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The role of inbreeding and outbreeding in herbivore resistance and tolerance in *Vincetoxicum hirundinaria*

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• *Background and Aims* Inbreeding via self-fertilization may have negative effects on plant fitness (i.e. inbreeding depression). Outbreeding, or cross-fertilization between genetically dissimilar parental plants, may also disrupt local adaptation or allelic co-adaptation in the offspring and again lead to reduced plant fitness (i.e. outbreeding depression). Inbreeding and outbreeding may also increase plant vulnerability to natural enemies by altering plant quality or defence. The effects of inbreeding and outbreeding on plant size and response to herbivory in the perennial herb, *Vincetoxicum hirundinaria*, were investigated.
• *Methods* Greenhouse experiments were conducted using inbred and outbred (within- and between-population) offspring of 20 maternal plants from four different populations, quantifying plant germination, size, resistance against the specialist folivore, *Abrostola asclepiadis*, and tolerance of simulated defoliation.
• *Key Results* Selfed plants were smaller and more susceptible to damage by *A. asclepiadis* than outcrossed plants. However, herbivore biomass on selfed and outcrossed plants did not differ. The effects of inbreeding on plant performance and resistance did not differ among plant populations or families, and no inbreeding depression at all was found in tolerance of defoliation. Between-population outcrossing had no effect on plant performance or resistance against *A. asclepiadis*, indicating a lack of outbreeding depression.
• *Conclusions* Since inbreeding depression negatively affects plant size and herbivore resistance, inbreeding may modify the evolution of the interaction between *V. hirundinaria* and its specialist folivore. The results further suggest that herbivory may contribute to the maintenance of a mixed mating system of the host plants by selecting for outcrossing and reduced susceptibility to herbivore attack, and thus add to the growing body of evidence on the effects of inbreeding on the mating system evolution of the host plants and the dynamics of plant–herbivore interactions.

Key words: Inbreeding depression, outbreeding depression, resistance, tolerance, *Vincetoxicum hirundinaria*, *Abrostola asclepiadis*, mating system.

INTRODUCTION

Inbreeding is a widespread and common phenomenon in natural plant populations (Vogler and Kalisz, 2001). It reduces heterozygosity and the contribution of overdominance, and increases the expression of recessive alleles within individuals, altering the distribution of genetic variation within populations (Falconer, 1981; Charlesworth and Charlesworth, 1987). These genetic changes usually incur fitness costs to offspring; so-called inbreeding depression (Husband and Schemske, 1996). Inbreeding depression is a potent evolutionary force that probably constrains the evolution of self-pollination in plants (Keller and Waller, 2002).

Although gene transfer between populations via pollen or seed migration seems to be rare in many species, it does to a certain extent contribute to the maintenance of genetic variation and the prevention of inbreeding in geographically isolated populations (Slatkin, 1985; Ellstrand and Elam, 1993). On the other hand, cross-pollination between markedly genetically dissimilar plants (e.g. individuals from different populations) may lead to reduced offspring fitness (Waser and Price, 1983; Lynch, 1991; Waser, 1993; Leimu and Fischer, 2010). This fitness decline, or outbreeding depression, may arise due to the disruption of adaptation to local conditions, or allelic co-adaptation within or across gene loci, or a combination of these mechanisms (Campbell and Waser, 1987; Lynch, 1991).

Due to its major impact upon plant fitness (Marquis, 1984), plants defend themselves against herbivory by reducing the amount of damage they experience (resistance) or the negative fitness impacts of herbivore damage (tolerance) (Strauss and Agrawall, 1999; Tiffin, 2000). Because variation in plant response to herbivory often has a genetic basis (Berenbaum *et al.*, 1986; Simms and Rausher, 1987; Marquis, 1990), reduced genetic variation is likely to limit the future adaptive evolution of herbivore resistance and tolerance (Ivey *et al.*, 2009). Consequently, inbreeding and between-population outbreeding may alter plant susceptibility to herbivore attack or its capacity to resist or tolerate herbivory (Carr and Eubanks, 2002; Ivey *et al.*, 2004; Stephenson *et al.*, 2004; Du *et al.*, 2008; Delphia *et al.*, 2009; Bello-Bedoy and Núñez-Farfán, 2010; Leimu and Fischer, 2010). However,

while a number of recent studies have reported inbreeding depression in plant resistance and/or tolerance to herbivory (Carr and Eubanks, 2002; Ivey *et al.*, 2004; Stephenson *et al.*, 2004; Du *et al.*, 2008; Delphia *et al.*, 2009; Bello-Bedoy and Núñez-Farfán, 2010), this is by no means universal (Núñez-Farfán *et al.*, 1996). Moreover, the severity of inbreeding depression on plant defence varies among populations (Carr and Eubanks, 2002; Ivey *et al.*, 2004; Leimu *et al.*, 2008), and complex interactions between genetic and environmental conditions, such as the history of inbreeding and spatio-temporal variation in herbivore pressure, influence how inbreeding affects plant defence (Leimu *et al.*, 2008).

