

# Pollination ecology of *Digitalis purpurea*

## - patterns and processes

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## Abstract

In this thesis I report on several aspects of the reproductive ecology of the short-lived, monocarpic plant *Digitalis purpurea* L. (Plantaginaceae). Observational field studies are combined with controlled crosses and greenhouse experiments to address questions on pollinator attraction, pollinator behaviour, and possible consequences thereof on mating patterns. Fitness consequences of different matings are investigated through the entire lifetime of progeny. In addition, I focus on the effects of display size and local plant density on pollination and florivory.

Bumblebee visitation rates at the plant level increased with display size and plant density, and although bout lengths also increased, the increase was too slow to balance the increased display size. The result was a decrease in per flower visitation rate with display size, indicating that pollinators do not mediate selection for larger display sizes in this population. A significant interaction between density and display size for the proportion of flowers visited per display suggests that density may modify the selective impact of pollinators.

The floral herbivore *Eupithecia pulchellata* Stephens (Lepidoptera: Geometridae) also showed an affinity towards large inflorescences and dense patches, but overall attack rate was low. However, the proportion of flowers attacked increased with inflorescence size, resulting in size-dependent floral damage that could select for smaller display. However, since overall attack rates were low, it is possible that the plants are capable of compensating floral loss at the observed attack rates.

The pollination system of *D. purpurea* (acropetal flower maturation of protandrous flowers in vertical racemes combined with pollinators that start foraging bouts at the base of the inflorescences), was shown to be effective in preventing geitonogamy, only 2.1% of plant visits of bout length two or more involved visitation to a male flower before a female flower. This system was also efficient in terms of both pollen import and export, independent of display size. In view of the short bout lengths (ca 2 flowers) it was surprising that pollen export did not decline with display size. This was due to increased skipping of flowers by pollinators on larger displays, resulting in high visitation rate to male flowers.

Inbreeding depression was significant for several life history traits. The 21% reduction in cumulative fitness was relatively small in view of the high outcrossing rate (estimated as 0.96) and may indicate that this population has experienced more selfing during periods of lower pollinator activity. Increased masking of deleterious alleles by the tetraploidy of *D. purpurea* may also contribute. However, when progeny with different inbreeding levels were grown in the presence of competitors, inbreeding depression was increased, indicating that inbred progeny will suffer larger fitness loss in natural environments. In this population, an optimal outcrossing distance was detected as a consequence of the outbreeding depression found at several life history traits in progeny from 30 m-crossings. Reports on within-population outbreeding depression are rare and my study suggests that outbreeding depression may be more common in autopolyploids than in diploids. The fact that the majority of plant species are believed to be polyploid, merits the inclusion of polyploids in future studies of mating system evolution.

## List of papers

- I. Grindeland, J. M., N. Sletvold & R. A. Ims 2005. Effects of floral display size and plant density on pollinator visitation rate in a natural population of *Digitalis purpurea*. **Functional Ecology** 19: 383-390.
  - II. Grindeland, J. M. & N. Sletvold. Darwin's pollination syndrome - a plant's dilemma resolved? Submitted manuscript
  - III. Sletvold, N. & J. M. Grindeland 2008. Floral herbivory increases with inflorescence size and local plant density in *Digitalis purpurea*. **Acta Oecologica**. In Press.
  - IV. Grindeland, J. M. 2008. Inbreeding depression and outbreeding depression in *Digitalis purpurea*: optimal outcrossing distance in a tetraploid. **Journal of Evolutionary Biology**. Published online 13 March 2008. [doi: 10.1111/j.1420-9101.2008.01519.x]
  - V. Grindeland, J. M. & N. Sletvold. Joint effects of inbreeding level and competition in F2 in tetraploid *Digitalis purpurea*. Manuscript
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# Introduction

Reproduction, this all-important event in the life of any organism, includes some evolutionarily determined decisions that are integral parts of the organism's life history, i.e. repeated reproduction or not and timing of the first reproduction, but also decisions that are more dynamic, i.e. with whom to mate. For plants, that are sessile and in most cases hermaphroditic, the latter decision involves how to increase the number of possible mates, whether selfing is an option, and if so, when to self. In order to maximize its reproductive output, a hermaphrodite must divide its resources between the sometimes conflicting interests of its two sexual functions. Moreover, many plant species have the possibility of selfing, but this requires cooperation between functions that the individual most of the time benefits from separating. Since the majority of plants experience inbreeding depression upon selfing, most species have traits believed to promote outcrossing. In this thesis I investigate different aspects of the pollination ecology and mating pattern of a self-compatible plant.

## **Display size and pollinator attraction**

The floral display is an important element of the animal-pollinated plant's pollination system and should be under selection to maximize the efficiency of pollen transfer between plants. Floral display is usually the combination of flower number and size, although other plant features may contribute to the overall attraction. Pollinators normally respond to larger displays by increasing their visitation rate (reviewed in Ohashi & Yahara 1999).

Once at the plant, the number of flowers visited before moving on to the next plant will be governed by the reward level of the flowers probed, rather than the display size. It is thus possible for the plant to separate the evolution of attraction from reward, in order to maximize pollen import and export. Such a separation is supported by the fact that nearly all studies investigating the effects of display size on pollinator attraction have found that larger displays increase pollinator visitation rates at the plant level, whereas results at the flower level are mixed. It is also commonly observed that although bout length (i.e. number of flowers visited per plant visit) increases with display size, the increase does not compensate for the

increased flower number (Mitchell et al. 2004 and references therein). The empirical results on the resulting per flower visitation rates vary from decrease to increase, with several reports on a constant relationship (e.g. Andersson 1988, Klinkhamer et al. 1989, Robertson & Macnair 1995, Ohashi & Yahara 2002). However, even if large displays are at a disadvantage as far as per flower visitation rate go, large displays may be advantageous if the quality of pollinations increase with display size.

