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PLANT-ANIMAL INTERACTIONS - ORIGINAL PAPER

Delayed response in a plant–pollinator system to experimental grassland fragmentation

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Abstract The fragmentation of natural habitat is considered to be a major threat to biodiversity. Decreasing habitat quality and quantity caused by fragmentation may lead to a disruption of plant–pollinator interactions and to a reduction in sexual reproduction in plant species. We conducted a 6-year field experiment to investigate the effects of small-scale fragmentation on plant–pollinator interactions and genetic diversity in the self-compatible *Betonica officinalis*. We examined the abundance and composition of pollinators, the foraging behaviour of bumblebees and the performance, outcrossing rate and genetic diversity of *B. officinalis* after 2 and 6 years in experimentally fragmented nutrient-poor, calcareous grassland in the northern Swiss Jura mountains. Fragments of different size (2.25 and 20.25 m^2) were isolated by a 5-m-wide strip of frequently mown vegetation. Control plots of corresponding size were situated in adjacent undisturbed grassland. Experimental grassland fragmentation altered the composition of *B. officinalis* pollinators and reduced their flower visitation rate. Furthermore, the foraging behaviour of bumblebees was changed in the fragments. After 6 years of fragmentation seed weight was higher in fragments than in control plots. However, the densities of *B. officinalis* rosettes and inflorescences, plant height and inflorescence length were not affected by fragmentation. The outcrossing frequency of *B. officinalis* growing in fragments was reduced by 15% after 2 years and by 33% after 6 years of experimental fragmentation. This resulted in a significant reduction of the genetic diversity in seedlings emerging in fragments after 6 years. Our study shows that small-scale habitat fragmentation can disturb the interaction between *B. officinalis* and pollinators resulting in a reduced outcrossing frequency and genetic diversity in plants growing in fragments. However, the response to fragmentation was considerably delayed. This finding strengthens the claim for long-term field experiments with proper replications and controls to assess delayed effects of habitat fragmentation.

Keywords *Betonica officinalis* · Genetic diversity · Pollinator foraging behaviour · Outcrossing frequency · Self-compatibility

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Introduction

The fragmentation of natural habitats is generally considered to be a major threat to biodiversity (Saunders et al. 1991). Fragmentation reduces the area suitable for organisms and leads to isolation and decreased size of remnant populations in plants and animals (Gilpin and Soulé 1986). The disadvantages suffered by small populations involve greater sensitivity to environmental and demographic stochasticity (Holsinger 2000), which together with a loss of genetic diversity and inbreeding depression result in a high risk of local extinction (Krauss et al. 2004). Furthermore, effects of habitat fragmentation may lead to the disruption of biotic interactions such as parasitism, seed dispersal or pollination and hence, can affect species with previously stable populations (Kearns et al. 1998; Groppe et al. 2001).

Pollinators are of crucial importance for ecosystem services (Klein et al. 2007). Worldwide approximately 80% of the flowering plant species depend on animals for their pollination (Buchmann and Nabhan 1996). Habitat fragmentation may affect the behaviour, abundance and community composition of pollinators (Aizen and Feinsinger 1994; Goverde et al. 2002). Alterations in behaviour, frequency and composition of flower visitors in turn might influence pollination efficiency which determines the quantity and/or quality of pollen deposited on the stigmas and therefore seed and fruit production (Aizen and Feinsinger 1994; Aguilar and Galetto 2004). Furthermore, habitat fragmentation may alter the spatial distribution of plants causing changes in foraging patterns of flower visitors (Ghazoul 2005). Increased distances between single plants to be pollinated could lead to pollination limitation (Waites and Ågren 2004). Studies addressing fragment size and isolation effects revealed that larger plant populations are more attractive for pollinators, which results in a higher pollination success (e.g. Jennersten 1988; Ågren 1996).

Isolated patches may lead to disruption of plant–pollinator interactions (e.g. Duncan et al. 2004; Ward and Johnson 2005). Pollinator limitation may not occur when the pollinators are able to fly long distances. However, after having moved a long distance, pollinators may stay in an isolated patch for a longer period and thereby repeatedly visit the same flowers (Goverde et al. 2002). The altered pollinator behaviour may increase inbreeding and/or geitonogamy in the plant population resulting in seeds of reduced quality (Lienert 2004).

Plant responses to habitat fragmentation are species specific and related to the degree of pollinator dependency for successful sexual reproduction (Murcia 1996). Theoretical models show that the plant breeding system and degree of pollinator specialization are key traits characterizing the susceptibility to habitat fragmentation (Waser et al. 1996). Self-incompatible plants with specialized pollination systems are highly dependent on pollinator mutualism (Bond 1994), and thus extremely vulnerable to changes in habitat quality and quantity caused by habitat fragmentation because any decrease in pollinator frequency may result in reproductive failure (Aizen et al. 2002). In contrast, self-compatible plants with a generalist pollination system are expected to be more resilient to fragmentation-related changes in their pollinator communities, because the absence of some pollinators can be buffered by the presence of others (Morris 2003). However, empirical evidence indicates that no generalization can be made concerning the susceptibility to habitat fragmentation of a particular plant species based either on the breeding system or on the specialization of pollinators (Ghazoul 2005; Aguilar et al. 2006).

A variety of plants rapidly respond to changes in habitat quality and connectivity (Lienert 2004). In many cases, however, there is a time lag in the response to the altered habitat (Helm et al. 2006). The magnitude of this time lag depends on the life history, reproductive mode and dispersal ability of the species involved (Ewers and Didham 2006). Perennial plant species with clonal propagation, short-distance dispersal and permanent seed banks exhibit a pronounced time lag in their response to habitat fragmentation (Pacha and Petit 2008). Similarly, plant–pollinator interactions may show a delayed response to habitat fragmentation due to different increases or decreases in the abundance of different pollinators and flower numbers in the varying environmental conditions of the succeeding years. Subtle effects on plant performance and outcrossing frequency and the resulting changes in the genetic diversity of the populations could therefore be overlooked by chance. Long-term field experiments with proper replications and controls are therefore essential to examine effects of habitat fragmentation on pollinator–plant interactions.

