

# Herbivores, pollinators and selection on flowering time in tetraploid and octoploid *Cardamine Pratensis*

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## Plants & Ecology

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The Department of Ecology,  
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Cover: *Cardamine pratensis*, buds and flowers damaged by herbivores, at the study site  
(Tovetorp Research station, Sweden) Photo: Elsa Fogelström, 2012

## Summary

Repeated polyploidization events are thought to be among the most important causes of sympatric speciation throughout evolutionary time. Changes in phenology and trait expressions that arise in polyploids have been shown to affect plant interactions with insect herbivores and pollinators. If these interactions are changed, selection subsequent to the polyploidization event could contribute to further divergence, or increased similarity, of cytotypes. This study was conducted to investigate plant interactions with insect herbivores and pollinators and patterns of selection in tetraploid and octoploid *Cardamine pratensis* L. (Brassicaceae), and to answer the following questions: i) Is there phenotypic selection on flowering phenology and number of flowers? ii) Is pollen limitation or herbivory most important for variation in reproductive output? iii) During what stages of ovary and ovule development do variation in fitness mainly arise? iv) Can the intensity of interactions be linked to phenology and number of flowers, suggesting that selection is mediated by pollen limitation or herbivory? v) Do intensity of interactions, and of interaction-mediated selection, differ between ploidy levels?

From a combination of an observational study of herbivory and a hand pollination experiment, selection was found for earlier flowering in both subspecies. Intensity of herbivory was an important determinant of plant reproductive success, while hand pollination had little effect. Positive effects of flowering time on intensity of herbivory suggest that selection for earlier flowering was mediated by herbivores.

Tetraploids initiated flowering later and suffered from more intense herbivory than did octoploids. The direction of selection was similar in both subspecies. However, differences strength of selection and intensity of herbivory suggest that there is a possibility of selection for their further divergence.

## Sammanfattning

Polyploidisering anses vara en av de viktigaste orsakerna till artbildning inom populationer. Skillnader i blomningskaraktärer, såsom fenologi och blomantal, som uppstår i och med polyploidiseringen har visat sig påverka växters interaktioner med såväl pollinatörer som herbivorer. Förändringar i växt-insektsinteraktioner till följd av polyploidiseringen kan leda till selektion för ökade skillnader, eller likheter, mellan cytotyper. Studiens syfte var att undersöka växters interaktioner med insekter i form av pollinatörer och herbivorer, samt att undersöka selektionsmönster hos tetraploida och oktoploida *Cardamine pratensis* L. (Brassicaceae). Jag ville även besvara följande frågor: i) Sker selektion på blomningstid och blomantal? ii) Vilken växt-insektsinteraktion är viktigast för variation i reproduktionsframgång? iii) Under vilka utvecklingsstadier uppstår den största variationen i fitness? iv) Kan intensiteten av interaktionerna kopplas till blomningsfenologi och blomantal, vilket skulle indikera att selektion förmedlas genom pollenbegränsning eller herbivori? v) Skiljer sig styrkan av interaktionerna, och av interaktionsförmedlad selektion, mellan ploidinivåer? Studien utformades som en kombination av en observationsstudie av herbivori och ett handpollineringsexperiment, och jag fann selektion för tidigare blomning hos de två underarterna. Reproduktionsframgång styrdes av herbivori snarare än pollenbegränsning. Förlust av potentiell fitness genom att fröämnen inte utvecklades till frön var den faktor som starkast påverkade variation i fitness, och detta var också den fas i utvecklingen som bidrog i störst utsträckning till kvantitativ förlust av fitness. Ett positivt samband mellan blomningsfenologi och herbivoriintensitet indikerar att selektion för blomningstid var förmedlad av herbivorer.

Tetraploider blommade senare och utsattes för mer intensiva herbivorattacker än oktoploider. Detta, samt skillnader i selektionsstyrka, indikerar att selektion skulle kunna leda till ökad divergens av underarterna.

## Introduction

Polyploidization is an important cause of sympatric speciation (Soltis *et al.* 1993). It is now considered a highly dynamic process, where polyploidization events take place repeatedly, contrary to views of polyploidization as solitary events (Soltis *et al.* 1993). Allopolyploids are the results of hybridization between related species, while in autopolyploids, chromosome doubling has taken place within a species (Ramsey & Schemske 2002).

Polyploids often differ from their diploid ancestors in phenology and morphological traits, as well as in habitat preferences, physiology and life history traits (reviewed by Ramsey & Schemske 2002). Later flowering and slower development is common in autopolyploid populations, compared to the lower ploidy level parent populations (reviewed by Ramsey & Schemske 2002). Additionally, differentiation in habitat preferences and spatial distribution of polyploids and their ancestors has been reported in several taxa (e.g. Lövkvist 1956, Thompson *et al.* 2004, Ramsey 2011).

There are many examples of changes in plant traits brought about by polyploidization affecting plant interactions with mutualist and antagonist insects (e.g. Segraves & Thompson 1999, Arvanitis *et al.* 2010). Insects play an important role as agents of selection plants both as herbivores (Herrera 2000; Arvanitis *et al.* 2008) and pollinators (e.g. Sletvold & Ågren 2010, Bartkowska & Johnston 2012). If the outcome of these interactions differs between ploidy levels, selection subsequent to polyploidization might promote further divergence, or convergence, of cytotypes.

Herbivores have been identified as important agents of selection on floral traits and phenology in plants, and changes in these targets of selection brought about by polyploidization are likely to affect plant-herbivore interactions (Thompson & Segraves 1997; Thompson *et al.* 2004). Herbivores have been shown to discriminate between cytotypes, and polyploidization can promote novel interactions, host switches and host range expansions in insect herbivores (Janz & Thompson 2002; Thompson *et al.* 2004; Münzbergová 2006; Arvanitis *et al.* 2010). Thompson & Segraves (1997) found that autotetraploid *Heuchera grossulariifolia* are subject to more attacks from a pollinating herbivore moth than diploids. Earlier flowering in tetraploid *H. grossulariifolia*, bringing time of flowering closer to that of a closely related species, is likely to have enabled host switches and host range expansions in local populations of a pollinating herbivore moth (Janz & Thompson 2002). Although studies

investigating the effects of herbivores on selection subsequent polyploidization are still scarce, there are examples showing that herbivore-mediated selection can promote further divergence of polyploids. For instance, in *H. grossulariifolia*, seed parasitizing moth larvae have been shown to mediate divergent selection on flowering phenology and scape length (Nuismer & Ridenhour 2008).