At the species level, increased asexual reproduction might have long-term implications for the macroevolution of plant resistance against insect herbivores (Johnson *et al.*, 2009). For example, in the Onagraceae, increased asexual reproduction frequently results in the evolution of increased susceptibility to generalist herbivores, whereas the effects of decreased sexual reproduction on specialist herbivores are likely to be more complex (Johnson *et al.*, 2009). Compared with the effects of inbreeding, the effects of between-population outbreeding on plant resistance are much less studied (but see Leimu and Fischer, 2010). Experiments on the effects of hybridization between species on plant resistance have either reported no effect upon resistance between hybrids and parental plants, an additive effect, hybrid susceptibility, or hybrid resistance that resembles that of the susceptible parental species (Fritz *et al.*, 1999). Between-population cross-fertilization also has positive effects on herbivore resistance as new gene combinations at the loci determining resistance may have a selective advantage over common local genotypes to which local herbivores are adapted (Strauss and Karban, 1994; Leimu and Fischer, 2010). However, spatial and temporal variation in gene flow and selection regimes, in addition to different population histories, are likely to contribute to the variation in outbreeding depression in plant fitness and herbivore resistance (Waser and Price, 1983; Waser, 1993). Thus, the plant mating system can affect plant–herbivore interactions, with further variation among populations of interacting species.

In this study, the effects of experimental inbreeding (self-fertilization) and outbreeding (within-population and between-population cross-fertilization) on plant size and on herbivore resistance and tolerance in the perennial herb, *Vincetoxicum hirundinaria*, were examined. In our study area, the distribution of *V. hirundinaria* is naturally fragmented and the species has a mixed mating system with relatively high levels of self-fertility (Leimu, 2004). This fragmented distribution and assumed inbreeding have not led to reduced genetic variation even in small populations (Leimu and Mutikainen, 2005), nor have any signs of inbreeding depression been reported on the reproductive success of *V. hirundinaria* (Leimu, 2004). However, self-fertilization may affect other fitness-related traits, or alter plant quality and/or defence and tolerance to herbivores (Carr and Eubanks, 2002; Ivey *et al.*, 2004; Stephenson *et al.*, 2004; Du *et al.*, 2008; Delphia *et al.*, 2009; Bello-Bedoy and Núñez-Farfán, 2010). If inbreeding results in reduced herbivore resistance or tolerance, outcrossing should be favoured in host–plant populations experiencing constant high levels of herbivory. In fact, recent studies indicate that herbivory may promote stable mixed mating systems in host plants since the relative fitness of selfed and outcrossed plants is influenced by the interactions with herbivores (Núñez-Farfán *et al.*, 2007; Steets *et al.*, 2007a, b; Bello-Bedoy and Núñez-Farfán, 2011). In addition, in small, isolated or newly colonized populations, inbreeding might be common and favoured as reproductive assurance. Thus, in a small population with a long history of inbreeding, the negative effects of inbreeding can be purged (Jarne and Charlesworth, 1993). Taken together, the spatial variation in the levels of herbivory and the distribution pattern of *V. hirundinaria* may contribute to the maintenance of a mixed mating system. Moreover, fragmented distribution, isolation and among-population variation in herbivore pressure may have enhanced adaptation to local conditions (L. Laukkanen, University of Turku, Finland, unpubl. res.). Consequently, between-population cross-fertilization could lead to outbreeding depression.

We examined here whether there is any evidence for inbreeding depression in *V. hirundinaria* size and resistance against its specialist folivore *Abrostola asclepiadis*, and whether inbreeding depression varies among populations or plant families. We also investigated whether inbreeding depression influences tolerance of defoliation and if any such effect varies among plant families. Finally, outbreeding depression in plant size and resistance against *A. asclepiadis*, and whether there is among-population or among-family variation in this outbreeding depression, was examined.

MATERIALS AND METHODS

Study species

Vincetoxicum hirundinaria (Asclepiadaceae) is a long-lived perennial herb. It has mainly a continental Eurasian distribution, but its natural range also covers the islands and coastal areas of the middle Baltic Sea. The number of stems per plant varies from one (young individuals) to more than a hundred, the average plant size in SW Finland being between 20 and 60 stems per individual (Muola *et al.*, 2010b). In our study area, flowering begins in the middle of June and lasts until the beginning of August. The number of flowers per plant varies from a few to several hundred depending on the plant size. *Vincetoxicum hirundinaria* is insect pollinated and the main pollinators are large flies, moths and bees (Timonin and Savitskii, 1997). Pollen is aggregated into pollen sacs (pollinia) and each flower has five pairs of pollinia. Pollination occurs when the pollinia are inserted into the stigmatic chambers, from which the pollen tubes grow towards the ovaries. Each flower contains five stigmatic chambers and two ovaries. Three of these chambers lead to one ovary and the remaining two chambers to the other ovary (Wyatt and Broyles, 1994). Although most of the Asclepiadaceae have a late-acting self-incompatibility system (Wyatt and Broyles, 1994; Lipow and Wyatt, 2000), varying levels of self-fertility have been observed in several species (Wyatt, 1976; Kephart, 1981; Bookman, 1984; Kahn and Morse, 1991; Wyatt and Broyles, 1994; Lipow *et al.*, 1999; Lipow and Wyatt, 2000). In our study populations *V. hirundinaria* has a mixed mating system (Leimu, 2004), meaning that individuals are capable

