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Research

Pigments, photosynthesis and photoinhibition in two amphibious plants: consequences of varying carbon availability

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Summary

• In the present study, we investigated the effects of CO_2 availability on photosynthesis, photoinhibition and pigmentation in two species of amphibious plants, *Lobelia cardinalis* and *Nesaea crassicaulis*.

• The plants were grown emergent under atmospheric conditions and submerged under low and high CO_2 availability.

• Compared with *Lobelia*, *Nesaea* had thin leaves and few stomata in all CO_2 treatments. While *Lobelia* expressed no variation in anthocyanin content among treatments, *Nesaea* produced high concentrations of anthocyanin when submerged. *Lobelia* photosynthesis increased in response to increasing CO_2 availability, and photoinhibition was negatively related to xanthophyll content. By contrast, *Nesaea* photosynthesis was highest under submerged conditions, and there was no relationship between photoinhibition and the xanthophyll content.

• We conclude that the response of *Lobelia* to varying CO_2 availability is similar to that of terrestrial plants and that this species relies on the xanthophyll cycle for nonphotochemical quenching (NPQ) and protection against photoinhibition. By contrast, the thin leaves, few stomata and low levels of chlorophylls and accessory pigments in *Nesaea*, relative to *Lobelia*, suggest adaptation to a submerged habitat. While *Nesaea* does not seem to rely on the xanthophyll cycle or other xanthophylls for NPQ, some role of anthocyanins in the protection against photoinhibition cannot be ruled out, owing to its effect as a sunscreen and as an efficient quencher of free radicals.

Key words: anthocyanin, aquatic macrophytes, chlorophyll fluorescence, CO_2, excess light energy, nonphotochemical quenching (NPQ), photoprotection, xanthophyll cycle.

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Introduction

Plants that grow in marshes or along the banks of streams and lakes are subject to alternating periods of flooding and emergence caused by changing water levels, and are often referred to as amphibious plants (Nielsen, 1993). The periodicity of alternating flooding and emergence can range from seasonal to more frequent changes in the order of days or weeks. Flooding may cause abrupt and significant changes in several important environmental parameters (e.g. the availability of light, water and carbon) (Maberly & Spence, 1989). While light is often regarded as probably the most important parameter in controlling plant growth in aquatic habitats (Kirk, 1994), amphibious plants are usually submerged in rather shallow water, and changes in irradiance as a consequence of flooding are often minor, although increases in turbidity associated with flooding can temporarily reduce light. The potential effects of changing water levels on water availability, as a result of flooding, are obvious (Maberly & Spence, 1989). However, amphibious plants are usually rooted in waterlogged sediment,

even when emergent at low water levels, and as such will have access to sufficient water until the sediment eventually dries up during periods of prolonged drought.

By contrast, carbon availability probably represents the most significant factor to which amphibious plants adapt during changes in water levels (Maberly & Spence, 1989). While CO_2 is not generally considered as a limiting factor for growth in terrestrial and emergent amphibious plants (Monteith & Elston, 1993), the situation is quite the opposite in aquatic habitats. Although water in equilibrium with atmospheric air has a CO_2 concentration almost equal to that of air (15 μM or 350 ppm) (Maberly & Spence, 1989), the high viscosity of water relative to that of air results in the formation of thick boundary layers around submerged leaves (Nobel, 1999), through which CO_2 diffuses at a rate 10 000-fold slower than in air (Proctor, 1982). Consequently, CO_2 may be limiting for photosynthesis and growth in submerged plants (Salvucci & Bowes, 1982; Maberly & Spence, 1989). Even in habitats that receive an influx of CO_2-rich groundwater (Rebsdorf *et al.*, 1991) or carbonates from the substrate (Maberly & Spence, 1989), CO_2 may be limiting for the growth of submerged plants (Maberly & Spence, 1989). Many aquatic plants have adaptations that will allow them to utilize carbon sources alternative or supplementary to free CO_2 in the water. These include CO_2 from the soil water taken up through the roots (Raven *et al.*, 1988), bicarbonate utilized by submerged leaves in addition to free CO_2 (Sand-Jensen, 1987), and atmospheric CO_2 utilized by leaves that grow above the surface of the water (Nielsen, 1993). In spite of these adaptations, the carbon availability in freshwater systems may often be limiting to the photosynthesis of aquatic plants (Sand-Jensen & Frost-Christensen, 1999) and can be expected to increase the susceptibility of these plants to photoinhibition (White *et al.*, 1996; Vestergaard, 2003).

Under conditions of low carbon availability, and/or high light irradiance, the absorbed excitation energy may be in excess of that required by processes downstream of light harvesting (e.g. the Calvin cycle for which CO_2 is a substrate). It is well documented that photosystem II (PSII) is vulnerable to conditions of excess excitation energy, during which photoinhibition may occur. Excess excitation energy may induce reactive oxygen species (ROS) in the presence of O_2: the ROS are products of oxidization of water by PSII (Oxborough & Baker, 2000). ROS may, in turn, cause direct physical damage (photodamage, i.e. chronic photoinhibition) to the photosynthetic apparatus, in particular the protein complex D1 of PSII. Photodamage may be prevented through the dissipation of excess excitation energy (photoprotection, i.e. dynamic photoinhibition) through the light-dependent conversion of the xanthophyll violaxanthin into antheraxanthin and zeaxanthin in the xanthophyll cycle (Demmig *et al.*, 1987; Gilmore, 1997). In plants growing fully exposed to the sun, up to 70% of absorbed photons may be dissipated in this way (Adams *et al.*, 1996). In addition to its role in the xanthophyll cycle, zeaxanthin has been shown to be an efficient quencher of free radicals (Demmig-Adams & Adams, 1992; Horton *et al.*, 1996).