### **Perils of display size: floral herbivory**

Although large displays are advantageous in attracting pollinators, they can also be disadvantageous due to increased attraction of antagonistic visitors. Animals expected to respond to display size include those species that seek out flowers as sites for oviposition, i.e. floral herbivores and predispersal seed predators. In terms of populations dynamics, the impact of floral herbivory and predispersal seed predation will be higher than that of herbivory on vegetative tissue, because the latter only destroys plant tissue that the plant normally is capable of regenerating (Kolb et al. 2007). Loss of seeds will also normally be more severe than the loss of flowers, anthers or ovules, but the demographic impact of loss of floral tissue can be large (Rose et al. 2005, McCall & Irwin 2006). The compensation of lost flowers is not always possible, and florivory may therefore represent a selective force opposing the potential pollinator-mediated selection for larger displays. Although several studies have reported that large plants have higher attack probability (Ehrlén 1996), the relationship between within-plant attack rate and display size has received less attention (Klinkhamer et al. 1997).

### **Plant density, animal behaviour and plant fitness**

Optimal foraging theory predicts animals to modify their behaviour according to the density of the resource sought after (Pyke 1984). Whether animals seek plants as immediate food sources or as resources for their offspring, they are expected to evolve strategies that reduce searching time. Accordingly, high density patches should be visited more often than low-density patches, and isolated plants should receive longer visits (i.e. more flowers probed). When to leave the individual plant



may be determined by the frequency of non-rewarding flowers, e.g. low nectar content in the case of pollination or evidence of prior oviposition in the case of floral herbivory, but these decisions may be influenced by the distance to the next plant with potential resources (flowers). Plant density could therefore modify animal behaviour in ways that change the selection on display size in dense vs. sparse patches.

### **Display size and fitness through male and female function**

If quality of pollinator visits are as important as quantity, animal-pollinated plants will benefit from receiving many short visits instead of few longer ones (Iwasa et al. 1995). Female function will suffer if bout lengths are long because pollinator pollen load becomes depleted, and male function will suffer because of pollinator saturation. Long bouts will also increase the amount of pollen lost before the pollinator leaves the plant, thereby reducing potential siring success (Rademaker et al. 1997). In hermaphroditic plants there is the added disadvantage of within-plant pollen transfer, resulting in stigma clogging if the plant is self-incompatible, and self-fertilisation if self-compatible (de Jong et al. 1993). Moreover, loss of outcrossing opportunities will result from pollen discounting in both cases, and in the case of self-compatible plants, from ovule discounting as well (Barrett 2002).

This is the backdrop for the “plant’s dilemma” described by Klinkhamer & de Jong (1993): Almost all plant features that lead to increased attractiveness to pollinators also lead to longer flower visitation sequences. The result is that larger displays receive more visits, but also increased potential for geitonogamy. A solution to this dilemma would be to receive as many pollinator visits as possible, each of them to a single flower (Klinkhamer & de Jong 1993). However, it is difficult to imagine how this could be achieved without disposing of reward altogether, thereby adopting a new set of problems experienced by non-rewarding species (e.g. frequency dependency, Jersakova et al. 2006). Other solutions to the dilemma could involve temporal separation of sexual functions or genetical incompatibility between pollen and stigma. One special example that utilises both spatial and temporal separation of sexual functions is the combinations of plant traits described by Darwin (1862) and coined “Darwin’s [pollination] syndrome” (McKone et al. 1995): Vertical inflore-

scences with acropetalous flower maturation, protandrous flowers and pollinators that start at the bottom of the inflorescence. Through sequential flower dichogamy these plants achieve inflorescence herkogamy, resulting in spatial separation of sexual functions that will ensure pollen import and export in the same bout if pollinators arrive at a lower, female flower, and continue upwards until visiting at least one male flower (Best & Bierzychudek 1982). Although this pollination syndrome seems to function as predicted under experimental conditions (Best & Bierzychudek 1982), it is not known how effective it is under natural conditions, nor is it known how the effectiveness may correlate with display size.

The long-held view that dichogamy has evolved as a mechanism reducing inbreeding has been challenged since many taxa exhibit both dichogamy and self-incompatibility (Lloyd & Webb 1986). Lloyd and Webb's alternative hypothesis of reduced interference between sexual functions has later been supported in the case of protandry because this trait is often found in self-incompatible taxa, but selfing-avoidance is still regarded as the most important reason for protogyny (Routley et al. 2004). Darwin's pollination syndrome should also warrant low sexual interference within individuals.

Attractiveness of plant display has also been considered in view of allocation to sexual functions (de Jong & Klinkhamer 2005). The application of Bateman's principle (1948), i.e. that female fitness is resource limited and male fitness is mate limited, to plants, gave the prediction that attractiveness of floral display should benefit male fitness more than female fitness (Bell 1985). This prediction has received some empirical support (Stanton et al. 1986, Mitchell 1993, Aizen & Basilio 1998, Carlson 2007), but has also initiated much debate on the generality of these relationships (e.g. Burd & Callahan 2000, Ashman & Morgan 2004). In plants with Darwin's pollination syndrome, nectar production is higher in female phase flowers (Best & Bierzychudek 1982, Galen & Plowright 1985) and higher flower visitation rate may accordingly be expected to female phase flowers. If so, this suggests stronger pollinator limitation of male function. This system also provides opportunities for investigating correlates of male reproductive success (i.e. opportunities for pollen

export), an aspect of mating systems greatly underrepresented in studies of pollination ecology (Barrett 2003).

