Butterflies and moths - agents of pollinator-mediated selection and species separation in the two closely related carnations *Dianthus carthusianorum* and *D. sylvestris*

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Prologue

The evolution of plant-pollinator interactions

Angiosperms represent the most diverse and dominant group of plant organisms. With more than 250’000 species worldwide they outnumber by far other plant groups like mosses, ferns or gymnosperms. Flowering plants conquered the world’s terrestial habitats within a short period of 70 million years, beginning in the early cretacous (140 million years ago) and reaching dominance about 65 million years ago (Niklas 1983, Friis and Crepet 1987). Interestingly, insects co-radiated (to some degree) during the same period (Grimaldi 1999). Does the novelty of plant-pollinator interactions explain their success story? Doubtlessly, the establishment of animal-mediated pollination represented a dramatic key innovation in the realm of plant organisms. Since plants are sessile, genetic exchange was restricted predominantly to the very proximate neighborhood in the pre-pollinator era. Thus, gametic exchange mediated by animal vectors must have revolutionized sexual reproduction of plants. For the first time pollen was transported rather target-oriented. The evolution of plant-pollinator interactions was only possible, since one group of these organisms is sessile whereas the other is mobile. All this certainly set the stage for a rapid co-evolution of one of the most common mutualistic relationships with tremendous ecological consequences. Darwin described beautifully his idea about the origin of plant-pollinator interactions:

> ”Certain plants excrete a sweet juice … by glands at the base of stipules …, and the back of the leaf… . This juice, though small in quantity, is greedily sought by insects. Let us now suppose a little sweet juice or nectar to be excreted by the inner bases of the petals of a flower. In this case insects in seeking the nectar would get dusted with pollen, and would certainly often transport the pollen from one flower to the stigma of another flower. The flowers of two distinct individuals of the same species would thus get crossed; and the act of crossing, we have good reason to believe (as will hereafter be more fully alluded to), would produce very vigorous seedlings, which consequently would have the best chance of flourishing and surviving. Some of these seedlings would probably inherit the nectar-excreting power. Those individual flowers which had the largest glands or nectaries, and which excreted most nectar, would be oftenest visited by insects, and would be oftenest crossed; and so in the long-run would gain the upper hand. Those flowers, also, which had their stamens and pistils placed, in relation to the size and habits of the particular insects which visited them, so as to favour in any degree the transportal of their pollen from flower to flower, would likewise be favoured or selected. (Darwin 1859)

Obviously, since its appearance plant-pollinator interactions promoted and maintained biodiversity in both the plants’ and animals’ evolutionary history. Whether pollinators alone can promote speciation in sympatry is highly debated (Waser 1998, Coyne and Orr 2004, Waser and Campbell 2004). In contrast, it is widely acknowledged that under para- or allopatric conditions pollinators can promote divergence, at least together with other separating forces. For instance adaptation to local conditions (ecological speciation) can be paralleled by divergent evolution of flower traits in response to a different pollinator fauna (Hodges and Arnold 1994, Coyne and Orr 2004, Waser and Campbell 2004). Biotically pollinated plant families reveal higher species richness compared to abiotically pollinated ones (Eriksson and Bremer 1992, Grimaldi 1999, Ricklefs and Renner 2000). Even more, the floral parts, i.e. flower shapes, colours and odours, show a tremendous diversity in contrast to the vegetative parts of plants, which certainly represents adaptation in response to pollinator-mediated selection. Thus, pollinator-mediated selection most likely represents an important driving force in the evolutionary history of flowering plants.

Pollinator crisis

Today plant-pollinator interactions represent one of the most important ecological interactions in terrestrial ecosystems. Virtually every life-form on land depends directly or indirectly on the primary production of angiosperms, whereas the majority of these flowering plants in turn depends intimately on myriads of animal pollinators belonging to diverse taxonomic groups (e.g. bats, bees, beetles, birds, butterflies, moths, primates, etc.). This enormous interconnected biodiversity provides innumerable benefits on which also human welfare is inescapably dependent. Economical, ecological and cultural significance of pollination is more and more recognized by public perception (media, politicians and economists). Although the recent anxiety about a worldwide collapse of honey bee populations might represent nothing but another medial hysteria, at least a substantial part of the population becomes aware of our own dependence on pollination. However, even scientists are still far from being able to quantify the risks for human welfare in response to a potential global pollination crisis (Bond 1995, Allen-Wardell et al. 1998, Kearns et al. 1998), i.e. the consequences of the rapid loss of pollinator species. It is high time to realize that we are on the way to experience a dramatic loss in biodiversity with unpredictable consequences. Loosing the pollinator species might even accelerate the threat.

Besides these anthropocentric perspectives on the immediate impact of human welfare in response to a potential global pollination crisis, other risks related to pollination are hardly less problematic. Since pollination is a key interaction in most ecosystems the rapid loss of biodiversity might even be accelerated in response to the interruption of plant-pollinator relationships, e.g. the more pollinators species disappear the greater the risk of extinction (Bond 1995, Allen-Wardell et al. 1998, Kearns et al. 1998). We are still far from knowing whether the complex webs of plant-pollinator interactions buffer ecosystem integrity by broad redundancy, e.g. if some pollinator species can compensate the tasks of another pollinator species that went extinct or if there exists a critical number of pollinator species for ecosystem integrity. How complex this task can be is recognized if we consider the plant-pollinator interactions of only one flowering plant species. For example, if we observe that the considered plant species is visited by numerous and diverse pollinator species, we are likely tempted to conclude that this plant species is able to cope with the loss of few of these pollinator species. This might be a fatal conclusion. What if only one species supplies the bulk of pollination necessary for sustainable reproduction of the considered plant species? This trivial and rather extreme example clarifies that we are facing a huge challenge if we want to get a general idea about the relevance of plant-pollinator interactions for ecosystem integrity. We first have to quantitatively explore numerous particular plant-pollinator relationships before we are able to deduce some general conclusions (Herrera 1989, Waser et al. 1996). Most important is the question about the degree of specialization versus generalization for the plant as well as for the involved pollinator species (Waser et al. 1996, Johnson and Steiner 2000). Obviously, the more specialized the more vulnerable a species will be, especially if both partners depend exclusively on each other. Recent research revealed a rather nested structure of flower visitation in plant-pollinator webs (Memmott 1999, Bascompte et al. 2003), i.e. one of the two interdependent organisms being rather specialized whereas the other seems to be more generalized, e.g. generalized pollinators visit a specialized plant species (vice versa). As mentioned above, this pattern needs confirmation beyond the level of simple visitation patterns. We need hard evidence for whether observed visitations are in fact an important contribution to the reproduction of the considered plant species. Accordingly, we need to measure carefully each component of pollinator-mediated fitness. Pollinator importance represents such a measure and corresponds to the proportional benefit contributed by a pollinator species to the total reproductive success of a plant (Young 1988, Herrera 1989, Pettersson 1991, Waser et al. 1996, Olsen 1997). Pollinator importance equals the product of the pollinator's abundance, visitation frequency and pollination efficiency relative to the total benefit from all involved pollinator species. Estimates of pollinator importance will quantify the relative dependence of a plant species on its pollinator species, and thus clarify if and how plant species are threatened by disappearing pollinator species.

Pollinator-mediated selection on flower traits

Estimates of pollinator importance do not only tell us about the actual ecological relationships, they also pave the way to the study of the evolutionary processes in plant-pollinator interactions. The relative contribution of each of the three pollinator-mediated components (abundance, visitation frequency and pollination efficiency) to plant reproduction will illuminate which mechanisms promote pollinator-mediated selection, and thus how flower traits evolve in response to pollinator-mediated selection (Waser et al. 1996). For instance, pollinators might be biased in their colour vision and thus show a higher visitation frequency to particular floral colors. Or a change in the abundance of pollinator species might alter the frequencies of tongue-lengths in the pollinator guild, which in turn then changes the pollination efficiency among the extant variation of floral depth. Thus, the components of pollinator importance further clarify which of them promote pollinator-mediated selection, and whether this leads to generalist or specialist flowers. Imagine a plant species which is pollinated by numerous pollinators belonging to rather different and diverse taxonomic groups (e.g. bees, butterflies, flies). If all these visitors represent more or less inefficient pollinators, which by their collective visitation frequency determine the reproduction of the plant, the plant species represents a generalist with respect to pollination. Specialization, in contrast, takes place if a plant species alters its floral traits in response to selection of a subset of pollinator species (e.g. by adapting floral shape toward the shape of butterflies, altering floral colour toward the preferences of a certain pollinator group or species). There exist several scenarios toward specialization in floral traits, but all of them have in common that pollinator-mediated selection will only lead to an evolutionary change if the selected phenotypes experience a fitness advantage. This can happen either by overcompensating trade-offs (gain in fitness by specialization overcompensates the loss of pollination success by other pollinators) or by gaining an additional benefit (floral alterations do not affect the pollination efficiency of other pollinators) (Aigner 2001).

Plant-pollinator interactions in the flowering plant genus Dianthus

In the present thesis we consider evolutionary, ecological and conservational aspects of plant-pollinator interactions using two closely related carnation species, *Dianthus carthusianorum* and *D. sylvestris* (Caryophyllaceae), and their pollinator species. The two species represent ideal model systems for studying the plant-pollinator interactions. Still most of the profoundly investigated model systems in pollination biology are based on only few plant species. It is thus important to expand the range of study organisms of which we gain a deep understanding on their pollination. Both species are rather specialized in pollination (Lepidoptera) and restricted to fairly narrow ecological conditions, i.e. dry and nutrient poor habitats. Such habitats are more and more threatened due to human activities (nutrient input, habitat destruction and fragmentation). Additionally, these ecological conditions are also associated with rather threatened pollinator species, e.g. butterflies and solitary bees. Thus, these circumstances provide ideal premises to investigate plant-pollinator interactions in the context of the main contemporary challenges in conservation biology. It was thus one out of other aims to investigate whether the carnations might be threatened by disappearing pollinator species.

As mentioned above the two carnation species are closely related. Despite their obvious similarity they possess some very distinctive features, e.g. they differ in flower depth, inflorescence architecture and color. These differences are likely, at least in part, the result of differential pollinator-mediated selection regimes (Vogel 1954, Faegri and L. 1979). Such speculation however demands verification with respect to the modes of pollinator-mediated selection and its contribution during speciation. Since the two carnation species occasionally occur in sympatry, where some rare hybrids are found, we are in the fortunate position to investigate pollinator-mediated selection, the mechanisms upholding species barriers as well as the processes that potentially lead to the completion of speciation. The recorded rare hybrids indicate that the two carnation species are interfertile. However, the rareness of hybrids indicates also mechanisms impeding that the two species interbreed randomly. Observations suggest that the pollinators might play a crucial role in upholding the species isolation in sympatry (Grant 1949, 1994).

In the following four studies we investigated aspects of pollinator-mediated selection, extinction risk with respect to the plant-pollinator relationship, and isolation mechanism between the two closely related carnation species.

CHAPTER 1 - The evolution toward shorter flowers

In this study we experimentally tested whether and how pollinator-mediated selection shapes floral traits in response to differential pollination efficiency (one of the three components of pollinator importance). Variation in the depth of the tubular flower and the length of the proboscis of lepidopteran pollinators potentially causes differential pollination efficiency through mechanical fit. “Mechanical fit” implies a mechanism based on intimate physical contact between flower and pollinator. This evolutionary mechanism implies a mechanical function of flower shape with respect to pollination. It claims that the tremendous diversity of shapes observed in angiosperms is in part the result of selection in response to the optimization of pollination efficiency conducted by pollinators. To date, several studies have shown pollinator-mediated selection on tubular flowers (Nilsson 1988, Schemske and Horvitz 1989, Robertson and Wyatt 1990, Johnson and Steiner 1997, Maad 2000, Alexandersson and Johnson 2002, Castellanos et al. 2003). However, only one has explicitly shown selection toward improved mechanical fit (Campbell et al. 1996). In our experiment we were the first to do this by including at the same time the quantitative traits of the pollen donor flower (male phase), the pollinator and the pollen accepting flower (female phase), i.e. we have controlled for all traits responsible for the physical properties of pollen transfer.

CHAPTER 2 - Pollinator importance and pollination crisis

In this field study we identified the relevant pollinators of *D. carthusianorum* in a sympatric population with *D. sylvestris*. We estimated components of pollinator importance, i.e. pollination efficiency and visitation frequency under field conditions (Young 1988, Herrera 1989, Pettersson 1991, Waser et al. 1996, Olsen 1997). Furthermore, we clarified whether reproduction is limited by pollen supply mediated by the recorded pollinator species. Finally, we attempted to answer whether *D. carthusianorum* is rather specialized than generalized in pollination and whether its local persistence is vulnerable with respect to its relevant pollinator species. Since two of the potentially most important pollinator species disappeared at numerous sites during the last decades in the southwestern region of Switzerland (Rhone valley), where we conducted our study, local extinction is a realistic scenario for these pollinator species (Gonseth 1987) and hence also for *D. carthusianorum*.

CHAPTER 3 - Intra- and interspecific reproductive barriers

In this study we explore whether *D. sylvestris* is separated by intra- and interspefic reproductive barriers. For that purpose we applied several intra- and interspecific (*D. carthusianorum*) pollinations by hand and recorded reproductive success in terms of fruit set, number seeds per fruit, seed weight and germination success. These measurements indicate whether postmating prezygotic isolation and/or intrinsic postzygotic isolation (until F1-viability) contributes to species separation in the two sympatric carnation species. Furthermore, intraspecific crosses clarify whether selfing leads to inbreeding effects and whether local differentiation leads to outbreeding effects.

CHAPTER 4 - Ethological isolation

Preferential visitation of pollinators to flowers of one carnation species over the other might significantly separate the two sympatrically occurring plant species (Grant 1949, 1994). Since flower visitation belongs to the first isolation mechanisms in a sequence of diverse other isolation barriers, this mechanism might represent a strong absolute as well as relative isolation barrier (Ramsey et al. 2003). Observations during several seasons of field work suggested that the diurnal butterflies visiting *D. carthusianorum* show a high fidelity to this carnation species (personal observations). From this we hypothesized that gene flow from *D. carthusianorum* to *D. sylvestris* is strongly limited by the pollinator species' preferences of *D. carthusianorum*.

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Chapter 1

Selection toward shorter flowers by butterflies whose probosces are shorter than floral tubes

Daniel Bloch and Andreas Erhardt

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SELECTION TOWARD SHORTER FLOWERS BY BUTTERFLIES WHOSE PROBOSCES ARE SHORTER THAN FLORAL TUBES

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Abstract. Darwin's meticulous observations on the function of floral shape led to his famous prediction of a long-tongued pollinator, which he believed to be the evolutionary trigger for the long-spurred flowers of the Madagascar star orchid. Although tubular flowers are common, long tubes or spurs are an exception, suggesting that selection maintaining short flowers is widespread. Using the butterfly-pollinated carnation *Dianthus carthusianorum* and two butterfly species differing in proboscis length (*Melanargia galathea* and *Inachis io*) as model organisms, we experimentally demonstrate a reduction in pollinator efficiency with an increasing difference between proboscis length and floral tube length. Such a relationship is a prerequisite for the evolution of floral shape in response to pollinator morphology.

Key words: butterfly pollination; coevolution; Dianthus carthusianorum; *generalization;* Inachis io *L.; mechanical fit;* Melanargia galathea *L.; pollination efficiency; pollinator effectiveness; pollinator-mediated selection; proboscis length; specialization.*

Introduction

The great diversity of flowering plants is generally viewed as a result of key innovations and (co-) evolutionary processes with pollinators. The primary task of flowers is to promote pollen transfer, a physical process based on the intimate contact of the interacting organisms. The shape of flowers should influence the efficiency in pollen transfer. The diversity in flower shapes reflects this adaptive "mechanical fit" to pollinator morphology (Darwin 1862, Grant and Grant 1965, Stebbins 1970). "Mechanical fit" implies a functional relationship between the morphologies of flowers and pollinators with regard to pollen transfer. This apparent "fit" was an essential argument for the general formulation of "pollination syndromes" (Vogel 1954, Faegri and van der Pijl 1979, Fenster et al. 2004, Wilson et al. 2004). Recently this appealing theory has been doubted (Wilson 1995, Ollerton 1996, 1998, Waser et al. 1996, Johnson and Steiner 2000), as most pollinators have been found to be generalists in flower visitation and most flowers seem to have a broader spectrum of pollinators than their floral characters might suggest. In addition, some recent analyses of plant–pollinator webs (Memmott 1999), in particular flower visitation patterns, have revealed broad spectra of visited plant taxa, suggesting that the initial view of a tightly knit system determined by mechanical fit may not hold up. However, flower visitation does not necessarily imply successful pollination (Olsen 1997). Flower visitors can deplete nectar resources without a substantial contribution to pollination and may rather be classified as floral parasites than as mutualists. Thus, the actual fitness contribution of a pollinator species is the product of its pollination efficiency (removal and deposition of pollen grains), its abundance, and its visitation rate (Sugden 1986, Galen and Newport 1987, Herrera 1987, Armbruster 1988, Young 1988, Herrera 1989, Waser et al. 1996, Olsen 1997, Fulton and Hodges 1999). Consequently, pollinator-mediated selection is driven either by one or a combination of the three components, e.g., flowers traits could be selected by pollinator's preferences or by higher pollination efficiencies related to a better mechanical fit. Pollination efficiency is a function of mechanical fit; the better the physical fit between pollinator and flower morphology, the higher the pollination efficiency is. Hence, pollinator-mediated selection towards higher pollination efficiency is related to adaptive alterations in flower morphology toward a better fit to the pollinator's morphology. Documentation of evolutionary steps in "mechanical fit" is a complex task involving three morphological levels, i.e., both male and female reproductive floral parts and pollinator traits relevant for pollen transfer. Since the depth of tubular flowers is a morphological key trait with respect to pollination efficiency, tubular flowers provide an excellent model system for investigating pollinator-mediated selection on the mechanical properties of quantitative morphological floral traits.