of reproducing via self-fertilization and outcrossing. Fruits normally ripen at the end of August or early September. Each pod contains approx. 20 wind-dispersed seeds (Leimu, 2004).

Vincetoxicum hirundinaria is highly toxic to mammals and many generalist insects (Solbreck and Sillén-Tullberg, 1990) due to the possession of several types of secondary metabolites (Eibler *et al.*, 1995; Staerk *et al.*, 2000; Leimu *et al.*, 2005; Muola *et al.*, 2010*b*). Despite its toxicity, *V. hirundinaria* is the host plant for the monophagous folivorous moth, *Abrostola asclepiadis* (Noctuidae). The female moth oviposits on the leaves of *V. hirundinaria* (Förare and Engqvist, 1996), the eggs hatching about 10 d later, and the five larval instars are completed in about 5–6 weeks (Förare, 1995). Damage levels vary between years and among populations, ranging from no damage to almost complete defoliation (Leimu and Lehtilä, 2006; Muola *et al.*, 2010*b*). Chemical defences appear not to be rapidly inducible (A. Muola, University of Turku, Finland, unpubl. res.), but do significantly affects both the preference and performance of *A. asclepiadis* larvae (Muola *et al.*, 2010*b*).

Plant material

Twenty large plant individuals (>10 stems; hereafter maternal plants) were randomly selected from four different populations: Lammasluoto (60°14·059′, 21°56·589′), Naantali (60°27·8′, 22°1·091), Seili (60°14·193′, 21°57·413) and Mörkö (58°59·55′, 17°41·36). These populations were chosen because they vary in size from 100 to 10 000 individuals. Moreover, the distance between the Mörkö population, which is situated on the south-eastern coast of Sweden, and the other three populations situated on separate islands of the Archipelago Sea (SW Finland) is around 260 km. The distance between the Lammasluoto and Naantali populations is 26 km and that between the Lammasluoto and Seili populations 0·7 km. A previous study by Leimu and Mutikainen (2005) suggests that populations situated in the SW archipelago of Finland are genetically differentiated. The selected maternal plants grew at least 3 m apart from each other to ensure that they represented different genotypes. Plants were removed from the field and transported into a greenhouse at the Ruissalo Botanical Garden, University of Turku, in May 2006

and 2007. The maternal plants were planted into 5·0 L pots (Kekkilä, Karkea ruukutusseos) and they were allowed to grow in greenhouse conditions until 2008. All plants were fertilized with water-soluble full fertilizer (Nutri S – B, N:P:K 16:4:25) twice during the summers 2006, 2007 and 2008.

Hand pollination

To obtain selfed and outcrossed offspring from the 80 maternal plants, hand pollination was conducted in June 2007. Five flowers from each maternal plant were self-fertilized with pollen from another flower of the same plant and five flowers were outcrossed with pollen from a different plant randomly selected from the same population by inserting two pairs of pollinia into the opposite stigmatic chambers of a flower with a needle. In addition, 15 flowers per plant (five flowers per each pollen donor population) from the Lammasluoto and Mörkö populations received between-population cross-pollination from three other populations (Mörkö, Naantali and Seili for Lammasluoto, and Lammasluoto, Naantali and Seili for Mörkö). Each individual was used as a pollen donor only once in pollination treatment, and the donors and recipients were randomized. The pollinated flowers were tagged to distinguish the different pollination treatments. Each pollination treatment was conducted on a separate randomly selected stem. Ripe pods were harvested before they were completely open and seeds were first dried at room temperature and then kept at + 8°C until germinated in the greenhouse in March 2008. Following germination, seedlings were allowed to grow for at least 4 weeks. We then randomly selected two to three seedlings per maternal plant and pollination treatment from every population (except the Naantali population from where we selected 10–20 juvenile plants per maternal plant and pollination treatment), and planted them in separate 0·15 L pots (Kekkilä, Karkea ruukutusseos). The plants used in the experiment were thus small juvenile plants with relatively few leaves (4–12 leaves per plant).