### **Genetical costs of mating**

Inbreeding depression, i.e. the reduction in fitness of offspring from matings between related individuals, has been regarded as the most important factor driving the evolution of mating systems since Darwin's seminal work (Darwin 1876). The fact that most angiosperms are hermaphroditic and thus capable of the most extreme form of inbreeding (i.e. selfing) has given rise to an enormous amount of theoretical and empirical work on the existence and severity of inbreeding depression (Lande & Schemske 1985, Schemske & Lande 1985, Charlesworth & Charlesworth 1987, Husband & Schemske 1996, Armbruster & Reed 2005). Lande and Schemske (1985) showed that inbreeding depression is expected to evolve jointly with selfing rate and that the stable situation would be predominately selfing populations with low degree of inbreeding depression ( $\delta < 0.5$ ) and predominately outcrossing populations with high degree of inbreeding depression ( $\delta > 0.5$ ). However, their conclusions depend on the underlying mechanisms of inbreeding depression.

Two alternative genetical mechanisms have been proposed to account for inbreeding depression: the partial dominance hypothesis and the overdominance hypothesis (Schierup & Christiansen 1996). The former explains inbreeding depression as the expression of deleterious alleles, at least partially recessive, in homozygous form, and the latter states that heterozygosity *per se* is advantageous. Investigations on the evolution of selfing rates and inbreeding depression under the overdominance hypothesis predicts that in some situations intermediate selfing rates may be stable (Charlesworth & Charlesworth 1990). The two hypotheses also differ in their predictions on the level of inbreeding depression if inbreeding increases: With overdominance, inbreeding depression will increase with increased selfing, whereas with partial dominance, inbreeding depression may decrease at some point (Charlesworth & Charlesworth 1990, Barrett & Charlesworth 1991). In the latter case the genetic load will be reduced as more deleterious alleles are exposed to and removed by selection (Charlesworth & Charlesworth 1987) and such purging has been reported

(Barrett & Charlesworth 1991, Crnokrak & Barrett 2002, but see Byers & Waller 1999). At present, the partial dominance hypothesis has received most support (Charlesworth & Charlesworth 1999, Crnokrak & Barrett 2002, Carr 2003).

Inbreeding depression may also be influenced by the ploidy level of the species. Polyploid species have multiple copies of their nuclear genome and the consequences for the expression and evolution of inbreeding depression in natural populations is not agreed upon. For autopolyploid species both reduction and increase in expressed inbreeding depression compared to diploid species has been predicted (Bennett 1976, Ronfort 1999), but empirical investigations in natural populations are few (Galloway & Etterson 2007, and references therein).

Due to limited gene flow distances, plant populations may be structured with related plants growing in the vicinity of each other. Matings over short distances may therefore result in biparental inbreeding depression and offspring quality will improve with distance between mates. However, at some distance the fitness of offspring will decline because mates will be genetically dissimilar due to genetic drift or natural selection. The outbreeding depression that results when genetically dissimilar individuals mate has been observed in many species, but the distance where it is detected varies greatly between species (Price & Waser 1979, Sobrevila 1988, Waser & Price 1989, Fischer & Matthies 1997, Fenster & Galloway 2000, Montalvo & Ellstrand 2001). Outbreeding depression after matings between plants separated by large distances is interesting in terms of elucidating the mechanisms of hybrid breakdown, but in order to affect the evolution of populations, effects must be present at distances comparable to those of gene flow. Outbreeding depression at relevant scales has been detected in several species (review in Waser 1993), and it has been suggested that the population subdivision this represents could be the early stages of sympatric speciation (Waser et al. 2000). The mechanisms invoked to explain outbreeding depression are the so-called ecological mechanism (i.e. selection yields adaptation to different microhabitats and progeny are maladapted to both habitats) and the genetic mechanism (i.e. coadapted gene complexes arisen through drift and separated by low gene flow, will be broken up in progeny) (Schierup & Christiansen 1996). In

populations simultaneously experiencing inbreeding depression and outbreeding depression there exists an optimal outcrossing distance (Price & Waser 1979).

Inbreeding depression has been shown to vary through life history stages and investigations should cover as large a part of the life history as possible (Husband & Schemske 1996). In the case of outbreeding depression comparatively little is known of how the expression of outbreeding depression vary through the life history of progeny (Waser et al. 2000). The expression of inbreeding depression has also been shown to vary with environmental conditions. Studies comparing progeny grown in greenhouse or common garden with those grown in field conditions have found more severe effects in the latter conditions (Dudash 1990, Armbruster & Reed 2005). In polyploids, knowledge on how inbreeding and outbreeding depression is expressed through the life cycle is very limited, especially in the case of outbreeding depression (Etterson et al. 2007, Galloway & Etterson 2007).

## **Aims of the thesis**

With data from observations in a natural population and from greenhouse-grown progeny of controlled crossings, I investigate several aspects of the reproductive ecology of short-lived, monocarpic *Digitalis purpurea* L.

