

Selection on phenotypic plasticity of morphological traits in response to flooding and competition in the clonal shore plant *Ranunculus reptans*

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Abstract

Adaptive evolution of phenotypic plasticity requires that plastic genotypes have the highest global fitness. We studied selection by spatial heterogeneity of interspecific competition and flooding, and by temporal heterogeneity of flooding on morphological plasticity of 52 genotypes of the clonal shore plant *Ranunculus reptans*. Competition reduced clone size, rosette size, leaf length and stolon internode thickness. Flooding had similar effects and reduced competition. Differences in selection between environments imply potential for either local adaptation or for indirect evolution of phenotypic plasticity. We also detected direct selection for plastic reductions in internode length in response to flooding and for a plastic increase in internode length in response to competition. Plastic responses of some morphological traits to flooding were in line with selection thereon, suggesting that they indeed are adaptive and might have evolved in response to direct selection on plasticity.

Introduction

Many species grow along environmental gradients and differences in selection pressures along such gradients may result in locally adapted genotypes. Evidence for adaptive intra specific genetic differentiation has been found over distances of only a few metres (Linhart, 1988; Prati & Schmid, 2000; van Kleunen *et al.*, 2000a; van Kleunen & Fischer, 2001; Lenssen *et al.*, 2004a). As an alternative to genetic specialization, plants may respond to spatial and temporal environmental heterogeneity by plasticity in physiological and morphological traits (Bradshaw, 1965; Dudley & Schmitt, 1996; van Kleunen & Fischer, 2001), which implies that these plastic responses

have evolved themselves and may still be under selection.

Phenotypic plasticity may evolve indirectly as a correlated response to selection on trait values in single environments (Via & Lande, 1985; Falconer, 1990; van Hinsberg, 1996; van Kleunen & Fischer, 2003). However, selection could also act directly on phenotypic plasticity when highly related individuals grow in different environments (McLellan *et al.*, 1997; van Kleunen & Fischer, 2005), i.e. when they experience spatial heterogeneity. This is especially likely for vegetatively spreading plants, which constitute the majority of most floras (Klimeš *et al.*, 1997). In such plants, the genet (i.e. the genetic individual), which may be represented in several environments, is the evolutionary unit rather than its constituent ramets (i.e. the physiological individuals) (Sackville Hamilton *et al.*, 1987; Vuorisalo *et al.*, 1997; Fischer & van Kleunen, 2002). Moreover, when individuals experience different environmental conditions over time (i.e. temporal heterogeneity) or small scale spatial

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environmental heterogeneity, selection could act directly on plasticity of the individual (Schlichting & Pigliucci, 1998). This is especially likely in modular organisms, such as plants, in which each new module can respond to its local environment (Winn, 1996; de Kroon *et al.*, 2005).

Selection on phenotypic plasticity has generally been inferred from comparisons of selection on trait values in different environments (Scheiner, 1989; Dudley, 1996; Dudley & Schmitt, 1996; Donohue *et al.*, 2000; Dorn *et al.*, 2000; Steinger *et al.*, 2002; Huber *et al.*, 2004). These studies are powerful in determining whether a plastic response *per se* would be beneficial. However, they do not unequivocally prove that the plastic genotypes are selected for rather than two groups of specialist genotypes (Releya, 2002; van Kleunen & Fischer, 2005). This is because evolution of adaptive plasticity requires that plastic genotypes have the highest global fitness averaged over the environments (Releya, 2002; van Kleunen & Fischer, 2005) rather than the highest fitness in each environment separately. Studies testing for direct selection on phenotypic plasticity of replicated genotypes grown in spatially heterogeneous environments, however, are very scarce (Weis & Gorman, 1990; van Kleunen & Fischer, 2001; Stinchcombe *et al.*, 2004) and none of them studied plasticity in response to more than one environmental cue and in response to temporal environmental heterogeneity.

Here, we test for plastic responses in the number and length of leaves, and the length and specific length of internodes in response to flooding and competition, and for selection imposed by these environments on these morphological traits and their plasticities for the clonal shore plant *Ranunculus reptans*. This species grows in its natural populations in central Europe along a *c.* 10 m long gradient from low elevation 'lakeside' microhabitats with long flooding periods and the absence of competing grasses to higher elevation 'landside' microhabitats with shorter flooding periods and the presence of the competing grass *Agrostis stolonifera* (Prati & Peintinger, 2000). Previous studies have shown local adaptation of lakeside genotypes to flooding (Lenssen *et al.*, 2004a) and local adaptation of landside genotypes to competition with *A. stolonifera* (Prati & Schmid, 2000; van Kleunen & Fischer, 2001). Adaptive responses to flooding and competition are likely to entail a reduction of respiration by reducing the size of rosettes, leaves and internodes (Lenssen *et al.*, 2004a), and elongation of leaves and internodes (van Kleunen & Fischer, 2001), respectively. Although gradients of flooding and of competition covary in natural shore situations the effects of competition and flooding have not been assessed simultaneously and there have been no tests for selection on plasticity in response to both environmental factors simultaneously.

To test for plasticity in response to spatial heterogeneity in competition and in flooding and selection thereon, we grew a total of 624 plants representing 52 genotypes

of *R. reptans*, half from lakeside and half from landside microhabitats, in a factorial design with and without competition and flooding. Because it is impossible to experimentally control competition and flooding conditions in the natural habitat of *R. reptans*, this study was carried out under controlled greenhouse conditions. To test for plasticity in response to temporal heterogeneity in flooding and selection thereon, we grew flooded plants for an additional period without flooding (drained conditions). We asked the following questions: (1) How are plant morphology and fitness affected by competition and flooding? (2) Is there variation in these traits and their plasticities among genotypes, and if yes is it related to the microhabitat of origin? (3) Do flooding and competition impose selection on morphological traits? (4) Does spatial heterogeneity in flooding and in competition select for plasticity in morphological traits? (5) Does temporal heterogeneity in flooding select for plasticity in morphological traits?

