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Constraints on the evolution of phenotypic plasticity in the clonal plant *Hydrocotyle vulgaris*

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Abstract

The evolution of phenotypic plasticity of plant traits may be constrained by costs and limits. However, the precise constraints are still unclear for many traits under different ecological contexts. In a glasshouse experiment, we grew ramets of 12 genotypes of a clonal plant *Hydrocotyle vulgaris* under the control (full light and no flood), shade and flood conditions and tested the potential costs and limits of plasticity in 13 morphological and physiological traits in response to light availability and flood variation. In particular, we used multiple regression and correlation analyses to evaluate potential plasticity costs, developmental instability costs and developmental range limits of each trait. We detected significant costs of plasticity in specific petiole length and specific leaf area in response to shade under the full light condition and developmental range limits in specific internode length and intercellular CO_2 concentration in response to light availability variation. However, we did not observe significant costs or limits of plasticity in any of the 13 traits in response to flood variation. Our results suggest that the evolution of phenotypic plasticity in plant traits can be constrained by costs and limits, but such constraints may be infrequent and differ under different environmental contexts.

Introduction

Plants that experience spatial and temporal environmental heterogeneity often express morphological and physiological plasticity (van Kleunen *et al.*, 2007; Hirsch *et al.*, 2013; Venâncio *et al.*, 2016). In particular, adaptive plasticity can be favoured by natural selection, enabling organisms to match their phenotypes to local conditions and overcome environmental stresses (Dorn *et al.*, 2000; Weinig *et al.*, 2004). In an ideal world, an individual always matches its phenotype to the optimum in a given environment (Scheiner & Berrigan, 1998). However, this property is hard to realize in nature owing to some constraints, such as limits to the phenotypic expression of plastic genotypes or costs of the ability to be plastic (DeWitt *et al.*, 1998; Lind & Johansson, 2009; Bongers *et al.*, 2017). Constraints on the evolution of plasticity may reduce the degree of plasticity and shift the competitive advantage between fixed and plastic taxa, further leading to the evolution of specialist genotypes, instead of highly plastic generalist genotypes (Tienderen, 1991; DeWitt *et al.*, 1998; van Kleunen *et al.*, 2000).

The cost of plasticity is depicted as the fitness reduction in plastic organisms relative to fixed organisms with the same phenotypes in a focal environment (Dorn *et al.*, 2000; Bell & Galloway, 2008). It may derive in part from an increased energy requirement for plastic genotypes to maintain the developmental and physiological machinery, produce novel structures or acquire information about the environment (Steinger *et al.*, 2003; Caruso *et al.*, 2009). Moreover, developmental instability costs, that is, reduced developmental stability for more plastic genotypes than less plastic genotypes, can further lead to reduced fitness in plastic organisms (DeWitt *et al.*, 1998; van Kleunen *et al.*, 2000). Plasticity limits are the functional

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constraints that reduce the benefits of plasticity, resulting in suboptimal phenotypes in plastic organisms compared with those with fixed development (DeWitt *et al.*, 1998). Without developmental range limits, plastic genotypes are more likely to produce extreme phenotypes than fixed genotypes. However, when developmental range limits exist, less plastic genotypes can produce more extreme phenotypes than highly plastic genotypes (DeWitt *et al.*, 1998; Auld *et al.*, 2010). This may be generated by more developmental baggage for more plastic genotypes to carry (van Kleunen *et al.*, 2000).

Owing to the ecological and evolutionary implications, constraints on plasticity have attracted much attention (DeWitt *et al.*, 1998; Lind & Johansson, 2009). Some theoretical models have explored the effects of different constraints on the evolution of plasticity, but there is limited empirical information with which to evaluate these constraints (Valladares *et al.*, 2007; Caruso *et al.*, 2009; Lind & Johansson, 2009). Specially, developmental instability costs and developmental range limits are still underappreciated components of the evolution of plasticity (DeWitt *et al.*, 1998). Therefore, additional empirical work on a range of systems is necessary to gain a deeper understanding of the constraints on the evolution of plasticity.

Hydrocotyle vulgaris is considered a potentially invasive species in China owing to its high phenotypic plasticity, rapid clonal reproduction, strong adaptability and competitive exclusion of native species (Miao *et al.*, 2011; Dong *et al.*, 2014; Liu *et al.*, 2014). It often appears on the waterside under the forest canopy where it experiences shade and flood variation; thus, light and flooding are two important ecological factors affecting its survival. In this study, we explored the phenotypic plasticity of *H. vulgaris* in response to light and flood variation. Regression and correlation analyses were then used to estimate the potential costs and limits of the plasticity. Specifically, we asked the following questions: (1) What are the morphological and physiological responses of *H. vulgaris* to different light and flood conditions? (2) Does phenotypic plasticity of these traits vary among genotypes? (3) Are there any costs of plasticity of traits in response to light and flood variations? (4) Are there developmental instability costs and developmental range limits of traits associated with their plasticity?

Materials and methods

The species

Hydrocotyle vulgaris L. (Araliaceae) is a perennial clonal herb, with plagiotropic stems along which each node has the capacity to produce a ramet consisting of a petiolate leaf and adventitious roots (Dong *et al.*, 2013). It originated from Europe and was introduced to China in the 1990s as an ornamental plant (Liu *et al.*, 2014). The species mainly relies on vegetative propagation via stem fragments to form large clones and spread, although it still can flower and may produce viable seeds (Dong *et al.*, 2014; Liu *et al.*, 2014).

Plant material

In November 2014, a total of 216 *H. vulgaris* plants were collected from 20 populations in Zhejiang Province, China. They were cultivated and propagated vegetatively in the glasshouse at Forestry Science Co., Ltd, of Beijing Forestry University in Beijing. After using amplified fragment length polymorphism (AFLP) to identify the genotype of each plant, we selected 12 genotypes for the experiment described below.

Experimental design

In early July 2015, we selected 18 ramets of similar size from each of the 12 genotypes, thus 216 ramets in total. Each ramet consisted of one node, one leaf (10–15 cm in petiole length) and some adventitious roots. The ramets were each planted in a plastic pot (9.5 cm in bottom diameter, 13.5 cm in top diameter and 11.5 cm in height) filled with a 10-cm-deep mixture of turf, vermiculite and quartz sand at 1 : 1 : 1 volume ratio.