Photoprotection may also be provided by anthocyanins, a group of pigments derived from the flavonoid pathway (Timmins *et al.*, 2002). Anthocyanins can act as a sunscreen that absorbs light before it reaches the chloroplasts and PSII (Gould *et al.*, 1995; Timmins *et al.*, 2002). They are also efficient quenchers of free radicals (Wang *et al.*, 1997; Gould *et al.*, 2002a; Timmins *et al.*, 2002). Anthocyanins are found in the abaxial epidermis of forest understorey plants, where they provide protection from free radicals, produced during sudden periods of high light in sunflecks, without hampering light absorption in the more normal deep-shade environment in which these plants grow (Lee, 2002). Anthocyanins are also found in expanding and senescing leaves where they are thought to provide photoprotection during synthesis and breakdown of photosystems (Gould *et al.*, 2002b; Lee, 2002), and they are produced in response to environmental stresses such as nutrient deficiency, high irradiance and plant diseases (Chalker-Scott, 1999).

Here, we studied the effect of different CO_2 availabilities on the photosynthesis, photoinhibition and pigmentation of two species of amphibious plants [*Lobelia cardinalis* L. and *Nesaea crassicaulis* (Guill. & Perr.) Koehne]. The plants were grown emergent under atmospheric conditions and under two different submerged treatments, with either high or low concentrations of free CO_2. The effect of these treatments on photosynthesis and photoinhibition, as well as on the content of xanthophyll cycle components and other pigments that may be involved in photoprotection, including anthocyanins, was investigated.

Materials and methods

L. cardinalis L., a North American member of the Lobeliaceae, and *N. crassicaulis* (Guill. & Perr.) Koehne, an African member of the Lythraceae, were supplied by Tropica A/S (Hjortshøj, Denmark). Under natural conditions, both plants are emergent in marshes and along stream banks. Both species are popular among aquarists for their red coloration and are readily available throughout the year. The plants represent two different strategies of adapting to varying environmental conditions. *L. canadensis* has leaves that are green with a red coloration of the abaxial epidermis, especially when grown emergent. *N. crassicaulis* has leaves that achieve an overall reddish coloration when grown submerged (Kasselmann, 2003).

The plants were grown in aquaria under an irradiance of 170 μmol m^{-2} s^{-1} (photosynthetically active radiation) in a 16 h : 8 h light/dark cycle at 20°C. They were rooted in mineral wool that, in itself, is inert with respect to nutrient content. To ensure sufficient nutrient supply, both macro- and micronutrients were added to the rooting medium at regular intervals, using commercially available slow-release fertilizers

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(ASB-Grünland, Vallensbæk Strand, Denmark). The water in the aquaria was a 50 : 50 mixture of tap water and demineralized water, which was replaced twice per week to keep the aquaria free of algal growth. The plants were grown for 1 month under three different carbon-availability treatments – emerged and exposed to atmospheric air, or submerged in water containing either a high or a low CO_2 concentration – after which the measurement program was initiated. The water level in the aquaria containing emergent plants was maintained so that the surface of the rooting medium was just covered and the leaves had access to atmospheric air. The CO_2 availability of the aquaria containing submerged plants was controlled by adjusting the pH to 6.2 and 7.7, respectively, with CO_2 concentrations of 1.5 mM and 40 μM, as measured by Gran-titration (Stumm & Morgan, 1996). The pH, and thus the CO_2 concentration, were maintained by aerating the water with CO_2 from a gas cylinder controlled by an aquarium CO_2-fertilizer device (JBL Proflora vario 500; JBL, Neuhofen, Germany). Three plants of each of the two species were planted in each of three replicate aquaria of the three treatments.

The carbon affinity of the plants was measured on detached leaves in closed bottles mounted on a rotating wheel in a thermostatic incubator (20°C) with a photon flux of 390 μmol m^{-2} s^{-1}. The experimental water was aerated with atmospheric air, before the experiment, to bring it to equilibrium with atmospheric O_2 and CO_2. Carbon affinity was determined by measuring alkalinity and total carbon concentration in the bottles before and after at least 24 h of continuous illumination. The carbon affinity is expressed as the ratio of the final concentration of total inorganic carbon (C_T) to initial alkalinity (Alk) (Maberly & Spence, 1983).

