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Geographically distinct *Ceratophyllum demersum* populations differ in growth, photosynthetic responses and phenotypic plasticity to nitrogen availability

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Abstract. Two geographically distinct populations of the submerged aquatic macrophyte *Ceratophyllum demersum* L. were compared after acclimation to five different nitrogen concentrations (0.005, 0.02, 0.05, 0.1 and 0.2 mM N) in a common garden setup. The two populations were an apparent invasive population from New Zealand (NZ) and a noninvasive population from Denmark (DK). The populations were compared with a focus on both morphological and physiological traits. The NZ population had higher relative growth rates (RGRs) and photosynthesis rates (P_{max}) (range: RGR, 0.06–0.08 per day; P_{max}, 200–395 μmol O_2 g^{-1} dry mass (DM) h^{-1}) compared with the Danish population (range: RGR, 0.02–0.05 per day; P_{max}, 88–169 μmol O_2 g^{-1} DM h^{-1}). The larger, faster-growing NZ population also showed higher plasticity than the DK population in response to nitrogen in traits important for growth. Hence, the observed differences in growth behaviour between the two populations are a result of genetic differences and differences in their level of plasticity. Here, we show that two populations of the same species from similar climates but different geographical areas can differ in several ecophysiological traits after growth in a common garden setup.

Additional keywords: acclimation, hornwort, phenotypic plasticity, photosynthesis, submerged macrophyte.

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Introduction

Ceratophyllum demersum L. (common hornwort) is a cosmopolitan submerged macrophyte found in fresh waters in most parts of the world. Its geographic distribution extends from cold temperate regions to the tropics (Les 1989) and from oligotrophic to hypertrophic waters (de Winton *et al*. 2009). It is well known that separate populations of individual species from geographically distinct regions may behave differently in their respective local environments (Cook 1990; Santamaría *et al*. 2003). This may be because the plants have evolved into local ecotypes adapted to the specific environment (i.e. genetically determined differences or ecotypic differentiation), but can also be due to phenotypic plasticity (Sultan 2000). Phenotypic plasticity is the propensity of a genotype to express distinct phenotypes when exposed to different environmental conditions (Richards *et al*. 2006). The production of distinct phenotypes occasionally results in plants behaving invasively if a new habitat outside the native range is colonised. Invasive plants (i.e. non-native introduced species or populations which, due to vigorous growth and spreading potential, cause negative economic or ecological effects in the introduced area (Richardson *et al*. 2000; Lockwood *et al*. 2007)) can occupy other niches, have a different growth form, grow significantly faster become larger, have different morphology and to be more competitive than plants in their natural ranges (Dawson and Warman 1987). Also, invasive populations or species are often better at exploiting limiting resources, such as nitrogen (Pattison *et al*. 1998).

Non-native species of aquatic plants often behave invasively in introduced ranges as they create monocultures through high fecundity and competition, negatively affecting the biodiversity and ecological functions of aquatic ecosystems (Dawson and Warman 1987; Cook 1990; Maron *et al*. 2004; Clayton and Edwards 2006). In contrast, the behaviour of the same species is often relatively benign in its natural range, even though it may also create monocultures in those regions. When 'invasive' refers intraspecifically to a population of a species rather than the whole species, it is not an inherent property of the population genotype; rather, it is a description of the phenotypic characteristics of the population in the invaded area. In other words, the invasive population may not necessarily behave invasively if it was moved or spread to other regions. Common garden experiments, where plants from different regions are grown under similar conditions, may be used to distinguish between these two scenarios (i.e. local ecotypes *vs* high phenotypic plasticity).

In this study, we therefore compared the response to nitrogen enrichment of two geographically distinct populations of *C. demersum*. We used an invasive introduced population from New Zealand (NZ) and a noninvasive native population from Denmark (DK). Although *C. demersum* occurs in most parts of the world, it was introduced to New Zealand only 60 years ago, where it now causes serious problems for indigenous biodiversity and is considered one of the worst aquatic pest plants (de Winton *et al*. 2009). By comparing two populations of the same species, we aimed to explore physiological differences between slow-growing and fast-growing genotypes of the same species, and how these relate to aspects of growth and fitness that may be important in invasions. We hypothesised that (1) the NZ population with the invasive behaviour would have higher growth rates than the DK population after growth in a common garden, and (2) that the NZ population would have higher plasticity in key traits that enable plants to exploit enhanced nitrogen levels. We focus on the differences between the two populations across all treatment levels, but we also evaluate the plasticity of single plant parameters in relation to nitrogen availability.

Materials and methods

Plant material

Shoots of the NZ *Ceratophyllum demersum* L. were collected in the Waikato region of the North Island, New Zealand, and shipped to Denmark in January 2008. Danish *C. demersum* was collected in September 2008 from a small pond in Risskov (Aarhus, Jutland, Denmark). For the common garden setup, plants were rinsed carefully in tap water and grown free-floating in separate 21-L tanks with two-thirds demineralised water and one-third tap water. A commercial nutrient solution for aquarium plants (TropicaAquacare Plant Nutrition+, Tropica Aquarium Plants, Egaa, Denmark) containing all essential macro- and micronutrients was added three times weekly to reach a nitrogen concentration of 99.5 μM and a phosphorus concentration of 3.4 μM. The alkalinity of the medium was 1.0 meq L^{-1} and pH ranged from 7.5 to 8.0. The tanks were continuously aerated with atmospheric air and placed in a growth chamber at ~19°C and a PPFD of 130 μmol m^{-2} s^{-1} (400–700 nm) at the water surface, as measured with a calibrated Li-Cor LI-190SA quantum sensor (Li-Cor Inc. Lincoln, NE, USA), which is a light regime typical for populations of this species and other similar submerged macrophytes in the field (Sand-Jensen 1989). Light was provided by metal halide bulbs in a 14 h light–10 h dark cycle until initiation of the preacclimation period. The water was renewed weekly to minimise growth of algae.