In this paper, we report results from a 6-year habitat fragmentation experiment in natural mesocosms (Shrivastava et al. 2004) with a treatment versus control design to examine changes in the interaction between the plant *Betonica officinalis* and its associated pollinators. In particular, we compare the performance of *B. officinalis*, its flower visitation rate and the species composition of pollinators in fragments and corresponding control plots 2 and 6 years after the initiation of the experimental fragmentation. Using random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) technique, we also assess the outcrossing rate in *B. officinalis* and the genetic diversity of the seedlings in fragments and control plots 2 and 6 years after the beginning of the experiment. Our sampling design allows an assessment of the time lag in fragmentation-related changes in the genetic diversity of the plants. As fragmentation may alter the pollinator–plant interaction, we expect both a decrease in outcrossing rate and in genetic diversity of *B. officinalis* 6 years after experimental fragmentation.

Materials and methods

Study species

Betonica officinalis (Lamiaceae) is a perennial, self-compatible herb. It is distributed in Europe from Norway and Sweden to Spain and Bulgaria (Hegi 1964). In Switzerland, it can mainly be found in south-facing nutrient-poor calcareous meadows and pastures. The plant spreads vegetatively by producing juvenile rosettes at the bases of mature rosettes. Each rosette can produce one or two spike-like

inflorescences that are composed of up to 70 purple, protandrous zygomorphic flowers (length 10–12 mm, width 3–4 mm) that contain up to 2 µl nectar (Hegi 1964; Rusterholz and Erhardt 1998). Flowering occurs from middle of June to the end of August in the northwestern Swiss Jura mountains. The flowers are visited by a variety of generalist pollinators, including bees, bumblebees, hoverflies, butterflies and wasps. Pollinated flowers produce one to four seeds in a nutlet. Up to 20% of the seeds are fertilized by selfing (H.-P. Rusterholz, unpublished data).

Study sites and experimental design

The experiment was carried out in three calcareous grasslands in the northwestern Swiss Jura mountains: Nenzlingen (47°28′N, 7°34′E; elevation 510 m a.s.l.), Vicques (47°22′N, 7°26′E; 590 m a.s.l.) and Movelier (47°25′N, 7°20′E; 770 m a.s.l.). The study sites were located within 20 km from each other. Originally covered by beech forest, the grasslands have been grazed by cattle for many centuries, leading to the characteristic *Mesobromion* alliance (Zoller 1954). A study site description can be found in Baur et al. (1996).

The experimental fragmentation of the grasslands was created in April 1993 by mowing the vegetation around the fragments. One experimental unit, hereafter a "block", measured 32 × 29 m and contained two small (0.5 × 0.5 m), one medium (1.5 × 1.5 m) and one large fragment (4.5 × 4.5 m), each separated by a 5-m-wide strip of mown vegetation, as well as the mirror-symmetrically arranged control plots of equal size in the undisturbed vegetation of the other half of the block (Fig. 1). Within each block, the positions of the different sizes of fragment-control plot pairs as well as the fragment and control halves were randomised. The blocks were part of larger study areas (1.5–2 ha) that were enclosed by a fence to exclude large herbivores. The experimental fragmentation was maintained from April 1993 to November 1999 by frequently mowing (6–12 times per year) the area between the fragments in the period from March to October. The entire area of the study sites was mown in November every year to prevent succession.

In the present study, we focused on medium and large fragments and the corresponding control plots. Small plots were not considered because they contained too few *B. officinalis*. Thus, the experimental set-up consisted of 12 blocks with 24 fragments (12 medium and 12 large) and the corresponding 24 control plots distributed over the three study sites. The mean density of *B. officinalis* inflorescences in fragments was 3.8 (individuals/m^2) and 3.1 in the control plots. No *B. officinalis* plants were flowering in the frequently mown isolation zones. In 1993, 1995 and 1999, the performance and seed characteristics of *B. officinalis*

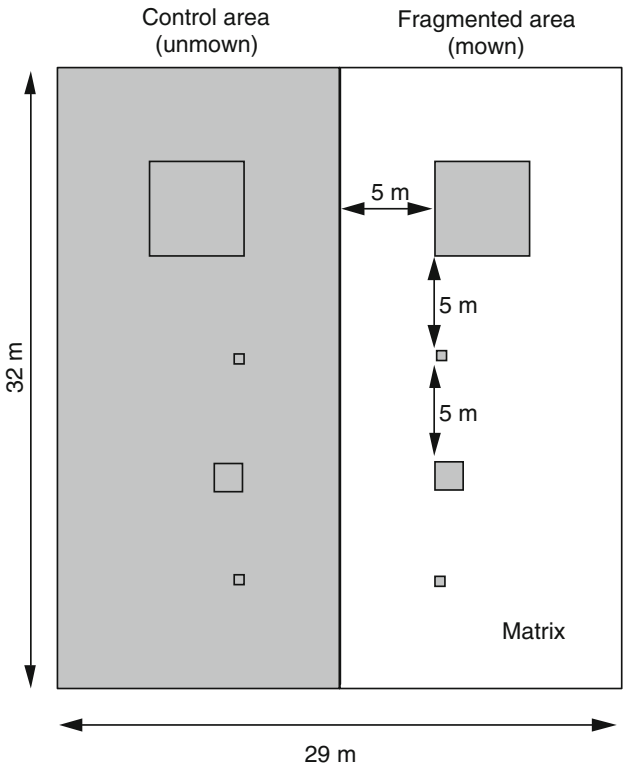

Fig. 1 Layout of one block (out of 12) set up in the field in April 1993. Each block contained four small (0.25 m^2), two medium (2.25 m^2) and two large plots (20.25 m^2). Half of each block served as the control, the other half was experimentally fragmented by frequent mowing between plots. Distance between plots and between plots and continuous vegetation was 5 m. The position of each pair of plots of a given size as well as the position of control and fragmented halves were randomly chosen in each of the 12 blocks. Data from small plots are not considered here

were investigated in all 12 blocks. In 1995 and 1999, flower visitation pattern of *B. officinalis,* foraging behaviour of pollinators and genetic diversity were assessed in a subset of seven blocks situated in Movelier and Vicques.