- Does display size and plant density influence the plant and flower visitation rate of pollinators and the probability of floral herbivory? *Paper I & III.*
- Is the pollination system of *D. purpurea* efficient in promoting outcrossing and how does it relate to display size and plant density? *Paper II.*
- Is there an optimal outcrossing distance in this population and how does the expression of inbreeding depression and outbreeding depression vary throughout the life cycle of progeny? *Paper IV*
- How does expression of inbreeding depression vary with inbreeding level and level of competition? *Paper V.*

# Species and population

## ***Digitalis purpurea***

*Digitalis purpurea* L. (Plantaginaceae, sensu APG II 2003), the common foxglove (Fig. 1c), is in Norway native to coastal areas from the south-western parts northwards to Nord-Trøndelag county (Lid & Lid 2005). It is found on naturally and man-made disturbed areas, e.g. pasture, roadsides, clear-cuttings, screes and tree uprootings. The species has a persistent seed bank (van Baalen 1982) that can survive more than 80 years in the soil (K. Fægri, pers. comm.). Recruitment is restricted to bare soil because germination is inhibited by low light conditions (van Baalen & Prins 1983). Germination usually takes place in spring, and a rosette is produced the first season that may flower the following summer after vernalization during winter, but flowering can be delayed several years (Sletvold & Grindeland 2007). *D. purpurea* has traditionally been termed a biennial plant, but because it may delay flowering beyond the second summer it is more accurately described as a short-lived perennial (Sletvold 2005, Sletvold & Grindeland 2007). In Norway the species is considered strictly monocarpic in natural habitats, but survival through secondary rosettes and repeated flowering is common elsewhere (van Baalen & Prins 1983). Many populations are transient, i.e. populations are established after some disturbance, and disappear in a few years. However, the species may have long-lived populations in areas where small, frequent disturbances occur, i.e. pastures (Sletvold & Rydgren 2007).

Plants produce one or a few racemose inflorescences on average around 1 m height (mean±sd = 101±28 cm, N= 133, Paper I) with an average of 50 flowers each (mean±sd = 51±11, N= 133, Paper I). The pink to purple flowers are 5 to 6 cm long with nectar guides in the shape of dark spots surrounded by lighter areas on the lower lip of the bell-shaped flower. The inside of the lower lip of the flower have hairs, several mms long, which prevent small insects from entering the flower and removing nectar (pers. obs.). The flowers are self-compatible (Darwin 1876), nectar producing and visited mainly by bumblebees (Best & Bierzychudek 1982, Paper I). There is little spatial separation of the sexes within flowers, but distinct temporal separation (protandry). Acropetal flowering combined with protandry results in

inflorescences in which the sexes are spatially separated: below the topmost buds follow newly opened, prefertile flowers, then male flowers, neuters (flowers that are neither male nor female), female flowers, and developing capsules. When pollen removal is low, flowers simultaneously expressing male and female functions may replace some or all neuters (pers. obs.). Nectar production per flower increases with flower age, resulting in decreasing reward upwards in the inflorescence (Percival & Morgan 1965). Higher rewards in lowermost flowers induce bumblebees to visit the lowest, female flowers first, thereby promoting crosspollination (Best & Bierzychudek 1982). In the absence of pollinators *D. purpurea* is able to produce seeds by delayed selfing, although fruit set and seed set is variable and generally low (Darwin 1876, N. Sletvold, pers. comm.). Each flower may produce more than 1000 seeds each weighing ca. 90 µg (Salisbury 1942). Seeds are ballistically dispersed, i.e. via a passive ballistic mechanism (van der Pijl 1972) and most seeds are likely to fall within a few meters of the maternal plant (van Baalen 1982).

### **Study population**

Field work was carried out in an established population in a pasture located at Øydvinstod in Ulvik (Hordaland County, 60° 34' N, 6° 58' E) in south-western Norway (Fig. 1a, b). The site was located on the steep south-facing slopes towards the Hardangerfjord, (5 – 60 m a.s.l.). The pasture was grazed by sheep in spring and late summer. The population of *D. purpurea* had been roughly constant in size for several years prior to this (N. Øydvin, pers. comm.). The total area of ca 6000 m<sup>2</sup> consisted mainly of grassland scattered with patches of *D. purpurea* of varying size and density. Individual patches of *D. purpurea* may be transient and disappear as the gap closes, but new patches of *D. purpurea* will appear as new gaps in the vegetation are formed (Sletvold & Rydgren 2007).

### **Pollinators and other visitors**

The study population was probably exclusively pollinated by bumblebees, *Bombus* spp. (Paper I). In total four species of *Bombus* (taxonomy follows Løken 1992) were

observed, but one species, *B. hortorum*, was the main pollinator in both years with more than 50% of visits.