To measure photosynthesis, individual leaves were incubated at 20°C and at eight incident light intensities, ranging from 0 to 1200 μmol m^{-2} s^{-1}, provided by a Hansatech (King's Lynn, UK) LS2 high-intensity light source fitted with precalibrated neutral filters. Emergent photosynthesis was measured as CO_2 consumption using an ADC-225-Mk3 IR gas analyzer (IRGA; Analytical Development Company, Hoddesdon, UK) with an ADC-LSC-2 leaf chamber. Submerged photosynthesis was measured as O_2 evolution in a stirred Hansatech DW3 leaf chamber fitted with a Clark-type O_2 electrode. The water in the O_2 chamber was the same as used in the growth aquaria, so that photosynthesis was measured under conditions equivalent to those for growth. Photosynthesis was calculated as mmol CO_2 consumed or mmol O_2 produced m^{-2} leaf surface as well as g^{-1} of chlorophyll (Chl) *a* h^{-1}. For comparisons of emergent and submerged photosynthesis, it was assumed that the photosynthetic quotient had a value of 1 (PQ = 1).

Photoinhibition was estimated from chlorophyll fluorescence parameters, in particular the ratio of variable to maximum fluorescence (F_V/F_M) and electron transport rate (ETR), measured on a pulse-amplitude modulated fluorometer based on image analysis technology (Image-PAM, Walz, Germany). The parameter F_V/F_M has optimal values of approx. 0.83 for most species of higher plants (Maxwell & Johnson, 2000), and values lower than this indicate stress, in particular photoinhibition (Maxwell & Johnson, 2000).

The plant content of Chl *a* and *b* and carotenoids was measured by high-performance liquid chromatography (HPLC). Plant extracts were prepared from lyophilized and pulverized leaf samples that were transferred to 96% ethanol and sonicated in an ice bath for 5 min. Analyses were performed on a Dionex Summit HPLC system (Dionex, Hvidovre, Denmark) applying a gradient method, using methanol : ammonium acetate (80 : 20, v/v) as eluent A and methanol : acetone (90 : 10, v/v) as eluent B. The gradient started with a 50 : 50 mixture of eluents A and B, changing to 100% eluent B after 10 min. After 30 min the gradient was changed back to a 50 : 50 mixture of the two eluents, which was achieved in 1 min. The method included 9 min of equilibration time, so that the total run time was 40 min. The column was a Waters Spherisorb 3 μm ODS2 4.6 × 150 mm C18 column, cooled to 6°C. The flow rate was set to 0.3 ml min^{-1}. Standards were provided by DHI Water and Environment (Hørsholm, Denmark).

The total anthocyanin content of the plants was determined by the pH-differential method (Giusti & Wrolstad, 2000). Approx. 30 mg of pulverized leaf samples was extracted for 45 min in 5 ml of methanol : acetic acid (50 : 50 v/v). Subsequently, the liquid phase was evaporated and the sample resuspended in 10 ml of nanopure water. The sample was then divided into two parallel samples and passed through solid-phase extraction tubes (Strata-X; Phenomenex, Torrance, CA, USA), thus fixing the anthocyanin. The anthocyanin was subsequently eluted into test tubes with methanol that was evaporated to provide clean anthocyanin samples. Eventually, the two parallel samples were resuspended in potassium chloride buffer (0.025 M KCl, pH 1 with HCl) and sodium acetate buffer (0.4 M $CH_3CO_2Na{\cdot}3H_2O$, pH 4.5 with HCl). The absorbances of the samples (*A*) were measured spectrophotometrically at 520 and 700 nm and calculated according to the following equation:

$$A = (A_{520} - A_{700})_{pH1} - (A_{520} - A_{700})_{pH4.5}$$

The anthocyanin concentration in the sample was calculated according to the following equation:

$$\text{Anthocyanin concentration} = (A \times MW)\varepsilon^{-1},$$

[MW, the molecular weight (449.2 g mol^{-1}) of cyanidin-3-glucoside; ε, the absorbance (26 900 mol^{-1}) of cyanidin-3-glucoside.]

Stomatal density was measured by microscopy on casts made on the abaxial leaf surfaces using GT5 casting paste (P-B Miljø, Bjerringbro, Denmark). Stomata were counted in 0.14 mm^2 fields of vision in the microscope. Ten fields of

vision were counted on each leaf and nine leaves were counted for each of the three treatments.

Dry weight was measured after lyophilizing the leaves. For surface area measurements, the leaves were scanned on a flatbed scanner and the area was measured using SIGMASCANPRO image-analysis software (Jandel Scientific, Erkrath, Germany).

As a result of the experimental set-up, data analysis was performed as a nested analysis of variance (ANOVA) design (Sokal & Rohlf, 1995) with Tukey's test as a posthoc test (Sokal & Rohlf, 1995). The significance level was set to $P = 0.05$ in all cases. Data were tested for normal distribution using the Kolmogorov–Smirnov test (Sokal & Rohlf, 1995), and homogeneity of variance was analysed using Bartlett's test (Sokal & Rohlf, 1995). Relationships between F_V/F_M and xanthophylls and anthocyanins were tested using least-squares linear regression (LSLR) (Sokal & Rohlf, 1995), also using a significance level of 0.05.

