Effects of experimental inbreeding on herbivore resistance and plant fitness: the role of history of inbreeding, herbivory and abiotic factors

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Abstract
Inbreeding is common in plant populations and can affect plant fitness and resistance against herbivores. These effects are likely to depend on population history. In a greenhouse experiment with plants from 17 populations of *Lychnis flos-cuculi*, we studied the effects of experimental inbreeding on resistance and plant fitness. Depending on the levels of past herbivory and abiotic factors at the site of plant origin, we found either inbreeding or outbreeding depression in herbivore resistance. Furthermore, when not damaged experimentally by snail herbivores, plants from populations with higher heterozygosity suffered from inbreeding depression and those from populations with lower heterozygosity suffered from outbreeding depression. These effects of inbreeding and outbreeding were not apparent under experimental snail herbivory. We conclude that inbreeding effects on resistance and plant fitness depend on population history. Moreover, herbivory can mask inbreeding effects on plant fitness. Thus, understanding inbreeding effects on plant fitness requires studying multiple populations and considering population history and biotic interactions.

Keywords
Experimental inbreeding, fitness, herbivore resistance, herbivory, inbreeding depression, outbreeding depression, purging of genetic load.

INTRODUCTION
Inbreeding is common in natural plant populations and can have considerable demographic and evolutionary consequences (Keller & Waller 2002), largely because inbred offspring have lower fitness than outcrossed offspring (Falconer 1981; Charlesworth & Charlesworth 1987; Husband & Schemske 1996). Inbreeding depression can be lower in populations with a long history of inbreeding due to purging of the genetic load (Lande & Schemske 1985; Charlesworth & Charlesworth 1987; but see Keller & Waller 2002; Lienert & Fischer 2004; Willi et al. 2005). Moreover, inbreeding depression can be more apparent under stressful biotic and abiotic conditions (Dudash 1990; but see Willi et al. 2007).

Given that inbreeding affects the expression of genetic variation and that variation in plant responses to natural enemies, such as herbivores, often has a genetic basis (e.g. Marquis 1984, 1990; Berenbaum et al. 1986; Simms & Rausher 1987, 1989), inbreeding is not only likely to affect plant fitness directly but also indirectly by modifying interactions between plants and herbivores (Carr & Eubanks 2002; Hayes et al. 2004). However, inbreeding effects on plant–herbivore interactions have started to receive attention only recently (Nunez-Farfán et al. 1996; Carr & Eubanks 2002; Hayes et al. 2004; Ivey et al. 2004; Stephenson et al. 2004). Inbreeding effects on plant defence can manifest themselves as altered suitability, resistance or tolerance to natural enemies (e.g. Strauss & Karban 1994; Carr & Eubanks 2002; Ivey et al. 2004; Stephenson et al. 2004).

Of the few studies addressing inbreeding and herbivory in combination, some have found inbreeding depression in plant resistance and/or tolerance to herbivores and other natural enemies (Ouborg et al. 2000; Carr & Eubanks 2002; Hayes et al. 2004; Ivey et al. 2004; Stephenson et al. 2004), whereas others have not (e.g. Nunez-Farfán et al. 1996). However, because most of these studies were based on one or very few study populations, their results may not be generalizable. Indeed, the studies using more than one...
population found the severity of inbreeding depression in resistance to vary among host-plant populations (Carr & Eubanks 2002; Ivey et al. 2004). This can arise due to among-population differences in levels of past inbreeding or in selection by past herbivory or the abiotic environment. The effects of such potential causes for among-population variation have, however, not been addressed. Thus, it is not known whether inbreeding effects on resistance are influenced by past selection by herbivores or whether they have been purged from populations with a long history of inbreeding. Furthermore, it is not known whether adaptation to abiotic conditions, which may shape the genetic constitution of populations, influences effects of inbreeding.