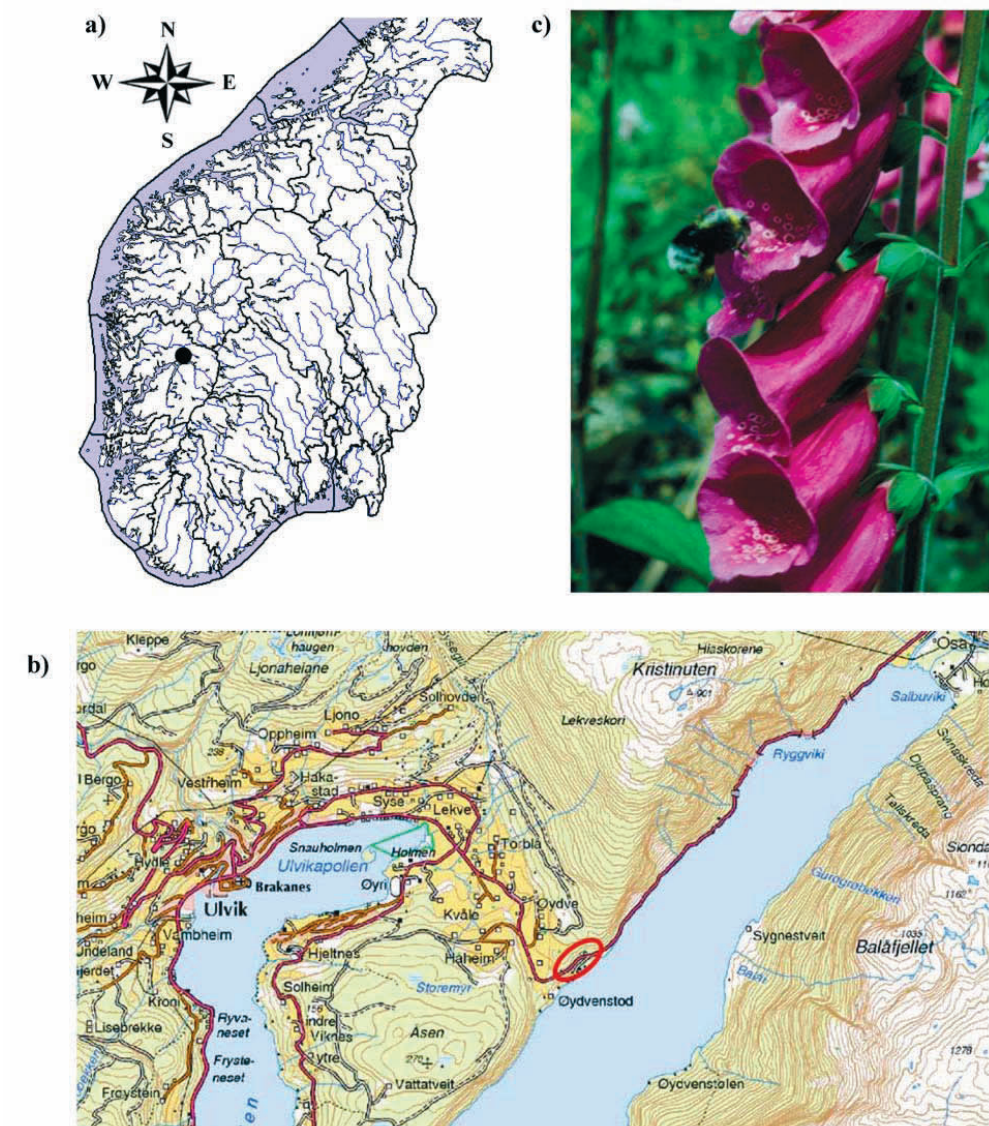


Figure 1. a) Map of Southern Norway with Ulvik indicated with black dot. b) Detailed map of Ulvik-area with study area indicated with red ellipse. c) *Digitalis purpurea* and its principal visitor, *Bombus hortorum*.



The year-to-year variation in species abundance was probably influenced by the composition of surrounding plants that bloomed during the observation period. Especially, flowers of various *Rubus* species were much visited by the smaller *B. hypnorum* and *B. lucorum*. The majority of *Bombus* individuals observed on *D. purpurea* collected nectar, but some pollen-collection was observed. When collecting pollen, the bumblebee turns upside-down inside the corolla and actively removes pollen from the anthers (pers. obs.). The only other visitor seen entering a flower of *D. purpurea* was a single *Apis mellifera* L. individual, but as it did not carry visible pollen, it is uncertain whether *A. mellifera* contributes to pollination. However, *A. mellifera* individuals frequently visited *D. purpurea* inflorescences collecting nectar from flowers where the corolla recently had fallen off.

Several plants had flower buds containing larvae of the moth *Eupithecia pulchellata* Stephens (Lepidoptera: Geometridae) (Paper III). Oviposition takes place on immature buds and one larva develops inside the bud. The larva feeds on developing anthers and ovaries, but does not stop the bud from growing, although infected flowers will not open because the larva seals the flower lips with silk.

## Methods

### **Pollinator observation (I and II)**

Data on pollinator visitation patterns were collected through field observations of a population of *D. purpurea* in 1998 and 1999. Natural patches, 12 and 14 in 1998 vs. 1999, respectively, were haphazardly selected to meet predefined criteria of plant density (3 and 2 levels in 1998 and 1999, respectively). Natural variation in daily inflorescence size was used. Pollinator species and sequence of flowers visited were recorded during three 10 min periods in 1998 and five 15 min periods in 1999, resulting in 6 h vs. 17.5 h total observation time. In 1999 the actual sexual phase of each flower was recorded during one of the observation days.

### **Floral herbivory (III)**

In 1997, 88 plants at a roadside were inspected and the number of flower buds containing an *E. pulchellata* larva was recorded during three days late in the flowering season. Data on the presence/absence of attacked buds in the 133 plants used for the pollinator observation study in 1999 (Paper I) were also included in this study.

### **Crossing distance (IV)**

In 1997, thirty randomly selected plants were pollinated with pollen from four different distances: 0 m (self), 1 m, 6 m and 30 m. At maturity fruits were collected, brought to the laboratory and seed set was determined. Germination and growth experiments were conducted under greenhouse conditions and the following life history traits were recorded: germination percent, germination speed, juvenile survival, juvenile size, flowering time, and flower number.