Plants rely on pollen vectors for sexual reproduction, and their reproductive success is often limited by pollen availability (reviewed by Ashman *et al.* 2004). Pollinators play an, potentially, important role as agents of selection on plant reproductive traits. Pollinator mediated selection due to pollen limitation is likely to affect traits that increase plant attractiveness to pollinators (Haig & Westoby 1988), such as inflorescence size and number of flowers (reviewed by Wyatt 1982). Flowering phenology is also likely to be subject to selection, due to the importance of timing flowering with the occurrence of pollinators (e.g. Parra-Tabla & Vargas 2007).

Pollinator-mediated selection has been shown to promote divergence and reproductive isolation of cytotypes. In *H. grossulariifolia*, diploids and tetraploids differ in floral morphology as well as in flowering phenology and attract different suites of pollinating species, resulting in divergent selection for phenotypic traits and further reproductive isolation of cytotypes (Segraves & Thompson 1999; Nuismer & Cunningham 2005). Husband & Schemske (2000) identified pollinator fidelity do diploid and tetraploid *Chamerion angustifolium*, respectively, as an important factor enabling cytotypes to coexist in the field without hybridizing. As gene flow between cytotypes is held low, divergent phenology and morphology of cytotypes is maintained in mixed populations (Husband & Schemske 2000).

Several authors have pointed out the importance of taking both mutualist and antagonist interactions into account when analysing plant-insect interactions (e.g. Herrera 2000, Bartkowska & Johnston 2012, Burkhardt *et al.* 2012). For example, Bartkowska & Johnston (2012) found that both pollinators and herbivores contributed to selection on floral traits in *Lobelia cardinalis*, even though pollinators were identified as the main agents of selection. As herbivores and pollinators often create opposed selection on plant traits, both types of interactions are crucial in understanding selection and speciation subsequent the polyploidization event.

The aim of this study is to investigate plant-insect interactions and patterns of selection in tetraploid and octoploid *Cardamine pratensis* L. (Brassicaceae). Polyploidization in the *Cardamine* system has been extensively studied by Lökvist (1956) and Franzke & Hurka (2000). Additionally, interactions between tetraploid *C. pratensis* ssp *pratensis*, octoploid *C. pratensis* ssp *paludosa* and their main herbivores in the study area are well understood from studies conducted by Arvanitis and colleagues (Arvanitis 2007).

In this system, patterns of plant-herbivore interactions have been shown to differ between ploidy levels and habitats (Arvanitis 2007). Polyploidization in *C. pratensis* is likely to have resulted in a habitat switch of octoploids to moist and semi-shaded habitats, subjecting them to attacks by a specialist gall midge, and reducing attacks by a butterfly seed predator (Arvanitis 2007). The shift to semi-shaded conditions is likely to have enabled a host range expansion of the gall midge from the closely related plant that flowers simultaneously, and occur in the same type of habitats as octoploid *C. pratensis* (Arvanitis *et al.* 2010). Investigating seed predation in different habitats, tetraploids were found to be the main targets of butterfly attacks, and no effect was found of seed predation on phenology (Arvanitis *et al.* 2007), while growing in open mixed ploidy level populations, octoploids were more frequently attacked by butterflies than tetraploids, and selection for later flowering was found in both cytotypes (Arvanitis *et al.* 2008). Additionally, in mixed ploidy level populations, gall midges are almost completely specialized in octoploids, imposing selection for earlier flowering in the higher ploidy level (Arvanitis *et al.* 2010). Thus, differences between the ploidies can be explained by habitat and herbivore-mediated selection, in addition to differences that has arisen through polyploidization.

Studies of cruciferous plants have identified a wide variety of insects as actual and potential pollinators, including flies (Diptera), bees (Hymenoptera), butterflies (Lepidoptera) and beetles (Coleoptera) (e.g. Gómez *et al.* 2009, Robertson & Leavitt 2011, Fulkerson *et al.* 2012). However, interactions between *C. pratensis* and its pollinators have been poorly described and hence, little is known about the relative importance of pollinators and herbivores on reproductive success in the two subspecies. This study simultaneously investigates effects of herbivory and pollination on reproductive success in *C. pratensis*, keeping individuals of the two subspecies mixed in the same environment within their natural distribution range, subjected to their natural pollinators and herbivores. More specifically, I will try to answer the following questions; i) Is there phenotypic selection on flowering



phenology and number of flowers? ii) Is pollen limitation or herbivory more important for variation in reproductive output? iii) During what stages of ovary and ovule development do variation in fitness mainly arise? iv) Can the intensity of interactions be linked to phenology and number of flowers, suggesting that selection is mediated by pollen limitation or herbivory? And v) do intensity of interactions, and of interaction-mediated selection, differ between ploidy levels?

## Material and Methods

### *Study system*

*Cardamine pratensis* L. (Brassicaceae) is a perennial rosette herb with rhizomatous growth. It belongs to a phylogenetically young clade in the *Cardamine* complex, originated in Southern Europe in post-glacial times (Franzke & Hurka 2000). Its current distribution covers the northern hemisphere, from Europe, through North and East Asia to North America and Greenland. Several subspecies with different cytotypes are described, from diploid to dodecaploid, and polyploidization events are likely to have occurred repeatedly through autopolyploidy (Franzke & Hurka 2000). Three subspecies are found in Sweden, ssp. *pratensis*, ssp. *paludosa* and spp. *poleminioides* (Lövkvist 1956; Arvanitis 2007). The last is an alpine plant, while the other two subspecies have partially overlapping distributions in lowland areas throughout Sweden, where they are found in pastures, meadows, ditches and damp woods (Lövkvist 1956; Arvanitis 2007). The only cytotype of ssp. *pratensis* found in Sweden is tetraploid while octo- to dodecaploid cytotypes are known of ssp. *paludosa* (Lövkvist 1956; Arvanitis 2007). This study will focus on tetraploid ssp. *pratensis* ( $2n=30$ ) and octoploid ssp. *paludosa* ( $2n=56$  or  $64$ ) that co-occur and can be distinguished in the field.