So far, only positive correlations between pollinator tongue length, floral tube/spur depth, and plant reproductive success have been documented (Schemske and Horvitz 1989, Maad 2000, Alexandersson and Johnson 2002), and experimental manipulations in the field have shown that long-spurred orchid flowers are

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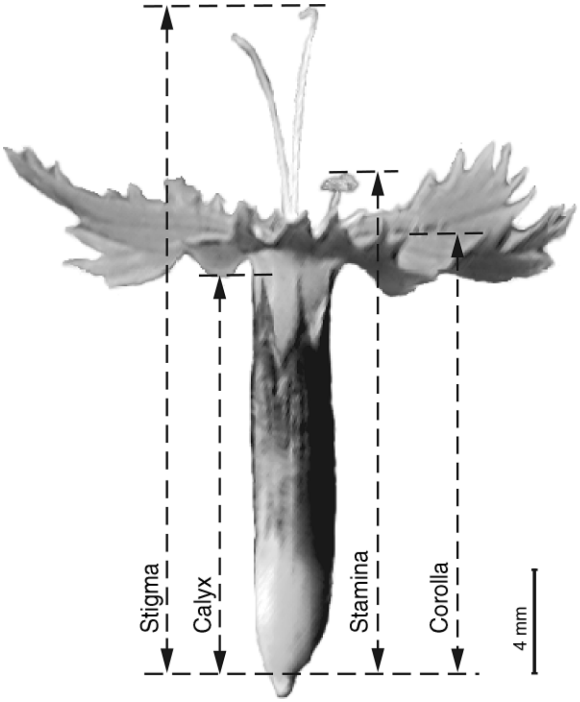

FIG. 1. Illustration of the measured floral traits of the protandrous and tubular flower of *Dianthus carthusianorum*.

better at exporting and receiving pollinia than shorter-spurred conspecific flowers (Nilsson 1988, Robertson and Wyatt 1990, Johnson and Steiner 1997). Furthermore, studies on plant species with variable floral phenotypes have shown that different pollinator types, e.g., hummingbirds vs. bumble bees, are most effective at pollinating different morphological floral types (Campbell et al. 1996, Castellanos et al. 2003). However, pollinator-mediated selection on tube or spur length was not confirmed in several studies involving lepidopteran species (Pettersson 1991, Herrera 1993, Luyt and Johnson 2001).

Evidence for selection improving "mechanical fit" is rare (Campbell et al. 1996). So far, no study has combined simultaneously the continuous variation of pollinator traits, a critical component of the mutual "mechanical fit," with the variation of the related flower traits in an experimental approach. Here we examined pollinator-mediated selection on the floral morphology in *Dianthus carthusianorum* L. by measuring pollen transfer between flowers varying in calyx tube, corolla depth, and stigma and anther length using two butterfly pollinators with different proboscis lengths, the long-tongued *Inachis io* L. and the shorter-tongued *Melanargia galathea* L. Since *D. carthusianorum* is exclusively pollinated by lepidopteran pollinators (Bloch et al. 2006), tubular width of the flower plays a less critical role for mechanical fit as for plant species that are pollinated by different types of pollinators, e.g., hummingbirds vs. hawkmoths (Campbell et al. 1996, Temeles 1996). Therefore, we consider mechanical fit as a one-dimensional problem, i.e., the fit of flower depth and proboscis length. With our straightforward approach consisting of an experimental unit made up of a butterfly, a flower in male phase, and a flower in female phase, we received a direct and causally linked measure of pollen transfer. This allowed us to control for the morphological effects of the flower in the male phase, the flower in the female phase, and the involved butterfly individual on pollen transfer. We expected to find differential pollination efficiencies, and thus pollinator-mediated selection, in response to continuous morphological variation in the lengths of male and female floral traits and the pollinator's proboscides.

MATERIAL AND METHODS

Organisms

Dianthus carthusianorum L. (Caryophyllaceae) is a gynodioecious perennial herb. The protandrous flowers consist of a calyx tube, protruding unfused petals, stamens, and stigma lobes (Fig. 1). The flowers are pollinated mainly by butterflies (Knuth 1898, Hegi 1979, Bloch et al. 2006). The tubular flower architecture of *D. carthusianorum* is an excellent model for studying the evolutionary process affecting flower depth mediated by pollinators. Protandry allows convenient separation of male and female function under experimental conditions. Natural populations of *D. carthusianorum* show continuous variation in flower depth that is distinct between individuals, populations, and sympatric congeners (D. Bloch, *unpublished data*).

The butterfly species *Inachis io* L. (Nymphalidae) and *Melanargia galathea* L. (Satyridae) served as experimental pollinators, because their proboscides differ in length (15.1 ± 0.4 mm and 12.7 ± 0.7 mm, respectively [mean ± SD]; *t* test, $P < 0.0001$), but both remain within the flower depth range of *D. carthusianorum*. *Melanargia galathea* (Bloch et al. 2006) and *I. io* (A. Erhardt, *unpublished data*) are both recorded pollinators of *D. carthusianorum*.

Experiment

Experimental plants were raised in pots from seeds and held at the facilities of the Botanical Institute of the University of Basel. The seeds were collected at Bitsch (VS) in Switzerland. Each experimental flower was cut off and put into a vial. Floral traits (length of calyx, 14.71 ± 1.47 mm; length of corolla, 15.66 ± 1.46 mm; length of stamen, 19.44 ± 1.35 mm; length of stigma, 22.03 ± 2.21 mm; diameter of corolla, 18.35 ± 2.73 mm) were measured using digital calipers (Fig. 1), and the number of exposed stamina was counted. To measure pollen transfer, we first offered a flower in the male phase and then an emasculated flower in the female phase to each butterfly. Flowers for measuring female function were emasculated during their male phase 2–3 d before the experiment. Each flower in either male or in female phase was used only for one experiment ($N = 362$

male and female flowers). Time spent for each visit was held as constant as possible by interrupting visits after ~2 min, if butterflies had not left the experimental flowers earlier (88.3% of all visits within 110–130 s). We did not control for nectar reward, but checked whether time spent for visits influenced the number of pollen grains transferred, assuming that time spent could have been the result of the amount of nectar present in the experimental flower. Adults of *M. galathea* were caught in dry grasslands at Nenzlingen (BL) and Leuk (VS) in Switzerland. Butterflies of *I. io* were raised from larvae collected near Rheinfelden, Germany, and fed stinging nettle. Adults of both species were kept in a climate chamber under conditions that forced them to stay inactive (low light and temperature at 15°C) and were daily fed by hand with a sugar solution. Butterflies were numbered individually and randomly chosen for each experiment (*I. io*, $N = 37$; *M. galathea*, $N = 39$). Butterflies that refused to visit a flower were replaced by other randomly selected individuals. Experiments were performed under constant climatic conditions in a transparent lighted climate chamber (26°C, 60% air humidity). After the experiment, stigmas with deposited pollen grains were fixed on slides with glycerol jelly that contained safranin ($C_{20}H_{19}ClN_4$) to stain pollen grains (Beattie 1971). Deposited pollen grains were counted under a microscope with the aid of photographs of different focus levels of the microscope, laid over one another using Adobe Photoshop 7 (Adobe Systems, San Jose, California, USA), which allowed us to accurately identify pollen grains. Finally, the proboscis length of each individual butterfly was measured.

Statistics

Statistical analyses were calculated with R statistical software (R Development Core Team 2003). We employed linear mixed-effects models (LME; Pinheiro and Bates 2000) to analyze the influence of the two butterfly species and floral traits on the transferred number of pollen grains. The dependent variable (number of grains) was square-root transformed to adjust for heteroscedasticity. Since the lengths of the considered floral traits are strongly correlated to one another, we included only one floral trait per flower in our different analyses to rule out ambiguous results from colinearity. The correlation of floral traits was separately analyzed by Pearson correlations for all trait combinations (Lertzman and Gass 1983). Furthermore, we performed model selection on the basis of the Akaike Information Criterion (AIC), i.e., terms were excluded from a model if $P > 0.2$ (corresponds to the decision based on AIC). Additionally, we neglected serial correlations for the within-group experimental order of repeatedly used butterfly individuals, since we could not find any serial dependence (coefficient for autocorrelation, $\rho = -0.012$; likelihood ratio test, $P = 0.87$). Since time was held quite constant for each experimental flower visit, time was not related to the number of pollen grains transferred (donor flower, $F_{1,353} = 0.015$, $P = 0.90$; acceptor flower, $F_{1,353} = 0.033$, $P = 0.86$), and therefore, we excluded time from our analyses.

Single traits.—The main hypothesis was tested with a model consisting of the butterfly species as fixed factor, the floral traits (stigma and stamen length and number of exposed stamina) as covariates, and the butterfly individuals as repeated random factor, i.e., butterflies are nested within pollinator species (model A). As most of the variation in proboscis length appeared between the two pollinator species, we analyzed two further alternative models with respect to the main hypothesis. In the first model we replaced the factor pollinator species by the covariate proboscis length (model B), and in the second model we weighted the dependent variable number of pollen grains by the mean proboscis length, i.e., we divided the number of pollen grains by the proboscis length (model C). The former tested for an overall effect of proboscis length and the latter indicates whether the pollinator species effect is caused by proboscis length (or correlated traits of the butterfly).

Further statistical analyses were conducted testing the effect of floral traits and differences thereof (stamina, stigma, calyx, corolla) on pollination efficiency. Because of the multiple interdependencies of the variables, we did not correct the *P* values for multiple statistical analyses. Thus, these results have to be considered as exploratory. We analyzed these relationships with the same error structure given by butterfly individuals as random factor (nested within butterfly species). In every statistical model we consistently incorporated the same floral traits for the flower in male as well as the flower in female phase within a statistical model, e.g., we considered the influence of calyx length on the number of pollen grains transferred for the flower in male (donor) as well as the flower in female (acceptor) phase. Exploratory analyses cover three qualitatively distinctive relationships with the first being the influence of calyx and corolla length (complements the single-trait analyses analogous to the main analysis with stamina/stigma length, models D–E).

Trait length differences.—The second class of analyses investigates the influence of the difference in length of flower traits within the flower (models F–H). The third class of analyses covers the influence of the difference between the length of a flower trait and the involved length of the proboscis on pollen transfer function (models I–K). Models without significance for the incorporated floral traits (based on AIC, $P > 0.2$) were then analyzed separately for each butterfly species.

Selection differentials.—We also estimated standardized selection differentials (S') as the covariance of each character divided by the character's standard deviation (Lande and Arnold 1983). Fitness was assumed to be represented by the amount of pollen transfer (Bloch et al. 2006), i.e., relative fitness was calculated by dividing the number of transferred pollen grains through the mean number of transferred pollen grains. Selection differentials were estimated either for data pooled with both pollinator species (common slope indicated by nonsignificant interaction) or for only one pollinator species (floral traits are only significant when considered for only one species). Since analyses for selection differentials deviated from distributional assumptions, we did not perform tests. Whether fitness is related to the explored traits is appropriately tested in the models (A–K). Furthermore, the considered traits within the same flower are strongly correlated and therefore cause ambiguous colinearity. Thus, selection gradients, which reveal direct and indirect (correlated) as well as concave/convex selection, were not calculated (Lande and Arnold 1983, Mitchell-Olds and Shaw 1987, Phillips and Arnold 1989). Consequently, we were not able to separate direct from indirect selection and could not test whether fitness minima or maxima for different traits on the same flower exist.

Results

The axial lengths of the four floral morphology traits within a flower were all correlated (for all, $P < 0.0001$); calyx length and corolla length, $r = 0.78$ (± 0.043 CI, $t_{360} = 16.6$); calyx with stamen, $r = 0.66$ (± 0.068 CI, $t_{360} = 16.6$); calyx with stigma, $r = 0.62$ (± 0.068 CI, $t_{360} = 14.9$); corolla with stamen, $r = 0.74$ (± 0.051 CI, $t_{360} = 20.8$); corolla with stigma, $r = 0.68$ (± 0.060 CI, $t_{360} = 17.7$).

Single traits.—The main analysis (Table 1, model A) revealed that longer stigmas received less pollen and longer anthers exported less pollen after a single visit of either butterfly species (Fig. 2A, B). The long-tongued butterfly species *I. io* transferred more pollen than did the shorter-tongued *M. galathea* (Fig. 2C). Variation in the number of exposed stamina (Table 1) did not significantly influence pollen export. Interaction terms and the diameter of the corolla were not related to pollen transfer ($P > 0.2$) in this and all forthcoming analyses and thus have been removed from the respective final models (Table 1).

In an alternative model (Table 1, model B), when the covariate proboscis length was used in a model to replace the factor butterfly species (Table 1, model A), the higher F value of the covariate proboscis length indicates a better fit, supporting the hypothesis that the longer tongue of *I. io* was responsible for the higher pollination efficiency of this butterfly species. Moreover, the species effect of butterflies became nonsignificant when the dependent variable, number of pollen grains, was weighted (divided) by the butterflies' proboscis length (Table 1, model C). Furthermore, calyx and corolla length were only affecting pollen export mediated by the shorter-tongued *M. galathea* (Table 1, models D and E), i.e., the traits were nonsignificant when separately analyzed for *I. io*.

Trait length differences.—A greater difference between floral traits within a single flower (Table 1, models F–H) significantly reduced pollen export and deposition, but not for pollen deposition (model F, acceptor) relative to the difference between corolla and calyx length. Again, only visits of the shorter-tongued *M. galathea* resulted in differential pollen export in response to the difference between corolla and calyx length (model F), i.e., the trait differences were nonsignificant if separately analyzed for *I. io*.

A greater difference between proboscis and stamina/stigma length significantly reduced pollen export and deposition (Table 1, model I), but not for pollen export (model J, donor) relative to the difference between proboscis and calyx length. Again, only for the shorter-tongued *M. galathea* did the differences between proboscis and calyx length and proboscis and corolla length result in differential pollen deposition (models J and K), i.e., the trait differences were nonsignificant if separately analyzed for *I. io*.

The estimated selection differentials range from 0.043 to 0.238 and are for all single traits and trait length differences listed in Table 1. Their signs and magnitudes are in good correspondence with the estimated coefficients of the LME analyses and the respective significance. Note that selection differentials are estimated either from pooled data of both pollinator species or, if indicated, from data of only one butterfly species.

Discussion

The results of the present study suggest that overall these two pollinator species may exert directional selection toward shorter male (stamen length) and female (stigma length) reproductive organs (Table 1, model A; Fig. 2). These floral traits are strongly correlated with other depth-determining flower components. Floral phenotypic variation has been documented for *D. carthusianorum* at the level of individuals and populations (Bloch 2000). Some of this variation is likely heritable, which is supported by numerous studies examining heritabilities. Mean heritabilities in other plant species ranged from 0.2 for traits under strong selection (life history) up to 0.45 for traits under less selection (morphological traits; Mousseau and Roff 1987, Campbell 1996). To the extent that floral depth traits are at least partly genetic, our results suggest a potential evolutionary response, either through direct or indirect selection (Campbell et al. 1994), toward shorter flowers, at least when pollinated by species whose proboscis were shorter than the floral tubes. Nonsignificant interaction terms (butterfly species : stigma length, butterfly species : stamina length) indicate that these two pollinator species do not exert significantly different selection on floral traits.

To date, pollinator-mediated selection on flower depth has only been documented by correlational studies from field surveys (Nilsson 1988, Robertson and Wyatt 1990, Johnson and Steiner 1997, Maad 2000, Alexandersson and Johnson 2002). One field study reported of a temporally labile fitness advantage of flowers with shorter tubes, hypothesizing that the high abundance,

TABLE 1. Details on the conducted linear mixed-effects (LME) model analyses for flower and pollinator traits.