Nitrogen treatment

In September 2009, plants were removed from the common garden tanks and transported to a temperature-controlled room (18°C) in the laboratory. We used 6-L aquaria illuminated by parabolic banks of Philips TL5 High Output fluorescent growth tubes (Philips Electronics NV, Eindhoven, The Netherlands) that provided a PPFD of 200 μmol m^{-2} s^{-1} at the water's surface. The basic growth medium used throughout all subsequent preincubation and experimental treatments was the standard N- and P-free general purpose culture solution of Smart and Barko (1985), but with the alkalinity raised from 0.85 to 1.2 meq L^{-1}. To this solution, we added 0.02 mM P as KH_2PO_4 plus a commercial micronutrient solution for aquarium plants (Plant Nutrition *liquid*, TropicaAquacare) and added N as NH_4NO_3 to generate five distinct N treatments (0.005, 0.02, 0.05, 0.1 and 0.2 mM N). For each treatment, 15 apical shoots of each population were mounted upright in plastic nets and grown in separate duplicate aquaria. The aquaria were continuously aerated with atmospheric air and the water was renewed three times weekly.

The plants were allowed to acclimate to the treatment N concentrations over a 2-week preincubation period. The experiment was then initiated by cutting the apical shoots to 50 mm in length and removing any attached algae with a fine brush. Fresh mass (FM) was then determined for all shoots. From the 30 shoots of each population from each nutrient level, 10 were frozen for later dry mass (DM) determination to estimate initial DM : FM ratio, and the remaining 20 were divided into two groups and were again mounted upright in plastic nets and placed in two separate aquaria. Throughout the 3-week experimental period, all growth conditions were constant and identical to the preincubation period as described above.

Growth and physiological responses

Morphology

Specific leaf area (SLA) was determined on the same shoots as used for light response curves (see the 'Light response curves' section). We used 10–12 mature leaves from the apical part of four shoots from each treatment. One-sided leaf area was analysed using a scanner (HPScanjet G4050, Hewlett-Packard Company, Palo Alto, CA, USA) and the WinFolia Leaf Area Analysis software (Regent Instruments, 2005, www.regentinstruments.com). Subsequently, the leaves were freeze-dried and weighed. The length of the main shoot and the number and length of sideshoots were determined for all shoots used for photosynthesis measurements (see below).

Total carbon and nitrogen concentration

For measurements of total tissue N and C contents, plants were harvested and stored in liquid nitrogen until analysis. Total tissue N and C concentrations were determined on ~2 mg homogenised freeze-dried material using a NA 2000 N-protein CN analyser (Fisons Instruments SpA, Rodano, Italy).

Gas exchange

After 20–22 days of growth at the different N levels, light-saturated rates of photosynthesis (P_{max}) were measured as oxygen (O_2) production during incubation at 18°C. Dark respiration (R_d) was measured as O_2 consumption during incubation in total darkness for 2 h at the ambient CO_2 concentration (16 μM free CO_2) before photosynthesis measurements. Two to three shoots from each of the 20 aquaria were used for these measurements (i.e. five shoots in total per N level per population). Apical shoots were cut to ~25 mm in length and incubated in separate 30-mL glass-stoppered bottles containing modified standard medium (Smart and Barko 1985) with an alkalinity of 1.2 meq L^{-1} and a saturating

CO_2 (700 µM free CO_2) after adjustment to pH 6.2 with 1 M HCl. The remaining part of each shoot was weighed (FM) and frozen for later DM determination. The incubation time was adjusted to allow a change in O_2 concentration of ~50–100 µM during incubation. The bottles were mounted on a rotating wheel in a thermostatic incubator at a saturating PPFD of 250 µmol m^{-2} s^{-1} provided by fluorescent tubes. Five glass beads (diameter = 5 mm) were added to each bottle to ensure stirring. The incubation medium in the bottles was completely renewed with temperature-adjusted and aerated fresh medium between measurements of R_d and P_{max}. After each incubation, the O_2 concentration was measured with a temperature-calibrated Clark-type oxygen microelectrode (Type OX-500, Unisense A/S, Aarhus, Denmark). Oxygen production was subsequently calculated by subtracting the O_2 concentration in control bottles incubated without plant material. The apical shoots were weighed (FM) and frozen and were later freeze-dried for DM and chlorophyll determination (see below).

Light response curves

Photosynthetic light responses were measured from the O_2 exchange rates of the apical 25 mm of shoots in Rank oxygen electrode chambers (Rank Brothers Ltd, Cambridge, England), using 15-W white halogen light sources (Osram AG, Munich, Germany) to provide six PPFDs in the range of 0–420 µmol m^{-2} s^{-1}. Experiments were conducted at 20°C using 20 mL of the medium described above (alkalinity = 1.2 meq L^{-1}, pH = 7.9, $[CO_2] = 0.034$ mM, $[HCO_3^-] = 1.19$ mM). The O_2 concentrations in the chambers were in the range of 250–350 µM (85–120% saturation) during experiments. Photosynthesis–irradiance curves were fitted to the experimental data in SigmaPlot 8.0 (SPSS Inc., Chicago, IL, USA) using the tangential hyperbolic formula of Jassby and Platt (1976) modified to include respiration (Schwarz *et al.* 1996) to produce estimates of the light-saturated rate of net photosynthesis (P_{sat}), the initial slope of the curve (α), the onset of light saturation (I_k) and the light compensation point (I_c).

pH drift experiment

The influence of N level on the ability of *C. demersum* to utilise bicarbonate (HCO_3^-) was assessed by means of simple pH drift experiments (Allen and Spence 1981). Following measurements of R_d and P_{max}, the medium in the incubation bottles was renewed with a temperature-adjusted medium that had been aerated with atmospheric air to ensure equilibrium with atmospheric CO_2 and O_2. A small air bubble was left in each incubation bottle to prevent oversaturation of O_2 during the long incubation time. The shoots were incubated in the light for 20–24 h as described for the gas exchange measurements above. Final pH was measured using a combination pH-electrode (PHC2001–8, PHM 92 LABORATORY pH meter, MeterLab, Radiometer Analytical A/S, Copenhagen, Denmark), and alkalinity was analysed by Gran plot titration, using a TIM 850 Titration Master (Radiometer Analytical SA, Villeurbanne, France). After the measurements, each shoot was weighed (FM) and then frozen and later freeze-dried for DM determination and chlorophyll extraction.

