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

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**Summary**

- The river floodplain species *Rorippa amphibia*, *Rorippa sylvestris*, and their hybrid *Rorippa × aniceps* 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 × aniceps*, underwater photosynthesis, waterlogging and submergence responses.


**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 aquaticus (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 Wisła (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 & Matthys, 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 groups R. amphibia 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 (Voesez, 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 (F1) 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 F1 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 F1 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.
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.

**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

<table>
<thead>
<tr>
<th>Pair</th>
<th>River</th>
<th>Location¹, country</th>
<th>Latitude</th>
<th>Longitude²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Meuse</td>
<td>Herbricht, Belgium</td>
<td>N: 50°54′43″ E: 05°42′26″</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Meuse</td>
<td>Swalmen, the Netherlands</td>
<td>N: 51°14′36″ E: 06°00′31″</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>IJssel</td>
<td>Doesburg, the Netherlands</td>
<td>N: 52°01′25″ E: 06°08′42″</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Zwarte Water</td>
<td>Zwartsluis, the Netherlands</td>
<td>N: 52°37′30″ E: 06°04′48″</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Elbe</td>
<td>Darchau, Germany</td>
<td>N: 53°14′01″ E: 10°54′18″</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Elbe</td>
<td>Darchau, Germany</td>
<td>N: 53°14′01″ E: 10°54′18″</td>
<td></td>
</tr>
</tbody>
</table>

¹Settlements closest to the exact sampling location.

²WGS84 coordinates.
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 = 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 $0^\circ$ (prostrate orientation) to $90^\circ$ (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 (RGshoot $= \log_e DWS_{\text{end}} - \log_e DWS_{\text{start}}$; RGroot $= \log_e DWR_{\text{end}} - \log_e DWR_{\text{start}}$). Furthermore, we calculated the shoot weight fraction (SWF = DWS/(DWS + DWR)) and the shoot dry matter content (SDMC = 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. amphibia 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 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. amphibia 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. amphibia 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. amphibia 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 $(\hat{H} \pm 95\% \text{ CI})$ for the number of leaves formed was $0.56 \pm 0.20$. Finally, we evaluated whether $H = 0$ (R. amphibia dominance), $H = 0.5$ (additivity) and $H = 1$ (R. sylvestris dominance) were part of $\hat{H} \pm 95\% \text{ CI}$. If not, we rejected dominance and additivity, and further evaluated whether there was partial dominance of R. amphibia ($0 < \hat{H} \pm 95\% \text{ CI} < 0.5$) or R. sylvestris ($0.5 < \hat{H} \pm 95\% \text{ CI} < 1$), or overdominance ($\hat{H} \pm 95\% \text{ CI} < 0 \ or 1 < \hat{H} \pm 95\% \text{ CI}$).

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. Rotippa amphibia total DW was around twice that of R. sylvestris, and R. amphibia...
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

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**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, $+1 \text{ SE} \ (n=48)$.
Table 2: Summary of response variable comparison (ANOVA) between the parental species and the hybrids for each of the treatments and reaction slopes