Variable	S'	LME *n*	df	*F*	*P*	Estimated coefficient	SE
Single traits							
Model A							
Pollinator species		76	1, 74	5.71	**0.020**	49.86	7.22
(*Melanargia galathea*)						−1.73	0.87
Stamina length donor	−0.104	362	1, 283	4.95	**0.027**	−0.59	0.30
Stigma length acceptor	−0.203	362	1, 283	19.72	**<0.0001**	−0.82	0.18
No. exposed stamina		362	1, 283	1.76	0.186	−0.36	0.27
Model B							
Proboscis length	0.145	76	1, 74	7.13	**0.009**	0.68	0.31
Stamina length donor	−0.104	362	1, 283	5.09	**0.025**	−0.60	0.29
Stigma length acceptor	−0.203	362	1, 283	18.89	**<0.0001**	−0.80	0.18
No. exposed stamina		362	1, 283	1.68	0.196	−0.35	0.27
Model C							
Pollinator species		76	1, 74	0.40	0.530	49.16	7.16
(*Melanargia galathea*)						−0.21	0.86
Stamina length donor	−0.104	362	1, 283	4.99	**0.026**	−0.59	0.29
Stigma length acceptor	−0.203	362	1, 283	20.41	**<0.0001**	−0.82	0.18
No. exposed stamina		362	1, 283	1.72	0.191	−0.35	0.27
Model D†							
Calyx length donor	−0.001	171	1, 130	0.03	0.850	0.00	0.47
Calyx length acceptor	−0.104	171	1, 130	3.85	*0.052*	−0.68	0.35
Model E†							
Corolla length donor	−0.059	171	1, 130	2.83	*0.095*	−0.63	0.40
Corolla length acceptor	−0.169	171	1, 130	8.19	**0.005**	−1.03	0.36
Trait length differences							
Model F†							
Corolla-calyx donor	−0.083	171	1, 130	5.60	**0.019**	−1.02	0.48
Corolla-calyx acceptor	−0.128	171	1, 130	0.37	0.544	−0.42	0.50
Model G							
Pollinator species		76	1, 74	5.84	**0.018**	28.87	2.31
(*Melanargia galathea*)						−1.71	0.86
Stamina-calyx donor	−0.155	362	1, 284	11.21	**0.001**	−0.92	0.36
Stigma-calyx acceptor	−0.181	362	1, 284	8.45	**0.004**	−0.96	0.23
Model H							
Pollinator species		76	1, 74	11.78	**0.001**	24.63	1.72
(*Melanargia galathea*)						−1.83	0.86
Stamina-corolla donor	−0.092	362	1, 284	11.21	**0.001**	−0.21	0.19
Stigma-corolla acceptor	−0.182	362	1, 284	8.45	**0.004**	−0.89	0.31
Model I							
Pollinator species		76	1, 74	5.91	**0.018**	24.95	1.68
(*Melanargia galathea*)						1.47	1.20
Proboscis-stamina donor	0.174	362	1, 284	6.04	**0.015**	0.52	0.28
Proboscis-stigma acceptor	0.238	362	1, 284	18.28	**<0.0001**	0.75	0.18
Model J†							
Proboscis-calyx donor	0.043	171	1, 130	0.26	0.612	−0.03	0.45
Proboscis-calyx acceptor	0.131	171	1, 130	4.24	**0.041**	0.67	0.33
Model K†							
Proboscis-corolla donor	0.095	171	1, 130	3.22	*0.075*	0.35	0.40
Proboscis-corolla acceptor	0.186	171	1, 130	8.29	**0.004**	0.93	0.32

Notes: The first column lists the variables used for donor (male phase) and acceptor (female phase) flowers in the respective model. All listed traits represent trait lengths (mm) along the flower axis. The dependent variable equals the number of transferred pollen grains (square-root transformed), except in model C. In model C, before taking the square root, we divided the number of transferred pollen grains by the overall mean proboscis length of the respective butterfly species. Significant results appear in boldface type, and statistical trends appear in italic type. Standardized selection differentials (S') were estimated as the covariance of each character and fitness divided by the character's standard deviation. As floral traits considered in models A–C are identical, S' values do not differ. Note that the selection differentials for the models without daggers were estimated from data pooling both pollinator species, whereas those with daggers were estimated only from data containing the indicated pollinator species. Where models are based on data including both pollinator species, the species in parentheses (*M. galathea*) represents the difference to the reference group (*Inachis io*).

† Models including only *M. galathea.*

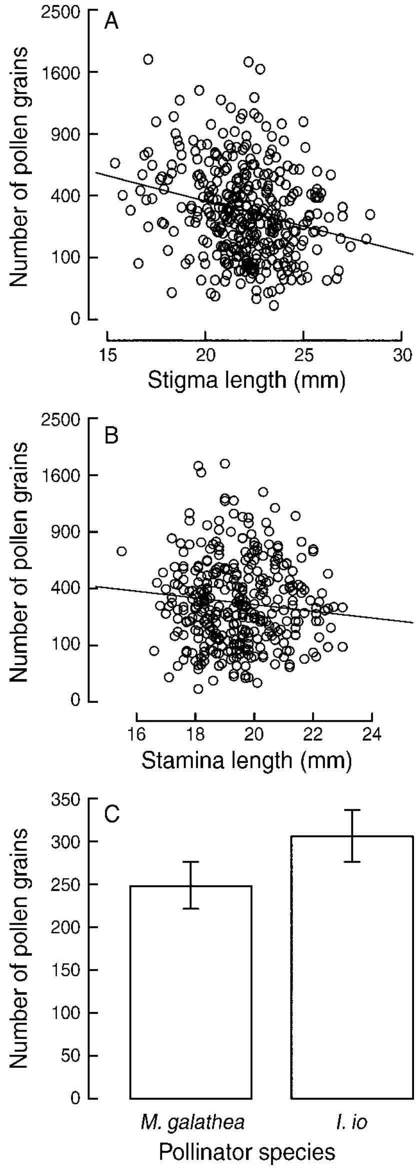

FIG. 2. Pollen grains transferred by butterflies visiting a flower in the male phase and subsequently a flower in the female phase. All graphs represent estimates from main hypotheses considered in model A, analyzed by a linear mixed-effects (LME) model (Table 1). (A) Influence of stigma length of the receiving flower in the female phase ($F_{1,283} = 19.72$, $n = 362$, $P < 0.0001$; square-root transformed, adjusted for mean stamina length and mean pollen transfer of *Inachis io*) and (B) influence of stamina length of the donor flower in male phase ($F_{1,283} = 4.96$, $n = 362$, $P = 0.0268$) on the amount of pollen transfer (square-root transformed, adjusted for mean stigma length and mean pollen transfer of *I. io*). (C) Pollen transfer (mean ± SE) of the two butterfly species *Melanargia galathea* and *I. io*, adjusted for the mean stamina and stigma length ($F_{1,74} = 5.68$, $n = 76$, $P = 0.0197$).

though temporally unstable, of short-tongued bumble bees was responsible for it (Schemske and Horvitz 1989).

In the present study, *I. io* transferred more pollen than *M. galathea*. Since the number of pollen grains deposited was positively related to proboscis length (Table 1, model B) and the species effect became nonsignificant (Table 1, model C) when the dependent variable (i.e., number of pollen grains) was divided by the proboscis length, differential pollination efficiency was likely caused by the difference in proboscis lengths of the two butterfly species. However, we cannot rule out a causal relationship of pollination efficiency with other pollinator traits that are potentially correlated with proboscis length. An effect of proboscis length on the amount of pollen transfer is also supported by the observation that the stigmatic surface contains papillae, which are concentrated toward the tip of the stigma, and this enlarged surface presumably improves adhesion of pollen grains (D. Bloch, *personal observation*). Thus, butterflies with shorter proboscides may have missed this prominent part of the receptive surface as they were mainly touching the lower part of the stigma. Furthermore, both tested butterfly species had shorter proboscis than the lengths of the stigmas (difference, *I. io*, 6.7 ± 2.17 mm; *M. galathea*, 9.62 ± 2.45 mm) and stamina (*I. io*, 4.26 ± 1.38 mm; *M. galathea*, 6.91 ± 1.47 mm). These observations may at least partly explain why even though we observed directional selection for shorter flowers, rendering flowers accessible for pollinators with shorter proboscides, *I. io*, the pollinator with the longer proboscis, was more efficient than *M. galathea.* These results suggest that pollinators, which have longer tongues than *I. io*, have once selected the length of the flower traits of the experimentally used plants.

The greater the difference between the length of proboscis and flower size (Table 1, models I–K), the lower the pollen transfer, supporting the notion that pollen transfer is a function of the mechanical properties of the interacting morphometric characters of both organisms. Species-specific differences are most likely caused by the fact that *M. galathea* often had shorter proboscides than the depth of the visited flower. To reach the bottom of the calyx tube *M. galathea* then had to dive almost perpendicularly to the flower axis, pushing apart the unfused petals. This change in position most likely reduced pollination efficiency.

All additional exploratory analyses and the estimated selection differentials (S') complement the findings of our main analyses (Table 1). They confirm consistently the direction of pollinator-mediated selection, i.e., selection toward shorter flowers, and support that the examined flower traits represent a functionally integrated entity with respect to their mechanical properties mediating pollen transfer. However, whether traits are directly or indirectly selected could not be differentiated by our experiments, because colinearity, caused by the strongly correlated traits within a flower, confounds the estimation of selection gradients and thus conceals potential selection for covariation of flower traits (Mitchell-Olds and Shaw 1987).

Field experiments demonstrated that the variation in the number of transferred pollen grains (i.e., pollination efficiency) documented in this experiment leads indeed to differential fecundity in *D. carthusianorum* under natural conditions and thus generates pollinator-mediated selection through differential pollinator effectiveness (Bloch et al. 2006). Even though we offered emasculated flowers in vials in our experimental setup, flower-visiting behavior of experimental butterflies did not differ from that of butterflies in the field (D. Bloch, *personal observation*). However, we cannot completely rule out that our experimental flowers may have experienced different pollination efficiencies compared to natural conditions (Price and Waser 1982, Hurlbert et al. 1996).

The scatterplots in Fig. 2A, B illustrate considerable amounts of unexplained variation that indicate high levels of noise inherent to the mechanical properties of pollen transfer and/or reduced levels of intraspecific variation in flower traits purged by pollinator-mediated selection. Intraspecific variation of flower traits affecting the mechanical fit of flowers and pollinators is often reduced compared to other flower traits (Cresswell 1998), implying that nonadaptive phenotypic variation of floral traits has been eliminated. Thus, the proportion of unexplained variation will increase if the variation in fitness related to pollination efficiency decreases.

We know of only one study with an analogous focus as our investigation (Campbell et al. 1996). In that study, pollen export was related to the fit of flower width of *Ipomopsis aggregata* with the depth of insertion of hummingbird bills. Both studies provide rare evidence for pollinator-mediated selection involving explicitly the "mechanical fit" between pollinator and flower morphology.

Whether such pollinator-mediated selection can generate a directional evolutionary response or even promote specialization in flower traits depends strongly on the long-term composition of the pollinator guild (Sugden 1986, Galen and Newport 1987, Herrera 1987, 1989, Armbruster 1988, Young 1988, Waser et al. 1996, Olsen 1997, Fulton and Hodges 1999, Aigner 2001, Price et al. 2005) and thus on the abundance and visitation rate of the involved pollinator species. However, specialization in flower traits can also occur alternatively to the most effective pollinator principle (Stebbins 1970, Waser et al. 1996), i.e., without trade-off, if a pollinator species contributes an additional benefit (Aigner 2001). In other words, very different constellations of pollinators can set the stage for pollinator-mediated selection toward an amelioration of mechanical fit (e.g., number of pollinator species, homogeneity of pollinator attributes, abundances and preferences of involved butterfly species, variation in pollination efficiencies). Finally, improvement in mechanical fit will only occur if variation in pollination efficiencies leads to differential reproductive success under the regime of the given pollinator guild. Thus, with these two pollinators, we would expect a reduction in flower depth for *D. carthusianorum* (for plants with similar variation in flower depth) if *M. galathea*, or a pollinator with the same proboscis length, is the dominant pollinator species. Pollinator-mediated selection toward shorter flowers renders the flowers accessible for shorter-tongued pollinators and hence broadens the potential spectrum of pollinator species. This could lead to a "re-generalization" in the pollination system (Armbruster and Baldwin 1998) and would correspond to the inversion of a coevolutionary race toward deep corolla and long-tongued pollinators (Nilsson 1988). As specialization toward deep flowers excludes short-tongued pollinators, it involves a trade-off to be compensated for in terms of visitation frequency and/or pollination efficiency by the longer-tongued pollinator species.

It is generally accepted that the evolution toward deeper flowers represents a process of increasing specialization. Consequently, a process in the opposite direction could be considered a "re-generalization" in the pollination system, to date exclusively demonstrated in a field survey (Schemske and Horvitz 1989) and by phylogenetic reconstruction (Armbruster and Baldwin 1998). We provide evidence for a mechanism that likely obstructs a coevolutionary race of plants and pollinators toward deep flowers and long tongues and thus a plausible explanation for the rarity of very deep flowers among the common phenomenon of tubular or spurred flowers.

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Chapter 2

Pollination crisis in the butterfly-pollinated wild carnation *Dianthus carthusianorum*?

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Research

Pollination crisis in the butterfly-pollinated wild carnation *Dianthus carthusianorum*?

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Summary

- Knowledge of pollination services provided by flower visitors is a prerequisite for understanding (co)evolutionary processes between plants and their pollinators, for evaluating the degree of specialization in the pollination system, and for assessing threats from a potential pollination crisis.
- This study examined pollination efficiency and visitation frequency of pollinators – key traits of pollinator-mediated fecundity – in a natural population of the wild carnation *Dianthus carthusianorum*.
- The five lepidopteran pollinator species observed differed in pollination efficiency and visitation frequency. Pollinator importance, the product of pollination efficiency and visitation frequency, was determined by the pollinator's visitation frequency. Pollination of *D. carthusianorum* depended essentially on only two of the five recorded pollinator species. Seed set was pollen-limited and followed a saturating dose–response function with a threshold of *c.* 50 deposited pollen grains for fruit development.
- Our results confirm that *D. carthusianorum* is specialized to lepidopteran pollinators, but is not particularly adapted to the two main pollinator species identified. The local persistence of *D. carthusianorum* is likely to be at risk as its reproduction depends essentially on only two of the locally abundant, but generally vulnerable, butterfly species.

Key words: pollination efficiency, visitation frequency, pollinator limitation, pollination crisis, pollinator-mediated selection, tubular flowers, *Dianthus carthusianorum*.

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Introduction

Flowering plants rely primarily on insect vectors for efficient pollen transfer (Bond, 1995; Kearns & Inouye, 1997; Waser & Campbell, 2004). Since Darwin, the tremendous diversity of flower shapes, colours and odours has been interpreted as an adaptation to pollinators (Darwin, 1862). Flowers and pollinators were implicitly assumed to be coadapted because of their obvious associations. Eventually, the descriptive concept of pollination syndromes established a seemingly sound explanation for the functionally related diversity of flowers and pollinators (Vogel, 1954; Faegri & van der Pijl, 1979). Altogether, this led to the biased perception that plant–pollinator relationships are rather specialized. In recent years this paradigm has come under scrutiny (Ollerton, 1996; Waser *et al.*, 1996; Ollerton, 1998; Johnson & Steiner, 2000); for example, analyses of plant–pollinator webs revealed that most plant species within a community were visited by numerous pollinators stemming from a broad taxonomic spectrum (Memmott, 1999). However, visitation does not imply successful pollination (Olsen, 1997). Rather, the relevance of a pollinator species is its effective contribution to the plant's fecundity. Therefore only detailed investigations will uncover whether a plant's flowers are specialized or generalized with respect to the pollination system. The degree of specialization vs generalization in plant–pollinator relationships has implications for conservation biology. Recent speculation about the effect of a decreasing pollinator fauna on plant fitness (pollination crisis: Bond, 1995; Allen-Wardell *et al.*, 1998; Kearns *et al.*, 1998; Karrenberg & Jensen, 2000) makes it even more important to identify the essential factors of the interaction between plants and their pollinators.

The carnation *Dianthus carthusianorum* (Caryophyllaceae) is a prime example of a plant species that appears to have a specialized pollination. The most obvious characteristic feature of *D. carthusianorum* are its conspicuous, tubular shaped flowers which provide a mechanical barrier to nectar consumption for flower visitors with too short proboscides. By quantitatively analysing the dependence on its pollinator species, the risk of extinction through potentially decreasing pollinator species can be assessed.

A pollinator's importance to a plant equals its relative contribution to the plant's reproduction, and consists of its abundance, visitation rate and pollination effectiveness (Waser *et al.*, 1996; see also Sugden, 1986; Herrera, 1987; Armbruster, 1988; Young, 1988; Herrera, 1989; Olsen, 1997). These separate components of pollinator importance provide a detailed qualitative and quantitative characterization of the plant's dependence on its flower visitors. For instance, a plant species is considered to be highly specialized if only one out of several visiting species effectively pollinates the plant's flowers. In contrast, a plant is considered generalized if a diverse guild of flower visitors are similar in their pollination effectiveness. Therefore the components of pollinator importance tell us how and to what a degree a plant species depends on its different pollinator species, and eventually indicates whether a plant species is generalized or specialized with respect to its pollinators. Furthermore, the relative contributions of the different components of pollinator importance provide insight into the current selection regime, and hence the evolutionary processes of the plant–pollinator relationship (Waser *et al.*, 1996). For instance, only frequently visiting pollinator species can exert ample selection pressure to cause an evolutionary response toward specialization (an increase in pollination effectiveness).

In this study we recorded the flower visitors, estimated their components of pollinator importance, and evaluated their relative contribution to fecundity of the butterfly-pollinated carnation *D. carthusianorum.* The aims were: (1) to identify the relevant pollinator species in terms of pollinator importance; (2) to estimate for each pollinator species the relative influence of the separate components (visitation frequency and pollination efficiency) on its pollinator importance; (3) to judge the degree of specialization in the pollination system; and (4) to assess whether the loss of pollinator species might present a risk to the local persistence of *D. carthusianorum.* We further investigated the relationship between different pollen quantities deposited on the stigma and the resulting reproductive success in *D. carthusianorum.* This provides the link between pollination efficiency, the raw pollination effect and pollination effectiveness, the pollinator's contribution to plant fecundity.