Both subspecies produce one to several flowering shoots with white to pink flowers. Flowering usually starts in May and lasts for 6-7 weeks (Arvanitis 2007). The cytotypes are almost completely reproductively isolated and individual plants are mostly self-incompatible (Lövkvist 1956). They are, however, capable of extensive clonal reproduction in moist conditions, producing adventitious shoots from the rosette leaves (Lövkvist 1956). Tetraploids generally initiate more and smaller flowers and fruits (siliques), and start flowering earlier than octoploids, while octoploids tend to produce larger flowers, fruits and

flowering shoots (Lövkvist 1956; Arvanitis 2007). The subspecies also differ in local distribution, *ssp. paludosa* growing in localities that are more moist and shaded than the localities preferred by *ssp. pratensis* (Arvanitis 2007).

### *Study design*

This study was designed as a combination of an observational study of herbivory and a pollination experiment. The design made it possible to simultaneously estimate the effects of herbivory and pollinator interactions on plant reproductive success during several sequential developmental stages. It also allowed linking reproductive success to plant phenotypic traits.

Healthy looking plants with known ploidy level and origin were chosen in April 2012. They had all been cultivated and vegetatively propagated in the common garden at Stockholm University, Sweden. The majority of the plants used in this study were derived from natural populations in Ludgo parish, Sweden, while a few were derived from localities in the southernmost and northeast parts of Sweden. Some of these plants were chosen as outcross pollen sources. All plants in the study were given individual numbers for recognition.

The plants were transported to Tovetorp research station, Ludgo parish, Sweden, in the beginning of May 2012. The experiment was set up in an outdoor enclosure to keep out large grazers, while it allowed free access for insect herbivores and pollinators. Within the enclosure, all plants were replanted into equally sized pots. Due to the roughness of the ground, pallets were put out to stabilize the pots. The enclosure went from fairly open in the beginning of May to increasingly shaded from the start of emergence of canopy in early- to mid-June. The plants were haphazardly arranged onto the pallets, and the hand pollination experiment started May 10 with the flowering of the first plant. All plants that initiated flowering during May, 306 plants in total, were included in the experiment as treatment or control individuals. Plants were randomly assigned to receive hand pollination treatment or to be kept as controls. Controls were left without treatment. Randomization was carried out in Microsoft Excel 2011 for Mac, and was done separately for individuals of each ploidy level to get an equal number of control- and experimental plants from each cytotype.

In total, 280 plants could be followed over the entire study, excluding plants that were damaged in ways that prevented counting of flowers, or applying pollen by hand. The most common cause of exclusion was breakage of the inflorescence. Of the remaining 152

octoploids, 77 received hand pollination and 75 were controls. Of the remaining 128 tetraploids, 66 plants received supplementary pollen and 62 were controls. I monitored all plants included in the experiment from the first day of flowering until flowering shoot senescence. During this period, day of first flowering was recorded for all plants, and all open, healthy flowers in the pollination treatment group were given supplementary pollen on at least one occasion. Pollen availability was, in some cases, limited by harsh weather conditions (rainfall and low temperatures), desynchronized development of anthers and stigmas and of pollen only being available in sufficient amount on an anther for a very short time. To solve the problem of pollen shortage, pollen for hand pollination treatment was in some instances taken from any plant of the same ploidy level, be it a control plant, pollination plant or belonging to the pollen source group. No flower received supplementary pollen from a plant descending from the same genetic individual as the treatment plant.

The development of reproductive parts was categorized into four stages; bud, flower, initiated fruit and mature fruit. Flowers were counted as they opened, and fruits were regarded as initiated when the petals had fallen off and the ovary had started to elongate. Fruits were considered mature when fully elongated and swollen. All reproductive parts were scored every 2-4 days from May 10 until the end of June and fruits were collected just prior to seed dispersal. After collection, fruits were transported to the lab where the seeds of each fruit were counted.

### *Statistical analyses*

#### Selection analyses

Analyses of selection differentials and selection gradients were carried out in order to investigate whether traits were under selection and if patterns of selection differed between cytotypes. I included the number of flowers and flowering phenology, defined as day of first flowering, as traits in my models, and the analyses were carried out using the following standard methods, described by Lande & Arnold (1983).

Relative fitness and standardized traits were calculated separately for the two cytotypes. Relative fitness was calculated by dividing the number of seeds per plant with the mean of that ploidy level. Standardized trait measures were obtained by subtracting the population mean values of the respective traits from the original values. Those measures were then divided with the standard deviation of the respective traits.

The cytotypes were analysed separately, and the analyses of selection were performed in three steps. First, I estimated the selection differentials. In order to do so, I built one linear model for each trait, with the standardized trait as a single explanatory variable. The estimated slopes of standardized traits, the selection differentials, provide information of the direction and strength of direct and indirect selection for that trait. Secondly, I estimated the selection gradients in multiple linear regressions including both traits.  $\beta$ -estimates from this regression represent the linear selection gradient estimates and are measures of the direct directional selection on that trait. In the final step, I added quadratic and interaction terms to the models described above, and extracted the estimates for those terms. Quadratic and interaction terms estimate non-linear components of selection, and whether selection on one trait depends on values of the other trait, respectively.

### Pollen limitation and herbivory

Effects of pollen limitation and herbivory on fitness were analysed with GLM:s. I used three fitness estimates as response variables; the number of mature fruits per flower, the mean number of seeds per fruit and the total number of seeds per plant. Pollination treatment (yes/no) and intensity of herbivory on flowers and fruits were used as predictor variables, where herbivory was estimated as the proportion of flowers and initiated fruits that were lost to herbivory in each individual.

I made two models for each fitness estimate, one separate model for each subspecies, in order to examine the effects within each subspecies and to compare effects between the ploidies. Number of mature fruits per flower was square root arc sine transformed and number of seeds per plant was log transformed to improve homoscedasticity before analyses. No model selection was performed, since I wanted to estimate the relative importance of both predictor variables.