Understanding how genetic and environmental characteristics of populations influence inbreeding depression is of fundamental interest and also essential considering responses to current anthropogenic environmental changes, such as habitat alteration and fragmentation. Habitat fragmentation has changed the genetic structure and ecological conditions of populations of numerous plant species worldwide. Reductions in population size commonly reduce genetic variation, increase levels of inbreeding and, as a consequence, reduce fitness (Leimu et al. 2006). Moreover, fragmentation alters the abundance and composition of communities of natural enemies of plants (e.g. Kéry et al. 2001; Steffan-Dewenter & Tscharntke 2002). Such changes clearly reflect the potential for changes in both selective pressures exerted by herbivores and the abilities of plants to respond. However, the evolutionary consequences of habitat fragmentation on interactions between plants and herbivores have not been examined.

Here we examined how population level variation in past natural herbivory, history of inbreeding and abiotic environmental conditions influence effects of experimental inbreeding on herbivore resistance and plant fitness. We conducted greenhouse experiments with plants originating from 17 Swiss Lychnis flos-cuculi populations. We used L. flos-cuculi as our study plant because of its mixed mating system (Biere 1996; Galeuchet et al. 2005b) and because it is known to suffer from inbreeding depression in fitness after experimental selfing (Hauser & Loeschcke 1994, 1996). Moreover, its population sizes differ due to habitat fragmentation, and smaller populations have lower heterozygosity and reduced plant fitness (Galeuchet et al. 2005a,b). Finally, its populations occur in habitats that differ in the levels of herbivore damage and in abiotic conditions. In our experiment, we used plants resulting from two generations of experimental selfing and within-population outcrossing grown in a greenhouse. The generalist snail, Arianta arbustorum, was used as the herbivore in the experiments.

We addressed the following questions: (i) Are inbred plants less resistant against herbivory than outcrossed plants? (ii) Do inbreeding effects on resistance depend on past herbivore pressure, history of inbreeding or environmental factors at the populations of origin? (iii) Do inbreeding effects on fitness depend on past herbivore pressure, history of inbreeding or environmental factors at the populations of origin and do these effects differ between damaged and undamaged plants?

**Materials and Methods**

**Study species**

Lychnis flos-cuculi (Caryophyllaceae) is a polycarpic perennial herb. It is still widespread and abundant throughout its distribution range where it occurs in sunny and moist habitats such as wet hay meadows and calcareous fens (Galeuchet et al. 2005a). However, in recent decades, its populations have become smaller and more isolated due to the loss and fragmentation of suitable habitats. Plants grow up to 70 cm tall flowering stems from leaf rosettes, and produce up to 60 insect-pollinated flowers.

A number of generalist herbivores, including snails, leaf miners and lepidopteran larvae (Wiorkos & Plassmann 1999), and some specialists (e.g. Galeuchet 2003) have been observed to attack the plants in the field. We used the generalist hermaphroditic snail, A. arbustorum (Gastropoda: Helicidae), as herbivore in our experiment. It occurs commonly throughout Europe (Burla & Stahel 1983; Speiser & Rowell-Rahier 1991), is found on L. flos-cuculi in our study populations (D. Galeuchet, pers. comm.) and is known as important herbivore of related plant species (Elmqvist & Gardfjell 1988). Moreover, it is known to be sensitive to several plant secondary compounds (Hägge & Rahier 2001), and thus it can be expected to respond to variation in plant defence.

**Population characteristics**

We used plants from 17 L. flos-cuculi populations located in calcareous fens in North-East Switzerland. We obtained information on population characteristics from previous studies (Galeuchet 2003; Galeuchet et al. 2005a; Hoehn 2006). As a measure of herbivory in the field, we used the average number of leaves damaged by herbivores in the field populations during the 3 years 2000–2002 (Galeuchet 2003), including the time when the initial selfing and outcrossing hand-pollinations (see below) were conducted in 2000 (Perret 2003). Observed heterozygosity of the populations was determined with microsatellite markers based on 9–19 plants from each population (Galeuchet et al. 2002, 2005b). In the field, plants of L. flos-cuculi grow best in moist, full-light and nutrient-rich habitats at low altitudes (Galeuchet 2003; Perret 2003). Altitudes of our study populations were obtained using Swiss topographic maps, and mean
ecological indicator values for levels of moisture, light and nutrients were calculated from vegetation records according to Landolt (1977) for each population (Galeuchet et al. 2005a). The environmental population characteristics were not significantly correlated with each other (light vs. moisture: \( r = -0.059, P = 0.841 \); light vs. nutrients: \( r = -0.305, P = 0.289 \); moisture vs. nutrients: \( r = -0.326, P = 0.255 \)).