The experiment was a randomized design consisting of three treatments (i.e. control, shade and flood) for each genotype. Each treatment had six replicates. The control treatment received full light in the glasshouse, and the shade treatment received only 30% of full light, which was regulated using a black shading net (without changing the light quality). For the flood treatment, pots of 12 different genotypes belonging to the same replicate were placed in one of six independent plastic boxes (66.5 cm in length, 45 cm in width and 35 cm in depth) filled with tap water. The water level was 10 cm above the soil surface; therefore, plant leaves in the flood treatment protruded from the water to receive the same light intensity as that in the control treatment.

The experiment lasted from 13 July to 25 September 2015. During the experiment, tap water was supplied three times per week to keep sufficient water for plant growth. Offspring ramets produced by the same initial (parent) ramet were allowed to root while confined within the pots to prevent them from growing into other pots.

Harvest and measurements

To determine the photosynthetic and transpiration characteristics of *H. vulgaris*, on September 21, an adult leaf in each pot was randomly selected and the net photosynthetic rate, stomatal conductance, intercellular

CO_2 concentration and transpiration rate were measured. The measurements were obtained between 07:00 and 08:00 at 1200 μmol m^{-2} s^{-1} with the built-in LED light source of a portable photosynthesis system LI-6400 (LI-COR, Lincoln, NE, USA). Moreover, two similar adult leaves from each individual were plucked and transported to the laboratory to measure malondialdehyde, superoxide dismutase and soluble sugar content on 9 March 2016. Malondialdehyde, superoxide dismutase and soluble sugar content were determined by thiobarbituric acid test, the nitro blue tetrazolium photoreduction method and the anthrone colorimetry method, respectively (Giannopolitis & Ries, 1977; Li *et al.*, 2000).

At harvest, plants in each pot were sorted into four parts: petioles, stem internodes, leaves and roots. After measuring petiole length, internode length and leaf area in each pot, the four parts were dried separately at 70 °C for 48 h and weighed. Number of ramets (an indicator of vegetative reproductive ability) and total biomass were used as fitness measures. Specific petiole length was calculated as total petiole length divided by total petiole dry mass, specific internode length as total internode length divided by total internode dry mass and specific leaf area as total leaf area divided by total leaf dry mass. Mean petiole length, specific petiole length, mean internode length, specific internode length, mean leaf area and specific leaf area were used as morphological traits.

Statistical analysis

All analyses were implemented in SPSS 19.0 (SPSS, Chicago, IL, USA), except permutation tests, which were carried out using R (version 3.3.2, R Core Team).

Treatment effects

We used MANOVA to assess the effects of treatment (control, shade and flood; fixed effect), genotype (random effect) and treatment × genotype (random effect) on the overall response of *H. vulgaris*. In the MANOVA model, the independent variables were all traits of fitness (number of ramets and total biomass), morphology (mean petiole length, specific petiole length, mean internode length, specific internode length, mean leaf area and specific leaf area) and physiology (net photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, transpiration rate, malondialdehyde, superoxide dismutase and soluble sugar content) of *H. vulgaris*. Following the MANOVA model, the treatment effect was further separated into the shade effect (control vs. shade) and the flood effect (control vs. flood) by two orthogonal planned contrasts, and the treatment × genotype effect was also separated into the shade × genotype effect [(control vs. shade) × genotype] and the flood × genotype effect [(control vs. flood) × genotype] by two planned contrasts (Sokal & Rohlf, 1981). Following MANOVA, the results of ANOVA for each variable were also produced. Data on internode length, specific internode length and soluble sugar content were transformed to logarithms before these analyses.

Plasticity costs

We calculated phenotypic plasticity of a trait i of a genotype j in response to shade and flood ($\bar{P}_{ij}$), respectively, according to the formula of Valladares *et al.* (2000):

$$\bar{P}_{ij} = \frac{\left|\bar{Z}_{ij,1} - \bar{Z}_{ij,2}\right|}{\mathrm{Max}\left\{\bar{Z}_{ij,1}, \bar{Z}_{ij,2}\right\}} \quad (1)$$

where $\bar{Z}_{ij,1}$ is the mean value of a trait i of a genotype j across the replicates in the control treatment, and $\bar{Z}_{ij,2}$ is the mean value of the trait i of the genotype j across the replicates in the shade or flood treatment. In a target environment, costs of plasticity of a trait occur when plasticity of genotypes is negatively correlated with fitness of the genotypes in this environment after ruling out the effect of the difference in trait mean values among genotypes (DeWitt *et al.*, 1998; van Kleunen *et al.*, 2000). The latter is because plasticity of a trait is commonly positively related to mean value of the trait (van Kleunen *et al.*, 2000). To test for potential costs of plasticity of a trait after accounting for the contribution of mean value of the trait to fitness, we thus used the multiple regression model of van Kleunen *et al.* (2000):

$$\overline{W}_{j,k} = \mathrm{Constant}_{ik} + \alpha_{ik}\bar{Z}_{ij,k} + \beta_{ik}\bar{P}_{ij} \quad (2)$$

where $\overline{W}_{j,k}$ is the mean fitness measure of a genotype j in a target environment k (k = the control, shade or flood treatment), $\bar{Z}_{ij,k}$ is the mean value of a trait i of the genotype j in the environment k, $\bar{P}_{ij}$ is plasticity of the trait i of the genotype j in response to the treatment (shade or flood), and $\mathrm{Constant}_{ik}$, α_{ik} and β_{ik} are the constant and regression coefficients of the model for the trait i in the environment k. A significant negative value of the regression coefficient β_{ik} means that the fitness measure is negatively related to plasticity of the trait, suggesting that a cost of plasticity occurs (van Kleunen *et al.*, 2000). As we tested for the significance of only a negative value of β_{ik}, we used one-tailed tests. Moreover, we applied a sequential Bonferroni procedure to P-values to correct for multiple testing (Rice, 1989).