Plant performance

We counted the number of rosettes and inflorescences of *B. officinalis* in each fragment and control plot in the first year of the experiment in July/August 1993 and 2 and 6 years after the initiation of the experimental fragmentation in 1995 and 1999. Total plant height and inflorescence length of each blooming *B. officinalis* were measured during the flowering peak in the same years.

Flower visitation pattern

Flower visitation rates were assessed in fragments and control plots after 2 and 6 years of experimental fragmentation by counting the number of insects visiting *B. officinalis* inflorescences. During the flowering peak of *B. officinalis*

in July/August of 1995 and 1999, each plot was examined for six periods of 20 min on 6 different days, resulting in a total observation time of 2 h for each plot in both years. The observed flower visitors were assigned to one of six groups (bees, bumblebees, hover flies, butterflies, wasps and others). In bumblebees, which are frequent pollinators of *B. officinalis,* we assessed the time an individual spent in a plot including the searching time for food plants and the duration of the flower visits. Flower visitors that were already in a plot at the beginning of an observation period were not considered. Furthermore, the duration of bumblebee visits to single *B. officinalis* inflorescences was recorded. Observations were made under weather conditions favourable for insect foraging activity (air temperature $\geq$18°C, wind speed $\leq$1 m/s, cloudiness $\leq$25%) between 10 a.m. and 4.30 p.m. The order of surveys in fragments and control plots was randomised on each observation day to avoid any bias due to time-dependent differences in pollinator behaviour and activities. To assess the effect of flower abundance on flower visitation rate of *B. officinalis*, the number of plant species in bloom and the total number of open flowers in the plots were recorded on three occasions during the flowering peak of *B. officinalis* in 1995 and 1999.

Seed set and outcrossing rate

To examine the effects of experimental grassland fragmentation on seed characteristics of *B. officinalis*, we collected two to five inflorescences in each medium fragment and control plot and five to 12 inflorescences in each large fragment and control plot in September 1993, 1995 and 1999. To assess the outcrossing rate of *B. officinalis*, leaf samples of rosettes of the mother plants were collected in seven blocks situated in Movelier and Vicques in 1995 and 1999. Leaf samples were stored at –80°C until required for genetic analysis.

We assessed seed set by counting the number of aborted and developed seeds of 20 randomly selected *B. officinalis* fruits of each inflorescence. Mean seed weight was determined in each inflorescence. Thirty randomly selected seeds from each collected inflorescence were then placed in a pot of 10 cm diameter filled with a mixture of sand and sieved soil obtained from the study site. Seeds were germinated under controlled environmental conditions (24°C, 16 h light/16°C, 8 h darkness). To estimate the germination rate, the number of newly emerged seedlings was counted every 3 days for 5 weeks. Then three seedlings were randomly chosen in each pot, while the remaining seedlings were cut. The chosen seedlings were allowed to grow for 3 weeks under the conditions described above. For the genetic analysis, one of the three juvenile plants was randomly chosen in each pot.

DNA isolation and RAPD amplification

High-molecular-weight DNA from leaf tissue was isolated following the method of Doyle and Doyle (1987). Template quality and quantity were measured using a spectrophotometer.

To ensure the reproducibility of our RAPD-PCR analysis, 12 randomly chosen leaf and seedling samples from the study sites were screened for decamere primers (Operon Technologies, USA). Out of the 40 primers examined, five primers were selected yielding polymorphic and reproducible bands. DNA was amplified twice with the selected primers to assess reproducibility between runs. The reproducibility of the primers selected ranged from 96.4 to 100% (Table S1). These five primers were used in the subsequent analyses of the 560 samples collected in 1995 and the 738 samples collected in 1999. One DNA sample served as an additional control to the blanks in every PCR run.

The RAPD-PCR mixture was 25 ng DNA, 0.2 μM primer, 200 μM of each dNTPs (Pharmacia) and 0.5–1.0 unit Taq polymerase (Promega) depending on the specific primer. Amplification was achieved in a PCT-100 cycler (MJ Research, USA) under the following conditions: 40 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C. PCR was finished with an extension of 5 min at 72°C.

The amplified products were separated on 1.2% agarose gels in 0.5 M TRIS–borate–EDTA buffer containing ethidium bromide (concentration: 0.0001%), using a molecular size standard (Bio-Rad). Each DNA sample was repeated once in separate amplification reactions. DNA from a few leaf samples showed poorly reproducible banding patterns. DNA from these leaves was extracted again and the RAPD-PCR procedure was repeated. However, it was impossible to obtain clear RAPD-PCR patterns for a number of leaf samples (46 out of 560 samples in 1995 and 108 out of 738 samples in 1999). Individuals that did not provide clear or reproducible signals were excluded from the analysis.

For data scoring, the banding patterns were recorded using the gel documentation system AlphaImager (Alpha Innotech, CA, USA). The image profile and molecular weight of each band were determined with the fingerprinting software of Bio-Rad (Bio-Rad Laboratories, CA, USA).

Statistical analyses

We used R statistic (version 2.6.2; R Development Core Team 2008) for all analyses. To avoid pseudoreplication, analyses were performed with the mean values of data gathered in each fragment or control plot. ANOVA and analysis of covariance (ANCOVA) were used because the experiment had a hierarchical split–plot design. If necessary, data were log or arcsin transformed to obtain normally distributed residuals and homogeneous group variance.