### Key factor analysis

In order to identify the development stage in which the plants would be most vulnerable to predation of reproductive parts, or more specifically, where loss of reproductive parts will contribute the most to variation in total plant fitness, a key factor analysis was performed. I used the method described by Podoler & Rogers (1975), and plant fitness was measured as number of seeds per plant.

First, I estimated  $n_i$ , the maximum potential number of seeds that an individual could produce in each development stage. Each  $n_i$  was calculated as the product of the largest seed number recorded for an individual in the dataset and the number of remaining ovaries in a development stage for an individual. The maximum number of seeds produced by an individual fruit was 20, why each individual count of reproductive parts was multiplied with that number. This was done for each individual and each development stage, so that every individual got an estimated maximum number of seeds that could be produced from its number of buds, flowers, initiated- and mature fruits, respectively.

Second, the killing power  $k_i$ , was calculated by subtracting the logarithm of  $n_{i-1}$ , the maximum potential number of seeds that an individual could produce in a development stage, from the logarithm of  $n_i$ , the maximum number of seeds that could be produced from the next stage. The obtained  $k$ -value is a measure of mortality from one development stage to another, and of the effect this mortality will have on the fitness of an individual:

$$k_i = \log_{10}(n_i) - \log_{10}(n_{i-1})$$

$k_1$  thus represents the killing power, the loss of potential seeds, due to loss of buds that did not develop into flowers.  $k_2$  is the killing power of flowers not initiating fruits, and  $k_3$  the killing power of initiated fruits being lost and not developing into mature fruits. The fourth and final  $k$ -value represents the fitness loss due to ovules in the remaining mature fruits not developing into seeds.

Third, the individual loss in potential fitness due to loss of reproductive parts over the entire growing season,  $K$ , was calculated as the sum of the separate  $k$ -values:

$$K = k_1 + k_2 + k_3 + k_4$$

For each subspecies, I made separate plots of each  $k$ -vector as a function of  $K$ , as suggested by Podoler & Rogers (1975). This was done in order to visually inspect the relationship between fitness loss in each stage and total loss of fitness. The slope of each relationship was obtained by regressing each vector of  $k$ -values on  $K$ , separately. The stage with the largest regression coefficient will have the killing power that contributes most to variation in total potential loss of fitness. I also calculated the arithmetic mean for each  $k$ -value as an estimate of proportional, quantitative fitness loss in each development stage.

### Phenotype and intensity of interactions

Plants were not pollen limited, why intensity of pollen limitation was not analysed. Effects of phenotypic traits on losses to herbivory were analysed separately for four sequential developmental stages. Intensity of herbivory was defined as the proportional loss of reproductive parts due to herbivory during a developmental stage. The first three stages were defined as the transitions from buds to flowers, from flowers to initiated fruits and from initiated to mature fruits. For the fourth transition, I calculated the proportion mature fruits lost to herbivory, as herbivory in this stage precluded the dispersion of seeds.

I then used GLM:s in order to analyse the effects of flowering phenology and number of flowers on herbivory in each stage. Analyses were performed separately for each subspecies. All possible interactions were included in the full models, as were the quadratic terms of the main effects. All response variables were square root arc sine transformed to reduce variance heterogeneity in the models, and models were compared and reduced with pairwise ANOVA:s. With this method, an ANOVA is used to compare the model with a version of that same model, where one explanatory variable is removed. A non-significant effect means that the variable can be removed, since it did not add any explanatory power to the model.

Statistical analyses were carried out in R version 2.15.1 (2012-06-22), using RStudio version 0.96.330 for Mac. Regressions and generalized linear models (GLM:s) were performed using the `lm` function. Slopes were estimated with the `summary` function, and GLM:s containing factors were analysed with the `Anova` function in the `car` package in R. As preliminary analyses showed variance heterogeneity in predictor variables between cytotypes, and as the

**Table 1.** Results from linear, and generalized linear and mixed models, analysing effects of standardized traits on relative fitness in tetraploid and octoploid *C. pratensis* plants.

Standardized trait	Tetraploids				Octoploids			
	$\beta$ -estimate	SE	t	p-value	$\beta$ -estimate	SE	t	p-value
<u>Selection differentials</u>								
Flowering phenology	-1.10	0.16	-7.01	<b>&lt;0.001</b>	-0.70	0.09	-7.69	<b>&lt;0.001</b>
Number of flowers	0.72	0.19	3.74	<b>&lt;0.001</b>	0.77	0.09	9.02	<b>&lt;0.001</b>
<u>Selection gradients</u>								
Flowering phenology	-1.00	0.14	-7.04	<b>&lt;0.001</b>	-0.42	0.09	-4.55	<b>&lt;0.001</b>
Number of flowers	0.54	0.14	3.82	<b>&lt;0.001</b>	0.56	0.09	6.12	<b>&lt;0.001</b>
(Flowering Phenology) <sup>2</sup>	0.31	0.10	3.22	<b>0.002</b>	-0.12	0.07	-1.71	0.091
(Number of Flowers) <sup>2</sup>	0.25	0.08	3.00	<b>0.004</b>	-0.10	0.06	-1.54	0.126
Number of flowers x Flowering Phenology	-0.41	0.12	-3.46	<b>0.001</b>	-0.42	0.11	-3.79	<b>&lt;0.001</b>

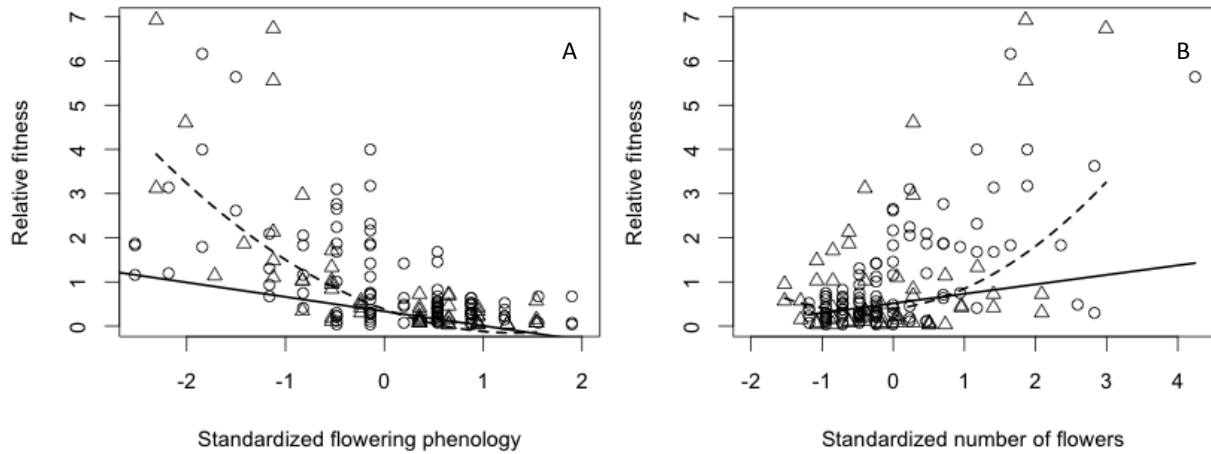
main purpose of this study was to compare effects between subspecies, the subspecies were analysed separately.

