ANNE WEBER

EVOLUTIONARY CONSEQUENCES OF HABITAT FRAGMENTATION: SELECTION ON PLANT PHENOTYPIC TRAITS

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Evolutionary Consequences of Habitat Fragmentation: Selection on Plant Phenotypic Traits

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Anne Weber

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Gutachter der Dissertation:

PD Dr. Annette Kolb
Arbeitsgruppe Vegetationsökologie und Naturschutzbiologie
Institut für Ökologie
Universität Bremen

Prof. Dr. Johan Ehrlén
Plant Ecology
Department of Ecology, Environment and Plant Sciences
Stockholm University

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Table of contents

In all four papers integrated in this thesis, Annette Kolb contributed to ideas, discussion and improvement of the manuscripts, while I carried out the field work and was responsible for data analysis and the writing of the manuscripts.

Summary

Habitat fragmentation is considered to be one of the major threats to plant population viability, species survival and biological diversity worldwide. Small and isolated populations of formerly more widespread plant species are restricted to the remnants of previously connected habitats and are often exposed to changes in environmental conditions. Any change in abiotic or biotic factors may affect the selective environments that plants in fragmented populations are subjected to. For example, plant-animal interactions have often been found to be disrupted by habitat fragmentation, and mutualists as well as antagonists are known to exert selective pressures on plant phenotypic traits. However, only recently researchers have begun to examine evolutionary change in response to habitat fragmentation and altered plant-animal interactions and knowledge about the adaptive potential of small and isolated populations and their long-term persistence in highly fragmented habitats is still scarce. The two major aims of this thesis were therefore to investigate fragmentation effects on phenotypic selection on relevant plant traits and to determine if fragmented plant populations are able to respond to such selection pressures. The study was conducted with the perennial, self-incompatible plant species *Phyteuma spicatum* (Campanulaceae), which occurs in highly fragmented, base-rich, deciduous hardwood forests in north-western Germany.

The overall hypothesis was that selection on plant visual traits is stronger in small compared to large populations, because pollen limitation due to decreased visitation rates in fragmented plant populations could lead to the evolution of trait values that attract pollinators. To determine whether habitat fragmentation influences the strength and direction of phenotypic selection on floral display size and flowering phenology, trait-fitness relationships (i.e. selection gradients) were assessed in 16 natural populations of varying size and density in an observational study over two consecutive years. Additionally, supplemental hand-pollinations were performed in a subset of the populations to experimentally quantify the significance of pollinators in contributing to the patterns of selection. The general assumption that phenotypic trait selection may be affected by plant population size and density was confirmed for floral display size, although only in one of the two study years. Selection for increased inflorescence size decreased with increasing population size and density. Contrary to our expectation, however, this appeared not to be related to changes in pollination intensity. This finding was supported by the hand-pollination experiment: only weak evidence was obtained that selection for increased inflorescence size may have been driven by pollinators. Therefore, abiotic or biotic factors other than pollinators may have contributed to selection on inflorescence size in this study system. Furthermore, population size and density did not affect selection on flowering phenology.

In a next step, small-scale conspecific density effects on pollinator foraging behaviour and trait-fitness relationships were investigated within a large population of varying local plant density. Results indicated that pollinators prefer plants with a more showy floral display and that this preference may depend on local plant density. At low densities pollinator

visitation rates were low, but increased with increasing floral display size, while this relationship was not found at high flowering plant densities, where visitation rates were overall higher. These patterns, however, did not translate into density-dependent trait-fitness relationships and differences in seed production again did not appear to be related to differences in pollination. Nevertheless, selection gradients for several phenotypic traits in *P. spicatum* were significant and phenotypic selection appeared to be spatially and temporally variable. Gradients for floral display size were always positive, with seed production increasing with increasing inflorescence size. The strength of selection differed among populations and years and analyses also revealed non-linear relationships in a few cases. Furthermore, we detected linear selection on flowering state, i.e. early flowering plants produced more seeds than late flowering plants. In some populations and years also non-linear relationships were significant, with intermediate flowering plants having a slightly higher fitness. Consistent with this, linear selection on flowering synchrony suggested an advantage of plants flowering together with many conspecifics. The results obtained for the species studied here are consistent with the view that natural selection often varies in strength and form as well as over space and time.

Finally, to gain a more conclusive insight into the potential of plants in fragmented populations to respond to such phenotypic selection pressures, offspring of plants of ten natural populations of varying size were grown from seed under common environmental conditions. We assessed if the amount of heritable variation for several phenotypic traits was affected by the size and abiotic environmental conditions of the populations of origin. Results showed that small plant populations may indeed have a reduced capacity to respond and adapt to changes in the environment: in two of the measured traits, namely flowering duration and mean seed mass, broad-sense heritabilities (H^2) decreased with decreasing population size. Also environmental conditions of the populations of origin significantly affected H^2-estimates of some of the measured traits. However, mean values of heritable trait variation were rather low and, consistent with findings of other studies, patterns were overall quite variable.

In conclusion, selection pressures on plant phenotypic traits may be stronger in small or low-density populations and small populations may suffer from reduced heritable trait variation compared to large populations. Thus, fragmented plant populations may have a reduced capacity to respond and adapt to novel selection pressures caused by further anthropogenic impacts such as species introduction or climate change and may thus face an increased risk of extinction. However, patterns in *P. spicatum* were variable and we can not conclude from the results obtained here that habitat fragmentation in general will lead to divergent plant evolutionary trajectories in present-day landscapes. It is obvious that evolutionary consequences of habitat fragmentation are complex and further research is certainly needed to answer some of the open questions.

Zusammenfassung

Habitatfragmentierung gilt als eine der größten Bedrohungen für das Vorkommen von Pflanzenpopulationen, für das Überleben von Arten und den Erhalt der biologischen Vielfalt weltweit. Ursprünglich weit verbreitete Arten bestehen heute oftmals aus kleinen und isolierten Populationen, beschränkt auf verbliebene Fragmente ehemals großer zusammenhängender Lebensräume. Solche Populationen sind daher häufig veränderten Umweltbedingungen ausgesetzt, wobei jegliche Änderung von abiotischen oder biotischen Faktoren zu veränderten selektiven Einflüssen auf die Individuen fragmentierter Populationen führen kann. Beispielsweise ist bekannt, dass insbesondere Pflanzen-Tier-Interaktionen durch die Fragmentierung von Lebensräumen beeinträchtigt werden können, sowie dass Mutualisten und Antagonisten Selektionsdrücke auf die Eigenschaften von Pflanzen ausüben. Dennoch werden die evolutionären Veränderungen im Zusammenhang mit Habitatfragmentierung und veränderten biotischen Interaktionen erst seit kurzem erforscht. Zudem gibt es nur wenige Untersuchungen darüber, ob kleine und isolierte Pflanzenpopulationen in der Lage sind sich an neue Selektionsdrücke anzupassen, um so langfristig in stark fragmentierten Lebensräumen zu überleben. Die Hauptziele dieser Arbeit waren daher, zum einen herauszufinden ob phänotypische Eigenschaften von Pflanzen in fragmentierten Lebensräumen durch Bestäuberselektion modifiziert werden, und zum anderen festzustellen inwieweit Pflanzen durch genetische Variabilität in der Lage sind auf einen solchen Selektionsdruck zu reagieren. Die Untersuchungen wurden am Beispiel der mehrjährigen, fremdbestäubten Pflanzenart *Phyteuma spicatum* (Campanulaceae) in stark fragmentierten, basenreichen Laubwäldern in Nordwestdeutschland durchgeführt.

Grundsätzlich wurde erwartet, dass die Selektion auf Pflanzeneigenschaften in kleinen Populationen stärker ist als in großen. Aufgrund geringerer Besuchsraten sind kleine Populationen eher pollenlimitiert, was zur Ausprägung von auffälligeren Pflanzenmerkmalen führen kann, um vermehrt Bestäuber anzulocken. Um zu bestimmen in welchem Maße Habitatfragmentierung die Stärke und Richtung phänotypischer Selektion auf Blütenstandsgröße sowie Blühzeitpunkt beeinflusst, wurden die Zusammenhänge zwischen Merkmalen und Fitness (d.h. Selektionsgradienten) in 16 natürlichen Populationen unterschiedlicher Größe und Dichte in zwei aufeinander folgenden Jahren untersucht. Zusätzlich wurden in einem Teil der Populationen Handbestäubungen durchgeführt, anhand derer die Bedeutung von Bestäubern für die Selektionsmuster experimentell ermittelt werden sollte. Die allgemeine Annahme, dass phänotypische Selektion durch Populationsgröße und -dichte beeinflusst wird, konnte für die Blütenstandsgröße bestätigt werden, jedoch nur in einem der beiden Untersuchungsjahre. Der Selektionsdruck auf eine hohe Blütenanzahl nahm mit zunehmender Populationsgröße und -dichte ab. Entgegen unserer Erwartung wurde kein Zusammenhang mit der Bestäubungsintensität gefunden, was durch die Ergebnisse des Handbestäubungsexperiments untermauert wurde. Hier wurden lediglich schwache Hinweise darauf gefunden, dass Selektion auf Blütenstandsgröße durch Bestäuber verursacht wird.

Demzufolge übten wahrscheinlich andere Umweltfaktoren als Bestäuber einen Selektionsdruck auf die Blütenanzahl aus. Ferner wurde die Selektion auf die Blühphänologie in *P. spicatum* nicht durch Fragmentierung beeinflusst.

In einem nächsten Schritt wurden lokale Dichteeffekte von blühenden Artgenossen auf das Bestäuberverhalten und die Zusammenhänge zwischen Pflanzenmerkmalen und Fitness innerhalb einer großen Population mit unterschiedlicher lokalen Individuendichte untersucht. Die Ergebnisse zeigen, dass Bestäuber vermehrt Pflanzen mit auffälligen Blütenständen anfliegen, sowie dass diese Präferenz von der lokalen Pflanzendichte abhängt. Bei geringen Dichten waren die Besuchsraten niedrig, nahmen aber mit zunehmender Blütenstandsgröße zu. Bei hohen Pflanzendichten mit insgesamt höheren Besuchsraten wurde dieser Zusammenhang hingegen nicht gefunden. Diese Verhaltensmuster spiegelten sich jedoch nicht in dichteabhängigen Selektionsdrücken wieder, da es scheinbar keinen Zusammenhang zwischen Samenproduktion und veränderter Bestäubung gab. Dennoch sprachen die Untersuchungsergebnisse insgesamt dafür, dass die phänotypischen Eigenschaften von *P. spicatum* Selektionsdrücken ausgesetzt waren, und dass die Selektionsmuster räumlich und zeitlich variierten. Die Selektionsgradienten für Blütenstandsgröße waren positiv, d.h. mit zunehmender Blütenanzahl nahm die Samenproduktion zu. Allerdings unterscheiden sich die einzelnen Populationen und Untersuchungsjahre in der Stärke der Selektion und die statistischen Analysen zeigten in wenigen Fällen auch nicht-lineare Zusammenhänge auf. Ferner fanden wir lineare Selektion auf den Blühzeitpunkt, was bedeutet, dass Pflanzen, die zu einem frühen Zeitpunkt blühten mehr Samen produzierten als später blühende Individuen. Darüber hinaus gab es zum Teil auch nicht-lineare Zusammenhänge, aus welchen deutlich wurde, dass Pflanzen, die zur artspezifischen Hauptblühzeit blühten, eine leicht erhöhte Fitness hatten. Hiermit übereinstimmend wurde lineare Selektion auf synchrones Blühen gefunden, was einen Vorteil für diejenigen Individuen bedeutet, die zeitgleich mit möglichst vielen Artgenossen blühen. Die Ergebnisse dieser Untersuchung bestätigen, dass natürliche Selektion oftmals sowohl in Stärke und Ausprägung, als auch räumlich und zeitlich variiert.

In einer weiteren Untersuchung wurden Nachkommen aus Samen von Pflanzen aus zehn natürlichen und unterschiedlich großen Populationen gezogen, um das Potential der Pflanzen fragmentierter Populationen zu ermitteln auf solche Selektionsdrücke zu reagieren. Hierzu wurde der Anteil an vererbbarer Variation für mehrere Pflanzeneigenschaften bestimmt und ermittelt, ob die Vererbbarkeit (Heritabilität) von Eigenschaften von der Populationsgröße oder den Umweltbedingungen abhängt. Die Ergebnisse zeigten, dass das Reaktionsvermögen und die Anpassungsfähigkeit kleiner Populationen eingeschränkt sein kann. Für zwei der aufgenommenen Pflanzenmerkmale, nämlich Blühdauer und mittlere Samenmasse, sank die Heritabilität mit abnehmender Populationsgröße. Zudem unterlag die Vererbbarkeit einiger Pflanzenmerkmale dem Einfluss der Umweltbedingungen der Ursprungspopulationen. Im Großen und Ganzen waren die mittleren Werte der Heritabilität jedoch vergleichsweise niedrig und die gefundenen Muster generell recht variabel.

Zusammenfassend lässt sich sagen, dass Pflanzenmerkmale in Populationen von geringer Größe und/oder Dichte stärkeren Selektionsdrücken ausgesetzt sind als in großen Populationen. Ebenso kann es in kleinen Populationen zu einer geringeren Heritabilität von Merkmalen kommen. Demnach könnten fragmentierte Populationen weniger in der Lage sein sich langfristig anzupassen und auf zukünftige Selektionsdrücke, wie z.B. durch die Einführung neuer Arten oder den Klimawandel, zu reagieren. Dies wiederum kann eine erhöhte Aussterberate fragmentierter Populationen zur Folge haben. Insgesamt waren die Ergebnisse für *P. spicatum* jedoch recht inkonsistent, was es erschwert generelle Aussagen über die Auswirkungen von Habitatfragmentierung auf evolutionäre Veränderungen in heutigen Landschaften zu treffen. Die Ergebnisse dieser Arbeit machen umso mehr deutlich, dass weitere Forschungsarbeit notwendig ist, um den Kenntnisstand zu vervollständigen.

Chapter 1

Introduction

Forest fragment (Brest, Elbe-Weser region). Photograph by A. Weber.

General introduction

Human activities have changed between one-third and one-half of the earth's land surface, with habitat destruction and fragmentation being two of the major anthropogenic impacts on natural landscapes (Vitousek et al. 1997; Lienert 2004). Also natural processes such as glacial advances, volcanic activity, geologic faulting and major sea level rise contributed to the reduction and isolation of natural habitats. However, the extent of anthropogenic induced habitat changes far exceeds natural fragmentation rates and is operating at a much faster time-scale (Lienert 2004). For the last hundreds of years, habitat loss and fragmentation have mainly been caused by changes in land use, rural development and urbanization (Henle et al. 1996). Decreased habitat size and increased habitat isolation may cause changes in the abiotic and biotic environment of the remnant patches and may affect species abundance and diversity (e.g., Saunders et al. 1991; Steffan-Dewenter & Tscharntke 1999; González-Varo et al. 2009). Fragmentation is generally associated, for example, with small population size, reduced gene flow among populations as well as the disturbance of plant-animal interactions (e.g., Aizen & Feinsinger 1994; Lennartsson 2002; Aguilar et al. 2008). Moreover, increases in the edge-to-interior ratio and deterioration of habitat quality following fragmentation may affect individual growth and reproduction as well as the performance of populations (Murcia 1995; Vergeer et al. 2003; Valdés & García 2011). On the whole, habitat fragmentation may increase population vulnerability due to genetic, demographic and environmental stochasticity, thereby threatening the long-term viability of small and isolated populations (Lacy 2000; Oostermeijer 2003).

Ecological consequences of altered plant-animal interactions in response to habitat fragmentation

Today, many formerly more widespread species and especially sessile organisms such as plants are thus restricted to the remnants of previously connected habitats and are exposed to changes in abiotic and biotic conditions of their environment. It is likely that such changes may affect individual plant fitness, e.g. germination, seedling establishment, plant growth, flowering probability and reproductive success. However, in many cases the breaking up of continuous habitat into small and isolated patches may have more indirect effects. Among others, fragmentation and its consequences for abiotic conditions as well as species abundance, diversity, distribution and behaviour (e.g., Settele et al. 1996; Hunter 2002; Tscharntke & Brandl 2004) may alter plant-animal interactions which in turn may strongly influence plant performance (Mustajärvi et al. 2001; Ghazoul 2005). For instance, forest clearing and increases of edge effects in small compared to large patches may cause an increase in wind flux, light intensity, temperature and nutrient influx from the surrounding agricultural land (Saunders et al. 1991), resulting in negative effects on species composition or abundance. Insects may no longer find enough resources (pollen and nectar) in small plant populations (Winfree et al. 2009), which may result either in the avoidance of small plant

populations or changes in their foraging behaviour and movement among patches and individual plants (Sih & Baltus 1987; Jennersten 1988; Steffan-Dewenter & Tscharntke 1999; Lennartsson 2002). Furthermore, low plant density can result in longer residence times of visitors on individual inflorescences because of increased flying distances to the next flowering conspecific (Ghazoul 2005). Plants in small or low-density populations may therefore suffer from significant pollen limitation and reductions in reproductive success caused by fewer flower visits, reduced pollen deposition or lower pollen quality (Jennersten 1988; Ehlers 1999; Aguilar & Galetto 2004; Waites & Ågren 2004; Kolb 2005). Not only pollinators but also seed dispersers, seed predators, herbivores or pathogens can be affected by fragmentation (Jennersten & Nilsson 1993; Lienert & Fischer 2003; Ouborg & Biere 2003; McConkey et al. 2012). For example, plants in small populations have been found to suffer from higher levels of herbivory near the edges of populations (Elzinga et al. 2005). On the other hand, fragmentation may also have beneficial effects on plant fitness when disrupting antagonistic interactions, thereby releasing plants from their herbivores or pathogens (Groom 2001; Ehlers & Olesen 2003; Colling & Matthies 2004). Hence, environmental changes may affect both mutualistic and antagonistic plant-animal interactions, which may result in complex ecological patterns with very different consequences for individual plant fitness as well as population performance and viability.

Evolutionary consequences of altered plant-animal interactions in response to habitat fragmentation

There is increasing evidence that evolutionary change may occur on relatively short timescales and also in response to human-induced environmental change (Palumbi 2001; Stockwell et al. 2003; Strauss et al. 2006; Franks et al. 2007). Thus, apart from ecological consequences, habitat fragmentation may also have evolutionary consequences (Hoffmeister et al. 2005; Jacquemyn et al. 2012). Reduced population size and increased spatial isolation can have detrimental effects on the genetic structure and diversity of natural plant populations (e.g., Willi et al. 2006; Aguilar et al. 2008). Small and isolated populations are expected to experience an increased impact of genetic drift, i.e. the random change in allele frequency from one generation to the next, resulting in the loss of genetic variants from the population. Levels of inbreeding may be higher in small compared to large plant populations and may lead to an increase in homozygosity as well as the expression of recessive deleterious alleles (Ellstrand & Elam 1993; Young et al. 1996; Oostermeijer 2003). These phenomena may cause inbreeding depression, i.e. a reduction in fitness of individuals (Keller & Waller 2002; Lienert 2004). The loss of genetic variation in fragmented populations may reduce their ability to respond to future environmental change, which might limit their adaptive potential and finally even lead to extinction (Young et al. 1996; Booy et al. 2000; Jump et al. 2009). Furthermore, the degree of inbreeding may also affect biotic interactions. For example, negative effects of competitors, herbivores or pathogens have been shown to be more

pronounced in inbred than in outcrossed plants (Cheptou et al. 2000; Carr & Eubanks 2002; Kariyat et al. 2012). This indicates that habitat fragmentation may alter biotic interactions through effects on the genetic population structure of the species involved in these interactions.

Habitat fragmentation may also lead to evolutionary change via alterations in the encounter rates of plants with their mutualists and antagonists. Plant-pollinator interactions may not only be influenced by plant population size and density but also by plant visual traits. Pollen limitation due to decreased visitation rates in fragmented plant populations could thus lead to the evolution of reduced reliance on pollinators or enhancement of traits that attract pollinators (Haig & Westoby 1988; Ashman et al. 2004; Knight et al. 2005). Several studies suggest that self-compatibility and increased clonal growth are likely to evolve in response to decreased pollen availability (Eckert 2002; Moeller & Geber 2005; Kennedy & Elle 2008). However, changes in pollinator visitation rate might also select for increased investment in plant visual traits such as floral display size or flowering duration (Johnston 1991; Elzinga et al. 2007; Murúa et al. 2010; Sletvold & Ågren 2010). Furthermore, resistance or tolerance of plants to natural enemies in response to fragmentation might evolve when the abundance of the antagonists or their negative impacts increase (Ouborg & Biere 2003; Muola et al. 2010). Taken together, effects of habitat fragmentation and therewith associated environmental changes may be either direct, affecting plant and population performance, or indirect via altered plant-animal interactions, which might induce plant characteristics to evolve differently among populations.

Phenotypic selection and heritable trait variation

The idea of phenotypic selection goes back to Darwin & Wallace (1858), and today natural selection is generally accepted as the main cause of adaptive evolution within wild populations (Kingsolver & Pfennig 2007). For natural selection to result in evolution three conditions must be fulfilled: first, that there is phenotypic variation for the trait of interest, second, that there is some consistent relationship between phenotypic trait variation and variation in fitness and third, that some part of the phenotypic variation is caused by genetic variation, i.e. that the trait is heritable (Endler 1986; Falconer & Mackay 1996). Any environmental factor that results in differential fitness among plant phenotypes can thus be the cause of natural selection (e.g., Strauss & Whittall 2006; MacColl 2011). Many plant species rely on insect visitors for sexual reproduction, while pollinators often prefer certain plant and floral phenotypes to others (Goulson et al. 1998; Lortie & Aarssen 1999; Fenster et al. 2004). If their preference differentially affects the fitness among plants that vary in a heritable trait, pollinators may function as agents of selection and thus influence trait evolution in plants (Alexandersson & Johnson 2002; Sapir 2009). Not only pollinators, but also seed dispersers, competitors, herbivores, predators, pathogens as well as abiotic conditions (e.g., temperature, water, light, and soil nutrients) have been suggested to act as

selective agents on plant phenotypic traits (Dudley 1996; Totland 1999; Caruso 2000; Caruso et al. 2003; Strauss & Whittall 2006).

About 30 years ago regression-based approaches for measuring natural selection in terms of selection gradients, i.e. trait-fitness relationships, were introduced (Lande & Arnold 1983; Arnold & Wade 1984). Since then numerous studies investigated selection gradients in natural plant populations (Kingsolver et al. 2001 and references therein). These methods comprise the regression of some fitness component (e.g. total seed or fruit production per plant) on the phenotypic trait of interest, with the slope measuring the strength and direction of selection (Lande & Arnold 1983). By including linear and quadratic terms, the strength and direction of both linear (i.e., directional; selection gradient β) and non-linear (quadratic) selection can be assessed. The quadratic selection gradient (γ) may describe a convex relationship ($\gamma < 0$; e.g., stabilizing selection) or a concave relationship ($\gamma > 0$; e.g., disruptive selection) between trait values and relative fitness. However, to visualize the nature of the curvature and to detect if peaks or valleys are actually included, graphical analyses of the relationships are needed (Brodie et al. 1995; Siepielski et al. 2009). To compare selection gradients among populations, which is relevant since natural selection is known to vary spatially (Caruso et al. 2003; Gómez et al. 2008), absolute fitness is relativized to have a mean of one, and the phenotypic traits are standardized to have a mean of zero and a standard deviation of one (Lande & Arnold 1983).

By altering abiotic and biotic environmental conditions habitat fragmentation may thus lead to novel selection pressures on plant phenotypic traits, and may differently affect evolutionary processes in populations of varying size or isolation (Hoffmeister et al. 2005; Carroll & Fox 2008). However, to determine evolutionary consequences of human-induced environmental change requires not only the assessment of phenotypic selection but also of the heritable basis of phenotypic variation in traits (Geber & Griffen 2003). Heritability of a trait is defined as the proportion of phenotypic trait variation in a population that is attributable to genetic variation among individuals (Lynch & Walsh 1998). Response to selection can be constrained by the lack of genetic variation, i.e. the population mean can not change over generations (Conner & Hartl 2004), yet, genetic variation has been found for many traits (Geber & Griffen 2003; Ashman & Majetic 2006). It has also been suggested that the amount of heritable trait variation may differ among populations, and especially so if they vary in size (Willi et al. 2007; but see Ellmer et al. 2011). Heritability is commonly estimated via the degree of resemblance among related individuals, for example by conducting offspring-parent regressions or sibling analyses (Falconer & Mackay 1996; Conner & Hartl 2004).