Rubisco activity

The activity of Rubisco was analysed in plant material sampled in the light at mid-afternoon and frozen immediately in liquid N_2. Five shoots per N level per population were harvested. The frozen plants were ground to powder in liquid N_2 with a mortar and pestle. Subsequently, a portion of the powder was homogenised using a prechilled Ten Broeck homogenisator (Wheaton, Millville, NJ, USA) with 5 mL of extraction buffer, stored on ice, containing 50 mM CO_2-free Bicine (pH 8.0; Sigma-Aldrich, St Louis, MO, USA), 10 mM $MgCl_2$, 5 mM DTT, 10 mM isoascorbate, 0.1 mM EDTA and 2% (w/v) polyvinyl-pyrrolidone-40. Aliquots of the homogenates were taken for chlorophyll determination. Assays were performed at 18°C in 6-mL plastic vials containing 300 µL of the assay solution (50 mM CO_2-free Bicine (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT and 0.1 mM EDTA) and 50 µL 192.5 mM $NaH^{14}CO_3$ (specific activity 16×10^6 kBq $mmol^{-1}$). Nonactivated Rubisco activity ($Rubisco_{non}$) was determined by adding 100 µL of the plant extract and 50 µL 5 mM D-Ribulose-1,5-bisphosphate sodium salt hydrate (RuDP) simultaneously to the assay mixture. Total Rubisco activity ($Rubisco_{tot}$) was determined by incubating the plant extract in a buffer solution for 5 min at 18°C before addition of RuDP. The reactions were stopped after 60 s by addition of 100 µL 6 M HCl. The samples were evaporated to desiccation at 60°C for 24 h and then redissolved in two drops of 6 M KOH and 1.2 mL milli-Q water (Milli-Q Gradient, Millipore A/S, Copenhagen, Denmark) and then 3.2 mL scintillation liquid (Insta-gel Plus, PerkinElmer Life and Analytical Sciences, Shelton, CT, USA) was added. The radioactivity of the bound acid-stable ^{14}C was determined by liquid scintillation counting (Liquid Scintillation Analyzer, Tri-CARB 2100 TR, Packard Instruments Company, Meriden, CT, USA). The chlorophyll concentration in each sample was determined by spectrometry after extraction in 96% ethanol in darkness for several hours to enable expression of Rubisco activity on a DM basis using the chlorophyll concentration determined for plant material used for photosynthesis measurements (see below).

Dry matter and pigments

Fresh mass, DM and chlorophyll concentration were determined for all shoots used in the photosynthesis measurements. The DM of the shoots was measured after freeze-drying. Subsequently, the plant material was ground and the concentrations of chlorophyll *a* and *b* were determined spectrophotometrically after extraction overnight in the darkness in 96% ethanol according to Lichtenthaler (1987).

Data analysis

Data were analysed by two-way ANOVA using the software Statgraphics Centurion XV (Statpoint Technologies, Inc., Warrenton, VA, USA) with N level and population as the main factors. Variance homogeneity was tested using Levene's test and data were log-transformed if necessary. The final pH values from the pH drift experiments were converted to H^+ concentration before the data analysis was conducted. Tukey's Honestly Significant Difference (HSD) procedure was used as a *post hoc* multiple range test to identify significant differences between mean values at the 5% significance level. A nested

analysis, with the two replicate aquaria nested in the population for each N level, was used. The aquarium effect was only significant for the DM : FM ratio. For all other parameters, the two replicate aquaria were pooled for the statistical analysis.

To assess the degree of plasticity, the environmentally standardised plasticity index (ESPI) was calculated for selected parameters as follows: $ESPI = (max - min)\ distance^{-1}$ mM^{-1} N, where *min* and *max* were the minimum and maximum values of the population means of the respective parameter, and *distance* was the absolute distance between the N treatment levels at which maximum and minimum mean values were found (Valladares *et al*. 2006). The ESPI is often used for quantifying the phenotypic change for a given environmental change, in this case, N concentration in the media.

Relative growth rate (RGR) was calculated as $(\ln M_2 - \ln M_1)\ t^{-1}$, where M_1 and M_2 were the initial and final DM of the shoot, respectively, and t was the incubation time in days. M_1 was estimated from initial FM and the DM : FM ratios determined for plants harvested at the initiation of the experiment. Photosynthetic nitrogen use efficiency (PNUE) was calculated as P_{max} divided by the tissue N concentration of the plant using treatment mean values, as tissue N concentrations and photosynthesis were measured on different plant individuals within each treatment. Respiration efficiency (RE) was calculated as $P_{max}\ R_d^{-1}$.

Results

Nitrogen concentration in the growth medium significantly affected the growth, morphology and ecophysiology of the two *C. demersum* populations, but overall differences between the DK and NZ populations were also apparent (Table 1). For most growth parameters, the DK and the NZ populations responded differently to the N treatments, as indicated by the significant interaction terms in the ANOVA (Table 1).

Relative growth rate and morphology

The RGRs of the two populations of *C. demersum* were significantly affected by N level (Table 1). Generally, RGR was lowest for both populations when grown at low N and increased at higher N levels, although maximum RGR was found at 0.05 mM N and 0.1 mM N for the NZ and DK populations, respectively. The plot of RGR versus tissue N concentration shows that the RGRs were not severely reduced at the lowest N level, and it was therefore not possible to determine a critical tissue N concentration for growth (Fig. 1*a*).

