and low-malate leaves deviated significantly at 300 mmol CO_2 m^{-3} and below, underwater net photosynthesis being lower in the low-malate leaves. The high rates of net underwater photosynthesis in leaves with high malate were presumably related to the greater availability of CO_2 resulting from more decarboxylation of malate.

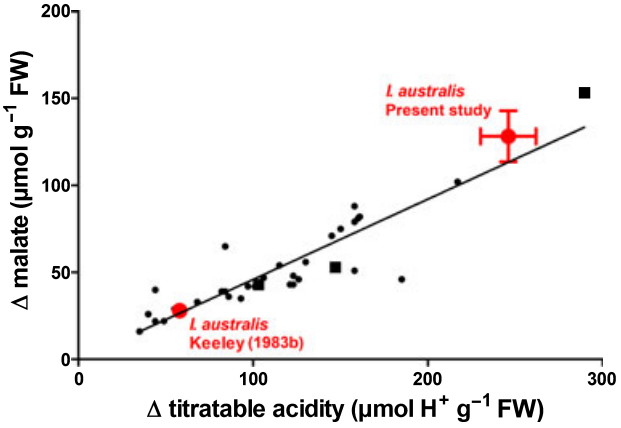


Fig. 3 Changes (Δ) in tissue titratable acidity vs Δ malate in leaves of species of the genus *Isoetes* (*Isoetes* spp., small circles; *Isoetes australis*, large circles; *Isoetes howellii*, squares). Δ titratable acidity and Δ malate were calculated as dawn (am) minus dusk (pm) values. The regression line is based on the entire data set and has a slope of 0.46. Values are from Keeley (1983b) and the present study (mean ± SE for both parameters); only values where titratable acidity was determined by titration to pH 6.4 are included. The figure is modified and redrawn from Keeley (1998a) with values from the present study added to emphasize the range in both tissue malate and titratable acidity within species. Thus, *I. australis* and *I. howellii* are highlighted using large symbols.

Assessment of apparent photorespiration; response of underwater net photosynthesis to O_2 in leaves of contrasting malate status

Photorespiration can be high in submerged plants, especially when CO_2 is depleted and O_2 increases well above air equilibrium, so that a low CO_2 : O_2 ratio occurs at Rubisco. As CAM can suppress photorespiration in terrestrial plants (Lüttge, 2002), we evaluated whether leaves of *I. australis* with high or low malate differ in photorespiration. Apparent photorespiration was assessed by investigating the effect of increasing O_2 on the rate of underwater net photosynthesis (Fig. 5).

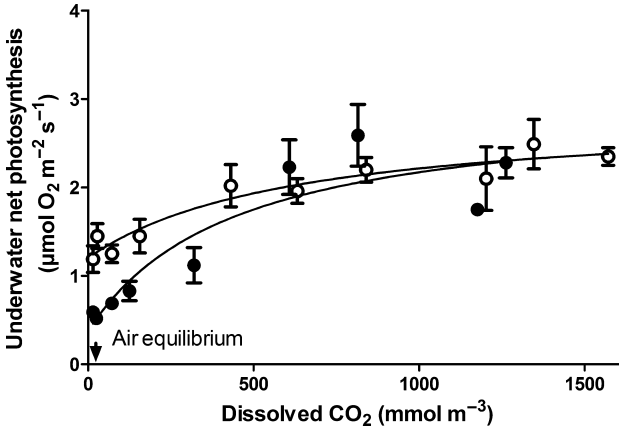


Fig. 4 Underwater net photosynthesis vs external CO_2 concentration in leaves taken from submerged *Isoetes australis* and incubated with 140 mmol O_2 m^{-3} at 20°C. Tissue was sampled at dawn (high malate, open circles) following 16 h of preceding darkness or at dusk (low malate, closed circles) after 8 h in the light. Each data point represents the mean ± SE (n = 5). Data are fitted to a Michaelis–Menten model with a y-axis constant.