Outline of the thesis

Although it is well known that habitat fragmentation may strongly affect biotic interactions (Tscharntke & Brandl 2004 and references therein), and that animals exert selective pressures on plant phenotypic traits (e.g., Campbell 2009; Harder & Johnson 2009), only in recent years

researchers have begun to examine evolutionary changes in response to habitat fragmentation and altered plant-animal interactions (Moeller & Geber 2005; Cheptou et al. 2008; Jacquemyn et al. 2012). Moreover, we still lack knowledge if small or isolated plant populations are able to adapt to novel selection patterns and persist even in highly fragmented habitats. The two major aims of this thesis were therefore to investigate fragmentation effects on the strength and direction of phenotypic selection on relevant plant traits and to determine the potential of plants in fragmented populations to respond to such selection pressures by estimating the amount of heritable trait variation. Thereby, this thesis wishes to contribute to a more conclusive insight into the effects of habitat fragmentation on plant evolutionary trajectories in present-day landscapes.

As study system highly fragmented populations of the self-incompatible, insect-pollinated plant species *Phyteuma spicatum* (Campanulaceae) were chosen. The species is restricted to remnant deciduous hardwood forests in north-western Germany. Within the study area these small and isolated forest patches are integrated in a landscape mainly used for agricultural purposes. A detailed description of the study area and study species is given in CHAPTER 2.

As described above, habitat fragmentation may limit pollination service and may thus lead to reductions in plant reproductive success. This could result in novel selection pressures and the evolution of a more conspicuous floral display to attract pollinators in fragmented populations. The overall hypothesis of this thesis is that pollinator-mediated selection on floral display size and flowering phenology is stronger in small compared to large plant populations. In CHAPTER 3 it was therefore explored whether habitat fragmentation, in terms of differences in population size as well as in mean density, influences the strength and direction of selection on plant phenotypic traits by examining selection gradients, i.e. trait-fitness relationships, in 16 natural plant populations of *P. spicatum* over two consecutive years. The traits of main interest were floral display size (i.e., inflorescence height and size) and flowering phenology (i.e., flowering time and synchrony; "flowering time" corresponds to the "flowering state" in CHAPTER 4).

It is known that pollinators may be a driving force in the evolution of plant visual traits (e.g., Sandring & Ågren 2009). However, also other biotic or abiotic factors may affect trait evolution in plants and experimental manipulations are needed to disentangle the effects of pollinators from those of other agents of selection (Sletvold et al. 2010; MacColl 2011). To complement the study presented in the previous chapter, which was purely observational, in CHAPTER 4 the strength of pollinator-mediated selection was experimentally quantified to directly link differences in plant fitness to differences in pollination intensity along a population size gradient. Supplemental hand-pollinations in six differently-sized *P. spicatum* populations over two consecutive years were carried out and estimates of selection gradients were compared for plants receiving additional pollen to those obtained for open-pollinated control plants.

Plants surrounded by many flowering conspecifics may be more attractive to pollinators than plants growing with few conspecific neighbours (e.g., Field et al. 2005). Thus, also local plant density may affect plant-pollinator interactions and may play a vital role in trait selection. However, only little is known about how plant density affects trait-fitness relationships via changes in pollinator activity. In extension to the analyses presented in the last two chapters, which focused on among-population differences, in CHAPTER 5 density effects were investigated at a much smaller spatial scale. To examine how local plant density and plant phenotypic traits affect pollination and plant reproductive success, pollinator observations were conducted within a large population of *P. spicatum* with strong variation in the spatial distribution of individual plants.

Habitat fragmentation may influence trait evolution in plants through modification of environmental factors that act as selective agents (Jacquemyn et al. 2012). However, the degree to which plants in fragmented populations are able to respond to such altered selective pressures is not well studied. Low genetic variation as well as increased environmental heterogeneity may affect the amount of heritable trait variation in fragmented plant populations (Falconer & Mackay 1996). Thus, the evolutionary potential may be reduced in small compared to large populations due to reduced trait heritability. To explore this, in CHAPTER 6 offspring of plants of ten populations of varying size were grown under common environmental conditions and the amount of heritable variation was estimated for several phenotypic traits.

Finally, in CHAPTER 7 the results of the studies included in this thesis are discussed and linked to findings of other studies. Conclusions are drawn and future research approaches are suggested.

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CHAPTER 2

STUDY AREA AND STUDY SPECIES

Left: Part of the study area with forest fragments (Elbe-Weser region). Satellite photograph from: http://earth.google.de/ (20.12.2012).
Right: Phyteuma spicatum *growing in a natural population (nature reserve* "*Im Tadel*"*, Elbe-Weser region). Photograph by A. Weber.*

Study area

The area chosen for this study consists of highly fragmented forest patches integrated in the agricultural landscape of north-western Germany. More specifically, the study area (*ca.* 425 km^2) is situated between Bremen and Hamburg in the Elbe-Weser region (Fig. 1) and belongs to the rural districts of Rotenburg (Wümme) and Stade. The area is mostly covered by Pleistocene deposits of the Saale glaciation (200–100 ka ago) (Wulf & Kelm 1994), with till deposits characterized by a relatively high content of clay, silt and limestone (Ehlers 1983).

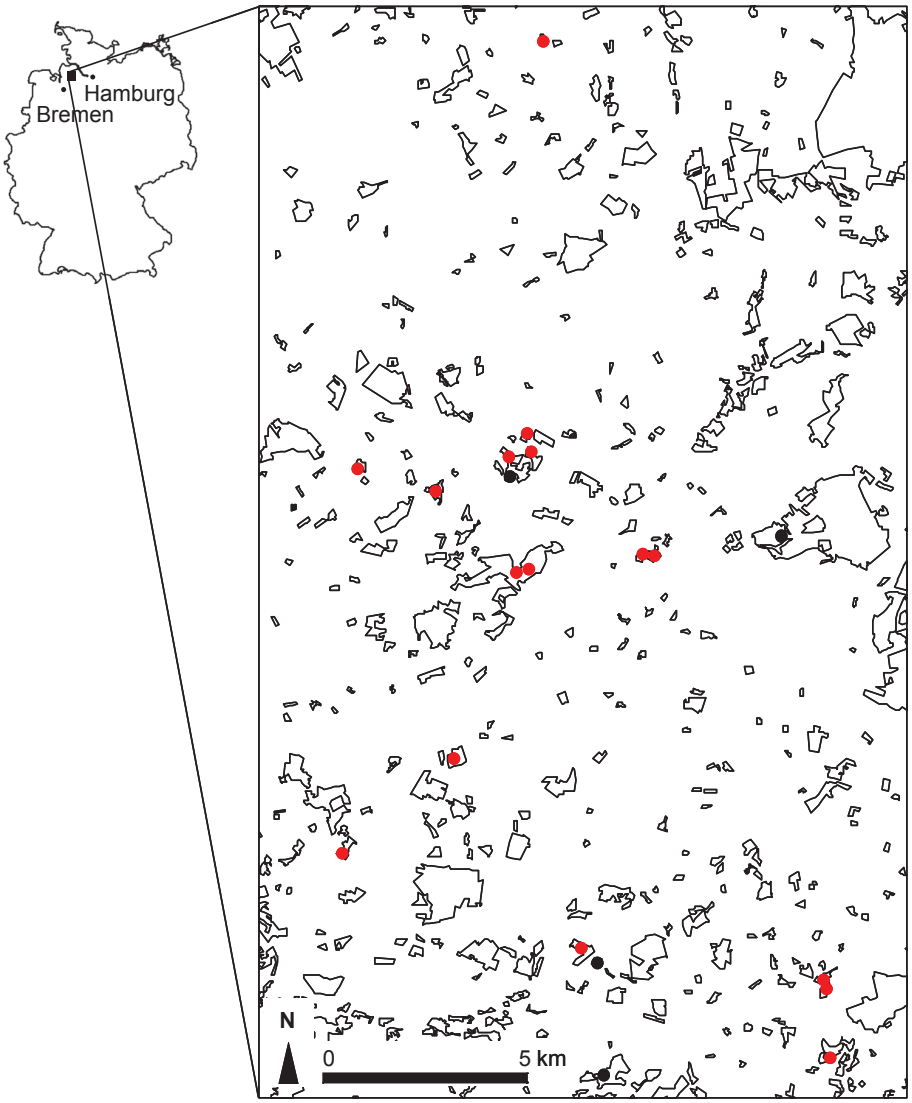

Fig. 1 Forest patches (only those > 1ha) and populations of *Phyteuma spicatum* in the study area, situated between Bremen and Hamburg in north-western Germany. Populations that were part of this study are indicated by red symbols (n = 16), populations not used in this study are indicated by black symbols (n = 4) (base figure by A. Kolb).

Sand and gravel amount to over 50% of the till, which is in sum not very nutrient-rich (Behre 1976). Different soil types (e.g., gley, pseudogley, podsol, para-brown earth and brown earth soils) have developed depending on the relief and the level of the groundwater table (Roeschmann 1971). The landscape is flat to slightly undulating with elevations between

10 and 40 m above sea level. The region is characterized by a (sub-)atlantic climate with mild winters and relatively high amounts of rainfall over the year. Mean monthly temperature is approximately 8.7°C, with a mean of ca. 16°C in June, and mean annual precipitation is about 738 mm (Heinemann 2006; http://www.dwd.de/, 29.12.12).

Human activities have drastically transformed the natural landscape and reduced the forest cover especially since the middle ages (Kelm 1994). Forests in the study area have been highly fragmented for more than 250 years. Main causes were deforestation, grazing, fire and forest-litter utilization. In the 18th century the total forest cover in the Elbe-Weser region constituted merely 4.6% of the landscape and increased to about 9.8% until the end of the 20th century (Kelm 1994; Wulf & Kelm 1994). This increase was primarily due to reforestations with conifers (Behre 1976), and coniferous forests still represent the majority of the forest types in the region. Today the total forest cover in the study area amounts to about 13% of the landscape, of which only 25% are deciduous hardwood forest (Kolb & Diekmann 2004).

The forest fragments in the Elbe-Weser region contain different woodland communities and also areas of coniferous forest. In the study area, base-rich woodland communities are rare and mostly confined to lower-lying areas that have contact to groundwater, thereby assessing additional nutrients (Wulf 1992). However, especially the base-rich deciduous hardwood forest patches (Fig. 2) are of conservation interest, since they represent last refuges

Fig. 2 Base-rich, deciduous hardwood forests in the study area (Elbe-Weser region). Photographs by A. Weber.

for numerous rare and endangered forest herbs (Wulf & Kelm 1994; Garve 2004; Kolb 2005b). The base-rich forest patches that were part of this study vary in size between ca. 1 and 50 ha, and are separated from other deciduous forests by about 50–750 m (A. Kolb, unpublished data). Obligate forest herbs occurring in the base-rich forest fragments are thus restricted to relatively small and isolated patches in the study area.

Study species

To investigate effects of habitat fragmentation (in terms of differences in population size and density) on phenotypic selection, *Phyteuma spicatum* L. (Campanulaceae) was chosen as

study species. There were two main reasons for this choice. First, the species is relatively rare in the study area compared to other parts of the country. Within the study area only 20 populations of *P. spicatum* are still present (Fig. 1), with larger forest fragments supporting larger populations of this species (Kolb et al. 2010). Therefore, the species shows a highly fragmented distribution in the area associated with considerable variation in population size (6–2235 flowering individuals; means based on counts from 2006–2009, with data collected by A. Weber and A. Kolb). Second, results from previous studies suggested that there may be pollinator-mediated selection on specific phenotypic traits and that this selection might be affected by habitat fragmentation (A. Kolb, unpublished data). Kolb (2005a) found that across populations of *P. spicatum*, floral display size and seed output were positively correlated. This relationship, however, differed among populations with stronger phenotypic selection in smaller populations (A. Kolb, unpublished data). *Phyteuma spicatum* is self-incompatible and depends on insect pollination for seed production (Huber 1988). Individuals in small populations produced fewer seeds than individuals in larger populations and this was mainly caused by pollen limitation (Kolb 2005a). Furthermore, experimental populations indicated that small populations with on average more showy plants may be more attractive to pollinators than small populations with less conspicuous plants, while large populations may attract pollinators irrespective of mean floral display size (A. Kolb, unpublished data). Accordingly, pollinators might act as selective agents on phenotypic traits in *P. spicatum*, and selection patterns might differ among populations of varying size.

Phyteuma spicatum occurs in Central and Atlantic Europe (Wheeler & Hutchings 2002). In the study area *P. spicatum* is restricted to fresh or moist, base-rich deciduous hardwood forests (Wulf & Kelm 1994; Kolb & Diekmann 2004). Dominant tree species in these forests include *Alnus glutinosa*, *Carpinus betulus*, *Fagus sylvatica*, *Fraxinus excelsior* and *Quercus robur*, and relatively common species of the field layer are, for example, *Anemone nemorosa*, *Carex sylvatica*, *Galium odoratum*, *Hedera helix*, *Lamium galeobdolon*, *Sanicula europaea* and *Viola reichenbachiana*.

The species is an iteroparous, perennial hemicryptophyte that produces annual rosettes of petiolate, basal leaves (Wheeler & Hutchings 2002) and when flowering, one (sometimes ≥ 2) inflorescence with about 20–100 flowers on an upright stalk of 10–70 cm (data based on individuals growing in the study area) (Fig. 3). *Phyteuma spicatum* remains a vegetative, non-reproductive plant during the first two years and generally reaches sexual maturity in the third year or later (Wheeler & Hutchings 2002). Plants are able to flower each year, but often reproductive parts are only produced every second year or even less frequently (my personal observation). In the study area, flowering usually takes place in May and June, with individual plants flowering between ca. 5 and 20 days, depending on number of flowers, pollination intensity and weather conditions. The hermaphroditic, protandrous flowers lack a pedicel and are densely packed within each inflorescence (Wheeler & Hutchings 2002). Flowers open sequentially in the inflorescence from the bottom to the top of the inflorescence.

Fig. 3 *Phyteuma spicatum*, left: in a natural population within the study area, right: with its main pollinator *Bombus pratorum*. Photographs by A. Weber.

During the male phase of a flower, the pollen covers the style where it is available to insects (Wheeler & Hutchings 2002). Flowers also produce nectar from an epigynous disc and are mainly pollinated by bumblebees (Huber 1988), in the study area especially by *Bombus pratorum* L. (Fig. 3). Spontaneous autogamy and geitonogamy have been observed, but no or very few seeds were produced, presumably due to gametophytic self-incompatibility (Huber 1988). Seeds are oval and small, with a mean air-dry seed mass of 0.12 mg ± 0.02 (data based on individuals growing in the study area). Mean seed production per capsule and per plant is 7.5 ± 4.1 (mean ± SD) and 307 ± 270, respectively. Seed dispersal takes place from late June to the beginning of July. The seeds lack any apparent adaptations to long-distance dispersal with seeds being mainly gravity-dispersed, mostly within a distance of less than 1 m to the parent plant (Maier et al. 1999). Consequently, plants tend to have a clumped distribution within a given population. Seedling establishment depends on vegetation and litter cover as well as on light intensity (Kolb & Barsch 2010), and recruitment success within populations is low (Wheeler 1997).

Adult plants may be severely damaged by herbivores, with roe deer (*Capreolus capreolus*) often removing entire inflorescences, while slugs and snails usually cause less damage (Kolb 2008, 2012). In some populations within the study area, plants have been observed to wilt prior to seed maturity (producing no or only few viable seeds), which is suspected to be caused by fungal pathogens (Kolb 2012).

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CHAPTER 3

Evolutionary consequences of habitat fragmentation: population size and density affect selection on inflorescence size in a perennial herb

Weber, A. & Kolb, A. (2011)
Evolutionary Ecology 25: 417–428.

Inflorescence of Phyteuma spicatum*. Photograph by K. Barsch.*

Abstract

Habitat fragmentation is considered to be one of the major threats to biological diversity worldwide. To date, however, its consequences have mainly been studied in an ecological context, while little is known about its effects on evolutionary processes. In this study we examined whether habitat fragmentation affects selection on plant phenotypic traits via changes in plant-pollinator interactions, using the self-incompatible perennial herb *Phyteuma spicatum*. Specifically, we hypothesized that limited pollination service in small or low-density populations leads to increased selection for traits that attract pollinators. We recorded mean seed production per capsule and per plant as a measure of pollination intensity and assessed selection gradients (i.e., trait-fitness relationships) in 16 natural populations of varying size and density over two years. Mean seed production was not related to population size or density, except for a marginal significant effect of density on the mean number of seeds per capsule in one year. Linear selection for flowering time and synchrony was consistent across populations; relative fitness was higher in earlier flowering plants and in plants flowering synchronously with others. Selection on inflorescence size, however, varied among populations, and linear selection gradients for inflorescence size were negatively related to plant population size and density in one year. Selection for increased inflorescence size decreased with increasing population size and density. Contrary to our expectation this appeared not to be related to changes in pollination intensity (mean seed production was not related to population size or density in this year), but was rather likely linked to differences in some other component of the abiotic or biotic environment. In summary, our results show that habitat fragmentation may influence selection on plant phenotypic traits, thereby highlighting potential evolutionary consequences of human-induced environmental change.

Keywords: floral display size, flowering phenology, *Phyteuma spicatum*, phenotypic selection, pollination, seed production

Introduction

Habitat fragmentation is considered to be one of the major threats to biological diversity worldwide (Primack 2006). To date, its consequences have mainly been studied in a purely ecological context, namely in terms of effects on fitness components, population viability and long-term survival of species in fragmented landscapes (e.g., Settele et al. 1996; Lienert 2004). It has only recently been acknowledged that fragmentation and other forms of human-induced environmental change may also affect evolutionary processes (Palumbi 2001; Stockwell et al. 2003; Hoffmeister et al. 2005). Habitat fragmentation could, for example, influence trait evolution by affecting abiotic or biotic environmental factors that are known to act as selective agents.

In plants, pollinators are of fundamental importance to trait evolution (Fenster et al. 2004). Flower visiting animals often prefer certain plant and floral phenotypes to others and

can therefore select for specific plant traits (Goulson et al. 1998; Alexandersson & Johnson 2002; Parra-Tabla & Vargas 2007; Sandring & Ågren 2009). For example, it has been shown that pollinators visit plants with a larger floral display more often than plants with a less showy display and that bout lengths increase with the number of open flowers (Ohara & Higashi 1994; Grindeland et al. 2005), which in turn may lead to a higher reproductive success (Ohara & Higashi 1994; Parra-Tabla & Vargas 2007). Also flowering phenology, both in terms of the timing of flowering and the degree of flowering synchrony, may be important for pollination and subsequent seed production and has therefore been suggested to be of adaptive value (Augspurger 1981; Elzinga et al. 2007; Sandring & Ågren 2009).

Habitat fragmentation often disrupts the interactions between plants and their pollinators (e.g., Olesen & Jain 1994). In small and isolated habitat fragments and populations, the abundance and species richness of pollinators may be lower and their foraging behaviour may be altered (Sih & Baltus 1987; Steffan-Dewenter & Tscharntke 1999; González-Varo et al. 2009). Plants in small populations may therefore receive fewer flower visits, smaller pollen loads or pollen of poorer quality, and in consequence suffer from pollen limitation and reductions in seed output (Jennersten 1988; Byers 1995; Ågren 1996; Steffan-Dewenter & Tscharntke 1999; Aguilar & Galetto 2004; Kolb 2005). Pollen limitation due to fragmentation is especially detrimental in self-incompatible plant species which are highly dependent on pollinators for sexual reproduction (Aguilar et al. 2006).

We thus know that pollinators may exert strong selective pressure on plant phenotypic traits and that habitat fragmentation may affect plant-pollinator interactions. However, almost nothing is known about how fragmentation mediates patterns of selection on plant phenotypic traits via changes in pollinator availability. Limited pollination service in small populations could lead to the evolution of reduced reliance on pollinators or enhancement of traits that attract pollinators (Haig & Westoby 1988; Ashman et al. 2004; Knight et al. 2005). For example, pollen limitation has been predicted to select for mechanisms providing reproductive assurance such as self-fertilization or increased clonal growth (Lloyd 1992; Eckert 2002; Moeller & Geber 2005; Kennedy & Elle 2008; Eckert et al. 2009). Pollen limitation could also lead to selection for more synchronous flowering or a more conspicuous floral display (Johnston 1991; Elzinga et al. 2007). Optimal flowering phenology and floral display may therefore differ among populations of varying size or density because of variation in the abundance of pollinators.

The main aim of this study was to examine potential evolutionary change in response to habitat fragmentation and altered plant-pollinator interactions by investigating current phenotypic selection pressures along a population size gradient. We have chosen a highly fragmented habitat type as study system, remnants of temperate deciduous forest in north-western Germany, and the self-incompatible, perennial forest herb *Phyteuma spicatum* L. as model species. Previous studies with this species have shown that individuals in small populations produce fewer seeds than individuals in larger populations and that this is mainly

caused by pollen limitation (Kolb 2005, 2008; Kolb et al. 2010). Furthermore, small populations with on average more showy plants (in terms of a tall inflorescence and of an inflorescence with many flowers) appeared to be more attractive to pollinators than small populations with less conspicuous plants, while large populations attracted pollinators irrespective of mean floral display size (A. Kolb, unpublished data). These data were not collected on the individual plant level, but demonstrate that the effect of floral display traits on pollination may differ depending on plant population size. To examine in more detail whether habitat fragmentation affects pollinator-mediated selection on plant phenotypic traits, we investigated trait-fitness relationships in 16 natural *P. spicatum* populations of varying size. In addition, we examined effects of population density, as this is also known to affect plant-pollinator interactions (Ghazoul 2005). Our specific objectives were 1) to investigate effects of population size and density on mean seed production, using the latter as a measure of pollination intensity (Kolb 2005, 2008); 2) to test whether phenotypic selection on inflorescence size, time of flowering and flowering synchrony varies among populations and; if so, 3) to examine whether among-population variation in selection is related to plant population size or density. To the best of our knowledge, this is one of the first studies examining how habitat fragmentation affects plant evolutionary trajectories in present-day landscapes.

Materials and methods

Study species and study area

Phyteuma spicatum L. (Campanulaceae) is an iteroparous, perennial hemicryptophyte endemic to Central and Atlantic Europe (Wheeler & Hutchings 2002). It produces annual rosettes of basal leaves and normally one inflorescence with about 20–100 flowers on an upright stalk of 10–70 cm (data based on individuals growing in our study area). *Phyteuma spicatum* remains a vegetative, non-reproductive plant during the first two years and reaches sexual maturity in the third year or later (Wheeler & Hutchings 2002; A. Kolb, unpublished data). Flowering takes place in May and June, with individual plants flowering between 5 to 15 days. The hermaphroditic, protandrous flowers are sessile and densely packed within each inflorescence, and open sequentially in the inflorescence from the bottom to the top. The flowers are mainly pollinated by bumblebees (Kolb 2008). Spontaneous autogamy and geitonogamy result in no or very few seeds, presumably due to gametophytic self-incompatibility (Huber 1988). Seed production per capsule and per plant range from 0–34 (mean ± SD; 2008: 7.5 ± 4.1, n = 831 individuals; 2009: 7.2 ± 4.2, n = 752) and 0–3303 (2008: 307 ± 270, n = 831; 2009: 287 ± 266, n = 752), respectively. Roe deer, and to a lesser extent slugs and snails, may damage plants (Kolb 2008; our personal observation). In some populations, we observed plants to wilt prior to seed maturity, which appears to be caused by pathogens.

In our study area, situated between the cities of Bremen and Hamburg in north-western Germany (Kolb 2005), the species is relatively rare (see http://www.floraweb.de for a Germany-wide distribution map) and restricted to fresh or moist, base-rich deciduous hardwood forests. Forests in this area are highly fragmented and cover ca. 13% of the landscape, of which only 25% are deciduous hardwood forest. Larger forest fragments support larger populations of the species (r = 0.67, P = 0.005, n = 16, area and mean population size log-transformed).

Data collection

Data were collected from May to July in 2008 and 2009 in 16 populations of *P. spicatum*. Population size was estimated as the number of flowering individuals during peak flowering (end of May – early June) and varied between 10 and 2982 (2008) and 6 and 1718 (2009) flowering plants (Appendix 1). Population density was determined as the mean number of intact inflorescences of *P. spicatum* within a radius of 50 cm around each plant that was included for study (see below), and expressed as inflorescence number per 0.8 m^2 (2.3–22.1 in 2008, and 1.9–16.7 in 2009; Appendix 1). Population size and density were significantly correlated in 2008 (Pearson correlation, r = 0.54, P = 0.031), but not in 2009 (r = 0.41, P = 0.118). The populations, some of which are located in the same forest fragment, are separated by ca. 100 m to 10 km from each other. Population isolation does not appear to affect pollination or reproductive success in this system (Kolb 2005, 2008).