Results

Both *Lobelia* and *Nesaea* expressed a strategy for CO_2 acquisition that is indicative of their truly amphibious life form, and there were no significant treatment–species interactions for the ability of the plants to extract carbon from the water. Both species achieve an end pH in the range 8.62–8.74 in the pH-drift experiments, corresponding to C_T : Alk values in the range 0.89–1.09, which is indicative of photosynthetic CO_2 use only, with no contribution of bicarbonate. In contrast to the carbon affinity, there were significant morphological differences and also slight differences in the morphological response of plants to treatments. *Nesaea* had significantly higher specific leaf areas (thinner leaves) than *Lobelia* (Fig. 1, ANOVA, $P < 0.001$), regardless of treatment, and both species achieved higher specific leaf areas in low-CO_2 water than in high-CO_2 water (Fig. 1, ANOVA, $P < 0.001$). *Nesaea* in air had a relatively low specific leaf area, equal to that of high-CO_2 water plants, whereas *Lobelia* in air had leaves with a high specific leaf area, equal to that of low-CO_2 water plants (Fig. 1). In addition to having thicker leaves than *Nesaea*, *Lobelia* had a higher stomatal density than *Nesaea*, in all treatments (Fig. 1, ANOVA, $P < 0.001$). Both species had a significantly lower stomatal density in water than in air (Fig. 1, ANOVA, $P < 0.001$); in fact, stomata were almost absent in submerged *Nesaea*. No differences were observed for the two submerged treatments in either species (ANOVA, $P > 0.05$ for both species), so these data are pooled in Fig. 1.

Both species exhibited lower Chl *a* contents when grown submerged compared with emerged (Fig. 2, ANOVA, $P < 0.001$), and no significant differences in the Chl *a* content were found for either species among the two submerged treatments (Fig. 2, ANOVA, $P > 0.05$ in both cases). *Nesaea* had a lower Chl *a* content than *Lobelia* in all treatments (Fig. 2, ANOVA, $P < 0.0001$). While both species expressed the same Chl *b* : Chl *a* ratio when growing in air (Fig. 3, ANOVA, $P > 0.05$), this

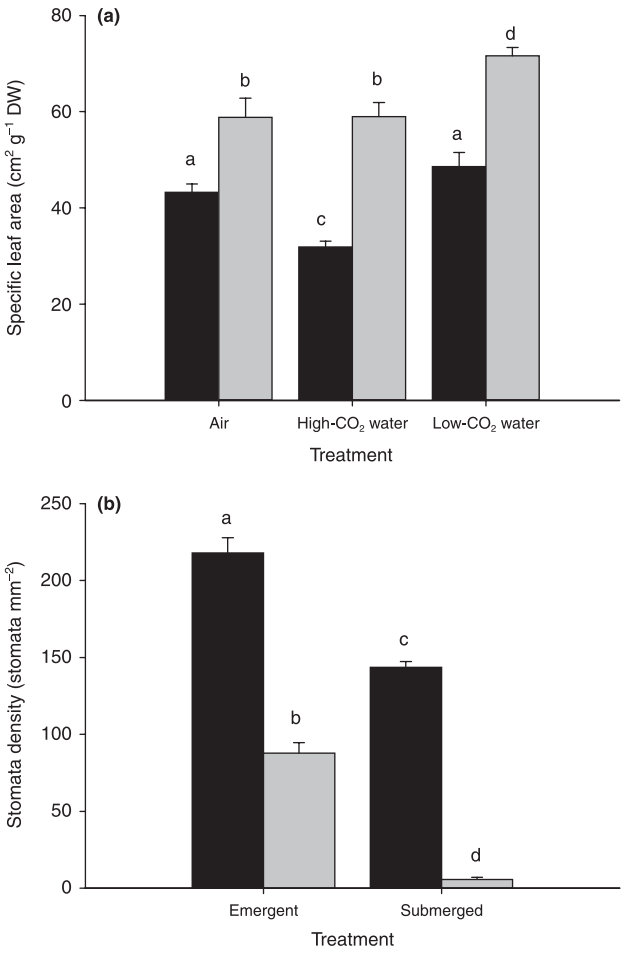

Fig. 1 Specific leaf area (a) and stomata density (b) for *Lobelia cardinalis* (black bars) and *Nesaea crassicaulis* (grey bars). The standard error is shown above each bar. Values that are significantly different ($P < 0.05$) are indicated by different letters. DW, dry weight.

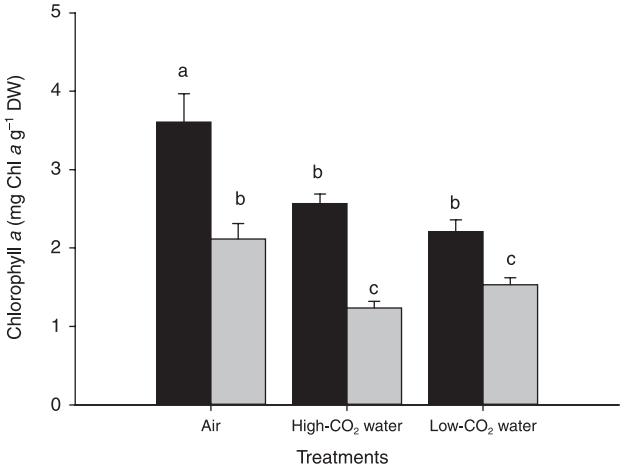

Fig. 2 Chlorophyll a (Chl *a*) content in *Lobelia cardinalis* (black bars) and *Nesaea crassicaulis* (grey bars). The standard error is shown above each bar. Values that are significantly different ($P < 0.05$) are indicated by different letters. DW, dry weight.