## Results

### *Selection analyses*

Octoploids initiated flowering earlier than tetraploids, and mean flowering date differed significantly between the ploidy levels (Welch Two Sample t-test,  $t_{253.84} = 4.46$ ,  $p < 0.001$ ). Tetraploids produced an average of 9.63 flowers, while mean flower number was 5.40 in octoploids (Welch Two Sample t-test,  $t_{250.79} = 8.10$ ,  $p < 0.001$ ).

Selection differentials were significant, indicating directional selection towards more flowers, as well as earlier flowering in both cytotypes (Table 1). Estimates of directional selection gradients were significant in both ploidy levels, indicating that the effects of traits on fitness was direct, and that selection was directional for earlier flowering and a larger number of flowers. These estimates showed stronger selection on flowering time in tetraploids than in octoploids. Selection on flowering time and number of flowers was directional in octoploids (Fig. 1), as the non-linear estimates of selection gradients were not significant for the higher ploidy level. In tetraploids, non-linear gradients were significant for both traits. Fitness decrease with later flowering was most pronounced in tetraploids that initiated flowering



**Figure 1.** Graphs showing selection gradients of flowering phenology (A) and number of flowers (B) in octoploid (○, solid line) and tetraploid (△, dashed line) *C. prantensis* plants. Gradients for octoploids are obtained from multiple regressions of standardized traits on relative seed number with quadratic and interaction terms included.

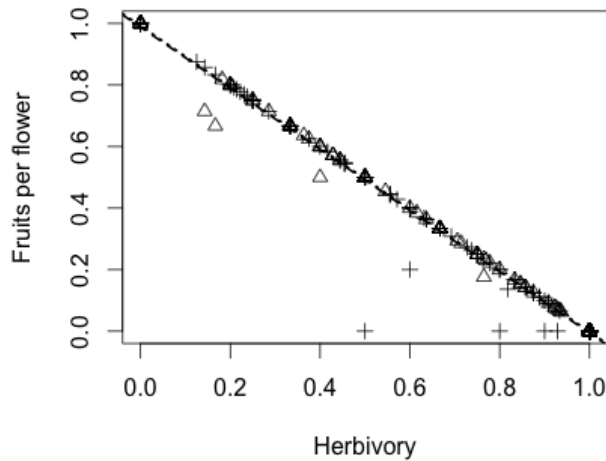
earlier than the cytotype average (Fig. 1A), while fitness increase with number of flowers accelerated in tetraploids that produced an above-average number of flowers (Fig. 1B). Significant interaction terms flowering time x number of flowers found in both subspecies suggest that focal trait-fitness relationships varied with the other trait (Table 1).

#### *Pollen limitation and herbivory*

Herbivory was the main determinant of reproductive success in both cytotypes, as it had significant effects on all three fitness measures (Table 2). Herbivory was also the main overall reason of flowers not developing into mature fruits in both cytotypes (Fig. 2). Octoploids initiated flowering earlier than tetraploids. As a result, tetraploids suffered higher losses to herbivory of reproductive parts than did octoploids (Welch Two Sample t-test,  $t_{257.17} = 8.93$ ,  $p < 0.001$ ), and both cytotypes produced approximately the same average number of mature fruits (Welch Two Sample t-test,  $t_{231.60} = -1.64$ ,  $p = 0.10$ ), despite the larger average number of buds ( $t_{242.82} = 9.50$ ,  $p < 0.001$ ), flowers and initiated fruits ( $t_{225.16} = 7.37$ ,  $p < 0.001$ ) produced in tetraploids.







**Figure 2.** ANCOVA graph showing the effects of herbivory on the proportion of flowers developing into mature fruits in *C. pratensis* plants receiving hand pollination ( $\Delta$ , dashed line) and in control plants (+, longdashed line). Herbivory was estimated as the proportion of flowers and initiated fruits lost to herbivory per individual. Individuals in which loss of flowers and initiated fruits are affected by factors other than herbivory, or a combination of factors, fall below the regression lines.

The majority of the observed herbivores were identified as gall midges (Cecidomyiidae) and aphids (Aphidoidea). The gall midge and butterfly that have previously been identified to specialize in *Cardamine* were not observed. Hand pollination had no significant effect on reproductive success in the two cytotypes (Table 2).

#### *Key factor analyses*

In both subspecies, non-development of ovules was the key factor influencing total variation in loss of potential seeds, followed by loss of initiated fruits (Table 3). The largest quantitative loss of fitness,  $\bar{k}$ , was also found for the phase of ovule development, followed by loss of initiated fruits.

#### *Phenotype and intensity of interactions*

Insect herbivore hatching started around May 25, after which all flowers were infested with larvae. Flowering phenology was an important determinant of intensity of herbivory in both subspecies (Table 4). In tetraploids, losses of buds and initiated fruits to herbivory increased linearly with later flowering, and there was a weak tendency of a similar relationship for loss of mature fruits ( $p = 0.084$ ). The significant interaction flowering phenology x number of

**Table 3.** Summary of the results from a key factor analysis<sup>+</sup>, as well as calculations of mean  $k$ -values, for tetraploid and octoploid *C. pratensis* plants.