**Plant material**

Initially, in each population 12 randomly selected plants were assigned as maternal plants and bagged in 2000. To obtain inbred F1 offspring, altogether 204 maternal plants were selfed by hand pollinations between two flowers of the same plant. To obtain outbred F1 offspring, the 204 plants were hand-pollinated with another plant from the same population. For the outcrosses, each plant served once as maternal plant and once as paternal one and different pollen donors were used for different maternal plants. The distances between crossed plants were between 5 and 10 m. The pollinated flowers were bagged again, and mature capsules were collected when the seeds had ripened. The seeds were germinated, and two randomly selected seedlings per fruit were transplanted and grown in the greenhouse until they flowered.

The F1 plants resulting from selfing were further selfed by hand-pollination to obtain the inbred F2, i.e. seeds resulting from two generations of selfing. The F1 plants resulting from outcrossing were further outcrossed with unrelated offspring of the same population to obtain the outbred F2, i.e. seeds resulting from two generations of outcrossing within the same population. For our experiment, we used these seeds to grow inbred and outbred F2 plants; i.e. for each of the 17 populations of origin, we used plants that resulted from two generations of inbreeding and plants that resulted from two generations of outcrossing. Using the F2 rather than the F1 plants has the advantage of reducing potential maternal environmental carry-over effects. For three of the 17 populations, we only had either inbred or outbred families. However, as the results did not depend on whether these populations were included or excluded, we took them into account in the analyses. Further details on the crossing procedures are provided in Hoehn (2006).

**Experimental design**

We sowed seeds of the inbred and outbred F2 families from each of the 17 populations in the greenhouse in February 2006. We reported 2–4 seedlings of each of 44 inbred families and of 47 outbred families and grew the plants until June 2006. All plants were well watered every day or every second day and grown under natural light conditions corresponding to the full-sun wet-grassland conditions in natural populations. In June 2006, we randomly assigned half of the plants of each family to the herbivory treatment (143 outbred and 133 inbred plants) while the other half served as control (130 outbred and 129 inbred plants). Before conducting the treatments, we counted the number of rosette leaves as a measure of initial plant size.

We collected snails for the herbivory treatment from a neutral environment, the park Sanssouci in Potsdam, Germany. This ensured that potential adaptation of snails to our study populations could not affect our results. The snails were kept in plastic containers and starved for 4 days before use in the experiment. After the experiment, all snails were released at the site of collection.

For the herbivory treatment, we covered the plants with cellophane bags permeable to air, put one snail in each bag and allowed it to feed on the plant for 6 days. The snails were weighed immediately before and after use in the experiment. The control plants were also covered with cellophane bags for the same 6 days after which all bags were removed. After removing the snails, we counted the number of damaged leaves and estimated the proportion of leaf area damaged by the snails visually to the nearest 1%. These proportions were similar to the ones recorded in the field by Galeuchet et al. (2005a). We used the common measures of resistance, 1- the proportion of leaf area damaged by the herbivores and inverse of snail performance (see Statistical analyses).

After the treatments, the plants were allowed to grow in the greenhouse for another 8 weeks, when most plants had started flowering again. At this time, we counted the number of rosette leaves and measured the rosette diameter as the measure of plant size and counted the number of flowering stalks, and the combined number of buds, flowers and fruits. Because 89.6% of all flower buds, flowers and fruits were fruits and most of the remaining flower buds and flowers turned into fruit capsules later, we added their numbers and considered them as number of fruits. Fruit production in the greenhouse is mainly due to selfing. The number of flowers produced correlated strongly positively with the number of fruits produced (\( r = 0.9804, P = 0.0001 \)). This indicates that there were no differences in selfing rates between families or populations, which would have influenced our analyses of inbreeding effects on the number of fruits.