Within each population and year, we randomly marked about 80–90 flowering plants (except when fewer were present; Appendix 1) with small flags stuck into the ground. To avoid excessive damage by deer, we protected all marked individuals with fences or deer repellent; pollinators were not affected by this. Still, some of the plants were later damaged by herbivores (mainly by slugs) and in a few populations, some plants had already completely wilted prior to seed maturity; these plants were therefore excluded from analysis.

We measured a number of traits for each plant (mean population values ± SD of all traits included in the analyses are shown in Appendix 1). During peak flowering we determined the number of inflorescences per plant and, as a measure of the time of flowering, assigned each plant to one of 15 phenological states based on the proportion of open flowers, from late (state 1, all flowers still in bud stage) to early flowering (state 15, all flowers already in fruit). We used the mean difference in phenological state between a focal individual and all other marked individuals in the population as a measure of flowering synchrony, calculated as:

$$S_j = \frac{\sum_{i=a}^{b} n_i \left| i - j \right|}{N - 1}$$

where S_j is the synchrony value of a plant in phenological state j, a and b are the minimum and maximum phenological states in the population, respectively, n_i is the number of plants in phenological state i and N is the total number of plants in the population. This index thus expresses the degree of asynchrony: plants with a low value flower together with many other individuals, while plants with a high value flower when no or only few other plants are flowering. Given limited resources and the relatively large number of populations and individuals included in our study, we were not able to collect the data needed to calculate more standard measures of flowering synchrony (see e.g. Elzinga et al. 2007). While the index used here serves as an indicator for the degree of flowering synchrony, other estimates may have yielded more accurate results.

Shortly after flowering we measured the height of each inflorescence stalk (including the inflorescence) as well as the size of each inflorescence, i.e. the portion with flowers. At the time of seed maturity (end of June, early July), we collected all inflorescences. For each inflorescence, we determined the mean number of seeds per capsule (means being based on ten randomly chosen capsules), the total number of seed capsules as well as the total number of seeds (calculated as the mean number of seeds per capsule × the total number of capsules). For plants with more than one inflorescence, we calculated the mean inflorescence height and size as well as the mean number of seeds per capsule. Total seed production per plant was calculated as the sum of the total number of seeds produced by each inflorescence.

Data analysis

To examine whether population size and density have an influence on mean seed production (and thus likely on pollination intensity; Kolb 2005, 2008) in 2008 or 2009, we regressed the mean number of seeds per capsule and per plant on log-transformed population size and density. We also investigated seed number per capsule because this measure is less dependent on plant size than seed number per plant, and may therefore better reflect pollination success. As the number of ovules may differ between flowers (Wheeler & Hutchings 2002), differences in seed set per capsule may be caused both by variation in ovule number and by variation in seed : ovule ratios. Results from a previous study, however, suggest that pollen-limitation in individuals of small populations results in fewer seeds both per capsule and per plant (Kolb 2005).

To test whether phenotypic selection on plant traits varies among populations, we conducted standard selection gradient analyses for each year by regressing relative fitness on standardized trait values (Lande & Arnold 1983). We used the total number of seeds per plant as fitness estimate. Within each population, absolute fitness was relativized to have a mean of one (by dividing the number of seeds by the mean number of seeds), and the plant traits were standardized to have a mean of zero and a standard deviation of one (by subtracting the mean and dividing by the standard deviation). We did not include inflorescence number as a trait because most plants had only one inflorescence (82.1% in 2008, and 84.4% in 2009). As

mean inflorescence height and size were strongly correlated within populations (*r* ranging from 0.33 to 0.90 in 2008, and from 0.61 to 0.97 in 2009), we restricted our analyses to inflorescence size. The size of an inflorescence is a measure for both flower number and duration of flowering and might therefore be under stronger pollinator-mediated selection than inflorescence height. We tested for differences in selection gradients among populations by fitting a general linear model with standardized inflorescence size and flowering time as well as the population × standardized size and population × standardized flowering time interactions as predictor variables, and relative fitness as response variable. The main effect of population was not included in the model because fitness values were relativized within populations prior to analysis. We used separate models to test for effects on flowering synchrony because flowering synchrony and time were strongly correlated (*r* ranging from 0.35 to –0.94 in 2008, and from –0.47 to –1.0 in 2009) and because flowering synchrony is not a "pure" individual plant trait as it also depends on the phenological state of the other flowering individuals in the population. Analyses were conducted separately for 2008 and 2009 because we had data for both years only for a subset of plants; individuals flowering in 2008 but not in 2009 were replaced by other flowering individuals in 2009 to obtain the desired sample size.

To test if the observed among population-variation in linear selection on inflorescence size in 2008 and 2009 (see Results, Table 2) was related to population size or density, we regressed linear selection gradients (β's) for inflorescence size on population size and density. Selection gradients were obtained from within-population multiple regressions of relative fitness on standardized inflorescence size and flowering time.

In all cases, we only present models including linear terms because the relatively low sample size in the small populations placed an upper limit on the number of possible predictors in our statistical models. We also did not find much evidence for non-linear selection, as tested in populations with ≥ 12 marked individuals using models including quadratic terms; quadratic terms were significant ($P < 0.05$) only in a few cases (inflorescence size: in two populations in 2008 and 2009, flowering time: in one population in 2009, flowering synchrony: never).

All analyses were conducted in R 2.7.2 (R Development Core Team 2008), using the "lm" function for all statistical models and the "Anova" function of the "car" package for F-tests.

Results

Contrary to our expectation, the mean number of seeds produced per capsule and per plant were mostly not reduced in plants of small or low-density populations (Table 1). We only observed a marginal significant positive effect of population density on the mean number of seeds per capsule in 2009 (Table 1; Fig. 1). Seed production per capsule was not affected by

density in the other year and also not by population size (Table 1). Likewise, the mean number of seeds produced per plant was not related to population size or density (Table 1).

Table 1 Effects of log-transformed population size and density on the mean number of seeds produced per capsule and per plant in 16 populations of *Phyteuma spicatum*. Values of $P < 0.1$ are in italics.

		Linear regression on population size (log)		Linear regression on population density (log)	
	Year	Regression equation	*P*	Regression equation	*P*
Number of seeds / capsule	2008	$y = 0.23\,x + 6.21$	0.446	$y = 1.04\,x + 5.32$	0.202
	2009	$y = 0.01\,x + 7.03$	0.979	$y = 1.10\,x + 5.31$	*0.061*
Number of seeds / plant	2008	$y = 7.36\,x + 260.14$	0.693	$y = 39.10\,x + 220.85$	0.452
	2009	$y = 3.70\,x + 248.03$	0.811	$y = 66.74\,x + 159.41$	0.116

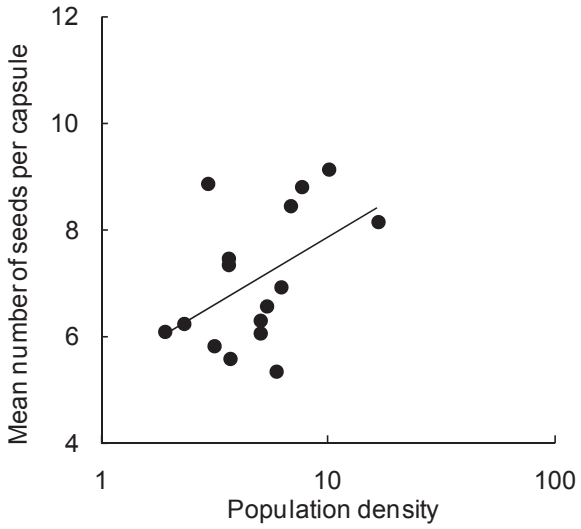

Fig. 1 Relationship between log-transformed population density and mean number of seeds produced per capsule in 16 populations of *Phyteuma spicatum* in 2009 (see Table 1 for regression statistics).

Linear selection on inflorescence size varied among populations, the population × inflorescence size interaction was significant in both 2008 and 2009 (Table 2). Inflorescence size-fitness relationships were positive in all populations, but differed in slope (range of selection gradients, 2008: 0.34–1.01, 2009: 0.29–1.05). In both study years, we detected linear selection on flowering time, the effect was marginally significant in 2008 and highly significant in 2009 (Table 2). Relative fitness was higher in earlier flowering plants, and this relationship was consistent across populations. In both 2008 and 2009, we also found evidence for linear selection on flowering synchrony (Table 3). More synchronous plants had a higher relative fitness than plants flowering off-peak, and again this relationship was consistent across populations.

Table 2 Linear selection on inflorescence size and flowering time in 16 populations of *Phyteuma spicatum* in 2008 (n = 831 individuals) and 2009 (n = 752). Values of P are bold if < 0.05 and in italics if < 0.1.

Source of variation	df	F	P	Parameter estimate
2008				
Inflorescence size	1	487.79	**<0.001**	
Flowering time	1	2.80	*0.095*	0.039
Population × inflorescence size	15	1.92	**0.019**	
Population × flowering time	15	0.43	0.972	
2009				
Inflorescence size	1	387.38	**<0.001**	
Flowering time	1	13.19	**<0.001**	0.089
Population × inflorescence size	15	4.09	**<0.001**	
Population × flowering time	15	0.31	0.995	

Note: Results are from linear models (type II sums of squares), with standardized traits and population as predictors and relative fitness as response variable. Parameter estimates (from models without interaction terms) are given for main effects where interaction is not significant (P > 0.05).

Table 3 Linear selection on flowering synchrony (here expressed as the degree of asynchrony) in 16 populations of *Phyteuma spicatum* in 2008 (n = 831 individuals) and 2009 (n = 752). Values of P < 0.05 are in bold.

Source of variation	df	F	P	Parameter estimate
2008				
Flowering synchrony	1	38.61	**<0.001**	–0.167
Population × synchrony	15	0.90	0.569	
2009				
Flowering synchrony	1	51.43	**<0.001**	–0.231
Population × synchrony	15	0.44	0.967	

Note: Results are from linear models (type II sums of squares), with standardized traits and population as predictors and relative fitness as response variable. Parameter estimates are from models without interaction terms.

Selection for increased inflorescence size decreased with increasing plant population size and density in 2008; the relationships between linear selection gradients and the two population parameters were significant (Figs. 2a, c). In 2009, among-populations differences in selection on inflorescence size, however, were not related to population size or density (Figs. 2b, d).

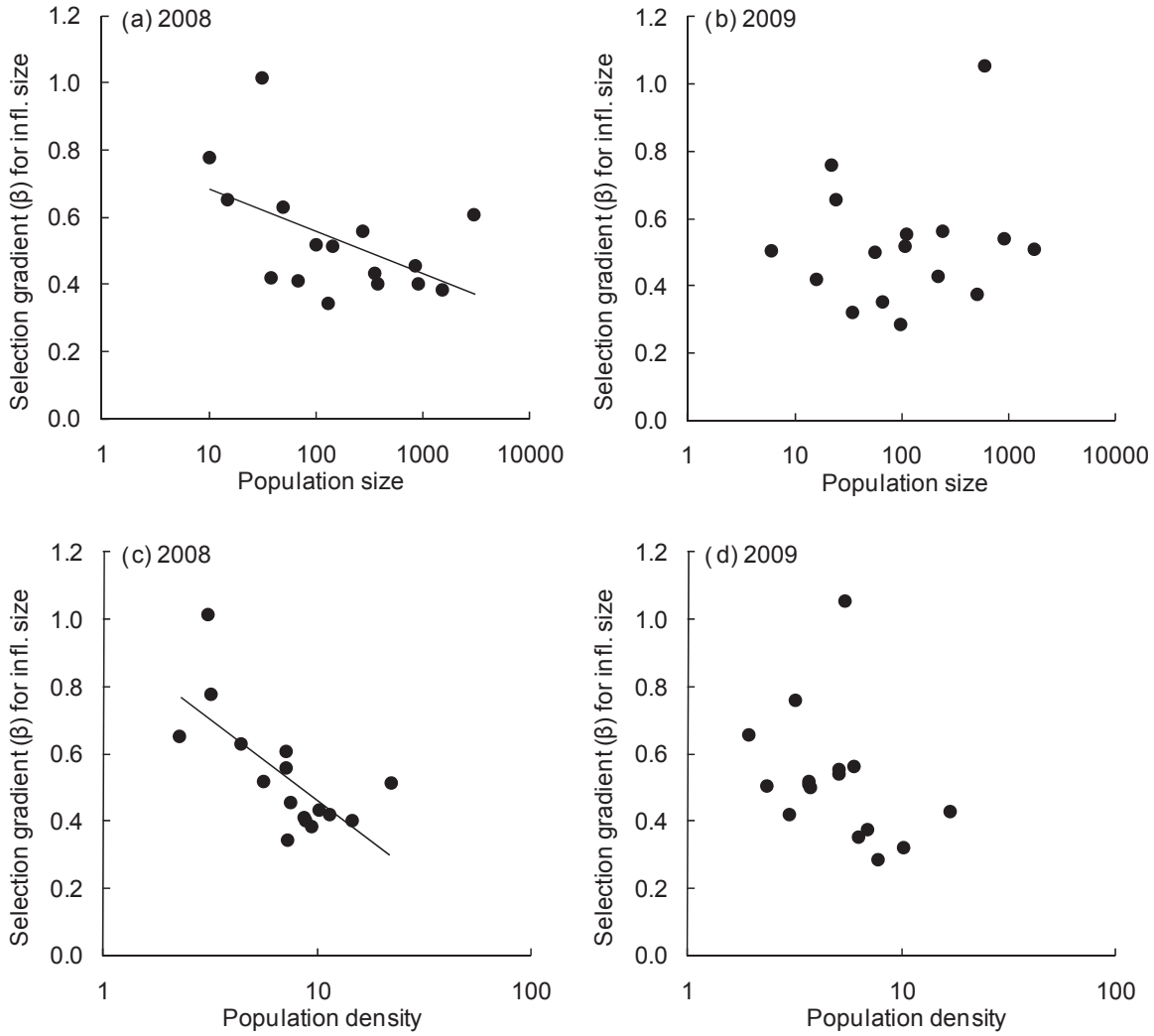

Fig. 2 Relationships between log-transformed population size (a, b) or density (c, d) and phenotypic selection gradients for inflorescence size (*β*'s, based on the total number of seeds per plant) in 16 populations of *Phyteuma spicatum* in 2008 and 2009. Population size: 2008, $y = -0.05\,x + 0.80$, $P = 0.044$; 2009, $y = 0.02\,x + 0.44$, $P = 0.584$; population density: 2008, $y = -0.21\,x + 0.94$, $P = 0.002$; 2009, $y = -0.12\,x + 0.71$, $P = 0.175$.

Discussion

The results of our study show that habitat fragmentation may affect plant evolutionary trajectories. In one of the two study years, linear selection gradients for inflorescence size were negatively related to plant population size and density; selection for increased inflorescence size decreased with increasing number of flowering individuals. Contrary to our expectation, however, this appeared not to be related to changes in pollination intensity; mean seed production was not related to population size or density in this year.

In contrast to previous studies with this species (Kolb 2005, 2008; Kolb et al. 2010), seed production was not affected by population size in the two study years. Our data thus

provide no evidence for pollen limitation of plant reproduction in small populations. In 2008, during peak flowering of *P. spicatum*, the weather was dry and warm (fewer rainy days and relatively high temperatures compared to previous years; data from www.dwd.de), conditions which are considered beneficial for pollinators. Lundberg (1980), for example, found that foraging activity of bumble bees was generally higher during dry conditions and that the number of foragers was strongly influenced by temperature. Furthermore, in most populations the number of flowering individuals was higher during this year compared to 2009 (Appendix 1) and other years, possibly leading to the attraction of more pollinators and increasing the availability of conspecific pollen (Sih & Baltus 1987). Favourable weather conditions and high flowering rates may thus have caused pollen not to be limiting in small and low-density populations; population size and density were significantly correlated in this year. A pollen supplementation experiment conducted in several of the populations also suggested that plants were not pollen limited in this year (data not shown). In 2009, weather conditions during peak flowering were less favourable and also within-population flowering rates were relatively low. Correspondingly, we found a marginal significant effect of population density on the number of seeds produced per capsule in this year. Population size (which was not related to population density in this year), however, did not affect seed production. In general, temporal variation in plant-pollinator interactions (Herrera 1988; Alarcón et al. 2008) and seed production (Wärner 2009) are common. Herrera (1988), for example, found among-year variation in diversity, composition, abundance and behaviour of pollinators of an evergreen shrub, which may translate into differential fitness of the plants.

In both years, we found phenotypic selection on inflorescence size as well as on flowering time and synchrony. Relative fitness was higher in plants with larger inflorescences, in early-flowering plants and in plants flowering synchronously with conspecifics, which corresponds to the findings of numerous other studies (e.g., Augspurger 1981; Ohara & Higashi 1994; Sandring & Ågren 2009). Ohara & Higashi (1994) observed for *Corydalis ambigua* that plants with larger inflorescences received longer bumble bee visits and exhibited higher fecundity than plants with smaller inflorescences. Similarly, Sandring & Ågren (2009) documented pollinator-mediated selection for many flowers in *Arabidopsis lyrata*. In *Phyteuma nigrum*, early flowering plants received more bumble bee visits per inflorescence than plants flowering later in the season (Kwak et al. 1991), which seems to be a common pattern in plants (Elzinga et al. 2007). Within populations of *Hybanthus prunifolius*, individuals flowering synchronously with other individuals of the population attracted more pollinators and set more seeds than individuals flowering asynchronously with the population (Augspurger 1981). Selection on floral display traits and flowering phenology can also be affected by factors other than interactions with pollinators. For example, abiotic conditions or interactions with antagonists may affect selection on floral display size and flowering phenology (see Elzinga et al. 2007; Franks et al. 2007 and further below).

While linear selection on flowering time and synchrony did not differ among populations, we found spatially variable selection for inflorescence size in both study years. Such spatial variation in selection appears to be common and often related to differences in the abiotic or biotic environment (Gómez & Zamora 2000; Caruso et al. 2003; Thompson 2005). A major finding of our study was that in one of the study years patterns of selection were related to population size and density. This, however, did not appear to be driven by changes in pollinator availability. Thus, contrary to our hypothesis that pollen limitation in small and sparse populations would select for a larger floral display in such populations, other selective agents (that may also be related to population size or density) must have been responsible for the observed patterns. Abiotic environmental conditions, for example, may vary with habitat fragment and population size (Saunders et al. 1991) and cause differential selection on plant phenotypic traits. Previous studies with *P. spicatum* have shown that light availability (Kolb 2005; 11 of the 14 populations included in that study were also part of the present study) and base saturation in terms of soil Ca content (A. Kolb, unpublished data) were significantly and marginally significantly related to population size, respectively. Relative light intensities decreased and soil Ca content tended to increase with increasing population size. Thus, selection for increased inflorescence size may be stronger under higher relative light intensities and low base saturation in small populations. Several studies suggest that the availability of abiotic resources such as water, nutrients, light or temperature can influence phenotypic selection on plant traits (Dudley 1996; Totland 1999; Caruso et al. 2003). For example, Caruso et al. (2003) found selection on floral traits in *Lobelia cardinalis* and *L. siphilitica* to be influenced by soil water availability. Furthermore, interactions with antagonists such as herbivores and pathogens, which have been shown to select for small plant and floral display size (Gómez 2003; Sletvold & Grindeland 2008), may be disrupted by habitat fragmentation (Groom 2001; Colling & Matthies 2004). Increasing damage intensities with increasing population size and density could thus lead to selection for decreased inflorescence size. However, previous studies in this system suggested that the intensity of herbivory is not related to population size (Kolb 2008; Kolb et al. 2010), and we investigated phenotypic selection pressures in the absence of the main antagonists, namely roe deer. The effects of pathogens, though, are at least partly included in our data (we only excluded plants severely affected by pathogens), and pathogen damage appeared to be highest in the four largest populations (our personal observations). Analyses conducted with only completely healthy plants, however, resulted in qualitatively very similar results (not shown).

In summary, our results show that habitat fragmentation may influence selection on plant phenotypic traits. In our study system, selection for increased inflorescence size decreased with increasing population size and density in one of two years. This appeared not to be caused by differences in pollinator availability as initially hypothesized, but was likely related to differences in some other component of the abiotic or biotic environment. Further research is thus needed to understand what ultimately drives the observed spatial, and

temporal, variation in selection. Our findings are nonetheless interesting, as they highlight potential evolutionary consequences of habitat fragmentation and thus human-induced environmental change.

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CHAPTER 4

Population size, pollination and phenotypic trait selection in *Phyteuma spicatum*

Weber, A. & Kolb, A. (2013)
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Hand-pollination of Phyteuma spicatum*. Photograph by K. Barsch.*

Abstract

Plants in small populations may receive fewer visits, smaller pollen loads or pollen of poorer quality and suffer from reduced reproductive success compared to plants in larger populations. Consequently, pollen limitation of plants in small populations has been suggested to result in the evolution of reduced reliance on pollinators or the enhancement of traits that attract pollinators. The main aim of this study was to experimentally quantify the strength of pollinator-mediated selection on floral display size and flowering phenology in populations of varying size, using the self-incompatible, perennial herb *Phyteuma spicatum* as study species. We conducted supplementary hand pollinations in six populations (ranging in size between ca. 20–3000 flowering individuals) over two consecutive years and assessed selection gradients (i.e., trait-fitness relationships) in open- and hand-pollinated plants. Our results show that some populations are pollen limited in some years, but, contrary to our expectation, the degree of pollen limitation was not significantly related to population size. We found phenotypic selection for increased inflorescence size (in most populations and in both years), but we obtained no or no strong evidence that selection was pollinator-mediated or that the strength of selection was related to population size. This may have been the result of low statistical power, an inherent problem of studies examining effects of population size that require the inclusion of populations with only few individuals. In addition, given that selection appeared to be spatially and temporally variable, abiotic or biotic factors other than pollinators may have contributed to selection on inflorescence size.

Keywords: flowering phenology, habitat fragmentation, hand pollination, inflorescence size, pollen limitation

Introduction

The size of natural plant populations has been shown to affect interactions between plants and their mutualists and antagonists (Olesen & Jain 1994; Ehlers & Olesen 2003; Colling & Matthies 2004; Ghazoul 2005). In small populations, for example, plant-pollinator interactions may be disturbed (Ågren 1996; Kolb 2005, 2008), which may impact plant fitness and population viability (Kolb 2005; Lennartsson 2002; but see Kolb et al. 2010) and which also has been suggested to affect evolutionary processes (Jacquemyn et al. 2012). It is well known that pollinators are a driving force in the evolution of plant visual traits and flowering phenology (Ohara & Higashi 1994; Sandring & Ågren 2009). However, other biotic as well as abiotic factors may also affect trait evolution in plants (Dudley 1996; Totland 1999; Caruso et al. 2003; Gómez 2003; Sletvold & Grindeland 2008), and experimental manipulations are therefore needed to disentangle the effects of pollinators from those of other agents of selection (MacColl 2011).

Specifically, large plant populations are expected to be more attractive to pollinators because of a larger supply of pollen and nectar. Smaller and more isolated populations, in

turn, often experience a lower abundance and species richness of pollinators with altered foraging behaviours (Sih & Baltus 1987; Steffan-Dewenter & Tscharntke 1999; González-Varo et al. 2009). Plants in small populations may therefore receive fewer flower visits, smaller pollen loads or pollen of poorer quality compared to plants in larger populations (Jennersten 1988; Byers 1995; Steffan-Dewenter & Tscharntke 1999; Aguilar & Galetto 2004; Kolb 2005, 2008). Consequently, pollen limitation may increase and seed output may decrease with decreasing population size, which may ultimately result in the evolution of reduced reliance on pollinators or the enhancement of traits that attract pollinators (Haig & Westoby 1988; Ashman et al. 2004; Knight et al. 2005). However, to date only few studies investigated population size effects on pollinator-mediated selection (Sun et al. 2010; Weber & Kolb 2011), and, to the best of our knowledge, none have explored this experimentally.

In a previous study, we assessed selection gradients (i.e., trait-fitness relationships) in 16 natural populations of varying size of the perennial forest herb *Phyteuma spicatum* (Weber & Kolb 2011). We found that selection for increased inflorescence size decreased with increasing population size in one of two study years. That study, however, was purely observational and we could not link differences in selection gradients to differences in pollination intensity along the population size gradient. The main aim of the present study was therefore to experimentally quantify the strength of pollinator-mediated selection in populations of varying size. We conducted supplementary hand pollinations in six differently-sized *P. spicatum* populations over two consecutive years. Following the approach of Sletvold et al. (2010), we estimated the strength of pollinator-mediated selection by subtracting estimates of selection gradients for plants receiving supplemental hand pollination from estimates obtained for open-pollinated control plants.