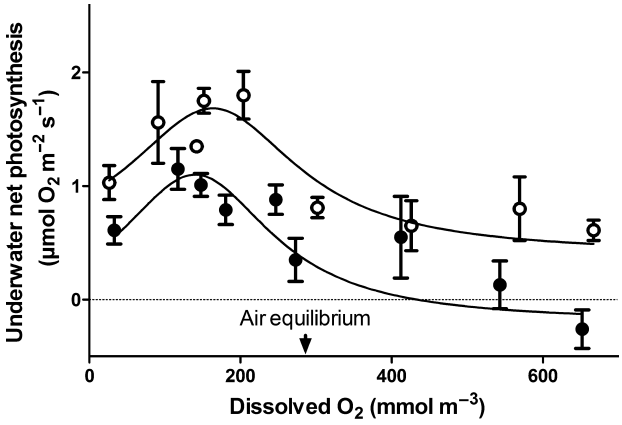


Fig. 5 Underwater net photosynthesis vs external O_2 concentration (mean value during incubation) in leaves taken from submerged *Isoetes australis*, and incubated with 30 mmol CO_2 m^{-3} at 20°C. Tissue was sampled at dawn (high malate, open circles) following 16 h of preceding darkness or at dusk (low malate, closed circles) after 8 h in the light. Each data point represents the mean ± SE (n = 5) and data are fitted to a Gaussian model. The air equilibrium of O_2 in water at 20°C is 284 mmol m^{-3}.

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Apparent photorespiration was substantially higher in leaves low in malate, as compared with those high in malate; when the dissolved O_2 concentration was 2.2-fold the air equilibrium concentration (i.e. 625 mmol m^{-3}), net photosynthesis was negative in low-malate tissues, whereas, although decreased, it remained positive in high-malate tissues (Fig. 5). Photorespiration was even substantial in low-malate leaves when dissolved O_2 was at air equilibrium; net photosynthesis at air equilibrium was only 31% of the maximal rate in these leaves – the rate of net photosynthesis was highest at 140 mmol O_2 m^{-3}, a value about half of the dissolved O_2 concentration at air equilibrium. In high-malate leaves, although net photosynthesis was maximal at a dissolved O_2 concentration of 160 mmol m^{-3}, when O_2 was at air equilibrium net photosynthesis was only reduced to 67% of the maximal rate. Moreover, maximal underwater net photosynthesis in this solution with 30 mmol CO_2 m^{-3} was 1.5-fold greater in high-malate than in low-malate leaves. The decrease in net O_2 production (i.e. apparent photorespiration) exceeded by 5.7-fold the R_d (R_d = 0.14 ± 0.02 μmol O_2 m^{-2} s^{-1} at 284 mmol O_2 m^{-3}; n = 4), so that the inferred photorespiration clearly exceeded R_d. In addition to declines in net photosynthesis at high O_2, it declined at the lowest O_2 concentration tested (25 mmol m^{-3}), presumably as a result of tissue hypoxia. In summary, as CO_2 : O_2 declined, leaves with high CAM (i.e. high-malate leaves) showed lower apparent photorespiration than those with low malate and hence low CAM decarboxylation.

Underwater net photosynthesis of leaves formed in air

When several *Isoetes* species emerge above water, leaves formed in air develop stomata and they utilize atmospheric CO_2 directly in C_3 photosynthesis, whereas CAM activity is lost, as evidenced by low Δ acidity (Keeley *et al.*, 1983a,b). *Isoetes australis* is also capable of growing with air-exposed shoots, but leaves formed in air still lack stomata, although leaf turnover is *c.* 1 leaf wk^{-1} (Keeley, 1983b). We evaluated the presence of stomata, CAM activity and underwater net photosynthesis for leaves formed in air (plants de-submerged for 6 wk), compared with those formed under water (continued submergence). For plants in waterlogged substrate with leaves in air for 6 wk, no stomata were present. Malate concentrations in leaves formed in air also remained high at dawn (103 ± 21 μmol g^{-1} FW; mean ± SE, n = 3; cf. 143 ± 15 μmol g^{-1} FW in leaves of submerged plants; Fig. 2). When leaves formed in air were tested for the effect of elevated O_2 on underwater net photosynthesis (Fig. 6), samples taken at dawn (high in malate) were found to be less affected than samples taken at dusk (low in malate), this being further evidence for substantial CAM activity in the leaves formed in air. Similarly to the pattern observed for submerged leaves, underwater net photosynthesis of low-malate leaves (dusk) was strongly restricted at elevated external O_2; these low-malate leaves showed net consumption of O_2 (high apparent photorespiration; Fig. 6). In summary, leaves formed in air maintained CAM activity.