Inbreeding depression in plant size and herbivore resistance

The incidence and effect of inbreeding depression on germination, plant size and herbivore resistance among plant families and populations were investigated using plants from

TABLE 1. *Effects of self-fertilization on plant performance*

Source of variation	Stem length				No. of leaves			
	d.f.	*F*	χ^2	*P*	d.f.	*F*	χ^2	*P*
Fixed effects								
Pollination treatment	1, 26·9	5·11		0·0321	1, 96·2	4·59		0·0347
Random effects								
Family (population)			2·6	0·0534			2·4	0·0607
Population			<0·0001	0·5000			0·1	0·3759
Family (population) × pollination treatment			0·5	0·2398			<0·0001	0·5000
Population × pollination treatment			<0·0001	0·5000			<0·0001	0·5000

The results of the linear mixed model analyses testing for the effects of pollination treatment (self-fertilization vs. within-population cross-fertilization), plant population, plant family and their interactions on the length of the stems and number of leaves of *Vincetoxicum hirundinaria*. Plant family was nested within plant population.

four populations and from 5–11 families per population in June 2009. Germination was recorded, and the lengths of the stems were measured and the number of leaves counted as two separate measures of plant performance. Two to three plants per maternal plant and per pollination treatment were randomly chosen to examine herbivore resistance, quantified both by the amount of damage inflicted by the herbivore and by the growth of F_2 *A. asclepiadis* larvae. A laboratory population of *A. asclepiadis* was established from 100 eggs collected from the Lammasluoto population in July 2007 and, subsequently, larvae from the parental and F_1 generation were fed on randomly chosen plants collected from Lammasluoto. Fifteen non-related female and male moths were mated in net cages (one mating pair in each net cage) in greenhouse conditions. Each net cage contained two *V. hirundinaria* individuals on which the moths laid their eggs after mating. To obtain the larvae for the experiment, the net cages were checked daily to detect and collect newly hatched larvae. In the experiment, experimental plants were covered with mesh bags and one randomly selected newly hatched *A. asclepiadis* larva was assigned to each plant. To control for genetic variation among *A. asclepiadis* families, larvae from each of the 15 full-sib families were assigned randomly to plants from each population, each plant family and on both selfed and outcrossed plants. The larvae fed on the plants for 4 d, after which they were weighed. Larval biomass was used to estimate herbivore performance and used as an inverse measure of plant resistance (Agrawal, 2005; Muola *et al.*, 2010a). After removal of the larvae, the percentage of leaf area consumed was estimated and used as the second measure of plant resistance.

To test for inbreeding depression in germination rates (the number of seeds germinated/the number of seeds produced) a two-way analysis of variance (ANOVA) was conducted, with pollination treatment (selfing vs. within-population outcrossing) and plant population as the explanatory factors. We analysed whether there was inbreeding depression in plant size and whether inbreeding depression in plant size varied among plant families and populations by conducting linear mixed models separately for the two size measures (stem length and number of leaves). Pollination treatment (self-fertilization vs. within-population cross-fertilization) was used as the fixed factor. Plant family nested within population, plant population and the interactions of pollination treatment with plant family (test for genetic variation in inbreeding) and plant population (test for among-population variation in inbreeding) were included as random factors. To obtain the statistical significance of the random factors, likelihood ratio tests were conducted and the *P*-values of the likelihood ratio tests were divided by two (Littell *et al.*, 2006). Inbreeding depression in plant resistance against *A. asclepiadis* was analysed with a similar linear mixed model, separately for the two resistance measures, i.e. the percentage of leaf area eaten and the performance of *A. asclepiadis*.

Inbreeding depression and defoliation tolerance

Inbreeding depression in plant tolerance of defoliation was investigated using selfed and outcrossed (within-population) offspring from ten families from the Naantali population in

July 2009. Between ten and 20 plants per maternal plant and pollination treatment were randomly chosen for the experiment. Half of the plants of each family and both crossing treatments were defoliated and the other half served as undamaged controls. All plant leaves were removed by cutting the petioles close to the stem. This was done to simulate the most comprehensive damage seedlings experience in field conditions, since they are often completely consumed by *A. asclepiadis* (R. Leimu and A. Muola, pers. obs.). Stem length and leaf number were measured 6 weeks after the defoliation treatment and tolerance was estimated by comparing
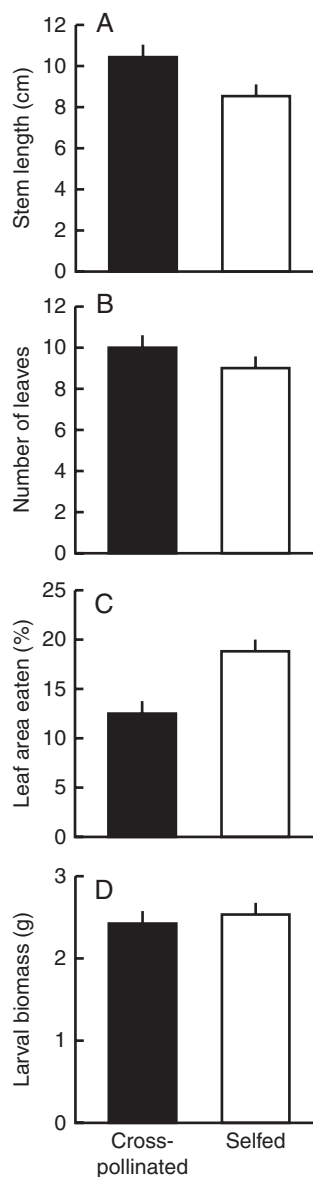