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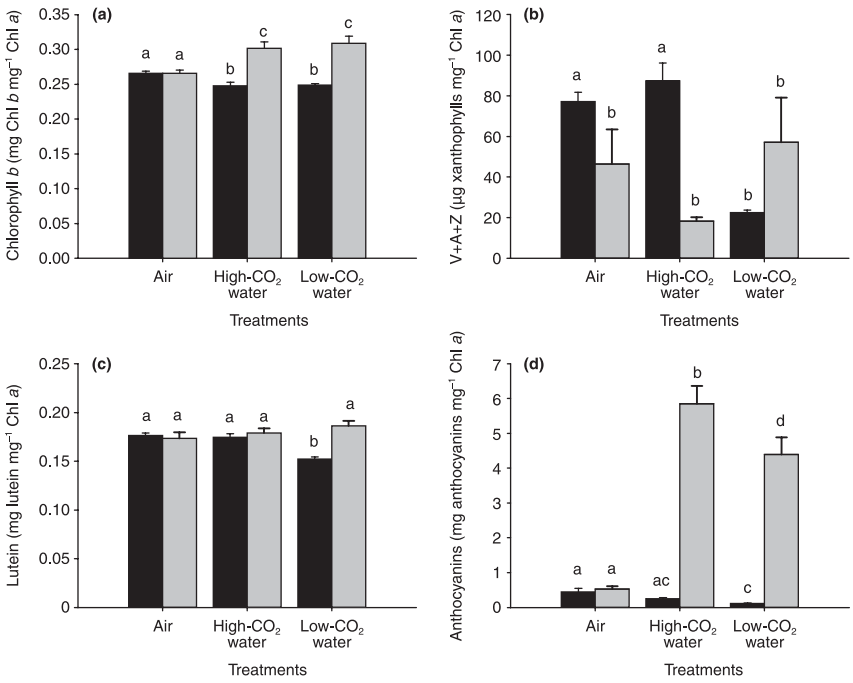

Fig. 3 Pigment contents, normalized to chlorophyll *a* (Chl *a*). (a) Chl *b*; (b) sum of xanthophyll cycle components, violaxanthin+antheraxanthin+zeaxanthin (V+A+Z); (c) lutein; (d) anthocyanins. Black bars, *Lobelia cardinalis*; grey bars, *Nesaea crassicaulis*. The standard error is shown above each bar. Values that are significantly different ($P < 0.05$) are indicated by different letters.

parameter was expressed differently by the two species during submergence. Compared with the air treatment, the Chl *b* : Chl *a* ratio of submerged *Lobelia* decreased significantly, whereas that of submerged *Nesaea* increased (Fig. 3, ANOVA, $P = 0.002$ and $P = 0.006$, respectively). No significant differences were found among the two submerged treatments for either species (Fig. 3, ANOVA, $P > 0.05$).

There were significant treatment–species interactions in the xanthophyll cycle components, violaxanthin, antheraxanthin and zeaxanthin (given as V+A+Z) relative to Chl *a* in Fig. 3. In *Nesaea* there were no significant changes in xanthophyll cycle components in response to the CO_2 availability (ANOVA, $P > 0.05$). By contrast, *Lobelia* had a significantly lower content of xanthophyll cycle components in low-CO_2 water than in air and high-CO_2 water treatments (ANOVA, $P < 0.001$), the two latter treatments inducing the same level of xanthophyll cycle components (ANOVA, $P > 0.05$). Consequently, while the content of xanthophyll cycle components was significantly higher in *Lobelia* than in *Nesaea* in air and in high-CO_2 water, the situation was different in low-CO_2 water, where a nonsignificant tendency towards a higher concentration of xanthophyll cycle components in *Nesaea* compared with *Lobelia* was seen. The content of lutein, another important photoprotective carotenoid, followed the same pattern as for xanthophyll cycle components in the low-CO_2 water treatment, whereas there was no significant differences in the lutein content among *Lobelia* and *Nesaea* in the air and in high-CO_2 water treatments (Fig. 3, ANOVA, $P > 0.05$ in both cases).

There were pronounced differences in the anthocyanin content among *Lobelia* and *Nesaea*. While there were no significant differences in the anthocyanin content among the two species in air (Fig. 3, ANOVA, $P > 0.05$), the anthocyanin content of *Nesaea* was significantly and several-fold higher than that of *Lobelia* when submerged (Fig. 3, ANOVA, $P < 0.0001$). In *Lobelia*, the relative anthocyanin content decreased in the water treatments compared with the air treatment. The lowest content was achieved in plants growing in low-CO_2 water (Fig. 3, ANOVA, $P = 0.004$), while the content in high-CO_2 water plants was intermediate and not significantly different from either of the two other treatments. In *Nesaea* the anthocyanin content increased dramatically when the plants were grown submerged compared with emerged. The highest content was achieved in plants growing in high-CO_2 water, while the content in low-CO_2 water plants was intermediate but statistically significantly different from both other treatments (Fig. 3, ANOVA, $P < 0.001$).