**Statistical analyses**

We conducted the analyses of covariance (ANCOVA; Type III sums of squares) to test for inbreeding depression in the resistance and plant fitness. ANCOVA allows comparing a series of regression models and thus to analyse data with
both categorical and continuous explanatory variables and their interactions (Littell et al. 2006). We analysed two measures of resistance: 1-proportion of leaf damage and final snail weight using initial weight as covariate. In the latter case, increased resistance is indicated by reduced herbivore performance. We also tested for differences in plant fitness (number of fruits) between the damaged and undamaged plants. In these analyses, we also tested whether the effects of cross or experimental herbivory were influenced by the characteristics of the populations of plant origin (levels of herbivore damage measured in the field, observed microsatellite heterozygosity and environmental characteristics). Because our main interest was to examine the effects of population characteristics, we used population mean values of inbred and outbred plants for all analyses.

First, we analysed a full model, which contained all two-way interactions between the population characteristics and cross (inbred vs. outbred). In the analyses of plant fitness, we initially also included all three-way interactions of cross and treatment (herbivory or bagged control) and the population characteristics. From the final models, we removed all non-significant two- and three-way interactions in a stepwise manner. Initial snail weight was included as a covariate in the analyses on the proportion of leaf area damaged and the final snail weight. Initial number of rosette leaves was, in turn, taken into account as a covariate in the analyses on the proportion of leaf area damaged and on plant fitness. We found a weak negative correlation between plant size in terms of the number of rosette leaves and the proportion of leaf area damaged ($r = -0.129, P = 0.0007$), indicating that larger plant size does not stimulate the snail herbivores to consume more. All analyses were conducted using the PROC GLM procedure of the SAS 9.1 software (SAS Institute, Cary, NC, USA).

RESULTS

General effects of inbreeding on plant resistance and fitness

The snail herbivores damaged on average a lower proportion of the leaf area of the experimentally inbred plants compared to the outbred plants (Table 1; Fig. 1a). In contrast, the snails grew better on experimentally inbred plants than on outbred plants (Table 1; Fig. 1b), indicating inbreeding depression in resistance. In the control treatment, the experimentally inbred plants produced fewer fruits than the outbred plants, indicating inbreeding depression when the plants were not damaged by the snail herbivores (Table 2; Fig. 1c). In contrast, because damage by the snail herbivores reduced fruit production of the outbred plants to a greater extent (57.2%) than that of the inbred plants (35.9%), the fitness differences between inbred and outbred plants were equalized under snail herbivory, resulting in no detectable inbreeding depression (Fig. 1c).

Inbreeding effects in relation to natural levels of herbivory

The proportion of leaf area damaged of the experimentally outbred plants was independent of past herbivory at the site of origin (Fig. 2a). In contrast, the proportion of leaf area damaged of the inbred plants increased, i.e. their resistance decreased, with the past level of herbivory at the site of origin (Table 1; Fig. 2a). The lower resistance of inbred plants from populations with high past herbivory indicates inbreeding depression in resistance for these populations (Table 1; Fig. 2a).

The fitness of the outbred plants decreased with higher level of past herbivory in the population of origin whereas the fitness of the inbred plants was independent of the level of past herbivory (Table 2; Fig. 3a). Hence, inbreeding depression in fitness was found only for populations with low levels of past herbivory (Fig. 3a).

Inbreeding effects in relation to history of inbreeding

Inbreeding depression in resistance was not statistically significantly related to the observed heterozygosity of the