FIG. 1. Mean effects of selfing on plant performance and resistance against a specialist folivore. Average performance of cross-pollinated (within population) and self-pollinated *Vincetoxicum hirundinaria* was measured as (A) length of the stems [cm, least-square mean (lsmean) $\pm$ s.e.] and as (B) number of leaves (lsmean $\pm$ s.e.). Average resistance against the folivore, *Abrostola asclepiadis*, was measured as (C) percentage of leaf area eaten (lsmean $\pm$ s.e.) and as (D) herbivore performance (larval biomass, lsmean $\pm$ s.e.).

them with the estimates of the same variables before the defoliation treatment was imposed. The period of 6 weeks was chosen since in the field *V. hirundinaria* seedlings have about 8–9 weeks to recover from defoliation before the end of the growing season. Defoliation tolerance was estimated by dividing the mean performance of the defoliated plants by the mean performance of the control plants of the same plant family for both crossing treatments (Strauss and Agrawal, 1999; Muola *et al.*, 2010*a*).

A linear mixed model was used to elucidate any effect of inbreeding depression on plant size. Pollination treatment (self-fertilization vs. within-population cross-fertilization) was used as a fixed factor, and seedling family and the interaction between pollination treatment and seedling family were included as random factors. To obtain the statistical significance of the random factors, likelihood ratio tests were conducted and the *P*-values of the likelihood ratio tests were divided by two (Littell *et al.*, 2006). Separate analyses were conducted for the length of the stems and number of leaves. Inbreeding depression in plant tolerance was analysed using pairwise *t*-tests to compare the tolerance of selfed and outcrossed offspring of each plant family.

Outbreeding depression in plant size and herbivore resistance

Outbreeding depression was investigated by comparing the size of offspring from the within-population outcrosses and between-population outcrosses of plants from the Lammasluoto and Mörkö populations. Altogether, our data included measurements of replicates from nine families from Lammasluoto and seven families from Mörkö. We also investigated whether there are among-family or among-population differences in how outbreeding affects plant size. Plant size was estimated as described above.

Outbreeding depression in herbivore resistance and whether outbreeding depression varies between plant families or among plant populations was investigated using the same plants as described above. The experiment and statistical analysis were also conducted as described above (see 'inbreeding depression . . . and herbivore resistance'). However, in contrast to the first experiment, plant population was included as a fixed factor in the model as we used plants from only two populations to investigate outbreeding depression. We only tested for the differences between plants from within- and between-

population outcrosses ignoring the origin of the pollen donor population used. This was done because there were no statistically significant differences in either the performance or resistance of the offspring depending on the pollen donor population (data not shown). All statistical analyses were conducted using the SAS statistical package version 9·2.

RESULTS

Inbreeding depression in plant size and herbivore resistance

Selfed seeds had a lower relative germination rate than seeds originating from outcrossing ($0·19 \pm 0·04$ for selfed and $0·70 \pm 0·05$ for outcrossed seeds; d.f. = 1, $F = 67·59$, $P < 0·0001$). Relative germination rates of selfed and outcrossed seeds did not differ among populations (d.f. = 3, $F = 0·43$, $P = 0·6494$). Outcrossed offspring had longer stems and produced more leaves than selfed offspring, indicating inbreeding depression in plant size (Table 1, Fig. 1A, B). However, there was no among-family or among-population variation in how self-fertilization affected plant size (Table 1).

Selfed offspring suffered significantly more herbivore damage than outcrossed offspring, indicating inbreeding depression in herbivore resistance (Table 2, Fig. 1C). The proportion of leaf area eaten varied among plant families, ranging from $7·0 \pm 1·2$ to $28·0 \pm 6·0$ (mean $\pm$ s.e.), indicating some genetic variation in plant resistance (Table 2). We found little among-family or among-population variation in how inbreeding affected leaf consumption, as indicated by the non-significant pollination treatment by family and pollination treatment by population interactions (Table 2). In contrast to the results based on the amount of damage, however, we found little variation in larval biomass linked to plant inbreeding (Table 2, Fig. 1) and no significant among-family or among-population variation in how inbreeding affected the larval biomass, as indicated by the non-significant pollination treatment by family and pollination treatment by population interactions (Table 2).

