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Different flooding responses in *Rorippa amphibia* and *Rorippa sylvestris*, and their modes of expression in F₁ hybrids

Marc Stift^{1,3}, Pieterella C. Luttikhuisen¹, Eric J.W. Visser² and Peter H. van Tienderen¹

¹Institute for Biodiversity and Ecosystem Dynamics, Experimental Plant Systematics, Universiteit van Amsterdam, P.O. Box 94062, 1090 GB

Amsterdam, the Netherlands; ²Department of Experimental Plant Ecology, Radboud University Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, the

Netherlands; ³Present address: Division of Environmental & Evolutionary Biology, Graham Kerr Building, University of Glasgow, Glasgow G12 8QQ, UK

Summary

Author for correspondence:

Marc Stift

Tel: +44 141 330 5346

Fax: +44 141 330 4952

Email: m.stift@bio.gla.ac.uk

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- The river floodplain species *Rorippa amphibia*, *Rorippa sylvestris*, and their hybrid *Rorippa* × *anceps* were studied here, with the aim of identifying potential species differences with respect to flooding tolerance, and of assessing their expression in F₁ hybrids.
- Parents and their F₁ hybrids were subjected to three flooding treatments mimicking natural conditions, and growth-related and leaf morphological traits were compared.
- In contrast to *R. sylvestris*, *R. amphibia* responded to waterlogging by forming specialized roots, and its growth was not reduced. These traits were dominantly expressed in hybrids. Both species and the hybrids established shoot growth over 2 wk of complete submergence. Only in *R. sylvestris* was this not at the expense of root biomass, suggesting that *R. sylvestris* can photosynthesize underwater. *Rorippa sylvestris* also showed a hyponastic response. Hybrids were intermediate to the parents in this respect.
- This study shows that phenotypic expression of parental traits in F₁ hybrids is mostly additive, but can also be dominant. This suggests that a large overlap in habitat use of parents and hybrids is likely. If such an overlap occurs, the main evolutionary consequences of hybridization in *Rorippa* will be the introgression of genes, as the hybrids are fully fertile.

Key words: hybrid habitat preference, hybridization and introgression, hyponastic response, *Rorippa* × *anceps*, underwater photosynthesis, waterlogging and submergence responses.

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Introduction

Interspecific hybridization is considered to be a process with important evolutionary consequences, both in plants and in animals (Stebbins, 1950; Anderson & Stebbins, 1954; Arnold, 1992, 1997; Dowling & Secor, 1997; Rieseberg, 1997; Seehausen, 2004; see also Schemske, 2000). A first and crucial step in the process involves the creation of F₁ hybrids. Their viability, fertility and ecological position combined with the availability of a suitable habitat determine whether hybridization is incidental and without further consequences, or leads to further hybrid or backcross generations. These

could eventually form a new evolutionary lineage (hybrid speciation; see Arnold, 1992, 1997; Rieseberg, 1997), or a permanent hybrid swarm maintained by repeated backcrossing (Barton & Hewitt, 1985). Such backcrossing may result in the introgression of genes (Anderson & Hubricht, 1938; Anderson, 1949; Stebbins, 1950; Rieseberg & Wendel, 1993; Arnold, 2004) giving rise to new allelic combinations and novel phenotypes (Arnold, 1992, 1997; Rieseberg & Carney, 1998; Rieseberg *et al.*, 2000).

Hybrid swarms are often found in disturbed habitats (Anderson, 1948; Stebbins, 1950; Anderson & Stebbins, 1954). Flooding disturbance has played an important role in the

formation and maintenance of hybrid swarms, for example involving *Iris fulva* and *Iris brevicaulis* (e.g. Johnston *et al.*, 2004; Martin *et al.*, 2006), *Jacobaea vulgaris* (formerly *Senecio jacobaea*) and *Senecio aquatica* (Kirk *et al.*, 2005) and the *Rorippa* × *anceps* hybrid complex (Jonsell, 1968; Bleeker, 2007). In the latter system, the parental species are the self-incompatible perennials *Rorippa amphibia* and *Rorippa sylvestris*, both common along all major rivers in Europe. The distributions of the species within the floodplain suggest that they have become adapted to microhabitats with different flooding regimes (Blom, 1999). *Rorippa amphibia* occurs in sites with relatively stable water tables, mostly as emergent plants, or in dense, constantly moist grassland vegetation. *Rorippa sylvestris* prefers more open, ephemeral sites on riverbanks that can be flooded for longer periods, but can also dry out completely in summer (Jonsell, 1968; Blom, 1999).

Rorippa amphibia (mainly diploid and tetraploid) and *R. sylvestris* (mainly tetraploid and hexaploid) often grow in sympatry, presumably because sedimentation and erosion shape river floodplains into a mosaic of habitats. The species are interfertile at the tetraploid level and the hybrid *Rorippa* × *anceps* can easily be generated and backcrossed with both parents in the glasshouse (Jonsell, 1968; Bleeker, 2004; Stift, 2007). At several locations along the rivers Elbe (Germany) and Wisla (Poland), the presence of a range of intermediate morphologies suggests that hybridization and backcrossing have led to the formation of hybrid swarms (Bleeker, 2007). Molecular analyses of amplified fragment length polymorphism (AFLP) and chloroplast *trnL/F* intergenic spacer DNA sequences (Bleeker & Hurka, 2001; Bleeker & Matthies, 2005) and patterns of microsatellite variation of both parental species and putative hybrids (E. H. McLean, unpublished data) have confirmed these morphological indications of introgressive hybridization along the Elbe river. The availability of hybrids and the position of *Rorippa* within the tribe Cardamineae (Al-Shehbaz *et al.*, 2006), which belongs to the same phylogenetic lineage (I) as the genomic model species *Arabidopsis thaliana* (Beilstein *et al.*, 2006), provide excellent opportunities for comparative genomics (Schranz *et al.*, 2007). Thus, *Rorippa* may function as a model system with which to unravel the genetic basis of traits that are associated with flooding tolerance.

The first aim of this research was to quantify the reactions of *R. amphibia* and *R. sylvestris* in coping with different flooding regimes. We measured growth, biomass allocation, dry: fresh weight ratios and leaf morphological traits under well-drained, waterlogged and fully submerged conditions. In concordance with its stable wet to waterlogged habitat, we predicted that *R. amphibia* would grow well in both drained and waterlogged treatments, but that *R. sylvestris* would exhibit the most vigorous growth under well-drained conditions and a growth reduction under waterlogged conditions. We expected submergence to reduce growth in both species and to affect leaf morphology and positioning (Voesenek *et al.*, 2006). Moreover, we hypothesized that *R. amphibia* shoots would grow more when submerged

in an attempt to reach the surface, thus possibly depleting available carbohydrate resources (Groeneveld & Voesenek, 2003); such a strategy would be advantageous in a habitat where flooding tends to be shallow and predictable. By contrast, we expected that *R. sylvestris* would arrest growth (thus storing carbohydrate reserves) and passively wait for better times, perhaps in a state of anaerobic dormancy (Laan & Blom, 1990; Vartapetian & Jackson, 1997). This would be advantageous in a habitat where flooding is deep and less predictable in duration. We also expected that *R. sylvestris* would generally allocate more biomass to root and rhizome storage tissue, to fuel its more vigorous regeneration from rhizomes (Jonsell, 1968).