Material and methods

Study species

Ranunculus reptans L. (Ranunculaceae) is a stoloniferous, rosette forming, perennial herb with a circumpolar distribution (Hess *et al.*, 1980). The species mainly grows on periodically inundated shores of rivers and lakes with low vegetation cover. Within populations in central Europe at Lake Constance (latitude: 47°40'N, longitude: 9°20'E) plants that are close to the relatively low winter water level of the lake (lakeside microhabitat), experience an average summer inundation period of 150 days and little competition from other species. Plants that are 5–10 m further land inward and *c.* 30 cm higher up the shore (landside microhabitat) experience an average inundation period of 80 days and competition with grasses, mainly *A. stolonifera* (Prati & Peintinger, 2000).

Rosettes of *R. reptans* may have up to 20 leaves with leaf blades 10–50 mm long and 1–5 mm wide. When flooded, plants form short filiform leaves instead of the larger terrestrial leaves. Rosettes form stolon branches, which consist of rooted and unrooted rosettes connected by stolon internodes. When the plants are not flooded, each rosette may produce one flower (Prati & Peintinger, 2000).

Plant material and precultivation

We used 54 plants collected in 12 populations around Lake Constance in 1995, 1997 and 2002. Previous RAPD (Fischer *et al.*, 2000) and allozyme (Willi *et al.*, 2005) studies showed that these 54 plants belong to different genotypes. Within each population, equal numbers of genotypes had been collected from the lakeside and the landside microhabitat. To reduce possible maternal environmental carry over effects, we propagated the 54

genotypes vegetatively at least three times. Both pre cultivation and the experiment were carried out in a controlled greenhouse of Radboud University Nijmegen at *c.* 20 °C with additional lighting to extend the daily light period to 16 h.

Experimental set-up

To test for effects of flooding, competition and their interaction, we used a full factorial design with four treatment combinations (Fig. 1): (+F–C) flooding in the absence of competition, (+F+C) flooding in the presence of competition with *A. stolonifera*, (–F–C) no flooding in the absence of competition and (–F+C) no flooding in the presence of competition.

We measured all plants after 9 weeks (hereafter referred to as census A). Moreover, we grew the plants that had been flooded (i.e. the ones in treatments +F–C and +F+C; Fig. 1) for another 5 weeks in the absence of flooding and measured them again (hereafter referred to as census B). This served to test for differences in the rate of recovery from flooding between competition

treatments and among genotypes, and to test for plasticity in morphological traits of individual plants in response to temporal heterogeneity in flooding.

We used a randomized block design with three blocks corresponding to the three basins used for the flooding treatment. We filled 72 trays (length × width × depth = 46 × 28.5 × 8 cm) with a 3 : 1 mixture of sand and compost, and added 20 g slow release fertilizer (3.0 g N, 2.2 g P, 2.6 g K and 0.4 g Mg; Osmocote Plus, Grace Sierra International, Heerlen, the Netherlands) to each tray. For each treatment by block combination, we randomly assigned one of 12 rosettes of each of the 54 genotypes to one of nine positions in each of six trays (Fig. 1; totalling 648 plants).

Trays assigned to the competition treatments (i.e. treatments –F+C and +F+C; Fig. 1) had been planted with tillers of *A. stolonifera* 2 months before the start of the experiment. To reduce effects of transplantation on *R. reptans*, we clipped the dense mat of *A. stolonifera* back to soil level immediately before planting the rosettes of *R. reptans*. Two weeks later, when the plants were well established, we submerged the trays assigned to the treatments with flooding (i.e. treatments +F–C and +F+C; Fig. 1) for 9 weeks in one of three circular basins with a diameter of 180 cm and a depth of 90 cm filled with nonchlorinated tap water. The water layer transmitted 37% of the incident light. To prevent growth of algae in the water, we added *Daphnia* sp. and used a filtering system. Weekly measurements in all basins showed that water temperature remained between 21 and 24 °C, and that the oxygen concentration remained constant at 9.7 mg L⁻¹. Nonflooded plants were watered at least every second day. During the experiment, all individuals of one landside genotype and of one lakeside genotype died, reducing the data set to 52 genotypes.

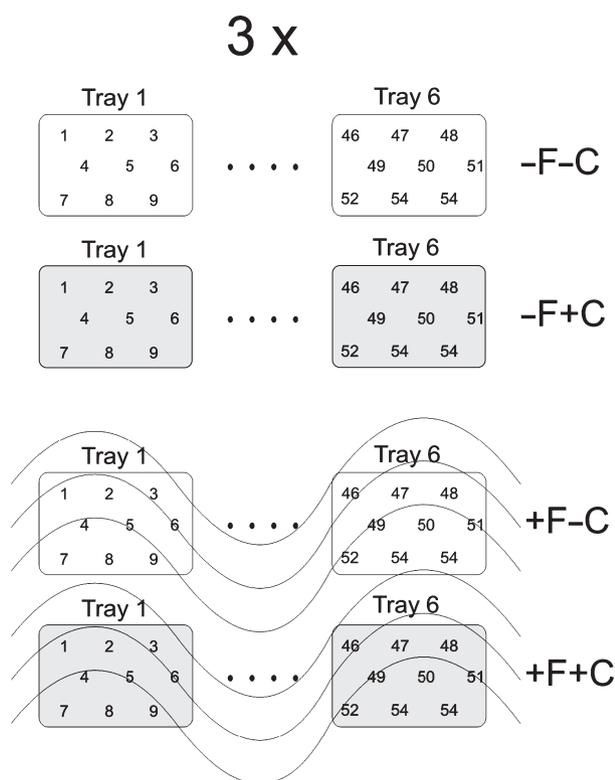


Fig. 1 Schematic illustration of the experimental set up. For each of the four treatment combinations (–F–C: without flooding and without competition, –F+C: without flooding and with competition, +F–C: with flooding and without competition, +F+C: with flooding and with competition) in each of the three blocks, one replicate of each of the 54 genotypes was randomly assigned to one of 54 planting positions distributed over six trays.