Effects of fragmentation on density of rosettes and inflorescences of *B. officinalis* (n/m^2), plant height, inflorescence length and seed set (%), seed weight and germination rate (%) were examined using a five-way ANOVA including the factors year, site, block, plot size, fragmentation and their interactions. The block effects were tested against the variation of interaction of fragmentation and block nested within site to eliminate spatial variation within study sites and site effects were tested against the variation between blocks within sites. The effects of fragmentation were tested against the variation of the interaction of block and fragmentation.

Flower visitation rate was defined as the number of insect visits observed in a plot during 20 min divided by the number of *B. officinalis* inflorescences. Spearman rank correlation analysis showed that the number of plant species in bloom (per m^2) and density of total open flowers of co-flowering plant species were highly correlated: $r_s = 0.56$, $n = 56$, $P = 0.001$. Furthermore, the density of all open flowers of co-flowering plant species and the density of *B. officinalis* inflorescences were negatively correlated ($r_s = -0.34$, $n = 54$, $P = 0.017$). Therefore, the effects of fragmentation on flower visitation rate were analysed using a five-way ANCOVA considering the five factors listed above and the density of *B. officinalis* inflorescences (n/m^2) as co-factor.

Regression analysis was performed to examine the relationship between the time bumblebees spent on an inflorescence and the length of the inflorescence (log/log transformed, $R^2 = 0.70$, $n = 54$, $P < 0.0001$). The residuals of this regression analysis were used to evaluate the effects of fragmentation on flower visitation behaviour when applying ANOVA. The same ANOVA model was used to examine the effects of fragmentation on the time bumblebees spent in the plots. Furthermore, contingency analysis was used to test whether the experimental fragmentation changed the species composition of flower visitors in the plots.

Outcrossing frequencies were calculated using the multilocus estimation procedure of Shaw et al. (1981) and van Treuren et al. (1993). This model compares mother-offspring combinations by assigning offspring into one of two classes: discernible outcrosses (progeny exhibit at least one band not present in the maternal phenotype), and ambiguous mating (progeny showing the same banding pattern as the maternal phenotype, and thus may result from self-fertilization). We assumed that outcrossing can be identified by the presence of a band in the offspring (seedling) that was absent in the mother plant (rosette). Outcrossing was only considered to occur if all five primers showed the same result. Outcrossing frequencies were assessed on the plot level by calculating the sum of discernible outcrossing and ambiguous mating events relative to the sample size. We also determined the genetic diversity of the seedlings using Popgene version 1.32 (Yeh et al. 1997). The effects of fragmentation on outcrossing frequency and genetic diversity were examined using the same five-way ANCOVA with the co-factors total flower visitation rate and density of *B. officinalis* inflorescences (n/m^2). The ANOVA and ANCOVA models were stepwise reduced as recommended by Crawley (2007).

Results

Plant performance

Densities of neither *B. officinalis* rosettes nor inflorescences differed between fragments and control plots or between years (Table 1; Table S2). However, densities of both *B. officinalis* rosettes and inflorescences were affected by plot size (Table 1). Medium plots (fragments and controls) had higher densities of rosettes and inflorescences than large plots. Total plant height and inflorescence length of *B. officinalis* did not differ between fragments and control plots (Table 1). However, the plants were taller and the inflorescences bigger after 2 and 6 years of fragmentation than at the beginning of the experiment (Table 1). Inflorescence length of *B. officinalis* was also affected by plot size (Table 1). The inflorescences were larger in both medium fragments and control plots than in the corresponding larger ones. The significant interactions between site and year on inflorescence density, total plant height and inflorescence length of *B. officinalis* indicated a high site-related variation in these plant parameters during the period of investigation.

Flower visitation pattern

Flower visitation rate was not affected by fragmentation after 2 years ($F_{1,6} = 1.41$, $P = 0.28$). After 6 years of experimental fragmentation, however, flower visitation rate of *B. officinalis* was significantly reduced in fragments ($F_{1,6} = 19.51$, $P < 0.001$). Overall, there was a significant fragmentation effect on flower visitation rate (Table 2). Furthermore, there were significant effects of the study sites and plot sizes on the flower visitation rate (Table 2). Comparing the study sites, the overall flower visitation rate was reduced by 19% at Vicques. Flower visitation rate was reduced by 40% when medium fragments were compared with medium control plots and by 25% when large fragments were compared with large control plots (Fig. 2). However, the density of *B. officinalis* inflorescences did not influence the flower visitation rate of *B. officinalis* (Table 2).

Experimental fragmentation caused a shift in the composition of flower visitors (after 2 years, $\chi^2 = 141.29$, $df = 4$,

Table 1 Summary of ANOVAs testing the effects of year, site, experimental fragmentation, block and plot size on the densities of rosettes and inflorescences, total plant height and inflorescence length of *Betonica officinalis*

	Density of rosettes			Density of inflorescences			Total plant height			Inflorescence length		
	df	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Year (*Y*)	2,105	0.18	0.834	2,105	1.09	0.162	**2,92**	**58.02**	**<0.0001**	**2,92**	**24.63**	**<0.0001**
Site (*S*)	2,9	0.43	0.663	2,9	0.93	0.429	2,9	2.08	0.181	2,9	0.21	0.814
Fragmentation (*F*)	1,11	0.02	0.891	1,11	0.11	0.746	1,11	0.94	0.353	1,11	0.08	0.785
Block(site) [*B*(*S*)]	9,11	1.67	0.209	9,11	2.14	0.117	9,11	1.23	0.367	9,11	1.98	0.142
Plot size (*P*)	**1,105**	**13.00**	**<0.0001**	**1,101**	**6.19**	**0.014**	1,92	0.30	0.558	**1,92**	**12.19**	**<0.0001**
F × *B* (*S*)	**11,105**	**2.33**	**0.013**	**11,101**	**2.13**	**0.025**	**11,92**	**7.07**	**<0.0001**	**11,92**	**3.57**	**<0.0001**
F × *P*		Excl.			Excl.			Excl.		1,92	2.17	0.144
Y × *S*		Excl.		**4,101**	**2.96**	**0.0001**	**4,92**	**15.95**	**<0.0001**	**4,92**	**6.14**	**0.0001**
Y × *F*		Excl.			Excl.		2,92	5.03	0.131		Excl.	