### **Inbreeding levels (V)**

A second generation of seeds with four expected levels of inbreeding ( $f = 0.028, 0.069, 0.14, 0.31$ ) was produced through controlled crossings of some of the plants from the crossing distance experiment. Six maternal families were randomly selected and seeds from each inbreeding level were sown in the greenhouse. Germination speed was recorded and a growth experiment with two competition levels (no competitor vs. four conspecifics per pot) was set up. Eight weeks after sowing the plants were harvested, dried, and dry mass was measured.

### **Statistical analyses**

Data were analysed with general linear models, generalized linear models and in some cases with non-parametrical models (Kruskal-Wallis) using various versions of SPSS (9.0-15.0, SPSS Inc, Chicago, USA) and SAS (8.0-9.1, SAS Institute, Cary, USA).

# Results and discussion

## **Pollinator visitation rate, floral display size and plant density**

There was an increase in plant visitation rate with floral display size in both years and across all densities investigated (Fig. 1, Paper I). In 1999, plants in dense patches received significantly more visits. These findings are in accordance with theory and most empirical reports (Pyke et al. 1977, Ohashi & Yahara 1999). The expected increase in bout lengths with increasing display size was supported by the data in 1999, but not in 1998. Moreover, the increase found in 1999 was quite slow, from 1.7 to 3.2 flowers per bout for display size of 3 vs. 12 (Fig. 2, Paper I), and it is interesting to note that in roughly half the plant visits, the pollinator left after probing one flower only (59% vs. 43% in 1998 and 1999, respectively. Paper I). In this population *D. purpurea* seems close to fulfilling Klinkhamer and de Jong's (1993) solution to "the plant's dilemma". Although Best and Bierzychudek's (1982) model predicted bumblebees to visit 46% of the flowers before leaving and this was confirmed from their observations on plants protected from visit prior to observation, they reported 63% one-flower visits on unprotected plants. The data collected from my population (e.g. 3.1 visits per hour per flower in 1999, Paper I), indicate that nectar standing crop will be low most of the time and predictions on bumblebee behaviour must take this into account.

A result that was consistent between years was that flower visitation rate decreased significantly with display size (Fig. 4, Paper I). Most earlier studies have found flower visitation rate to be independent of display size. The pollinator's behaviour may be explained in terms of risk averse foraging and the fact that variation in floral reward level has been found to correlate positively with display size (Biernaskie & Cartar 2004, Biernaskie & Gegeer 2007). It has also been suggested that inflorescences with flowers that require long handling time by the pollinator will receive shorter bouts (Ohashi 2002). Handling time on *D. purpurea* is quite long because the bumblebee must crawl into the pendant flower.

If male or female reproductive success is limited by per flower visitation rate, large displays are disadvantageous. Using per flower visitation rate as a measure of

success may, however, be misleading, since selection acts on the whole reproduction of the plant (Andersson 1988). Moreover, the lowest flower visitation rates observed were 1.2 per flower per hour (12-flower display, 1998, Fig. 4a, Paper I) which combined with the duration of male vs. female phases (2.1 and 1.5 d, paper II) and the duration of visitation period (10h) yields 25 vs. 18 visits to male and female flowers, respectively. Combining plant visitation rate in 1999 with probability of pollen import or export, the resulting pollen import/export frequency to the plant was quite high (Fig. 2). Even the lower values observed in 1998, are probably sufficient to achieve the necessary transport of pollen, and selection on display size should be mediated through quality rather than quantity of visitations.

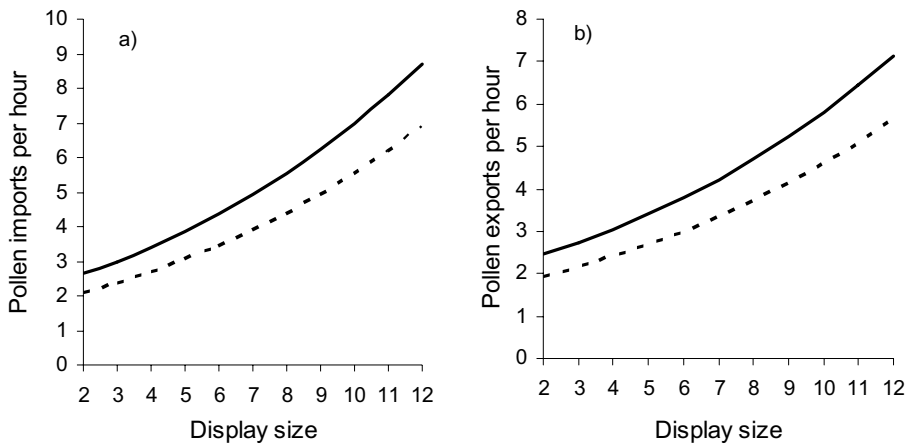


Figure 2. Pollen import (a) and pollen export (b) to and from inflorescences of *D. purpurea* in 1999 based on predicted plant visitation rate (Paper I) and predicted probability of import and export (Paper II). Lines represent high and low density patches, solid vs. dotted lines, respectively.

Patch density influenced plant visitation rate and bout length as expected: more plant visits in dense patches, but longer bouts in sparse patches (Figs 1 & 2, Paper I). More surprisingly, there was an interaction between density and display size for proportion of the display visited (Fig. 3b, Paper I), meaning that the proportion of flowers visited per display decreased faster in high density patches. This suggests that

selective pressures on display characters and mating patterns may be influenced by local plant density.