Plant measurements

To obtain nondestructive estimates of initial plant size for each plant after the 2 week acclimation period, we recorded the number of leaves and the lengths of the longest leaf and of all stolon branches. From these data, we estimated plant dry mass [mg] as

$$0.07 \times [\text{no. of leaves} \times \text{length of longest leaf (mm)}] + 0.14 \times \text{total stolon branch length (mm)} \quad (1)$$

($R^2 = 0.64$, $n = 30$; Lenssen *et al.*, 2004a).

After the 9 week flooding period (i.e. at census A), we counted the total number of rosettes and the number of rooted rosettes for each plant as measures of plant size and vegetative reproduction, respectively. For nonflooded plants, we also counted the number of flowers as a measure of sexual reproduction. We did not do this for the flooded plants because they did not flower. On all plants, we counted the number of leaves and measured

the length of the longest leaf of the first rosette that had been produced after the 2 week acclimation period. On the newly produced clone parts, we also measured the lengths of the first three internodes. For nonflooded plants, to determine specific internode length we harvested the measured internodes and weighed them after drying for at least 48 h at 70 °C. For flooded plants, we marked all rosettes produced during the flooding period with toothpicks to distinguish them from rosettes produced during the subsequent 5 week recovery period.

At the end of the recovery period (i.e. at census B), we counted the number of rosettes and rooted rosettes again, and additionally the number of flowers for each remaining plant (i.e. plants in treatments +F–C and +F+C). On the first rosette that had been produced during the recovery period, we counted the number of leaves and measured the length of the longest leaf. On the clone part produced during the recovery period, we also measured the lengths of the first three stolon internodes. We harvested these internodes and the ones that had been measured at census A and weighed them after drying for at least 48 h at 70 °C. We calculated the specific internode length as the ratio of the combined lengths of the three internodes to their dry mass.

Analyses

We first analysed the data to test for effects of flooding and competition and genetic variation. Then we did three different sets of selection gradient analyses. In the first set of analyses, we tested for selection on morphological traits in each of the four environments. In the second set of analyses, we tested for selection on plasticity of morphological traits of genets in response to competition and flooding (i.e. on plastic responses to spatial environmental differences). In the third set of analyses, we tested for selection on plastic changes of morphological traits during recovery from flooding (i.e. on plastic responses to temporal environmental changes).

We did all analyses with the statistical software SPSS (SPSS Inc., Chicago, IL, USA). To achieve normality and homoscedasticity, we log₁₀ transformed number of leaves per rosette, specific internode length, and number of rosettes, rooted rosettes and flowers prior to analyses. For the selection gradient analyses, however, we used untransformed values for the measure of fitness, the number of rosettes, as recommended by Lande & Arnold (1983).

Test of treatment effects and genotypic variation

To test for effects of flooding and competition on and genotypic variation in the traits measured on all plants at census A, we used hierarchical (type I SS) mixed model analyses of variance (ANOVA). We included variation among blocks, trays, populations and genotypes as random factors, and the effects of microhabitat of origin (land and lakeside), competition and flooding as fixed factors. Tray

was nested within block, flooding and competition. Genotype was nested within microhabitat of origin and population. To correct for differences in initial clone size, we used log₁₀ transformed estimates of initial plant dry mass (Eqn 1) as a covariate in the analyses of the number of rosettes and rooted rosettes. Because the flowers of the nonflooded plants were counted at census A and the ones of the flooded plants at census B (they did not flower at census A), these data were analysed separately for each group of plants.

For the flooded plants, we used repeated measurements analyses of variance to test for changes in number of rosettes and number of rooted rosettes and differences in morphological traits between clone parts produced during the flooding period and the parts produced afterwards during the recovery period. The ANOVA model was similar to the one described above but did not include the factor 'flooding' and its interactions. Because *F* values for the population effect were always nonsignificant, and because we did not aim at studying genetic differentiation among populations, we removed it from the final models.

Test of selection on morphological traits

To test whether there is selection on the number of leaves per rosette, leaf length and the length and specific length of stolon internodes in each of the four environments, we used selection gradient analyses by regressing fitness on each of these morphological traits (Lande & Arnold, 1983). Because genets rather than their ramets are the evolutionary units of clonal plants, we used genotypic values instead of values of individual plants. This has the additional benefit that it avoids bias in the selection gradients by environmentally induced covariation between a morphological trait and fitness (Rauscher, 1992). We present results with the number of rosettes at census A as measure of fitness, which were qualitatively similar to the results obtained for the number of rooted rosettes or flowers. To compare selection gradients among treatments, we calculated the fitness of each genotype relative to the average fitness of all genotypes, and expressed regression coefficients in units of standard deviations (Lande & Arnold, 1983). To test whether selection differed between environments, we analysed relative fitness (i.e. relative number of rosettes) with analyses of covariance in which we included the four morphological traits as covariates and their interactions with competition and flooding.