Response		Tetraploids				Octoploids			
Cause of fitness loss		$\bar{k}$	$\beta$ -estimate	t	p-value	$\bar{k}$	$\beta$ -estimate	t	p-value
$k_1$	Loss of buds	0.08	-0.02	-2.56	<b>0.012</b>	0.05	-0.01	-0.95	0.34
$k_2$	Loss of flowers	0.05	-0.01	-1.82	0.072	0.02	0.01	0.89	0.37
$k_3$	Loss of initiated fruits	0.26	0.35	17.12	<b>&lt;0.001</b>	0.20	0.19	6.87	<b>&lt;0.001</b>
$k_4$	Non-developed ovules	0.51	0.68	30.93	<b>&lt;0.001</b>	0.65	0.82	26.84	<b>&lt;0.001</b>
K	Summed losses	0.90				0.92			

<sup>+</sup> Results from linear regressions of  $k$ -values (loss of potential seeds, due to loss of reproductive parts that did not develop into the next stage) on K (loss in potential fitness due to loss of reproductive parts over the entire growing season).

flowers for tetraploid bud loss to herbivory suggests that relationships between herbivory and day of first flowering differed between individuals with different flower number.

In tetraploids, number of flowers was a significant determinant of loss of flowers and initiated as well as mature fruits to herbivory (Table 4). Additionally, there was a weak tendency of an effect of flower number on loss of buds to herbivory ( $p = 0.078$ ). Loss of fruits to herbivory decreased linearly with number of flowers. The effect of flower number on proportional loss of flowers was non-linear, where loss to herbivory decreased more rapidly at higher flower numbers (Appendix). Loss of mature fruits to herbivory was lowest in tetraploids with 15-20 flowers, and increased at higher and lower flower numbers.

In octoploids, flowering phenology significantly affected loss of buds, flowers and initiated fruits to herbivory (Table 4). Bud loss was at its highest in late flowering individuals and lowest in individuals that initiated flowering just before mid-season, while loss of flowers was the lowest in individuals flowering mid-season (Appendix). Loss of initiated fruits increased linearly with later flowering. No relationship was found between the investigated traits and loss of mature fruits in octoploids. There was a tendency of loss of initiated fruits to increase linearly with flower number ( $p = 0.068$ ), but no significant effects could be found of flower number on losses to herbivory in octoploids.

**Table 4.** Results from generalized linear models investigating the relative effects of flowering phenology and number of flowers on loss of reproductive parts to herbivory during flower and fruit development. Herbivory was measured as the proportion of reproductive parts lost to herbivory in four developmental stages. All predictor variables were arc sine transformed prior to the analyses.

	Loss of Buds			Loss of Flowers			Loss of Initiated Fruits			Undeveloped Ovules		
	$\beta$	SE	t	$\beta$	SE	t	$\beta$	SE	t	$\beta$	SE	t
<b>Tetraploids</b>												
Flowering Phenology	0.048	0.014	3.52 ***				0.064	0.007	8.53 ***	0.034	0.019	1.76 .
Number of Flowers	0.024	0.013	1.78 .	0.037	0.015	2.39 *	-0.018	0.006	-3.25 **	-0.200	0.073	-2.74 **
(Flowering Phenology) <sup>2</sup>												
(Number of Flowers) <sup>2</sup>				-0.002	0.001	-2.83 **				0.006	0.002	2.47 *
Flowering Phenology x												
Number of Flowers	-0.004	0.001	-3.24 **									
<b>Octoploids</b>												
Flowering Phenology	-0.066	0.034	-1.94 .	-0.081	0.033	-2.44 *	0.086	0.015	5.55 ***			
Number of Flowers				-0.021	0.014	-1.45	0.021	0.011	1.84 .			
(Flowering Phenology) <sup>2</sup>	0.004	0.002	2.48 *	0.003	0.001	2.38 *						
(Number of Flowers) <sup>2</sup>												
Flowering Phenology x												
Number of Flowers				0.003	0.002	1.95 .						

Significance codes: \*\*\* < 0.001, \*\* < 0.01, \* < 0.05, . < 0.1

## Discussion

During the study year, there was selection for an earlier flowering phenology and a larger number of flowers in both subspecies. Variation in reproductive output could largely be explained as effects of herbivory, while hand pollination had no effect on fitness. Ovule development was the phase that explained most of the variation in loss of potential reproductive output, and also where the largest proportion of reproductive parts was lost. Intensity of herbivory was highest in late flowering individuals. These results strongly suggest that herbivory was the main interaction mediating selection on flowering traits in the study population this season. Effects of flower number on intensity of herbivory were found only in the lower ploidy level, suggesting that herbivores mediated selection on flower number in tetraploids but not in octoploids. Differences between ploidies were found in mean number of flowers, flowering time and intensity of herbivory. Tetraploids flowered later, produced fewer flowers and suffered more losses to herbivory than octoploids. The shapes of selection gradients and the strength of linear estimates of selection differed between the cytotypes.

### *Selection*

Octoploids initiated flowering earlier than tetraploids. Polyploids are generally thought to flower later than their lower ploidy level progenitors (reviewed by Ramsey & Schemske 2002), although herbivore mediated selection for earlier flowering in the higher ploidy level has been found in *H. grossulariifolia* (Nuismer & Ridenhour 2008). Later flowering in octoploid *C. pratensis* has been reported by Arvanitis *et al.* (2007), however, as habitat preferences differ between the two subspecies, observed differences in flowering phenology in the field could be mediated by habitat characteristics. Exposing the two cytotypes to the same environmental conditions, octoploids might in fact flower earlier than tetraploids. Additionally, as autopolyploids are often sturdier and less sensitive to low temperatures than their lower ploidy level progenitors (Levin 1983), low temperatures, rain and shading during the experiment could have made conditions more favourable to octoploids, that naturally occur in more shaded and moist localities (Lökvist 1956; Arvanitis 2007).

Number of flowers is expected to decrease with higher chromosome number in species with different ploidy levels (reviewed by Ramsey & Schemske 2002), and the results from this

study of octoploids producing a lower average number of flowers than tetraploids coincides with results from previous studies of the system (Lökvist 1956; Arvanitis 2007).