Table 1. Results of ANOVA (*F*-ratios) showing the effects of population (Pop), N level in the water (N) and their interaction (Pop × N) on growth, photosynthesis, Rubisco activities and pigment concentrations in *Ceratophyllum demersum*

df, degrees of freedom; P_{max}, maximum photosynthetic rate; R_d, dark respiration; RE, respiratory efficiency; P_{sat}, light-saturated rate of net photosynthesis; α, initial slope of the photosynthesis–irradiance curve; I_c, light compensation point; I_k, onset of light saturation; $Rubisco_{non}$, nonactivated Rubisco activity; $Rubisco_{tot}$, total Rubisco activity; DM : FM ratio, ratio of dry mass to fresh mass. Level of significance: NS, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Parameter	df	Pop	N	Pop × N
RGR	1, 4, 108	462.2***	16.6***	3.8**
Main stem length	1, 4, 112	243.8***	11.0***	2.6**
Total stem length	1, 4, 112	669.9***	7.5***	2.9*
SLA[A]	1, 2, 18	123.9***	51.9***	6.2**
Tissue parameter				
%N	1, 4, 36	43.5***	233.3***	9.1***
%C	1, 4, 36	0.2[NS]	1.0[NS]	4.6**
Photosynthesis				
P_{max}	1, 4, 40	194.6***	11.9***	2.1[NS]
R_d	1, 4, 40	22.9***	10.5***	0.4[NS]
RE	1, 4, 40	7.2*	1.1[NS]	1.2[NS]
P_{sat}[A]	1, 2, 18	72.7***	45.7***	4.7*
α[A]	1, 2, 18	24.0***	2.1[NS]	1.6[NS]
I_c[A]	1, 2, 18	4.6*	4.9*	0.5[NS]
I_k[A]	1, 2, 18	8.5**	16.6***	1.5[NS]
Rubisco				
$Rubisco_{non}$	1, 4, 38	104.3***	58.6***	7.2***
$Rubisco_{tot}$	1, 4, 38	112.3***	127.6***	5.4**
Rubisco activation level	1, 4, 38	14.5***	4.2**	0.9[NS]
Dry matter and pigment content				
DM : FM ratio	1, 4, 40	1.4[NS]	29.0***	4.6**
Chl *a*	1, 4, 64	6.5*	93.2***	0.1[NS]
Chl *a* + *b*	1, 4, 64	8.4**	97.3***	0.0[NS]
Chl *a*/*b* ratio	1, 4, 64	15.4***	3.8**	1.6[NS]

[A]Only measured for plants grown at 0.005, 0.05 and 0.2 mM N.

The RGRs of the NZ population were consistently higher (1.6–3.8 times higher) than the RGRs of the DK population. The NZ population also had significantly longer main stem lengths (1.4–2.0 times that of the DK population; Table 2) and a significantly higher number of sideshoots (3–12 sideshoots in the NZ population compared with 0–1 in the DK plants). Consequently, the NZ plants had significantly higher total stem length (2.9–4.5 times) for all N levels (Table 1, Fig. 1*b*). Also the leaves of the NZ population were generally thinner than those of the DK population as indicated by the significantly higher (1.5–2.4 times) SLA of the NZ population at all N levels (only determined at 0.005, 0.05 and 0.2 mM N) (Table 1, Fig. 1*c*). The SLA increased significantly with N concentration in both populations (Fig. 1*c*).

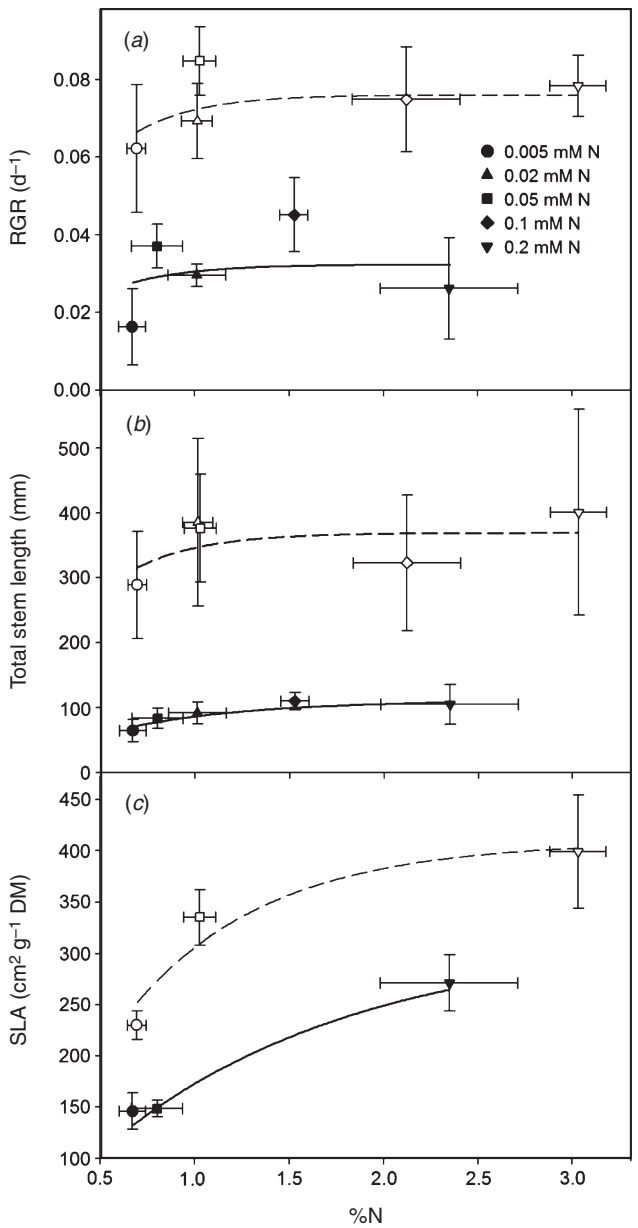

Fig. 1. (*a*) Relative growth rate (RGR), (*b*) total stem length and (*c*) specific leaf area (SLA) of two populations of *C. demersum* grown at five different levels of nitrogen plotted against tissue N concentration. Black symbols, Danish (DK) plants; white symbols, New Zealand (NZ) plants (mean values ± s.d.). Lines of best fit (exponential equations with two parameters, $y = a(1 - e^{-bx})$) have been added to illustrate trends in the responses.

Total carbon and nitrogen content

Concentrations of total C did not differ significantly between the two populations and were not affected by the treatment (tissue C range: DK, 40.5–41.5%; NZ, 40.3–41.2%, Table 1). Tissue N concentration was similar for the two population at 0.005, 0.02 and 0.05 mM N (0.67–1.02% N) but increased more for the NZ population at 0.1 and 0.2 mM N levels than for the DK population (Table 1; Figs 1, 2).