| Table 1 ANOVA summary of effects of characteristics of populations of plant origin on measures of resistance to snail herbivory (1-proportion of leaf area damaged, snail performance) of inbred and outbred plants |
|-----------------|---|-------|-------|
| Source          | d.f. | F     | P     |
| 1-damage        |     |       |       |
| Initial snail weight | 1  | 1.56  | 0.2259|
| Number of rosette leaves | 1  | 8.86  | 0.0075|
| Cross           | 1   | 6.29  | 0.0208|
| Observed heterozygosity | 1  | 0.33  | 0.5696|
| Herbivory in the field | 1  | 0.85  | 0.3666|
| Moisture        | 1   | 0.31  | 0.5833|
| Light           | 1   | 0.00  | 0.9511|
| Nutrients       | 1   | 0.38  | 0.5449|
| Altitude        | 1   | 0.62  | 0.4417|
| Herbivory in the field × cross | 1  | 7.22  | 0.0142|
| Residual        | 20  |       |       |
| Snail weight    |     |       |       |
| Initial snail weight | 1  | 8.25  | 0.0091|
| Cross           | 1   | 15.11 | 0.0008|
| Observed heterozygosity | 1  | 0.46  | 0.5054|
| Herbivory in the field | 1  | 1.09  | 0.3086|
| Moisture        | 1   | 0.99  | 0.3312|
| Light           | 1   | 0.85  | 0.3681|
| Nutrients       | 1   | 4.42  | 0.0477|
| Altitude        | 1   | 0.62  | 0.4414|
| Moisture × cross | 1  | 15.51 | 0.0008|
| Residual        | 21  |       |       |

Population mean values were used in this analysis.
plant populations of origin (Table 1), providing no evidence for purging of such inbreeding depression. When not exposed to snail herbivores, the fitness of the outbred plants increased with the level of heterozygosity at the population of origin (Table 2; Fig. 3c). The fitness of damaged and undamaged inbred plants was independent of the level of heterozygosity at the population of origin (Table 2; Fig. 3c). The higher fitness of undamaged outbred plants of populations with high heterozygosity compared to the inbred plants from these populations indicates inbreeding depression for those plants from these populations (Table 2; Fig. 3c). In contrast, when not damaged by the snails, the higher fitness of inbred plants from populations with low heterozygosity compared with the outbred plants from the same populations suggests outbreeding depression for plants from these populations (Table 2; Fig. 3c).

### Table 2 ANCOVA summary of effects of characteristics of populations of plant origin on fitness (assessed as number of fruits) of inbred and outbred plants (factor cross) damaged or undamaged by snail herbivory (factor treatment)

<table>
<thead>
<tr>
<th>Source</th>
<th>d.f.</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial no. rosette leaves</td>
<td>1</td>
<td>1.95</td>
<td>0.1703</td>
</tr>
<tr>
<td>Treatment</td>
<td>1</td>
<td>0.76</td>
<td>0.3901</td>
</tr>
<tr>
<td>Cross</td>
<td>1</td>
<td>20.84</td>
<td>0.0001</td>
</tr>
<tr>
<td>Treatment × cross</td>
<td>1</td>
<td>3.27</td>
<td>0.0781</td>
</tr>
<tr>
<td>Observed heterozygosity</td>
<td>1</td>
<td>11.16</td>
<td>0.0018</td>
</tr>
<tr>
<td>Herbivory in the field</td>
<td>1</td>
<td>5.05</td>
<td>0.0301</td>
</tr>
<tr>
<td>Moisture</td>
<td>1</td>
<td>1.75</td>
<td>0.1937</td>
</tr>
<tr>
<td>Light</td>
<td>1</td>
<td>0.46</td>
<td>0.5051</td>
</tr>
<tr>
<td>Nutrients</td>
<td>1</td>
<td>0.38</td>
<td>0.5429</td>
</tr>
<tr>
<td>Altitude</td>
<td>1</td>
<td>0.84</td>
<td>0.3643</td>
</tr>
<tr>
<td>Observed heterozygosity × cross</td>
<td>1</td>
<td>9.26</td>
<td>0.0041</td>
</tr>
<tr>
<td>Natural damage level in field × cross</td>
<td>1</td>
<td>8.02</td>
<td>0.0072</td>
</tr>
<tr>
<td>Moisture × cross</td>
<td>1</td>
<td>16.07</td>
<td>0.0003</td>
</tr>
<tr>
<td>Light × cross</td>
<td>1</td>
<td>4.50</td>
<td>0.0401</td>
</tr>
<tr>
<td>Altitude × cross</td>
<td>1</td>
<td>6.13</td>
<td>0.0176</td>
</tr>
<tr>
<td>Observed heterozygosity × treatment</td>
<td>1</td>
<td>10.32</td>
<td>0.0026</td>
</tr>
<tr>
<td>Light × treatment</td>
<td>1</td>
<td>3.18</td>
<td>0.0822</td>
</tr>
<tr>
<td>Altitude × treatment</td>
<td>1</td>
<td>0.23</td>
<td>0.6324</td>
</tr>
<tr>
<td>Observed heterozygosity × treatment × cross</td>
<td>1</td>
<td>6.00</td>
<td>0.0188</td>
</tr>
<tr>
<td>Light × treatment × cross</td>
<td>1</td>
<td>4.30</td>
<td>0.0446</td>
</tr>
<tr>
<td>Altitude × treatment × cross</td>
<td>1</td>
<td>3.31</td>
<td>0.0763</td>
</tr>
<tr>
<td>Residual</td>
<td>40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Population mean values were used in this analysis.