Materials and Methods

Study plant and study site

Dianthus carthusianorum L. (Caryophyllaceae) is a gynodioecious perennial herb, forming a rosette of grass-like leaves and one to several, mostly unramified shoots 30–45 cm high. As far as is known, *D. carthusianorum* is not clonal and reproduces only sexually (Hegi, 1979). During the flowering period (June–October) these shoots produce an inflorescence with numerous protandrous flowers. The crimson-coloured petals are enclosed in a narrow calyx tube and end in a flat rim, which serves as a landing platform for pollinators. The flowers have two stigma lobes and 10 stamens, and are mainly visited and pollinated by butterflies that feed on the nectar secreted from nectaries at the base of the filaments at the very bottom of the calyx (Knuth, 1898). The calyx tubes at our study site had a mean length of 15.3 ± 2.6 mm (1 SD) with a diameter of 3–5 mm. Anthesis of a single flower lasted for 2–5 d (D. Bloch, A. Erhardt, pers. obs.). Selfing is generally prevented by protandry. However, selfing is possible through a back-curling movement of the stigma lobes at the end of anthesis.

Field work was conducted in a large natural population of *D. carthusianorum* (> 1000 individuals) growing in a rocky steppe (Festucion valesiacae, according to Ellenberg, 1996), on a south-facing slope near Leuk (Valais) in the Rhone valley in the Swiss Alps. *Dianthus carthusianorum* was the dominant flowering plant at the study site during the observation period, which lasted from the end of June to the end of July 2002.

Pollination efficiency

Pollination efficiency of different insect visitors of *D. carthusianorum* was assessed by covering 40 randomly chosen plants with cages to exclude pollinators from the end of June until the end of July 2002. A cage consisted of a thin, cubic wooden frame (20 × 20 × 50 cm) covered with nylon mesh (pore diameter 0.25 mm). These caged plants provided the virgin flowers for measuring the pollination efficiency of the different pollinators. Plants with virgin flowers in the female stage of anthesis were exposed to foraging insects and were observed continuously under good weather conditions. Observed visitors were identified, and the stigma lobes were harvested immediately after the departure of each insect. The harvested stigma lobes were fixed on a slide with a jelly fixative containing glycerol, gelatine, distilled water and Safranin stain ($C_{20}H_{19}ClN_4$) (Beattie, 1971). Pollen grains adhering to the stigma lobes were counted under the microscope. As experimental flowers were not emasculated, the measurements may also include self-pollen transferred to the stigma lobes by movements of the foraging insect. Stigma lobes of unvisited caged flowers were harvested regularly at the end of anthesis to assess the number of pollen grains deposited by selfing under insect exclusion.

Visitation frequency

The visitation frequency of floral visitors was estimated by counting visits of foraging insects on *D. carthusianorum* within three equally sized observation areas (8 × 8 m). Each visit was recorded and the corresponding species identified. The observed visitation frequencies of pollinators represent their activity on *D. carthusianorum*, but do not necessarily reflect their general abundances. Observation periods were distributed randomly throughout the day (from 09 : 00 to 16 : 00 h, which covers the most important foraging period; D. Bloch, A. Erhardt, pers. obs.), and the three observation plots were distributed randomly within the *D. carthusianorum* population. Each observation period lasted 30 min, each observation area contained the same flower density (20 inflorescences), and observations were conducted during optimal weather conditions for foraging butterflies (sunny, at most a slight breeze). This standard setting is referred to as an 'observation unit'. During July 2002, a total of 16 observation units were conducted. Butterflies foraged primarily during the morning because of strong thermic winds in the afternoon.

Pollinator importance

Pollinator importance was calculated as the product of pollination efficiency and visitation frequency of a given pollinator species (for details see Statistical analysis). Thus pollinator importance indicates the significance of each pollinator species to the reproduction of *D. carthusianorum* at our study site.

Relation between pollen quantity deposited on stigma and seed set

The relationship between the quantity of pollen deposited on the stigma and seed set was investigated by measuring seed set after deposition of varying pollen loads. This allowed us to verify whether, and how, pollination efficiency (the number of pollen grains deposited by a single visit of a pollinator) is related to realized fecundity, and thus whether pollination efficiency is a proper estimate for pollination effectiveness, the contribution to fecundity by each pollinator visit. Twenty-four randomly chosen plants were covered with cages to exclude pollinators. Emasculated virgin flowers in female phase were hand-pollinated with three categories of pollen load (small, intermediate, large). The pollen was deposited on the stigma lobes using a small brush. Each of the three categories was deposited on three different flowers of each study plant. If a plant produced three more flowers, the procedure was repeated, allowing resource-rich plants to express their resource availability through production of further flowers and fruits, rather than measuring the development of an experimentally limited, low number of fruits. Stigma lobes were harvested 3 d after hand pollination (ensuring fertilization) and were fixed in Safranin jelly, and the number of pollen grains on the stigma lobes was counted under the microscope. An intermediate distance of 15 m between pollen donor plant and pollen acceptor plant was chosen to reduce the probability of inbreeding or outbreeding effects (Schemske, 1983; Waser & Price, 1983; Waser, 1993). Two to three unpollinated flowers per plant were cut off and used for counting the mean ovule number per plant. By mid-August, when the focal fruits were ripe, each of the 24 plants in the experiment had produced five to 12 flowers, and the plants soon ceased producing further flowers. Matured fruits were harvested, and seed set was determined dividing seed number by mean ovule number per plant. The mean per plant was used, as counting ovules is a destructive method and it is therefore impossible to count first ovules and then seeds of the same fruit. However, analysis of ovule distribution showed that ovule number is fairly stable within the flowers of a plant (mean of coefficients of variation = 13.9 ± 5.0% (1 SD), $n = 6$).

Pollen loads deposited on stigmas of flowers with unlimited exposure to pollinators were assessed using stigma lobes from uncaged plants that were harvested at the end of anthesis and fixed in Safranin jelly. These flowers were not emasculated, hence the number of pollen grains represents the sum of insect-deposited pollen including cross-pollination and selfing. This is regarded as the number of pollen potentially determining reproductive success in the lifetime of a flower under natural conditions.

Statistical analysis

Statistical analyses were calculated with R Statistical Software (R Development Core Team, 2003). Conclusions about pollination efficiency were based on a linear mixed-effects model (Pinheiro & Bates, 2000) with the pollinator species as fixed and plant individuals as random factors. Violation of homoscedasticity required the omission of three pollinator species with low sample sizes (*Papilio machaon* L., $n = 4$; *Macroglossum stellatarum* L., $n = 3$; *Thymelicus sylvestris* (Poda), $n = 2$) and the square-root transformation of the dependent variable 'number of pollen grains'. Pollen deposition by selfing was included for comparison with the numbers of pollen deposited by the pollinator species. P values were corrected for multiple testing according to sequential Bonferroni technique (Holm, 1979).

Visitation frequencies of pollinator species were analysed using a generalized linear model (McCullagh & Nelder, 1989). As visitation frequency, the response variable, consisted of counts, we employed a model based on a Poisson distribution and controlled for overdispersion (var = mean, link = log). Two of the five observed pollinator species, *M. stellatarum* and *T. sylvestris*, were excluded from the analysis because they were not recorded at any observation unit.

Pollinator importance was estimated as the product of pollination efficiency and visitation frequency. To prevent pseudoreplicated samples of pollinator importance, we multiplied each measure of visitation frequency with the mean of a random

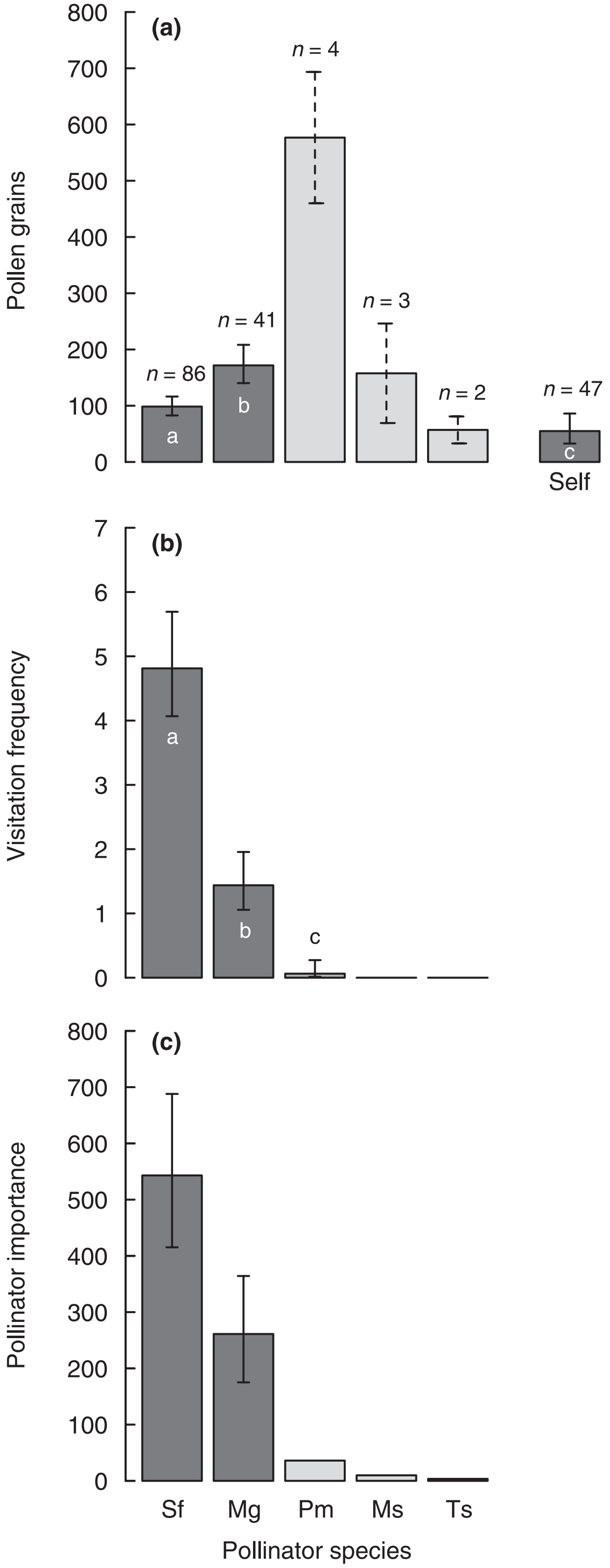

Fig. 1 Pollination estimates (means ± 1 SE) of the five main pollinator species of *Dianthus carthusianorum* at the study site (Untere Rotafen, Leuk VS, Switzerland). Sf, *Satyrus ferula*; Mg, *Melanargia galathea*; Pm, *Papilio machaon*; Ms, *Macroglossum stellatarum*; Ts, *Thymelicus sylvestris*. Dark bars, estimated parameters from statistical analysis (see Materials and Methods); light grey bars, calculated from raw data. Different letters between bars indicate significant differences. (a) Pollination efficiency (number of pollen grains deposited on stigma per visit and by selfing) analysed as a linear mixed-effects model (contrasts: ab, $P = 0.0029$; ac, $P = 0.0003$, bc, $P = 0.0210$, all corrected by sequential Bonferroni technique). (b) Visitation frequency per observation unit (1 observation unit = 20 inflorescences within an area of 8 × 8 m during 30 min observation time), analysed as a generalized linear model with Poisson distribution (contrasts: ab, $P = 0.0025$; ac, $P = 0.0005$; bc, $P = 0.0435$, all corrected by sequential Bonferroni technique). (c) Pollinator importance, the product of visitation frequency and pollinator efficiency. For comparison, we assumed the visitation frequencies of Ms and Ts to be equal to that of Pm, the least frequently observed pollinator in the observation units.

subsample of pollination efficiency (subsample for *Satyrus ferula* (Fabricius), $n = 5$; *Melanargia galathea* L., $n = 2$). Statistical analysis was conducted as a linear model for the two pollinator species *S. ferula* and *M. galathea*. Sample sizes of the other three pollinator species were too small for this analysis. The pollinator importance of *S. ferula* and *M. galathea* was square-root transformed to fulfil the assumptions of error distribution. For comparison, pollinator importance of the three omitted species was calculated from the means of their pollination efficiencies and visitation frequencies.

The relation between the number of pollen grains deposited on a stigma and seed set was analysed with a nonlinear mixed-effects model (Pinheiro & Bates, 2000). Prior examination of data distribution and calculations of the relationship between pollen quantity and seed set required log-transformation of pollen quantity, and indicated a threshold value for seed set. Therefore we fitted a model of the form $y = b \times (x - a)$ by constraining y to 0 for $x < a$, where y = seed set; x = number of pollen grains; b = slope; a = threshold. As different pollen quantities were applied to different flowers of the same plant, we defined plants as random factors. Thus the parameters of the dose–response-function were estimated for each plant individual. In some plants, herbivory critically reduced the number of flowers to sample sizes too small to provide reliable estimates. Model fitting was therefore based on 13 out of 24 plants. However, this did not cause a biased estimate, as we could not detect a systematic pattern of herbivory.

Results

Pollination efficiency

During the 4 wk of the study, 136 insect visits to the target flowers were recorded. All visits were made by individuals of five different lepidopteran taxa (Fig. 1a), most frequently by *S. ferula* (Satyridae, 63% of visits) and *M. galathea* (Satyridae, 30%), and rarely by *P. machaon* (Papilionidae, 3%), *M. stellatarum* (Sphingidae, 2.5%), and *T. sylvestris* (Hesperiidae, 1.5%). The number of pollen grains deposited per visit on stigma lobes ($F_{2,130}$ = 814.6, P = 0.0005) differed between *S. ferula* (9.92 ± 1.73; mean ± 95% CI), *M. galathea* (13.13 ± 2.67; mean ± 95% CI), and the number of pollen found on self-pollinated flowers (7.41 ± 1.90; mean ± 95% CI). Contrasts corrected by sequential Bonferroni technique revealed significant differences in pollen deposition between *S. ferula* and *M. galathea* (P = 0.0292) and between self-pollinated flowers and the two pollinator species (*M. galathea*, P = 0.0003; *S. ferula*, P = 0.0210).

Visitation frequency

In the observation plots, *S. ferula*, *M. galathea* and *P. machaon* differed in their visitation frequencies ($F_{2,45}$ = 23.557, P = 0.0009, dispersion = 2.18). In the 16 observation units conducted, *S. ferula* was missing only once, while *M. galathea* was absent five times and *P. machaon* as many as 15 times. *Satyrus ferula* was the most frequently observed visitor with a mean of 1.57 (± 0.11, 1 SE) individuals per observation unit, followed by *M. galathea* (0.36 ± 0.31, 1 SE) and the rare visits of *P. machaon* (–2.77 ± 0.10, 1 SE). Contrasts corrected by sequential Bonferroni technique revealed different visitation rates for *S. ferula* and *M. galathea* (P = 0.0025); for *S. ferula* and *P. machaon* (P = 0.0005); and for *M. galathea* and *P. machaon* (P = 0.0435). Visits by *M. stellatarum* and *T. sylvestris* were never recorded during the observation units (Fig. 1b). To calculate their pollinator importance, we assumed their visitation frequency to be 0.06 individuals per observation unit (mean visitation frequency of *P. machaon*).

Pollinator importance

Pollinator importance tended to be higher for *S. ferula* compared with *M. galathea* ($F_{1,30}$ = 2.93, P = 0.095; 23.3 ± 2.93 and 16.16 ± 2.93, respectively; mean ± 1 SE). The pollinator importance of *S. ferula* was by far higher than that of *P. machaon*, *M. stellatarum* and *T. sylvestris* (6.63%, 1.81% and 0.66% relative to *S. ferula*, respectively; Fig. 1c).

Relationship between pollen quantity deposited on stigma and seed set

Seed set of the study plants was positively related to pollen quantity deposited on the stigma lobes ($F_{1,71}$ = 38.10, n = 85, P < 0.0001). However, this relationship was observed only after a threshold of 3.92 ± 0.16 (mean ± 1 SE; $F_{1,71}$ = 669.45, n = 85, P < 0.0001; Fig. 2). Note that in a nonlogarithmic plot, the function would asymptotically approach an upper limit of approx. 40% seed set (Fig. 3). However, the semilogarithmic plot shows the threshold level as well as the linear relationship more clearly (Fig. 2). During the flowering period, 16.13% of freely accessible *D. carthusianorum* flowers accumulated < 50 pollen grains during their anthesis (Fig. 3), and 71% of the flowers accumulated < 200 pollen grains (187 ± 34; mean ± 1 SE; n = 31). Thus seed set of these flowers was clearly pollen-limited under natural conditions.