Test of selection on phenotypic plasticity in response to spatial heterogeneity in flooding and competition

To test whether there is selection on morphological plasticity of genotypes (i.e. genets) in response to spatial heterogeneity in flooding, we used selection gradient analyses on plasticity separately for the environments with and without competition. Similarly, to test whether there is selection on morphological plasticity of geno

types in response to spatial heterogeneity in competition, we used selection gradient analyses on plasticity separately for the environments with and without flooding. In the selection gradient analyses for each pair of environments, we regressed genotypic values of fitness (i.e. number of rosettes) averaged over the two environments on genotypic values of each morphological trait averaged over both environments (i.e. elevation of the reaction norm) and on genotypic values of plasticity in these traits to those two environmental factors (i.e. slope of the reaction norm; van Kleunen & Fischer, 2001). When there are differences in variation of fitness between both environments, the arithmetic mean will be biased towards the environment with the highest variation in fitness. To reduce this bias, we used the geometric mean instead (van Kleunen & Fischer, 2005). In these analyses, we assume equal frequencies of the different environments and equal representation of the genotypes among the environments, which was the case in our experiment but does not necessarily hold for natural populations. Both in the absence and presence of competition, values of trait plasticity in response to flooding were calculated by subtracting the genotypic mean value (X) in the treatment without flooding from the one in the treatment with flooding (i.e. $X_{+F-C} - X_{-F-C}$ and $X_{+F+C} - X_{-F+C}$). Similarly, both in the absence and presence of flooding, values of trait plasticity in response to competition were calculated by subtracting the genotypic mean value in the treatment without competition from the one in the treatment with competition (i.e. $X_{-F+C} - X_{-F-C}$ and $X_{+F+C} - X_{+F-C}$).

Test of selection on phenotypic plasticity in response to temporal heterogeneity in flooding

To test whether there is selection on morphological plasticity of individuals in response to temporal heterogeneity in flooding (i.e. only for individuals in the treatments +F-C and +F+C), we also used selection gradient analyses. We regressed total fitness at the end of the experiment (i.e. total number of rosettes at census B) on morphological traits averaged over both census dates (i.e. elevation of the reaction norm) and on plasticities in these traits (i.e. slopes of the reaction norms). We calculated plasticity for each plant by subtracting the trait value at census A from the one at census B. As for the other selection gradient analyses, we used genotypic values instead of values of individual plants. To compare between competition treatments, we expressed fitness of each genotype relative to the average over all genotypes for each of the environments and expressed regression coefficients in units of standard deviations. To test whether selection differed between competition environments, we analysed relative fitness (i.e. relative number of rosettes) with analyses of covariance in which we included the means and plasticities of the four morphological traits as covariates and their interactions with competition.

Results

Effects of competition and flooding on fitness measures and morphological traits

Nine weeks after the start of the experiment, i.e. at census A, competition had significantly decreased the production of rosettes and rooted rosettes of *R. reptans* (Fig. 2a,b; Table 1). Competition also reduced the production of flowers for both the nonflooded and flooded plants (Fig. 2c, $F_{1,30} = 168.58$, $P < 0.001$ and $F_{1,32} = 105.00$, $P < 0.001$ for nonflooded and flooded plants, respectively; Appendix 1). Flooding prevented plants from flowering and, averaged over both competition treatments, flooding also reduced the production of rosettes but increased the production of rooted rosettes (Fig. 2a,b; Table 1).

Flooded plants reduced their production of rosettes and rooted rosettes under competition significantly less than nonflooded plants did (Fig. 2a,b; Table 1). Moreover, after the 5 week recovery period of the plants that had been flooded (i.e. at census B), not only the number of rosettes and rooted rosettes increased (Fig. 2a,b; time effect: $F_{1,162} = 7.11$, $P = 0.008$ and $F_{1,159} = 17.40$, $P < 0.001$ for the number of rosettes and rooted rosettes, respectively; Appendix 2) but also the negative effect of competition became stronger (Fig. 2a,b; time by competition interaction: $F_{1,32} = 68.68$, $P < 0.001$ and $F_{1,32} = 209.82$, $P < 0.001$ for the number of rosettes and rooted rosettes, respectively; Appendix 2). These results indicate that flooding reduces competition.

Nine weeks after the start of the experiment, i.e. at census A, plants grown in the presence of competition had rosettes with significantly fewer and shorter leaves and a higher specific internode length than plants grown in the absence of competition (Fig. 2d,g; Table 1). Averaged over both competition treatments, flooded plants produced significantly fewer and shorter leaves, and stolons with a higher specific internode length than nonflooded plants (Fig. 2d,e,g; Table 1), indicating that flooding and competition had similar effects on plant morphology. Moreover, flooded plants produced significantly shorter internodes than nonflooded plants (Fig. 2f; Table 1). The reduction in the number of leaves per rosette and the increase in specific internode length in response to competition were significantly smaller for flooded plants than for nonflooded plants (Fig. 2d,g; Table 1), and indicate that flooding alleviates the negative effect of competition on rosette size and stolon thickness.

After the 5 week recovery period of the plants that had been flooded (i.e. at census B), the number of leaves per rosette and the length of the longest leaf had significantly increased (Fig. 2d,e; time effect: $F_{1,160} = 65.94$, $P < 0.001$ and $F_{1,140} = 225.79$, $P < 0.001$, respectively; Appendix 3), and the specific internode length had significantly decreased (Fig. 2g; time effect: $F_{1,131} = 81.66$, $P < 0.001$; Appendix 3). Moreover, the negative