Inbreeding effects in relation to the abiotic environment

When fed on outbred plants, snail growth decreased with increasing moisture level at the sight of plant origin (Fig. 2b). This indicates lower resistance of inbred plants.

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compared to outbred plants for populations with higher moisture levels and thus inbreeding depression in resistance for these populations (Fig. 2b). The better growth of the snails on inbred compared to outbred plants when the plants originate from populations with low moisture levels indicates, in turn, lower resistance of the outbred plants for these populations and thus outbreeding depression in resistance for plants from populations with higher moisture levels (Fig. 2b). Inbreeding depression in resistance, assessed via snail growth or leaf damage, did not depend on light conditions or altitude of the populations of origin.

Figure 2 Inbreeding effects on resistance in relation to (a) past herbivory in the field (number of damaged leaves) and (b) moisture indicator value (Landolt 1977) at the site of plant origin. Residual variation after correcting for covariates is presented in the figures. The black dots and black line denote outbred plants, and the grey dots and grey line denote inbred plants.

Figure 3 Effects of inbreeding and experimental snail herbivory on plant fitness in relation to (a) past herbivory in the field (number of damaged leaves), (b) moisture indicator value (Landolt 1977), (c) observed heterozygosity, (d) light intensity and (e) altitude (metres above sea level) at the site of origin. Residual variation after correcting for covariates is presented in the figures. The black dots and black line denote outbred plants, and the grey dots and grey line denote inbred plants. Filled symbols and solid lines denote undamaged plants, and open symbols and dashed lines denote damaged plants.
When not damaged by the snail herbivores, the fitness of the outbred plants decreased with increased levels of moisture and light, and the altitude of the population of plant origin (Fig. 3b,d,e). In contrast, the fitness of undamaged inbred plants tended to increase with higher levels of these abiotic factors of the populations of origin (Fig. 3b,d,e). Therefore, the higher fitness of the outbred compared to the inbred plants indicates inbreeding depression for these populations when not under experimental herbivory. The association of inbreeding effects with moisture levels at the site of origin did not differ between undamaged and damaged plants. In contrast, the fitness of damaged plants was independent of the cross type and the levels of light and altitude of the population of origin (Fig. 3d,e).

These findings highlight the importance of correcting for the effects of the abiotic environment at the site of plant origin when testing for effects of natural herbivory and heterozygosity on potential inbreeding depression in herbivore resistance.

**DISCUSSION**

**Inbreeding effects on resistance and plant fitness**

Overall, we found that the snails grew larger on inbred plants, but consumed more leaf material of the outbred plants (Fig. 1a,b). The former suggests inbreeding depression in plant resistance while the latter suggests outbreeding depression. This adds to the idea that the levels of inbreeding in plants influence their resistance against herbivores (Nunez-Farfán et al. 1996; Carr & Eubanks 2002; Hayes et al. 2004; Ivey et al. 2004; Stephenson et al. 2004). However, the observed opposing patterns for the two resistance measures appear contradictory and call for explanation.