Specifically, we examined whether 1) the degree of pollen limitation decreases with increasing population size, 2) selection on floral display size and flowering phenology can be attributed to interactions with pollinators, and 3) pollinator-mediated selection is stronger in small compared to large populations.

Materials and methods

Study system

The spiked rampion, *Phyteuma spicatum* L. (Campanulaceae), is an iteroparous, perennial herb occurring in Central and Atlantic Europe (Wheeler & Hutchings 2002). In our study area in north-western Germany, the species is relatively rare and restricted to fresh or moist, base-rich deciduous hardwood forests (Kolb 2005). *Phyteuma spicatum* produces annual rosettes of basal leaves and, when flowering, one (sometimes ≥ 2) inflorescence with about 20–100 flowers on an upright stalk of 10–70 cm (data based on individuals growing in our study area). Flowering usually takes place in May and June, with individual plants flowering between 5 and 15 days. The hermaphroditic, protandrous flowers lack a pedicel and are densely packed within each inflorescence. Flowers open sequentially from the bottom to the

top. During the male phase of a flower the pollen covers the style where it is available to insects. The flowers are mainly pollinated by bumblebees, especially by *Bombus pratorum* L. (Kolb 2008; Weber & Kolb 2013). Spontaneous autogamy and geitonogamy result in no or very few seeds, presumably due to gametophytic self-incompatibility (Huber 1988). Seed production per capsule and per plant range from 0 to 34 (mean ± SD: 6.6 ± 3.7, n = 432 individuals) and 0–1632 (210 ± 183, n = 432) in 2008, and from 0 to 24 (mean ± SD: 7.2 ± 4.0, n = 380 individuals) and 0–875 (233 ± 173, n = 380) in 2009, respectively. *Phyteuma spicatum* lacks aids to seed dispersal and most seeds land close to the parent plant (Wheeler & Hutchings 2002). Herbivory by roe deer, and to some extent by slugs, can be severe (Kolb 2008, 2012).

Data collection

Data were collected from May to July in 2008 and 2009 in six populations of *P. spicatum*. Population size varied between 31–2982 (2008) and 22–1718 (2009) flowering individuals (Table 1). To examine whether seed production was pollen limited and to assess the strength of pollinator-mediated selection, we conducted supplementary hand pollinations throughout the flowering period (end of May – mid June). We aimed for ca. 40 plants per population receiving supplemental pollen (hand-pollination treatment, HP) and for ca. 40−80 open-pollinated control plants (control, C). In the small populations fewer plants were available, and we therefore included all for study. Sample size in the hand-pollination treatment can be lower based on the expectation of reduced variation in seed production following supplemental pollination (Sletvold & Ågren 2010). Within each population, plants were randomly chosen and assigned to one of the two treatments. We only included plants with one inflorescence; in the study area, relatively few plants (≤ 15%) produce more than one inflorescence. Some plants were lost due to herbivore damage, reducing the number of plants available for analysis. In 2008 (values for 2009 are given in parentheses), a total of 432 (380) plants were available, with 177 (154) plants being assigned to the supplemental hand-pollination treatment and 255 (226) serving as open-pollinated controls (population-level sample sizes are given in Table 1).

Because all plants were included for study in the small populations, we used plants from a nearby large population as pollen donors for the hand pollinations. For comparability, we followed the same protocol in the large populations. A previous study showed that there was no difference in seed production between plants receiving supplemental pollen from the same population or from another large population (Kolb 2005). Pollen-covered styles were collected from the donor plants, stored in Eppendorf tubes and then wiped over the receptive stigmas of the experimental plants until the stigmas were visibly covered with pollen. Because the flowers open sequentially in the inflorescence from the bottom to the top, every plant was hand pollinated at least 3–6 times, approximately every 2–3 days, using different pollen

donors at each hand-pollination event. Individual flowers received supplemental pollen at least once.

During peak flowering (early June) we assigned each plant to one of 15 phenological states, from early (state 1) to late flowering (state 15). The 15 flowering states were assigned as follows: 1–2, all or most flowers already in fruit (thus, these plants flowered early in the season, before phenology measurements were taken), with no or only few receptive stigmas remaining at the top of the inflorescence, respectively; 3–12, open flowers present, with the proportion of open flowers in the inflorescence ranging from 100% to 10%; and 13–15, all flowers still in bud stage, with decreasing differentiation of the buds (thus, these plants flowered late in the season).

Shortly after flowering we measured the height of each inflorescence stalk (including the inflorescence), and the size of each inflorescence, i.e. the portion with flowers. Size was expressed as the length of the inflorescence and corresponded to the vertical measurement from the base of the inflorescence to its tip.

At the time of seed maturity (early to mid July), we collected the inflorescence of each plant. For each inflorescence, we determined the mean number of seeds per capsule (based on ten randomly chosen capsules), the total number of seed capsules as well as the total number of seeds per plant (calculated as the mean number of seeds per capsule × the total number of capsules).

Data analysis

To test whether the degree of pollen limitation differed among populations of varying size, we first calculated a standardized index of pollen limitation (*PLI*) for each population and then regressed *PLI* on log-transformed population size. Following Larson & Barrett (2000), *PLI* was calculated as $1 - (P_C/P_{HP})$, where P_C is the mean number of seeds produced per plant for open-pollinated controls and P_{HP} is the mean for plants that received supplemental hand pollination. $PLI = 0$ and 1 indicate no or complete pollen limitation, respectively.

To test whether selection can be attributed to interactions with pollinators, we first quantified selection within pollination treatments and populations by conducting standard selection gradient analyses (Lande & Arnold 1983), and then assessed differences in selection gradients among treatments by using analysis of covariance (ANCOVA).

In selection gradient analysis relative fitness (here, the total number of seeds per plant) is regressed on standardized trait values (here, inflorescence traits and flowering state). As inflorescence height and size were strongly positively correlated (Pearson correlation, r ranging from 0.59 to 0.80 among populations in 2008, and from 0.48 to 0.88 in 2009), we only included inflorescence size to avoid problems of collinearity. The size of an inflorescence is a measure for both flower number and duration of flowering (Weber & Kolb 2013) and might therefore be under stronger pollinator-mediated selection than inflorescence height. Within each population and treatment, absolute fitness was relativized to have a mean

of one, and the plant traits were standardized to have a mean of zero and a standard deviation of one. Models were fitted separately for each year, since some of the plants were included in both years and were therefore not independent from one another. We initially included quadratic terms in the models to also quantify non-linear selection. These were never statistically significant, and we therefore report results from analyses including only linear terms. Because of non-normality of the model residuals, we tested statistical significance of selection gradients (β's) by estimating 95% confidence intervals from 3000 bootstrap replicates using the R package "boot" (Canty & Ripley 2012). We present bias-corrected accelerated confidence intervals (BCa); the use of other methods yielded qualitatively very similar results.

To test whether selection gradients differed between the two pollination treatments, we used ANCOVA. The models included relative fitness as the dependent variable, and the standardized traits (inflorescence size and flowering state), pollination treatment (open-pollinated control *vs*. hand pollination) as well as all trait × treatment interactions as independent variables. Again, separate models were fitted for each population and year. A significant trait × treatment interaction in the ANCOVA would indicate that pollinators contribute to selection on that particular trait. To quantify the strength of pollinator-mediated selection, we subtracted for each trait the estimated selection gradient for plants receiving supplemental hand pollination (β_{HP}) from the estimate obtained for open-pollinated controls (β_C), $\Delta\beta_{poll} = \beta_C - \beta_{HP}$ (Sletvold et al. 2010). To examine whether the strength of pollinator-mediated selection was related to population size, we regressed $\Delta\beta_{poll}$ on log-transformed population size.

All analyses were performed using R 2.15.1 (R Development Core Team 2012).

Results

Degree of pollen limitation

The degree of pollen limitation differed among populations and years (Fig. 1), being relatively low in 2008 (*PLI* in all populations ≤ 0.11) and higher in 2009 in at least some of the populations (values up to 0.49). The degree of pollen limitation was not related to population size (2008: $P = 0.542$, 2009: $P = 0.628$).

Phenotypic selection

In all populations and in both years, we found linear selection for increased inflorescence size; selection gradients ranged between 0.350 and 1.014 in the open-pollinated controls and between 0.287 and 0.586 in the hand-pollinated plants [values refer to significant relationships (all except three), Table 1]. Pollinators did not significantly contribute to selection on inflorescence size; the inflorescence size × pollination treatment interactions of the ANCOVAs were never significant (Table 1). However, in most populations and years, selection on inflorescence size was weaker in the hand-pollination treatment than in the open-

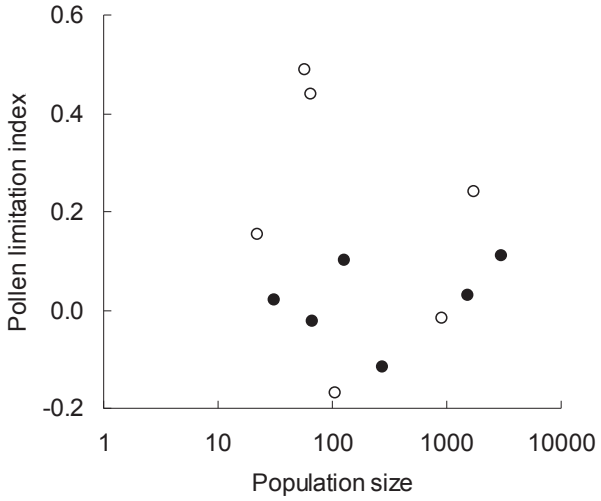

Fig. 1 Relationships between log-transformed population size and the standardized index of pollen limitation (see Methods) in six populations of *Phyteuma spicatum* in 2008 (closed symbols) and 2009 (open symbols).

Table 1 Linear selection gradients (± SE) for open-pollinated control plants (β_C) and for plants receiving supplemental hand pollination (β_{HP}) in six populations of *Phyteuma spicatum* in 2008 and 2009. $\Delta\beta_{poll}$ ($\beta_C - \beta_{HP}$) is the strength of pollinator-mediated selection. β_C, β_{HP} and $\Delta\beta_{poll}$ are in bold when 95% confidence intervals (CI, calculated from 3000 bootstrap replicates) do not include zero; for $\Delta\beta_{poll}$, CI refers to the trait × pollination treatment interaction of an ANCOVA examining effects of inflorescence size (Infl. size), flowering state (Flow. state) and pollination treatment on relative fitness. Population sizes (number of flowering individuals) and sample sizes for the control (C) and hand-pollination treatments (HP) are given in parentheses for each population and year. Flowering state was assessed in 15 states, from 1 (early flowering) to 15 (late).

Trait	2008			2009		
	β_C	β_{HP}	$\Delta\beta_{poll}$	β_C	β_{HP}	$\Delta\beta_{poll}$
Population 1 [2008: 31 (C: 17, HP: 13), 2009: 22 (C: 9, HP: 9)]						
Infl. size	**1.014**±0.418	0.293±0.151	0.720	**0.679**±0.305	0.318±0.390	0.362
Flow. state	0.020±0.225	–0.182±0.230	–0.202	–0.103±0.209	–0.265±0.316	–0.162
Population 2 [2008: 68 (C: 20, HP: 18), 2009: 66 (C: 11, HP: 9)]						
Infl. size	**0.462**±0.201	**0.287**±0.111	0.176	**0.350**±0.259	0.187±0.181	0.163
Flow. state	–0.102±0.123	–0.136±0.101	–0.034	0.107±0.187	0.181±0.224	0.074
Population 3 [2008: 129 (C: 42, HP: 37), 2009: 57 (C: 26, HP: 23)]						
Infl. size	**0.385**±0.072	**0.439**±0.135	–0.054	**0.467**±0.170	**0.470**±0.082	–0.003
Flow. state	–0.0003±0.065	–0.088±0.085	–0.087	–0.155±0.110	–0.081±0.136	0.074
Population 4 [2008: 273 (C: 55, HP: 36), 2009: 106 (C: 44, HP: 34)]						
Infl. size	**0.735**±0.185	**0.586**±0.134	0.149	**0.583**±0.067	**0.311**±0.162	0.272
Flow. state	0.024±0.064	–0.032±0.132	–0.056	–0.001±0.064	–0.105±0.089	–0.104
Population 5 [2008: 1524 (C: 66, HP: 38), 2009: 914 (C: 75, HP: 40)]						
Infl. size	**0.406**±0.074	**0.499**±0.057	–0.093	**0.600**±0.074	**0.412**±0.071	0.189
Flow. state	0.032±0.052	–0.056±0.079	–0.088	–0.023±0.041	0.085±0.092	0.108
Population 6 [2008: 2982 (C: 55, HP: 35), 2009: 1718 (C: 61, HP: 39)]						
Infl. size	**0.564**±0.052	**0.423**±0.074	0.141	**0.568**±0.072	**0.341**±0.112	0.227
Flow. state	0.079±0.047	–0.026±0.057	–0.105	–0.045±0.067	–0.146±0.078	–0.101

pollinated controls (Table 1), suggesting that selection may have at least partly been driven by pollinators. Furthermore, in two small populations, estimates of selection gradients for inflorescence size were significantly different from zero only in the open-pollinated controls (population 1 in 2008 and 2009, population 2 in 2009), with pollinator-mediated selection ($\Delta\beta_{poll}$) ranging from 0.163 to 0.720 (Table 1, Fig. 2). Population size had no significant effect on $\Delta\beta_{poll}$ (2008: $P = 0.207$, 2009: $P = 0.909$). There was no statistically significant selection on flowering state in any of the populations in 2008 or 2009 (Table 1).

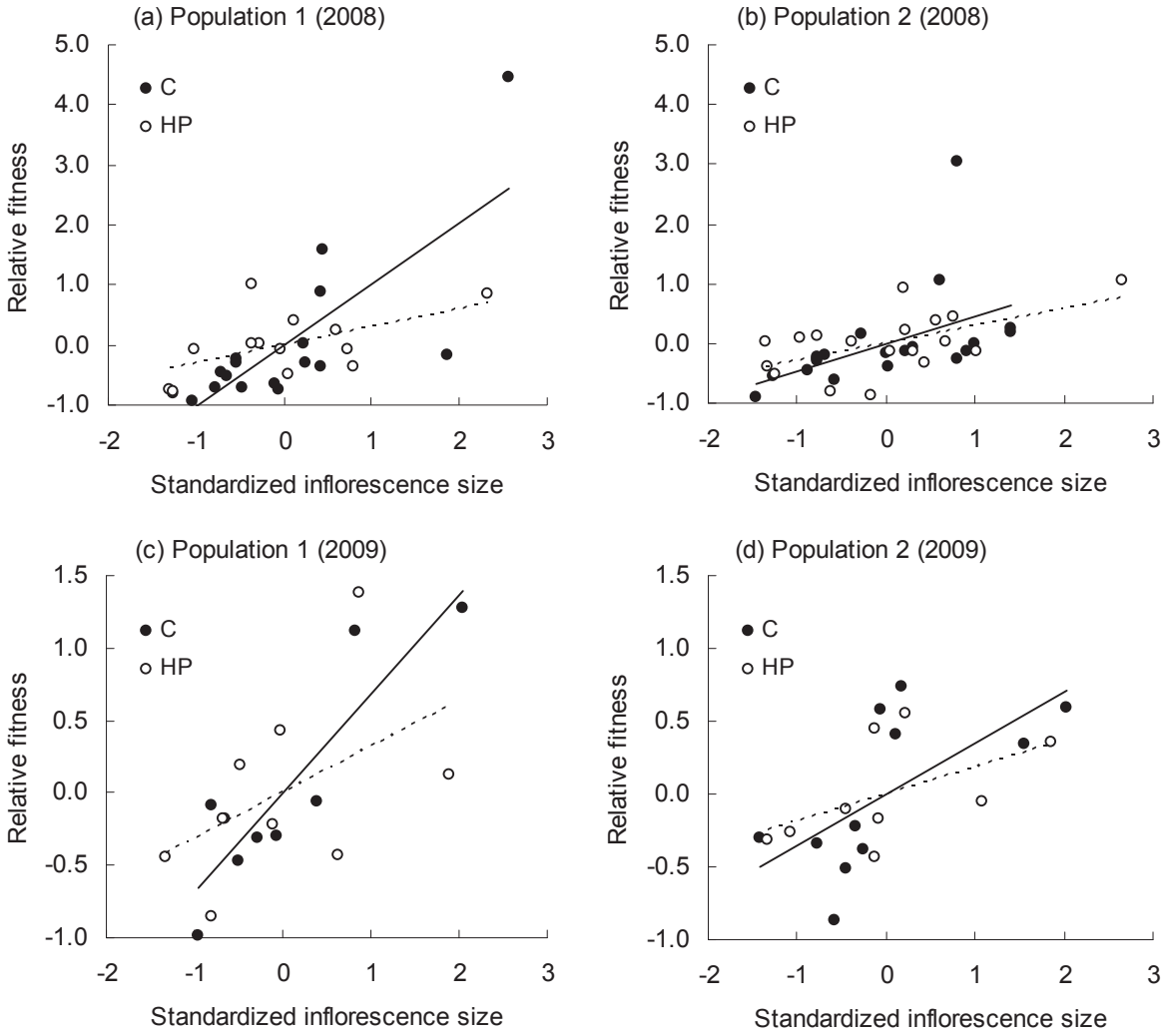

Fig. 2 Standardized linear phenotypic selection gradients for inflorescence size in open-pollinated control plants (C, closed symbols, solid line) and in plants receiving supplemental hand pollination (HP, open symbols, dashed line) in population 1 (a, c), and population 2 (b, d) in 2008 and 2009. The selection gradients are illustrated with added-variable plots, in which the residuals from a linear regression model of relative fitness on standardized flowering state are plotted against the residuals from a regression model of standardized inflorescence size on standardized flowering state.

Discussion

Our results show for the perennial herb *P. spicatum* that some populations are pollen limited in some years, but, in contrast to previous work with this species (Kolb 2005), the degree of pollen limitation was not significantly related to population size. The fact that the degree of pollen limitation differed considerably between the two study years in most populations and also in comparison to some of the levels observed in Kolb (2005; study conducted in 2003 in partly the same populations, with e.g. *PLI* = 0.38 in population 4 compared to –0.12 and –0.17 in the present study), suggests strong temporal variation in pollinator abundance and potentially also in other environmental factors. This is in line with the results of other studies (Alexandersson & Ågren 1996; Goodwillie 2001; Vanhoenacker et al. 2006). Goodwillie (2001), for example, found temporal variation in the magnitude of pollen limitation in the self-incompatible, annual herb *Linanthus parviflorus*, and suggested that this may have been caused by variation in both pollinator abundance and resource availability. In our case, weather conditions during peak flowering of *P. spicatum* differed considerably between the two study years. In 2008 (the year with overall lower *PLI* values), the weather was dryer and warmer compared to 2009, conditions which are considered beneficial for pollinators (Lundberg 1980). Correspondingly, Fernández et al. (2012) found that pollen limitation intensity was positively related to annual rainfall in *Erysimum popovii*. Furthermore, the number of flowering individuals of *P. spicatum* was up to twice as high, or even higher, in 2008 (compare population size estimates between 2008 and 2009 in Table 1), which too may have led to the attraction of more pollinators, increasing pollen availability and causing pollen not to be limiting in this year. The degree of pollen limitation was also variable among populations, especially in 2009. Such spatial differences in the degree of pollen limitation appear to be common, often being linked to differences in the pollinator fauna or in other environmental factors (Goméz et al. 2010; Fernández et al. 2012). While we lack data on overall environmental conditions, we can largely rule out that differences in the pollinator community caused differences in the magnitude of pollen limitation. As mentioned above, *P. spicatum* is mainly pollinated by *Bombus pratorum* in our study area (Kolb 2008; Weber & Kolb 2013).

While there was no phenotypic selection on flowering state, we found phenotypic selection for inflorescence size in all populations and in both years; relative fitness was higher in plants with larger inflorescences than in plants with a smaller floral display. This corresponds to results of earlier studies with this (Weber & Kolb 2011, 2013) and other species (Sandring & Ågren 2009; Cuartas-Domínguez & Medel 2010; Sletvold et al. 2010). Large inflorescences may be more attractive to pollinators and selection on floral display size has therefore often been suggested to be mediated by interactions with pollinators (Ohara & Higashi 1994; Gómez 2003). In our study, however, pollinators did not significantly contribute to selection on inflorescence size; the inflorescence size × pollination treatment interaction was never significant. Yet, results suggest that selection for an increased

inflorescence size may have at least partly been driven by pollinators. Supplemental hand pollination reduced the slope of the selection gradients of inflorescence size in most populations and years, suggesting that pollinators contributed to the observed selection on inflorescence size. Furthermore, in the two smallest populations, selection gradients were only significant in the open-pollinated controls, and not in the hand-pollinated plants. Low sample sizes, which are inherent to studies examining effects of population size and thus also including populations with only few individuals, may have precluded the detection of significant inflorescence size × pollination treatment interactions in this study. In addition, other selective agents may have been at play (see e.g., Strauss & Whittall 2006), and the positive relationship between inflorescence size and seed production may have at least partly been due to that plants with more flowers are likely to produce more seeds than plants with fewer flowers.

Furthermore, our data show that the overall degree of pollen limitation in a population must not necessarily be linked to the strength of selection in that population. For example, the degree of pollen limitation was relatively high in population 3 in 2009, but the strength of selection on inflorescence size was similar in both pollination treatments. Similarly, Andersson (1996) found little evidence for pollinator-mediated selection in *Saxifraga granulata* despite pollen limitation. It needs to be kept in mind that the degree of pollen limitation is an average estimate for a population and that individual plants may or may not experience the same pollen limitation. Only when the degree of pollen limitation varies with trait expression, pollinator-mediated selection will take place. Furthermore, pollen limitation could be related to traits other than inflorescence size or flowering phenology.

Contrary to our expectations, we did not find significant population size effects on the magnitude of pollen limitation or on the strength of selection in *P. spicatum*, nor did pollinators significantly contribute to trait selection. Still, both the degree of pollen limitation and the strength of selection on inflorescence size were spatially and temporally variable and supplemental hand pollination reduced the strength of selection on inflorescence size in most populations and years, the latter suggesting that selection may have at least partly been driven by pollinators. While low sample sizes may have precluded the detection of significant effects, abiotic or biotic factors other than pollinators may too have contributed to selection on inflorescence size (see also Weber & Kolb 2011). Studies incorporating multiple possible selective agents would provide more conclusive evidence for such effects.

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CHAPTER 5

Local plant density, pollination and trait-fitness relationships in a perennial herb

Weber, A. & Kolb, A. (2013)
Plant Biology 15: 335–343.

Bombus pratorum *pollinating a large inflorescence of* Phyteuma spicatum*. Photograph by A. Weber*.

Abstract

Both differences in local plant density and phenotypic traits may affect pollination and plant reproduction, but little is known about how density affects trait-fitness relationships via changes in pollinator activity. In this study we examined how plant density and traits interact to determine pollinator behaviour and female reproductive success in the self-incompatible, perennial herb *Phyteuma spicatum*. Specifically, we hypothesized that limited pollination service in more isolated plants would lead to increased selection for traits that attract pollinators. We conducted pollinator observations and assessed trait-fitness relationships in a natural population, whose individuals were surrounded by a variable number of inflorescences. Both local plant density and plant phenotypic traits affected pollinator foraging behaviour. At low densities, pollinator visitation rates were low, but increased with increasing inflorescence size, while this relationship disappeared at high densities, where visitation rates were higher. Plant fitness, in terms of seed production per plant and per capsule, was related to both floral display size and flowering time. Seed production increased with increasing inflorescence size and was highest at peak flowering. However, trait-fitness relationships were not density-dependent, and differences in seed production did not appear to be related to differences in pollination. The reasons for this remain unclear, and additional studies are needed to fully understand and explain the observed patterns.

Keywords: flowering phenology, inflorescence size, *Phyteuma spicatum*, pollinator behaviour, reproductive success, spatial variation

Introduction

The spacing of individuals can vary greatly within natural plant populations. Such differences in local density may affect ecological processes such as mutualistic and antagonistic interactions. For example, pollination, seed dispersal, mycorrhizal symbiosis, herbivory, seed predation, pathogen infection or competition among plants all have been shown to be influenced by plant density (Levin & Kerster 1969; Jennersten et al. 1983; Brody 1992; Mustajärvi et al. 2001; Schroeder & Janos 2004; Ghazoul 2005 and references therein; Carlo & Morales 2008; Sletvold & Grindeland 2008). By influencing the abundance of biotic agents, or by changing their behaviour, local plant density often determines plant reproductive success and plays a vital role in the selection of plant phenotypic traits (e.g., Kunin 1993; Steven et al. 2003; Sletvold & Grindeland 2008).