Gas exchange

P_{max}, measured as maximum rates of photosynthesis at saturating light intensity and 700 µM free CO_2, differed significantly between the two populations at all N levels (Table 1, Fig. 2*a*). P_{max} increased with increasing N concentration in the water; however, this was only significant for the NZ population. The highest P_{max} was recorded for NZ plants growing at 0.2 mM N (395 µmol O_2 g^{-1} DM h^{-1}) which was 2.3 times higher than P_{max} of the DK population at the same N level. R_d was also affected by N level in both populations and increased at higher N (Tables 1, 2). Respiration was generally higher in the NZ population. RE differed significantly between populations, though with no significant differences at the individual N levels (Tables 1, 2). Photosynthetic nitrogen use efficiency (PNUE) was consistently approximately two times higher in NZ plants than in DK plants (1.8–2.4 times). Both for NZ and DK plants, the PNUE decreased at higher N levels (range: DK, 7.2–13.5 µmol O_2 mg^{-1} N h^{-1}; NZ, 13.0–29.3 µmol O_2 mg^{-1} N h^{-1}).

Light response curves

P_{sat} measured at ambient inorganic C concentrations resembled the response of P_{max}, with increasing rates at increasing N levels and NZ plants having significantly higher rates than DK plants, except at 0.005 mM N (Table 1 and 2, Fig. 3*a*, *b*). Overall, I_c, I_k and α were all significantly higher for the NZ population (Table 1), but not at the individual N levels (Table 2).

Rubisco activity

The activities of nonactivated Rubisco ($Rubisco_{non}$) and total Rubisco ($Rubisco_{tot}$) were significantly affected by N level for both populations (Tables 1, 2; Fig. 2*b*). The Rubisco activity generally increased with increasing N levels. The highest activities of $Rubisco_{non}$ and $Rubisco_{tot}$ were recorded at 0.1 and 0.2 mM N, respectively, for the DK population and at 0.2 mM N for the NZ population (Table 2, Fig. 2*b*). The Rubisco activity was 1.2–2.4 times higher for the NZ population than the DK population. The activation state of Rubisco (calculated as $Rubisco_{non}$ $Rubisco_{tot}^{-1}$) was generally higher for the NZ population, but this was not statistically significant at the individual N levels (Table 1 and 2). No clear

Table 2. Mean values (± s.d.) for different physiological and morphological parameters of two populations of *C. demersum* (from Denmark (DK) and New Zealand (NZ)) grown at five different levels of nitrogen (0.005, 0.02, 0.05, 0.1 and 0.2 mM N) for 20–22 days

Different superscript letters indicate statistical significant differences between mean values at the 5% significance level (*post hoc* Tukey's Honestly Significant Difference test, one-way ANOVA). P_{sat}, light-saturated rate of net photosynthesis; α, initial slope of the photosynthesis–irradiance curve; I_c, light compensation point; I_k, onset of light saturation; R_d, dark respiration; RE, respiration efficiency; $Rubisco_{non}$, nonactivated Rubisco activity; DM : FM ratio, ratio of dry mass to fresh mass; DM, dry mass

Parameter	DK population					NZ population				
	0.005	0.02	0.05	0.1	0.2	0.005	0.02	0.05	0.1	0.2
Main stem length (mm)	$63^{a} \pm 14$	$92^{bc} \pm 17$	$78^{ab} \pm 13$	$103^{c} \pm 9$	$102^{bc} \pm 26$	$126^{d} \pm 16$	$151^{e} \pm 27$	$141^{de} \pm 17$	$141^{de} \pm 21$	$144^{de} \pm 17$
Photosynthesis										
P_{sat} (μmol O_2 g^{-1} DM h^{-1})[A]	$68^{a} \pm 15$	–	$102^{a} \pm 35$	–	$178^{bc} \pm 30$	$128^{ab} \pm 52$	–	$233^{c} \pm 36$	–	$337^{d} \pm 19$
$\alpha^{A,B}$	$1.8^{ab} \pm 0.2$	–	$1.4^{a} \pm 0.3$	–	$2.4^{abc} \pm 0.6$	$2.7^{bc} \pm 0.9$	–	$2.9^{bc} \pm 0.5$	–	$3.0^{c} \pm 0.2$
I_c (μmol photons m^{-2} s^{-1})[A]	$12.1^{a} \pm 5.1$	–	$16.5^{ab} \pm 2.7$	–	$15.2^{ab} \pm 1.2$	$14.4^{ab} \pm 2.6$	–	$17.8^{ab} \pm 2.9$	–	$19.4^{b} \pm 1.5$
I_k (μmol photons m^{-2} s^{-1})[A]	$51^{a} \pm 10$	–	$90^{ab} \pm 8$	–	$92^{abc} \pm 8$	$67^{ab} \pm 31$	–	$101^{bc} \pm 27$	–	$134^{c} \pm 15$
R_d (μmol O_2 g^{-1} DM h^{-1})	$20^{a} \pm 6$	$38^{ab} \pm 16$	$35^{ab} \pm 7$	$55^{bc} \pm 5$	$49^{bc} \pm 15$	$44^{abc} \pm 12$	$52^{bc} \pm 12$	$48^{bc} \pm 20$	$67^{c} \pm 10$	$65^{c} \pm 5$
RE	$4.9^{ab} \pm 1.8$	$3.9^{ab} \pm 2.0$	$3.3^{ab} \pm 1.7$	$2.0^{a} \pm 0.6$	$4.0^{ab} \pm 2.4$	$4.6^{ab} \pm 0.9$	$5.8^{ab} \pm 1.1$	$9.1^{b} \pm 9.0$	$4.4^{ab} \pm 0.6$	$6.1^{ab} \pm 0.8$
Rubisco										
$Rubisco_{non}$ (μmol C g^{-1} DM h^{-1})	$48^{a} \pm 8$	$139^{bc} \pm 23$	$118^{ab} \pm 37$	$218^{d} \pm 21$	$182^{bcd} \pm 43$	$112^{ab} \pm 41$	$197^{cd} \pm 17$	$183^{bcd} \pm 32$	$363^{e} \pm 47$	$383^{e} \pm 59$
Rubisco activation state (%)	$34^{a} \pm 3$	$45^{ab} \pm 4$	$44^{ab} \pm 9$	$46^{ab} \pm 9$	$35^{a} \pm 6$	$42^{ab} \pm 12$	$53^{b} \pm 4$	$47^{ab} \pm 7$	$53^{b} \pm 5$	$50^{ab} \pm 7$
Dry matter and pigment content										
DM : FM ratio	$0.20^{c} \pm 0.04$	$0.11^{ab} \pm 0.03$	$0.12^{ab} \pm 0.03$	$0.08^{a} \pm 0.01$	$0.08^{a} \pm 0.02$	$0.14^{b} \pm 0.02$	$0.11^{ab} \pm 0.02$	$0.12^{ab} \pm 0.02$	$0.09^{a} \pm 0.01$	$0.09^{a} \pm 0.01$
Chl *a* (mg chl g^{-1} DM)	$2.2^{a} \pm 0.4$	$3.8^{bc} \pm 0.7$	$3.6^{bc} \pm 0.8$	$5.4^{de} \pm 0.7$	$6.5^{ef} \pm 1.1$	$2.6^{ab} \pm 0.5$	$4.1^{cd} \pm 0.8$	$4.1^{cd} \pm 0.4$	$5.7^{e} \pm 1.0$	$7.1^{f} \pm 0.8$
Chl *a* + *b* (mg chl g^{-1} DM)	$2.9^{a} \pm 0.5$	$5.2^{bc} \pm 0.9$	$4.9^{bc} \pm 1.1$	$7.4^{de} \pm 0.9$	$8.9^{ef} \pm 1.4$	$3.6^{ab} \pm 0.7$	$5.8^{cd} \pm 1.1$	$5.6^{cd} \pm 0.5$	$7.9^{ef} \pm 1.3$	$9.7^{f} \pm 1.1$
Chl *a*/*b* ratio	$2.9^{c} \pm 0.1$	$2.7^{abc} \pm 0.08$	$2.8^{bc} \pm 0.1$	$2.7^{abc} \pm 0.1$	$2.8^{abc} \pm 0.1$	$2.7^{ab} \pm 0.2$	$2.6^{ab} \pm 0.1$	$2.8^{abc} \pm 0.2$	$2.6^{a} \pm 0.1$	$2.7^{abc} \pm 0.1$