Excl. Excluded from analysis due to the stepwise reduction procedure

Statistical significance indicated by *bold* letters

Table 2 Summary of analyses of covariance testing the effects of the factors year, site, experimental fragmentation, block and plot size on total flower visitation rate of *B. officinalis,* outcrossing frequency and genetic diversity of seedlings

	Total flower visitation rate			Outcrossing frequency			Genetic diversity		
	df	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Year (*Y*)	1,38	2.05	0.161	**1,33**	**7.37**	**0.011**	**1,34**	**256.7**	**<0.0001**
Site (*S*)	**1,5**	**8.23**	**0.035**	**1,5**	**7.75**	**0.039**	1,5	0.78	0.418
Fragmentation (*F*)	**1,6**	**10.69**	**0.017**	**1,6**	**31.14**	**0.001**	1,6	1.89	0.218
Block(site) [*B*(*S*)]	5,6	0.99	0.494	5,6	0.28	0.908	5,6	0.75	0.615
Plot size (*P*)	**1,37**	**11.58**	**0.002**	1,33	0.21	0.644	**1,34**	**6.06**	**0.019**
Total flower visitation rate		n.a.		**1,33**	**4.52**	**0.041**	1,34	0.14	0.709
Density of *B. officinalis* inflorescences	1,38	1.02	0.319	1,33	0.21	0.648	1,34	1.89	0.178
F × *B* (*S*)	6,38	0.17	0.980	6,33	0.80	0.573	6,34	0.49	0.811
F × *P*		Excl.		1,33	0.85	0.364		Excl.	
Y × *S*		Excl.			Excl.			Excl.	
Y × *F*	1,38	1.23	0.275	1,33	1.50	0.217		Excl.	

Depending on the test the co-factors were density of *B. officinalis* inflorescences (n/m^2) or total flower visitation rate. *n.a.* not included in the analysis

Statistical significance indicated by *bold* letters

$P < 0.0001$; after 6 years, $\chi^2 = 55.61$, $df = 4$, $P < 0.0001$). After 2 years of experimental fragmentation, flowers in medium fragments were more frequently visited by wasps (42.4 vs. 20.0%) and less frequently visited by bees (18.8 vs. 50.8%) than flowers in control plots (Fig. 3). In large fragments, flowers were more frequently visited by hover flies (28.9 vs. 8.1%) and less frequently visited by bumblebees (23.1 vs. 48.0%) than flowers in the corresponding control plots (Fig. 3). After 6 years of fragmentation, flowers in medium fragments were more frequently visited by wasps (19.6 vs. 4.7%) and less frequently visited by bees (19.2 vs. 52.5%) than flowers in control plots (Fig. 3). In large fragments, flowers were more frequently visited by bumblebees (35.7 vs. 28.4%) and less frequently visited by hover flies (2.4 vs. 6.8%) than flowers in the control plots (Fig. 3).

The foraging behaviour of bumblebees (the most abundant pollinator of *B. officinalis*) changed in the course of the fragmentation experiment. Bumblebees did not differ in the time spent in fragments and control plots (9.7 ± 0.6 s vs. 8.9 ± 0.5 s; $F_{1,6} = 1.66$, $P = 0.25$) after 2 years of fragmentation. After 6 years, however, foraging bumblebees stayed longer in fragments than in control plots (21.2 ± 3.6 s vs. 12.7 ± 2.4 s; $F_{1,6} = 10.1$, $P = 0.019$). The duration of an inflorescence visit did not differ between fragments and control plots after 2 and 6 years of fragmentation ($P > 0.37$).

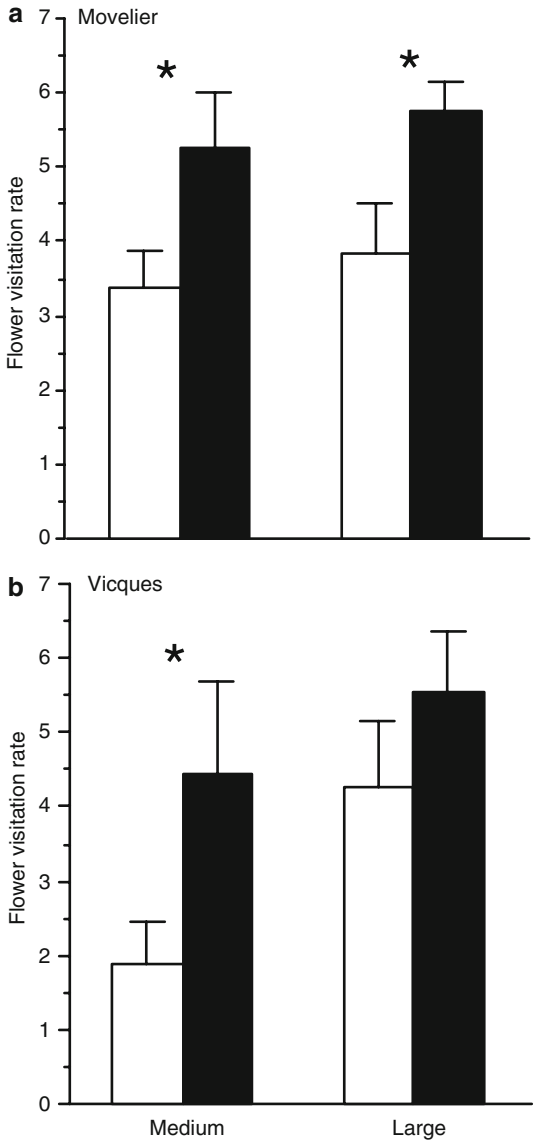

Fig. 2 Flower visitation rate of *Betonica officinalis* (number of visits per inflorescence during 20 min) in medium and large fragments (*open bars*) and the corresponding control plots (*black bars*) at the study sites in **a** Movelier and **b** Vicques [means ± SE (*error bars*) are shown; $n = 14$]. * $P < 0.05$ (Wilcoxon rank sum test)