The relationships between F_V/F_M, usually regarded as expression of photoinhibition (Maxwell & Johnson, 2000), and the xanthophyll cycle components (V+A+Z) and anthocyanins, are shown in Fig. 4. F_V/F_M is related to the V+A+Z content in *Lobelia*, but not in *Nesaea* (*Lobelia*: slope = –0.0015, $P = 0.002$; *Nesaea*: slope = 0.0003, $P = 0.311$). The relationships between F_V/F_M and lutein are not shown, but follow the same trends as for xanthophyll cycle components, albeit with weaker correlations. F_V/F_M was not related to the anthocyanin content in either of the two species, although the *Nesaea* data were indicative of a relationship between the two parameters (*Lobelia*: slope = –0.129, $P = 0.395$; *Nesaea*: slope = –0.0087, $P = 0.063$).

The photosynthesis–irradiance (P–I) curves for the two species revealed very different response patterns (Fig. 5). In *Lobelia*, air-grown plants had higher apparent quantum yields

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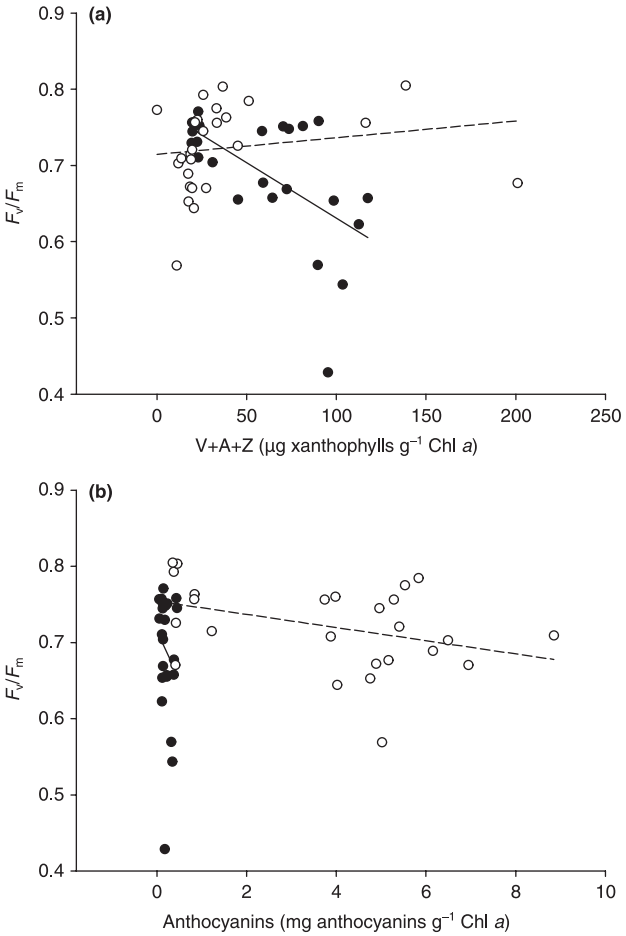

Fig. 4 Relationships between F_V/F_M and total xanthophyll cycle components (a) and total anthocyanins (b) for *Lobelia cardinalis* (black circles, solid line) and *Nesaea crassicaulis* (white circles, dashed line).

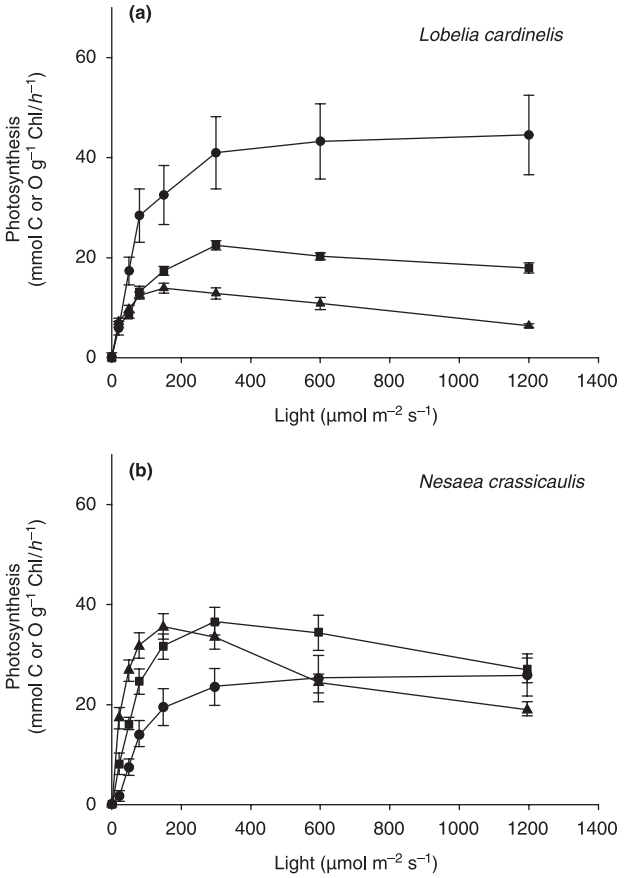

Fig. 5 Photosynthesis–light curves for *Lobelia cardinalis* (upper panel) and *Nesaea crassicaulis* (lower panel). Treatments: air (circles), high-CO_2 water (squares), low-CO_2 water (triangles). The standard errors are shown.