Inbreeding depression and defoliation tolerance

Plants used in the defoliation tolerance experiment showed inbreeding depression in size. Stems of selfed offspring were shorter ($9·6 \pm 0·6$ cm) than those of outcrossed offspring

TABLE 2. *Effects of self-fertilization on plant resistance against a specialist folivore*

Source of variation	Percentage of leaf area eaten				Herbivore performance			
	d.f.	F	χ^2	P	d.f.	F	χ^2	P
Fixed effects								
Pollination treatment	1, 94·6	9·58		0·0026	1, 26·7	0·21		0·5483
Random effects								
Family (population)			3·8	0·0257			0·1	0·3759
Population			<0·0001	0·5000			<0·0001	0·5000
Family (population) × pollination treatment			<0·0001	0·5000			0·8	0·1855
Population × pollination treatment			<0·0001	0·5000			<0·0001	0·5000

The results of the linear mixed model analyses testing for the effects of pollination treatment (self-fertilization vs. within-population cross-fertilization), plant population, plant family and their interactions on percentage of leaf area eaten by *A. asclepiadis* and herbivore performance (larval biomass) on *Vincetoxicum hirundinaria*. Plant family was nested within plant population.

($11 \cdot 1 \pm 0 \cdot 6$ cm; $F = 10 \cdot 02$, d.f. = 1, $9 \cdot 58$, $P = 0 \cdot 0106$) and had fewer leaves ($9 \cdot 5 \pm 0 \cdot 3$ for selfed compared with $10 \cdot 2 \pm 0 \cdot 3$ for outcrossed; $F = 4 \cdot 99$, d.f. = 1, 345, $P = 0 \cdot 0261$). Although there was significant among-family variation in size ($\chi^2 = 5 \cdot 7$, $P = 0 \cdot 0085$ for stem length and $\chi^2 = 3 \cdot 6$, $P = 0 \cdot 0289$ for leaf number), we found no among-family variation in how inbreeding affected the plant size, as indicated by the non-significant pollination treatment by family interaction ($\chi^2 = 0 \cdot 7$, $P = 0 \cdot 2014$ for stem length and $\chi^2 < 0 \cdot 0001$, $P = 0 \cdot 5000$ for leaf number).

In spite of the inbreeding depression effect observed for stem length and leaf number, neither estimate of herbivore tolerance indicated inbreeding depression: stem length selfed = $1 \cdot 06 \pm 0 \cdot 05$ and outcrossed = $1 \cdot 06 \pm 0 \cdot 05$, ($t = 0 \cdot 03$, d.f. = 9, $P = 0 \cdot 980$); leaf number selfed = $0 \cdot 85 \pm 0 \cdot 05$ and outcrossed = $0 \cdot 79 \pm 0 \cdot 05$, ($t = -0 \cdot 90$, d.f. = 9, $P = 0 \cdot 393$). Based on these figures, all plants appeared to be compensating equally for the effects of artificial defoliation.

Outbreeding depression in plant size and herbivore resistance

Neither stem length nor leaf number varied for plants produced from within-population or between-population outcrosses ($F = 0 \cdot 04$, d.f. = 1, $34 \cdot 5$, $P = 0 \cdot 8385$ for stem length; $F = 0 \cdot 41$, d.f. = 1, $35 \cdot 2$, $P = 0 \cdot 5262$ for leaf number), providing no evidence for outbreeding depression in plant size. Likewise, there was no significant among-family or among-population variation in how outbreeding affected plant size (plant family by pollination treatment: $\chi^2 = 0 \cdot 6$, $P = 0 \cdot 2193$ for stem length and $\chi^2 = 0 \cdot 2$, $P = 0 \cdot 3274$ for leaf number; population by pollination treatment $F = 1 \cdot 61$, d.f. = 1, $34 \cdot 5$, $P = 0 \cdot 2133$ for stem length; $F = 1 \cdot 31$, d.f. = 1, $35 \cdot 2$, $P = 0 \cdot 2602$ for leaf number; Fig. 2A, B).

Herbivore resistance, measured either as the percentage of leaf area eaten or as a reduction in larval biomass, did not vary for between- and within-population outcross plants (Table 3, Fig. 2C, D). The amount of damage and herbivore performance did not differ among plant families (Table 3) and there was no among-family or among-population variation in how pollination treatment affected resistance (Table 3).