Interactions with pollinators are especially important in plant species that depend on animals for successful reproduction (Richards 1997). As predicted by optimal foraging theory (Pyke et al. 1977), the availability of floral resources may strongly influence the behaviour of pollinators (Kunin 1993). To maximize their rate of net energy intake, pollinators tend to fly short distances relative to the plant spacing distances encountered, thus flying predominantly between nearest neighbour plants and avoiding energetically expensive long-distance flights

(Zimmerman 1981). Plants surrounded by many flowering conspecifics may therefore be more attractive to pollinators than plants growing with few conspecific neighbours because pollinators require less energy and time to move between flowers (Klinkhamer & de Jong 1990; Field et al. 2005). Accordingly, pollination intensity and plant reproductive success often increase with increasing plant density (Kunin 1997; Ghazoul 2005).

Pollinator visitation differences not only arise from differences in local plant density, but are to a large extent also determined by individual plant characteristics. Flower visiting animals often prefer certain plant and floral phenotypes to others, thereby acting as selective agents (Alexandersson & Johnson 2002; Sandring & Ågren 2009). For example, pollinators have been shown to visit plants with a larger floral display more often than plants with a less showy display, which may increase plant reproductive success (Ohara & Higashi 1994). Also flowering time may be important for pollination and subsequent seed production and has therefore been suggested to be of adaptive value (Gross & Werner 1983; Elzinga et al. 2007; Sandring & Ågren 2009).

Variation in both plant traits and plant spacing may thus influence plant reproduction by affecting interactions with pollinators (Grindeland et al. 2005; Makino et al. 2007; Jacquemyn & Brys 2010). Little, however, is known about how local plant density affects trait-fitness relationships via changes in pollinator activity (but see Schmitt 1983 and Aspi et al. 2003). Isolated plants that experience pollen limitation may have a lower fitness than less isolated plants that experience less pollen limitation. Ultimately, this could thus result in directional selection for a more conspicuous floral display in more isolated plants. Klinkhamer et al. (1989), for example, found that locally isolated plants attracted more pollinators when having more flowers, which increased the absolute number of flowers pollinated. Plants flowering off-peak, i.e. early or late in the season, may have fewer mates and therefore less available pollen in their environment than at peak flowering (Elzinga et al. 2007), an effect that may be aggravated when growing with few conspecific neighbours. Selection for an intermediate flowering time could thus also be stronger in more isolated plants. The strength of selection on floral display size and flowering time may therefore differ among plants growing at different densities because of variation in the abundance of pollinators and changes in their foraging behaviour.

The main aim of this study was therefore to examine how local plant neighbourhood and plant phenotypic traits interact to determine pollinator behaviour as well as plant reproductive success, using the self-incompatible, perennial herb *Phyteuma spicatum* as study species. We hypothesized that isolated plants are less attractive to pollinators than plants with many conspecific neighbours and, as a result, are more likely to be pollen limited. We therefore expected trait-fitness relationships to be stronger in more isolated plants than in plants growing with many conspecific neighbours. In a previous study with *P. spicatum*, we compared selection gradients (i.e., trait-fitness relationships) between populations and found that the strength of selection on both inflorescence size and flowering phenology varied with

population size and mean population density, although we have no evidence that this was linked to differences in pollination intensity (Weber & Kolb 2011). While that study solely focused on among-population differences, we here investigated effects at a much more local scale, i.e. within a population that showed strong variation in the spacing of individual plants. One goal of this new study was to examine whether patterns observed at the population level are similar to those detected at a more local scale. In contrast to our previous study, in which we used the level of seed production as a measure of pollination intensity, we now conducted actual pollinator observations and can therefore directly link potential differences in trait-fitness relationships to differences in pollination intensity.

Our specific objectives were 1) to examine pollinator response to differences in plant phenotypic traits and in local plant density, 2) to test if plant phenotypic traits influence fitness (in terms of seed production per capsule and per plant) and if trait-fitness relationships vary with density, and 3) to examine the direct effects of traits and density on seed production as well as their indirect effects via changes in pollination.

Material and Methods

Study system

Phyteuma spicatum L. (Campanulaceae; Fig. 1) is an iteroparous, perennial hemicryptophyte occurring in Central and Atlantic Europe (Wheeler & Hutchings 2002). In our study area, situated between the cities of Bremen and Hamburg in north-western Germany, the species is relatively rare and restricted to fresh or moist, base-rich deciduous hardwood forests (Kolb 2005). *Phyteuma spicatum* produces annual rosettes of basal leaves and, when flowering, one to several inflorescences on upright stalks of *ca*. 10–70 cm (Fig. 1a). Flowering usually takes place in May and June, with individual plants flowering between 5 to 15 days. The hermaphroditic, protandrous flowers (*ca*. 20–100 per inflorescence) are sessile (i.e., lacking a pedicel) and densely packed within each inflorescence, and open sequentially from the bottom

Fig. 1 *Phyteuma spicatum*, left: in a natural population within the study area (labels indicate the traits examined in this study), right: with its main pollinator *Bombus pratorum*. Photographs by D. Kolb (left) and A. Weber (right).

to the top. The flowers are mainly pollinated by bumblebees (Kolb 2008 and this study) (Fig. 1b). Spontaneous autogamy and geitonogamy result in no or very few seeds, presumably due to gametophytic self-incompatibility (Huber 1988). Seed production per capsule and per plant range from 0–22 (mean ± SD: 7.1 ± 3.7) and 0–1860 (255 ± 224), respectively (data from this study, n = 344 individuals). *Phyteuma spicatum* lacks aids to seed dispersal and most seeds land close to the parent plant (Wheeler & Hutchings 2002). Herbivory can be severe (Kolb 2008); roe deer may remove entire inflorescences, while slugs and snails usually cause less damage.

Data collection

Data were collected from May to July in 2010 in a natural population of *P. spicatum* located in a forest with about 85–90% canopy cover. The population was selected because it contained a sufficient number of flowering individuals (2328 of a total of *ca*. 5000 adult individuals that were patchily distributed over an area of *ca*. 6 ha), showed variation in local plant density and was easily accessible. We randomly marked 359 flowering plants, and tagged each plant with a small flag stuck into the ground. To avoid excessive damage by deer, we protected all marked individuals with fences or deer repellent (ARBIN Wildabweiser, Dr. Stähler). Pollinators moved freely between protected and non-protected plants, and pollinator visitation rates did not differ between fenced individuals and those protected by the repellent ($P > 0.1$). Still, some of the plants were later damaged by herbivores (mainly by slugs), giving 344 plants for analysis. For each plant we determined the local conspecific plant density by counting the total number of inflorescences of *P. spicatum* within a radius of 5 m (excluding the focal plant), and expressed density as inflorescence number per 78.5 m² (hereafter referred to as "total" plant density). The number of inflorescences per 78.5 m² ranged from 0–94 (mean 27.1 ± SD 19.6) and the distance between marked plants and their nearest flowering neighbour ranged from 3 cm to 34 m (mean 145.2 cm ± SD 465.4).

During peak flowering (mid June), we determined the number of inflorescences of each plant and assessed its time of flowering by assigning it to a phenological state based on the proportion (estimated in 10% classes, from 0 to 100%) of already wilted flowers, open flowers and flowers still in the bud stage. Plants were then sorted according to the percentage of wilted flowers, open flowers and buds (in this order), which resulted in a total of 59 different states, ranging from late (state 1, all flowers still in bud) to early flowering (state 59, all flowers already in fruit). Shortly after flowering we measured the width of the broadest leaf, the height of each inflorescence stalk (including the inflorescence) and the size of each inflorescence, i.e. the portion with flowers (Fig. 1a). Size was expressed as the length of the inflorescence and corresponded to the vertical measurement from the base of the inflorescence to its tip (hereafter referred to as "total" inflorescence size). For plants with more than one inflorescence (13.9% of all plants), we calculated the mean inflorescence height and size.

At the time of seed maturity (early to mid July), we collected all inflorescences of each plant included in our study. For each inflorescence, we determined the mean number of seeds per capsule (means being based on ten randomly chosen capsules), the total number of seed capsules as well as the total number of seeds (calculated as the mean number of seeds per capsule × the total number of capsules). Total seed production per plant was calculated as the sum of the total number of seeds produced by each inflorescence.

To examine whether plant traits and local plant density affected pollinator behaviour, we conducted pollinator observations during 9 – 23 June 2010. Because we had limited time and resources, we were only able to observe pollinators on a subset of the marked plants (n = 143). Plants were growing in a range of different local densities (1–69 inflorescences per 78.5 m²). Individual plants were observed at a similar phenological state, i.e. when many or all flowers were already open, some still in the bud stage and none or only few wilted. Observations were made between 10.00 and 18.00 on sunny, although sometimes partly cloudy and/or windy days with at least 16°C. Plants of different conspecific densities were observed simultaneously by A. Weber and A. Kolb or a field assistant. Each plant was observed twice for one hour during different times of the same day to account for daytime changes in pollinator behaviour, resulting in a total of 286 observation hours. To avoid observational bias, observers were rotated among different densities and between the two observation hours. We recorded the number of inflorescences with at least one open flower in a 5 m radius around each plant at the time of the observations and used this as a measure of "current" plant density. We also measured the "active" inflorescence size of each observed plant, which was defined as the vertical length of the portion of the inflorescence with open flowers (Fig. 1a). During each observation period (2 × 1 hour), we recorded for each plant (1) the total number of visits (bumblebees and other insect visitors), and (2) the time each visitor spent on the plant. Because the flowers of *P. spicatum* are relatively small and densely packed within each inflorescence, it is very difficult if not impossible to observe insect visits to individual flowers. Therefore, we collected data only at the plant level. Visitors that contacted flowers of the focal plant were considered as pollinators. Visitation times were used to calculate both the total time (summed across all visitors) and the average time (total time divided by the number of visitors) visitors spent foraging on the focal plant during the two hours (hereafter referred to as total and average bout length).

Data analysis

We used generalized linear models to test for effects of inflorescence height and active inflorescence size as well as current plant density on visitation rate, i.e. the number of pollinator visits per plant and observation period (Poisson error distribution, log link function) as well as on total and average bout lengths per plant (Gamma error distribution, inverse link function). Inflorescence height and active inflorescence size were positively, but not very strongly correlated (r = 0.49, P <0.001, n = 143). Interaction terms (i.e., between

inflorescence height or size and density) were only retained in the models when significant ($P < 0.05$). As bumblebees were the most important pollinators (91.4% of all visitors; see also Results), we only included bumblebees in our analyses. Results for bumblebees and all other visitors combined were qualitatively similar (not shown). Analyses were conducted using the "glm" function as well as the "Anova" function of the "car" package for likelihood-ratio χ^2-tests in R 2.13.1 (R Development Core Team 2011).

To test for linear and non-linear effects of inflorescence traits and flowering time on seed production, we conducted multiple regression analyses similar to standard selection gradient analyses by regressing relative fitness on standardized trait values (Lande & Arnold 1983). By including either linear or both linear and quadratic terms, one can draw conclusions about the nature of the relationship between a specific trait and fitness (and thus about the potential strength and direction of linear and/or non-linear phenotypic selection). By including density as well as all trait × density interactions in the models, one can test whether trait-fitness relationships vary with density. We thus interpret regression coefficients for density as environmental covariates (e.g., Mitchell-Olds & Shaw 1987), while the effects of (potentially evolvable) traits are interpreted in the presence of density in the model.

As total inflorescence size and inflorescence height were strongly positively correlated (Pearson correlation, $r = 0.82$, $P < 0.001$, $n = 344$), we restricted the analyses to inflorescence size to avoid problems of collinearity. The focus on total inflorescence size was based on our finding that pollinator response to differences in active inflorescence size depended on local plant density, while the response to differences in inflorescence height did not (see Results). Total inflorescence size and active inflorescence size were positively correlated ($r = 0.66$, $P < 0.001$, $n = 143$). Furthermore, the size of an inflorescence is a measure of both flower number (as reflected in positive correlations between total inflorescence size and capsule number, $r = 0.87$, $P < 0.001$, $n = 344$) and duration of flowering (as tested during a different study in the same population in 2011: $r = 0.48$, $P < 0.001$, $n = 305$). Total inflorescence size and flowering time were only weakly ($r = 0.12$, $P = 0.029$, $n = 344$) correlated. We used the mean number of seeds per capsule as well as the total number of seeds per plant as estimates for female fitness. While total seed production per plant more adequately estimates fitness in terms of the number of offspring produced, it is also likely to be closely related to inflorescence size (i.e., plants with larger inflorescences and thus more flowers may produce more seeds than plants with smaller inflorescences and fewer flowers). We therefore used seed production per capsule, which is likely to be more independent of inflorescence size, as an additional fitness estimate. As in selection gradient analyses, fitness was relativized to have a mean of one [by dividing the number of seeds per capsule (or per plant) by the mean number of seeds per capsule (or per plant)], and the plant traits were standardized to have a mean of zero and a standard deviation of one (by subtracting the mean and dividing by the standard deviation).

To estimate linear and non-linear regression coefficients, we run two sets of models. Linear regression coefficients were quantified with models that included linear terms only,

whereas quadratic regression coefficients were quantified with models that included both linear and non-linear terms. Thus, we either included only standardized total inflorescence size and flowering time as predictor variables or both total inflorescence size and flowering time as well as their quadratic terms (i.e., inflorescence size^2 and flowering time^2). In all cases, relative fitness (either based on the number of seeds per capsule or on the number of seeds per plant) was included as the response variable. To test whether trait-fitness relationships varied with density, we included total local plant density as main effect as well as all trait × density interactions in our models. Density was not correlated to inflorescence size ($r = –0.06$; $P = 0.280$; $n = 344$) or flowering time ($r = –0.04$; $P = 0.509$; $n = 344$).

We included the width of the broadest leaf in our analyses to account for differences in resource state among individuals and thus to reduce problems associated with environmental covariance (Scheiner et al. 2002), i.e. that correlations between traits and fitness were not causal but due to growing conditions influencing both fitness and trait expression in individuals. Leaf width is a suitable measure for overall plant size in this species, and is positively correlated to inflorescence height ($r = 0.42$, $P < 0.001$, $n = 344$) and size ($r = 0.43$, $P < 0.001$, $n = 344$). It also explains differences in growth, survival and flowering better than alternative size measures (Kolb et al. 2010). Furthermore, we have some indication that leaf width reflects differences in local environmental conditions; across 24 sites, mean leaf width of flowering individuals significantly decreased with increasing C:N ratios ($r = –0.60$, $P = 0.002$; A. Kolb, unpublished data). As *P. spicatum* is an iteroparous perennial, results could theoretically also be a function of the relationship between plant size and plant age. Plant size (in terms of inflorescence height, total inflorescence size and width of the largest leaf), however, is not related to plant age in *P. spicatum* (tested across 70 individuals that flowered in at least three years during 2006–2010 in the same population; Kolb et al., 2010 and A. Kolb, unpublished data).

Starting with full models, non-significant interactions were removed from the models in a backward stepwise procedure. The analyses were conducted using the "lm" function (R Development Core Team 2011). Because of non-normality of the model residuals, we tested statistical significance of regression coefficients by estimating 95% confidence intervals from 3000 bootstrap replicates using the R package "boot". We present bias-corrected accelerated confidence intervals (BCa), which, according to Crawley (2007), is the interval preferred by statisticians (the use of other methods yielded qualitatively very similar results).

One potential drawback of the analyses described above is that they were not performed on the same group of plant individuals (i.e., while trait-fitness relationships were assessed using the complete set of plants, pollinator observations were only carried out in a subset of plants). We therefore selected all individuals for which we had a complete set of data ($n = 134$) and conducted a path analysis to examine the direct and indirect relationships between standardized traits (inflorescence height, active inflorescence size and leaf width), current local plant density, pollinator visitation rates and relative fitness. This is a powerful analytical

approach and has been used in similar contexts (e.g., Cariveau et al. 2004). The full, conceptual model is shown in Fig. 4a and was developed based on findings of our own and other studies (e.g., Cariveau et al. 2004; Grindeland et al. 2005; Weber & Kolb 2011). Given that larger plants may produce more seeds, inflorescence size, inflorescence height and leaf width directly influence seed production. Inflorescence traits may also have an indirect effect on reproduction via changes in pollinator visitation rates. Plant density affects both plant size and seed production via intraspecific competitive effects and it also influences pollinator visitation rates, which in turn affect seed production. Finally, inflorescence traits and leaf width are intercorrelated due to genetic and/or environmental conditions. We identified possible paths using the "Build" module implemented in the software TETRAD III (Scheines et al. 2011). The significance level for the inclusion of paths was set to $P < 0.1$ (recommended for samples sizes between 100 and 300), and we assumed causal sufficiency, i.e. did not fit latent variables. The software uses prior background knowledge and covariation of supplied variables to determine conditional independence of these variables and outputs a set of causal relationships (for statistical tests and algorithms used by the software see Scheines et al. 2011). Given the conceptual model shown in Fig. 4a, we thus assigned variables to different tiers, where variables in higher tiers could not be causes of those in lower tiers, and also specified a number of forbidden links. Two path models were identified, the first included seed production per plant as fitness estimate, the second seed production per capsule. The degree of fit between the observed and expected covariance structure of each model was tested using the functions "specify.model" and "sem" in the R package "sem", which fits models by the method of maximum likelihood (R Development Core Team 2011). A non-significant goodness-of-fit chi-square indicates that the model provides a reasonable fit to the data. Standardized path coefficients were calculated using the function "std.coef".

Results

Pollinator behaviour and local plant density

In total, i.e. during the 286 observation hours, we counted 428 visitors, of which 91.4% were bumblebees [mainly *Bombus pratorum* L. (Fig. 1b); Hymenoptera]. The remaining flower visitors were hoverflies and very few other non-identified insect species. Both current plant density and plant phenotypic traits affected pollinator visitation rates per plant (Table 1). Visitation rates increased with density (Fig. 2) and inflorescence height (Table 1). The effect of active inflorescence size varied with local plant density; the active inflorescence size × density interaction was significant (Table 1).

At low densities (< 10 inflorescences per 78.5 m²), visitation rates were overall low, but increased with increasing active inflorescence size (Fig. 2). For example, at a density of 1 (corresponding to about 3 inflorescences per 78.5 m²), plants with an inflorescence size of 2 and 4 cm received about 1 and 2 visits per observation period, respectively. This relationship disappeared at high densities, where visitation rates were generally higher. At a density of

Table 1 Effects of inflorescence height, active inflorescence size and current plant density [log-transformed (x+1)] on the total number of visits per plant and observation period in a natural population of *Phyteuma spicatum* (*n* = 143 plants). Results are from a generalized linear model (Poisson errors, log link function, type II SS). Values of *P* are in bold if < 0.05. The height × density interaction was not significant and therefore removed from the model. Est. = parameter estimate.

Source of variation	χ^2	df	*P*	est.
Inflorescence height	4.15	1	**0.042**	0.012
Active inflorescence size	0.63	1	0.429	0.542
Current plant density	135.73	1	**<0.001**	1.045
Active infl. size × current plant density	10.42	1	**0.001**	–0.201

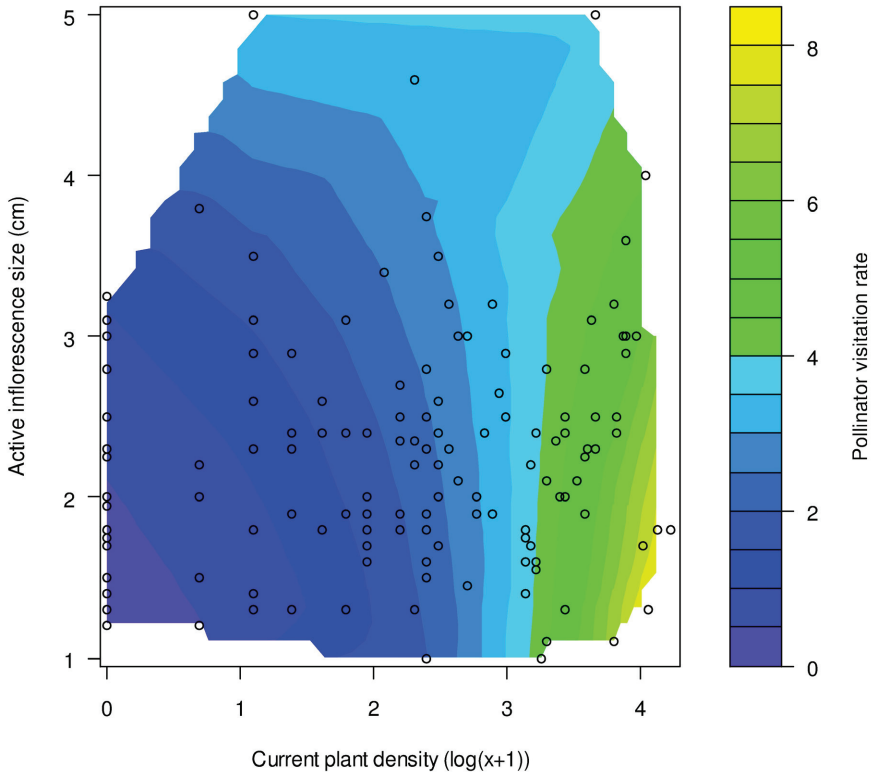

Fig. 2 Visitation rates per plant and observation period (2 h) in relation to active inflorescence size and current plant density in a natural population of *Phyteuma spicatum* (*n* = 143 plants). Fitted values from a generalized linear model (Poisson errors, log link function) are shown, with values ranging from 0 (dark blue) to >8 visits per observation period (yellow). The figure was generated with the "interp" function of the R package "akima" (linear interpolation) and with the "filled.contour" function contained in "graphics" (R Development Core Team 2011). The latter function was modified to show also the original data points for active inflorescence size and density (open circles).

3 (about 20 inflorescences) plants were visited about 3 times, irrespective of their inflorescence size. Both the total bout length and the average bout length were positively affected by inflorescence height, but not related to active inflorescence size (Table 2). Local plant density positively affected total bout length, whereas average bout length decreased with increasing plant density (Table 2).

Table 2 Effects of inflorescence height, active inflorescence size and current plant density [log-transformed (x+1)] on a) total and b) average bout length per plant and observation period in a natural population of *Phyteuma spicatum* (*n* = 104 plants; only plants with at least one visit were included).

	a) Total bout length				b) Avg. bout length			
Source of variation	χ^2	df	*P*	est.	χ^2	df	*P*	est.
Inflorescence height	5.38	1	**0.020**	–0.0003	11.89	1	**0.001**	–0.001
Active inflorescence size	0.33	1	0.566	0.001	0.51	1	0.474	0.003
Current plant density	8.27	1	**0.004**	–0.004	4.13	1	**0.042**	0.006

Results are from a generalized linear model (Gamma errors, inverse link function, type II SS). Values of *P* are in bold if < 0.05. All trait × density interactions were not significant and therefore removed from the model. Negative parameter estimates (est.) correspond to a positive relationship between inflorescence height or density and bout length due to the inverse link function used in the model.

Table 3 Effects of standardized traits on relative fitness in terms of the number of seeds per capsule and the total number of seeds per plant. Parameter estimates from models including only linear (a) and both linear and quadratic terms (b) are shown (with 95% confidence intervals, CI, calculated from 3000 bootstrap replicates). Estimates with 95% CI that do not include zero are in bold. Local plant density was log-transformed for analysis (x+1). Leaf width was included in the models to account for differences in individual resource state. All trait × density interactions were non-significant and therefore removed from the models.