Selection for earlier flowering was found in both cytotypes. In tetraploids, selection on flowering phenology was non-linear, where fitness decrease with day of first flowering was most pronounced in early flowering individuals. In octoploids, selection gradients were linear, indicating directional selection for earlier flowering. Patterns of selection in *C. pratensis* have been shown to differ between ploidy levels and habitats (Arvanitis 2007). Herbivore-mediated selection for earlier flowering has been reported in octoploid *C. pratensis* in semi-shaded localities (Arvanitis *et al.* 2010), while selection for later flowering has been found in open habitat mixed ploidy level populations (Arvanitis *et al.* 2007). The relationships between day of first flowering and fitness found in this study are likely to have been highly dependent on local interactions and habitat characteristics at the study site the study year. Evolution of flowering phenology can occur rapidly on a local scale, mediated by both abiotic and biotic interactions (reviewed by Elzinga *et al.* 2007). Given that there was a sufficient amount of genetic variation for flowering phenology in the study individuals, it is possible that selection could have resulted in earlier flowering in the study population, were they to have remained at the study site after the experiment was ended.

Phenotypic selection for a larger number of flowers was found in both subspecies. In tetraploids, the fitness increase in plants that produced an above average number of flowers could be explained by successful pollinator attraction, but is more likely a result of positive density dependence of flowers and herbivore satiation (Crawley 1989). If the number of flowers damaged by insects was constant, the number of flowers damaged by herbivores would decrease with flower number after a certain amount of flowers produced per plant, since herbivores would then be satiated. In octoploids, a positive, linear selection gradient suggests directional selection for a larger number of flowers. Positive direct effects of flower number on fitness have been found in octoploid *C. pratensis* in a study conducted by Arvanitis *et al.* (2010). However, in their study, negative indirect effects of flower number mediated by herbivory were found as well (Arvanitis *et al.* 2010). In plants, the larger the number of flowers, the more seeds could potentially be produced. Hence, positive direct effects of flower number on fitness are expected, and the patterns found in this study could, to some extent, be due to flower number per se. However, the number of flowers and seeds that a plant can produce is ultimately limited by resource availability, why selection for more flowers might

not lead to increased flower production in *C. pratensis*. Hence, flowering phenology is likely to have been the main driver of selection this year.

### *Pollen limitation and herbivory*

Herbivory proved to be the main predictor of reproductive success in both cytotypes, while hand pollination had no effect. Herbivory did not only affect the number of fruits produced per flower and total seed production, but it also affected the number of seeds produced per fruit. As almost all flowers were infested with gall midge larvae from the time of hatching until pupation, and since most of the fruits that developed and matured seeds were intact at the time they were collected, it is likely that herbivores caused damage to reproductive structures in the flowers early in the season, during flowering.

The non-significant results of hand pollination treatment are somewhat surprising, but are likely due to plants being limited by resources rather than pollen availability. As *C. pratensis* plants are self-incompatible (Lövkvist 1956), the lack of differences in fitness between hand pollinated and control plants cannot be explained by selfing in control plants. Reproduction comes at a cost, and trade-offs between allocating limited resources to reproduction and to survival or growth are common (reviewed by Obeso 2002). For example, Totland & Eide (1999, in Lundemo & Totland 2007) found that supplementary pollen only increased reproductive success in *Ranunculus acris* when temperatures during the growing season were higher than normal, indicating that seed production was mainly limited by environmental conditions. It is possible that hand pollination in this study did not result in increased fitness of *C. pratensis* plants, since reproduction was already limited by low temperatures and herbivory during the study year.

Zimmerman & Aide (1989) found that, although hand pollination resulted in increased seed production in *Aspasia principissa*, fruit production in one year was associated with decreased vegetative growth in the next year, and large fruit production was associated with smaller fruit size, showing that plants were simultaneously limited by pollen and resource availability. It is possible that the plants used in this study will be able to compensate for fitness loss due to herbivory this season with increased reproduction next flowering season.

### *Key factor analyses*

Loss of reproductive parts during the phase of ovule development was the key factor explaining variation in plant reproductive success in both cytotypes, followed by loss of initiated fruits. This was also the phase where the largest proportion of losses occurred. Non-development of ovules in octoploid *C. pratensis* plants has been explained with lack of space for the large seeds in the pod (Lövkvist 1956). In this case, however, it was found to be the key factor determining variation in reproductive success in tetraploids as well, why a general lack of space could be ruled out as the main explanation.

### *Phenotype and intensity of interactions*

Flowering phenology was a significant predictor of the intensity of herbivory during bud and fruit development in both subspecies, where the intensity of herbivory was found to increase with later flowering, in accordance with the results from the selection analyses. As mentioned above, it is likely that ovules were damaged by herbivores during flowering. If this is indeed the case, it supports the results of plants benefiting from early or late flowering, since early fruit set will make less flowers targets to herbivory from the time of hatching until pupation of insect larvae. For octoploids, this agrees with previous results of increased gall midge attacks in later flowering individuals (Arvanitis *et al.* 2010). However, during this experiment tetraploids were attacked by gall midges as well, which was not the case in the study conducted by Arvanitis *et al.* (2010). The shape of the galls, which did not swell, and the fact that both subspecies were attacked, indicates that the plants were attacked by a different gall midge species than the specialist gall midge whose interactions with *C. pratensis* were investigated by Arvanitis (2007). Gall midge attacks on both subspecies might explain the similarity in patterns of herbivory and flowering phenology in the two cytotypes. The effects of flowering phenology on the intensity of herbivory during flowering (due to possible damages to reproductive structures, limiting the development of ovules during this phase) and fruit initiation, suggests that selection on time of flowering was mediated by herbivores in both subspecies during these phases of development.

Herbivore mediated selection is expected to target flowering phenology, as desynchronizing flowering with the incidence of herbivores could provide an escape from herbivory in time (Kawagoe & Kudoh 2010). Patterns and intensity of selection could be highly variable,



differing between populations and years, and highly dependent on the incidence of different herbivore species (Kawagoe & Kudoh 2010). In *C. pratensis*, herbivore mediated selection on flowering traits has been shown to vary between populations and years, as well as with habitat and herbivore identity (Arvanitis 2007). Hence, the patterns found this year could largely have been determined by the herbivore species involved, as well as by the choice of locality for the experiment.

