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 CO₂ 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 CO₂ and O₂ 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 but also lower apparent photorespiration. Suppression of CAM by apparent photorespiration was evident at a range of O₂ concentrations, including values below air equilibrium. At a high O₂ 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 O₂ and CO₂ concentration ranges in floodwaters, via increased CO₂ 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 CO₂ uptake from the water (Maberly & Madsen, 2002). In addition, some plants can utilize alternative CO₂ sources; for example, floating leaves directly access atmospheric CO₂, and large permeable root systems access sediment CO₂ (Maberly & Madsen, 2002). Physiological adaptations to deal with low CO₂ availabilities in floodwaters include those that increase CO₂ to Rubisco, commonly referred to as carbon-concentrating mechanisms (CCMs) (Maberly & Madsen, 2002; Raven *et al.*, 2008); these are bicarbonate use (Prins & Elzenga, 1989), C₄ photosynthesis (Magnin *et al.*, 1997), C₃–C₄ 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 CO₂ : O₂ ratio (Ogren, 1984; Sage, 2004). The low CO₂ availability in aquatic environments itself lowers the CO₂ : O₂ ratio; moreover, as O₂ is 28.5-fold less soluble in water than is CO₂ (Baranenko *et al.*, 1990), O₂ tends to build up inside gas spaces in photosynthetic tissues during the daytime as escape to the...
surrounding solution can be slow (Bowes, 1993). Thus, with CCMs such as CAM, a more favourable CO₂ : O₂ ratio presumably can be maintained at Rubisco and thus diminish photorespiration. Inferences regarding reduced photorespiration in aquatic plants with CCMs are based on lower CO₂ 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 CO₂ into malate, followed by daytime decarboxylation of the malate so that CO₂ is supplied to the C₃ pathway without the need for concurrent inward diffusion of external CO₂. 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 CO₂ 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 CO₂ 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 CO₂, 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 CO₂ concentrations, compared with leaves low in malate. Secondly, the long-standing hypothesis that CAM reduces photorespiration, based on low CO₂ compensation points (Salvucci & Bowes, 1983; Maberly & Madsen, 2002), was tested in experiments where underwater net photosynthesis and apparent photorespiration were manipulated while external CO₂ and O₂ concentrations were kept constant. The data show that tissues high in malate have enhanced underwater net photosynthesis at low external CO₂ 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

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**Fig. 1** Temporary granite rock pools in south-western Australia (a) and typical underwater vegetation (b). The temporary rock pools are rain-water-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.
turf 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 Ca²⁺, 0.10 Mg²⁺, 0.10 K⁺, 0.30 Cl⁻, 0.10 SO₄²⁻ and 0.10 HCO₃⁻. 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 – CO₂ response**

Underwater net photosynthesis by leaves was measured using the method described in Colmer & Pedersen (2008). Leaf tips (5–10 mm), without the chlorophyllous 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 KHCO₃ (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 O₂ concentration in the incubation medium was set at 50% of air equilibrium, by bubbling in 1 : 1 v/v of N₂ and air (before adjustment of dissolved CO₂); this avoided build-up of high O₂ during the measurements. As vials were incubated in the light immediately after adding the leaves, and as these produce O₂ when in the light, there was no risk of tissue hypoxia. Dissolved CO₂ treatments were imposed by adding specific concentrations of KHCO₃ to the incubation medium with pH always adjusted to pH 6.00 using various amounts of KOH depending on KHCO₃ added, to provide a range of CO₂ concentrations from 30 to 2000 mmol m⁻³ (Stumm & Morgan, 1996). K₂SO₄ was added as required in the various treatments so that K⁺ concentration was equal across treatments. For each CO₂ concentration, vials without leaves served as blanks.

Following incubations of known durations (60–75 min), dissolved O₂ concentrations in the vials were measured using a Clark-type O₂ 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 – O₂ response**

Underwater net photosynthesis by the leaves was measured using a system identical to the one already described for the CO₂ response; however, initial O₂ concentration was manipulated rather than CO₂. The initial O₂ concentration was adjusted by bubbling containers with N₂ or O₂ and then mixing these as required to obtain the desired O₂ concentration. After mixing, KHCO₃ was injected to obtain 30 mmol CO₂ m⁻³ at pH 6.00 in the incubation medium in all vials. Increasing apparent photosynthesis relative to photosynthesis was evident as a decline in net O₂ production rate, below the maximal rate when O₂ was optimal. In some samples (i.e. tissues low in malate), net O₂ consumption occurred (i.e. O₂ production from photosynthesis became less than O₂ consumption), as photosynthesis 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 O₂ concentration in the vial during the incubation, as measured with an O₂ microelectrode.

**Underwater dark respiration (R₉)**

Respiration by leaves when under water in darkness was measured, using a system identical to that already described for the CO₂ response, but with the lights off. The initial O₂ concentration of the medium was adjusted by bubbling containers with N₂ or O₂ and then mixing these as required to obtain the desired O₂ concentrations of half air equilibrium, air equilibrium and double air equilibrium. After mixing, KHCO₃ was injected to obtain 30 mmol CO₂ 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 O₂ uptake in the vials
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 N₂, freeze-dried, ground in a ball mill, and then extracted in iced 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 iced cold 5% (v/v) perchloric acid. The combined supernatant from the two extractions was pH-adjusted to 3.0–3.5 using K₂CO₃ 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) using a mobile phase consisting of 25 mM KH₂PO₄ 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, maleic, shikimic, lactic, acetic, malic, 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 Isoetes 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 CO₂ 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 CO₂-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 Bonferroni 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 CO₂ or O₂ 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 Isoetes 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).](image-url)
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 CO₂ fixed during the night (Fig. 2).

Underwater net photosynthesis in response to dissolved CO₂ for leaves of contrasting malate status

The presence of CAM has been hypothesized to enable maintenance of net photosynthesis when external CO₂ is low in submerged plants (Madsen, 1987). We tested the potential benefits of CAM activity by comparing CO₂ response curves for leaves either high (dawn) or low (dusk) in malate. The CO₂-saturated rate of underwater net photosynthesis did not differ between high- and low-malate leaves, being 2.3 μmol O₂ m⁻² s⁻¹ in both cases (Fig. 4). The response curves of high- and low-malate leaves deviated significantly at 300 mmol CO₂ 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 CO₂ resulting from more decarboxylation of malate.

Assessment of apparent photorespiration; response of underwater net photosynthesis to O₂ in leaves of contrasting malate status

Photorespiration can be high in submerged plants, especially when CO₂ is depleted and O₂ increases well above air equilibrium, so that a low CO₂ : O₂ 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 O₂ on the rate of underwater net photosynthesis (Fig. 5).
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 $R_d$ ($R_d = 0.14 \pm 0.02 \mu$mol 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 desubmerged 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.

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$_2$
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 equilbria 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 *Elodea*, 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 achorophyllous 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 califomica* and *Orcuttia viecida* (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
apparent photorespiration is also suppressed by CAM. Suppression of apparent photorespiration was evident at a range of dissolved O₂ 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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