(Φ) and higher maximum photosynthesis rates (P_{max}) than submerged plants and expressed no signs of photoinhibition. For submerged *Lobelia*, low-CO_2 plants had a significantly lower P_{max} than high-CO_2 plants and expressed photoinhibition at light intensities of > 150 µmol m^{-2} s^{-1}. Submerged high-CO_2 *Lobelia* only showed signs of photoinhibition at light intensities of > 300 µmol m^{-2} s^{-1}. The photosynthetic response of *Nesaea* to the three CO_2 treatments was quite different from that of *Lobelia* (Fig. 5). While emergent *Nesaea* had the lowest values of Φ, submerged *Nesaea* grown in low-CO_2 water archived the highest Φ. Similarly, the P_{max} of submerged *Nesaea* was significantly higher than that of air-grown *Nesaea* and independent of the CO_2 level in the water. Similarly to *Lobelia*, *Nesaea* grown in high-CO_2 water showed evidence of photoinhibition at light intensities of > 300 µmol m^{-2} s^{-1}, while *Nesaea* grown in low-CO_2 water showed evidence of photoinhibition at light intensities of > 150 µmol m^{-2} s^{-1}.

In *Lobelia*, ETRs (Fig. 6) were lowest for plants grown in high-CO_2 water, intermediate for air-grown plants and highest for plants grown in low-CO_2 water. Air-grown *Lobelia* showed incipient photoinhibition at light intensities of > 365 µmol m^{-2} s^{-1}, while submerged *Lobelia* showed incipient photoinhibition at light intensities of > 225 µmol m^{-2} s^{-1}. Only *Lobelia* grown in high-CO_2 water experienced complete photoinhibition at light intensities of > 1000 µmol m^{-2} s^{-1}. In *Nesaea*, the ETR was generally higher than in *Lobelia*, but *Nesaea* also showed much higher variation in ETRs than *Lobelia*. *Nesaea* showed incipient photoinhibition at the same light intensities as found in *Lobelia*, but only emergent plants had any ETRs at the highest irradiance of 1210 µmol m^{-2} s^{-1}. The ETR for *Nesaea* generally mirrored the P–I curves, while there was some discrepancy between the ETR and P–I curves for *Lobelia*.

Discussion

While both species studied grow as amphibious plants in their natural habitats (Kasselmann, 2003), a number of differences between them suggest that *Nesaea* is adapted to more submerged

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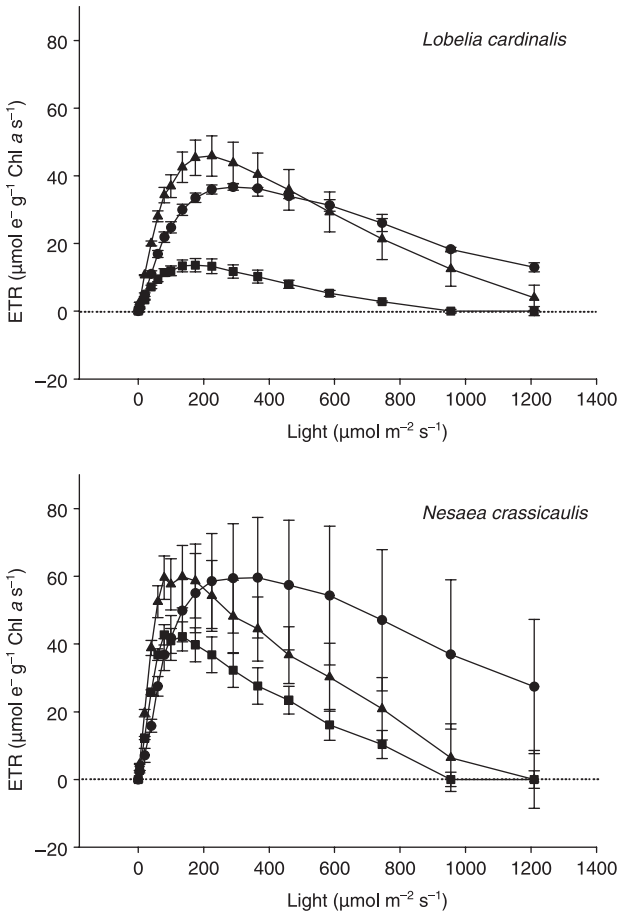

Fig. 6 Electron transport rates (ETRs) as a function of irradiance for *Lobelia cardinalis* (upper panel) and *Nesaea crassicaulis* (lower panel). Treatments: air (circles), high-CO_2 water (squares), low-CO_2 water (triangles). The standard errors are shown.

conditions than *Lobelia*. In contrast to *Lobelia*, *Nesaea* has thin leaves and very few stomata, even when growing emergent, features that are normally associated with submerged plants (Sculthorpe, 1967). The differences found in Chl *a* content are also in accordance with adaptation to different niches in the aquatic environment. *Nesaea* always had a lower Chl *a* content than *Lobelia*, which is consistent with a submerged rather than an emergent life form (Björkman, 1981; Nielsen & Sand-Jensen, 1989). Similarly, both species had significantly lower Chl *a* contents when growing submerged. This is consistent with the submerged form of an amphibious species having a lower Chl *a* content than the emergent form of the same species (Nielsen & Sand-Jensen, 1997). The increased Chl *b* : Chl *a* ratio of submerged *Nesaea* may be related to the light absorption properties of the leaf in general (e.g. thinner leaves), as it has been observed that the Chl *b* : Chl *a* ratio increases with increasing specific leaf area (Dale & Causton, 1992). While the Chl *b* : Chl *a* ratio is also known to increase under light limitation (Dale & Causton, 1992), in the present study all plants grew under the same light conditions and therefore the differences identified cannot be ascribed to variation in light climate. Although increasing specific leaf area in terrestrial plants is an adaptation to low-light conditions, in aquatic and amphibious plants it also represents an adaptation to the limited carbon availability of the submerged life form (Sculthorpe, 1967; Nielsen, 1993).