[A]Only measured for plants grown at 0.005, 0.05 and 0.2 mM N.
[B](μmol O_2 g^{-1} DM h^{-1}) (μmol photons m^{-2} s^{-1})$^{-1}$.

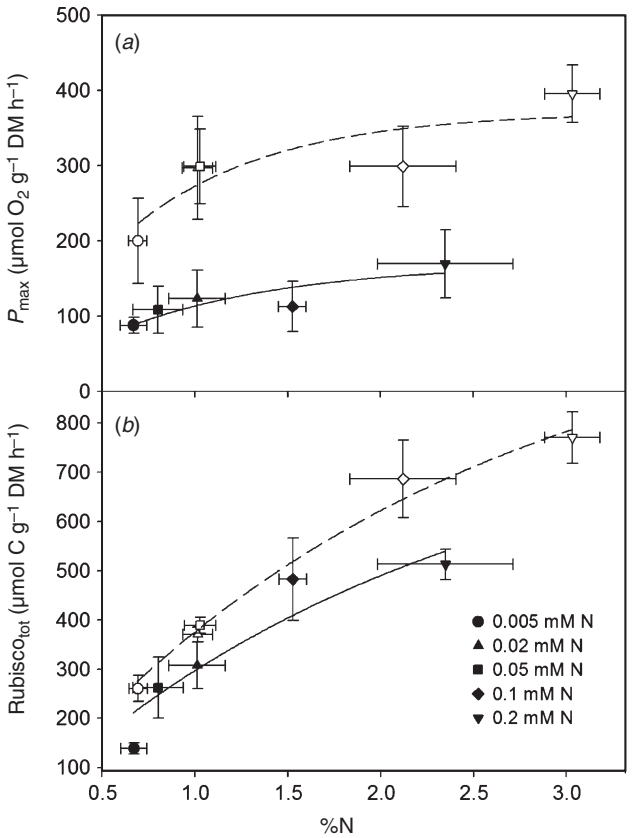

Fig. 2. (*a*) Maximum net photosynthesis rate measured at 700 μM free CO_2 (P_{max}) and (*b*) total Rubisco activity of two populations of *C. demersum* grown at five different levels of nitrogen plotted against tissue N concentration. Black symbols, Danish (DK) plants; white symbols, New Zealand (NZ) plants (mean values ± s.d.). Lines of best fit (exponential equations with two parameters, $y = a(1 - e^{-bx})$) have been added to illustrate trends in the responses.

trend in the response of Rubisco activation state to N level was observed for the two populations.

Dry matter and pigment content

Overall, the DM : FM ratio did not differ between the two populations but it was significantly higher for the DK population at 0.005 mM N (Tables 1, 2). The DM : FM ratios decreased at higher N levels. The concentration of chl *a* increased for both populations at higher N levels and was overall significantly higher for the NZ population (Tables 1, 2). The response of total chlorophylls (Chl $a + b$) to N was similar to chl *a* (Table 2). Overall, the chlorophyll *a/b* ratio was significantly higher for the DK population than for the NZ population (Tables 1, 2).

Bicarbonate utilisation

The N treatments affected the ability of the plants to utilise bicarbonate, as the final pH in the pH drift experiment was significantly higher for plants grown at high N compared with plants grown at low N (two-way ANOVA; *F*-ratios: $F_{Population}$, 2.26 (not significant), $F_{N\ level}$, 15.25 (significant at $P = 0.001$), $F_{Pop \times N\ level}$, 0.94 (not significant)). The average final pH increased from 10.52 to 10.78 in the DK population and from 10.55 to 10.74 in the NZ plants when the lowest and highest N treatments were compared. All plants had a very high HCO_3^- affinity, and there were no significant differences between the two populations at the individual N levels.