### **Floral herbivory**

The probability of herbivore attack increased strongly with inflorescence size and the probability was much larger in dense patches (Paper III). Total level of herbivory was low, only 16% of attacked plants lost more than 10% of their flowers. The proportion of damaged flowers increased with inflorescence size, indicating that this herbivore might select for smaller displays, which in the case of this monocarpic species could mean lower threshold size for flowering (Sletvold & Grindeland 2007). However, overall attack levels were low and in most cases probably within the range of flower numbers that the plant is able to compensate for. At the end of flowering, the majority of *D. purpurea* plants still have a number of small buds that have not opened (pers. obs.) and it is possible that these represent a surplus of flowers that can compensate for flowers lost to herbivory or other damage.

Pollinator visitation patterns and florivore oviposition patterns indicate that both plant visitors respond to increased display size with increased visitation rate, but the outcome of these increases are different: The herbivore's impact is binary (i.e. attacked flower or not attacked flower) while the pollinator's impact is quantitative and harder to assess the consequences of. Given the low level of herbivore attack and the high pollinator visitation rate, both visitors probably represent weak selection on display size. However, this may change in years with more severe herbivore attack or less pollinator abundance.

### **Plant's dilemma**

Pollinator visitation patterns in this population confirm the efficiency of Darwin's pollination syndrome in reducing opportunities for geitonogamous pollen transfer independent of display size (Paper II). Downward movements were relatively common (23% of bouts to two or more flowers), but only 2.1% of these involved visitation to a male flower prior to a female flower. In addition, 36% of all plant visits were to a single flower only. The presence of dual phased flowers may increase the rate of

pollinator mediated selfing, but the frequency of such flowers was very low on the investigated day in this population (2 of 364 open flowers, Paper II). In sum, these results support that Darwin's pollination syndrome secures a high outcrossing rate in this population of *D. purpurea*.

Plants probably received adequate pollinator service to both sexual functions (Fig. 5, Paper II). A high rate of pollen import is expected given the starting point of visiting bouts (70% of arrivals are to lower third of display, Table 2, Paper II). However, the result for pollen export is surprising; since most bumblebees start foraging at a lower flower and most bouts are quite short (Paper I), pollinators should rarely reach male flowers. The resulting model predicts more than half of the bouts to result in pollen export regardless of display size (Fig 5b, Paper II). The high pollen export rate can be accounted for by the skipping behaviour of the pollinators: skipping flowers is quite common and the rate increases with display size. When moving between flowers on vertical inflorescences, bumblebees may follow the simple rule of choosing the next flower vertically above the present one (Pyke 1978) in order to minimize the chance of revisitation. Large inflorescences of *D. purpurea* may therefore have several separate routes the pollinators can follow, leading to increased skipping of flowers. This system would be interesting to investigate in terms of the influence of inflorescence architecture on plant-pollinator interactions (Jordan & Harder 2006).

### **Outcrossing distance**

In this population significant inbreeding depression and outbreeding depression was detected at five vs. three life history traits. Inbreeding depression was found for seed mass, germination speed, juvenile size, flowering start, and flower number (Paper IV). Outbreeding depression was found for germination speed, juvenile size, and flowering start. Inbreeding depression increased at later life stages, whereas outbreeding depression was relatively constant. An estimate of cumulative fitness (germination per cent  $\times$  juvenile survival  $\times$  flower number) gave 21% inbreeding depression and 12% outbreeding depression, the latter marginally non-significant

( $P=0.10$ ). This demonstrates that intermediate distances (i.e. more than a few metres and less than 30 m) represent the optimal crossing distances in this population.

Outbreeding depression was found at several points in the early life history of progeny. Germination took on average 7% longer for 30-m progeny than for 6-m progeny, and this is probably a critical point in the life history of *D. purpurea*, since intraspecific competition is intense at this stage (seedling density of up to 0.5 million / m<sup>2</sup>, van Baalen & Prins 1983, Sletvold 2005). The inferior performance of 30-m progeny was upheld at juvenile size and flowering start. (Early flowering in this greenhouse experiment is viewed as a measure of how well the plant tolerated the vernalization conditions; whether early flowering constitutes a fitness advantage in natural environments is uncertain.) The reduction in flower number (23 flowers) of 30-m progeny was not statistically significant (Paper IV), but whether this represents a reduced difference between treatments at this stage or is due to the lower power of the statistical test (lower plant number because of space requirements) is not known. However, this experiment illustrates that outbreeding depression exists in this population and that the effects can influence mating pattern in this species.

Genetic substructuring of populations, resulting from selection or drift, is the proposed cause of outbreeding depression, but which of these processes that are responsible for the genomic incompatibilities in this population is not known. Planting progeny in parental sites could inform on the possible maladaptation at parental sites of progeny from long crosses, but in this species with its long-lived seedbank such an experiment may not be decisive, since one or both parental plants may be adapted to a microhabitat that existed several decades ago. Regardless of the origin of the genomic incompatibilities the increased number of gene copies in this autopolyploid species opens for more epistatic interactions (Etterson et al. 2007). Unfortunately, too few studies on outbreeding depression at within-population scale exist in general, and for polyploids in particular, to do more than raise the question of whether outbreeding depression is more common in polyploids.