Source of variation	Seeds per capsule		Seeds per plant	
a)				
Total inflorescence size	**0.184**	(0.121, 0.256)	**0.580**	(0.463, 0.762)
Flowering time	**–0.084**	(–0.131, –0.040)	**–0.067**	(–0.143, –0.005)
Total plant density	0.029	(–0.027, 0.080)	0.050	(–0.008, 0.107)
Leaf width	**0.069**	(0.009, 0.126)	**0.105**	(0.022, 0.185)
b)				
Total inflorescence size	**0.146**	(0.073, 0.216)	**0.443**	(0.357, 0.528)
Total inflorescence size^2	0.027	(–0.013, 0.068)	**0.105**	(0.025, 0.195)
Flowering time	–0.021	(–0.087, 0.047)	0.011	(–0.077, 0.083)
Flowering time^2	–**0.107**	(–0.194, –0.023)	–**0.107**	(–0.200, –0.010)
Total plant density	0.039	(–0.016, 0.088)	**0.063**	(0.003, 0.118)
Leaf width	**0.077**	(0.015, 0.138)	**0.126**	(0.054, 0.198)

Trait-fitness relationships and local plant density

Plants with a larger floral display had a higher fitness (Table 3). The number of seeds per capsule increased linearly with increasing inflorescence size (Table 3a; Fig. 3a), while the number of seeds per plant increased non-linearly with increasing floral display size (positive quadratic term, Table 3b; Fig. 3c). We also found significant non-linear effects of flowering time on seed production (Table 3b). Visual inspection of the relationships between

standardized flowering time and relative fitness, however, revealed no strong patterns (Figs. 3b, d). Plants with intermediate flowering times appeared to have a slightly higher fitness compared to plants flowering early or late. Local plant density positively affected the number of seeds per plant only in the model including quadratic terms, and had no effect on seed number per capsule (Table 3). We found no evidence for trait-fitness relationships to be density-dependent; all trait × density interactions were not significant (Table 3).

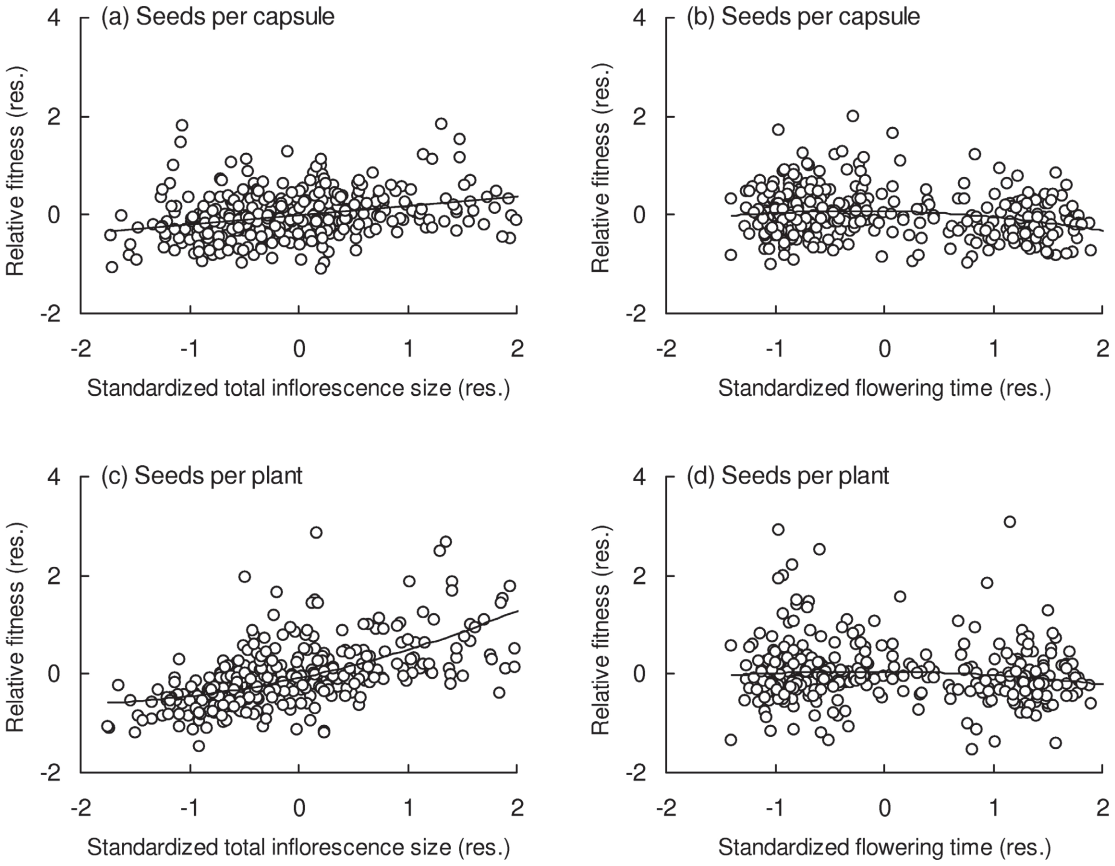

Fig. 3. Relationships between fitness and (a, c) total inflorescence size and (b, d) flowering time in a natural population of *Phyteuma spicatum* (*n* = 344 plants). Relationships are illustrated with added-variable plots, in which the residuals (res.) from a regression model of relative fitness (top: number of seeds per capsule, bottom: number of seeds per plant) on all traits [total inflorescence size, flowering time and their quadratic terms (except in (a) where the quadratic term was not significant, Table 3) as well as leaf width] except the focal trait are plotted against the residuals from a regression model of the focal trait on the other traits. Regression models including local plant density resulted in qualitatively similar plots.

Relationships between phenotypic traits, density, pollination and fitness

Six relationships were retained in the best path model of the causal relationships between inflorescence traits, leaf width, local plant density, pollinator visitation and seed production per plant (Fig. 4b). There were strong positive interrelationships between inflorescence

height, active inflorescence size and leaf width. We also detected (relatively) strong positive effects of inflorescence traits on seed production and of current plant density on pollinator visitation. Furthermore, inflorescence height positively influenced pollinator visitation rates. Very similar results were obtained when including the number of seeds per capsule as fitness estimate, except that the path between active inflorescence size and seed production was no longer included in the best model (Fig. 4b).

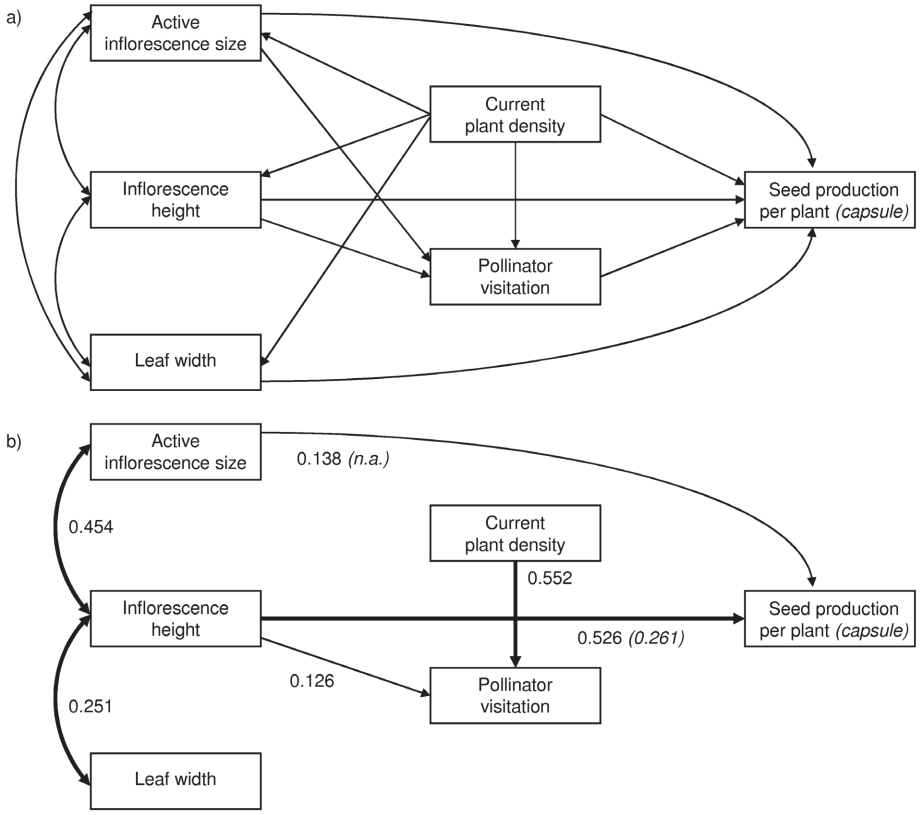

Fig. 4. Path diagrams describing the relationships between inflorescence traits (active inflorescence size and inflorescence height), plant size (leaf width), current local plant density, visitation by pollinators and seed production per plant (or per capsule, shown in parentheses) in a natural population of *Phyteuma spicatum* (*n* = 134 plants). One-headed arrows represent causal relationships, double-headed arrows represent free correlations. In a) and b) the full and best path models are shown, respectively. The best path models provided a reasonable fit to the data; observed and predicted covariances did not differ significantly (seeds per plant: χ^2 = 10.8, df = 9, *P* = 0.292, with paths explaining 36.1% of the variation; seeds per capsule: χ^2 = 10.0, df = 10, *P* = 0.443, 6.8%). All effects were significant at *P* < 0.1. Values are standardized path coefficients, with arrow width reflecting their magnitude (< or > 0.25).

Discussion

The results of our study show that local plant density may modify the relationship between plant phenotypic traits and pollination. The effect of active inflorescence size on pollinator visitation rate varied with the number of flowering conspecifics in the local neighbourhood.

Both inflorescence size and flowering time affected plant reproductive success in terms of the number of seeds produced per capsule or per plant, suggesting that there may be selection acting on these traits. Trait-fitness relationships, however, were not density-dependent, and differences in pollination also did not translate into differences in seed production.

As has also been observed in other species (Kunin 1993; 1997), pollinator visitation rates increased with increasing local plant density. Since long distance flying is energetically expensive, pollinators tend to minimize flight distances and flight times when foraging, which may explain the higher plant visitation rates in patches with many individuals (Zimmerman 1981; Klinkhamer & de Jong 1990). Dense flower patches may thus attract more pollinators, but competition for pollinators may also be higher (Steven et al. 2003). It has been predicted that pollinators spend less time on a plant when other food sources are nearby (Charnov 1976; Klinkhamer & de Jong 1990), while pollinators that forage in areas with only few individuals may stay longer on a given plant and visit more flowers (Kwak et al. 1998) because of the greater distance to the next plant neighbour. Correspondingly, we found that average bout length increased with decreasing plant density. This may increase within plant pollen transfer and reduce seed set especially in self-incompatible species (de Jong et al. 1993). In *P. spicatum*, geitonogamous pollination (i.e., pollination of a flower with pollen from another flower of the same plant) resulted in reduced growth of pollen tubes and very low seed production (Huber 1988). In our study, isolated plants thus received fewer pollinator visits that lasted longer, as also reported by Klinkhamer et al. (1989) for *Cynoglossum officinale*, suggesting that plants may suffer from low pollen quantity and poor pollen quality. This, however, was not supported by our data, as we found no significant effects of local plant density on seed production, except for a weak positive effect of local density on seed number per plant in the quadratic model (see Table 3 and results of the path analysis).

Pollinators often prefer plants with a larger floral display (Ohara & Higashi 1994; Gómez 2003). In our study, pollinator preference for active inflorescence size (i.e., the portion with open flowers), however, varied with local plant density. As expected, visitation rates increased with increasing active inflorescence size only at low densities. Display signals, as for example conveyed by a large inflorescence, may be more important for pollinator attraction in patches with few plants. Conversely, pollinators foraging in patches with more plants may not discriminate between inflorescences of different size (Mustajärvi et al. 2001; but see Schmitt 1983), because the cost of foraging between plants is low (Totland & Matthews 1998). Also bout length was affected by inflorescence traits; this, however, was not dependent on local plant density. Surprisingly, bout length was positively related only to inflorescence height in our study, and not affected by active inflorescence size. The positive relationship between bout length and inflorescence height may have been an effect of the correspondingly larger total inflorescence size. Bout lengths increased with increasing total inflorescence size ($P < 0.05$, analyses not shown), which in turn was positively related to

active inflorescence size. Among-plant variation in active inflorescence size was only about half of the variation in total inflorescence size [active and total inflorescence size varied between 1 and 5 cm (mean ± SD: 2.3 ± 0.8), and 1 and 10 cm (3.8 ± 1.6), respectively], possibly making effects more difficult to detect. In general, the relative attractiveness of inflorescence height versus size is difficult to distinguish in plants in which the two traits are correlated.

In our study population, plants with larger inflorescences produced more seeds than plants with a smaller floral display, suggesting that there may be phenotypic selection for increased floral display size. This corresponds to the findings of a previous study with the same species (Weber & Kolb 2011) and of numerous other studies (Harder & Johnson 2009; Sandring & Ågren 2009; Sletvold et al. 2010; Vanhoenacker et al. 2010). Inflorescence size is likely to be closely related to total seed production, because plants with more flowers may produce more seeds than plants with fewer flowers. Our finding that plants with larger inflorescences also produced more seeds per capsule, however, could imply that other mechanisms may have been at play. Selection on inflorescence size may also arise because a large inflorescence may increase attractiveness to pollinators (Ohara & Higashi 1994; Gómez 2003). In our study, pollinator visitation rates increased with increasing active inflorescence size at low densities, and pollinators generally preferred plants with taller inflorescences. Active inflorescence size and inflorescence height were both positively correlated to total inflorescence size (see Methods). Correspondingly, analyses examining effects of total inflorescence size on pollination also showed that pollinators preferentially visited plants with larger inflorescences ($P < 0.05$, analyses not shown). Alternatively, seed production per capsule could also simply increase with inflorescence size because flowers in larger inflorescences may have more ovules than flowers in smaller inflorescences. Together with the fact that the best path models did not include the path between pollinator visitation rates and seed production, our results therefore suggest that trait effects on pollination did not translate into differences in seed production.

We also found that plants with an intermediate flowering time produced more seeds than plants flowering early or late, although the relationship appeared to be relatively weak. Still, this could suggest that there is selection acting on this trait. This corresponds to the results obtained in 2009 in a previous study (where we also observed selection for an intermediate flowering time), but not to those obtained in 2008 (where there was no selection on flowering time) (data for the same population, contained in Weber & Kolb 2011). Stabilizing selection on flowering time is common (reviewed in Elzinga et al. 2007). O'Neil (1999) and Widén (1991), for example, showed for *Lythrum salicaria* and *Senecio integrifolius*, respectively, that seed production was lower in early and late flowering plants and highest at peak flowering, at least in some years. In general, temporal variation in trait-fitness relationships is common (e.g., Maad 2000; Caruso et al. 2003), which ultimately may result in more diffuse patterns of selection. Due to the nature of our pollinator observation

study (we only observed plants at a particular phenological state), we cannot draw any conclusions about whether selection on flowering time was mediated by pollinators.

Pollinator preferences for plants with specific inflorescence traits thus depended on local plant density. Contrary to our expectation, however, this did not translate into density-dependent variation in trait-fitness relationships, and in general, differences in seed production did not appear to be related to differences in pollination. Similarly, Schmitt (1983) observed that pollinator preference for plants with specific traits was affected by density, but that this did not translate into effects on seed set. There appear to be several possible explanations for our findings. First, our study focused on female reproductive success and neglected male fitness. Because *P. spicatum* is hermaphroditic, plants obtain half of their fitness through male function. Male and female fitness need not to be correlated and selection through female function (pollen import, seed production) or male function (pollen export, siring success) may therefore be very different (Lankinen & Larsson 2009). Albeit we have no evidence for this, pollinators may contribute to selection on inflorescence size via male fitness. Emms et al. (1997), for example, found for the lily *Zigadenus paniculatus* that the evolution of inflorescence size may partly be driven by selection for increased male success. Second, plants are often subject to multiple selection pressures, possibly masking or even counteracting pollinator-mediated selection. For example, antagonists such as seed predators and herbivores have been shown to select for small plant and floral display size (Ehrlén et al. 2002; Gómez 2003). However, we investigated trait-fitness relationships in the absence of the main antagonists (see Methods), and this explanation must therefore be treated with caution. Third, the observed effects may simply not have been strong enough, i.e. seed production may not have been pollen-limited even at the lowest observed pollinator visitation rates and bout lengths.

In summary, the results of our study illustrate that both the spacing of individuals in natural plant populations and plant phenotypic traits may affect pollination and, at least to some extent, also plant reproductive success. The number of flowering conspecifics in the local neighbourhood modified the relationship between active inflorescence size and pollinator visitation rate. This, however, did not translate into density-dependent variation in trait-fitness relationships as initially expected, and differences in seed production did not appear to be related to differences in pollination. The reasons for this remain unclear, and additional studies are needed to fully understand and explain the observed patterns.

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CHAPTER 6

Differences in heritable trait variation among populations of varying size in the perennial herb *Phyteuma spicatum*

Weber, A. & Kolb, A., manuscript.

Offspring of Phyteuma spicatum *(left) in a common garden of the University of Bremen and (right) transplanted into a forest site (Boitzenbostel, Elbe-Weser region). Photographs by A. Weber.*

Abstract

Habitat fragmentation may affect trait evolution in plants through changes in the abiotic or biotic environment. Evolutionary change, however, may be limited when fragmented populations suffer from genetic or environmental deterioration. In this study, we therefore examined the potential of plants in fragmented populations to respond to altered selective pressures by estimating the amount of heritable variation in important phenotypic traits, using the perennial forest herb *Phyteuma spicatum* as study species. We grew offspring of plants of ten natural populations of varying size under common environmental conditions and assessed if population trait means or heritability estimates were affected by the size and abiotic environmental conditions of the populations of origin. Most traits differed significantly among populations and maternal families, suggesting that genetic effects were responsible for the observed trait variation. Broad-sense heritabilities (H^2) ranged between 0 and 0.57, depending on trait and population of origin. Both size and environmental conditions of the populations of origin significantly affected means and H^2-estimates of some of the measured traits. Most importantly, heritabilities for flowering duration and mean seed mass decreased with decreasing population size, suggesting that plants in small populations may have a reduced capacity to respond and adapt to changes in the environment which alter selective pressures on these traits. Still, mean H^2-estimates were low to moderate, and, consistent with findings of other studies, patterns were generally quite variable. Further studies are therefore needed to gain more conclusive insights into the adaptive potential of small plant populations. Such knowledge is important if we want to understand how habitat fragmentation and associated changes in the environment affect patterns of trait evolution.

Keywords: Broad-sense heritability, environment, floral display size, flowering phenology, light intensity, plant traits, population size, seed production, soil conditions

Introduction

Habitat fragmentation may influence population viability and species survival in present-day landscapes and is therefore considered to be one of the major threats to biological diversity worldwide (Oostermeijer et al. 2003; Primack 2006). Potential evolutionary consequences of habitat fragmentation have been less well studied (Hoffmeister et al. 2005; Jacquemyn et al. 2012), but have received increasing attention in recent years (e.g., Moeller & Geber 2005; Cheptou et al. 2008; Weber & Kolb 2011). Habitat fragmentation could, for example, influence trait evolution through modification of abiotic or biotic environmental factors that act as selective agents (see below), although evolutionary change may be limited when fragmented populations suffer from genetic and environmental deterioration (Jump & Peñuelas 2005).

Several studies suggest that the availability of abiotic resources such as water, nutrients, light or temperature can influence trait selection in plants (Dudley 1996; Totland 1999; Caruso et al. 2003). Such abiotic factors may vary with habitat fragment and population size (Saunders et al. 1991), potentially leading to altered selection scenarios in fragmented landscapes. Weber & Kolb (2011), for example, presumed that selection for increased inflorescence size in small compared to large populations of *Phyteuma spicatum* may have been related to higher relative light intensities and lower base saturation in the small populations. Biotic components of the environment such as pollinators, herbivores or seed predators are also of fundamental importance to trait evolution in plants, being known to exert selective pressures on plant and floral display size, flowering phenology, floral scent and other traits (e.g., Majetic et al. 2009; Sandring & Ågren 2009; Kolb & Ehrlén 2010; Sletvold et al. 2010; Bodbyl-Roels & Kelly 2011). Because habitat fragmentation may disturb both mutualistic and antagonistic plant-animal interactions (Colling & Matthies 2004; Tscharntke & Brandl 2004; Ågren et al. 2008; Kolb 2008), it may also modify patterns of animal-mediated selection. For example, Murúa et al. (2010) found for *Viola portalesia* that selection on flower number and shape differed between native and transformed habitats with high and low population densities of the species, respectively, and that differences in selection were likely linked to changes in pollinator composition and visitation rate. Muola et al. (2010) observed that the impact of herbivory and seed predation on plant fitness of *Vincetoxicum hirundinaria* varied among fragmented plant populations, indicating spatial variation in phenotypic selection on herbivore resistance. Taken together, there is thus accumulating evidence that patterns of selection on plant phenotypic traits may be modified by habitat fragmentation. However, there is still only little knowledge about the degree to which plants in fragmented populations are able to respond to such altered selective pressures.

In general, evolutionary response to selective pressures will not only depend on phenotypic trait variation and fitness differences among trait variants, but also on trait heritability (Endler 1986; Johnson et al. 2009), with heritability being defined as the proportion of phenotypic variation in a trait that is attributable to genetic variation (Lynch & Walsh 1998). Heritability is a property not only of a trait but also of the population, depending both on the gene frequencies within a population and on the environmental circumstances to which individuals in a given population are subjected (Falconer & Mackay 1996). Fragmented populations that differ in their genetic structure and environmental conditions may thus also differ with respect to trait variation and heritability.

Several mechanisms may affect the genetic structure of fragmented populations (Ellstrand & Elam 1993; Young et al. 1996). Genetic drift, i.e. the random change in allele frequency from one generation to the next, may cause gene variants to be lost, an effect that will be most pronounced in small populations. In small populations with low genetic diversity, levels of inbreeding, i.e. the mating between closely related individuals, will be higher, increasing homozygosity as well as the expression of recessive deleterious alleles.

These processes may thus lead to a lower heritable trait variation (Lynch & Walsh 1998) and limit the evolutionary potential of populations to respond and adapt to changes in the environment (Booy et al. 2000; Jump et al. 2009). In addition to these negative genetic effects, also changes in local environmental conditions may reduce the capacity for evolutionary response (Charmantier & Garant 2005; Willi et al. 2006). Fragmentation often leads to increased heterogeneity of the environment as well as to unfavourable, often stressful conditions, for example through edge effects (Saunders et al. 1991). If environmental variation in small populations is high, the heritability of trait variation will be relatively low (Falconer & Mackay 1996; Charmantier & Garant 2005). In summary, small plant populations are thus expected to have lower heritabilities and consequently a reduced evolutionary potential compared to large populations (Ellstrand & Elam 1993; Young et al. 1996; Willi et al. 2007).

The main aim of this study was to examine the potential of plants in fragmented populations to respond to altered selective pressures by growing offspring of plants of populations of varying size under common environmental conditions and by estimating the amount of heritable trait variation for several important phenotypic traits. As model system we used the self-incompatible, perennial forest herb *Phyteuma spicatum*, which is restricted to remnants of deciduous forest in our study area (Kolb & Diekmann 2004; Kolb 2005). Previous studies with this species have shown that population size may influence trait selection (Weber & Kolb 2011) and that small populations suffer from lower levels of genetic diversity (Kolb & Durka 2013). Our specific objectives were (1) to test if there is among-population phenotypic variation in several traits such as plant size, floral display size, flowering phenology and seed traits, and if so, (2) to examine whether the observed variation is heritable, by estimating broad-sense heritabilities (H^2) for each trait and population. Furthermore, we tested (3) if population trait means and heritabilities are linked to the size or environmental conditions (light, soil parameters) of the populations of origin. While most previous studies assessing the influence of maternal environmental conditions on heritable trait variation were performed using only a small number of populations (Willi et al. 2006; but see Ellmer et al. 2011), we here examine patterns along continuous population size and environmental gradients.

Materials and methods

Study species and study area

Phyteuma spicatum L. (Campanulaceae) is an iteroparous, perennial hemicryptophyte that occurs in Central and Atlantic Europe (Wheeler & Hutchings 2002). It produces annual rosettes of basal leaves and, when flowering, one to several inflorescences on upright stalks. Flowering takes place in May and June, with individual plants usually flowering between 5 to 20 days. The hermaphroditic, protandrous flowers are densely packed within each inflorescence and open sequentially from the bottom to the top, being mainly pollinated by

bumblebees (Weber & Kolb 2013). Spontaneous autogamy and geitonogamy result in no or very few seeds, presumably due to gametophytic self-incompatibility (Huber 1988). Mean seed production per capsule and per plant in natural populations are 7.5 ± 4.1 (mean ± SD) and 307 ± 270, respectively (n = 831 individuals, data from 2008; Weber & Kolb 2011). Seeds are small (0.12 mg ± 0.02, n = 752; A. Weber, unpublished data) and lack special dispersal devices. The species is diploid (Huber 1988).

In our study area, situated between the cities of Bremen and Hamburg in north-western Germany, the species is relatively rare and restricted to fresh or moist, base-rich deciduous hardwood forests (Kolb 2005). Forests in this area have been highly fragmented for more than 250 years (Kelm 1994) and today cover ca. 13% of the landscape, of which only 25% are deciduous hardwood forest (Kolb & Diekmann 2004). Larger forest fragments support larger populations of the species (Weber & Kolb 2011).