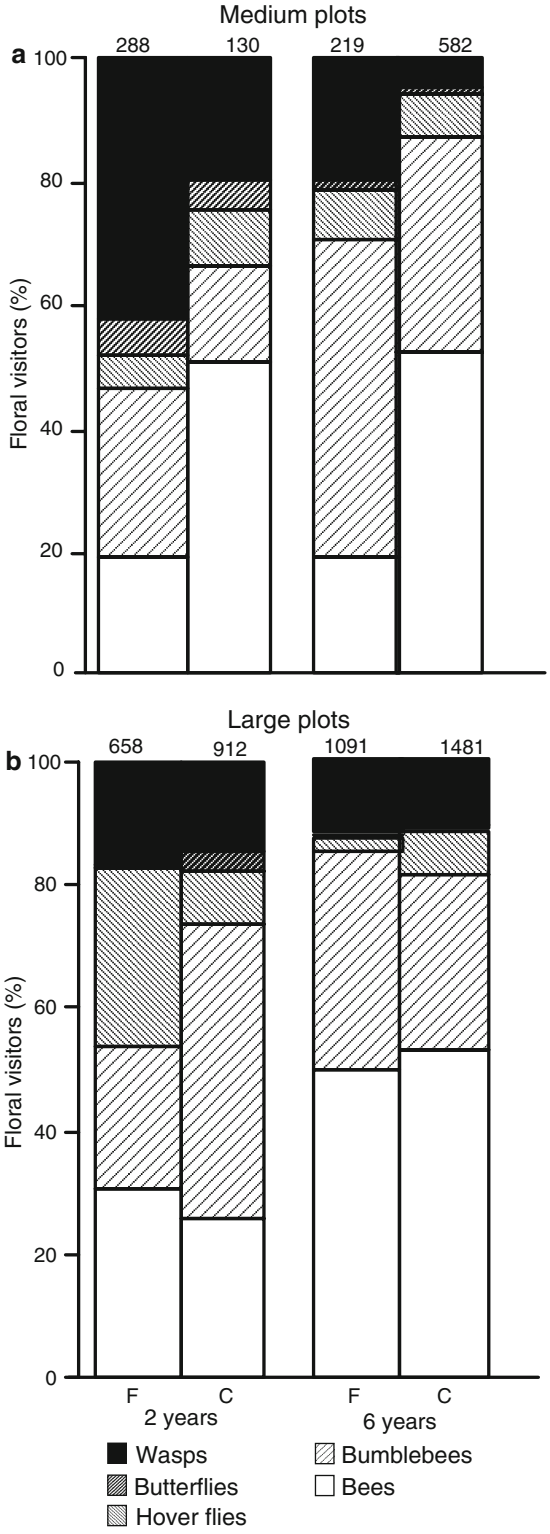

Fig. 3 Frequency of flower visitors of *B. officinalis* in **a** medium and **b** large fragments (*F*) and the corresponding control (*C*) plots after 2 and 6 years of experimental fragmentation. Values *above bars* indicate the number of flower visitors recorded in the plots

Seed set, seed weight and germination rate

Seed set, seed weight and germination rate of *B. officinalis* were not influenced by the experimental fragmentation (Table 3; Table S3). Seed set and seed weight differed among years. Both traits were larger after 6 years of fragmentation than at the beginning of the experiment and after 2 years of fragmentation. The significant interactions between year and site indicated that seed set and seed

Table 3 Summary of ANOVAs testing the effects of year, site, experimental fragmentation, block and plot size on seed set, seed weight and germination rate of *B. officinalis*

	Seed set (%)			Seed weight (mg)			Germination rate (%)		
	df	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Year (*Y*)	**2,92**	**56.19**	**<0.0001**	**1,91**	**28.96**	**<0.0001**	2,98	0.39	0.678
Site (*S*)	**2,9**	**9.82**	**0.0001**	**2,9**	**6.41**	**0.002**	2,9	3.70	0.067
Fragmentation (*F*)	1,11	1.89	0.196	1,11	0.02	0.890	1,11	1.54	0.240
Block(site) [*B*(*S*)]	9,11	0.68	0.714	9,11	1.61	0.225	9,11	1.16	0.401
Plot size (*P*)	1,92	2.58	0.111	1,91	0.77	0.380	**1,98**	**4.39**	**0.039**
F × *B* (*S*)	**11,92**	**4.21**	**<0.0001**	**11,91**	**2.31**	**0.015**	**11,98**	**2.84**	**0.002**
F × *P*	**1,92**	**10.79**	**0.001**	1,91	1.69	0.196		Excl.	
Y × *S*	**4,92**	**15.40**	**<0.0001**	**4,91**	**10.68**	**<0.0001**		Excl.	
Y × *F*		Excl.		**2,91**	**8.35**	**0.0005**		Excl.	

Statistical significance indicated by *bold* letters

weight varied differently at the study sites and in different years (Table 3). Germination rate was not affected by fragmentation, but was higher in large plots (fragments and controls) than in medium plots (Table 3).