The second aim of this research was to assess how the parental traits associated with the flooding regimes are expressed in first-generation (F_1) hybrids obtained from glasshouse crosses between wild-collected plants of the two species. We asked whether their phenotypic expression is conducive for ecological divergence. More specifically, we tested whether F_1 hybrids are intermediate with respect to the parental species (i.e. whether the expression of parental traits showed 'genomic additivity'), mostly resemble one of the parents ('genomic dominance'), or have trait values beyond those of either parent ('genomic overdominance').

Knowledge of how putatively adaptive traits (in this case associated with the different habitat preferences of *R. amphibia* and *R. sylvestris*) are expressed in F_1 hybrids will contribute to answering broader evolutionary questions regarding the long-term consequences of hybridization. Are hybrids merely present because of their constant formation in disturbed habitats (Schemske, 2000), or do they have the potential to occupy a specific novel niche (Arnold, 1997)? Do the ecological characteristics of first-generation hybrids promote reproductive isolation, which might eventually lead to a separate hybrid lineage or even a new species (Rieseberg, 1997; Buerkle *et al.*, 2000)? Or is it more likely that they will set the stage for backcrossing (to one or both parental species), making introgression and gene exchange the most important consequences of hybridization (Anderson & Hubricht, 1938; Whitney *et al.*, 2006; Gross *et al.*, 2007)?

Materials and Methods

Plant material

Even in a vegetative state, the *Rorippa* species can be distinguished easily. *Rorippa amphibia* (L.) Bess. has large leaves with big endlobes, while *Rorippa sylvestris* (L.) Bess. has smaller, more pinnate leaves. Their hybrid *Rorippa* × *anceps* (Wahlenb.) Rchb. has an intermediate leaf morphology (Jonsell, 1968). Both species and hybrids can regenerate vegetatively from rhizomes and stem fragments, albeit *R. sylvestris* does so more vigorously (Jonsell, 1968). During the growing seasons of 2002 and 2003, rhizomes of tetraploid *R. amphibia* (denoted 'A' in figures and tables) and *R. sylvestris* (denoted 'S') were collected from several locations throughout Europe and grown in a glasshouse environment.

Table 1 Sampling details for the six reciprocally crossed pairs of tetraploid genotypes of *Rorippa amphibia* and *Rorippa sylvestris* that were used in this study

Pair	<i>R. amphibia</i>			<i>R. sylvestris</i>		
	River	Location ¹ , country	Latitude Longitude ²	River	Location ¹ , country	Latitude Longitude ²
1	Meuse	Herbricht, Belgium	N: 50°54'43" E: 05°42'26"	Elbe	Hitzacker, Germany	N: 53°09'16" E: 11°02'47"
2	Meuse	Swalmen, the Netherlands	N: 51°14'36" E: 06°00'31"	Sure	Reisdorf, Luxembourg	N: 49°52'07" E: 06°16'04"
3	Ijssel	Doesburg, the Netherlands	N: 52°01'25" E: 06°08'42"	Rhine	Millingerwaard, the Netherlands	N: 51°52'02" E: 05°59'18"
4	Zwarte Water	Zwartsluis, the Netherlands	N: 52°37'30" E: 06°04'48"	Stour	Child Okeford, UK	N: 50°54'05" W: 02°15'01"
5	Elbe	Darchau, Germany	N: 53°14'01" E: 10°54'18"	Loire	Mesnil, France	N: 47°49'43" E: 02°15'38"
6	Elbe	Darchau, Germany	N: 53°14'01" E: 10°54'18"	Loire	Montjean, France	N: 47°23'36" W: 00°52'08"

¹Settlements closest to the exact sampling location.

²WGS84 coordinates.

In the summer of 2004, we created six independent pairs of *R. amphibia* and *R. sylvestris* of different origins (Table 1). Each pair was crossed reciprocally to obtain reciprocal F₁ hybrids (denoted 'AS' and 'SA' in figures and tables; AS, *R. amphibia* mother; SA, *R. sylvestris* mother). In December 2005, for each of these 12 crosses, five randomly chosen seeds were germinated on sterile filter paper moistened with 2 ml of a 3 µM gibberellic acid solution. Seedlings were transferred to soil and placed in the same glasshouse compartment as the parental genotypes. In March 2006, we selected one genotype from each of the 12 F₁ sibling groups (six genotypes of *R. amphibia* × *R. sylvestris* and six genotypes of *R. sylvestris* × *R. amphibia*). From these, and the 12 corresponding parental genotypes (six genotypes of *R. amphibia* and six genotypes of *R. sylvestris*), we obtained uniform, similar-sized rosettes by placing rhizome fragments washed in a 0.5 g l⁻¹ hypochlorite solution into Petri dishes containing half-strength Murashige and Skoog basal medium with Gamborg's vitamins (Sigma-Aldrich, Zwijndrecht, the Netherlands) at pH 5.6 with 0.8% purified agar (Hispanagar, Burgos, Spain). The rhizomes were left to sprout for 5 d in a growth cabinet (Sanyo MLR-350; Sanyo, Etten-Leur, the Netherlands) at 16-h light (36 µmol photosynthetically active radiation (PAR) m⁻² s⁻¹), 23°C, and 8-h dark, 15°C. For each genotype, 40 similar-sized single sprouts were cut off and transferred to trays with net pots (55 mm diameter) containing sterilized sand (0.5–1.0 mm grain size; Filcom BV, Papendrecht, the Netherlands) drained with 0.5 g l⁻¹ nutrient solution (Peat Lite Special, Peters Professional, Scotts, Heerlen, the Netherlands). After 3 wk in the glasshouse, 16 rosettes of each genotype were selected, based on uniformity in leaf number (four–five leaves), and transferred to individual pots (100 mm diameter) containing the same sterilized sand with 5 grains per pot of controlled release fertilizer (Osmocote Plus

15+11+13+2MgO+Trace Elements; Peters Professional). Four of the 16 rosettes were randomly assigned to each of three water treatments and an initial harvest group. Plants were left for 2 d before treatments started, so that plants were exactly 4 wk old at that point. The whole procedure was repeated 2 wk later so that eight replicates were obtained per genotype.