Developmental instability costs

Developmental instability of a genotype in a target environment is referred to as phenotypic variation within the target environment and measured as the standard deviation (SD) of the trait value among its replicates in the target environment (van Kleunen *et al.*, 2000). Genotypes of higher plasticity (in response to a different environment) may be developmentally less stable (within the same environment) than

genotypes of lower plasticity, and developmental instability may negatively affect fitness (DeWitt *et al.*, 1998; van Kleunen *et al.*, 2000). Therefore, we need to test first whether developmental instability and plasticity of the trait are positively related and then whether developmental instability of the trait is negatively related to fitness. Only if both relationships exist, then plasticity is considered to be associated with developmental instability costs. We thus calculated the correlation coefficient between measure of developmental instability (SD) and plasticity for each trait. To avoid spurious correlations caused by mathematical associations of these parameters (van Kleunen *et al.*, 2000), a permutation test was applied by randomly resampling observations with regard to genotypes within a target environment 1000 times. The significance of the correlation was estimated according to the distribution of the resulting 1000 correlation coefficients. If a positive correlation was detected, we further examined the relationship between measure of developmental instability and measure of fitness. We used one-tailed significance tests.

Developmental range limit

For each trait, we calculated the correlation between plasticity of the trait and the average value of the trait over the two environments (control and shade or control and flood). We also carried out second-order polynomial regressions between plasticity of the trait and the average value of the trait over the two environments. A limit of plasticity is indicated by a significant negative correlation or a concave-down relationship (i.e. a negative regression coefficient for the quadratic term) between plasticity and the average value of the trait (van Kleunen *et al.*, 2000). A permutation test was used to examine the significance of the relationships. The significance of coefficients was estimated based on the distribution of the resulting 1000 coefficients for the correlation analysis and the resulting *t*-values for the quadratic term of the polynomial regression (van Kleunen *et al.*, 2000). One-tailed significance tests were used.

Results

Treatment effects and genotypic variation

There were highly significant overall effects of treatment (MANOVA result: Wilk's $\lambda = 0.016$, $F_{30,144} = 33.12$, $P < 0.001$), genotype (Wilk's $\lambda = 0.029$, $F_{165,665} = 1.95$, $P < 0.001$) and their interaction (Wilk's $\lambda = 0.006$, $F_{330,943} = 1.46$, $P < 0.001$) on the traits of *H. vulgaris* (Appendix 1). Plants grown in the shade treatment exhibited lower fitness values (biomass and ramet number), net photosynthetic rate, stomatal conductance and intercellular CO_2 concentration, but higher specific petiole length, specific internode length and specific leaf area than those of plants grown in the control treatment (Table 1, Appendix 1). Variation among genotypes was detected for fitness measures, petiole length, specific petiole length, leaf area and specific leaf area (Appendix 1).

Plants grown in the flood treatment had lower fitness values, soluble sugar content, net photosynthetic rate, stomatal conductance and transpiration rate, but higher specific petiole length, internode length and specific internode length (Table 1, Appendix 1). Moreover, the significant flood–genotype interaction was detected in ramet number, leaf area and specific leaf area (Appendix 1).

Plasticity costs

With respect to variation in light availability, we detected a significant negative regression coefficient for

Table 1 Fitness, morphological and physiological traits of *H. vulgaris* under the three treatments.

Trait	Control	Shade	Flood
Ramet number	39.8 ± 1.5 a, A	20.3 ± 0.9 b	26.2 ± 2.4 B
Biomass (g)	3.83 ± 0.14 a, A	1.51 ± 0.10 b	2.11 ± 0.19 B
Petiole length (cm)	14.9 ± 0.4	14.5 ± 0.3	15.5 ± 0.5
Specific petiole length (m g^{-1})	5.61 ± 0.15 b, B	7.52 ± 0.16 a	7.81 ± 0.16 A
Internode length (cm)	6.26 ± 0.16 B	6.22 ± 0.19	13.6 ± 0.6 A
Specific internode length (m g^{-1})	2.46 ± 0.10 b, B	3.52 ± 0.13 a	5.21 ± 0.17 A
Mean leaf area (cm^2)	13.2 ± 0.3	13.3 ± 0.3	13.6 ± 1.6
Specific leaf area (cm^2 g^{-1})	381.7 ± 8.0 b	511.1 ± 14.3 a	485.6 ± 41.4
Malondialdehyde (μmol g^{-1})	0.214 ± 0.009	0.233 ± 0.008	0.228 ± 0.010
Superoxide dismutase (μmol g^{-1})	1.48 ± 0.06	1.28 ± 0.08	1.61 ± 0.09
Soluble sugar content (μmol g^{-1})	7.65 ± 0.44 A	6.73 ± 0.27	5.38 ± 0.48 B
Net photosynthetic rate (μmol CO_2 m^{-2} s^{-1})	14.7 ± 0.4 a, A	9.35 ± 0.40 b	8.04 ± 0.59 B
Stomatal conductance (mol H_2O m^{-2} s^{-1})	0.352 ± 0.010 a, A	0.268 ± 0.014 b	0.282 ± 0.013 B
Intercellular CO_2 concentration (μmol CO_2 mol^{-1})	343.9 ± 3.2 a	329.8 ± 4.0 b	374.2 ± 4.5
Transpiration rate (mmol H_2O m^{-2} s^{-1})	7.00 ± 0.18 A	6.99 ± 0.32	4.92 ± 0.18 B

Mean ± SE ($n = 12$) are shown. Within the same row, different small letters indicate significant difference between the control and shade treatment and different capital letters indicate significant difference between the control and the flood treatment.

plasticity in specific petiole length and specific leaf area in the control environment, indicating that genotypes with higher plasticity in these traits had lower fitness (Table 2, Fig. 1). However, with respect to flood variation, we detected a significant negative regression coefficient for plasticity in none of the 13 traits in neither the control nor the flood environment (Appendix 2).