Sampling of seeds and offspring cultivation

Plants were grown from seed and kept in a common garden and greenhouse for one year before they were transplanted into a forest site. We used seeds from plants of ten natural populations of varying size. All populations were part of a previous study in which we examined effects of population size on trait-fitness relationships (Weber & Kolb 2011). In that study, we collected seeds from 10 to 93 mother plants per population, depending on population size. Due to resource constraints, we were not able to include all plants in the current study, and therefore used the seeds from 10 to 28 randomly chosen plants. Seeds were collected at the beginning of July in 2008 and stored in the refrigerator at 4°C.

In September 2008, we sowed approximately 20–30 seeds (or fewer seeds when fewer were available) of each plant into each of 6 pots (9 × 9 cm) filled with a 78:22 mixture of garden soil ("Einheitserde" Type T, Terreau Professional / Gepac, Germany) and sand. Also, a slow release fertilizer (Osmocote) was added to the soil-sand mixture (0.1%). Pots were kept in the common garden until spring 2009 for seed stratification and germination. In April 2009, seedlings were reduced to one per pot resulting in 6 offspring per mother plant (in the following referred to as maternal family). In a number of cases (33%), fewer offspring were available due to low initial seed numbers or germination failure. Still, most maternal families were represented by ≥ four offspring individuals (see Data analysis). From April to Mai, all pots were kept in the greenhouse to facilitate growth and then transferred back to the common garden until the end of September 2009. To avoid location effects, pots were arranged in a randomized block design with 6 blocks, each containing one plant of each maternal family (unless fewer offspring were available). Watering was carried out as needed and plants were fertilized twice with water soluble fertilizer (Osmosol 614R).

At the beginning of October 2009, plants were transplanted to a 20 × 20 m area in a forest containing one of the *P. spicatum* populations (population "BB", Appendix 2) to subject the plants to more natural environmental conditions. Individuals were again planted

into 6 blocks, each containing one plant of each maternal family. Within each block, the distance between plants was 40 cm, and blocks were spaced 1–2 m apart from each other. The area was surrounded by a 1.6 m tall fence (Ursus Wildgatter AS 160/23/15 L) to protect plants from damage by deer.

Trait measurements

In 2010 several traits were measured for each plant. Most plants flowered (94.5%), and data collection was restricted to those flowering. At the beginning of May we recorded the number of inflorescences per plant. During the flowering period (May – June 2010), plants were visited every second day to determine the first and the last day of flowering (Julian day). Flowering duration was quantified as the number of days a given plant was in bloom. Shortly after flowering, we measured the height of each inflorescence stalk (including the inflorescence), the size of each inflorescence, i.e. the portion with flowers, as well as the number of leaves and the width of the broadest leaf. At the time of seed maturity (early – mid July), we collected one randomly chosen inflorescence per plant. We counted the number of seed capsules and the number of seeds to calculate the mean number of seeds per capsule. Furthermore, we determined the mean seed mass for each plant (mean mass of 50 randomly chosen seeds per plant).

Size and environmental conditions of the populations of origin

To characterize the populations of origin, we used population size and abiotic environmental data from previous work (Kolb et al. 2010; Weber & Kolb 2011; A. Kolb, unpublished data). Population size was determined as the mean number of flowering individuals and ranged between 6 and 2235 individuals (data from 2006–2009, Appendix 2). In each population, 5–15 (depending on population size) 4-cm-deep soil cores were collected from below the litter layer and pooled. Each sample was air-dried to constant mass and passed through a 2-mm sieve. Using standard techniques, all samples were analysed for pH (determined from a solution of 10 g of soil and 25 mL of 0.01M $CaCl_2$ with a standard glass electrode), plant available phosphorus (P), calcium (Ca), magnesium (Mg) and potassium (K) (extraction with ammonium lactate and photometric determination by flow injection analysis for P and Atomic Absorption Spectroscopy (AAS) for cations; all in mg per 100 g soil), carbon (C) and nitrogen (N) (in %; elemental analyser EuroEA 3000, HEKAtech, Germany). Light intensity was measured as photosynthetic photon flux density (PPFD) of photosynthetically active radiation (μmol s^{-1} m^{-2}; LI-COR Quantum Sensor, USA) at 8–24 locations in the forest and simultaneously outside each forest patch in the open (15-s averages each), on days with an overcast sky. The measurements were averaged and expressed as $PPFD_{forest}$ /$PPFD_{open}$ ×100, for a measure of relative light intensity.

Data analysis

We only included maternal families with 4 or more flowering offspring individuals in the analyses (146 families were represented by 6 offspring, 51 by 5 and 13 by 4, resulting in a total of 1183 individuals). To examine whether there is phenotypic variation among populations and among maternal families in the measured traits, we used linear models with a nested design, where maternal family was nested within population. Analyses were conducted using the "glm" (number of inflorescences; Poisson errors, log link function) or "lm" (all other traits) functions as well as the "Anova" function of the "car" package for likelihood-ratio χ^2-tests (glm) and F-tests (lm) in R 2.15.1 (R Development Core Team 2012; Fox & Weisberg 2011). For plants with more than one inflorescence, we used the maximal observed values for inflorescence height and size. The number of inflorescences was strongly correlated to the number of leaves (r = 0.89, P <0.001, n = 1183), and we therefore only examined effects on the number of inflorescences. As measures of flowering phenology we used the first day of flowering (flowering onset hereafter) and flowering duration.

To partition trait variation into its among-family (V_f) and within-family (V_w) components (Lynch & Walsh 1998), we used variance component analyses based on the restricted maximum likelihood (REML) method, using the "lmer" function contained in the R package "lme4" (Bates et al. 2012). We calculated broad-sense heritability at the population level (following Falconer & Mackay 1996) as $H^2 = 2V_f/(V_f + V_w)$ to examine whether the observed variation is heritable. Given the possible inclusion of non-additive genetic variance, H^2-estimates set an upper limit to the amount of additive variation available for selection (Falconer & Mackay 1996). Because progeny within families could be full- or half-sibs, we treated the progeny within families as full-siblings because this yields the most conservative estimate of heritability (Herlihy & Eckert 2007).

Lastly, to assess whether population size or environmental conditions affect population trait means or heritable trait variation (H^2), we used multiple regression models including population size (log-transformed), light intensity and soil parameters as predictor variables. Because we examined several soil parameters that were partly correlated, we first performed principal component analysis (PCA) with varimax-rotation to reduce the number of variables, using the "principal" function implemented in the "psych" package for R (Revelle 2012). We used sample scores of the principal components with eigenvalues > 1 for further analysis (PC1: C:N, Mg and K and PC2: pH, Ca, P; factor loadings were in each case > |0.62| and positive except for P), which together explained 63% of the total variance. Starting with full models (including population size, light intensity, PC1 and PC2 as predictors), non-significant ($P \geq 0.1$) terms were successively removed in a backward stepwise procedure. The four descriptors were not significantly correlated, except for population size and PC1 (r = 0.66, P = 0.036). The analyses were conducted using the "lm" function in R (R Development Core Team 2012).

Results

All measured traits differed significantly among populations and maternal families, with the exception of the number of inflorescences, which differed only among populations (Table 1).

Table 1 Effects of population and maternal family (nested within population) on phenotypic trait variation of *Phyteuma spicatum* plants grown from seed (n = 1183 individuals in n = 210 families from n = 10 populations). All plants were grown under common environmental conditions (abbreviation: pop. = population). Results are from generalized linear (number of inflorescences, χ^2) or linear models (all other traits, F) (see methods for details).

	Population[a]		Maternal family (pop.)[b]	
Source of variation	χ^2/F	P	χ^2/F	P
Number of inflorescences	145.99	<0.001	191.15	0.661
Maximal inflorescence height	4.03	<0.001	1.32	0.005
Maximal inflorescence size	11.20	<0.001	1.40	<0.001
Leaf width	6.38	<0.001	1.76	<0.001
Flowering onset	12.33	<0.001	1.75	<0.001
Flowering duration	5.46	<0.001	1.20	0.044
Number of seeds per capsule	3.90	<0.001	1.48	<0.001
Mean seed mass	6.35	<0.001	1.62	<0.001

[a] Degrees of freedom = 9

[b] Degrees of freedom = 200

Table 2 Broad-sense heritabilities (H^2) for eight phenotypic traits, estimated from offspring of plants of ten populations of *Phyteuma spicatum* (abbreviations: caps. = capsules, flow. = flowering, infl. = inflorescence, max. = maximal, no. = number, pop. = population). H^2 were calculated from restricted maximum-likelihood (REML) estimates of the variance in phenotypic traits among and within maternal families for each study population.

Pop. ID	No. of infl.	Max. infl. height	Max. infl. size	Leaf width	Flow. onset	Flow. duration	Mean no. of seeds/caps.	Mean seed mass
F	0.00	0.00	0.14	0.00	0.24	0.00	0.00	0.13
WN	0.23	0.30	0.17	0.21	0.57	0.14	0.00	0.00
R	0.25	0.00	0.22	0.00	0.00	0.00	0.17	0.00
WW	0.00	0.00	0.00	0.33	0.43	0.00	0.25	0.19
TO	0.38	0.00	0.19	0.31	0.26	0.00	0.00	0.00
TN	0.00	0.00	0.21	0.21	0.29	0.00	0.15	0.34
IP	0.35	0.00	0.00	0.28	0.15	0.21	0.00	0.24
HH	0.18	0.13	0.00	0.41	0.00	0.22	0.00	0.19
TW	0.00	0.00	0.00	0.00	0.00	0.22	0.49	0.32
BB	0.00	0.34	0.15	0.34	0.34	0.23	0.22	0.30

Differences within maternal families accounted for most of the variation in the measured traits (72–100%), whereas among-family variation was quite low (0–28%). Broad-sense heritability estimates (H^2) ranged between 0 and 0.57, depending on trait and population of origin (Table 2). Across populations, flowering onset, leaf width and mean seed mass had the highest H^2-estimates, means being moderate with 0.23, 0.21 and 0.17, respectively. All other traits, i.e. the number of inflorescences, maximal inflorescence height and size, flowering duration and the mean number of seeds per capsule, had lower mean H^2-estimates (0.08–0.14).

Table 3 Effects of mean size (based on counts from 2006–2009), light intensity and soil parameters (PC1 = C:N, Mg, K; PC2 = pH, Ca, P) of the populations of origin (n = 10) on mean values and heritability estimates of several phenotypic traits in offspring of *Phyteuma spicatum* (abbreviations: caps. = capsules, flow. = flowering, infl. = inflorescence, max. = maximal, no. = number, pop. = population). Parameter estimates are from multiple regressions (backwards stepwise procedure). Estimates are in bold if $P < 0.05$ and in italics if $P < 0.1$. Each column corresponds to a separate regression analysis either for trait means or heritabilities. Estimates for the number of inflorescences, maximal inflorescence height and leaf width were not affected by any of the four population descriptors and are therefore not shown.

Descriptor	Response variable				
	Max. infl. size	Flow. onset	Flow. duration	Mean no. of seeds/caps.	Mean seed mass
Trait means					
Pop. size		**0.52**		**-0.47**	
Light	**82.95**				
PC1	*0.32*	**-0.99**		**0.74**	
PC2		**0.47**			**-0.004**
R^2-adj.	0.593	0.693		0.486	0.373
F	7.552	7.756		5.246	6.362
P	0.018	0.017		0.041	0.036
df	7	6		7	8
Heritabilities					
Pop. size			**0.04**		
Light					**-16.43**
PC1		**-0.13**			
PC2					**-0.08**
R^2-adj.		0.385	0.432		0.540
F		6.627	7.857		6.272
P		0.033	0.023		0.028
df		8	8		7

Both population size and abiotic environmental conditions of the populations of origin significantly affected offspring trait variation (Table 3). The size of the populations of origin positively affected flowering onset, i.e. offspring of larger populations flowered later than offspring of smaller populations. Also, the mean number of seeds per capsule depended on population size; seed production decreased with increasing population size. Soil parameters of the populations of origin had positive effects on maximal inflorescence size (PC1: C:N, Mg, K), flowering onset (PC2: pH, Ca, P) and the mean number of seeds per capsule (PC1), and

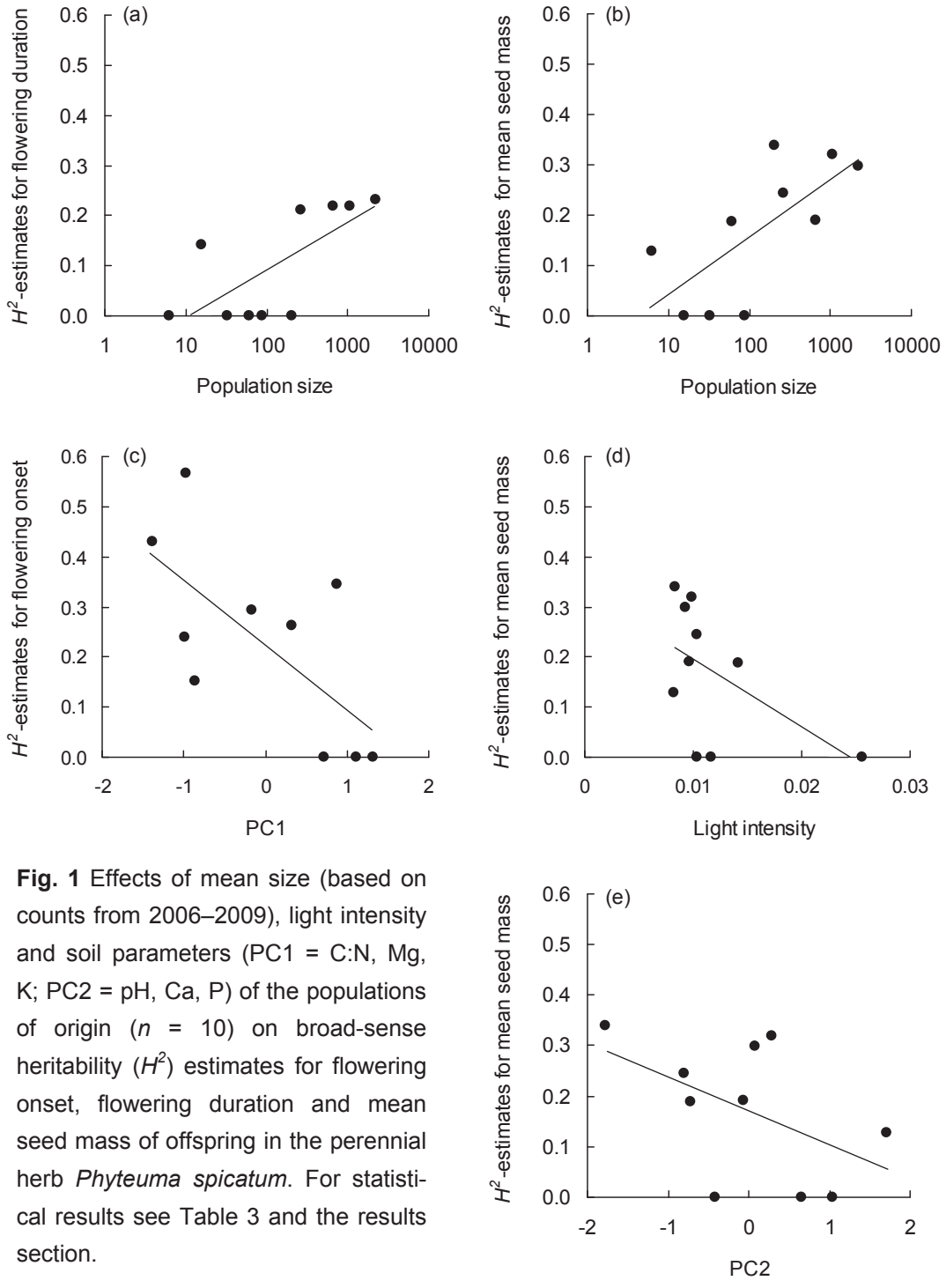

Fig. 1 Effects of mean size (based on counts from 2006–2009), light intensity and soil parameters (PC1 = C:N, Mg, K; PC2 = pH, Ca, P) of the populations of origin (n = 10) on broad-sense heritability (H^2) estimates for flowering onset, flowering duration and mean seed mass of offspring in the perennial herb *Phyteuma spicatum*. For statistical results see Table 3 and the results section.

negative effects on flowering onset (PC1) and mean seed mass (PC2). Furthermore, light intensity of the maternal environment positively affected maximal inflorescence size. Means of all other traits were not significantly influenced by any of the four population descriptors.

Size and environmental conditions of the populations of origin also had significant effects on heritability (H^2) estimates (Table 3). As expected, H^2-estimates for flowering duration increased with increasing population size, i.e. they were lower in smaller populations (Fig. 1a). Population size did not significantly affect H^2-estimates of any of the other traits in the multiple regressions; however, in a univariate analysis, heritabilities for mean seed mass were significantly related to population size, again being lower in smaller populations (R^2 = 0.436, F = 7.968, P = 0.022, df = 8, Fig. 1b). Furthermore, H^2-estimates for flowering onset were negatively affected by PC1 (Fig. 1c) and those for mean seed mass were negatively influenced by light intensity and PC2 (Figs. 1d, e).

Discussion

The results of our study suggest that plants in fragmented populations may have a reduced capacity to respond and adapt to changes in the environment. As expected, offspring plants of small populations were characterized by lower heritabilities at least for two of the measured traits (flowering duration and mean seed mass) than plants of large populations. Also, soil conditions and light availability in the populations of origin appeared to influence the amount of heritable trait variation. However, for several traits and populations we did not detect any heritable variation, and mean H^2-estimates were generally low to moderate, suggesting that environmental variance might often be more important than genetic differences for phenotypic trait variation in *P. spicatum*.

Nearly all measured traits differed significantly among populations and maternal families. Given that offspring plants were grown under common environmental conditions, this indicates that genetic effects may have been responsible for the observed trait variation. Phenotypic differences among populations suggest that these populations may have followed different evolutionary trajectories over time, resulting in different "genotype pools" over time. Similarly, differences among maternal families were likely due to genetic differences among individuals within populations. These results are consistent with those of Mazer & Delesalle (1996), who detected among population and among maternal family variation in several floral traits of *Spergularia marina*. Also the effects of size and environmental conditions of the populations of origin on mean offspring trait values suggest genetic adaptation to local environmental conditions. For example, in our study high light intensity environments appear to have selected for plants with large inflorescences, and plants growing in base-rich habitats may have a developmental advantage and thus flower earlier. Similarly, Totland (1999) found that differences in temperature conditions selected for different plant phenotypes in *Ranunculus acris*. In addition to genetic effects, non-genetic maternal effects may also influence offspring trait values (Roach & Wulff 1987) and could thus be partly responsible for

the observed among-population and among-family differences. However, environmentally-induced maternal effects are often only expressed during early stages of development and less so in traits of later life-cycle stages (Ouborg et al. 1991; Mazer & Delesalle 1996). Given that we measured traits in the year after germination and when plants were already flowering, the genotypes of the offspring likely contributed more to the observed trait variation than any maternal effects.

Our estimates of broad-sense heritability also suggest a genetic contribution to the observed phenotypic variation in several of the measured traits. Most traits were characterized by H^2-estimates of >0.30 in at least some of the populations, values which are considered to be moderate or even high by some authors (e.g., Ellmer et al. 2011). Similar broad-sense heritabilities were found for example by Worley & Barrett (2001), who report values between 0.14 and 0.53 for leaf area, flower number and inflorescence number in *Eichhornia paniculata*, and by Whitaker et al. (2012), who detected H^2-estimates between 0.18 and 0.53 for several traits of *Fragaria xananassa*. Still, mean H^2-estimates were relatively low across populations (0.08–0.23), with several of the individual H^2-estimates being zero or close to zero due to very low observed among-family phenotypic variation. Similarly, Waldmann & Andersson (1998) obtained heritability estimates of zero for some traits and populations of two *Scabiosa* species, and Schwaegerle & Levin (1991) detected heritability values between 0 and 0.15 for a large number of morphological traits in a population of *Phlox drummondii*. Furthermore, Mazer et al. (2004) reported low among-family variation in a subspecies of *Clarkia xantiana*. Low heritabilities, however, do not necessarily indicate that the populations are genetically invariant. As discussed by Schwaegerle & Levin (1991), this may simply reflect large environmental effects on plant phenotype that obscure genetic differences among plants. In general, it needs to be kept in mind that broad-sense heritability estimates are usually higher than narrow-sense heritabilities, given that they include all genetic contributions to the observed variance and not only that due to additive (i.e., allelic) genetic effects (Lynch & Walsh 1998).

Our results show that the amount of heritable trait variation varied among populations and that this variation was at least partly related to the size and abiotic environmental conditions of the populations of origin. Size of the population of origin positively affected heritability estimates for flowering duration and mean seed mass. Plants of small populations might therefore be less able to respond and adapt to changes in the environment and in selective pressures acting on these traits than plants of larger populations. The size of the population of origin, however, did not affect H^2-estimates for any of the other measured traits and in some cases heritabilities were relatively high in the small populations. Previous work suggests that there is considerable variation in the relationship between the adaptive potential of plant populations and their size (e.g., Widén & Andersson 1993; Waldmann & Andersson 1998; Willi et al. 2007; Ellmer et al. 2011). For example, Ellmer et al. (2011) found relatively high levels of heritable variation for traits related to plant size and reproduction in *Briza*

media, and that heritabilites were not associated with habitat fragment size. The authors concluded that the structuring of quantitative genetic variation may be relatively insensitive to changes in landscape structure in this species. Waldmann & Andersson (1998), on the other hand, found negative correlations between population size and heritabilities for several traits (leaf size, flower size, flowering onset) in two *Scabiosa* species, as well as a positive correlation between population size and heritable variation in leaf number in one of the two species. In addition to population size effects, also the maternal environment, in terms of soil parameters and light availability, affected heritable variation; H^2-estimates for flowering onset and mean seed mass were negatively related to light intensity and soil parameters of the populations of origin. Plants growing in populations with low light or specific soil conditions might therefore be better able to adapt to further changes in the environment, provided that these alter selection on flowering time and seed traits.

In summary, our results from *P. spicatum* indicate that the capacity of small plant populations to evolve in response to novel environmental conditions may be reduced. Heritabilities of two of the measured traits decreased with decreasing population size. However, heritable variation in many traits was very low in at least some of the populations, and patterns were generally quite variable. This is consistent with the diverse findings obtained in other study systems (see above) and more studies examining heritable variation in phenotypic characters in fragmented populations are certainly needed to obtain more conclusive evidence for that the adaptive potential of small populations may be reduced. Such information is needed if we want to understand the effects of habitat fragmentation and associated changes in the environment on patterns of trait evolution in plants.

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CHAPTER 7

SYNTHESIS AND PERSPECTIVES

Phyteuma spicatum. *Photograph by A. Weber.*

Synthesis

The effects of habitat fragmentation on phenotypic trait selection in plants is of general interest in order to get a better understanding of potential evolutionary consequences of human-induced environmental change (Jacquemyn et al. 2012). The main intention of this thesis was to obtain new insights into the effects of habitat fragmentation on evolutionary trajectories of plant populations in present-day landscapes. More specifically, the two major aims were: first, to investigate how fragmentation affects patterns of selection on plant phenotypic traits via altered plant-animal interactions, and second, to determine the potential of plants in fragmented populations to respond to such selection pressures (see CHAPTER 1). In order to achieve these aims, four studies were conducted (CHAPTERS 3–6) using the insect-pollinated, perennial herb *Phyteuma spicatum* (Campanulaceae) as model species, which occurs in fragmented forest patches in the study area in north-western Germany (see CHAPTER 2).

A major finding of this thesis was that patterns of phenotypic selection were related to plant population size and mean population density in one of two study years (CHAPTER 3). Selection for increased inflorescence size decreased with increasing population size and density, confirming our initial hypothesis that selection pressures on visual plant traits are stronger in small or low-density populations. However, in the second year we did not find such a relationship. Furthermore, patterns of selection on flowering phenology were consistent across the 16 study populations in both years (CHAPTER 3). Selection on flowering state (corresponds to "flowering time") as well as on synchrony was not significantly related to population size and mean density (CHAPTER 3), and selection on flowering state was also not affected by local plant density (CHAPTER 5). Thus, habitat fragmentation did not affect selection on optimal flowering time, which was expected since local environmental conditions (i.e., light intensity, temperature, water availability) and pollinator environments are likely to differ among fragmented habitat patches (Saunders et al. 1991; see also CHAPTER 1). The general assumption, that phenotypic trait selection may be affected by plant population size or density, was therefore partly confirmed for floral display size (in 2008 but not in 2009), but not for flowering phenology within this study system.