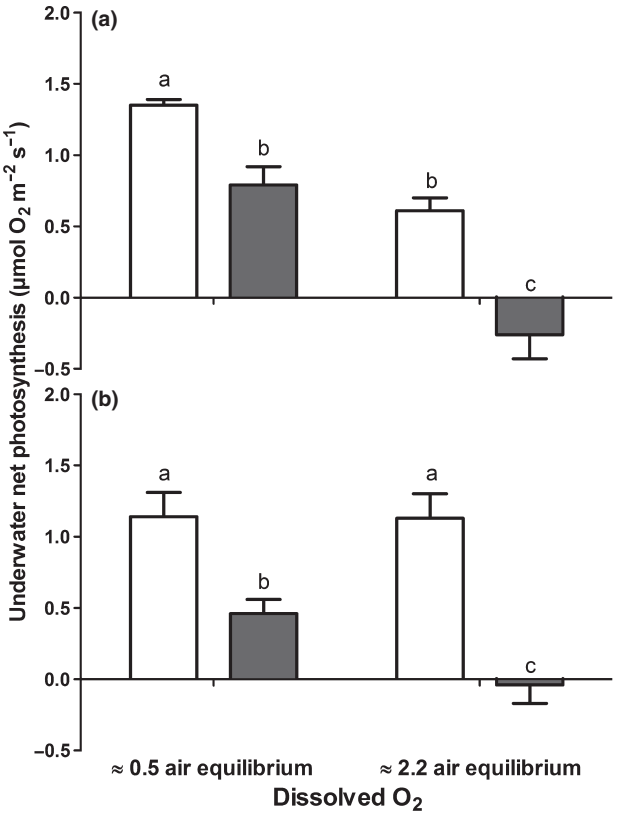


Fig. 6 Underwater net photosynthesis in leaves taken from *Isoetes australis* when submerged (a) or in leaves formed in air (b), measured at low (*c.* 0.5 air equilibrium; 140 mmol m^{-3}) or high (2.2-fold air equilibrium; 625 mmol m^{-3}) external O_2 concentration. Measurements were taken at 20°C for leaves collected at dawn (high malate, open bars) after 16 h in darkness or at dusk (low malate, closed bars) after 8 h in the light. Bars represent the mean ± SE (n = 4) and letters indicate significant differences (P < 0.05) between tissue types and treatments.

Discussion

The present study evaluated the influence of CAM on underwater net photosynthesis in plants challenged by low CO_2 and high O_2 concentrations, conditions often found in wetlands and in rock pools, which can potentially lead to high photorespiration. Leaves from the aquatic species *I. australis* with high and low malate concentrations (i.e. expected to have high and low decarboxylation rates, respectively) were compared. Leaves with high CAM activity maintained higher underwater net photosynthesis, particularly at low external CO_2 concentrations, and had lower apparent photorespiration, particularly when the O_2

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concentration was increased. These findings demonstrate the potential benefit of CAM photosynthesis for submerged plants, as fluctuations in underwater CO_2 and O_2 concentrations can be substantial (Van *et al.*, 1976). For example, the CO_2 concentration often decreases and the O_2 concentration increases in the water with time after sunrise, and then after sunset the opposite occurs (Keeley & Rundel, 2003).

Diminished photorespiration as a result of CAM would be of relevance to *I. australis* and other aquatic species growing in habitats with dynamic CO_2 and O_2 concentrations. Shallow seasonal pools experience dramatic diel changes in dissolved O_2, with high afternoon concentrations (35 kPa in Western Australian temporary granite rock pools (data not shown), and 30 kPa in a Californian vernal pool (Keeley, 1983a)). Such supersaturation of the water column would significantly restrict underwater photosynthesis in *I. australis*, but would have less effect in leaves containing high malate concentrations (Figs 5 and 6). *In situ* protection against photorespiration conferred by CAM activity, however, would be expected to fall within the extremes demonstrated in Figs 5 and 6, as the O_2 concentration in the water does not reach 2.2 times the air equilibrium concentration, and when the O_2 concentration is highest in the late afternoon much of the malate would typically already have been decarboxylated (Keeley *et al.*, 1983a). Nevertheless, as restriction of photorespiration by CAM occurs even at subatmospheric equilibria of O_2 (Fig. 5), the beneficial effect of CAM in suppressing photorespiration would still be expected to occur earlier in the day. The high CAM activity in *I. australis* may indicate that Australian temporary granite rock pools are environments where CCMs, and CAM in particular, are of importance for maintaining high underwater photosynthesis. The importance of CCMs in rock pools is further supported by the photosynthetic pathways in co-occurring species (*Crassula natans*, CAM; species of *Eleocharis*, CAM and sometimes C_4; species of *Marsilea*, unknown but not CAM; Keeley, 1998a,b).