Water treatments

Plants were subjected to three treatments (lasting 2 wk) mimicking conditions prevailing in the natural habitat: well drained (DRN), waterlogged (LOG), with constantly inundated soil and an emerging shoot, and completely submerged (SUB). Treatments were applied in white plastic buckets (diameter, 30 cm; height, 27 cm; volume, 16 l; Nipak BV, Papendrecht, the Netherlands) with overflow holes drilled at an appropriate height. Each bucket contained one plant. The experiment ended with a harvest after 2 wk. The height and diameter of the buckets were such that leaves neither touched the wall nor reached the water surface (in the SUB treatment) throughout the experiment. At the start of the experiment, all buckets were filled simultaneously with rain water. After 1 wk, and thereafter daily, the water in all buckets was simultaneously flushed for 3 h (at a low flow rate of 3 l h⁻¹) in order to control algal growth and nutrient accumulation. Although this procedure temporarily created thinner boundary layers and probably negatively affected plant volatile accumulation during this 3-h period, these effects were certainly much less severe than those of the low light availability and extra oxygen consumption at night caused by considerable algal growth. Throughout the experiment, temperature was regulated to between 20 and 23°C during the 16 h of daylight (SON-T Agro 400W; Philips, Eindhoven, the Netherlands), and to between 15 and 17°C during the night.

Response variables

Response variables were assessed for each of the experimental plants individually at the start and end of the treatment period, unless otherwise specified. We specifically chose to look at traits that allowed comparison between the parental species and hybrids at the rosette stage. We estimated the length (mm) of the above ground roots (roots protruding from the soil surface) and counted the number of adventitious roots (roots developing from the leaf axils), and the number of leaves. From the latter, we calculated the number of leaves that formed during the experiment. Furthermore, for the longest leaf (LLF), we assessed the following leaf morphological traits: the total length including the petiole (LENLLF; mm), width (WIDLLF; mm) and petiole length (PETLLF; mm). From these, we calculated the blade width:length ratio (BLADE SHAPE \equiv WIDLLF/(LENLLF – PETLLF)) and the petiole length relative to the total length (PETLLF/LENLLF). We measured the angle between the horizontal plane and the basal part of the petiole of the longest leaf (degrees) and of the youngest leaf (degrees), such that angles could range from 0° (prostrate orientation) to 90° (upright orientation). Then, after washing off all sand particles from roots and shoots and surface-drying all material by softly pressing with dry tissue paper, we immediately assessed the fresh weights of roots (FWR; g) and shoots (FWS; g). Finally, two random plants of each genotype were dried at 65°C for 72 h for assessment of the dry weights (DWs) of shoots (DWS; g) and roots (DWR; g). Similarly, DW had been assessed at the start of the experiment for the four plants of each genotype that had been assigned to the initial harvest group. From the DW measures, we calculated the relative growth of the shoot and root in the 2-wk period ($RG_{shoot} \equiv \log_e DWS_{end} - \log_e DWS_{start}$; $RG_{root} \equiv \log_e DWR_{end} - \log_e DWR_{start}$). Furthermore, we calculated the shoot weight fraction (SWF \equiv DWS/(DWS + DWR)) and the shoot dry matter content (SDMC \equiv DWS/FWS) for the plants at the end of the experiment. Arcsine square root or logarithmic transformations were applied where appropriate in order to approach normal distribution and variance homogeneity within groups.

Statistical analysis

Experimental design The experiment had a split plot design with genomic class (A, S, AS and SA) and treatment (DRN, LOG and SUB) as the main fixed factors. Each glasshouse table contained one replicate of each set (i.e. one crossed pair of *R. amphibibia* and *R. sylvestris*, and their reciprocal F₁ hybrid offspring) within each treatment. Table and set were treated as random factors.

Analysis of variance (ANOVA) All analyses were performed with SPSS version 11.0 for Windows (SPSS Inc., Chicago, IL, USA). Because of the skewed distribution of observations (i.e. many zeros), the number of adventitious roots and the length

of the aboveground roots were analysed in a separate nonparametric Kruskal–Wallis ANOVA procedure, using post-hoc Mann–Whitney *U*-tests to test for pairwise differences among parents and hybrids. For all other traits, we used ANOVA independent contrasts to compare trait expression of parents and both hybrid classes. First, we contrasted the trait values of the parental species (A vs S) for each treatment separately. Then, we tested for differences in the slope of the reaction norms of both parental species going from DRN to LOG and from LOG to SUB treatments. In the same manner, we contrasted the two reciprocal hybrid genomic classes (AS vs SA).

Evaluation of expression of parental traits in F₁ hybrids: the dominance coefficient If the parental species differed in their trait values and/or reaction norms, we proceeded to evaluate the hybrid trait values. We specifically tested hypotheses of dominance or partial dominance (i.e. hybrids resemble either *R. amphibibia* or *R. sylvestris*), additivity (i.e. hybrids are intermediate) and overdominance (i.e. hybrid trait values lie beyond the parental trait range). For this purpose, we defined *H* as the dominance coefficient for the trait of the hybrid, with *H* = 0 and *H* = 1 representing the situation in which the hybrid is identical to parent *R. amphibibia* and *R. sylvestris*, respectively, and *H* = 1/2 representing the situation in which the hybrid is exactly intermediate. We then calculated this value for our experimental data (denoted \hat{H}) by rescaling the estimated mean trait values for the hybrids and their 95% confidence interval (CI) limits to the difference between the mean parental trait values. For instance, in the DRN treatment, *R. amphibibia* and *R. sylvestris* formed a mean number of 1.35 and 2.83 leaves, respectively, and the hybrids on average 2.18 ± 0.30 leaves. To obtain \hat{H} , we calculated $(2.18 - 1.35)/(2.83 - 1.35) = 0.56$. Similarly, for the lower and upper bounds of the CI: $(2.18 - 0.30 - 1.35)/(2.83 - 1.35) = 0.36$; $(2.18 + 0.30 - 1.35)/(2.83 - 1.35) = 0.76$. Hence, the estimated dominance coefficient ($\hat{H} \pm 95\%$ CI) for the number of leaves formed was 0.56 ± 0.20 . Finally, we evaluated whether *H* = 0 (*R. amphibibia* dominance), *H* = 0.5 (additivity) and *H* = 1 (*R. sylvestris* dominance) were part of $\hat{H} \pm 95\%$ CI. If not, we rejected dominance and additivity, and further evaluated whether there was partial dominance of *R. amphibibia* ($0 < \hat{H} \pm 95\%$ CI < 0.5) or *R. sylvestris* ($0.5 < \hat{H} \pm 95\%$ CI < 1), or overdominance ($\hat{H} \pm 95\%$ CI < 0 or $1 < \hat{H} \pm 95\%$ CI).