<table>
<thead>
<tr>
<th>Response variable</th>
<th>Treatment/reaction slope</th>
<th>F-value contrast A vs S, conclusion</th>
<th>$H \pm 95%$ CI</th>
<th>$H = 0$ within CI?</th>
<th>$H = 0.5$ within CI?</th>
<th>$H = 1$ within CI?</th>
<th>Conclusion hybrid phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of leaves</td>
<td>DRN 48.0*** A &lt; S</td>
<td>0.56 ± 0.20</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Additivity</td>
<td></td>
</tr>
<tr>
<td>formed (count)</td>
<td>LOG 16.0*** A &lt; S</td>
<td>0.82 ± 0.35</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Add/dom S</td>
<td></td>
</tr>
<tr>
<td>(\textit{MS}_error 1.046, d.f. 1; 542)</td>
<td>SUB 0.95 A = S</td>
<td>Not calculated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DRN-LOG 4.29* A &lt; S</td>
<td>0.20 ± 0.95</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No power</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LOG-SUB 4.58* A &lt; S</td>
<td>1.10 ± 0.92</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Add/dom S</td>
<td></td>
</tr>
<tr>
<td>Relative shoot growth</td>
<td>DRN 3.74 A = S</td>
<td>Not calculated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(log$_e$, g kg$^{-1}$)</td>
<td>LOG 5.78* A &gt; S</td>
<td>0.97 ± 0.58</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Add/dom S</td>
<td></td>
</tr>
<tr>
<td>(\textit{MS}_error 0.287; d.f. 1; 254)</td>
<td>SUB 1.92 A = S</td>
<td>Not calculated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DRN-LOG 9.45** A &lt; S</td>
<td>0.45 ± 0.64</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No power</td>
<td></td>
</tr>
<tr>
<td>Relative root growth</td>
<td>DRN 8.49** A &lt; S</td>
<td>−0.22 ± 0.48</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Dom A</td>
<td></td>
</tr>
<tr>
<td>(log$_e$, g kg$^{-1}$)</td>
<td>LOG 0.79 A = S</td>
<td>Not calculated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\textit{MS}_error 0.372; d.f. 1; 254)</td>
<td>SUB 8.51** A &lt; S</td>
<td>0.25 ± 0.48</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Add/dom A</td>
<td></td>
</tr>
<tr>
<td>Shoot weight fraction</td>
<td>DRN 7.25* A &lt; S</td>
<td>0.46 ± 0.73</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No power</td>
<td></td>
</tr>
<tr>
<td>(arc sin $\sqrt{g}$, g kg$^{-1}$)</td>
<td>LOG-SUB 7.27* A &gt; S</td>
<td>0.82 ± 0.73</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Add/dom S</td>
<td></td>
</tr>
<tr>
<td>Shoot dry matter content</td>
<td>DRN 11.7** A &gt; S</td>
<td>0.71 ± 0.41</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No power</td>
<td></td>
</tr>
<tr>
<td>(log$_e$, mm)</td>
<td>LOG 5.03* A &gt; S</td>
<td>0.62 ± 0.62</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No power</td>
<td></td>
</tr>
<tr>
<td>(\textit{MS}_error 0.0228; d.f. 1; 254)</td>
<td>SUB 5.07* A &gt; S</td>
<td>0.67 ± 0.62</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Add/dom S</td>
<td></td>
</tr>
<tr>
<td>Length of LLF</td>
<td>DRN 23.1*** A &gt; S</td>
<td>0.01 ± 0.41</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Dom A</td>
<td></td>
</tr>
<tr>
<td>(log$_e$, mm)</td>
<td>LOG-SUB &lt; 0.01 A = S</td>
<td>Not calculated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Petiole:length ratio for LLF</td>
<td>DRN 81.3*** A &lt; S</td>
<td>0.54 ± 0.15</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Additivity</td>
<td></td>
</tr>
<tr>
<td>(arc sin $\sqrt{g}$, mm mm$^{-1}$)</td>
<td>LOG 59.2*** A &lt; S</td>
<td>0.49 ± 0.18</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Additivity</td>
<td></td>
</tr>
<tr>
<td>(\textit{MS}_error 0.0751, d.f. 1; 542)</td>
<td>SUB 170*** A &lt; S</td>
<td>0.38 ± 0.11</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Partial dom A</td>
<td></td>
</tr>
<tr>
<td>Width:length ratio blade for LLF</td>
<td>DRN-LOG 14.3*** A &lt; S</td>
<td>0.22 ± 0.52</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Add/dom A</td>
<td></td>
</tr>
<tr>
<td>(log$_e$, mm mm$^{-1}$)</td>
<td>LOG-SUB 31.1*** A &lt; S</td>
<td>0.70 ± 0.25</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Additivity</td>
<td></td>
</tr>
<tr>
<td>(\textit{MS}_error 0.0792; d.f. 1; 542)</td>
<td>SUB 32.5*** A &lt; S</td>
<td>0.52 ± 0.24</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Additivity</td>
<td></td>
</tr>
<tr>
<td>Angle of LLF</td>
<td>DRN-LOG 15.7*** A &gt; S</td>
<td>0.50 ± 0.50</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Add/dom S</td>
<td></td>
</tr>
<tr>
<td>(arc sin $\sqrt{g}$, °)</td>
<td>LOG-SUB &lt; 0.01 A = S</td>
<td>Not calculated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angle of YLF</td>
<td>DRN 142.4*** A &gt; S</td>
<td>0.47 ± 0.12</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Additivity</td>
<td></td>
</tr>
<tr>
<td>(arc sin $\sqrt{g}$, °)</td>
<td>LOG-SUB 19.4*** A &gt; S</td>
<td>0.43 ± 0.45</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Add/dom A</td>
<td></td>
</tr>
<tr>
<td>Angle of YLF</td>
<td>DRN-LOG 2.38 A = S</td>
<td>Not calculated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(arc sin $\sqrt{g}$, °)</td>
<td>LOG-SUB 28.6*** A &gt; S</td>
<td>0.40 ± 0.26</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Additivity</td>
<td></td>
</tr>
<tr>
<td>Angle of YLF</td>
<td>DRN 6.02* A &lt; S</td>
<td>0.69 ± 0.80</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No power</td>
<td></td>
</tr>
<tr>
<td>(arc sin $\sqrt{g}$, °)</td>
<td>LOG-SUB 7.24* A &lt; S</td>
<td>0.82 ± 0.73</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Add/dom S</td>
<td></td>
</tr>
</tbody>
</table>