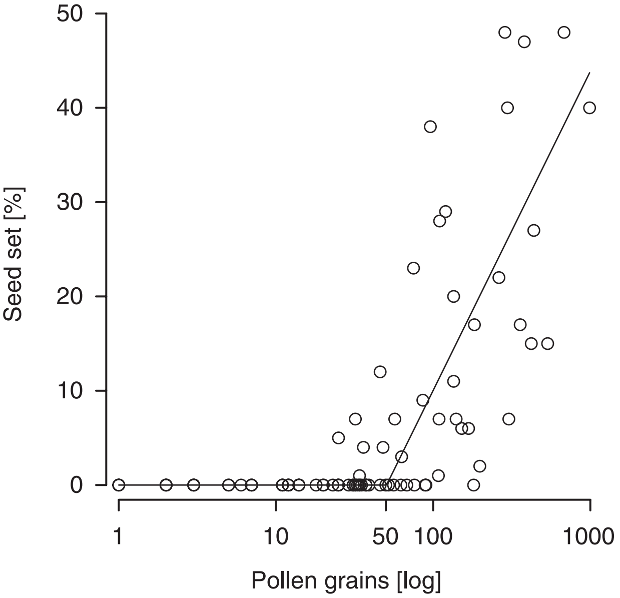

Fig. 2 Mean dose–response curve for pollen quantity (log-transformed) deposited on stigma lobes and seed set in *Dianthus carthusianorum* calculated from 85 flowers on 13 plants at the study site (Untere Rotafen, Leuk VS, Switzerland). This relationship links estimates of pollination efficiency with realized fecundity and thus with pollination effectiveness. Seed set = b × (pollen – a); a = 3.92 ± 0.16 (1 SE), b = 0.15 ± 0.2 (1 SE). The threshold is located at a = 50.57 pollen grains (back-transformed).

Discussion

Pollinator importance, visitation frequency and pollination efficiency

In our research population, the bulk of the reproductive success of *D. carthusianorum* was mediated by the visitation frequency of two pollinator species. We also confirmed that *D. carthusianorum* is mainly, if not exclusively, pollinated by diurnal Lepidoptera (Knuth, 1898; Hegi, 1979). Furthermore, we found considerable differences in pollination efficiency, visitation frequency and hence pollinator importance among the five observed pollinator species. Pollinator importance was determined by visitation frequency rather than by the pollination efficiency of pollinator species. The most important pollinator in this study was *S. ferula*. Although this species was only third in the hierarchy of pollination efficiency (Fig. 1a), it was the most important pollinator of *D. carthusianorum* because of its high visitation frequency (Fig. 1b). Earlier studies (Schemske, 1983; Spears, 1983; Waser & Price, 1983; Olsen, 1997) have also demonstrated that a pollinator's visitation frequency was the dominating factor for assessing its final level of pollinator importance. Thus the visitation frequency of pollinators was the principal determinant of plant fecundity regarding

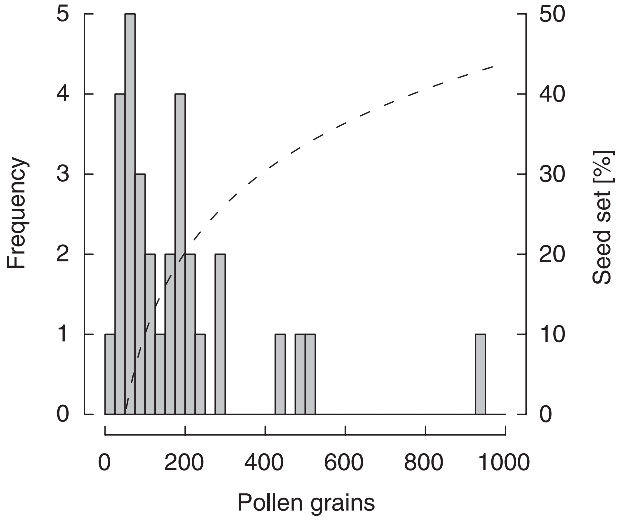

Fig. 3 Frequency distribution (left axis) of pollen quantities deposited on stigma lobes of *Dianthus carthusianorum* flowers ($n = 31$) with unlimited access to pollinators during anthesis at the study site (Untere Rotafen, Leuk VS, Switzerland). Inserted back-transformed dose–response function (dashed line, right axis) from Fig. 2 indicates pollen-limited seed set from naturally deposited pollen grains.

pollination, as seed set under natural conditions was strongly limited by pollen supply, be it from selfing and/or provided by pollinators. Our results also suggest that *D. carthusianorum*, although adapted to butterfly pollinators, is not particularly specialized to its main two butterfly pollinators at the study site, and thus appears to be rather a generalist with respect to its pollinating butterfly species. However, seasonal stability in visitation frequencies of the two main pollinator species (D. Bloch, A. Erhardt, pers. obs. at the study site, 2000–05) would probably promote specialization through pollinator-mediated selection, that is, adaptation for increased pollination effectiveness of the main pollinator species (Waser *et al.*, 1996).

Pollinator-limited fecundity and pollination effectiveness

Seed set of the study population was positively related to the number of pollen applied, a pattern found in many plant taxa (Jaquemart, 1997; Johnson & Bond, 1997; Bosch & Waser, 1999, 2001; Pflugshaupt *et al.*, 2002). This result confirmed our assumption that pollen deposition on the stigma by a single pollinator visit is a fair estimate of pollination effectiveness, the contribution to fecundity of a single pollinator visit. Seed set of flowers with unlimited access to pollinators was strongly pollen-limited (Fig. 3): 70% of the flowers received pollen quantities, which correspond to only 20% seed set. Sixteen per cent of the flowers accumulated even fewer pollen grains than necessary for the development of any seeds. However, resource availability is also relevant for realized fecundity, as indicated by the presence of a threshold value of pollen quantity deposited on the stigma lobes for fruit formation. A plant's investment policy in fruits is obviously to abort fruits of flowers that accumulate only a low pollen quantity, to the advantage of better-pollinated flowers in which limited resources are invested. As differences in microhabitat quality and/or genotype also affect seed set (Waser, 1993), resource availability is expected to influence seed set (Campbell & Halama, 1993; Baker *et al.*, 2000). A similar threshold between pollen quantity and seed number, as found in the present study, was described in a *Hibiscus* species almost 250 yr ago by Koelreuter (1761). He also noted that the observed threshold varied considerably according to environmental conditions. Thus differences in microhabitat and/or genotypic differences may affect responses in seed formation to pollen availability on an individual scale. The threshold itself, a refusal or incapacity to develop small numbers of seeds with the high costs of fruit formation, can also be viewed as a response to resource limitation on the habitat scale – an adaptation to the overall nutrient poor soil conditions of dry grasslands. The evolution of such a threshold in *D. carthusianorum* could correspond to the life strategy of stress-tolerant plants according to the CSR model described by Grime (1977).

Pollination crisis

Recently, several authors have claimed that the sexual reproduction of many plant species is threatened by declining pollinator frequencies because of human impact (Allen-Wardell *et al.*, 1998; Kearns *et al.*, 1998; Karrenberg & Jensen, 2000). Although various butterfly species visit *D. carthusianorum* in the investigated population, a decline in abundance, or a loss of one or even both of the two main pollinator species, could cause a serious threat to the local persistence of *D. carthusianorum.* Such a threat is realistic as both species, although locally abundant, are generally rare, weak dispersers, and are restricted to the ecological conditions of the local environment of rocky steppes (Benz *et al.*, 1994), itself a rare, species-rich habitat drastically reduced by viticulture, housing development and fertilization (Delarze *et al.*, 1999). In the Rhone valley of south-eastern Switzerland both butterfly species, *M. galathea* and *S. ferula*, have vanished from 29 of 67 and 31 of 62 sites, respectively, since 1970 (Gonseth, 1987). Thus the population density of both pollinator species shows a tendency to collapse or to go locally extinct. Furthermore, the observed pollinator limitation does not suggest that pollinator species compete significantly for nectar in *D. carthusianorum.* It therefore seems unlikely that the remaining pollinator species could compensate for a decrease in the primary pollinator species. However, as *D. carthusianorum* is self-compatible (no apomixis), a minimal sexual reproduction could be assured by selfing, although pollen quantity deposited by selfing is precariously close to the threshold for fruit formation. Thus the risk of local extinction will eventually depend on the demographic consequences of inbreeding

(Schemske, 1983; Waser, 1983; Waser & Price, 1983; Bond, 1995). Furthermore, flight distances of pollinators between visited flowers affect the progeny's fitness (Price & Waser, 1979; Levin, 1981). Butterflies cover greater flight distances than other insects, and therefore are able to sustain a higher effective population size (Beattie & Culver, 1979; Schmitt, 1980). Hence a stronger inbreeding depression is likely if a plant population becomes dependent on selfing because of a lack of pollinators. Consequently, *D. carthusianorum* may face serious inbreeding depression in the case of decreasing pollinator abundances. Other impacts of human activities, whether habitat deterioration or destruction and fragmentation (Jennersten, 1988), further threaten this plant–pollinator system.

Conclusions

This study shows that realized fecundity in the *D. carthusianorum* population investigated depends strongly on pollinator service. Two of the five lepidopteran pollinator species are by far the most important pollinators. Visitation frequency rather than pollination efficiency of pollinators was the main determinant of pollinator importance. Although pollination efficiencies do not indicate a particular specialization to the two main pollinator species, *D. carthusianorum* is specialized to pollination by diurnal Lepidoptera. Seed set under natural field conditions was strongly limited by pollen supply, whether by selfing or provided by pollinators. The local persistence of *D. carthusianorum* is at risk, as its reproduction depends essentially on two locally abundant, but generally vulnerable, pollinator species.

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Chapter 3

Exploring intra- and interspecific postmating barriers in two closely related sympatric *Dianthus* species

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Evolution, submitted

Abstract

Coexisting closely related plant species offer the opportunity to investigate mechanisms contributing to and maintaining species isolation. A variety of pre- and postzygotic isolation mechanisms known to reduce or interrupt gene flow, thus upholding species distinctiveness or even promoting species differentiation. In this study we investigate isolation barriers from postmating prezygotic to intrinsic postzygotic isolation. We hand-pollinated *Dianthus sylvestris* flowers with self-pollen, pollen from the same population, pollen from two distant populations and finally with interspecific pollen from the sympatrically occurring *D. carthusianorum*. In a first experiment we applied the different treatments to different plants, whereas in the second experiment we applied the different treatments to different flowers of the same plant. In both experiments we could neither detect postmating prezygotic nor intrinsic postzygotic isolation barriers. However, we recorded differential resource allocation to seeds, if the different pollination treatments were applied to different flowers of the same plant, in contrast to the first experiment. Two mechanisms, maternal choice among progenies or differential competitive abilities of progenies, are likely to have directed the allocation patterns, potentially reflecting weak in- and outbreeding effects. These results suggest that other isolation mechanisms (e.g. pollinator preferences) hold up species isolation in sympatry between the investigated species.

Introduction

Complete reproductive isolation through incompatibility represents the final, irreversible step in speciation. However, complete reproductive incompatibility between closely related plants is not the norm. In contrast, hybridization is a common phenomenon and itself a route toward speciation (Grant 1981; Rieseberg 1997; Ramsey and Schemske 1998, 2002; Mallet 2007). On the other hand, already low rates of gene flow between populations or species can prevent or dissolve genetic differentiation (Falconer and Mackay 1996). However, strong selection associated with different ecological conditions can compensate for vast gene flow and maintain species distinctiveness in flowering plants (Goulson and Jerrim 1997). Furthermore, numerous isolating mechanisms are known to be vital in upholding distinctiveness or even in promoting further differentiation of populations and species. For example, the conspecific *Mimulus lewisii* and *M. cardinalis* are separated by extrinsic prezygotic (habitat isolation and pollinator isolation), postmating prezygotic (interspecific pollen competition) as well as intrinsic postzygotic isolation (F_1 hybrid inviability and sterility). Although these mechanisms represent strong absolute isolation barriers, only the first two extrinsic prezygotic mechanisms contribute primarily to total isolation in the two *Mimulus* species (Ramsey et al. 2003).

In this study we consider intra- and interspecific isolation barriers in the carnation *Dianthus sylvestris*. We focus on postmating processes, i.e. postmating prezygotic (gametic isolation) and intrinsic postzygotic isolation. The former is mediated by non-competitive mechanisms, e.g. reduced pollen germination on “foreign” stigmas and reduced fertilization either through slow pollen tube growth and/or intrinsic gametic incompatibility (Levin 1978; Heslop-Harrison 1982; Williams and Rouse 1988, 1990; Niklas 1997), and competitive mechanisms, e.g. differential siring success from mixed pollen loads (Marshall 1991; Johnston 1993; Karron and Marshall 1993; Rieseberg et al. 1995; Carney et al. 1996; Marshall 1998; Diaz and Macnair 1999;

Niesenbaum 1999). In contrast, intrinsic postzygotic isolation manifests itself through hybrid inviability and/or sterility. Since *D. sylvestris* and its congener *D. carthusianorum* considered in this study are diploid, two mechanisms represent such candidates for intrinsic postzygotic isolation, i.e. different chromosomal rearrangements (Noor et al. 2001; Rieseberg 2001; Navarro and Barton 2003) and genetic incompatibilities (within and/or between locus). Genetic incompatibilities are more likely the cause of between locus effects, since its mechanism is based on the Dobzhansky-Muller model (though established by Bateson (Orr 1996)), which convincingly solves Darwin's dilemma (i.e. species (AA -> aa) evolve without passing the unfit heterozygote genotype (Aa)).

To explore whether postmating prezygotic (gametic) isolation and intrinsic postmating isolation contribute to intra- and interspecific barriers in *D. sylvestris*, we investigated reproductive success in terms of fruit set, number of seeds per fruit, seed weight and germination success in response to different pollen origins. We hand-pollinated flowers of *D. sylvestris* with self-pollen, outcross pollen from the same population, pollen from a population with similar ecological conditions (rocky steppe), pollen from a population with dissimilar ecological conditions (rocky steppe versus alpine meadow), and finally with interspecific pollen from the sympatric congener *D. carthusianorum.* In a first experiment we applied pollen of the same treatment on different flowers within plants, whereas in second experiment we applied the different pollination treatments to different flowers on the same plant. The two experiments should determine whether resource allocation alters if the plants have a "choice" with respect to the pollination treatments. Differential resource allocation would allow to assess the probable mechanisms that control resource allocation (maternal effects, progenies competitive abilities, resource limitation) (Temme 1986; Queller 1994; Lipow and Wyatt 1999; Parker et al. 2002; Banuelos and Obeso 2003). This study reveals whether postmating prezygotic (gametic isolation) (Howard 1999) or intrinsic postzygotic isolation (until F1 hybrid inviability) hold up population or species differentiation. Furthermore, interspecific crosses from our experiments provide insight into whether the species are fully separated or whether they show at least to some degree postmating pre- and/or postzygotic isolation. Intraspecific crosses should clarify whether in- or outbreeding affects reproductive success, and specify whether population differentiation to different environments alters intraspecific reproductive compatibility or success.

Material and methods

Study species

Dianthus sylvestris Wulfen (Caryophyllaceae) is a gynomonoecious-gynodioecious and *D. carthusianorum* L. a gynodioecious perennial herb (Knuth 1898; Hegi 1979). Both carnations have protandrous flowers with a calyx tube made up of fused sepals, and protruding unfused petals, stamens and stigma lobes (Knuth 1898). Both, *D. carthusianorum* as well as *D. sylvestris,* are non-clonal and self-compatible (Hegi 1979). The tubular flower shape is interpreted as an adaptation to long-tongued Lepidopteran pollinators, excluding other nectar competitors with a short proboscis unable to reach the nectar at the bottom of the calyx tube. *Dianthus sylvestris* is common in the European Alps, occurring predominantly in rocky habitats and dry unfertilized grasslands (Erhardt 1988). *Dianthus carthusianorum* is widespread in Central Europe, but restricted to unfertilized dry grasslands. The two species can occur sympatrically and overlap in their phenology. However, hybrids are rarely found (personal observation), indicating that the two species are interfertile. *Dianthus sylvestris* is primarily pollinated by the noctuid moth *Hadena compta* (an important seed predator), and by the sphingid moth *Macroglossum stellatarum*. In contrast *D. carthusianorum* is mainly pollinated by diurnal butterflies (Müller 1881; Erhardt 1988; Bloch et al. 2006).

Study sites

The experiments were conducted in a large natural sympatric population of *D. carthusianorum* and *D. sylvestris* growing in a dry rocky steppe (Festucion valesiacae according to (Ellenberg 1996); field name: Rotafen) on a south facing slope near Leuk (VS; 7°40' east, 46°19' north, ~ 900 m a.s.l.) in the Rhone valley in the Swiss Alps. The two species are patchily distributed, i.e. they are associated with different ecological niches, *Dianthus carthusianorum* being more abundant in grassy patches and *D. sylvestris* in rocky patches. Both species overlap in their flowering phenology, which lasts from May to September, with a peak in June and July.
Pollen of plants from two distant populations, both about 5 km away from the experimental site, were used for two of the five experimental treatments (see below). One population was located near Bratsch (VS, 7°44' east, 46°19' north) growing under similar ecological conditions at the same elevation (~900 m a.s.l.) as the study site, whereas the other population, located at the alp Oberu (2120-2160 m a.s.l., 7°40 east, 46°21 north), served as a representative for different ecological conditions (alpine meadow) compared to the experimental population at Rotafen (rocky steppe). There are morphological differences between *D. sylvestris* plants from Rotafen and those from Oberu: At Oberu, stems are shorter, but flowers are larger and calyces longer (mean calyx length: 20.8 ± 0.28 (SE) mm at Oberu, 18.0 ± 0.19 mm at Rotafen, T-test (two sided): $t_{df=106} = 8.28$, $P = 3.87*10^{-13}$). Since the vegetation period is shorter at high elevation, the flowering period is also much shorter at Oberu (early July to mid August) compared to the populations at low elevations (end of May until beginning of September).