Results

Initial measurements

A summary of all initial measurements (before treatments) and statistical evaluations (ANOVA independent contrasts) is provided in Supporting Information Table S1. At the start of the treatments (plants were 4 wk old at that point) the parental species already showed some differences. *Rorippa amphibibia* total DW was around twice that of *R. sylvestris*, and *R. amphibibia*

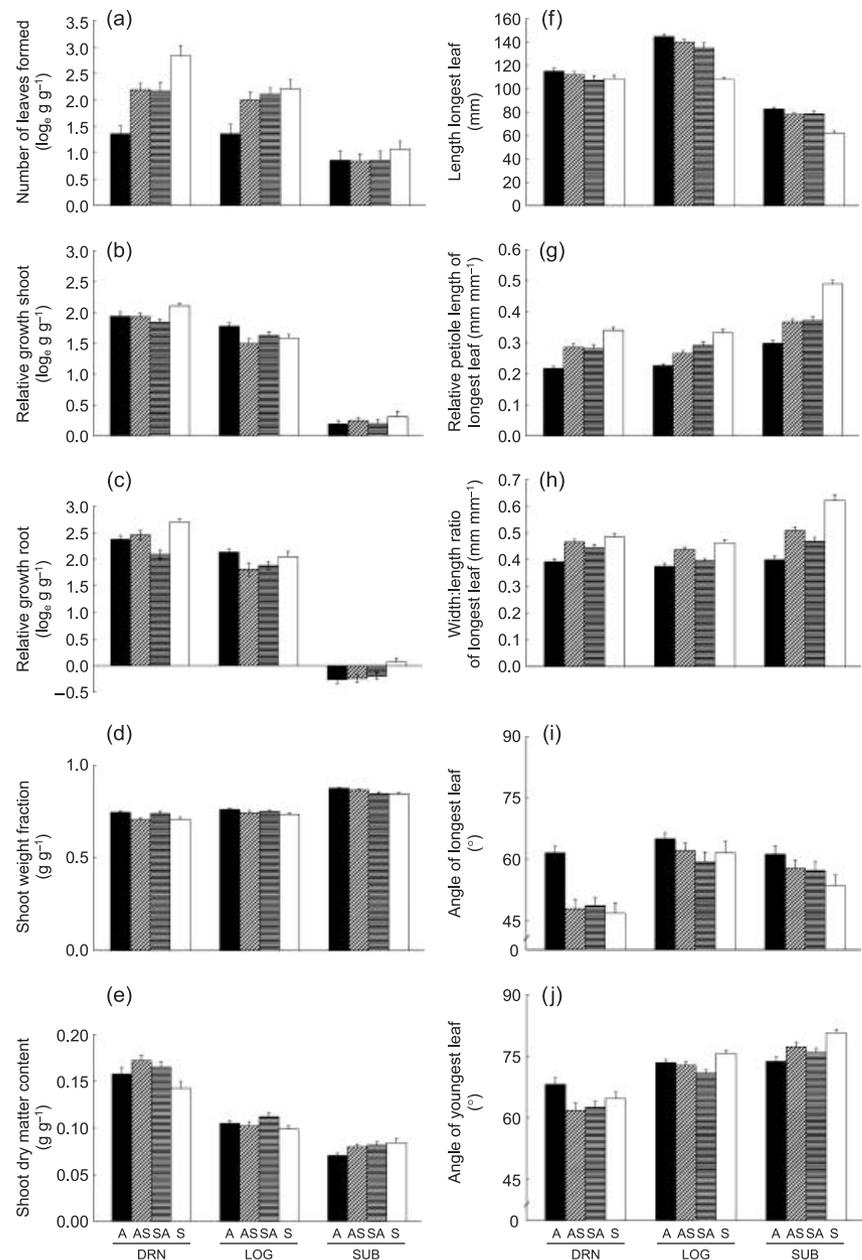


Fig. 1 Mean trait values (untransformed) for each flooding treatment for the different genomic classes. (a–e) Whole-plant traits and (f–j) leaf morphological traits. Treatments: DRN, well drained; LOG, waterlogged; SUB, submerged. Genomic classes: A, *Rorippa amphibia* (black); S, *Rorippa sylvestris* (white); AS, F₁ hybrid A × S (diagonal stripes); SA, F₁ hybrid S × A (horizontal stripes). Bars, + 1 SE ($n = 48$).

leaves had a shorter petiole and a more elongated blade shape while *R. sylvestris* had more leaves. In the F₁ hybrids all possible types of parental trait expression were observed: *R. amphibia* dominance (i.e. $H = 0$ within $\hat{H} \pm 95\%$ CI: total DW, blade shape); *R. sylvestris* dominance (i.e. $H = 1$ within $\hat{H} \pm 95\%$ CI: leaf number); additivity (i.e. $H = 0.5$ within $\hat{H} \pm 95\%$ CI: petiole length); and overdominance (i.e. $H = 0$, $H = 0.5$ and $H = 1$ not within $\hat{H} \pm 95\%$ CI: leaf length and leaf angle).

Measurements at harvest

Figure 1 shows the untransformed mean trait values that were assessed at the end of the experiment and Table 2 summarizes

the statistical evaluations (ANOVA independent contrasts A vs S). Irrespective of treatment, *R. sylvestris* invested relatively more biomass in the roots than *R. amphibia* (shoot mass fraction; Fig. 1d). The species also had inherently different leaf morphologies. *Rorippa sylvestris* had smaller leaves (Fig. 1f) with longer petioles (Fig. 1g) and a rounder leaf blade shape (i.e. higher width:length ratio; Fig. 1h).

Responses of parental species to waterlogging (LOG treatment)

Whole-plant traits The species differed markedly in their responses to waterlogging. *Rorippa amphibia* formed fewer

Table 2 Summary of response variable comparison (ANOVA) between the parental species and the hybrids for each of the treatments and reaction slopes