Developmental instability costs

For variation in light availability, we found significant positive correlations between developmental instability and trait plasticity for soluble sugar content in the control treatment (Table 3a, Fig. 2a) and for internode length and intercellular CO_2 concentration in the shade treatment (Table 3a, Fig. 2b,c). However, developmental instability of these traits was not negatively correlated with either of the two fitness traits (Fig. 2d–i). With respect to flood variation, developmental instability of specific leaf area and net photosynthetic rate in the flood treatment was significantly positively correlated with trait plasticity (Table 3b, Fig. 3a,b), but it was not significantly negatively correlated with fitness measures (Fig. 3c–f). These results suggested that there were no developmental instability costs of plasticity in any of the 13 traits in response to light availability or flood variation.

Developmental range limit

For variation in light availability, we observed significant negative correlations between trait plasticity and trait average over the control and the shade environments for specific internode length and intercellular CO_2 concentration (Table 4a, Fig. 4), suggesting that there was a developmental limit of plasticity in these two traits in response to light availability. With regard to flood variation, however, we did not find a negative correlation or a negative regression coefficient for the quadratic term of the second-order polynomial regressions between trait plasticity and trait average for any of the 13 traits (Table 4b), suggesting that there was no developmental limit of trait plasticity in response to flood variation.

Discussion

We found evidence for plasticity costs in specific petiole length and specific leaf area under full light and developmental range limits in specific internode length and intercellular CO_2 concentration in response to light availability variation. However, we did not observe significant costs or limits of plasticity in any of the 13 traits in response to flood variation.

Plastic responses to light availability and flood variation

When light availability is limited, the rate of photosynthesis in plants declines, leading to reductions in physiological activity and carbon loss (Steinger *et al.*, 2003; Martínez Pastur *et al.*, 2007; Trouwborst *et al.*, 2016; Puglielli *et al.*, 2017). Thus, the decreases in light availability under shade resulted in the decreases in net photosynthetic rate, stomatal conductance and intercellular CO_2 concentration, and consequently the sharp

Table 2 Analysis of plasticity costs in 13 traits of *H. vulgaris* in response to light variation. Costs were estimated by regressing the genotypic fitness (ramet number or biomass) within a target environment (control or shade) on the genotypic mean of a trait in that environment and the genotypic plasticity of that trait. A significant negative regression coefficient (β) for the plasticity term indicates costs of plasticity in that environment.

	(a) Ramet number						(b) Biomass					
	Control			Shade			Control			Shade		
Trait	β	t	P	β	t	P	β	t	P	β	t	P
Petiole length	0.165	0.620	0.276	0.118	0.384	0.355	0.217	0.920	0.191	0.174	0.605	0.280
Specific petiole length	**−0.974**	**−3.395**	**0.004**	0.131	0.736	0.241	**−0.920**	**−3.657**	**0.003**	0.115	0.821	0.217
Internode length	−0.205	−0.684	0.256	0.071	0.207	0.421	−0.311	−1.035	0.164	0.091	0.251	0.404
Specific internode length	0.045	0.145	0.444	0.254	1.484	0.086	−0.054	−0.270	0.397	0.304	2.791	0.011
Mean leaf area	−0.199	−0.702	0.251	−0.471	−1.629	0.069	−0.342	−1.122	0.146	−0.452	−1.732	0.059
Specific leaf area	**−1.078**	**−3.799**	**0.002**	0.508	1.383	0.100	**−1.081**	**−3.795**	**0.002**	0.465	1.279	0.117
Malondialdehyde	0.780	3.742	0.003	0.651	2.917	0.009	0.752	3.428	0.004	0.613	2.588	0.015
Superoxide dismutase	0.354	1.061	0.158	0.321	0.807	0.221	0.309	0.914	0.193	0.307	0.756	0.235
Soluble sugar content	−0.401	−1.216	0.128	−0.165	−0.423	0.341	−0.251	−0.714	0.247	−0.155	−0.395	0.351
Net photosynthetic rate	0.673	1.970	0.040	0.273	0.816	0.218	0.569	1.518	0.082	0.343	1.057	0.159
Stomatal conductance	−0.088	−0.209	0.420	−0.118	−0.204	0.422	0.040	0.096	0.463	−0.138	−0.238	0.409
Intercellular CO_2 concentration	0.435	2.277	0.025	−0.039	−0.079	0.470	0.480	2.161	0.030	0.171	0.375	0.359
Transpiration rate	0.036	0.172	0.434	0.654	2.870	0.009	0.346	1.534	0.080	0.642	2.638	0.014

Significant negative values of β are shown in bold after sequential Bonferroni correction ($\alpha < 0.05$).

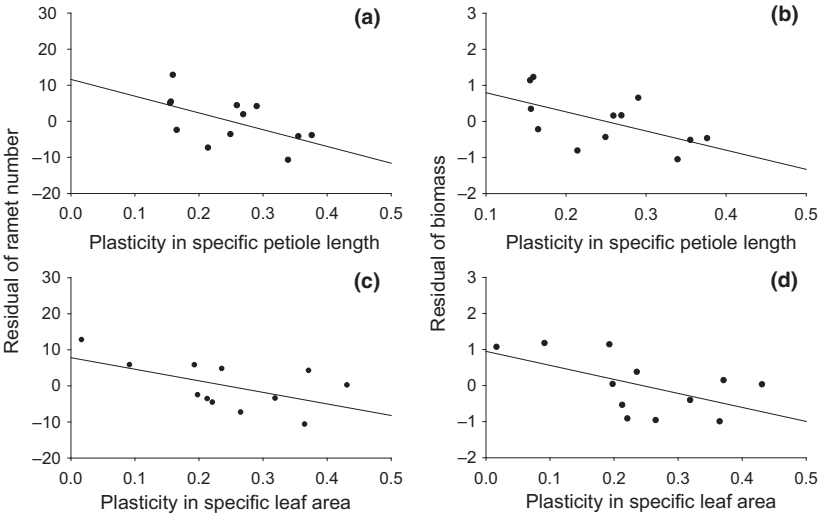

Fig. 1 Plasticity costs as detected by negative correlations between residual of fitness measures under full light (control) and plasticity in specific petiole length (a, b) and specific leaf area (c, d) in response to light availability variation. The residual fitness was calculated from the regression of the fitness measure on specific petiole length or specific leaf area under control treatment.