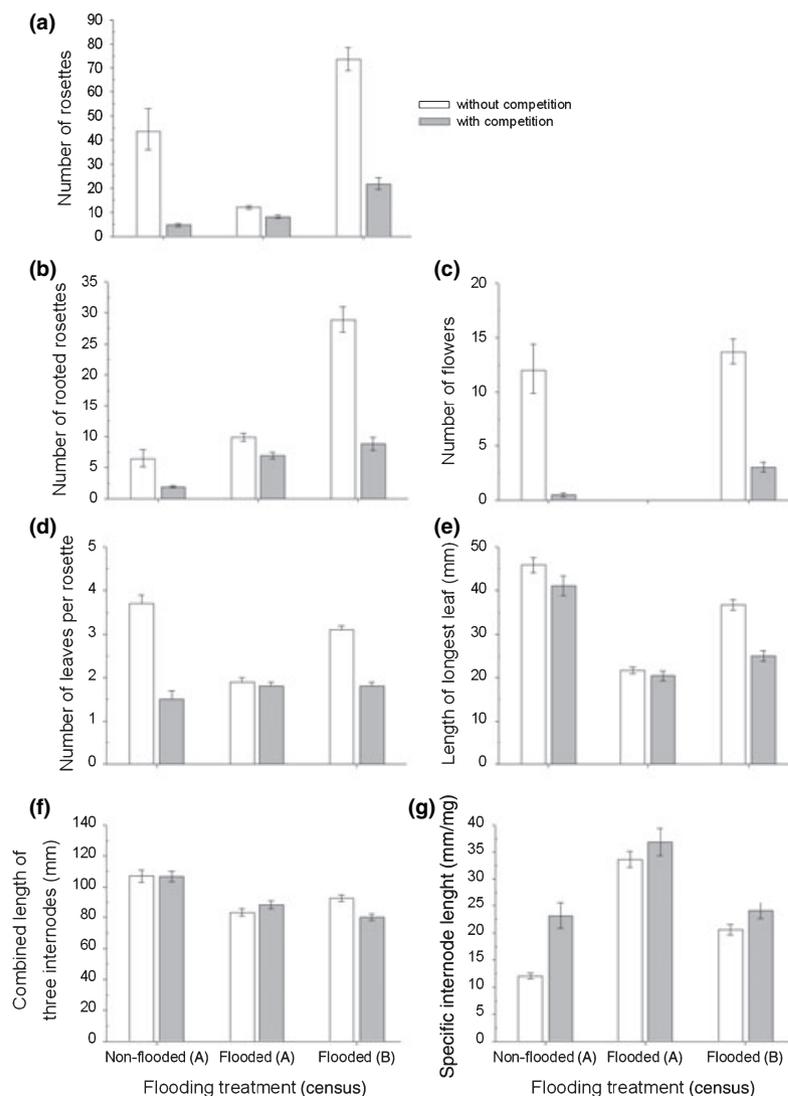


Fig. 2 Means (\pm SE) for fitness measures (a–c) and morphological traits (d–g) of *Ranunculus reptans* grown in the presence and absence of flooding and competition. Plants in the flooding treatment were measured directly after the flooding period (census A) and after a 5 week recovery period without flooding (census B). Open and filled bars represent plants grown in the absence and presence of competition, respectively.

effect of competition became more pronounced for both the number of leaves per rosette and the length of the longest leaf (Fig. 2d,e; time by competition interaction: $F_{1,32} = 24.90$, $P < 0.001$ and $F_{1,32} = 35.27$, $P < 0.001$, respectively; Appendix 3), and its effect on internode length had become significantly negative (Fig. 2f; time by competition interaction: $F_{1,32} = 19.01$, $P < 0.001$; Appendix 3). These results underline the reduction in competition because of flooding.

Genotypic variation in fitness measures and morphological traits

There was significant variation among genotypes in the number of flowers ($F_{50,123} = 1.67$, $P = 0.012$ and $F_{50,163} = 2.07$, $P < 0.001$ for nonflooded and flooded plants, respectively; Appendix 1). Moreover, genotypes differed significantly in their plastic response to flooding for the

number of rosettes (Table 1) and in their rate of increase after they had been flooded (time by genotype interaction: $F_{50,162} = 1.53$, $P = 0.025$; Appendix 2). For all of these traits and the plastic responses therein, the variation among genotypes was unrelated to the microhabitat of origin (Table 1).

There was significant variation among genotypes in the length of the longest leaf, the length of the first three internodes and the specific internode length (Table 1). Moreover, genotypes differed significantly in their response to flooding for the number of leaves per rosette and the length of the longest leaf (Table 1). The significant three way interaction between competition, flooding and genotype for the number of leaves per rosette (Table 1) indicates that the effect of flooding on the reduction in the number of leaves per rosette by competition differed among genotypes. Moreover, there was significant variation among genotypes in their plastic

Table 1 Summary of analyses of variance of fitness measures and morphological traits of *Ranunculus reptans* at the end of the 9 week flooding period (census A). Plants were grown in a factorial flooding by competition experiment with 52 genotypes representing two different microhabitats of origin. Numbers of rosettes, rooted rosettes and leaves and specific internode length were log10 transformed prior to analyses.