Effects of flower number on intensity of herbivory were only found in tetraploids, where loss of flowers and initiated fruits to herbivory were the lowest in individuals that produced a large number of flowers. The intensity of herbivory was the highest in tetraploids that produced a below-average number of flowers, indicating that selection for an increased number of flowers in tetraploids was partly herbivore mediated, while selection in octoploids was not. In general, octoploid *C. pratensis* are larger and produce fewer, and larger, flowers than do tetraploids (Lövkvist 1956). Hence, the size of the inflorescence, or of flowers, might have been more important in affecting herbivore attraction than flower number, and it is possible that flowering traits other than flower number were targets of selection in octoploids.

### *Differences between cytotypes*

Differences between the two subspecies were found in phenotypic traits, intensity of herbivory and in patterns of selection. Octoploids initiated flowering earlier, produced fewer flowers, and were less attacked by insects than tetraploids. Linear estimates of indirect and direct selection suggests that selection on flowering time was stronger in tetraploids, which could be explained with them being subjected to more intense herbivory than octoploids.

While selection gradients were linear in octoploids, the rates of fitness loss with later flowering, and of fitness gain with number of flowers, were the highest in late flowering tetraploids and in tetraploids that produced a larger number of flowers than the cytotype average. Selection for a larger flower number was likely to have been partly herbivore-mediated in tetraploids, while in octoploids, it was more likely to be caused by a positive effect of flower number per se. It is possible that the differences between ploidy levels include different strategies to cope with herbivory, and that the negative effects of a higher mean loss of reproductive parts to herbivory in tetraploids was counteracted by a larger

number of flowers. Overall, the direction of selection was found to be the same in both subspecies, subjecting them to the same, controlled, environment. However, the differences between the ploidies in strength of selection and intensity of herbivory suggest that there is a possibility for selection for their further divergence.

### *Concluding remarks*

The study year, selection on flowering phenology was found in both ploidy levels, and intensity of herbivory was the highest in individuals that initiated flowering late in the season. These results suggest that selection for earlier flowering was herbivore mediated. This study identifies flowering phenology as an important factor determining reproductive success in *C. pratensis* plants, as desynchronized flowering time and incidence of herbivores will allow plants to escape herbivory in time.

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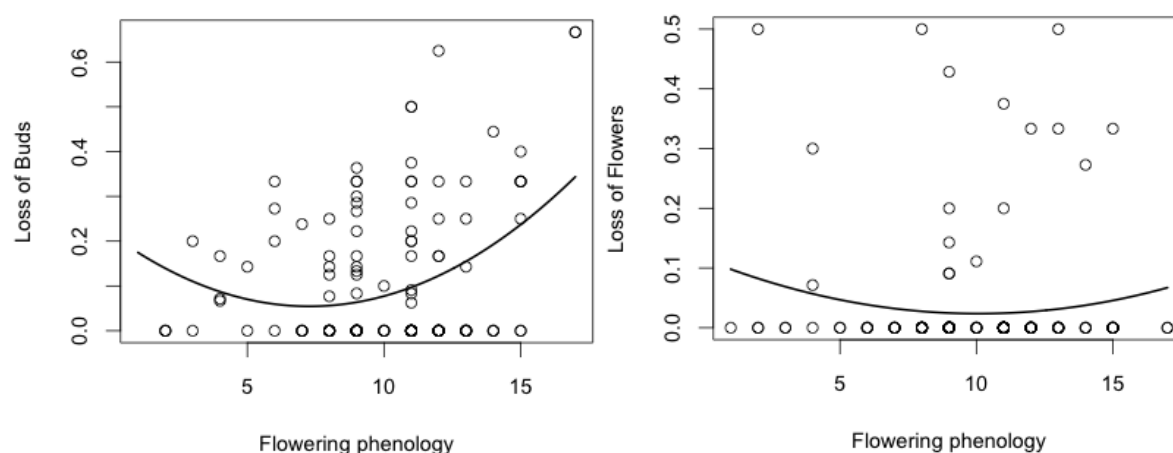
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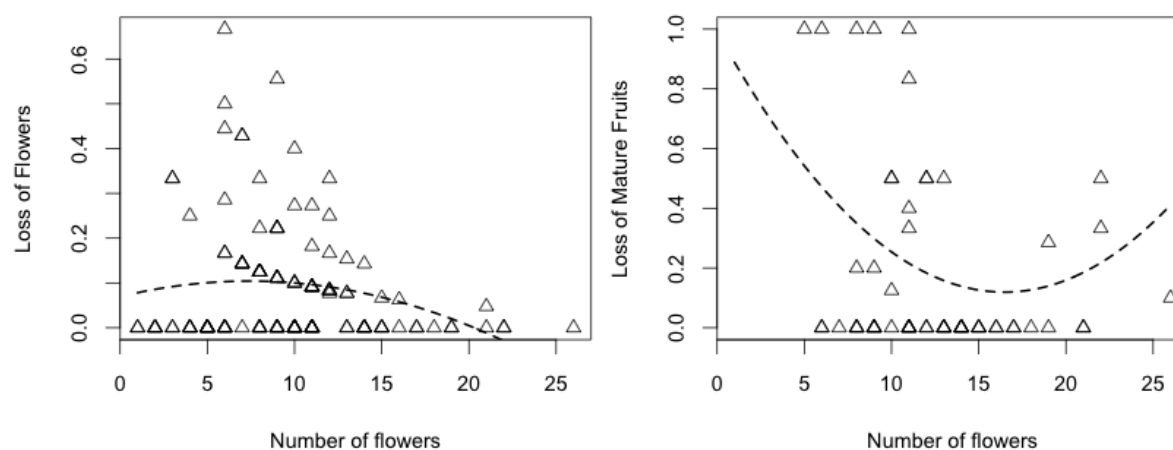
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## Appendix

Non-linear effects from generalized linear models investigating the relative effects of flowering phenology (Fig. 1) and number of flowers (Fig. 2) on intensity of herbivory during flower and fruit development in *C. pratensis* plants. Intensity of herbivory was measured as the proportion of reproductive parts lost to herbivory in four developmental stages.



**Figure 1.** Relative effects of flowering phenology on intensity of herbivory during flower and fruit development in octoploid *C. pratensis* plants.



**Figure 2.** Results from generalized linear models investigating the relative effects of number of flowers on intensity of herbivory during fruit development in tetraploid *C. pratensis* plants.