Table 3 Tests of the first prerequisite for developmental instability costs of plasticity based on correlation coefficients (*r*) between developmental instability and plasticity in response to light availability (a) and flood variation (b) in 13 traits of *H. vulgaris*.

	(a) Light availability				(b) Flood variation			
	Control		Shade		Control		Flood	
Plasticity in	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Petiole length	0.209	0.263	0.278	0.199	0.006	0.490	0.131	0.342
Specific petiole length	−0.424	0.089	0.424	0.080	−0.757	0.002	−0.274	0.206
Internode length	−0.307	0.165	**0.739**	**0.004**	0.058	0.426	0.431	0.077
Specific internode length	−0.471	0.065	−0.174	0.289	−0.898	<0.001	−0.072	0.414
Mean leaf area	0.444	0.074	0.256	0.206	0.429	0.078	−0.036	0.448
Specific leaf area	−0.319	0.156	0.404	0.107	−0.089	0.405	**0.654**	**0.008**
Malondialdehyde	0.417	0.078	−0.144	0.323	−0.158	0.313	−0.447	0.069
Superoxide dismutase	0.194	0.273	0.157	0.312	−0.047	0.444	0.207	0.252
Soluble sugar content	**0.634**	**0.001**	−0.396	0.105	0.236	0.236	−0.146	0.335
Net photosynthetic rate	−0.363	0.127	0.221	0.251	−0.184	0.280	**0.631**	**0.015**
Stomatal conductance	−0.384	0.104	−0.375	0.109	−0.023	0.477	−0.354	0.140
Intercellular CO_2 concentration	−0.168	0.317	**0.664**	**0.009**	0.427	0.078	0.223	0.246
Transpiration rate	0.431	0.093	0.265	0.220	0.059	0.431	−0.036	0.449

P-values were estimated by permutation tests. Significant positive values of *r* are shown in bold.

reduction in fitness measures. However, plastic responses to low light availability also have active components (Dorn *et al.*, 2000). We observed greater specific petiole length, specific internode length and specific leaf area under shade, consistent with previous reports indicating that shaded plants often increase their photon-harvesting surfaces by increasing the specific leaf area to optimize light capture and elongate their petiole length or internode length per unit biomass to improve light foraging (Steinger *et al.*, 2003; Mommer *et al.*, 2006; Weijschedé *et al.*, 2006; Bell & Galloway, 2008; Ivancich *et al.*, 2012; Guo *et al.*, 2016). Such types of plasticity in specific petiole length, specific internode length and specific leaf area are commonly assumed to be able to contribute to the fitness of plants growing in low light conditions and thus adaptive (van Kleunen *et al.*, 2000; Steinger *et al.*, 2003).

Plants under flood had lower soluble sugar content, net photosynthetic rate, stomatal conductance and transpiration rate. Stomatal closure of leaves in response to flood has been observed in several species (Pezeshki *et al.*, 1996; Glaz *et al.*, 2004; Li *et al.*, 2004). Moreover, the reduced transpiration rate may be the consequence of decreased moisture evaporation due to hampered gas exchange between the plant and the environment arising from stomatal closure (Mommer *et al.*, 2006; Ivancich *et al.*, 2012). In flood conditions, diminished photosynthetic processes may be caused not only by stomatal limitation, but also by adverse metabolic consequences of hypoxia, such as reduced Rubisco