Response variable	Treatment/ reaction slope	F-value contrast A vs S, conclusion	$\hat{H} \pm 95\%$ CI	H = 0 within CI?	H = 0.5 within CI?	H = 1 within CI?	Conclusion hybrid phenotype
Number of leaves formed (count) ($\sqrt{MS_{error}}$ 1.046; d.f. 1; 542)	DRN	48.0*** A < S	0.56 ± 0.20	No	Yes	No	Additivity
	LOG	16.0*** A < S	0.82 ± 0.35	No	Yes	Yes	Add/dom S
	SUB	0.95 A = S	Not calculated				
	DRN-LOG	4.29* A < S	0.20 ± 0.95	Yes	Yes	Yes	No power
	LOG-SUB	4.58* A < S	1.10 ± 0.92	No	Yes	Yes	Add/dom S
Relative shoot growth (\log_{e^r} g g ⁻¹) ($\sqrt{MS_{error}}$ 0.287; d.f. 1; 254)	DRN	3.74 A = S	Not calculated				
	LOG	5.78* A > S	0.97 ± 0.58	No	Yes	Yes	Add/dom S
	SUB	1.92 A = S	Not calculated				
	DRN-LOG	9.45** A < S	0.45 ± 0.64	Yes	Yes	Yes	No power
	LOG-SUB	7.20* A > S	0.79 ± 0.74	No	Yes	Yes	Add/dom S
Relative root growth (\log_{e^r} g g ⁻¹) ($\sqrt{MS_{error}}$ 0.372; d.f. 1; 254)	DRN	8.49** A < S	-0.22 ± 0.48	Yes	No	No	Dom A
	LOG	0.79 A = S	Not calculated				
	SUB	8.51** A < S	0.25 ± 0.48	Yes	Yes	No	Add/dom A
	DRN-LOG	7.25* A < S	0.46 ± 0.73	Yes	Yes	Yes	No power
	LOG-SUB	7.27* A > S	0.82 ± 0.73	No	Yes	Yes	Add/dom S
Shoot weight fraction ($\arcsin\sqrt{P}$, g g ⁻¹) ($\sqrt{MS_{error}}$ 0.0228; d.f. 1; 254)	DRN	11.7** A > S	0.71 ± 0.41	No	Yes	Yes	Add/dom S
	LOG	5.03* A > S	0.62 ± 0.62	Yes	Yes	Yes	No power
	SUB	5.07* A > S	0.67 ± 0.62	No	Yes	Yes	Add/dom S
	DRN-LOG	0.69 A = S	Not calculated				
	LOG-SUB	< 0.01 A = S	Not calculated				
Shoot dry matter content ($^{10}\log$, g g ⁻¹) ($\sqrt{MS_{error}}$ 0.0639; d.f. 1; 254)	DRN	7.43** A > S	-0.75 ± 0.51	No	No	No	Overdom (A)
	LOG	3.33 A = S	Not calculated				
	SUB	14.2*** A < S	0.88 ± 0.37	No	No	Yes	Dom S
	DRN-LOG	0.41 A = S	Not calculated				
	LOG-SUB	15.7*** A > S	0.50 ± 0.50	No	Yes	Yes	Additivity
Length of LLF (\log_{10^r} , mm) ($\sqrt{MS_{error}}$ 0.0713; d.f. 1; 542)	DRN	4.60* A > S	0.89 ± 0.65	No	Yes	Yes	Add/dom S
	LOG	80.1*** A > S	0.22 ± 0.16	No	No	No	Partial dom A
	SUB	78.4*** A > S	0.20 ± 0.16	No	No	No	Partial dom A
	DRN-LOG	23.1*** A > S	0.01 ± 0.41	Yes	No	No	Dom A
	LOG-SUB	< 0.01 A = S	Not calculated				
Petiole:length ratio for LLF ($\arcsin\sqrt{P}$, mm mm ⁻¹) ($\sqrt{MS_{error}}$ 0.0751; d.f. 1; 542)	DRN	81.3*** A < S	0.54 ± 0.15	No	Yes	No	Additivity
	LOG	59.2*** A < S	0.49 ± 0.18	No	Yes	No	Additivity
	SUB	170*** A < S	0.38 ± 0.11	No	No	No	Partial dom A
	DRN-LOG	0.88 A = S	Not calculated				
	LOG-SUB	14.3*** A < S	0.22 ± 0.52	Yes	Yes	No	Add/dom A
Width:length ratio blade for LLF ($^{10}\log$, mm mm ⁻¹) ($\sqrt{MS_{error}}$ 0.0792; d.f. 1; 542)	DRN	31.1*** A < S	0.70 ± 0.25	No	Yes	No	Additivity
	LOG	32.5*** A < S	0.52 ± 0.24	No	Yes	No	Additivity
	SUB	142.4*** A < S	0.47 ± 0.12	No	Yes	No	Additivity
	DRN-LOG	< 0.01 A = S	Not calculated				
	LOG-SUB	19.4*** A < S	0.43 ± 0.45	Yes	Yes	No	Add/dom A
Angle of LLF ($\arcsin\sqrt{P}$, °) ($\sqrt{MS_{error}}$ 0.172; d.f. 1; 542)	DRN	24.6*** A > S	0.91 ± 0.28	No	No	Yes	Dom S
	LOG	0.85 A = S	Not calculated				
	SUB	6.53* A > S	0.49 ± 0.54	Yes	Yes	Yes	No power
	DRN-LOG	8.13* A < S	0.75 ± 0.69	No	Yes	Yes	Add/dom S
	LOG-SUB	1.33 A = S	Not calculated				
Angle of YLF ($\arcsin\sqrt{P}$, °) ($\sqrt{MS_{error}}$ 0.113; d.f. 1; 542)	DRN	3.72 A = S	Not calculated				
	LOG	2.38 A = S	Not calculated				
	SUB	28.6*** A < S	0.40 ± 0.26	No	Yes	No	Additivity
	DRN-LOG	6.02* A < S	0.69 ± 0.80	Yes	Yes	Yes	No power
	LOG-SUB	7.24* A < S	0.82 ± 0.73	No	Yes	Yes	Add/dom S

DRN, well drained; LOG, waterlogged; SUB, submerged.

A, *Rorippa amphibia*; S, *Rorippa sylvestris*; AS, F₁ hybrid A × S; SA, F₁ hybrid S × A; LLF, longest leaf; YLF, youngest leaf; \hat{H} , dominance coefficient (see text for explanation); CI, confidence interval. Values in bold are significant at the indicated level: * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$. Add, additivity; dom, dominance; partial dominance A indicates that hybrid trait values were between additivity and A dominance (i.e. $0 < \hat{H} < 0.5$) and $H = 0$ and $H = 0.5$ were not within the 95% CI.

leaves than *R. sylvestris* in the well-drained (DRN) and the waterlogged (LOG) treatments (Fig. 1a). However, *R. sylvestris* responded more strongly to the LOG treatment, forming 25% fewer leaves when waterlogged, whereas *R. amphibia* leaf formation did not change relative to the DRN treatment. In the DRN treatment, the relative shoot growth was comparable (Fig. 1b), while relative root growth was higher in *R. sylvestris* (Fig. 1c). For these traits, *R. sylvestris* reacted more strongly to waterlogging than *R. amphibia*. As a result, the relative shoot growth of *R. sylvestris* in the LOG treatment became significantly lower than that of *R. amphibia*, and the relative growth of the roots became similar (Table 2). The shoot dry matter content of *R. amphibia* was higher than that of *R. sylvestris* in the DRN treatment, but there was no significant difference in the LOG treatment (Table 2), because of a stronger negative reaction slope of *R. amphibia* going from DRN to LOG (Fig. 1e). The most striking difference in response to waterlogging concerned the formation of specialized roots by *R. amphibia*. In the DRN treatment, adventitious and aboveground root formation were similar and low for the two species (adventitious roots: *R. amphibia*, 0.23 roots per plant; *R. sylvestris*, 0.50 roots per plant; Mann–Whitney *U*-test, $U = 1115$, not significant; aboveground root length: *R. amphibia*, 11 mm; *R. sylvestris*, 45 mm; Mann–Whitney *U*-test, $U = 1051$, not significant). However, *R. amphibia* formed significantly more adventitious roots when waterlogged (*R. amphibia*, 0.85 roots per plant; *R. sylvestris*, 0.29 roots per plant; Mann–Whitney *U*-test, $U = 822$, $P < 0.005$) as well as a significantly longer aboveground root length (*R. amphibia*, 184 mm; *R. sylvestris*, 31 mm; Mann–Whitney *U*-test, $U = 585$, $P < 0.0005$).