Effect	Number of rosettes		Number of rooted rosettes		Number of leaves per rosette		Length of longest leaf		Length of first three internodes		Specific internode length	
	d.f.	F	d.f.	F	d.f.	F	d.f.	F	d.f.	F	d.f.	F
Log(initial mass)	1	7.90**	1	1.24								
Block	2	4.57*	2	2.90	2	1.11	2	0.39	2	1.92	2	2.57
Flooding	1	4.58*	1	65.26***	1	8.02**	1	238.21***	1	48.27***	1	105.32***
Competition	1	133.97***	1	64.34***	1	34.73***	1	4.31*	1	0.37	1	21.28***
C × F	1	74.57***	1	20.67***	1	30.83***	1	2.70	1	1.02	1	15.78***
Tray[B,F,C]	64	4.03***	64	4.53***	65	2.59***	64	2.37***	64	3.44***	64	2.02***
Microhabitat	1	0.76	1	1.02	1	2.47	1	0.34	1	0.17	1	0.16
Genotype[M]	50	1.34	50	1.26	50	1.13	50	1.71**	50	2.41***	50	1.71**
F × M	1	0.39	1	0.33	1	0.32	1	0.47	1	1.73	1	0.00
F × G[M]	50	1.42*	50	1.29	50	1.63**	50	1.42*	50	1.39	50	1.03
C × M	1	0.13	1	0.40	1	0.56	1	1.08	1	0.00	1	0.62
C × G[M]	50	0.81	50	1.03	50	0.90	50	0.67	50	0.93	50	1.14
C × F × M	1	0.99	1	0.29	1	1.00	1	0.61	1	0.06	1	0.27
C × F × G[M]	49	0.87	49	1.11	50	1.50*	47	1.13	46	0.81	48	0.90
Residual†	288	0.08965	288	0.07126	283	0.03081	229	114.45	211	350.89	235	0.0546

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

†For the residual we give the MS instead of an F value.

change of leaf length during the recovery period after flooding (time by genotype interaction: $F_{50,140} = 1.64$, $P = 0.013$; Appendix 3). As for the numbers of flowers and rosettes, for all of these morphological traits and the plastic responses therein, variation among genotypes was unrelated to the microhabitat of origin (Table 1).

Selection on morphological traits in different environments

In all environments, selection gradients for the number of leaves per rosette were positive (Table 2), which was reflected in a marginally significant 'number of leaves per rosette' effect in ANCOVA for the number of rosettes ($F_{1,51} = 3.04$, $P = 0.087$). Also averaged over the four environments, there was selection for longer internodes.

This was reflected in a significant 'length of first three internodes' effect in ANCOVA for the number of rosettes (Table 2). In the presence of flooding selection gradients for internode length tended, however, to be lower (negative when competition was also absent) than in the absence of flooding (flooding by internode length interaction in ANCOVA for the number of rosettes: $F_{1,51} = 3.05$, $P = 0.087$, Table 2). For specific internode length, selection gradients were close to zero in the presence of flooding (+F-C and +F+C), positive in the absence of flooding and absence of competition (-F-C) and negative in the absence of flooding and presence of competition (-F+C; Fig. 3; Table 2). This was reflected in significant competition by specific internode length and competition by flooding by specific internode length interactions in ANCOVA for the number of rosettes

Table 2 Selection gradients of morphological traits of *Ranunculus reptans* under nonflooded and flooded conditions in the presence and absence of competition with *Agrostis stolonifera* and F values of analysis of covariance testing for overall selection (Cov) and differences in selection between flooding (F) and competition (C) treatments.

Trait	Nonflooded		Flooded		F values of ANCOVA			
	Without competition	With competition	Without competition	With competition	Cov	Cov × F	Cov × C	Cov × C × F
Number of leaves per rosette	0.025	0.133	0.279	0.181	3.04	0.03	0.00	1.10
Length of longest leaf	0.314	0.120	0.178	0.236	0.07	2.19	2.23	0.13
Length of first three internodes	0.224	0.245	0.189	0.205	8.99**	3.05	0.97	0.08
Specific internode length	0.238	0.287	0.048	0.026	2.41	0.12	4.39*	6.18*

Selection gradients are expressed as standardized regression coefficients after regression of the fitness measure relative number of rosettes at census A (i.e. directly after the flooding period) on the respective morphological trait.

* $P < 0.05$, ** $P < 0.01$.

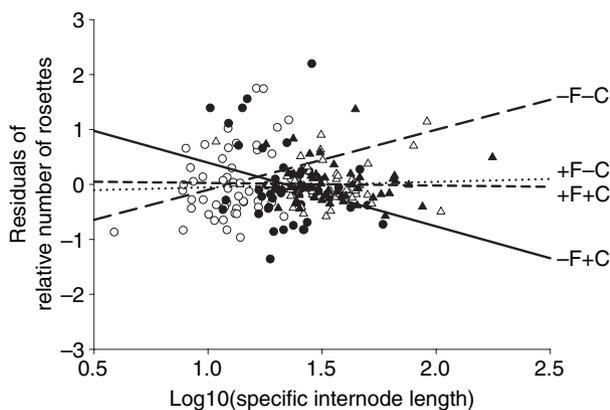


Fig. 3 Illustration of differences in selection gradients for specific internode length (mm mg^{-1}) between flooded (+F; triangles) and nonflooded (-F; circles) environments for genotypes of *Ranunculus reptans* grown in the presence (+C; closed symbols) and absence (-C; open symbols) of competition. The selection gradients differed depending on the presence and absence of competition, and these differences were large in the nonflooded environments and small under flooding. The number of rosettes was corrected for the effects of number of leaves per rosette, length of the longest leaf and length of first three internodes by using the residual number of rosettes after regression of the number of rosettes on these other three morphological traits.

(Table 2). Overall, these results suggest environment independent selection for more leaves per rosette, flooding dependent selection for absolute internode length, and selection depending on the combination of competition and flooding for specific internode length (Fig. 3).

Selection on plasticity in morphological traits in response to spatial heterogeneity in flooding and competition

In the absence of competition, spatial heterogeneity in flooding tended to select for a plastic increase in the

number of leaves per rosette in response to flooding ($\beta = 0.412$, $P = 0.051$). In the absence of competition, spatial heterogeneity in flooding significantly selected for a plastic reduction of internode length in response to the occurrence of flooding (Table 3). Further, spatial heterogeneity in competition selected for a plastic increase of internode length in response to the occurrence of competition (significant in the presence of flooding; Table 3). In the presence of competition, spatial heterogeneity in flooding selected for a plastic increase of specific internode length (Table 3). These results indicate direct selection for plastic responses to flooding and competition.