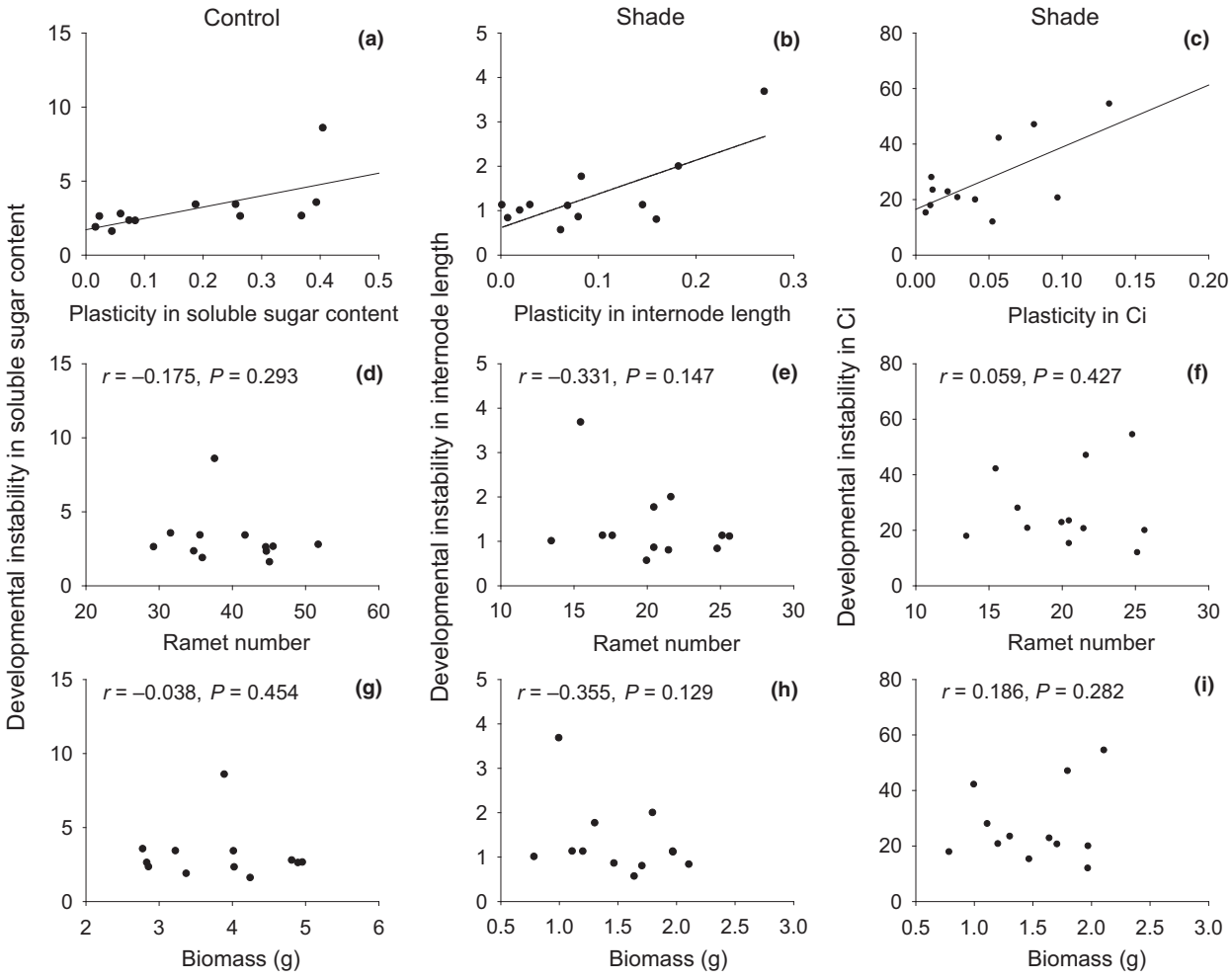

Fig. 2 Correlations of developmental instability in soluble sugar content under full light (control; a, d, g) and internode length (b, e, h) and intercellular CO_2 concentration (Ci) under shade (c, f, i) with (a–c) trait plasticity in response to light availability variation and (d–i) fitness measures.

activity, disruptions in photosynthate transport and chlorophyll degeneration (Pezeshki *et al.*, 1996; Campostrini *et al.*, 2001; Nicotra *et al.*, 2015). When photosynthesis is impeded by flood, plant growth is dominated by respiration; as a consequence, soluble sugar content is consumed to produce energy (van Kleunen *et al.*, 2007; McDowell *et al.*, 2008; Bongers *et al.*, 2017).

Flood excludes air from the soil, resulting in a low oxygen status within plants (Mommer *et al.*, 2006; Voesenek *et al.*, 2006). Therefore, plants tend to form aerenchyma, the air channels facilitating the diffusion of shoot- and leaf-derived oxygen to the roots (Mommer *et al.*, 2006; Voesenek *et al.*, 2006). Therefore, biomass allocated to per unit petiole or internode was decreased, with higher specific petiole length and specific internode length in the flood condition.

Plasticity costs and developmental instability costs

We only detected plasticity costs in specific petiole length and specific leaf area under full light, consistent with the wide consensus that costs of plasticity are rare relative to the number of traits measured (Dechaine *et al.*, 2007; Bell & Galloway, 2008). Costs of plasticity consist of both environmental and genetic effects. In this study, we only used 12 genotypes, and such small sample size may cause an insufficient genetic basis for plasticity, reducing the detection power of plasticity costs (Maherali *et al.*, 2010). However, the treatment × genotype interaction of those traits exhibiting plasticity costs was not significant, similar to the previous findings showing that it was possible to detect costs of plasticity without significant treatment × genotype interaction (Dorn *et al.*, 2000;

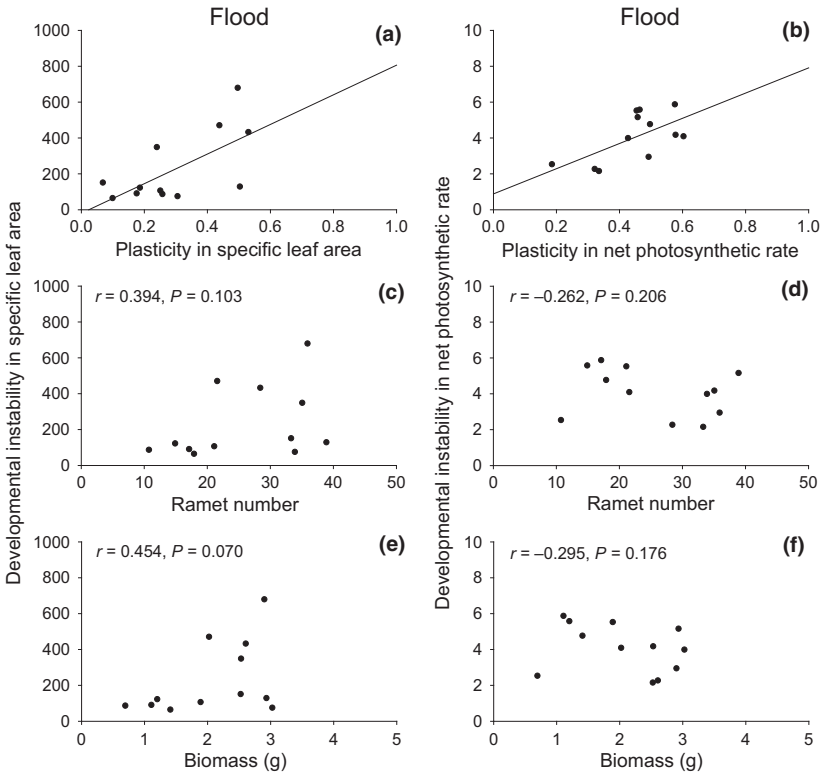

Fig. 3 Correlations of developmental instability in specific leaf area (a, c, e) and net photosynthetic rate under flood (b, d, f) with (a, b) trait plasticity in response to flood variation and (c–f) fitness measures.

Table 4 Tests for developmental range limits of plasticity in response to light availability (a) or flood variation (b) in 13 traits of *H. vulgaris* based on correlations and second-order polynomial regressions between trait plasticity and trait average over the two light environments (control and shade; a) or the two flood environments (control and flood; b). Developmental range limits of plasticity are indicated by a negative correlation coefficient (r) or a negative regression coefficient for the quadratic term (θ).