Dark-adapted values of the chlorophyll fluorescence parameter F_V/F_M reflect the potential quantum yield of PSII and may be indicative of photoinhibition (Maxwell & Johnson, 2000). The F_V/F_M parameter does not distinguish between chronic photoinhibition caused by damage to the photosynthetic apparatus (Demmig-Adams & Adams, 1992) and photoprotective dissipation of excess light energy as heat (Demmig *et al.*, 1987; Osmond, 1994; Gilmore, 1997). F_V/F_M is often found to correlate well with the content of xanthophyll cycle components in the leaves (Kurasova *et al.*, 2003). The xanthophyll cycle may be one of the most important mechanisms in nonphotosynthetic quenching (NPQ), which protects the plant against photodamage through dissipation of excess light energy as heat (Demmig *et al.*, 1987; Osmond, 1994). In *Lobelia* we found that there were significant changes in the content of xanthophyll cycle components as a result of different treatments, and we also found a linear relationship between the content of xanthophyll cycle components and F_V/F_M. By contrast, in *Nesaea* there was no variation in the content of xanthophyll cycle components as a consequence of treatments and there was no relationship between F_V/F_M and xanthophyll cycle components. We suggest that while *Lobelia* is dependent on the xanthophyll cycle and lutein for NPQ, as usually found in higher plants, the xanthophyll cycle plays little or no role in NPQ in *Nesaea*. The relatively high content of xanthophylls of *Lobelia* in high-CO_2 water under conditions where the risk of photoinhibition is expected to be lowest owing to the high CO_2 availability, may be an adaptation to the sensitizing of the photosynthetic apparatus to photoinactivation, which may occur in plants under elevated CO_2 (Kurasova *et al.*, 2003).

In addition to the role of carotenoids, anthocyanins may also be involved in photoprotection (Timmins *et al.*, 2002). Although anthocyanins are located in the cell vacuoles, rather than in the chloroplasts, they are able to absorb light before it reaches PSII in cells in lower cell layers in the leaf (Gould *et al.*, 1995; Timmins *et al.*, 2002). Anthocyanins are also efficient quenchers of free radicals (Wang *et al.*, 1997; Gould *et al.*, 2002a; Timmins *et al.*, 2002). Both *Lobelia* and *Nesaea* can achieve a more or less distinct red coloration under certain conditions, but while both species have similar anthocyanin contents when grown in air, the anthocyanin content of *Lobelia* decreases when it is grown submerged, while the anthocyanin content of *Nesaea* increases dramatically under the same conditions. In *Nesaea*, but not in *Lobelia*, there is also a near-significant linear relationship between anthocyanin content and F_V/F_M, indicating a possible role of anthocyanins in NPQ in this species.

While *Lobelia* has some ability to grow submerged, the increasing Φ and P_{max} with increasing CO_2 availability is characteristic of terrestrial plants with some capability of submerged growth (Sand-Jensen *et al.*, 1992; Nielsen, 1993). By contrast, the relatively high Φ and P_{max} values of *Nesaea* at low CO_2 availability probably result from the thinner leaves and the lower chlorophyll contents (i.e. smaller package effects), leading to higher chlorophyll-specific light absorption (Enríquez, 2005). Consequently the submerged leaves of *Nesaea* express an efficient light utilization under low light intensities, but also a significant photoinhibition under higher light intensities. This species is, as such, well adapted to submerged growth under low light intensities, but need a high CO_2 availability to avoid photoinhibition of photosynthesis in conditions of high light. At low light intensities, ETRs are higher in *Nesaea*, which shows signs of photoinhibition at lower light intensities than *Lobelia*. This is undoubtedly also a consequence of the lower chlorophyll contents in *Nesaea*, giving it a higher light utilization efficiency under low light intensities, but resulting in an earlier and more severe photoinhibition at higher light intensities, as has been observed in algae (Nielsen & Nielsen, 2005).

In general, the ETR is more closely coupled to photosynthesis in *Nesaea* than in *Lobelia*, where clear signs of photoinhibition are evident from ETRs, but not from the photosynthesis. This difference between the two species is probably a result of differences in leaf thickness, as has been shown in algae where the ETR is closely coupled to photosynthesis in thin-leaved species, but not in thick-leaved species (H. D. Nielsen & S. L. Nielsen, unpubl. obs.).

In conclusion, *L. cardinalis* can be considered a terrestrial plant, adapted to tolerate some submergence. It reacts like a terrestrial plant to varying CO_2 availabilities and relies on the xanthophyll cycle for NPQ and protection against photoinhibition. By contrast, *N. crassicaulis* seems to be better adapted to a more submerged habitat, with thin leaves, few stomata and a low content of chlorophylls and accessory pigments. Interestingly, *Nesaea* does not seem to rely on the xanthophyll cycle or other xanthophylls for NPQ, but some role of anthocyanins in the protection against photoinhibition cannot be ruled out.

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