Selection on plasticity in morphological traits in response to temporal heterogeneity in flooding

Neither in the absence nor in the presence of competition there was significant selection on plastic responses to temporal heterogeneity in flooding for any of the measured morphological traits (Table 4). However, averaged over both competition environments, there was significant selection for a plastic decrease in internode length during the recovery period from flooding (indicated by a significant 'length of first three internodes' effect in ANCOVA for number of rosettes; Table 4). This indicates that selection can act directly on phenotypic plasticity in morphological traits of genotypes of *R. reptans* exposed to temporal environmental heterogeneity in flooding.

Discussion

Effects of competition and flooding on fitness measures and morphological traits

Theory predicts that abiotic stress should reduce competitive interactions (Grime, 1979; Grace, 1990; Keddy, 1990). In line with this prediction, we found that

Table 3 Selection gradients of plasticity in morphological traits in response to flooding in the absence and presence of competition, and in response to competition in the absence and presence of flooding.

Environment	Number of leaves per rosette	Length of longest leaf	Length of first three internodes	Specific internode length
Plasticity in response to flooding				
Without competition	0.412	0.287	0.398*	0.800
With competition	0.056	0.325	0.025	0.306*
Plasticity in response to competition				
Without flooding	0.073	0.094	0.236	0.320
With flooding	0.132	0.224	0.459**	0.045

Selection gradients are expressed as standardized partial regression coefficients of the regression of the geometric mean in number of rosettes over pairs of environments at census A (i.e. directly after the flooding period) on the means of morphological traits over pairs of environments and differences (i.e. plasticities) between the two environments. Selection on a plastic increase and a plastic decrease of the morphological traits in response to flooding and competition are indicated by significant positive and negative selection gradients, respectively. * $P < 0.05$, ** $P < 0.01$.

Trait	Without competition	With competition	F values of ANCOVA	
			Cov	Cov × C
Number of leaves	0.092	0.256	0.02	1.62
Length of longest leaf	0.153	0.063	0.34	0.25
Length of first three internodes	0.101	0.219	7.22*	0.11
Specific internode length	0.030	0.027	0.05	0.05

Selection gradients are expressed as standardized partial regression coefficients of the regression of the relative number of rosettes at the end of the experiment (census B) on the mean values and differences (i.e. plasticities) between morphological structures produced during the 9 week flooding period (census A) and during the subsequent 5 week recovery period (census B). Selection on plasticity in the morphological traits is indicated by significant negative or positive selection gradients.

* $P < 0.05$.

although both competition and flooding reduced the overall growth of *R. reptans*, the strong negative effect of the presence of *A. stolonifera* was alleviated by flooding. This is most likely a consequence of the negative effect of flooding on the growth of the competing grass as indicated by the virtual absence of *A. stolonifera* at the end of the 9 week flooding period (J.P.M. Lenssen, personal observation). During the subsequent 5 week recovery period, *A. stolonifera* restored a high above ground biomass, and as a consequence the negative effect of competition on fitness of *R. reptans* had increased by the end of the experiment. Lenssen *et al.* (2004b) also found evidence in an experiment on six grass species that flooding may decrease competition. In conclusion, our study indicates that periodic floods in the natural habitat of *R. reptans* at Lake Constance are important for its survival by temporarily reducing interspecific competition.

In the presence of competition, *R. reptans* produced rosettes with shorter internodes, at least after flooding, and fewer and shorter leaves than the ones grown in the absence of competition. More generally, the effects of competition and flooding on morphological traits were in the same direction, as has also been found for *Rumex palustris* and *R. thyrsiflorus* (Mommer *et al.*, 2005). This, however, does not necessarily mean that these responses involve the same physiological mechanisms. Although elongation responses under shading most likely involve the phytochrome pathway (Schmitt & Wulff, 1993; but see Pierik *et al.*, 2004), elongation responses under flooding most likely involve the ethylene pathway (Voesenek *et al.*, 2004). This implies that selection on morphology by competition and flooding might act on different underlying physiological mechanisms (Mommer *et al.*, 2005).

Genotypic variation in fitness measures and morphological traits

There was significant variation among genotypes for most fitness measures and morphological traits. More

Table 4 Selection gradients of plasticity in morphological traits in response to temporal heterogeneity in flooding and F values of analysis of covariance testing for overall selection (Cov) and differences in selection between competition (C) treatments.

over, there was significant variation among genotypes in plastic responses to flooding, but not to competition. This might indicate that selection in the past has fixed plasticity of morphological traits in response to competition for *R. reptans* at Lake Constance. Overall, these results indicate that there is potential for future evolution of mean trait values, and of plasticity therein in response to flooding, whereas there is none for plasticity in response to competition.

In our experiment, genotypic variation in fitness measures and morphological traits was not related to the microhabitat of origin. This might suggest that either there are no differences in selection pressures on morphological traits along the natural flooding gradient at the shore of Lake Constance or that they have not resulted in genetic differentiation because of counteracting effects of gene flow (Stanton *et al.*, 1997), strong effects of genetic drift (van Kleunen *et al.*, 2005) or strong temporal heterogeneity. Previous studies on *R. reptans*, however, found that genotypes from the landside microhabitat perform better under competition (Prati & Schmid, 2000), had stronger plastic foraging responses under spatially heterogeneous competition (van Kleunen & Fischer, 2001), produced daughter ramets that remained physically integrated for a longer time (van Kleunen *et al.*, 2000a) and were less tolerant to flooding (Lenssen *et al.*, 2004a) than genotypes from the lakeside microhabitat. This clearly indicates that genetic differentiation between the landside and lakeside microhabitats is possible. Most likely we did not detect a land lakeside differentiation in the current experiment because we mainly used genotypes that had been collected in 2002 after an unusual extremely long flooding period, which may have sorted out most of the lakeside genotypes from the natural populations.