	(a) Light availability					(b) Flood variation				
Trait	r	P	θ	t	P	r	P	θ	t	P
Petiole length	0.303	0.173	−0.055	−0.010	0.497	0.271	0.195	−3.378	−0.695	0.235
Specific petiole length	−0.094	0.388	7.020	0.915	0.201	−0.418	0.101	−1.132	−0.117	0.461
Internode length	0.472	0.065	−2.789	−0.280	0.403	0.755	0.002	2.152	0.619	0.266
Specific internode length	**−0.564**	**0.022**	1.192	0.288	0.396	−0.369	0.124	4.621	0.713	0.243
Mean leaf area	0.427	0.087	9.628	0.924	0.194	−0.112	0.351	5.525	2.302	0.024
Specific leaf area	0.396	0.105	9.812	0.775	0.228	0.497	0.044	8.965	4.019	0.002
Malondialdehyde	0.262	0.194	−9.113	−0.987	0.467	0.174	0.298	−3.639	−0.696	0.147
Superoxide dismutase	−0.263	0.196	4.126	0.921	0.172	0.359	0.126	10.991	2.582	0.014
Soluble sugar content	−0.119	0.351	−5.902	−1.478	0.086	0.173	0.313	−2.341	−0.912	0.196
Net photosynthetic rate	0.225	0.251	−6.570	−0.954	0.171	−0.317	0.152	−8.883	−1.416	0.098
Stomatal conductance	−0.187	0.280	0.429	0.058	0.217	−0.345	0.141	2.539	0.453	0.123
Intercellular CO_2 concentration	**−0.705**	**0.005**	31.439	2.395	0.029	0.243	0.219	0.240	0.780	0.077
Transpiration rate	0.073	0.411	−5.610	−1.423	0.089	0.664	0.012	0.386	0.067	0.478

P-values were estimated by permutation tests. Significant negative values of r and θ are shown in bold.

van Kleunen *et al.*, 2000; Maherali *et al.*, 2010). Therefore, shortage of genetic variation for plasticity was not the entire reason for our failure of detecting some plasticity costs. Moreover, genotypes with costly plasticity may have been culled from populations by previous natural selection (DeWitt *et al.*, 1998; Weinig *et al.*, 2006; Bell & Galloway, 2008). Alternatively, there may be other underlying mechanisms leading to limited evidence for plasticity costs (Dechaine *et al.*, 2007; Lind & Johansson, 2009).

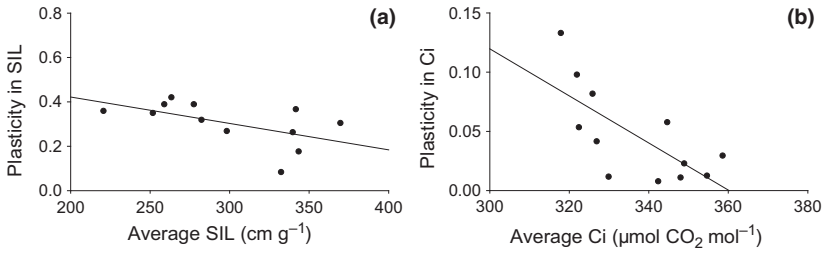

Fig. 4 Developmental range limits indicated by significant negative correlations between trait plasticity and trait average over the full light (control) and shade environments for (a) specific internode length (SIL) and (b) intercellular CO_2 concentration (Ci).

We found no evidence for developmental instability costs of phenotypic plasticity. Similarly, several studies have failed to detect such costs, including studies of predator-induced plasticity in the freshwater snail *Physa heterostropha* (DeWitt, 1998) and plasticity in the foraging characteristics of the stoloniferous herb *Ranunculus reptans* in response to competition (van Kleunen *et al.*, 2000). As plant individuals sample the environment less actively, information acquisition costs are probably not important (van Kleunen *et al.*, 2000).

According to Scheiner & Berrigan (1998), maintenance costs should exist in both environments, whereas production costs are measured as the difference in the regression coefficient of the plasticity term in two environments. Therefore, the detected environment-specific plasticity costs in specific petiole length and specific leaf area under full light are costs incurred by relatively more plastic genotypes to express the particular traits, over and above the costs that less plastic genotypes pay to express the same trait.

Developmental range limits

We detected developmental range limits of plasticity in specific internode length and intercellular CO_2 concentration in response to variation of light availability, suggesting that less plastic genotypes have a greater potential to produce extreme trait values than more plastic genotypes. Few empirical studies have found developmental range limits (DeWitt, 1998; van Kleunen *et al.*, 2000; Lind & Johansson, 2009), and our study provides additional evidence that this type of limit is rare. It is possible that alternating selection pressures would select against nonplastic extreme specialists. In addition, when directional or nonstabilizing selection occurs, the most extreme phenotype must also be the optimal phenotype expressed by the most highly plastic individuals (Lind & Johansson, 2009). Some researchers have proposed that the developmental range limit is a consequence of plasticity costs (Lind & Johansson, 2009; Auld *et al.*, 2010). However, in our study, the traits with developmental range limits were not based on plasticity costs, and these limits were likely generated by other mechanisms. Therefore, more studies are needed before it is possible to generalize about the existence and frequency of this type of plasticity limit.

Conclusions

Our results suggest that constraints on the evolution of traits towards the optimal reaction norm are infrequent and differ under different environmental contexts. One possible reason is that a genotype with higher plasticity in response to one environmental factor may be less plastic in response to other environmental factors (van Kleunen *et al.*, 2000). Although statistical methods provide us with an appropriate way to detect existence and frequency of plasticity constraints, we still need further theoretical and empirical studies to identify underlying mechanisms of these constraints.

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Conflict of interest

The authors declare no conflict of interests.

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Appendix 1

Results of MANOVA and ANOVAs for effects of treatments, genotypes and their interaction on (A) the overall response and (B) the response of each trait of *H. vulgaris*. The treatment effect was further separated into the shade effect and the flood effect by two planned contrasts, and the treatment × genotype effect was further separated into the S × G effect and the F × G effect by two planned contrasts.