Leaf morphological traits Upon waterlogging, the length of the longest leaf increased markedly in *R. amphibia*, but remained similar for *R. sylvestris* (Fig. 1f). The angle of the longest leaf of *R. amphibia* was not affected by waterlogging (Fig. 1i). In contrast, in *R. sylvestris*, the leaf angle was smaller in the DRN treatment (Table 2), but leaves turned upward in response to waterlogging (Fig. 1i) to attain an orientation similar to that of *R. amphibia* (Table 2). The leaf angle of the youngest leaf behaved in a similar fashion in the two species. Going from DRN to LOG, the leaf angle of the youngest leaf increased, but the reaction slope was steeper in *R. sylvestris* (Fig. 1j).

Responses of parental species to submergence (SUB treatment)

Whole-plant traits Submergence had a strong negative effect on relative growth in both species (both in shoots and in roots). Still, both species established significant shoot growth (Fig. 1b) over 2 wk of submergence (mean relative shoot growth \pm 95% CI (\log_e g g⁻¹; $n = 24$): *R. amphibia*, 0.189 ± 0.119 ; *R. sylvestris*, 0.310 ± 0.172). In *R. amphibia*, this was associated with a significant loss of root biomass (Fig. 1c; mean relative root growth \pm 95% CI (\log_e g g⁻¹; $n = 24$): *R. amphibia*, -0.263 ± 0.150),

whereas in *R. sylvestris* the root biomass remained constant (mean relative root growth \pm 95% CI (\log_e g g⁻¹; $n = 24$): *R. sylvestris*, 0.064 ± 0.164). Consistent with this, the number of newly formed leaves decreased in both species going from LOG to SUB, but more so in *R. sylvestris* (Fig. 1a).

In contrast to the response seen in the LOG treatment, in the SUB treatment *R. amphibia* formed significantly fewer (hardly any) adventitious roots than *R. sylvestris* (*R. amphibia*, 0.02 roots per plant; *R. sylvestris*, 0.29 roots per plant; Mann–Whitney *U*-test, $U = 1030$, $P < 0.05$), and had a shorter aboveground root length (*R. amphibia*, 8.3 mm; *R. sylvestris*, 45 mm; Mann–Whitney *U*-test, $U = 959$, $P < 0.05$).

Leaf morphological traits Both species showed a similar reaction going from LOG to SUB, that is, leaves became smaller upon submergence (Fig. 1f). Going from LOG to SUB, relative petiole length increased in both species, but the slope of the reaction was much steeper in *R. sylvestris* (Fig. 1g). At the same time, *R. sylvestris* leaves became rounder upon submergence (i.e. a higher width:length ratio; Fig. 1h). In both species the leaf angle of the longest leaf decreased upon submergence (Fig. 1i), but leaf positioning was significantly more prostrate in *R. sylvestris* in the SUB treatment. This contrasted with the pattern observed for the youngest leaf. In *R. sylvestris*, the leaf angle of the youngest leaf increased to an almost vertical orientation (i.e. more than 80°) in the SUB treatment, whereas in *R. amphibia* the leaf angle remained constant at *c.* 70° (Fig. 1j). Finally, we observed that *R. amphibia* leaf shape did not change in response to any of the treatments. Even in the submerged treatment, leaves remained simple.

Trait expression in F₁ hybrids

Comparing reciprocal hybrids To test for potential species-specific maternal effects, we compared the two reciprocal hybrid classes (ANOVA independent contrasts). For all response variables the reciprocal hybrids were similar in their trait values and responses to the treatments (data not shown). Therefore, the two reciprocal hybrid classes were considered together (referred to as ‘hybrids’) in subsequent contrast analyses.

Evaluating the dominance coefficient If the parental species did not differ significantly for a trait in a particular treatment, we did not calculate the dominance coefficient (indicated as ‘not calculated’ in Table 2). In all these cases, the hybrids did not show clear signs of overdominance (Fig. 1), although this was not formally tested. For traits for which the parental species differed significantly, but not markedly, the power to evaluate the dominance coefficient was limited because of widening confidence intervals (Fig. 2). Still, in most cases, it was possible to reject at least one of the hypotheses. Table 2 summarizes the statistical evaluations of hypotheses regarding the expression of parental traits in the hybrids ($H = 0$ corresponds to *R. amphibia* dominance; $H = 0.5$ corresponds to additivity; $H = 1$ corresponds