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

A, \textit{Rorippa amphibia}; S, \textit{Rorippa sylvestris}; AS, F, hybrid $A \times S$; SA, F, hybrid $S \times A$; LLF, longest leaf; YLF, youngest leaf; $\bar{H}$, 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. \(0 < \bar{H} < 0.5\)) 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. sylvestris* (Fig. 1f). If the parental species did not differ significantly for a trait in a particular treatment, they 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 to as ‘hybrids’) in subsequent contrast analyses.

**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* (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 to...
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 $H \pm 95\%$ CI; Table 2). In the SUB treatment, hybrids (similar to both parental species) established significant shoot growth (mean relative shoot growth $\pm 95\%$ CI $\log e g^{-1}$; $n = 48$): hybrids, $0.220 \pm 0.086$. 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 $\log e g^{-1}$; $n = 48$): hybrids, $-0.216 \pm 0.107$). 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 $H \pm 95\%$ CI; Table 2).

**Dominance of *R. sylvestris*** Hybrids only resembled *R. sylvestris* (i.e. $H = 1$ within $H \pm 95\%$ 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 $H \pm 95\%$ 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. \( H \pm 95\% \text{ CI} < 0 \)) 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 \( H \pm 95\% \text{ CI}; \) Table 2).

Discussion

The first aim of this paper was to compare \( R. \) amphibia (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 \( R. \) amphibia is better able to cope with waterlogging than \( R. \) sylvestris. In contrast to \( R. \) sylvestris, \( R. \) amphibia 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 \( R. \) amphibia 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 \( R. \) amphibia also showed an unexpected response. The leaf size of the longest leaf of \( R. \) amphibia 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 \( R. \) amphibia 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 \( R. \) amphibia shoot growth was associated with a loss of root biomass. This suggests that submerged \( R. \) amphibia 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 (\textit{Oryza sativa}; Raskin & Kende, 1984) and in \textit{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 \( R. \) amphibia 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 \( R. \) amphibia, 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 \( R. \) amphibia), the leaf petiole of \( R. \) sylvestris elongated and the orientation of the youngest leaf became almost vertical, suggesting a hypostatic 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 \textit{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 \textit{Rorippa} species calls for future experiments to test the effect of shade in this system. In addition to this, (micro)habitat differences between \( R. \) amphibia 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 \( R. \) amphibia, 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 (\textit{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 nuclear dominance, a phenomenon where the genome of one parent is silenced (see Pikaard (2000) for a review). Note also that tetraploid F1 hybrids consist of two complete sets of genes, one from each parent. Epistatic interactions in F1s 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 F1 and further hybrid phenotype may facilitate establishment of hybrids in a combination of traits of both parental species. This specific knowledge of flooding tolerance in these differences, making use of the extensive physiological are clearly different in their ways of coping with flooding. We . Taken together, our results indicate that the two species that 2006), the genomic resources available for R. sylvestris R. sylvestris Additionally, we have shown that shoot growth under submerged the occurrence of the parental species is mainly determined by mostly overlap with that of R. sylvestris (Anderson, 1948). In particular, the typical R. amphibia waterlogging responses were dominantly expressed in hybrids. As in R. sylvestris, 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. amphibia) 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. amphibia in locations where the occurrence of the parental species is mainly determined by flooding. In such a setting, introgression and (further) back-crossing would be more likely to happen in the direction of R. amphibia (Anderson & Hubricht, 1938).

In summary, our results supported our hypothesis that R. amphibia is better able to cope with waterlogging than R. sylvestris and provide insight into the traits that underlie the specialization of R. amphibia to waterlogged habitats. Additionally, we have shown that shoot growth under submerged conditions caused root biomass loss in R. amphibia, whereas R. sylvestris could prevent such loss. Furthermore, we have shown that R. amphibia 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. (Voesenek 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 × anceph R1 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. amphibia and R. sylvestris. Neutral microsatellite markers have revealed an interesting mixture of disomic and tetrasomic inheritance in F1 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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References