	Treatment (T)								T × G					
	Overall		Shade (S)		Flood (F)		Genotype (G)		Overall		S × G		F × G	
(A) MANOVA	$F_{30,144}$	*P*	$F_{15,72}$	*P*	$F_{15,72}$	*P*	$F_{165,665}$	*P*	$F_{330,943}$	*P*	$F_{165,665}$	*P*	$F_{165,665}$	*P*
All	**33.12**	**<0.001**	**43.29**	**<0.001**	**24.83**	**<0.001**	**1.95**	**<0.001**	**1.46**	**<0.001**	**1.26**	**0.027**	**1.51**	**<0.001**

	Treatment (T)								T × G					
	Overall		Shade (S)		Flood (F)		Genotype (G)		Overall		S × G		F × G	
(B) ANOVA	$F_{2,86}$	*P*	$F_{1,86}$	*P*	$F_{1,86}$	*P*	$F_{11,86}$	*P*	$F_{22,86}$	*P*	$F_{11,86}$	*P*	$F_{11,86}$	*P*
Ramet number	**44.07**	**<0.001**	**81.49**	**<0.001**	**37.84**	**<0.001**	**3.24**	**0.001**	1.33	0.176	0.47	0.919	**2.06**	**0.032**
Biomass	**67.30**	**<0.001**	**124.14**	**<0.001**	**58.37**	**<0.001**	**4.29**	**<0.001**	0.89	0.611	0.84	0.597	0.81	0.634
Petiole length	0.49	0.615	0.14	0.714	0.46	0.500	**2.15**	**0.025**	1.61	0.062	1.81	0.064	1.61	0.110
Specific petiole length	**29.47**	**<0.001**	**37.34**	**<0.001**	**46.17**	**<0.001**	**2.25**	**0.018**	0.73	0.799	0.77	0.664	0.40	0.951
Internode length	**101.93**	**<0.001**	0.76	0.385	**157.41**	**<0.001**	0.64	0.787	0.95	0.539	0.78	0.663	1.13	0.348
Specific internode length	**80.98**	**<0.001**	**31.52**	**<0.001**	**161.89**	**<0.001**	1.60	0.112	1.09	0.378	1.45	0.166	0.85	0.594
Mean leaf area	0.58	0.563	< 0.01	0.976	0.97	0.326	**2.61**	**0.006**	**3.48**	**<0.001**	0.24	0.994	**5.43**	**<0.001**
Specific leaf area	**8.72**	**<0.001**	**17.39**	**<0.001**	2.47	0.119	**2.24**	**0.019**	1.54	0.081	0.41	0.948	**2.44**	**0.011**
Malondialdehyde	1.13	0.326	2.26	0.136	0.51	0.478	1.05	0.415	0.59	0.920	0.77	0.673	0.43	0.938
Superoxide dismutase	1.77	0.177	1.92	0.169	0.32	0.574	1.60	0.113	0.88	0.621	0.55	0.860	1.17	0.318
Soluble sugar content	**9.01**	**<0.001**	0.02	0.902	**14.40**	**<0.001**	1.09	0.379	**1.81**	**0.028**	1.08	0.386	1.65	0.098
Net photosynthetic rate	**35.77**	**<0.001**	**56.60**	**<0.001**	**44.67**	**<0.001**	1.61	0.110	0.83	0.678	0.80	0.642	0.77	0.670
Stomatal conductance	**11.84**	**<0.001**	**22.60**	**<0.001**	**8.72**	**0.004**	0.48	0.910	0.85	0.662	0.73	0.712	0.85	0.594
Intercellular CO_2 concentration	**8.62**	**<0.001**	**6.31**	**0.014**	3.74	0.056	1.69	0.090	1.18	0.287	1.13	0.349	0.96	0.492
Transpiration rate	**8.01**	**0.001**	1.38	0.244	**15.71**	**<0.001**	0.79	0.647	0.96	0.519	0.81	0.633	1.11	0.360

Significant table entries ($P < 0.05$) are shown in bold.

Appendix 2

Analysis of plasticity costs in 13 traits of *H. vulgaris* in response to flood variation. Costs were estimated by regressing the genotypic fitness (ramet number or biomass) within a target environment (control or flood) on the genotypic mean of a trait in that environment and the genotypic plasticity of that trait. A significant negative regression coefficient (β) for the plasticity term indicates costs of plasticity in that environment.

	(a) Ramet number						(b) Biomass					
	Control			Flood			Control			Flood		
Trait	β	t	P	β	t	P	β	t	P	β	t	P
Petiole length	−0.074	−0.277	0.394	−0.432	−1.017	0.168	−0.112	−0.467	0.326	−0.426	−1.013	0.169
Specific petiole length	0.815	1.472	0.088	0.204	0.609	0.279	0.708	1.380	0.101	0.163	0.501	0.314
Internode length	−0.056	−0.184	0.429	0.043	0.076	0.471	0.078	0.244	0.407	0.187	0.381	0.356
Specific internode length	−0.183	−0.496	0.316	0.373	1.162	0.138	0.014	0.056	0.478	0.269	0.809	0.220
Mean leaf area	0.193	0.643	0.268	0.124	0.373	0.359	0.069	0.203	0.422	0.148	0.479	0.322
Specific leaf area	0.453	1.539	0.079	0.412	1.188	0.133	0.460	1.561	0.077	0.454	1.408	0.097
Malondialdehyde	−0.196	−0.598	0.283	−0.140	−0.455	0.330	−0.167	−0.508	0.312	−0.049	−0.155	0.440
Superoxide dismutase	0.493	1.702	0.062	0.459	1.408	0.097	0.547	1.972	0.040	0.397	1.139	0.142
Soluble sugar content	−0.389	−0.977	0.177	−0.332	−1.054	0.160	−0.187	−0.444	0.334	−0.310	−0.982	0.176
Net photosynthetic rate	0.292	0.917	0.192	−0.942	−1.562	0.077	0.245	0.736	0.241	−0.934	−1.527	0.081
Stomatal conductance	0.729	2.570	0.015	−0.837	−1.478	0.087	0.760	2.797	0.011	−1.045	−1.959	0.041
Intercellular CO_2 concentration	−0.397	−1.265	0.119	−0.174	−0.296	0.387	−0.171	−0.443	0.334	−0.206	−0.364	0.362
Transpiration rate	1.007	2.438	0.019	−0.266	−0.517	0.309	1.294	2.679	0.013	−0.487	−0.958	0.182

P-values were based on regression analyses. Significant negative values of β are shown in bold after sequential Bonferroni correction ($\alpha < 0.05$).

Significant table entries ($P < 0.05$) are shown in bold.