It has long been assumed, based on lower CO_2 compensation points for underwater net photosynthesis in aquatic species with CCMs, as compared with those lacking a CCM, that CCMs reduce photorespiration in aquatic plants (Salvucci & Bowes, 1983; Madsen, 1987). Here, measurements of underwater net photosynthesis at a range of O_2 concentrations for tissues differing in the amount of malate available for decarboxylation enabled assessment of apparent photorespiration (Fig. 5). Photorespiration was evident as declines in net O_2 production rates (photorespiration increasing relative to photosynthesis), and even the net consumption of O_2 at high O_2 : CO_2 in the medium (and therefore within the leaves). Apparent photorespiration was substantially higher in leaves low in malate, compared with those with high malate concentrations; for example, even in solution with O_2 at air equilibrium, photorespiration was already substantial (net O_2 production reduced to 31% of maximal) in the low-malate leaves. CAM is a very effective CCM; for example, decarboxylation of malate can result in a CO_2 concentration of up to 30 000 ppm in the lacunae of submerged *Littorella uniflora* (Madsen, 1987); this CO_2 concentration, 85-fold higher than atmospheric, would be expected to give a favourable CO_2 : O_2 ratio at Rubisco, reducing photorespiration.

CAM activity in leaves of *Isoetes* has been shown to vary significantly depending on both season and environment. For example, *Isoetes bolanderi* had much lower CAM activity in the spring compared with late summer (Keeley *et al.*, 1983b), and *Isoetes howellii* showed lower diurnal changes in tissue acidity when sampled from an oligotrophic lake as compared with a seasonal pool (Fig. 3, Keeley *et al.*, 1983a). Comparison of the present results with other published data also showed that CAM activity in *I. australis* can be highly variable (Fig. 3); Δ malate ranged from *c.* 25 (Keeley, 1983b) to 140 μmol g^{-1} FW (the present study), the latter value of Δ malate being the second-highest reported for the genus *Isoetes*. Some of this variation in Δ malate for *I. australis*, however, may be explained by our tissue analysis being conducted on the chlorophyllous portion of the leaves, whereas other studies may have used entire leaves including the achlorophyllous portion that sometimes makes up a substantial portion of the leaf (Keeley *et al.*, 1984). Even so, only *I. howellii* sampled from Mather Pool in California displayed higher Δ tissue acidity and Δ malate.

The conclusion that CAM is a significant CCM for underwater photosynthesis in aquatic plants is supported by its induction when plants are underwater, but its repression when the leaves emerge into the air; for example, in species of *Isoetes* (Keeley *et al.*, 1983a), *Littorella* (Robe & Griffiths, 2000), *Orcuttia calafornica* and *Orcuttia viscida* (Keeley, 1998b). In the present study, *I. australis* maintained CAM activity in leaves that formed in air (Fig. 6) and these leaves did not form stomata, a response similar to that of *Lobelia dortmanna* which also does not form stomata when in air (Pedersen & Sand-Jensen, 1992). The natural environment of *I. australis* may select for this more conservative 'constitutive CAM strategy' as the temporary shallow granite rock pools may dry up and refill in an unpredictable manner throughout the growth season, and when the dry season sets in, the emergent phase with leaves in air may be of short duration (days) before the sediment also dries up and plants desiccate. These two environmental characteristics of the habitat of *I. australis* might mean that switching back and forth between CAM and C_3 would be of little benefit.

In conclusion, using *I. australis*, a CAM species that inhabits seasonal pools on temporary granite rock outcrops, the present study showed not only that leaves high in malate for decarboxylation have enhanced underwater net photosynthesis at low external CO_2 concentrations but that

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apparent photorespiration is also suppressed by CAM. Suppression of apparent photorespiration was evident at a range of dissolved O_2 concentrations, including values below air equilibrium, and the present study demonstrated substantial suppression of photorespiration in an aquatic species possessing CAM.

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