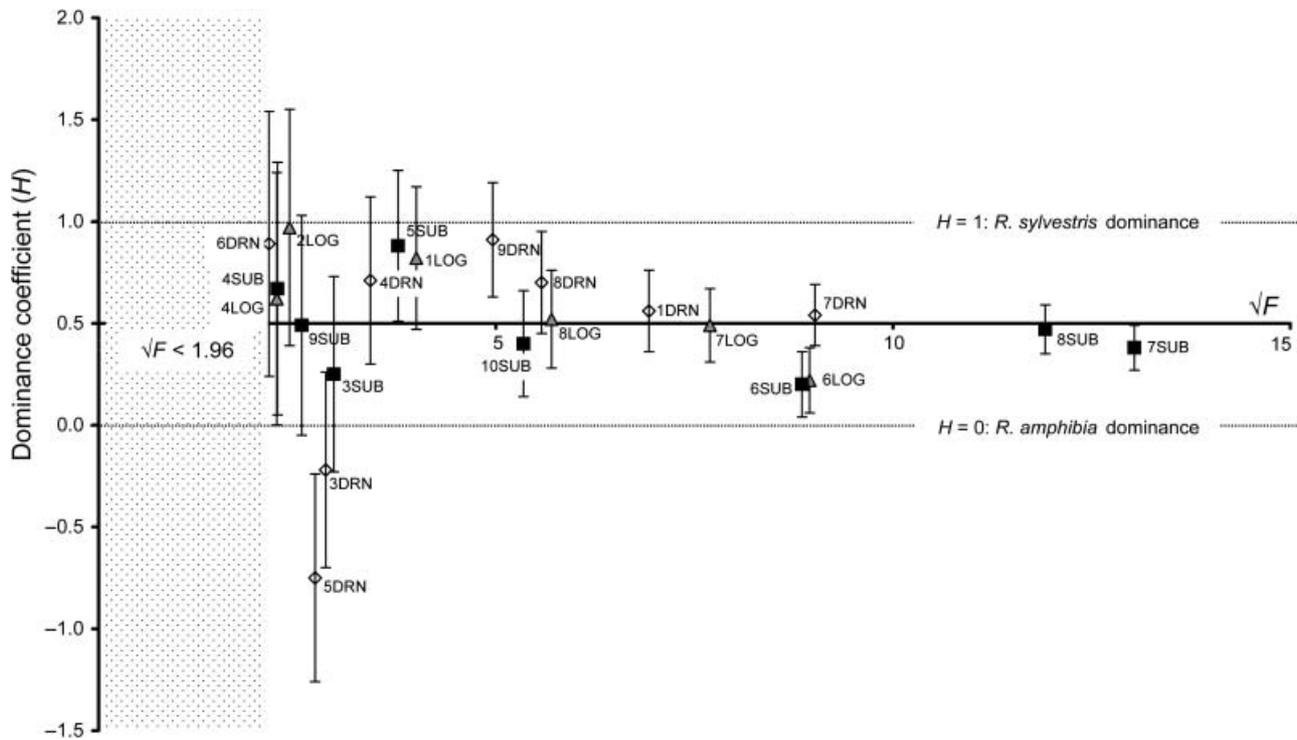


Fig. 2 Scatter plot of the dominance coefficient (H) and parental trait difference (expressed as \sqrt{F} , the square root of the ANOVA contrast A vs S). The shaded area ($\sqrt{F} < 1.96$) implies that the parents did not differ significantly. Bars, 95% confidence intervals. Labels indicate the treatment (diamonds, well drained (DRN); triangles, waterlogged (LOG); squares, submerged (SUB)) and response variable (1, number of leaves formed; 2, relative shoot growth; 3, relative root growth; 4, shoot weight fraction; 5, shoot dry matter content; 6, length of longest leaf; 7, relative petiole length of longest leaf; 8, width:length ratio of longest leaf; 9, angle of longest leaf; 10, angle of youngest leaf).

to *R. sylvestris* dominance). Figure 1 shows the untransformed hybrid mean trait values allowing comparisons with the parental means.

Dominance of *R. amphibia* Hybrids resembled *R. amphibia* in their lower relative root growth in the DRN treatment (i.e. $H = 0$ within $\hat{H} \pm 95\%$ CI; Table 2). In the SUB treatment, hybrids (similar to both parental species) established significant shoot growth (mean relative shoot growth $\pm 95\%$ CI ($\log_e g g^{-1}$; $n = 48$): hybrids, 0.220 ± 0.086). Similar to *R. amphibia*, this shoot growth was associated with a significant reduction in root biomass (Fig. 1c; mean relative root growth $\pm 95\%$ CI ($\log_e g g^{-1}$; $n = 48$): hybrids, -0.216 ± 0.107). Hybrids also showed the typical *R. amphibia* waterlogging response in forming more adventitious roots (*R. amphibia*, 0.85; *R. sylvestris*, 0.29; hybrids, 0.68; Mann–Whitney U -test for *R. amphibia* vs hybrids, $U = 2101$, not significant; for *R. sylvestris* vs hybrids, $U = 1844$, $P < 0.05$) and in increasing aboveground root length (*R. amphibia*, 184 mm; *R. sylvestris*, 31 mm; hybrids, 141 mm; Mann–Whitney U -test for *R. amphibia* vs hybrids, $U = 1979$, not significant; for *R. sylvestris* vs hybrids, $U = 1466$, $P < 0.0005$). The increasing length of the longest leaf of the hybrids going from DRN to LOG resembled the waterlogging response of *R. amphibia* (Fig. 1f). Hybrid leaf lengths resembled those of *R. amphibia* in the LOG and SUB treatments (partial

dominance of *R. amphibia*; Table 2). Finally, the relative petiole length of hybrids was more similar to that of *R. amphibia* in the SUB treatment (partial dominance of *R. amphibia*; Table 2), while for the other treatments it was intermediate to those of the parental species (i.e. 0.5 within $\hat{H} \pm 95\%$ CI; Table 2).

Dominance of *R. sylvestris* Hybrids only resembled *R. sylvestris* (i.e. $H = 1$ within $\hat{H} \pm 95\%$ CI) in their larger shoot dry matter content in the SUB treatment (Fig. 1e) and their more prostrate longest leaf orientation in the DRN treatment (Fig. 1i). In all other cases where hybrids resembled *R. sylvestris*, there was enough power to reject *R. amphibia* dominance ($H = 0$ not within $\hat{H} \pm 95\%$ CI), but not to distinguish between *R. sylvestris* dominance and additivity (Table 2). This was the case for the smaller relative shoot growth in the LOG treatment (Fig. 1c) and for the overall smaller shoot weight fraction (as compared with *R. amphibia*; Fig. 1d). Hybrids were also more similar to *R. sylvestris* in that the reaction slopes were steeper for the angle of the longest leaf from DRN to LOG (Fig. 1i) and for the angle of the youngest leaf from LOG to SUB (Fig. 1j). The larger number of leaves that hybrids formed in the LOG treatment (Table 2) and the negative reaction going from LOG to SUB (Fig. 1a) also indicated a tendency towards *R. sylvestris* dominance (Table 2).

Additivity and overdominance In the DRN treatment, hybrids had a higher shoot dry matter content than either parent (Fig. 1e), which was the only case in which overdominance (i.e. $\hat{H} \pm 95\% \text{ CI} < 0$) was observed (Table 2). In all remaining cases in which dominance of either of the parental species could be rejected, hybrids were intermediate in their trait values or reaction slopes (i.e. $H = 0.5$ within $\hat{H} \pm 95\% \text{ CI}$; Table 2).

Discussion

The first aim of this paper was to compare *R. amphibibia* (denoted 'A' in figures and tables) and *R. sylvestris* (denoted 'S' in figures and tables) in terms of their responses to different flooding regimes. The former species occurs in more stable, constantly wet to waterlogged habitats, and the latter in habitats with a more unpredictable regime of intermittent (often prolonged and deep) flooding and drought episodes. We set out with a specific set of expectations of species-specific responses to three treatments, chosen to be representative of the naturally prevailing water regimes (except drought). Our data indeed revealed pronounced differences between the species.

Our results support the hypothesis that *R. amphibibia* is better able to cope with waterlogging than *R. sylvestris*. In contrast to *R. sylvestris*, *R. amphibibia* growth hardly differed between the well-drained and waterlogged conditions. Leaf formation, morphology and positioning were not much affected by waterlogging either. This apparent waterlogging tolerance may be related to the ability of *R. amphibibia* to develop adventitious and aboveground roots in the water surface layer, which is a typical response for plants of waterlogged environments (Armstrong *et al.*, 1994; Blom & Voesenek, 1996; Vartapetian & Jackson, 1997). In *Rumex* spp. similar species differentiation was found in the ability to form adventitious roots (Visser *et al.*, 1996). *Rorippa amphibibia* also showed an unexpected response. The leaf size of the longest leaf of *R. amphibibia* increased significantly upon waterlogging, an effect not seen in *R. sylvestris*. Future studies should provide insight into the physiological basis of this leaf size difference, and its potential adaptive significance.

According to expectations, *R. sylvestris* showed the most vigorous growth under well-drained conditions, and high allocation to root growth. This corresponds to the hypothesis that under favourable conditions this species builds up carbohydrate reserves that are stored in the roots (Mooney, 1972), thereby increasing its regeneration capacity after adverse conditions (e.g. waterlogging or submergence).