Selection on morphological traits and their plasticities

Under all four flooding by competition conditions, plants with more leaves per rosette also tended to produce more

rosettes, indicating selection for large rosettes. In non flooded environments, both in the presence and absence of competition, there tended to be stronger selection for plants with long internodes than averaged over both flooded environments. Although this effect was only marginally significant, it suggests that long internodes do not only have a selective benefit under competition as both K selection (Pianka, 1970) and the shade avoidance hypothesis (Schmitt & Wulff, 1993) predict but that this is also the case in the absence of competition.

When photosynthesis of a plant is strongly reduced by flooding, its carbon balance is dominated by the respiratory costs required to keep the plant modules alive (Greenway & Gibbs, 2003). This would suggest that flooding should select for small plant modules. In a previous study, we indeed found selection for smaller rosettes and shorter internodes of *R. reptans* under long lasting flooding (Lenssen *et al.*, 2004a). In the present study, however, we did not find support for this hypothesis because, when plants were flooded, leaf and internode length were selectively neutral. Possibly, the duration of flooding was not long enough to cause major respiratory costs of larger modules or they were compensated by the benefits of producing leaves closer to the water surface where photosynthesis was possible (cf. Vervuren *et al.*, 2003; Mommer *et al.*, 2006).

Overall, we found evidence that selection on morphological traits of *R. reptans* differs between environments. This indicates that there is potential for local adaptation when there is sufficient genotypic variation and limited gene flow. When we compare selection gradients of the -F+C treatment (Table 2), which is most similar to the natural landside habitat, with the ones of the +F-C treatment, which is most similar to the natural lakeside habitat, we conclude that plants at the landside should evolve longer and thicker internodes than plants at the lakeside. These results are consistent with those of Lenssen *et al.* (2005) who showed for *A. stolonifera* that spatial dispersal of ramets via long stolon internodes provides a competitive advantage, especially in relatively open environments after flooding. Alternative to local adaptation, the observed different selection on morphological traits in different environments could lead to the evolution of phenotypic plasticity in morphological traits as a correlated response to selection in the single environments (e.g. Via & Lande, 1985).

Instead of as an indirect response, phenotypic plasticity can also evolve as a direct response to selection when plants grow in a spatially heterogeneous environment or when individuals are grown in a temporally varying environment (McLellan *et al.*, 1997; Schlichting & Pigliucci, 1998; van Kleunen & Fischer, 2005). Therefore, we also tested and found direct selection on phenotypic plasticity (Tables 3 and 4). One should keep in mind, however, that this indicates direct selection in the field only under the assumption that plants grow at equal frequencies in the different environments as was the case

in our experimental set up. Although this assumption does not necessarily hold for natural populations, around Lake Constance the areal extents of the landside and lakeside microhabitats are relatively similar. In other cases, one could use the frequencies of the environments as weighting factors when calculating the average fitness over environments (Via & Lande, 1985; van Tienderen, 1991; Sultan & Spencer, 2002; van Kleunen & Fischer, 2005).

In the case of competition, significant selection for a plastic increase in internode length in response to the occurrence of competition indicates that plastic internode elongation would be adaptive. The lack of an actual plastic response in internode length to competition therefore suggests that adaptive evolution of internode elongation in response to competition has been constrained by a lack of sufficient genetic variation or genetic correlations with other traits under selection. Alternatively, adaptive active plastic elongation of internodes may have been compensated by passive reductions in internode length because of resource limitation (Dorn *et al.*, 2000; van Kleunen *et al.*, 2000b).

Selection gradients for a plastic reduction of internode length to spatial heterogeneity in flooding were in line with the actual morphological changes observed in response to this environmental factor. This suggests that the observed plastic decrease in the length of internodes in response to the occurrence of flooding is adaptive. The observed plastic increase of specific internode length was also in line with the positive selection gradient for plasticity in this trait in response to flooding in the presence of competition. Significant selection for a plastic reduction of internode length during recovery from flooding (i.e. in response to temporal heterogeneity in flooding) was not in line with the lack of an overall plastic response of this trait during recovery from flooding. This indicates that although some of the plastic responses to flooding are adaptive this is not the case for all of them, and that it depends on whether the environments are heterogeneous in space or in time.

Conclusions

Although most studies have inferred selection on phenotypic plasticity from differences in selection between environments, the possibility for direct selection on phenotypic plasticity either in response to spatial or temporal heterogeneity has rarely been tested (Weis & Gorman, 1990; van Kleunen & Fischer, 2001; Stinchcombe *et al.*, 2004). We used both approaches in our selection gradient analyses with *R. reptans* which suggest that some of the observed plastic morphological responses to flooding are adaptive and might have evolved in response to direct selection on plasticity of genotypes growing in a highly heterogeneous environment.

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Supplementary Material

The following supplementary material is available for this article:

Appendix 1 Summary of analyses of variance of the number of flowers of flooded plants of *Ranunculus reptans* at the end of the 9 week flooding period (census A) and the subsequent 5 week recovery period (census B).

Appendix 2 Summary of repeated measures analyses of variance of fitness measures of flooded plants of *Ranunculus reptans* at the end of the 9 week flooding period (census A) and the subsequent 5 week recovery period (census B).

Appendix 3 Summary of repeated measures analyses of variance of morphological measures of flooded plants of *Ranunculus reptans* at the end of the 9 week flooding period (census A) and the subsequent 5 week recovery period (census B).

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