Experiments

Two experiments were conducted at the study site (Rotafen). In the first experiment we investigated the compatibility of pollen from different origins on different *D. sylvestris* plants. Plants of *D. sylvestris* were hand-pollinated with pollen from five different origins: (A) self pollen, (B) cross pollen from plants at the same site, (C) cross pollen from plants of the population near Bratsch (VS) with similar ecological conditions (same altitude, rocky steppe), (D) cross pollen from plants of the population at Oberu with distinct ecological conditions (alpine meadow), and (E) interspecific pollen from plants of the congener *D. carthusianorum* growing in sympatry with *D. sylvestris* at the study site. We randomly assigned 22 plants to each pollination treatment. On each plant 5 to 6 flowers were pollinated with pollen from the same origin (SPE = single pollen-donor experiment).
In the second experiment, we investigated whether the progenies from different paternal origins (treatments) are provisioned differently by the mother plants (MPE = multiple pollen-donor experiment). For that purpose different flowers on the same plant received pollen from the 5 different origins (treatments), i.e. we nested the pollen treatments within the plants. Each pollen treatment was replicated on 2 to 3 flowers per plant depending on whether all 5 treatments could be replicated. To reduce a potential bias from single paternity, the flowers on a plant received pollen from different plants (fathers) in both experiments. Other potentially confounding

factors such as time of pollination, phenology within and among plants, and position of flowers on the plant were randomly assigned to the different treatments.

To avoid contamination with non-experimental pollen we excluded pollinators by bagging experimental plants. Except for the selfing treatment (A), flower buds were emasculated with sharp tweezers just before flowering. Since emasculation was only possible by cutting a slit into the calyx, the calyces of the plant of the selfing treatment (A) have also been slit. When the stigma lobes were large and protruding out of the calyx, they were pollinated with pollen from the different sources. Pollen was collected in the morning, stored in Eppendorf tubes and used for pollination on the same day. Pollinations were conducted from mid-June until the end of August. The fruits were harvested when they were fully ripe. To avoid loosing seeds we wrapped each fruit with a small bag. Since some fruits did not set seeds we classified fruits into aborted and ripe fruits. Finally, we counted and weighed the seeds. We further determined ripe and aborted seeds by their differing colours. Ripe seeds were identified by their black, and aborted seeds by their light brown colour.

We measured germination success by taking a random sample of seeds from the different fruits, plants and treatments. A total of 389 seeds from SPE (78 per treatment, except 77 from treatment A), and a total of 314 seeds from MPE (62 from treatment A, 61 from B, 63 from C, 65 from D, and 63 from E) were randomly selected. Each seed was weighed and put into a separate pot, and these pots were then randomly arranged in six blocks in a common garden experiment at the botanical garden of the University of Basel. The garden bed with the pots was protected with a nylon mesh (pore diameter 0.25 mm).

Statistical analysis

Statistical analyses were calculated with R statistical software (R Development Core Team 2003). We analyzed the proportion of ripe fruits to pollinated flowers per plant, number of seeds and mean seed weight per fruit, averaged per plant. The two experiments (SME and MPE) were analyzed separately.

Fruit set (binary data) was analysed with a generalized linear model (GLM) (McCullagh and Nelder 1989) based on binomial distribution and logit transformed data. The number of seeds (count data) was modelled as another GLM based on Poisson distribution. Seed weight per fruit was analysed as ordinary linear regression based on least square estimation including the number of seeds per fruit as covariate to see whether the slope, i.e. the interaction between pollination treatment and number of seeds, of the linear relationship changes for the different pollen origins. Analyses were performed for pooled seeds (aborted “brown” and ripe “black”) as well as exclusively for ripe seeds.

We intended to reduce error variation by including other variables such as date of pollination (days since 1st of June), the number of pollinated flowers per plant (covariate), the number of flowers damaged by herbivores (covariate), plant diameter (covariate) and substrate where the plant grew in the starting models of the respective analyses. We then stepwise eliminated non-influential terms from the model (decision on AIC). Seed germination (binary data, logit transformed) was again analysed with GLM based on binomial distribution. Germination was analyzed for each experiment separately (SPE, MPE) as well as for the pooled dataset, each based on the block design according to the arrangements in the experiment. Predictors in the initial full model were block, seed weight, and pollination treatment.

Results

Fruit set for the SPE (final model) depended on the day of pollination ($\chi^2_1 = 14.105$, $P = 0.00017$) but was not influenced by the pollination treatment ($\chi^2_1 = 7.153$, $P = 0.12$). Estimated fruit set probabilities adjusted for mean date of pollination were 51.7% for selfing (A), 36.3% for cross-pollination with plants from the same site (B), 36.1% for cross-pollination with plants from the distant population with similar ecological conditions (C, same altitude), 34.3% for cross-pollination with plants from the distant population with distinct ecological conditions (D), and 40.4% for interspecific pollination with plants of the congener *D. carthusianorum* growing in sympatry (E). The final model was still characterized by overdispersion ($\chi^2_{94} = 160.0$, $P = 0.00003$). Since we knew of no further predictor we fitted the same final model as "quasibinomial" GLM, i.e. additionally estimated the dispersion parameter. The results remained similar (date of pollination, $P = 0.002$; pollination treatment, $P = 0.287$; scale parameter = 1.43).

Fruit set for the MPE (final model) depended on the day of pollination ($\chi^2_1 = 6.63$, $P = 0.010$) but was not influenced by the pollination treatment ($\chi^2_1 = 0.68$, $P = 0.95$). Estimated fruit set probabilities adjusted for mean date of pollination were 61.3% for (A), 65.1% for (B), 58.3% for (C, same altitude), 57.9% for (D), and for 40.4% (E). The final model was also characterized by overdispersion ($\chi^2_{84} = 122.18$, $P = 0.004$). A "quasibinomial" model did not alter the result (date of pollination, $P = 0.016$; pollination treatment, $P = 0.96$; scale parameter = 1.15).

The analyses of the number of seeds per fruit were in both cases, SPE and MPE, characterized by high levels of overdispersion (scale parameters: 5.3 and 10.5, respectively). Accounting for overdispersion no significant effects were detected in any of the two analyses.

Seed weight per fruit (SPE) tended to be different for the pollination treatments ($F_{1,88} = 2.03$, $P = 0.097$) when we controlled for the number of seeds ($F_{4,88} = 7842.8$, $P < 0.0001$). Since the linear relationship of seed weight depending on the number of seeds was not significantly different for treatments ($F_{4,88} = 0.22$, $P = 0.92$) the interaction term was omitted. In the MPE (Fig. 1) we found, in contrast, a highly significant effect for the pollination treatments ($F_{1,88} = 5.36$, $P = 0.0014$; adjusted for the overall mean of the number of seeds per fruit) when controlling for the number of seeds ($F_{4,88} = 562.75$, $P < 0.0001$) and the also relevant effect by the interaction ($F_{4,88} = 3.91$, $P = 0.0085$). Pairwise comparisons (TukeyHSD) showed significant differences in seed weight per fruit for some pollination treatments (**AD**: -1.11 < 3.75 < 8.61; **BD**: -0.52 < 4.60 < 9.73; **CD**: 0.19 < 5.17 < 10.15; **AE**: 0.52 < 5.53 < 10.54; .**BE**: 1.14 < 6.38 < 11.62; **CE**: 1.85 < 6.95 < 12.05).

Stepwise regression (based on AIC) for the pooled data (SPE, MPE) on seed germination eliminated all but the block variable ($\chi^2_5 = 18.71$, $P = 0.002$). The final model fits the model assumptions with respect to the binomial distribution ($\chi^2_{191} = 189.55$, $P = 0.52$). Neither seed weight nor pollination treatment had a significant effect on the germination success. Estimated germination probabilities (mean over all blocks per treatment) were 17% for A, 11.8% for B, 9.0% for C, 8.5% for D and 8.1% for E. The separate analysis on germination success for the seeds from the SPE ended up with a final model including the block effects ($\chi^2_5 = 12.60$, $P = 0.027$) as well as pollination treatments ($\chi^2_4 = 8.13$, $P = 0.067$). Again the final model did not violate the model assumptions with respect to the binomial distribution ($\chi^2_{79} = 76.1$, $P = 0.57$). Estimated germination probabilities (mean over all blocks per treatment) were 16.3% for A, 13.9% for B, 9.0% for C, 6.1% for D and 5.3% for E. In the separate analysis for the MPE stepwise regression eliminated all predictors from the model, presumably because our sample size (number of seeds) in the MPE was smaller than in the SPE and germination success in general was rather low. Pooling two neighboring blocks into one, ameliorated the structure of the statistical analyses. Stepwise regression retained the block effect ($\chi^2_2 = 8.98$, P = 0.039), the seed weight ($\chi^2_1 = 0.001$, P = 0.97) and their interaction term ($\chi^2_2 = 8.65$, P = 0.013) in the final model. Model assumptions were not violated with respect to the binomial distribution ($\chi^2_{39} = 50.67$, P = 0.10).

Discussion

Our results did not reveal incompatibility or strong reproductive barriers from postmating pre- and intrinsic postzygotic isolation related to the different pollen origins. Pollen from each treatment sired seeds, and those seeds successfully germinated and established viable seedlings. Therefore, the gene pool of the considered population of *D. sylvestris* is neither isolated from gene flow from other populations nor from the sympatrically coexisting congener *D. carthusianorum*. Neither fruit set nor the number of seeds set per fruit differed for the pollination treatment in either of the two experiments, i.e. the single (SPE) and the mixed (MPE) pollination applied to different flowers per plant. However, seed weight per fruit (controlled for the number of seeds set per fruit) depended on the applied pollen origin in the MPE. The rate of seed provisioning was significantly different for the applied pollination treatments, indicating a mechanism controlling for resource allocation (Fig 1). Progenies originating from selfing and from interspecific crosses (genetically most distinct) experienced least support from resource allocation of the mother plants in the MPE. These results suggest that differential allocation might not be an effect of genetic incompatibility - otherwise we would have expected similar effects in both experiments (SPE and MPE) - but might rather result from navigated allocation of resources. However, our experimental design does not allow to unambiguously separate whether allocation is controlled by maternal choice or by resource competition between progeny (Queller 1994; Lipow and Wyatt 1999; Banuelos and Obeso 2003). Nevertheless, we would also expect to find similar differences in the SPE, i.e. plants would allocate more resources for more demanding progeny (parent-offspring conflict, (Parker et al. 2002), if certain particularly competitive embryos would be able to enhance seed provisioning, e.g. by stronger or better fitting hormonal signals. It remains, however, speculative whether more exploitative embryos can boost the proportion of resources allocated for seed provisioning (reproduction) or whether the plant allocates a determinate proportion of resources (reproduction) to the progeny, which is then unequally allocated among progenies with different competitive abilities. The former would support maternal choice indicating that the plant's allocation strategy is only altered if the mother plant has a choice from a genetic diverse pool of progenies, and that the plant is able to recognize the genetic quality of its progenies. The latter would support in- and outbreeding effects affecting the embryo's competitive ability.

Mixed pollination, i.e. the pollination with different parental pollen, has been profoundly investigated. Most studies focused on mixed pollen loads on the same flower showing several competitive mechanisms that caused differential siring success and seed provisioning (Marshall 1991; Johnston 1993; Karron and Marshall 1993; Rieseberg et al. 1995; Carney et al. 1996; Marshall 1998; Diaz and Macnair 1999; Niesenbaum 1999). Since we have applied the pollination treatments (A-E) on different flowers, and did not vary pollen load, we can exclude these mechanisms causing the observed differential resource allocation, but we cannot rule out that interfering competition on the same flower could have altered the outcome of the experiment (Howard 1999). Nevertheless, we can exclude non-competitive postmating prezygotic isolation through reduced pollen germination on “foreign” stigmas, and reduced fertilization either through slow pollen tube growth and/or intrinsic gametic compatibility (Levin 1978; Heslop-Harrison 1982; Williams and Rouse 1988, 1990; Niklas 1997).

Whether the different allocation rates among the distinct progenies represent a stable allocation strategy across environmental conditions remains speculative. Phenotypic plasticity is a common phenomenon of plant’s allocation strategies (Schlichting and Pigliucci 1998; Kollmann et al. 2004; Obeso 2004), and therefore it is rather likely that the recorded allocation pattern will change under different environmental conditions. Furthermore, discrimination in resource allocation among progenies might only occur under harsh resource limitation (Sugiyama and Bazzaz 1997). The plants in our studied population are exposed to rather limiting conditions, since they are located in a very dry and nutrient poor environment, i.e. in a steep south sloping rocky steppe.

The pollination treatments had neither in the SPE, the MPE nor in the pooled data set a differential influence on germination success. Only effects from blocks and seed weights were detectable, and in the MPE even the interaction between block and seed weight was significant. Since the outdoor germination experiment was conducted during the extreme hot and dry summer 2003, the harsh conditions drastically reduced germination success. The block effects certainly reflect different drought stress among blocks. Thus the blocks represented different ecological conditions with respect to the strength of drought stress. The interaction term indicates that germination success altered for seeds with different weights under different ecological conditions with respect to drought stress. However, we cannot assign the magnitude of drought stress to the different blocks and hence the patterns of germination success are not interpretable. Nevertheless, germination success seems to alter for different seed weights under different ecological conditions. It has been repeatedly shown that seed weight is positively related to several fitness components during later life stages (Stanton 1984; Tremayne and Richards 2000; Halpern 2005). Therefore, we expect that the differential rate of resource allocation in seed provisioning would manifest during the plant’s later life stage.

Since relevant postmating pre- and intrinsic postzygotic isolation barriers could not be found, other mechanisms, maintaining the species barriers of the two closely related and sympatrically coexisting carnations, are yet to be determined. Naturally occurring hybrid plants indicate that interspecific gene flow occurs. Assuming that hybrids are easily recognized by their intermediate phenotype, which is supported by plants grown from interspecific crosses, we can conclude that hybrids are rare in the considered population. Isolation is in part maintained by the pollinators of the two carnation species. The two locally relevant diurnal butterfly pollinator species of *D. carthusianorum* (Bloch et al. 2006) show a clear preference (in preparation) for this species, and both butterflies were never recorded on flowers of *D. sylvestris* during four years of fieldwork. This implies a strong extrinsic prezygotic isolation (maybe also extrinsic postzygotic through discriminating intermediate hybrids) mediated through ethological isolation (Grant 1994; Fulton and Hodges 1999; Ippolito et al. 2004) at least asymmetrically upheld by the pollinators of *D. carthusianorum*. Instead, *D. sylvestris* is frequently visited by the long-tongued hawkmoth *Macroglossum stellatarum* (Sphingidae), which in turn does not show any preference to either of the two carnations (in preparation). However, observed visits suggest that this hawkmoth performs flower constancy (Waser 1986). More important, the two carnations are likely mechanically isolated (submitted), since the two carnation species differ in flower depth, and the proboscis of *M. stellatarum* fits well to the deeper flower of *D. sylvestris* (Grant 1994). Another important barrier might occur just after recombination, i.e. the F2-generation might experience a fitness breakdown by the disruption of positive epistatic interaction (Fenster and Galloway 2000; Tallmon et al. 2004). Investigations on the strength of different mechanisms separating species suggest that often a combination of mechanisms upholds the species barriers, but the actual relative contribution to of a mechanism to separation might deviate strongly form its strength if considered on its own (Ramsey et al. 2003; Kay 2006). However, if ecological selection is strong enough to eliminate non-adaptive alleles (Goulson and Jerrim 1997), then species may be effectively separated despite considerable gene flow.

Whether or not the mechanisms actually isolating the two species truly reflect those responsible for the speciation event itself, remains speculative. Nevertheless, the two carnations are not randomly distributed within the habitat, reflecting an association to different microhabitats (ecological isolation). *Dianthus carthusianorum* is associated with a pasture-like and *D. sylvestris* to a rocky habitat. These ecological niches might represent the main selective force during speciation, which more likely occurred under para- or allopatric conditions accompanied by divergent selection of a locally differing pollinator fauna (Waser and Campbell 2004).

In contrast to other studies (Waser and Price 1994; Fenster and Galloway 2000), we have not found any differences among the outcrossing treatments (B-C) among populations of *D. sylvestris*. Thus, outbreeding depression or discrimination by the mother plant among the different origins of offspring are not evident (but see above). Other studies have shown that heterosis often leads to fitness advantages in the F1 generation, especially in small inbred populations (Tallmon et al. 2004). Since our population is not small and *D. sylvestris* is predominantly outcrossing (Collin and Shykoff 2003), we would not expect strong effects from heterosis. Outbreeding depression occurs predominantly in the F2 generation when recombination breaks up the epistatic structure (Fenster and Galloway 2000; Tallmon et al. 2004). Thus, reciprocal transplants with plants from F2 generation would clarify this issue (Waser and Campbell 2004).