Outcrossing frequency and genetic diversity

The outcrossing frequency of *B. officinalis* was reduced by fragmentation and decreased with duration of the experiment (Table 2; Fig. 4). After 2 years, the frequency of outcrossing was 78.8% in fragments and 93.8% in control plots ($F_{1,6} = 7.73$, $P = 0.032$) and after 6 years, the frequency of outcrossing averaged 59.5% in fragments and 95.6% in control plots ($F_{1,6} = 27.93$, $P < 0.0001$). The outcrossing frequency also differed between the study sites. Compared to the control plots, the outcrossing frequency was reduced by 42% in fragments in Movelier and by 27% in Vicques. The outcrossing frequency was not influenced by plot size (Table 2). The flower visitation rate had a significant effect on the outcrossing frequency of *B. officinalis* (Table 2). In contrast, the density of *B. officinalis* inflorescences did not influence the outcrossing frequency (Table 2).

Overall, the genetic diversity of the *B. officinalis* seedlings did not differ between fragments and control plots (Table 2). However, when different years are analysed separately, genetic diversity of seedlings was significantly lower in fragments than in control plots (0.210 ± 0.029 vs. 0.271 ± 0.026; $F_{1,6} = 12.05$, $P = 0.014$) after 6 years of fragmentation. After 2 years, the genetic diversity of seedlings did not differ between fragments and control plots ($F_{1,6} = 3.39$, $P = 0.12$). The genetic diversity increased by 38% from 2 to 6 years of experimental fragmentation. The flower visitation rate and density of *B. officinalis* inflorescences did not influence the genetic diversity of seedlings (Table 2).

Discussion

Habitat fragmentation is expected to change the behaviour, frequency and composition of flower visitors which in turn might affect pollination efficiency determining the quantity and/or quality of pollen deposited on the stigmas and therefore seed and fruit production (Aguilar and Galetto 2004). Natural microcosms have been acknowledged as suitable tools for testing effects of habitat fragmentation, metacommunity theory and links between biodiversity and ecosystem processes (Shrivastava et al. 2004). Our study showed that experimental grassland fragmentation altered the composition of *B. officinalis* pollinators and reduced the flower visitation rate. Furthermore, the foraging behaviour of bumblebees, one of the most frequent pollinators of *B. officinalis,* was changed. As a consequence, the outcrossing rate of *B. officinalis* decreased in the fragments. In contrast, the experimental fragmentation only marginally affected the performance of *B. officinalis*.

The shift in pollinator composition and reduced flower visitation rate of *B. officinalis* in fragments recorded in this study are in line with the findings of several other studies (Jennersten 1988; Smith-Ramirez and Armesto 2003), although these studies were conducted on a much larger spatial scale. Some studies also provide evidence that the patchiness, size and density of floral resources are more important than habitat fragmentation in determining the flower visitation behaviour of pollinators (Jennersten and Nilsson 1993; Klinkhamer et al. 2001; Ishii et al. 2008). In our study, however, ANCOVA analyses revealed that neither the density of *B. officinalis* inflorescences nor the total density of open flowers of co-flowering plant species in the plots affected the flower visitation rate. This indicates that the reduced flower visitation rate in fragments was not caused by differences in floral resources between fragments and control plots. It is also possible that differences in the

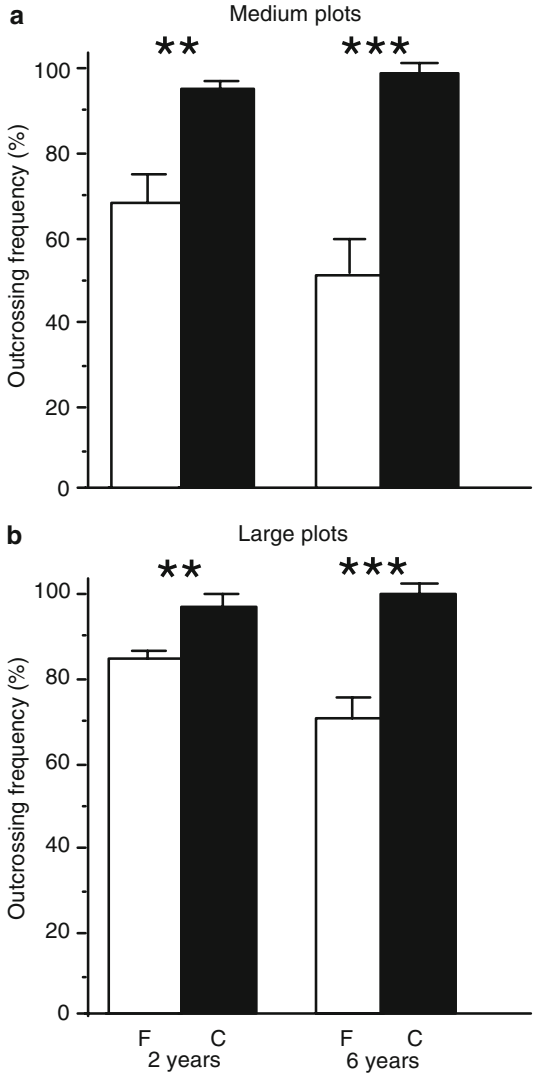

Fig. 4 Frequency of outcrossing in *B. officinalis* in **a** medium and **b** large F and C plots after 2 and 6 years of experimental fragmentation [means ± SE (*error bars*) are shown; $n = 7$]. For abbreviations, see Fig. 3. $^{**}P < 0.01$, $^{***}P < 0.001$ (Wilcoxon rank sum test)

total abundance of floral resources between the control part and the treatment part of a block could influence the flower visitation rate. However, in the present study the flower resources showed very similar spatial distribution patterns and abundances in the control parts and the treatment parts (fragments).