DISCUSSION

Inbreeding affected plant fitness directly by decreasing germination and the size of selfed offspring, and indirectly by affecting resistance against the specialist folivore, *A. asclepiadis*. Inbreeding effects on herbivore resistance are known to vary from positive to negative and depend on the type of damage and traits measured (Núñez-Farfán *et al.*, 1996; Carr and Eubanks, 2002; Ivey *et al.*, 2004; Stephenson *et al.*, 2004; Du *et al.*, 2008; Leimu *et al.*, 2008; Delphia *et al.*, 2009; Bello-Bedoy and Nunez-Farfan, 2010). Although larval biomass did not vary significantly between selfed or outcrossed plants, selfed plants did suffer more damage than outcrossed plants, indicating inbreeding depression in herbivore resistance in *V. hirundinaria*. While we cannot discount the possibility that the leaves of selfed plants were of lower nutritional quality than those of outcrossed plants, relative to the developmental time of the herbivore used (about 15 d in laboratory conditions), our experiment was a short-term trial
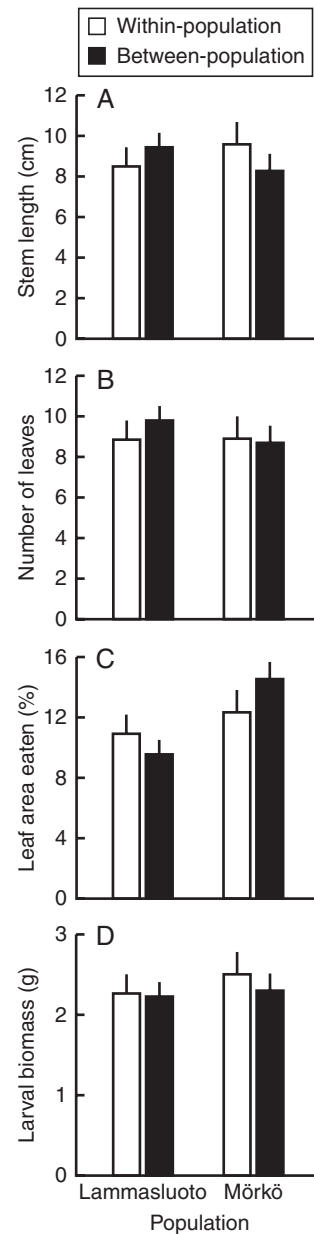

Fig. 2. Effects of within-population outcrossing and between-population outcrossing on performance and resistance of *Vincetoxicum hirundinaria* originating from Lammasluoto and Mörkö populations. Performance was measured as (A) length of the stems (cm, lsmean $\pm$ s.e.) and as (B) number of leaves (lsmean $\pm$ s.e.). Resistance against a specialist folivore, *Abrostola asclepiadis*, were measured as (C) percentage of leaf area eaten (lsmean $\pm$ s.e.) and as (D) herbivore performance (larval biomass, lsmean $\pm$ s.e.). Within-population outcrosses and between-population outcrosses as indicated in the key in (A).

(4 d due to the small size of the plants) and probably did not allow any substantial difference in individual biomass to emerge between larvae feeding on selfed and outcrossed plants. Moreover, some herbivores are able to increase consumption when feeding on low-quality diets in order to compensate for reduced post-ingestive physiological efficiency (Slansky, 1993; Kause *et al.*, 1999) and such compensatory consumption may lead to increased plant damage (Moran and Hamilton, 1980) as seen here. Although we cannot

TABLE 3. *The effects of between-population outcrossing on plant resistance against a specialist folivore*

Source of variation	Percentage of leaf area eaten				Herbivore performance			
	d.f.	F	χ^2	P	d.f.	F	χ^2	P
Fixed effects								
Pollination treatment	1, 97	0·08		0·7800	1, 16·9	0·64		0·4333
Population	1, 97	4·04		0·0572	1, 17·1	1·08		0·3128
Pollination treatment × population	1, 97	1·37		0·2443	1, 16·9	0·49		0·4918
Random effects								
Family (population)			<0·0001	0·5000			<0·0001	0·5000
Family (population) × pollination treatment			<0·0001	0·5000			<0·0001	0·5000

The results of the linear mixed model analyses testing for the effects of pollination treatment (within-population cross vs. between-population cross), plant population, plant family and their interactions on percentage of leaf area eaten by *A. asclepiadis* and herbivore performance (larval biomass) on *Vincetoxicum hirundinaria*. Plant family was nested within plant population.

disentangle whether the observed higher leaf consumption of selfed plants was due to reduced expression of defensive compounds or reduced nutritive quality, our results nonetheless show that selfed plants suffer increased herbivory.

Tolerance, another defensive strategy plants have evolved to reduce the negative fitness effects of herbivores, did not differ between selfed and outcrossed plants. Inbreeding depression is known to be more pronounced in poor environmental conditions (Dudash, 1990; Heschel and Paige, 1995; Armbruster and Reed, 2005), and plants in our experiment did not experience the level of resource limitation, or intra- or interspecific competition common to natural plant populations. It is also possible, especially when dealing with a perennial species like *Vincetoxicum*, that any inbreeding depression in plant tolerance can only be detected after a longer period of time. We also recognize here that the expression of defoliation tolerance can differ between artificial and natural damage, because they may pose different types of stress for the plants, and therefore may result in variable responses in plants (Karban and Baldwin, 1997; Lehtilä, 2003). Nonetheless, total defoliation by *A. asclepiadis* during the early ontogenetic phases of *V. hirundinaria* is common and the level of defoliation applied by artificial clipping appropriate for quantifying plant tolerance in this species. Moreover, survival and onward growth depend greatly on the plant's ability to compensate for such severe defoliation (Muola *et al.*, 2010*a*). Indeed, so severe is herbivory by *A. asclepiadis* during the juvenile stage that inbreeding depression for tolerance may have been purged by such a strong selective pressure.