In summary, we could detect neither interspecific incompatibility nor postmating prezygotic isolation. The observed differential resource allocation might represent a reduced competitive ability of hybrid progenies, and thus reflect an intrinsic postzygotic isolation barrier, but was irrelevant for fitness at least until germination. This suggests that other mechanisms, e.g. ethological isolation (submitted), mechanical isolation (submitted), ecological isolation, and/or outbreeding effects in F2-hybrids, hold up the barrier of the two carnation species *D. carthusianorum* and *D. sylvestris*. Neither of the three outcrossing treatments of *D. sylvestris* (B-C) differed in fruit set, the number of seeds per fruit, seed weight and resource allocation rate. In contrast, mean seed weight and resource allocation rate were higher compared to inbreed and interspecific crossed progeny, if the different pollination treatments were applied to different flowers of the same plant (MPE). This suggests that either maternal choice or the offspring's different competitive abilities in acquiring resources navigated resource allocation.

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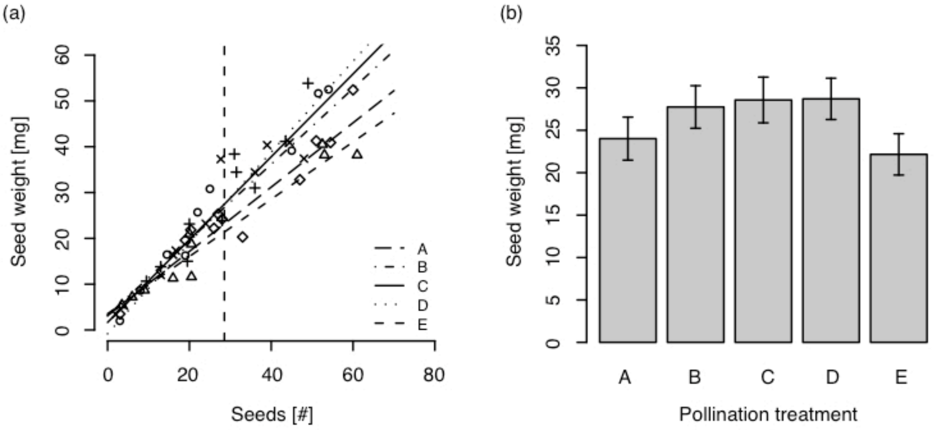

Figure 1 Resource allocation in terms of seed weight per fruit [mg] for the Mixed Pollination Experiment (MPE) for the different pollination treatments: (A) selfing, (B) cross-pollination with plants from the same site, (C) cross-pollination with plants from the distant population near Bratsch (VS) with similar ecological conditions (same altitude), (D) cross-pollination with plants from the distant population at Oberu (higher altitude) with distinct ecological conditions, and (E) interspecific pollination with plants of the congener *D. carthusianorum* growing in sympatry with *D. sylvestris* at the experimental site. (a) Seed weight per fruit [mg] in response to the number of seeds per fruit and pollination treatments. The vertical line marks the overall mean of number of seeds per fruit. (b) Mean (± 2 SE) seed weight per fruit [mg] adjusted for the overall mean of the number of seeds per fruit

Chapter 4

Frequency-dependent pollinator preferences and ethological isolation of two closely related sympatric carnations

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Evolution, submitted

Abstract

The establishment of complete intrinsic reproductive barriers between closely related plant species represents the final, irreversible step in speciation. However, closely related plant species often coexist despite being interfertile. In this study we explore the role of pollinator preferences for the separation of the two closely related, sympatrically occurring carnations *Dianthus carthusianorum* and *D. sylvestris*. In a previous study we found no isolation barriers from postmating prezygotic to intrinsic postzygotic isolation. However, observations suggest that pollinator preferences might cause an effective premating barrier. Since the short-tongued butterfly pollinators of *D. carthusianorum* cannot reach the nectar in the deeper flowers of *D. sylvestris*, ethological isolation as a side effect of mechanical isolation – according to Grant's categories of floral isolation - represents a likely mechanism of species isolation for pollinators of *D. carthusianorum.* In contrast, the long-tongued diurnal hawkmoth *Macroglossum stellatarum*, an important pollinator of *D. sylvestris*, can well reach the nectar of *D. sylvestris* flowers and does not experience such restrictions. We experimentally estimated the strength of flower preferences for the main recorded flower visitors of both carnation species in response to varying flower ratios of the two species. The tested butterfly species (*Melanargia galathea, Satyrus ferula* and *Ochlodes venatus*) showed strong, frequency-dependent preferences for *D. carthusianorum* remain representing an important interspecific reproductive barrier. In contrast, the hawkmoth *M. stellatarum* showed no preference. Species separation for this pollinator is likely caused by inefficient pollen transfer.

Introduction

Since the Cretaceous period (ca. 100 million years), angiosperms have co-radiated with insects into an enormous biodiversity. Biotically pollinated plant families reveal higher species richness compared to abiotically pollinated ones (Eriksson and Bremer 1992; Grimaldi 1999; Ricklefs and Renner 2000). Obviously, the appearance of plant-pollinator interactions represents a key innovation promoting and maintaining biodiversity in both the plants' and animals' evolutionary history. However, the role of pollinators for plant speciation is still discussed (Waser 1998). Whether pollinators alone can promote speciation in sympatry is highly debated (Coyne and Orr 2004; Waser and Campbell 2004). In contrast, it is widely acknowledged that under para- or allopatric conditions pollinators can promote divergence, at least together with other separating forces. For instance adaptation to local conditions (ecological speciation) can be paralleled by divergent evolution of flower traits in response to a different pollinator fauna (Hodges and Arnold 1994; Coyne and Orr 2004; Waser and Campbell 2004). Finally, floral traits represent one of several reproductive barriers that might facilitate the completion of speciation, e.g. in secondary contact through reinforcement and generating patterns of character displacement. Mechanical and ethological isolation are the two pollinator-mediated mechanisms that maintain the plants' reproductive barriers, and are classified by the term floral isolation (Grant 1949, 1994). Mechanical isolation is characterised by physical incompatibility in flower morphology with respect to pollinator-mediated transmission of pollen, i.e. either the plant species are adapted to pollinators differing in shape or pollen is deposited on different parts of the same pollinator. Ethological isolation is characterized by (1) hindered access to floral resources (side effect of mechanical isolation), (2) transient (learned) exploiting of certain flower resources (flower constancy) (Waser 1986), (3) floral secretion of sexual pheromones of pollinators, and (4) mono- and oligotrophic feeding of pollinators.

The investigation of closely related sympatrically co-existing plant species can reveal whether and to what degree mechanical and/or ethological isolation contribute to actual species separation (Ramsey et al. 2003; Kay 2006), and potentially clarify scenarios of the past driving mechanisms during speciation. Isolating barriers act sequentially over the entire plant's life cycle, with pollinator preferences acting prior to many of the other isolation mechanisms. Thus, pollinator preferences may contribute strongly to species separation (Schemske and Bradshaw 1999; Ramsey et al. 2003). In this study we explored whether pollinators show a preference for the flowers of either of two closely related, sympatrically occurring carnations, i.e. *Dianthus carthusianorum* and *D. sylvestris*, and we also measured the strength of these preferences. In the considered sympatric population, *Dianthus carthusianorum*'s reproduction depends vitally on only two diurnal butterfly species, i.e. *Satyrus ferula* and *Melanargia galathea* (Bloch et al. 2006). However, both butterfly species were not even once recorded on the congener *D. sylvestris* during four years of field work. In contrast, *D. sylvestris* is mainly pollinated by the diurnal hawkmoth *M. stellatarum*, which is also an occasional visitor of *D. carthusianorum*, and by the noctuid moth *Hadena compta* (Erhardt 1988; Collin and Shykoff 2003). We also considered whether potential preferences of pollinators could contribute to species separation. Since we could not find isolating pre- nor postzygotic barriers from post-pollination until the formation of F1 in a previous study (in prep), pollinator preferences are suspected to be a major source of species separation. Since the proboscis lengths of the pollinators of *D. carthusianorum* fit closely to its flower depth but are rather short for the deeper flowers of the congener *D. sylvestris*, Grant's first category of ethological isolation (Grant 1994), i.e. side effect of mechanical isolation, seems to enforce the preference. In contrast, the hummingbird hawkmoths (*Macroglossum stellatarum*) have longer proboscides, allowing them to take nectar from both, *D. carthusianorum* and *D. sylvestris*, as we could observe in the field. Furthermore, *M. stellatarum* is a generalist forager, visiting many different kinds of flowers, including ornamentals such as geraniums on balconies (personal observations). Therefore, we tested the hypotheses that (1) the pollinator species of *D. carthusianorum* show a clear preference to this carnation and (2) the pollinator species *M. stellatarum* of the congener *D. sylvestris* does not show a preference to either of the two carnation species. Furthermore, we measured the strength of the potential pollinator preferences by offering different flower ratios of the two carnation species.

Material and Methods

Study organisms

Dianthus carthusianorum L. (Caryophyllaceae) and *D. sylvestris* (Wulfen) are gynodioecious and gynomonoecious-gynodioecious perennial herbs, respectively. Both species form a rosette of lineal, grass like leaves and one to several, mostly unramified shoots. Their petals are enclosed in a narrow, fused calyx tube and end in a flat rim. The tubular flower structure inhibits flower visitors, except for long tongued insects, which can reach the nectar secreted at the base of the filaments at the very bottom of the calyx (Müller 1873). The two species differ in traits such as flower depth (calyx, corolla, stigma and stamina length), the colour, number and arrangement of flowers. *Dianthus sylvestris* has deeper flower tubes and pale pink petals, whereas *D. carthusianorum* has a shorter floral tube and crimson petals. Those trait combinations recall the classical concept of pollinator syndromes (Faegri and van der Pijl 1979), with *D. carthusianorum* and *D. sylvestris* representing “psychophily” and “phalaenophily” (Erhardt 1988), respectively. The two carnation species occur in sympatry, patchily distributed within the rocky steppes (Festucion valesiacae according to (Ellenberg 1996)) of the Rhone valley in southwestern Switzerland, *D. carthusianorum* being more abundant on grassy patches and *D. sylvestris* on rocky patches. These steppes extend over 80 km in isolated patches (in between dominant viniculture) along the south-facing slopes of the east-west running Rhone valley. Our study site (Rotafen, 4° 18′ 44″ N, 7° 39′ 46″ E) was located close to Leuk (VS, Switzerland), where we randomly collected 40 flowers per carnation species per day, and which we experimentally used on the same day.

The pollinator guild differs between the two carnation species at the study site (personal observations). Two butterfly species, i.e. *Satyrus ferula* (Fabricius) and *Melanargia galathea* L. (Satyridae), are the main pollinators of *D. carthusianorum* (Bloch et al. 2006). Further but distinctly less frequent visitors are hesperids (e.g. *Ochlodes venatus* (Turati), *Thymelicus sylvestris* (Poda)) and other diurnal butterflies. In contrast, *D. sylvestris* is mainly pollinated by the hummingbird hawkmoth (*Macroglossum stellatarum* L.) and the noctuid moth *Hadena compta* (Schiff.) (Erhardt, 1988). For our experiments we used all main pollinators of the two *Dianthus* species (*S. ferula*, *M. galathea*, *O. venatus* and *M. stellatarum*) except for *H. compta* because we were not able to catch sufficient individuals of this moth. All pollinators were caught the day before the experiments were made at the study site.

Experiment

We arranged six flowers of the two carnation species in a hexagon (side length = 10 cm) and tested the pollinators’ preference for every species (flower) ratio from 5:1 up to 1:5, respectively. Each flower was put into a small water filled vial (immediately after harvest). The vials were tied to a wire stick, which was stuck into the ground (height above ground level = 20 cm). Dome-shaped cages were put over the experimental arrangement. One pollinator was then placed into the cage and observed until the first flower visit. Only the first visit gives an unbiased estimate of flower preference, since the following flower choices depend on the experiences made on the previous flowers. We repeated the experiments at least six times for every flower ratio and every pollinator species resulting in a total of 122 trials. Experimental pollinators cannot be considered as naïve, since they were caught in the natural environment, where they most likely had already experienced flowers of the two sympatrically occurring carnation species.

Statistical analysis

All statistical analyses were conducted with R Software (R Development Core Team 2003). Preference per se and the strength of preference were analysed in two steps. Preferences per pollinator species were analysed by χ^2-tests comparing expected versus observed frequencies. Thus, the null-hypothesis was that pollinators randomly visit the offered flowers, i.e. they do not show a preference to either of the two carnation species. For that purpose we pooled the counts over all experimental flower ratios (from 5:1 up to 1:5 of *D. carthusianorum* versus *D. sylvestris*) per pollinator species and tested against the expected probability represented by the overall mean flower ratio, i.e. we tested a mean preference of all pollinators. Since the tested flower ratios are balanced, this equals testing against the null hypothesis of no preference represented by a visiting probability of 50% for each carnation species.

Difference of flower preference for the pollinator species was tested by generalized linear models (GLM) (McCullagh and Nelder 1989) using logistic regression (logit). The model consisted of the categorical variable pollinator species and the covariate flower ratio. We used *D. carthusianorum* as target species in our analyses, i.e. measurements corresponded to the frequencies of *D. carthusianorum*. However, the results do not depend on which plant species is chosen for the analyses, since, for example, less than expected visits to *D. sylvestris* would indicate the same as more than expected visits to *D. carthusianorum* and vice versa. Since we hypothesised that three of the four pollinator species (*M. galathea*, *S. ferula*, *and O. venatus*) would show a preference for *D. carthusianorum* and the fourth (*M. stellatarum*) for neither of the two carnation species, we once tested a model including every pollinator species as an individual level in the categorical variable pollinator species. In a second model we pooled the three pollinator species with the hypothetical preference within one level of the categorical variable pollinator species. Furthermore, we performed stepwise model selection on the AIC (Akaike Information Criteria) to infer the essential predictors of the two considered models. Tests corresponded to likelihood ratio test (LRT).

Results

All pollinator species but the hummingbird hawkmoth (*M. stellatarum*: $\chi^2_5 = 1.5$, $P = 0.91$) showed significant deviations from random flower visitation, i.e. they showed preferences for *D. carthusianorum* (*M. galathea*: $\chi^2_5 = 17.4$, $P = 0.0038$; *S. ferula*: $\chi^2_5 = 21.75$, $P = 0.0006$; *O. venatus*: $\chi^2_5 = 13.32$, $P = 0.0202$).

In the first model of the GLM analysis for the unpooled pollinator species, flower choice (probability to choose *D. carthusianorum*) depended on the flower ratio of the two carnation species ($\chi^2_1 = 32.67$, $P = 1.09*10^{-8}$). Pollinator species itself showed a significant difference in their flower choice ($\chi^2_3 = 19.951$, $P = 0.00017$), and the interaction term has been removed from the final model through stepwise elimination. In the second GLM analysis for the pooled data (pooled pollinator species, *M. galathea*, *S. ferula*, and *O. venatus*, with hypothesised preference for *D. carthusianorum*, Fig. 1) flower choice depended on the flower ratio of the two carnation species ($\chi^2_1 = 41.43$, $P = 3.67 * 10^{-10}$). Furthermore, the interaction term between pollinator species and flower ratio was kept in the model by the stepwise procedure, but revealed no significantly different relationship ($\chi^2_1 = 2.12$, $P = 0.145$) for flower choice in response to the offered flower ratio. The hummingbird hawkmoth (*M. stellatarum*) differed significantly ($\chi^2_1 = 12.69$, $P = 0.00047$) from the pooled pollinators in flower choice adjusted to the mean flower ratio (= 0.5). Since both models showed slight deviations in the scale parameter, we conducted further analyses by estimating the scale parameter (quasibinomial unpooled: 0.86, and pooled: 0.83) for the GLM's. These additional tests confirmed all our previous results, except for the interaction term in the pooled data analyses. However, the term tended only to be significantly different ($\chi^2_1 = 2.12$, $P = 0.079$).

Discussion

All pollinator species except the hummingbird hawkmoth (*M. stellatarum)* showed a strong preference in their flower visitation patterns. These preferences were always directed to flowers of *D. carthusianorum*, and thus confirm the hypothesis deduced from field observations. Furthermore, the strength of the preferences for *D. carthusianorum* was frequency-dependent, i.e. it depended on the offered flower ratio of the two carnation species (Fig. 1). Even if only one out of six flowers was a flower of *D. carthusianorum*, a preference for *D. carthusianorum* was still obvious. Since we caught the pollinators one day before the experiment in the field, they cannot be taken as naïve, i.e. they were likely influenced by previous experiences made in the field. We can therefore not separate innate from experienced preferences. However, the fact that we have not recorded any visits to *D. sylvestris* (but one short settling and taking off without even probing the flower) by the various pollinator species of *D. carthusianorum* during four seasons of fieldwork, suggests that if preference should be learned, this leads to a rather immediate determination. The observed preferences correspond also to preferences predicted from the classical concept of pollinator syndromes (Vogel 1954; Grant and Grant 1965; Faegri and van der Pijl 1979), with *D. carthusianorum* and *D. sylvestris* representing "psychophily" and "phalaenophily" (Erhardt 1988), respectively. The differences in colour and depth of the tubular flowers are two such conspicuous attributes. Thus, an innate bias in colour preference might to some degree explain the preferences observed in our experiments (Smithson and Macnair 1996; Weiss 1997). Probably more important, the shorter proboscides of the pollinators of *D. carthusianorum* compared to the deeper flowers of *D. sylvestris* cause some, if not a complete mechanical barrier, hindering access to the nectar at the very bottom of calyx tube of *D. sylvestris*. Negative experience will certainly encourage pollinators to avoid visiting these flowers in the future. Butterflies are able to learn in response to several cues, e.g. they can learn to efficiently handle food sources (Lewis 1986) and to associate colour, shape and odour with rewarding resources (Rausher 1978; Goulson and Cory 1993; Weiss 1995; Kandori and Ohsaki 1996; Weiss 1997; Kinoshita et al. 1999). In addition, they are able to associate different plant signals with different tasks, e.g. they can feed preferably on rewarding flowers with certain colours and simultaneously keep in mind the stimulus associated with plants suitable for oviposition (Weiss and Papaj 2003). Differences in colour and inflorescence architecture certainly provide conspicuous cues for discriminating between more and less rewarding carnation species. As the calyx tube of the flowers of *D. carthusianorum* is well within the reach of to the pollinators' proboscis length, enabling them to feed comfortably, their preference for *D. carthusianorum* would reflect ethological isolation as a by-product of mechanical isolation (Grant 1994).