It is likely that inbreeding influenced both resistance and the nutritional quality of the study plants. *Arianta arbustorum* snails are known to grow less when fed with plants that contained defence chemicals (Puutinen & Mutikainen 2001). Hence, the poorer growth of the snails on outbred plants may be due to higher levels of defensive compounds compared to inbred plants. Furthermore, the nutritional value of host plants is known to affect the consumption patterns of *A. arbustorum* (Speiser & Rowell-Rahier 1991). Certain herbivore species are known to increase consumption of nutrient-diluted or otherwise low-quality diets to compensate for reduced post-ingestive physiological efficiency (Slansky 1993; Kause et al. 1999). Thus, the fact that the snails consumed more leaf material of the outbred plants in our study can reflect lower leaf quality for the snails in terms of higher concentrations of defensive compounds and lower ratio of nutritive vs. defensive compounds. This could have forced the snails to consume more leaf material on outbred plants in order to gain the amount of nutritive compounds necessary for growth. Because nutritive quality and defensive compounds may be differently influenced by inbreeding (Hull-Sanders & Eubanks 2005), future studies on inbreeding effects on plant resistance should focus on the specific mechanisms of resistance, such as concentrations of defensive secondary chemicals.

Another main aim of our study was to examine whether herbivory influences inbreeding effects on plant fitness. It has been suggested that herbivory can alter inbreeding effects on fitness if damage by herbivores is analogous to stressful environmental conditions, under which inbreeding depression is commonly more pronounced (Dudash 1990; but see Willi et al. 2007). In contrast to this hypothesis, we observed inbreeding depression in plant fitness only for undamaged control plants, but not under experimental herbivory (Fig. 1c). This could suggest that herbivory may be more stressful for the plants than abiotic stress factors, such as low nutrient levels or drought, leading to poor performance of all plants irrespective of their genetic background, and therefore masking inbreeding effects of plants under herbivory. Alternatively, the herbivory applied in our study may have been qualitatively different from other environmental stress factors, and therefore it may have overridden the inbreeding effects observed in undamaged control plants. However, the effects of herbivory on inbreeding depression may depend on the type and amount of actual plant damage more than on the sheer presence of the herbivores. Therefore, as the outbred plants were damaged more by the snails than the inbred plants (Fig. 1a), the effect of herbivory on inbreeding depression may have been less pronounced than suggested by the disappearing fitness difference between inbred and outbred plants when they were damaged by the snails.

Inbreeding effects on different traits are likely to be linked. Our findings that inbreeding effects on resistance, as measured by leaf damage and snail weight (e.g. Figs 1a and 2a,b), were often opposing the observed inbreeding effects on fitness (e.g. the case of undamaged control plants in Figs 1c and 3a,b) support this idea. Due to limited resources, defending against herbivores can bring along allocation costs detected as trade-offs between resistance and fitness (Bergelson & Parrington 1996; Koricheva 2002; Strauss et al. 2002). Such costs can depend on the environment, genetic background and ecological context (Strauss et al. 2002). The fact that we found opposing effects of inbreeding on resistance, measured as inverse of leaf damage and as snail performance, and on fitness in response to population history after two generations in a common environment suggest a genetic basis of resource allocation to growth or defence (in the sense of Herms & Mattson 1992).
Effects of past herbivory on inbreeding depression in resistance and fitness

The resistance of outbred plants, as indicated by the amount of leaf area consumed by the snails, was independent of the level of past herbivory at the site of origin (Fig. 2a). This contradicts the expectation that selection by higher herbivore pressure at the site of origin would lead to higher resistance. It is likely that allocation trade-offs between resistance and fitness could have constrained the effect of such selection.

The fact that the resistance of the outbred plants, measured as inverse of damage, was independent of past herbivory at the site of origin whereas the resistance of inbred plants significantly decreased with increasing past herbivory (Fig. 2a) suggests inbreeding depression in resistance only for populations with high past levels of herbivory. It has been suggested that, under strong herbivory, plants with high resistance should favour inbreeding to avoid the break down of the resistance (Strauss & Karban 1994). Our finding suggests that such selection may be counteracted by inbreeding depression on resistance in populations experiencing high levels of herbivory.