Phenotypic plasticity

The ESPI was calculated for a set of important growth parameters (Fig. 4). The ESPI shows that the plasticity for parameters of importance for resource utilisation (RGR, SLA, total stem length, P_{max} and $Rubisco_{tot}$) was higher for the faster-growing NZ population than for the DK population. The index values were, however, lower in the NZ population for the rest of

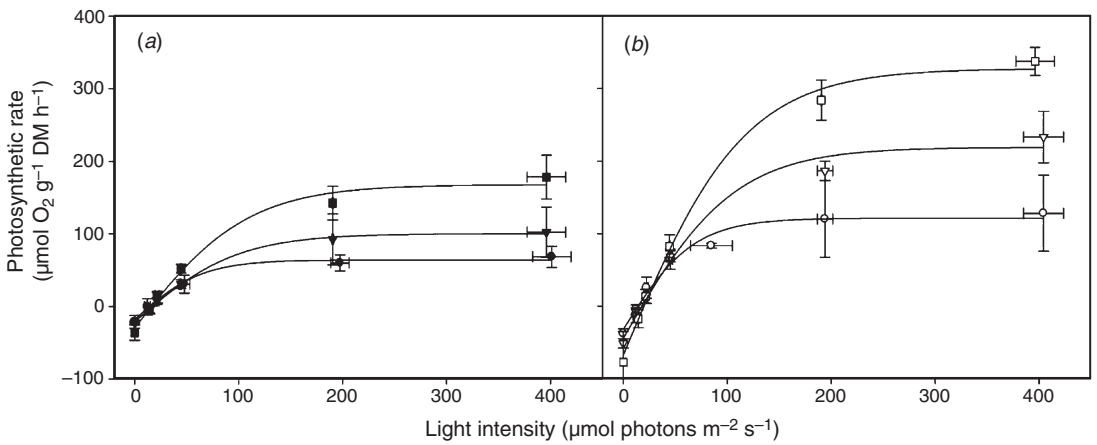

Fig. 3. Light response curves determined for two populations of *C. demersum* grown at 0.005 (circles), 0.05 (triangles) or 0.2 mM (squares) N using six different irradiance levels in the range of 0–420 μmol m^{-2} s^{-1} (PAR). (*a*) Danish (DK) plants; (*b*) New Zealand (NZ) plants (mean values ± s.d.). Photosynthesis–irradiance curves are fitted according to Jassby and Platt (1976), modified to include respiration $r > 0.98$; $P < 0.001$ in all cases.

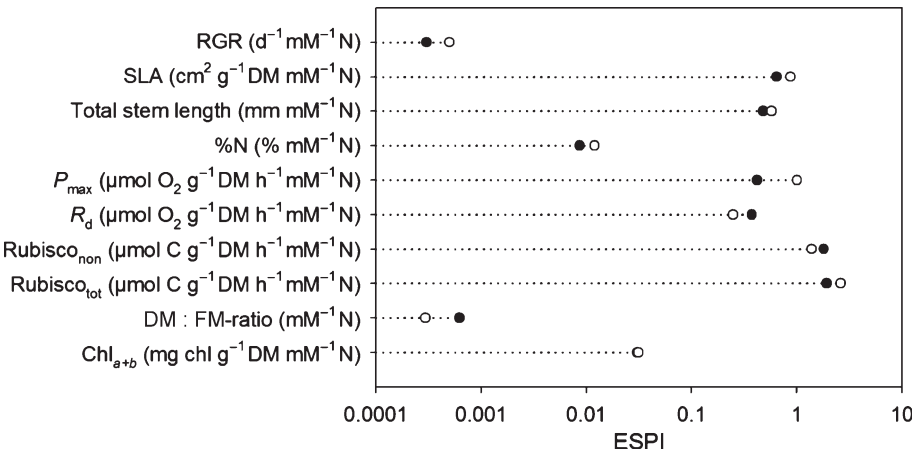

Fig. 4. The environmentally standardised plasticity index (ESPI) for a set of plant parameters (Valladares *et al*. 2006). Black symbols, Danish (DK) plants; white symbols, New Zealand (NZ) plants. RGR, relative growth rate; SLA, specific leaf area; P_{max}, maximum photosynthetic rate; R_d, dark respiration; DM : FM ratio, ratio of dry mass to fresh mass; $Rubisco_{non}$, nonactivated Rubisco activity; $Rubisco_{tot}$, total Rubisco activity.

the parameters. Hence, the plasticity was not consistently higher in the NZ population for all plant traits.

Discussion

The two populations of *C. demersum* with apparent invasive and noninvasive behaviour in their present local growth environments in New Zealand and Denmark, respectively, showed unambiguous differences in a range of ecophysiological plant traits, and they responded differently to nitrogen availability, despite an acclimation phase more than 1 year long under identical conditions in the laboratory before the experiment.

Apparent differences in geographically distinct populations may be caused either by phenotypic plasticity or by genetic differences. Phenotypic plasticity results in the production of different phenotypes when the plants experience different environmental conditions, whereas genetic differences arise as a result of evolution and creates local ecotypes with associated genetically determined differences in ecophysiological characteristics. It may also be a combination of these scenarios, where enhanced phenotypic plasticity develops in one of the populations after the separation of the two populations. Several studies have found genetically determined differences in morphology and physiology between populations of the same species that have been geographically separated (e.g. Clevering *et al*. 2001; Maron *et al*. 2004; Atkin *et al*. 2006; Eggert *et al*. 2006; Hansen *et al*. 2007; Hyldgaard and Brix 2012). As a consequence, the study of just a single population of a species and the comparison of different species, but only one population of each, might have limited value, and the results should be treated with caution, as they might have been different if another set of populations was studied. As an example, *C. demersum* has, in one study, been termed a 'nitrophilous' species (Goulder and Boatman 1971), but it may also be found in oligotrophic waters (de Winton *et al*. 2009). In the present experiment, we found no indications for N stress even at the lowest N levels in either of the *C. demersum* populations.