In contrast to the main pollinators of *D. carthusianorum* the hummingbird hawkmoth (*M. stellatarum*) has been observed rather frequently on both carnation species. Ethological isolation resulting from mechanical isolation does not apply for this pollinator species. Here, mechanical isolation as well as ethological isolation resulting from flower constancy (Grant 1950; Waser 1986) seem to reduce gene flow between the sympatric carnations. The hawkmoth's proboscis is just long enough to reach the bottom of *D. sylvestris* flowers but is considerably longer than the calyx tube of *D. carthusianorum*, so that if at all, only the proboscis touches the reproductive organs of *D. carthusianorum* flowers, whereas most of the pollen from the deep flowers of *D. sylvestris* is deposited on the hawkmoth's forehead. Consequently, most *D. sylvestris* pollen will not be deposited on the stigmas of *D. carthusianorum* (and vice versa) when *M. stellatarum* is switching from one carnation species to the other. Furthermore, a threshold of 50 pollen grains per stigma has to be reached to stimulate fruit production and seed set in *D. carthusianorum* under field conditions (Bloch et al. 2006). Interestingly, pollen grains transferred by *M. stellatarum* on *D. carthusianorum* have been estimated to be close to that threshold. The hawkmoths also exhibited flower constancy, i.e. they visited flowers of the same species in long sequences (Grant 1950; Waser 1986; Goulson and Cory 1993). In the field, *M. stellatarum* was only observed once to switch from one carnation species to the other. Thus, interspecific pollen deposition mediated by *M. stellatarum* likely falls below the minimum number of pollen to stimulate seed set (Bloch et al. 2006). It has however to be kept in mind that variation in resource limitation may alter the magnitude of the threshold (Sugiyama and Bazzaz 1997).

Two other factors might further promote isolation between the two carnation species regarding the frequency-dependent preference of the pollinators. First, *D. sylvestris* has single flowers, each on a separate stem, in contrast to the multi-flowered inflorescences of *D. carthusianorum*. Thus, in mixed stands flowers of *D. carthusianorum* will outnumber those of *D. sylvestris*. Second, since the two species are rather clustered in their own microhabitats (*D. carthusianorum* is associated to grassy patches and *D. sylvestris* to rocky patches) there exist actually few situations where flowers of both species are intermingled.

Non-random distribution into different microhabitats suggests that the two carnation species are adapted to different ecological conditions. Therefore, habitat isolation could represent a further potentially important barrier (Ramsey et al. 2003). However, it cannot be decided to what degree spatial segregation (ecological separation) and flower constancy contribute to the current isolation of the two species. The actual situation rather suggests that speciation was initiated as a result of ecological speciation (Hodges and Arnold 1994; Coyne and Orr 2004; Waser and Campbell 2004) accompanied by divergent pollinator-mediated selection (Bloch et al. 2006) under some spatial isolation (Waser and Campbell 2004). Whether pollinators alone can drive speciation in sympatry is still a matter of controversy (Coyne and Orr 2004; Waser and Campbell 2004).

In summary, we provide evidence for asymmetrical ethological isolation mediated by the butterfly pollinators of *D. carthusianorum*. We confirm the hypothesis that these diurnal butterfly pollinators show a strong preference for the flowers of *D. carthusianorum*. Since these pollinators have proboscides that are too short to reach the nectar of flowers of the congener *D. sylvestris*, we interpret these preferences as side effects caused by mechanical isolation, according to Grant's categorization of ethological isolation (Grant 1994). Furthermore, preferences were frequency-dependent in response to the ratio of the offered flowers from the two carnation species. Though the strength of the preference decreased the lower the ratio of flowers of *D. carthusianorum* was, preferences remained high across the entire range of the tested flower ratios. Although we did not investigate the relative contribution of innate versus learned preferences, we suspect a high innate component. In contrast, the longer tongued hawkmoth (*M. stellatarum*) did, as expected, not show preferences to either of the two carnation species. However, interspecific pollen transfer mediated through *Macroglossum stellatarum* is likely reduced by mechanical isolation (submitted) and flower constancy (personal observation).

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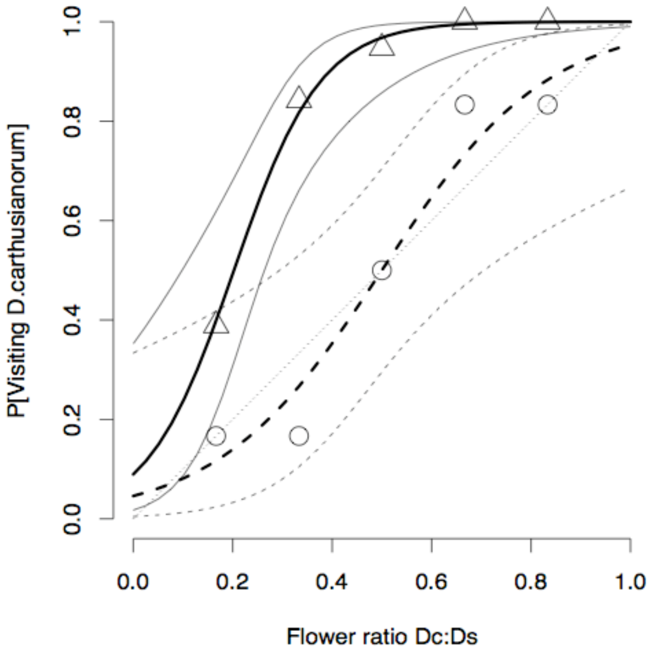

Figure 1. Probability of a pollinator visiting *D. carthusianorum* in response to the offered proportion of flowers from *D. carthusianorum* versus *D. sylvestris* (from 1:5 up to 5:1 flowers of *D. carthusianorum* versus *D. sylvestris*). The points display the data and the lines the estimated model: The pooled diurnal butterfly species (triangles and solid line: *M. galathea, S. ferula* and *O. venatus*) show a clear, though decreasing, preference. The estimates for the hawkmoth *M. stellatarum* (circles, dashed line) scatter around the dotted line, indicating visiting probabilities when there is no preference. The fine solid and dashed lines display the respective confidence intervals. All estimates represent probabilities predicted by logistic regression (Generalized Linear Model).

Epilogue

The aim of this thesis was to explore evolutionary, ecological and conservational aspects of plant-pollinator interactions. For that purpose we considered a new model system, i.e. the pollination biology of the two closely related carnation species *Dianthus carthusianorum* and *D. sylvestris*. Our investigations focused on questions concerning (1) pollinator-mediated selection promoting the evolution of flower traits, (2) the identification of the relevant pollinator species and analyses of their contribution to plant reproduction under natural conditions, and (3, 4) on the mechanisms separating the two closely related, sympatrically occurring carnation species. Our main questions were:

(1) Does pollinator-mediated selection occur in response to differential mechanical fit, i.e. differential pollination efficiency in response to variation in tubular flower depth and proboscis length?

(2) Which are the relevant pollinator species of D. carthusianorum*? Which components determine the magnitude of pollinator importance? Is* D. carthusianorum *rather specialized than generalized in pollination? Does differential pollen deposition produce differential reproductive success? Does* D. carthusianorum *experience an extinction risk due to its pollinator species?*

(3) Are there strong intra- and interspecific reproductive barriers from postmating prezygotic isolation up to intrinsic postzygotic isolation (until F1-viability) for the two closely related sympatric carnation species?

(4) Do preferences of pollinator species contribute to species separation of the two closely related carnation species? And if yes, what are the driving forces of the preferences?

The following paragraphs give the answers in short summaries of the main results of the conducted studies:

(1) Pollinator-mediated selection toward shorter tubular flowers

The main task of biotically pollinated flowers is to physically transfer pollen onto and take up pollen from the pollinator body. A prerequisite for successful pollen transfer is thus a tight mechanical fit adjusted to the three involved physical structures, i.e. the pollinator body, female (stigma) and male (stamina) reproductive organs. This study showed how this threefold interrelated fit evolves in response to the variation in pollination efficiency. The depth of tubular flowers of the carnations and the length of the pollinator's proboscides play a crucial role in the mutually adjusted mechanical fit. We recorded pollinator-mediated selection in response to differential pollination efficiency toward shorter floral reproductive organs. Even more, floral traits such as calyx and corolla experienced selection toward shorter phenotypes. Since all these depth determining floral traits are strongly correlated we were not able to disentangle whether all these traits are directly or indirectly selected. However, it is reasonable to see the whole flower as functional entity with respect to pollination efficiency. The corolla (unfused petals) represents a landing platform, which certainly has to be adjusted to the length of the reproductive organs. Furthermore, the tubular structure, which is build up by the fused sepals, stabilizes the whole flower, and thus the positioning of all other floral traits, especially if a pollinator lands on the flower. This is also illustrated by the differences mediated by the two experimental butterfly species. Interestingly the longer tongued *I. io* selected for shorter reproductive organs but not toward shorter corolla and calyx. In contrast to *M. galathea*, this butterfly had not to press its head into the corolla to reach the nectar at the bottom of the flower. *Melanargia galathea* had to push apart the petals because its proboscis was often shorter than the petals. These experimental results and observations underline the fine tuned adjustments of floral traits necessary for efficient pollination.

(2) Dianthus carthusianorum depended on only two butterfly species

Only two out of the five recorded pollinator species, the butterflies *S. ferula* and *M. galathea*, turned out to be by far the most important pollinators for *D. carthusianorum* in the considered sympatric population. Pollinator importance was mainly determined by visitation frequency of pollinators. The most efficient pollinators were not the most important ones. This is an indication that *D. carthusianorum* is not particularly specialized to its main pollinators. However, the fact that only butterflies were recorded as pollinators suggests that *D. carthusianorum* is specialized to butterfly pollination (but see also “mechanical fit” above). Differential hand-pollination revealed that the range of pollen deposition causes differential reproductive success and that flowers receiving pollen quantities below a threshold of fifty pollen grains do not set fruit. Since about the same amount of pollen grain is deposited by selfing, self-pollination seems to assure at least a minimal reproduction. Nevertheless, the reproduction of *D. carthusianorum* depends strongly on only two butterfly species. Although both butterfly species are locally abundant, they are generally rare. Furthermore, in the Rhone valley of south-eastern Switzerland the two butterfly species disappeared at nearly every second site during the last decades (Gonseth 1987). This underlines that local extinction of one or both pollinator species represents a likely scenario. Thus, *D. carthusianorum* is threatened to some degree by the potential loss of its pollinator species. However, the magnitude of the extinction risk depends on the demographic consequences of lowered fecundity and inbreeding effects (Bond 1995).

(3) No fitness disadvantages from inbreeding, outbreeding and hybridization

We could find neither complete reproductive barriers nor strong postmating prezygotic isolation or intrinsic postzygotic isolation (until F1-viability) between the two closely related sympatric carnation species. Although we recorded reduced resource allocation rates for seeds from self and interspecific pollination, these differences did not reduce fitness until germination. Interestingly, differential resource allocation occurred only if the mother plant experienced different pollination treatments among its flowers, and had the opportunity to choose among its offspring. This suggests that either the mother plant or the progeny can direct resource allocation. Thus, investigated isolation barriers are not relevant in the considered closely related sympatric carnation species. These results emphasize the role of the involved pollinator species for keeping the two carnation species isolated.

(4) Strong interspecific reproductive barriers mediated by pollinator preferences

During several seasons of field work we observed that some butterfly species showed a rather strong fidelity to the flowers of *D. carthusianorum*. We noticed but one settlement of *M. galathea* on a flower *of D. sylvestris*, followed by the immediate take-off, and this before the butterfly had even probed the flower. This observation suggests that the butterfly immediately realized to have erroneously landed on the flower *D. sylvestris*. Since we could not find relevant postmating prezygotic and intrinsic postzygotic isolation (see above), we hypothesized that ethological isolation represents a potential isolation barrier to hybridization (Grant 1949, 1994, Ramsey et al. 2003). In fact, the pollinators of *D. carthusianorum* showed a strong preference for the flowers of this carnation species. Thus, ethological isolation represents a relevant isolation mechanism separating the two closely related carnation species in sympatry.

General conclusions

Our research on the pollination biology of the two closely related carnation species *D. carthusianorum* and *D. sylvestris* established a new model system for the study of plant-pollinator interactions. Each of the conducted studies provided insight into different aspects of the considered plant-pollinator relationship.

In the first chapter we provided experimental evidence for pollinator-mediated selection by involving the quantitative traits of both, flower and pollinator. To date, the evolution of flower depth has been documented by correlation data (some included the manipulation of flower traits) from field studies (Nilsson 1988, Schemske and Horvitz 1989, Robertson and Wyatt 1990, Johnson and Steiner 1997, Maad 2000, Alexandersson and Johnson 2002). Others have also conducted experiments, but did not include quantitative traits of flower and pollinators (Campbell et al. 1996, Castellanos et al. 2003). We exemplified the intricate interplay of floral traits determining pollination efficiency in response to flower depth and pollinator's proboscis length. At the same time we were able to discriminate pollinator-mediated selection occurring via male and female reproductive success. Our results underline the function of floral shape as a physical property in pollen transfer. Furthermore, this is an experimental demonstration of how plants adapt their shape to mechanically fit to that of their pollinators.

Since we demonstrated pollinator-mediated selection via differential pollination efficiency we had to prove that differential pollen deposition, in fact, leads to differential fitness. In the second chapter we have shown this in a field study, where we measured the components of pollinator importance (pollination efficiency, visitation frequency), i.e. the relative contribution to plant reproduction, for each of the recorded pollinator species of *D. carthusianorum* (Young 1988, Herrera 1989, Pettersson 1991, Waser et al. 1996, Olsen 1997). From this we could confirm that *D. carthusianorum* is specialized to butterfly pollination, but not particularly adapted to the two main pollinator species. Furthermore, these measurements revealed that the reproduction of this carnation species depended on only two butterfly species (*M. galathea* and *S. ferula*). Since both pollinator species went locally extinct at several other sites in the Rhone valley (Gonseth 1987), the considered plant-pollinator interaction is under real threat (Bond 1995, Allen-Wardell et al. 1998, Kearns et al. 1998). Thus, the detailed information on the quantitative components of pollinator importance has clarified ecological, evolutionary as well as conservational aspects of the plant-pollinator interaction of this particular carnation species.

In the third and fourth chapter we focused on the advanced stage of speciation by investigating the species barriers between the two closely related carnation species. The sympatric occurrence of the two carnation species provides an exceptional opportunity to study the late phases of speciation. In the field we found only some rare hybrids indicating that some mechanisms must uphold species barriers between the two carnation species. Astonishingly, we could not find species barriers resulting from postmating prezygotic isolation to intrinsic postzygotic isolation, i.e. we could not find relevant fitness disadvantages for hybrids (chapter 3).

These results shifted our attention to the role of the pollinator species. Our observations during field work suggested that the pollinators of *D. carthusianorum* show a high fidelity to this plant species. In fact, we experimentally confirmed strong preferences for the butterfly pollinators of *D. carthusianorum*. Thus, ethological isolation represents an important premating prezygotic isolation barrier between the two closely related carnation species (Grant 1949, 1994, Ramsey et al. 2003). Since a main pollinator of *D. sylvestris*, the hawkmoth *M. stellatarum*, did not show such preferences to either of the two carnation species, other mechanisms must play a role too. Presumably *M. stellatarum* transfers only few pollen when shifting from one to the other carnation species. Intraspecific pollen deposition of *M. stellatarum* on *D. carthusianorum* was rather low and just reached the threshold of fruit set (chapter 2). This is certainly caused by the long proboscides of this hawkmoth, which prevents the contact of the head and labial palps of the hawkmoth with the reproductive organs of the flower. In contrast, when visiting the deeper flowers of *D. sylvestris* pollen is deposited on the head of the hawkmoth. From this and our first experiment on pollination efficiency (chapter1) we conclude that interspecific pollen transfer mediated by *M. stellatarum* must be even lower than the threshold required for fruit set as a result of mechanical isolation (Grant 1949, 1994).

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