The expression of inbreeding depression on plant fitness is likely to vary according to plant life history stage (Husband and Schemske, 1996; Du *et al.*, 2008), and *V. hirundinaria* appears to show such ontogenetic variation. For example, Leimu (2004) found no evidence of inbreeding depression on pod and seed production following hand pollinations (selfing and within-population outcrossing), suggesting that the effects of inbreeding depression in the later life history stages of *V. hirundinaria* may not be substantial. Similarly in their study on *V. hirundinaria* population genetics, Leimu and Mutikainen (2005) reported that despite high within-population levels of inbreeding (F_{IS}, mean 0·460), the inbreeding coefficient did not correlate with plant fitness, suggesting lack of inbreeding depression in terms of male and female

reproductive output. In the present study, however, in addition to a marked difference in germination rates between selfed and outcrossed seeds, we also found that seedlings originating from within-population outcrossing were taller and had more leaves than selfed offspring. Both observations indicate inbreeding depression in the early ontogenetic stages. It has been suggested that most inbreeding depression acting at early developmental stages is due to recessive lethal allele combinations and can be purged from selfing populations, whereas inbreeding depression acting on later developmental stages is due to weakly deleterious mutations, difficult to purge by selection (Husband and Schemske, 1996). The results of our study, combined with the previous work on *V. hirundinaria* (Leimu 2004; Leimu and Mutikainen, 2005), contradict this hypothesis as we found inbreeding depression in the juvenile stage but not in the later life history stages. However, it is also possible that the negative effects observed on growth during the juvenile stage may be reflected in later life history stages (Hanley and May, 2006). Thus to understand fully the negative effects of selfing, data are required on the potential negative effects of inbreeding on the reproduction of selfed plants. *Vincetoxicum hirundinaria* is a long-lived perennial and it takes several years for seedlings to reach sexual maturity. Consequently, data on inbreeding effects on propagule reproduction could not be included in this study.

A number of studies have reported variation in inbreeding depression between genotypes and among populations (Ouborg *et al.*, 2000; Ivey *et al.*, 2004; Leimu *et al.*, 2008; Bello-Bedoy and Núñez-Farfán, 2010). However, we found no among-population or genetic variation in inbreeding depression in plant size or herbivore resistance. This lack of variation in inbreeding depression might be explained by historical levels of inbreeding in our *V. hirundinaria* populations as the frequency of self-fertile plants within populations is relatively high and does not differ between populations in our study area (Leimu, 2004). The high level of genetic variation may also contribute to the observed lack of among-population and among-family variation in inbreeding depression. The amount of damage inflicted by *A. asclepiadis* also varies in our study area (Muola *et al.*, 2010*b*), which could potentially lead to varying selection pressure among the populations (Hanley and Sykes, 2009). However, the temporal variation observed in the level of folivory might counterbalance the spatial variation,

especially when we are dealing with a long-lived perennial species (R. Leimu and A. Muola, pers. obs.). Given that environmental stresses are known to exacerbate the effects of inbreeding (Dudash, 1990; Heschel and Paige, 1995), it is possible that our failure to detect among-family and among-population variation was an artefact of greenhouse conditions; in field conditions such variation might be more pronounced.

The lack of outbreeding depression observed in the present study may be explained by the low level of genetic differentiation and relatively high levels of gene flow among *V. hirundinaria* populations (Von Numers and van der Maarel, 1998; Leimu and Mutikainen, 2005). Moreover, although there seems to be local adaptation in some populations in our study area (L. Laukkanen, University of Turku, Finland, unpubl. res.), it might not be strong enough to facilitate outbreeding depression. Additionally, we used F_1 offspring from between-population crosses which may have benefited from heterosis, effectively masking any negative effects of outbreeding (Lynch and Walsh, 1998).

Our results firmly point to inbreeding depression in plant size and some evidence for inbreeding depression in herbivore resistance, but not in tolerance. Moreover, between-population outcrossing did not result in any manifestation of outbreeding depression. Our results thus suggest that inbreeding depression may play a role in the evolution of the plant–herbivore interactions. In fact, herbivory may even contribute to the maintenance of the mixed mating system of the host plant species if the relative fitness of selfed and outcrossed plants is influenced by the plant–herbivore interactions (Steets *et al.*, 2007*a*, *b*). Our results add to the growing body of evidence on the effects of inbreeding on the mating system evolution of the host plants and the dynamics of plant–herbivore interactions.

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