Effects of heterozygosity of the populations of origin on inbreeding depression

If inbreeding depression is due to dominance, it can be purged by natural selection from highly inbred populations (Lande & Schemske 1985; Charlesworth & Charlesworth 1987). In this case, populations with a longer history of inbreeding are more likely to show lower inbreeding depression under experimental inbreeding (Lande & Schemske 1985; Charlesworth & Charlesworth 1987). In contrast to such expectations, for resistance, measured either as inverse of snail performance or leaf damage, we found no effects of the history of inbreeding (indicated by observed heterozygosity) of the populations of origin on inbreeding depression.

When not damaged by the snails, the fitness of the outbred plants increased with increasing observed heterozygosity of the populations of origin (Fig. 3c), as is generally observed in studies conducted in common environments (Galeuchet et al. 2005a; Leimu et al. 2006). Moreover, we found evidence for inbreeding depression in plant fitness for undamaged plants when they originated from more heterozygous populations, i.e. from populations with low levels of past inbreeding. When plants originated from populations with low heterozygosity, the inbred plants had, in turn, higher fitness than the outbred plants indicating outbreeding depression and purging of the genetic load, but only when the plants were not damaged by the snails. These findings indicate that inbreeding effects on plant fitness can disappear when plants are damaged by herbivores.

The observed patterns of inbreeding depression suggest that fitness problems in populations of lower heterozygosity arise due to complex interactions of fragmentation effects on population genetics, the environment and on biotic interactions, such as those with herbivores. This is likely to be a general problem for highly fragmented populations. Moreover, the effects of intensive herbivore damage on plant fitness can largely diminish inbreeding depression in nature.

Effects of abiotic environmental characteristics on inbreeding depression in resistance and fitness

We found that inbreeding effects on resistance, measured either as inverse of snail performance or leaf damage, and on fitness can be influenced by the abiotic environmental conditions at the site of plant origin (Figs 2b and 3c–e). However, contradicting the idea of increased inbreeding depression under immediate stressful conditions (Dudash 1990), fitness differences between inbred and outbred plants were not apparent when plants were damaged by the snail herbivores in the experiment. Thus, also the modulating impact of past environmental conditions on inbreeding effects disappeared (Fig. 3d,e).

The finding that the effects of past environmental conditions can be detected after two generations under experimental conditions suggests a genetic basis of the observed patterns rather than maternal carry-over effects. Moreover, the role of past environmental conditions indicates that drawing conclusions from studies conducted on only one or few populations can be misleading and, even if conducted under controlled conditions, the outcome of such studies depends on the characteristics of the populations in question.

CONCLUSIONS

Our study highlights how inbreeding effects on herbivore resistance and plant fitness are influenced by the history of inbreeding, levels of herbivory and environmental conditions at the site of plant origin. This clearly demonstrates that generalizations on inbreeding effects from single-population studies can be very misleading. Our study suggests that complex interactions between the genetic and environmental conditions directly affecting resistance and fitness play a role for inbreeding depression and that allocation costs for resistance or growth and fitness also need to be considered.

Our study demonstrates that biotic stress, such as damage by herbivores, can diminish direct inbreeding effects on fitness. This raises the important question of whether
inbreeding depression is smaller in natural populations, where plants are exposed to herbivores and other natural enemies, than predicted from the commonly conducted controlled experiments where such biotic stress is not present. Alternatively, if natural enemies prefer more inbred plants in the field compared to more outcrossed plants, inbreeding depression could be more pronounced under natural conditions than estimated from controlled experiments where biotic stress is not considered.

Understanding the simultaneous evolution of plant mating systems, inbreeding effects and plant–herbivore interactions in different ecological settings is a new exciting field of evolutionary ecology, which deserves much more attention. It requires the combination of different approaches, including multipopulation studies, studies on the genetic mechanisms of inbreeding depression and resistance and on specific resistance traits, and studies addressing both specialist and generalist herbivores and multiple natural enemies.

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