The two populations of *C. demersum* used in this study were grown under similar conditions for at least 1 year before the nitrogen experiment. It is therefore justified to conclude that the ecophysiological differences observed between the populations are caused by genetic differences and not by differences in the local environments at the sites of collection. After growth at five different levels of N, the NZ plants differed overtly from the DK plants in several parameters, including both morphology and physiology. The morphological differences were clearly visible to the naked eye, as the DK plants were generally stiff and dark green with relatively thick crispy leaves and only few sideshoots, whereas the NZ plants had thinner and softer leaves and the stem was more branched. Physiologically, the NZ plants had markedly higher growth rates and photosynthesis rates compared with the DK plants, as also shown in an earlier study focussing on temperature acclimation (Hyldgaard and Brix 2012). Overall, the NZ plants were more vigorous and active, corresponding well with their invasive behaviour in New Zealand. Invasive plants frequently have higher growth rates and photosynthesis rates than noninvasive plants. However, most comparisons have been made between invasive and noninvasive species found in the same area (e.g. Baruch and Goldstein 1999; Durand and Goldstein 2001; Funk and Vitousek 2007; Kennedy *et al*. 2009). In the present study, the invasive characteristics were evident from the higher RGRs, photosynthetic rates (P_{max} and P_{sat}) and SLA. In addition, we also found more apical meristems per plant (more sideshoots), higher Rubisco activities, better light use efficiency (α) and PNUE in the invasive NZ population compared with the DK population. The combination of higher SLA (a greater leaf area produced per unit of DM), Rubisco activities and chlorophyll content per DM should lead to higher photosynthetic rates and higher growth rates, as was indeed seen here. Moreover, despite the high respiration rates we also saw in the NZ population, the REs were not lower in these plants due to the higher net photosynthesis rates compared with the DK plants. Also, the RGRs were relatively high in the NZ plants even at the lowest N levels where tissue N concentration was well below what is

considered normal for natural populations of freshwater macrophytes (Duarte 1992). Although reproductive output and success is often held to be the ultimate determinant of the fitness of a population, biomass is a useful fitness proxy because greater vegetative size is often associated with higher reproductive output (Weiner *et al.* 2009) and successful invaders must eventually accrue significantly more biomass than their native competitors (McKinley and Blair 2008). This is especially true for aquatic species such as *C. demersum*, which rarely ever set seed and whose invasive behaviour is strongly associated with prolific vegetative reproduction. Another typical characteristic of invaders is their ability to be efficient at exploiting limited resources (Funk and Vitousek 2007). NZ *C. demersum* was indeed more efficient than the DK plants at utilising the N taken up, as the PNUE of the NZ plants was high, even at the lowest N levels. The higher PNUE resulted from the higher photosynthetic rates of the NZ population compared with the DK population and were not a result of lower tissue N concentration in the NZ plants. Lastly, invaders have been reported to have high tissue N contents (Baruch and Goldstein 1999; Durand and Goldstein 2001), which was also seen for the NZ population in the present study, especially at the highest N levels. The PNUE values in this study are likely to be fully representative of field plants, as the light intensities under which we grew plants were typical of those in aquatic macrophyte stands in natural conditions. This amalgam of growth and physiological differences seen here between the two populations of *C. demersum* is in accordance with the results achieved by others comparing invasive and noninvasive plants. However, most of the prior studies compare different species, but here we show that it also applies within populations of the same species.

Species or populations possessing inherent high phenotypic plasticity can theoretically inhabit diverse new localities without undergoing genetic adaptation through natural selection, as high plasticity enhances the ecological breadth and thereby the environmental tolerance (Sultan 2000). Hence, an inherent high phenotypic plasticity may also result in differences in behaviour and is a common characteristic of invasive plants. It has therefore lately attracted attention as a possible explanation for the development of invasiveness in plants (Sakai *et al*. 2001; Hulme 2008; Riis *et al*. 2010; Davidson *et al*. 2011). A high level of phenotypic plasticity either enables the plant to make the necessary changes in order to keep their fitness unaltered in a broader spectrum of environmental conditions (the Jack-of-all-trades strategy) or allows individuals to exploit the advantages of favourable conditions better and thereby increase fitness (the master-of-some strategy), compared with individuals with lower levels of plasticity (Richards *et al*. 2006). The phenotypic response pattern of the NZ plants to nitrogen enrichment matches the third alternative strategy, which is a combination called 'Jack-and-master', conferring both robustness in stressful environments and the ability to exploit advantages in favourable conditions. This is seen here as higher fitness, approximated by RGR and photosynthesis, in the NZ plants across all treatment levels. A combination of RGR and photosynthetic rate is a good proxy for fitness for *C. demersum*, as this species reproduces primarily vegetatively through fragmentation (de Winton *et al*. 2009). In an earlier study conducted with the same two *C. demersum* populations, we found that the invasive NZ population exploited a 'master-of-some' strategy in response to temperature (Hyldgaard and Brix 2012). Plasticity in response to a change in the surroundings may differ between individual plant parameters and the environmental conditions in question (Richards *et al*. 2006). Moreover, plasticity in one plant parameter may result in homeostasis in others. We have demonstrated this in our two studies focussing on responses to a range of N concentrations in the media and water temperatures, respectively. In the present study, plasticity was not consistently higher in the invasive NZ population as we hypothesised. However, we found higher plasticity in the parameters that are essential for rapid growth such as RGR, SLA, P_{max}, total stem length and tissue N concentration, all of which are likely to assist the inherently faster-growing NZ population to respond rapidly and behave more invasively as N supply increases. High plasticity in these parameters also helps the plants to take advantage of environments with fluctuating resources and to exploit resources when these are limited. In our earlier study, the NZ plants showed higher plasticity in RGR and P_{max} in response to temperature (Hyldgaard and Brix 2012). Our results therefore show that the differences seen between the two populations in their present local environments are caused by a combination of higher phenotypic plasticity and genetic differences leading to more vigorous growth in the NZ population, differences which are maintained in the common garden experiment with similar environmental conditions.

Conclusions

We have unambiguous documented genetically determined differences in morphology and physiology between the two populations of *C. demersum* collected at geographically distinct locations. We found both higher growth rates and photosynthesis rates combined with higher plasticity in these key parameters, among others, in the NZ population compared with the DK plants. The characteristics of the NZ population agree well with its invasive behaviour in New Zealand. However, the origin of the invasive NZ population has not yet been discovered, and it is therefore not possible to reveal if evolution or the founder effect is involved in the differences seen.

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