The experimental habitat fragmentation also altered the foraging behaviour of bumblebees. Bumblebees were the most frequent pollinators of *B. officinalis*, responsible for more than 50% of all flower visits. Two years after the initiation of the experiment, we frequently observed bumblebees foraging in the matrix and rarely entering the fragments. After 6 years of fragmentation, however, bumblebees showed a preference to forage in fragments. The change in the foraging behaviour of bumblebees is confirmed by other studies demonstrating a remarkable patch constancy besides their ability to fly long distances (Cresswell 2000). Bumblebees visit repeatedly the same patches of floral resources (Osborne and Williams 2001) and frequently use specific plant-to-plant foraging routes (Thomson et al. 1987). However, the foraging behaviour of bumblebees is plastic and can easily be adjusted to changes in the environment (Chittka and Thomson 1997). Because long-distance flying is expensive in terms of energy, the bumblebees may optimize their net energy gain by adjusting their foraging behaviour to the changing spatial distribution of floral resources induced by the experimental fragmentation (Zimmerman 1982).

A reduced flower visitation rate of pollinators in fragments may decrease gene flow among plants and thus affect their breeding system (Grindeland 2008). In the present study, seed set of *B. officinalis* was only marginally affected by experimental fragmentation and seed weight was even higher in fragments than in control plots after 6 years of fragmentation. Thus no negative effects of inbreeding indicated by reductions in seed set, seed weight and/or germination rate were found in *B. officinalis*. This is not surprising because self-pollination usually accounts for up to 20% of *B. officinalis*' seed set in the wild (H.-P. Rusterholz, unpublished data). Our results are in line with the findings in various other plant species showing that neither seed set, seed weight nor germination rate were affected by habitat fragmentation (Costin et al. 2001; Aquirre and Dirzo 2008). Furthermore, our results confirmed the assumption that habitat fragmentation affects self-compatible plants with different pollinators less than self-incompatible plants with a single specialized pollinator (Waser et al. 1996; Aizen et al. 2002).

In self-compatible plants, the level of outcrossing is influenced by the number of outcrossing pollen deposited on the stigmas (Lloyd 1980). We found a reduced outcrossing frequency in *B. officinalis* growing in fragments. After 2 years of fragmentation, the outcrossing frequency decreased to 73% in fragments, and after 6 years to 60%, whereas in control plots the outcrossing frequency ranged from 94 to 97%.

Different types of genetic markers have been used to determine outcrossing rates in plant species (Lowe et al. 2004). Consequently, only studies using the same type of markers and the same resolution can be compared (Aguilar et al. 2008). The frequency of outcrossing found in our study is similar to the outcrossing rates of *Scabiosa columbaria* (van Treuren et al. 1994) and *Salvia pratensis* (van Treuren et al. 1993). The among-individual variation in outcrossing frequency in *B. officinalis* (0–83% in fragments and 78–100% in control plots) was larger than those reported for other self-compatible plant species including *Eucalyptus benthamii* (Butcher et al. 2005) or *Aquilegia canadensis*

(Routley et al. 1999). Apart from pollinator abundance, interfloral flight distance, body size and foraging pattern of the pollinators may also influence the plant mating system (Karron et al. 1995). In the present study, neither the number of flower visits nor flower offer affected the outcrossing frequency of *B. officinalis*. The reduced outcrossing frequency was most probably a result of the combined effects of an altered pollinator composition, a changed foraging behaviour of the pollinators and a reduced flower visitation rate. The reduced outcrossing frequency in the fragments could also be due to a higher rate of geitonogamy, because bumblebees spent more time in fragments than in the control plots. Low outcrossing rates reduce the genetic diversity of the remnant populations in subsequent generations. Within each population, the risk of losing alleles through genetic drift will increase with the level of inbreeding, leading to possible reductions in plant fitness and eventual extinctions (Fischer and Matthies 1997). The reduced genetic diversity in *B. officinalis* seedlings was not found until the sixth year of experimental fragmentation. This suggests that perennial plants like *B. officinalis* may show less genetic erosion due to genetic drift and enhanced inbreeding than annual or biennial plant species (Young et al. 1996).

In the present study, the plant–pollinator system showed a delayed response to experimental fragmentation. This delay could be a result of both year-to-year variation in the abundance and composition of pollinators and different weather conditions in the years of our investigation (Herrera 1995). In our study, observations of pollinators were only made under climatic conditions favourable for insect activity, thus reducing any bias due to different weather conditions. However, we found differences in the abundance and composition of pollinators among years (Fig. 3). The differences in pollinator guilds were mainly a result of variation in bee and bumblebee abundance. *B. officinalis* and other plant species pollinated by generalist insects might be less affected by among-year differences in the abundance and composition of pollinators than plants depending on specialized pollinators, because the absence of a given pollinator can be buffered by the presence of others (Morris 2003). Furthermore, different insects could differ in their efficiency to pollinate *B. officinalis* flowers. In the present study, however, the five pollinator guilds did not differ in their load of *B. officinalis* pollen (H.-P. Rusterholz, unpublished data) indicating that this factor may play a minor role in this plant–pollinator system. Repeated mowing of the isolation zones (6 times per year) changed the species composition and vegetation structure of the matrix (Fig. S4). The altered foraging behaviour of pollinators may therefore be the result of changes in the composition and structure of the vegetation growing in the matrix after 6 years of experimental fragmentation (Zschokke et al. 2000; Fig. S4). The finding that the quality of the matrix can be a key factor determining the response of plants and animals to habitat fragmentation was confirmed by other studies (Hirsch et al. 2003; Williams et al. 2006).

The present study showed that several plant breeding parameters were not affected by fragmentation-related changes in the reproductive system of *B. officinalis*. Furthermore, our study showed that long-term field experiments are necessary to disentangle slight changes in plant–pollinator interactions and to estimate the resulting effects on the outcrossing frequency and genetic diversity in plant populations.

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