The effect of submergence was comparable for the two parental species. Contrary to our expectation that *R. sylvestris* would arrest growth when submerged, both *R. amphibibia* and *R. sylvestris* established significant growth over 2 wk under submerged conditions. Growth was reduced compared with the well-drained and waterlogging treatments and restricted to the shoot. In *R. amphibibia* shoot growth was associated with a loss of root biomass. This suggests that submerged *R. amphibibia* plants cannot photosynthesize underwater at rates that are

sufficient to provide the carbon needed for the observed shoot growth. Possibly, they deplete their carbohydrate reserves in the taproot in an attempt to reach the surface. Mobilization of starch reserves upon submergence has been previously reported in rice (*Oryza sativa*; Raskin & Kende, 1984) and in *Rumex palustris* (Groeneveld & Voesenek, 2003). This will be advantageous if flooding is shallow so that restoring air contact is indeed possible. Alternatively, root growth in *R. amphibibia* may be hampered as a consequence of a limited diffusion of oxygen to the roots, as a result of lower porosities or substantial radial oxygen loss (Visser *et al.*, 2000). In contrast to *R. amphibibia*, the shoot growth in *R. sylvestris* was not accompanied by a reduction of root biomass. This suggests that *R. sylvestris* can survive (and even continue to grow) under submerged conditions without depleting its reserves. It appears that *R. sylvestris* may be capable of underwater photosynthesis (Mommer & Visser, 2005) at a rate sufficient to support overall growth. Moreover (in contrast to *R. amphibibia*), the leaf petiole of *R. sylvestris* elongated and the orientation of the youngest leaf became almost vertical, suggesting a hyponastic response (Voesenek *et al.*, 2006). Clearly, *R. sylvestris* does not passively wait for better times in a state of complete anaerobic dormancy (Laan & Blom, 1990; Vartapetian & Jackson, 1997), at least not under the (light) conditions in our experiment. In *Rumex* species, survival under submerged conditions was reduced in the absence of light (Nabben *et al.*, 1999). It was shown that shading may have an additional effect on growth in several waterlogging-tolerant species (Lenssen *et al.*, 2003). The apparent tolerance to both waterlogging and submergence of the *Rorippa* species calls for future experiments to test the effect of shade in this system. In addition to this, (micro)habitat differences between *R. amphibibia* and *R. sylvestris* could be explained by soil characteristics and differences in drought tolerance or drought avoidance strategies (Lenssen *et al.*, 2005; Touchette, 2007). Although this has not been addressed in the current study, preliminary results of ongoing work indicate that *R. sylvestris* is capable of surviving longer periods of drought than *R. amphibibia*, which may explain its occurrence in more drought-prone habitats (dykes, agricultural fields and roadsides) that are not necessarily affected by flooding.

Our second aim was to assess how parental traits associated with flooding are expressed in first-generation (F_1) hybrids. For each trait, we explicitly tested whether the average of the hybrids deviated from the hypothesis of intermediacy. If it did so, we subsequently tested for dominance of either parent and overdominance. In a study of gene expression in a maize (*Zea mays*) F_1 hybrid, additivity was found to be the most common mode of gene action between (inbred) parental lines (Swanson-Wagner *et al.*, 2006). We could not reject intermediacy for the majority of cases, which may indicate that the rule of additivity also applies if the hybridizing parents are outbred. However, for a number of traits we rejected the intermediacy hypothesis and observed dominance of parental species traits. This may be explained in several ways. First, the parental species may have fixed genetic differences for a few genes with a major effect on

growth and nonadditive gene action, similar to the situation of hybridizing two inbred parents (Swanson-Wagner *et al.*, 2006). Secondly, many individual genetic effects may be channelled to a common metabolic pathway which results in nonadditivity of the phenotypic expression. Finally, the observed patterns may be explained by nucleolar dominance, a phenomenon where the genome of one parent is silenced (see Pikaard (2000) for a review). Note also that tetraploid F_1 hybrids consist of two complete sets of genes, one from each parent. Epistatic interactions in F_1 s are therefore presumably different from those in later generations, where some parental genes could be absent, in particular as the mode of inheritance in the hybrid tetraploids is not disomic (Stift *et al.*, 2008). Current research is comparing the expression profiles of the parental species with those of F_1 and further hybrid (backcross) generations. From an ecological perspective, nonadditive expression of traits may have consequences for the habitat preference of hybrids. If hybrids resemble one of the parents (dominance), the hybrid habitat may overlap with that of the parent it resembles (Anderson, 1948). In particular, the typical *R. amphibibia* waterlogging responses were dominantly expressed in hybrids. As in *R. amphibibia*, waterlogging did not have an effect on growth and triggered the formation of adventitious roots, aboveground roots and a longer longest leaf. When submerged, hybrids also showed a root biomass reduction, indicating that (like *R. amphibibia*) hybrids are perhaps less efficient in terms of underwater photosynthesis compared with *R. sylvestris*. If so, the hybrid habitat may thus mostly overlap with that of *R. amphibibia* in locations where the occurrence of the parental species is mainly determined by flooding. In such a setting, introgression and (further) backcrossing would be more likely to happen in the direction of *R. amphibibia* (Anderson & Hubricht, 1938).

In summary, our results supported our hypothesis that *R. amphibibia* is better able to cope with waterlogging than *R. sylvestris* and provide insight into the traits that underlie the specialization of *R. amphibibia* to waterlogged habitats. Additionally, we have shown that shoot growth under submerged conditions caused root biomass loss in *R. amphibibia*, whereas *R. sylvestris* could prevent such loss. Furthermore, we have shown that *R. amphibibia* leaf morphology remained constant, while *R. sylvestris* leaf morphology changed markedly upon submergence. Taken together, our results indicate that the two species are clearly different in their ways of coping with flooding. We are currently further unravelling the mechanisms that underlie these differences, making use of the extensive physiological knowledge of flooding tolerance in *Rumex* spp. (Voisenek *et al.*, 2006), the genomic resources available for *A. thaliana* and the hybrids in our system. We found that hybrids combine a complex suite of traits from both parents, sometimes determined by additive, sometimes by dominant and rarely by overdominant expression of the parental phenotypes. This means that *Rorippa* × *anceps* F_1 hybrids possess a unique phenotype, consisting of a combination of traits of both parental species. This specific hybrid phenotype may facilitate establishment of hybrids in

free meandering rivers, particularly in the floodplains of rivers such as (amongst others) the Elbe and Wisla. Glasshouse crosses have corroborated field observations that hybrids are fertile and backcross readily to both parental species (Stift, 2007), making introgressive hybridization (Anderson & Hubricht, 1938) the most likely evolutionary consequence of hybridization between *R. amphibibia* and *R. sylvestris*. Neutral microsatellite markers have revealed an interesting mixture of disomic and tetrasomic inheritance in F_1 hybrids (Stift *et al.*, 2008), which raises questions concerning the extent of the potential for transgressive segregation in further hybrid generations (backcrosses). We are currently investigating these further generations under both natural and glasshouse conditions, at the phenotypic, genetic and transcriptional levels.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Summary of response variable comparison (ANOVA) between the parental species and the hybrids immediately before the start of the treatments

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