Turning to inbreeding depression, this topic is certainly more reported on than outbreeding depression in diploids, but reports from natural populations of autopolyploids are few (four according to Galloway & Etterson 2007) and my study

represents only the second species investigated through the whole lifecycle, *Campanulastrum americana* being the first (Galloway et al. 2003, Galloway & Etterson 2007). Significant inbreeding depression was found at all quantitative traits investigated, but no effect was found at any binary trait (i.e. where alternatives are survival/death, flowering/not flowering), indicating that the frequency of lethal mutations is low in this population. Although the autotetraploid genome represents more opportunities for recessives to hide from selection, autotetraploids are also expected to purge their genetic load (Ronfort 1999). The pattern of expressed inbreeding depression is similar to what is expected for predominately selfing diploids (Lande & Schemske 1985) and contrasts with the outcrossing rate inferred in this population (0.96, Paper IV). However, the estimate for outcrossing rate used in this study is based on pollinator observations during two weeks at peak flowering. Outcrossing rate is known to vary through the season and between seasons (Culley et al. 1999, Hirao et al. 2006). Observations in other seasons in this population indicate pollen standing crop to be correlated with weather conditions, and during and immediately after periods of rain there are ample opportunities for autogamous selfing or pollinator mediated within-flower selfing, resulting in lower outcrossing rates. It is therefore possible that this population of *D. purpurea* have purged most of its lethal mutations through periods of selfing, although the possibility of increased masking of lethals due to autopolyploidy cannot be ruled out.

A final point to consider for an autotetraploid species is the prevalence of tetrasomy in the genome. Populations that have experienced a chromosome doubling event are expected to go through a process of diploidisation that through time will render them effectively “diploid” (Wolfe 2001, Leitch & Bennett 2004). Polyploids may therefore be viewed as being in transition towards a diploid-like state and where a population can be found along this trajectory will depend on the time passed since polyploidisation. Tetrasomic inheritance was found for some isozymes in this population, but to what extent this is representative for the whole genome is unknown. *D. purpurea* is probably an ancient polyploid since most other species in the genus share the chromosome count with *D. purpurea* (Missouri Botanical Garden - W3 Tropicos 2007) and it is possible that some of the loci have disomic inheritance. Nevertheless,



since this study represents one of the first investigations on the effects of inbreeding depression and its possible ecological consequences, more studies are needed before more general conclusions on the effects of inbreeding depression in autopolyploids can be drawn.

### **Levels of inbreeding and ecological responses**

It is generally not known to what extent the observed inbreeding depression in benign greenhouse environments is representative for the field situation, but several studies have found increased inbreeding depression in more stressful environments (reviewed in Armbruster & Reed 2005). Different types of stress have been investigated, but results indicate that biotic stress (e.g. competition) increases inbreeding depression more than abiotic stress (e.g. water stress) (Cheptou et al. 2000a, Cheptou et al. 2000b). The expression of inbreeding depression for juvenile size in *D. purpurea* was more severe when inbred progeny were grown with competitors (Fig. 2, Paper V) and this corroborates the notion that inbreeding depression is stronger in stressful environments. Comparing the results with those found for the highly outcrossing (multilocus outcrossing rate of 0.94) autotetraploid *C. americana* is particularly interesting. This species showed a pattern of expressed inbreeding depression quite similar to my findings when grown in the greenhouse, resulting in a cumulative inbreeding depression of 0.23, albeit with significant inbreeding depression also for some binary traits (Galloway et al. 2003). When grown at native sites cumulative inbreeding depression rose to 0.94, again with some inbreeding depression at binary traits (Galloway & Etterson 2007). The detection of strong inbreeding depression already at the juvenile stage in *D. purpurea* when grown with competition ( $\delta = 0.37$ , Fig. 2, Paper V), suggests that it would translate into severe lifetime inbreeding depression in field conditions.

How the expression of outbreeding depression in greenhouse conditions relates to field conditions is not known (Edmands 2007), but if patterns resemble those found for inbreeding depression, the fitness loss following 30-m outcrossing could be large; greenhouse estimates of outbreeding depression was stronger than inbreeding depression for the key life history stage germination speed.

## Conclusions

I have demonstrated that plant visitation rate increases with floral display size in *Digitalis purpurea* across all plant densities and in both years investigated. Bumblebee bout length also increased with display size, but the rate of increase was slow, resulting in a decrease in proportion of flowers visited with display size. The resulting per flower visitation rate decreased with display size, a somewhat unexpected result, and possibly the consequence of increased variation in reward level on larger displays. Plants in high density patches received longer bouts than plants with equally sized displays in sparse patches, in keeping with optimal foraging theory. However, the proportion of flowers visited decreased faster in dense patches, indicating that density may influence mating patterns and selective pressures on display size.

The probability of floral herbivore attack to plants also increased with display size and plant density. The proportion of flowers attacked increased with display size, indicating florivore mediated selection for smaller display, but flower attack rate was probably within what most plants are able to compensate for by increasing total flowering. However, in years with higher herbivore densities significant selection for smaller display may result, which in the monocarpic *D. purpurea* could translate into lower threshold size for flowering.

Darwin's pollination syndrome was found to be effective in avoiding geitonogamy and in securing sufficient pollinator service to both sexual functions at all display sizes during the investigated period. The surprisingly high rate of pollen export across all display sizes is explained by the progressive skipping of flowers by bumblebees as displays get larger, thereby servicing male phase flowers even if bout lengths are short and foraging starts at the bottom of inflorescences.

Outbreeding depression was detected at several life history traits. If exacerbated in the natural environment, this could affect the fitness of progeny from longer crossings quite negatively, especially at the important seedling establishment stage. Inbreeding depression was significant at most quantitative life history traits and was stronger at later life stages. Although the pollinator visitation pattern indicated high outcrossing, the lack of expressed lethal recessives suggests a history of selfing, probably in periods of low pollinator activity. The performance of inbred progeny

under competition indicated that inbreeding depression found in the greenhouse is a lower limit to that experienced in the field. The existence of joint inbreeding depression and outbreeding depression implies the existence of an optimal outcrossing distance within this population.

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