It is well known, that small plant populations are less attractive or less apparent to insect pollinators and that plants in small populations might therefore suffer from increased pollen limitation (e.g., Ågren 1996; Mustajärvi et al. 2001). It is also known that pollinators preferentially visit large inflorescences with many flowers that provide a strong signal to attract pollinators and offer greater rewards in terms of pollen and/or nectar (Willson & Price 1977; Klinkhamer & de Jong 1990). Thus, in fragmented populations plants with a large floral display may be particularly favoured. Pollinators have been found to act as selective agents on floral display size in other studies (e.g., Ohara & Higashi 1994; Sandring & Ågren 2009). However, our data provide no evidence that the detected patterns of selection were related to changes in pollination intensity (CHAPTER 3); mean seed production was not related to

population size or density in *P. spicatum*. Our hand-pollination experiment produced weak evidence that selection for increased inflorescence size may have been driven by pollinators (CHAPTER 4). Supplemental pollen reduced the strength of selection in most populations and years, and in two small populations selection gradients were only significant in the open-pollinated controls. However, the degree of pollen limitation was not significantly related to population size. On the other hand, pollinator observations within a large population revealed that pollinators do prefer plants with a more showy floral display and that this preference may depend on local plant density (CHAPTER 5). At low densities, pollinator visitation rates were low, but increased with increasing floral display size, while this relationship disappeared at high flowering plant densities of *P. spicatum*, where visitation rates were overall higher. These results suggest that plant spacing may indeed be important for pollinator foraging behaviour and that pollinators may act as selective agents on plant phenotypic traits by affecting plant fitness differently in dense and sparse patches. Flowering plant density is known to influence the attraction of pollinators, their movement within and among patches, and thus the rate of pollen exchange and subsequent seed production (e.g., Kwak et al. 1998; Nattero et al. 2011). However, within our study system pollinator foraging patterns did not translate into density-dependent trait-fitness relationships and differences in seed production did not appear to be related to differences in pollination (CHAPTER 5). Taken our results together, we did not obtain clear evidence that pollinators are the driving force of the selection patterns detected here and the role of pollinators as selective agents within this system remains ambiguous.

Nevertheless, we detected selection on several phenotypic traits in *P. spicatum*. Patterns of selection were quite variable among populations and years (CHAPTERS 3 and 4). Harder & Johnson (2009) reviewed that it is common for phenotypic selection to vary among populations during the same year and between years within populations. Selection gradients for floral display size in *P. spicatum* were in fact always positive, yet, the magnitude of phenotypic selection varied temporally and spatially (CHAPTERS 3 and 4). Generally, the relationship between floral display size and seed production is linear (Harder & Johnson 2009; Kolb & Ehrlén 2010), which was also true for the species studied here. As expected, seed production increased with increasing inflorescence size. However, we also found significant quadratic terms in two of the 16 populations in 2008 and 2009 (CHAPTER 3) and within the population studied in 2010, indicating non-linear relationships (CHAPTER 5, Fig. 3c). Similarly, Johnston (1991) found within a population of *Lobelia cardinalis* significant positive quadratic selection on plant height. Since inflorescence size and plant height were always strongly positively correlated in *P. spicatum* (CHAPTERS 3, 4 and 5), the results obtained for inflorescence size mostly also apply to inflorescence height.

Furthermore, in most cases we detected linear selection on flowering state, with relative fitness being higher in earlier flowering plants (CHAPTER 3). However, within the largest population of *P. spicatum* we discovered in 2010 a significant non-linear effect of flowering

state on seed production (CHAPTER 5). Plants with intermediate flowering states appeared to have a slightly higher fitness compared to plants flowering early or late. Similar results were obtained in another population (data collected in 2010; A. Weber, unpublished data), emphasizing weak phenotypic selection for an intermediate flowering state in *P. spicatum*. Stabilizing selection on flowering time is common (Elzinga et al. 2007), but also early flowering seems to be often selected for (Munguía-Rosas et al. 2011 and references therein). Similar to our results, Widén (1991) detected phenotypic selection on early and intermediate flowering in the perennial herb *Senecio integrifolius* and patterns also varied temporally and spatially. Furthermore, Kwak et al. (1991) reported for *Phyteuma nigrum* that early flowering plants received more pollinator visits than plants flowering later in the season. Early flowering plants, however, may have a disadvantage compared to plants flowering during the population peak, since higher pollen availability in the neighbourhood during peak flowering makes a successful pollination more likely (Jakobsson et al. 2009). Consistent with this, we also detected linear selection on flowering synchrony in *P. spicatum*; plants flowering together with other individuals had a higher relative fitness than plants flowering asynchronously with the population (CHAPTER 3). A linear relationship is not surprising, because especially self-incompatible plants such as *P. spicatum* often benefit from the presence of flowering conspecifics. In summary, our results suggest that the strength of selection varies among populations and years in *P. spicatum* and might also differ to some extend in form; i.e. linear or non-linear selection (Brodie et al. 1995).

Several reviews indicate that it is quite common for natural selection to vary in strength, direction and form as well as over space and time (Kingsolver et al. 2001; Hereford et al. 2004; Siepielski et al. 2009). Such variable selection on floral display and flowering phenology is often found to be mediated by pollinators, for example, as a consequence of variation in the pollinator fauna (Schemske & Horwitz 1989; Gómez et al. 2008). We assumed that differences in weather conditions (2008 was dryer and warmer compared to 2009) might have induced temporal variation in selection on floral display size via changes in pollinator availability (CHAPTER 3). However, as mentioned above patterns of phenotypic selection were more likely linked to differences in some other, unmeasured component of the abiotic or biotic environment. Furthermore, there are some hints that abiotic conditions may have been responsible for the variation in selection among populations of varying size in *P. spicatum*. Relative light intensities decreased and calcium content in the soil tended to increase with increasing population size (CHAPTER 3). Spatial and temporal variation in selection on plant phenotypic traits seems to be induced quite often by differences in some abiotic factor (e.g., Totland 1999; Petit & Thompson 1998; Maad & Alexandersson 2004). But also biotic factors other than pollinators may be responsible for varying selection patterns (see CHAPTER 1). Certain plant characters that attract pollinators may also attract herbivores or seed predators, causing differences in seed production among patches of varying plant-animal interaction scenarios (e.g., Gómez & Zamora 2000; Toräng et al. 2008). For example,

Gómez (2003) found significant selection on several phenotypic traits in the herb *Erisymum mediohispanicum* when herbivores were absent. When they were present, selection on floral traits completely disappeared and selection strength on flower number and vegetative traits decreased. Within this study, however, herbivory is not likely to have contributed to selection patterns, since the main herbivore (*Capreolus capreolus*) was excluded. Additionally, an investigation in two large populations of *P. spicatum* showed that roe deer did not preferably consume large compared to small plants (measured via stem diameter in protected and control plants; A. Weber, unpublished data) and therefore may not act as selective agent on plant size. In summary, plant phenotypic traits may be shaped by different or even opposing selection pressures due to a complex network of interactions within plant populations (e.g., Galen 1999; Strauss & Irwin 2004; Ehrlén et al. 2012). Thus, the optimal phenotype may depend on the relative importance of the operating selective agents (Cariveau et al. 2004; Gómez 2008), with certain plant characteristics reflecting compromise adaptations (Herrera et al. 2002).

So far we have seen that there are selective pressures acting on several plant phenotypic traits in *P. spicatum*, that patterns are quite variable (CHAPTERS 3, 4 and 5) and that they might be affected by human-induced environmental change (CHAPTER 3). However, selection pressures on a phenotypic trait will lead to evolutionary change only when the trait under selection is heritable (Endler 1986; Johnson et al. 2009; see also CHAPTER 1). The analyses to estimate heritable trait variation were conducted to gain a more conclusive insight into how habitat fragmentation may affect patterns of trait evolution in plants (CHAPTER 6). Results showed that small plant populations may indeed have a reduced capacity to respond and adapt to changes in the environment: in two of the measured traits, namely flowering duration and mean seed mass, broad-sense heritabilities (H^2) decreased with decreasing population size. Our results for flowering duration in *P. spicatum* are in agreement with the view of Jacquemyn et al. (2012), who suppose that the optimal timing to reproduce might evolve differently depending on the degree of habitat fragmentation and associated changes in local environmental conditions. Thus, there is evidence of a possible effect of fragmentation on the evolvability of flowering phenology. Although selection gradients were not investigated for flowering duration within this thesis, there are some hints that flowering duration might be under selection. We found phenotypic selection on flowering state and synchrony (CHAPTER 3), indicating selection on phenology traits in this species. Furthermore, several authors detected selection on flowering duration in other species (e.g., O'Neil 1997; Giménez-Benavides et al. 2011; Internicola & Harder 2012), as well as heritable variation for the timing of flowering, the number of flowering events and flowering duration (Law et al. 1977; Mazer & Schick 1991; Widén 1991; Johnson 2007; Díaz & Merlo 2008). This suggests that phenology traits may often be prone to selection. In *P. spicatum* selection might act more strongly on first day of flowering than on flowering duration, since the latter may also be affected by the number of flowers and pollination intensity. Consistent with this, we found the highest mean heritability estimates for flowering onset whereas mean values for flowering

duration were much lower (CHAPTER 6). Thus, there is genetic potential for a selection response in flowering onset in *P. spicatum* and early flowering might be favoured, since selection on flowering state was mostly directional (CHAPTER 3; see above).

Furthermore, mean seed mass showed relatively high mean H^2-estimates compared to the other traits measured within this study and estimates were positively associated with plant population size, at least when analysed in an univariate analysis (CHAPTER 6). Consequently, plants of small populations might be less able to respond and adapt to changes in the environment and in selective pressures acting on mean seed mass than plants of larger populations. Ellmer et al. (2011) also found heritable variation for traits related to plant reproduction in *Briza media*, but heritability estimates were not related to habitat fragment size. We did not investigate selection on seed mass in *P. spicatum*, but several studies suggest that seed size may be under selection (Nelson & Johnson 1983; Jordano 1995). Abiotic as well as biotic environmental conditions may affect selection on seed characteristics. Large seeds have a higher probability of seedling emergence and establishment (Fenner 1985; Winn 1985), suggesting a selective force in the direction of large seed size. In contrast to this, seed-predators or dispersal distance may favour low seed mass in plants (Nelson & Johnson 1983; Volis 2009). In *P. spicatum* abiotic environmental conditions may affect the adaptive potential of plant populations; H^2-estimates for mean seed mass were negatively related to light intensity and soil parameters (CHAPTER 6). Thus, plants growing in populations with low light or specific soil conditions might be better able to respond to selection pressures caused by abiotic factors.

In general, mean values of heritable trait variation detected within this study were rather low, with several of the individual H^2-estimates being zero, possibly due to the presence of high environmental variance and non-heritable variation. Traits with low heritability will respond more slowly to selection pressures than traits with higher heritable variation (Falconer & Mackay 1996), and if genetic variation does not exist at all in a given trait, the population mean of this trait can not change over generations (Conner & Hartl 2004). For example, within this study no relevant H^2-estimates for floral display size (in terms of inflorescence height and size) were detected (CHAPTER 6), while display size was found to be under relatively strong linear selection in *P. spicatum* (CHAPTERS 3, 4 and 5). Thus, although floral display size in *P. spicatum* is strongly selected for, it may not evolve accordingly due to low heritable trait variation.

General conclusions and perspectives

On the whole, human-induced environmental changes such as habitat loss and fragmentation are considered to be a major threat to plant population viability, species survival and biological diversity (Eriksson & Ehrlén 2001; Oostermeijer 2003; Primack 2006). As outlined in this thesis, habitat fragmentation affects abiotic and biotic conditions which in turn may change the selective environments organisms are subjected to. Thus, evolutionary dynamics

may be altered, which may influence ecological processes and species persistence in present-day landscapes. Considering phenotypic selection pressures as well as the potential of natural populations to respond to such selection in conservation, restoration and management may become increasingly important in future (e.g., Rice & Emery 2003; Stockwell et al. 2003; Carroll & Fox 2008). Especially small plant populations might not have the potential to adapt to future anthropogenic threats, such as overharvesting, species introduction and climate change and may thus face an increased risk of extinction (e.g., Western 2001; Strauss et al. 2006; Leimu et al. 2010; Sedlacek et al. 2012).

Fragmentation may cause microevolutionary changes in natural plant populations, i.e. a change in the genetic constitution of a population over time (Merilä et al. 2001). Confirming our initial hypothesis, the results of this thesis show that selection pressures on plant phenotypic traits may be stronger in small or low-density populations (CHAPTER 3), but that small plant populations may also suffer from reduced heritable trait variation (CHAPTER 6). Thus, plants in fragmented populations may have a reduced capacity to respond and adapt to novel selection pressures caused by further changes in the environment. However, patterns of selection on floral display size and flowering phenology in *P. spicatum* varied spatio-temporally (CHAPTERS 3 and 4), and also patterns of trait heritability were generally variable (CHAPTER 6). Such variation, and especially temporal variation in phenotypic trait selection, might weaken the process of selection and thus prevent local adaptation of populations (Kawecki & Ebert 2004; Siepielski et al. 2009). Therefore, within this study system it is not likely that the measured plant characteristics will evolve differently among populations of varying size and we can not conclude from our results that habitat fragmentation in general will lead to divergent plant evolutionary trajectories in present-day landscapes.

Taken together, evolutionary consequences of habitat fragmentation are complex, and several questions are still in need of accurate answers. Further research is certainly needed, if we want to gain more conclusive insights into the evolutionary potential of fragmented plant populations to respond to rapid human-induced environmental changes. Concluding from the findings of this thesis as well as those obtained elsewhere, I recommend future studies investigating phenotypic selection in natural plant populations to take the following points into consideration.

First, investigation of natural selection over several years is necessary, because temporal variation in selection seems to be quite common (Siepielski et al. 2009). The results obtained within this thesis confirm this assumption: patterns of selection varied among years (CHAPTERS 3 and 4). According to Merilä et al. (2001), only long-term data sets provide time series long enough to be able to test for temporal variation in selection pressures and to eventually detect any response to selection. Results of one-year studies, on the contrary, might even lead to false conclusions of the strengths, direction or form of selection acting on phenotypic traits and must therefore be treated with caution.

Second, our results illustrate that environmental factors other than the most apparent might be responsible for the detected selection pressures. In *P. spicatum* we mostly could not link selection patterns to plant-pollinator interactions, and we were not able to identify other causes of selection within our study system (CHAPTERS 3, 4 and 5). To identify the agents of selection, experimental manipulations of environmental factors are especially helpful (Geber & Griffen 2003). Several scientists have already gone beyond conducting purely observational studies (e.g., Mauricio & Rausher 1997; Totland 1999; Lau et al. 2010; Sletvolt et al. 2010). Experiments assessing the relative importance of different selective agents may be able to discover conflicting selection pressures that shape and constrain plant evolutionary trajectories and may therefore give a more accurate picture of reality (e.g., Cariveau et al. 2004; Bartkowska & Johnston 2012).

Third, there is still only little knowledge about the degree to which plants in fragmented populations are able to respond to altered selection pressures. Thus, it might be constructive to combine investigations of natural selection with those of genetic variation (Wilson et al. 2006; Willi et al. 2006; Johnson et al. 2009), as attempted within this thesis. It would be useful not only to measure phenotypic selection pressures in natural populations but also to estimate heritable variation for the traits under selection. Otherwise, it is difficult to draw any conclusions about the evolutionary potential of plant populations and the long-term viability of species in changing landscapes. With further human impacts on natural habitats, such approaches might become more relevant and provide a better insight into plant evolutionary trajectories.

Fourth, our study focused exclusively on selection via female reproductive success. However, *P. spicatum* is hermaphroditic and obtains half of its fitness through male function, i.e. half of the genes are transmitted to the next generation through pollen. It is worth pointing out that selection may also act through male fitness, i.e. pollen export and siring success, in our study species as well as in many others. Male and female fitness do not necessarily have to be correlated (Devlin & Ellstrand 1990). Trait selection through female or male function may thus differ from one another (e.g., Campbell 1989; Hodgins & Barrett 2008; Lankinen & Larsson 2009), suggesting that measurements of selection through female fitness alone may be misleading. While we did not detect significant pollinator-mediated selection via female fitness (CHAPTER 4), our observations showed that pollinators preferred plants with a larger floral display (CHAPTER 5). Those findings suggest that pollinators might have contributed to selection on floral display size via male fitness in *P. spicatum*. To look further into this, we started a project that examines selection on plant phenotypic traits through both male and female function in our study system (A. Kolb, W. Durka and A. Weber, work in progress).

Although the detected patterns within this thesis were quite variable, I hope this work contributes to our understanding of the evolutionary consequences of habitat fragmentation and that it will encourage further research to answer some of the remaining open questions.

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Appendices

Appendix 1 Supplement to CHAPTER 3: Weber, A. & Kolb, A. (2011)
Evolutionary consequences of habitat fragmentation: population size and density affect selection on inflorescence size in a perennial herb.
Evolutionary Ecology 25: 417–428.

Appendix 2 Supplement to CHAPTER 6: Weber, A. & Kolb, A., manuscript
Differences in heritable trait variation among populations of varying size in the perennial herb *Phyteuma spicatum*.

Appendix 1 Population size, population density (± SD), number of individuals included in analysis (*n*) and mean trait values (± SD) in 16 populations of *Phyteuma spicatum* in 2008 and 2009. Traits include inflorescence size, flowering time and flowering synchrony.

Pop. ID	Pop. size[a]	Pop. density[b]	*n*[c]	Inflorescence size [cm]	Flowering time[d]	Flowering synchrony[e]
2008						
1	10	3.2 (1.9)	10	2.4 (0.9)	8.5 (3.6)	4.2 (1.3)
2	15	2.3 (1.0)	11	2.7 (0.9)	10.9 (3.1)	3.4 (1.4)
3	31	3.1 (1.7)	17	3.0 (1.7)	10.9 (4.0)	3.9 (2.2)
4	38	11.5 (4.6)	20	3.7 (1.4)	10.5 (3.8)	3.9 (2.0)
5	49	4.4 (3.9)	32	3.6 (1.7)	7.0 (3.7)	4.4 (0.7)
6	68	8.6 (5.6)	22	2.6 (1.0)	7.6 (4.7)	5.3 (1.0)
7	102	5.6 (4.4)	76	4.0 (1.9)	8.1 (4.0)	4.5 (1.2)
8	129	7.2 (3.4)	52	2.8 (0.9)	11.4 (2.6)	2.5 (1.7)
9	143	22.1 (15.5)	53	3.1 (1.5)	10.7 (3.2)	3.2 (1.8)
10	273	7.1 (4.0)	86	3.5 (1.7)	11.6 (2.8)	2.4 (2.0)
11	357	10.2 (7.1)	76	3.0 (1.2)	7.9 (4.2)	4.7 (1.0)
12	384	14.6 (10.8)	76	3.3 (1.4)	11.7 (2.5)	2.2 (1.7)
13	838	7.4 (4.6)	93	3.0 (1.3)	9.6 (3.6)	3.7 (1.9)
14	893	8.9 (5.3)	54	3.9 (1.6)	8.3 (4.2)	4.7 (1.2)
15	1524	9.5 (6.3)	80	2.9 (1.2)	11.2 (2.9)	2.8 (1.7)
16	2982	7.1 (4.9)	73	2.8 (1.1)	8.4 (4.3)	4.9 (1.1)
2009						
1	6	2.3 (0.8)	6	2.2 (0.8)	10.7 (3.3)	2.7 (2.6)
2	16	3.0 (1.1)	14	3.8 (1.7)	11.0 (2.5)	3.2 (1.3)
3	22	3.2 (1.0)	10	2.7 (1.9)	8.0 (4.7)	5.5 (0.8)
4	35	10.2 (4.8)	28	3.4 (1.3)	9.9 (3.4)	3.6 (1.6)
5	24	1.9 (1.1)	16	3.3 (1.6)	8.9 (4.6)	5.2 (1.3)
6	66	6.3 (3.0)	11	2.7 (1.0)	9.5 (3.7)	4.0 (1.2)
7	97	7.7 (5.1)	75	4.7 (1.9)	7.0 (3.8)	4.3 (0.9)
8	57	3.8 (2.4)	29	3.5 (1.2)	8.2 (4.2)	4.6 (1.3)
9	112	5.0 (3.2)	21	2.6 (0.8)	10.0 (3.1)	3.0 (1.8)
10	106	3.7 (2.6)	61	3.5 (1.4)	8.0 (3.8)	4.6 (1.1)
11	239	6.0 (5.5)	83	3.2 (1.1)	8.9 (4.2)	4.5 (1.5)
12	219	16.7 (10.7)	77	3.4 (1.3)	10.3 (3.3)	3.1 (1.9)
13	591	5.4 (3.9)	77	3.4 (1.3)	9.8 (2.6)	3.1 (0.8)
14	510	6.9 (4.9)	86	4.2 (1.6)	11.1 (3.2)	3.2 (1.8)
15	914	5.1 (3.2)	84	3.3 (1.3)	8.3 (3.7)	4.1 (1.3)
16	1718	3.6 (2.4)	74	2.9 (1.1)	8.5 (3.9)	4.4 (1.4)

[a] Number of flowering individuals.

[b] Mean number of intact inflorescences per 0.8 m^2.

[c] Our aim was to sample ca. 80–90 individuals per population (or all individuals in the small populations) (see Methods for details). Fewer individuals were available for analysis due to subsequent herbivore damage (most populations) and inclusion of plants in another experiment (populations 3, 6 and 8).

[d] Assessed in 15 states, from 1 (late flowering individuals) to 15 (early individuals).

[e] Calculated for each individual as the mean difference in phenological state between the focal individual and all other sampled individuals in the population, expressing the degree of asynchrony: plants with a low and high value flower together with many and few other individuals, respectively.

Appendix 2 Mean size (based on counts from 2006–2009) and mean trait values (± SD) of offspring plants from ten populations of *Phyteuma spicatum* (abbreviations: caps. = capsules, flow. = flowering, infl. = inflorescence, max. = maximal, no. = number, pop. = population).

Pop. ID	Pop. size	No. of infl.	Max. infl. height	Max. infl. size	Leaf width	Flow. onset	Flow. duration	No. of seeds/caps.	Mean seed mass
F	6	2.6 ± 1.6	55.0 ± 8.1	5.7 ± 1.9	4.8 ± 0.8	159.0 ± 2.1	13.5 ± 2.6	12.3 ± 3.1	0.116 ± 0.024
WN	16	1.7 ± 1.2	56.9 ± 11.4	6.8 ± 2.3	5.2 ± 0.7	158.9 ± 2.5	12.5 ± 2.1	10.4 ± 4.7	0.131 ± 0.025
R	32	2.3 ± 1.1	56.9 ± 8.1	7.8 ± 3.1	4.9 ± 0.7	157.5 ± 2.8	12.8 ± 2.2	11.7 ± 5.0	0.128 ± 0.022
WW	60	2.0 ± 1.0	53.1 ± 10.8	6.1 ± 2.2	5.2 ± 0.9	159.1 ± 2.8	12.7 ± 2.6	10.0 ± 4.8	0.131 ± 0.023
TO	89	2.3 ± 1.3	57.1 ± 12.3	6.8 ± 2.1	4.8 ± 0.8	158.7 ± 2.8	14.1 ± 3.3	10.4 ± 4.8	0.130 ± 0.028
TN	205	2.4 ± 1.2	55.9 ± 10.5	6.2 ± 1.9	4.7 ± 0.8	158.5 ± 3.3	13.6 ± 2.7	10.0 ± 3.8	0.137 ± 0.026
IP	266	4.0 ± 2.0	56.3 ± 8.3	5.3 ± 1.7	4.7 ± 0.7	159.6 ± 2.2	12.5 ± 2.5	9.3 ± 4.4	0.129 ± 0.027
HH	665	2.4 ± 1.2	55.2 ± 9.3	6.8 ± 2.1	5.1 ± 0.9	157.4 ± 2.5	13.7 ± 2.3	11.1 ± 4.8	0.123 ± 0.020
TW	1055	2.6 ± 1.4	54.8 ± 10.2	6.3 ± 1.9	4.8 ± 0.8	159.0 ± 2.4	13.2 ± 2.2	10.0 ± 4.0	0.128 ± 0.023
BB	2235	2.8 ± 1.3	51.7 ± 11.5	6.5 ± 2.1	5.0 ± 0.8	159.9 ± 3.3	13.5 ± 2.5	10.8 ± 4.4	0.121 ± 0.025