

# Herbivores and plant defences affect selection on plant reproductive traits more strongly than pollinators

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## Abstract

Pollinators and herbivores can both affect the evolutionary diversification of plant reproductive traits. However, plant defences frequently alter antagonistic and mutualistic interactions, and therefore, variation in plant defences may alter patterns of herbivore- and pollinator-mediated selection on plant traits. We tested this hypothesis by conducting a common garden field experiment using 50 clonal genotypes of white clover (*Trifolium repens*) that varied in a Mendelian-inherited chemical antiherbivore defence – the production of hydrogen cyanide (HCN). To evaluate whether plant defences alter herbivore- and/or pollinator-mediated selection, we factorially crossed chemical defence (25 cyanogenic and 25 acyanogenic genotypes), herbivore damage (herbivore suppression) and pollination (hand pollination). We found that herbivores weakened selection for increased inflorescence production, suggesting that large displays are costly in the presence of herbivores. In addition, herbivores weakened selection on flower size but only among acyanogenic plants, suggesting that plant defences reduce the strength of herbivore-mediated selection. Pollinators did not independently affect selection on any trait, although pollinators weakened selection for later flowering among cyanogenic plants. Overall, cyanogenic plant defences consistently increased the strength of positive directional selection on reproductive traits. Herbivores and pollinators both strengthened and weakened the strength of selection on reproductive traits, although herbivores imposed ~ 2.7× stronger selection than pollinators across all traits. Contrary to the view that pollinators are the most important agents of selection on reproductive traits, our data show that selection on reproductive traits is driven primarily by variation in herbivory and plant defences in this system.

## Introduction

Many phenotypic traits have evolved in response to mutualistic and antagonistic coevolutionary interactions. For example, plant–pollinator interactions are viewed as the primary driver of the evolutionary diversification of floral traits in angiosperms (Fenster *et al.*, 2004; Kay & Sargent, 2009). Pollinators exert direct selection on numerous reproductive traits that increase pollination success, including flower number (Conner

*et al.*, 1996; Parachnowitsch & Kessler, 2014), size (Benitez-vieyra *et al.*, 2016) and colour (Irwin & Strauss, 2005; Hopkins & Rausher, 2012; Sletvold *et al.*, 2016), flowering phenology (Sandring & Ågren, 2009), and mating system (Bradshaw & Schemske, 2003; Gervasi & Schiestl, 2017). In addition to pollinators, it is increasingly recognized that reproductive traits are subject to selection by antagonistic herbivores and that selection is altered by variation in plant defensive chemistry, although the relative effects of these three agents have yet to be investigated.

Coevolution between plants and herbivores has resulted in the diversification of chemical and physical defences used by plants to protect themselves from

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damage (Fraenkel, 1959; Ehrlich & Raven, 1964; Carmona *et al.*, 2011). Furthermore, herbivores are known to act as potent agents of selection on plant reproductive traits and herbivore-mediated selection often equals or exceeds the magnitude of selection imposed by pollinators (Johnson *et al.*, 2015). This occurs because herbivores and pollinators often use many of the same cues to locate plants (Strauss & Whittall, 2006). For example, larger flowers (Theis *et al.*, 2014), floral displays (Brody & Mitchell, 1997) and plant volatiles (Theis & Adler, 2012) can all attract herbivores and lead to direct herbivore-mediated selection on reproductive traits. Indirect selection by herbivores on reproductive traits can also occur when herbivores alter pollinator behaviour or influence patterns of resource allocation, thereby reducing plant investment in reproductive traits. For example, greater leaf herbivory in Missouri evening primrose (*Oenothera macrocarpa*) reduced flower size, resulting in lower pollinator visitation and reduced seed set of damaged plants (Mothershead & Marquis, 2000). Thus, herbivores can both directly and indirectly impose selection on plant reproductive traits.

Although herbivores and pollinators may independently impose selection on plant reproductive traits, plants often experience selection from both agents simultaneously. Simultaneous selection on floral traits by herbivores and pollinators can lead to reinforcing (i.e. same direction; Sletvold *et al.*, 2015), or more commonly conflicting (i.e. opposing directions) selection on reproductive traits (Johnson *et al.*, 2015). For example, in bird's-eye primrose (*Primula farinosa*), taller inflorescences attract more pollinators, but they are also more susceptible to grazing herbivores, resulting in opposing selection on inflorescence height (Ågren *et al.*, 2013). Despite advances in our understanding of the roles of herbivores and pollinators in imposing selection on plant reproductive traits, recent evidence suggests that variation in plant defences may modify interactions with these biotic agents and alter herbivore- and pollinator-mediated selection on plant reproductive traits.

Plant defences may modify the extent to which biotic agents impose selection on reproductive traits by altering the intensity of species interactions. For example, by reducing the strength of interactions with herbivores (i.e. through reduced herbivory), plant defences may reduce the strength of herbivore-mediated selection on plant traits. In addition, some studies have shown that damaged plants are less frequently visited by pollinators (Lehtila & Strauss, 1997; Mothershead & Marquis, 2000), leading to the possibility that defences may alter the strength of pollinator-mediated selection by reducing herbivore damage. Alternatively, pollinator-mediated selection may be altered by plant defences when defences expressed in floral structures or nectar influence pollinator attraction and/or pollination

success (Adler, 2001; Adler & Irwin, 2005). Finally, plant defences may alter selection on reproductive traits independent of their role in influencing biotic interactions; allocation costs (Strauss *et al.*, 2002) associated with the production of costly plant defences may trade-off with the production of particular reproductive traits (e.g. flower production, scent), rendering the expression of such traits below the phenotypic optimum. This process is expected to cause defence-mediated changes in selection on reproductive traits (Thompson & Johnson, 2016). Quantifying how plant defences, herbivores and pollinators interact to influence selection on plant reproductive traits would provide important insight into the causal agents responsible for driving the diversification of reproductive traits in flowering plants.

Recent work supports the hypothesis that pollinator- and herbivore-mediated selection could be contingent on a plant's defensive phenotype. Thompson & Johnson (2016) found that the presence of a chemical anti-herbivore defence (hydrogen cyanide [HCN]) altered selection on floral traits in white clover (*Trifolium repens*) and speculated that altered interactions with herbivores and pollinators – rather than trade-offs in resource allocation – were responsible for differences in selection between defended and undefended genotypes. A second study in common milkweed (*Asclepias syriaca*) corroborated the hypothesis that variation in defence can alter selection on inflorescence number and size (Thompson *et al.*, 2017). A shortcoming of these previous studies is that they did not experimentally manipulate biotic interactions, making it difficult to disentangle whether observed differences in selection between defended and undefended plants were due to allocation costs associated with defence or altered interactions with herbivores and/or pollinators. The simultaneous manipulation of plant defences, pollination and herbivory is necessary to test the hypothesis that variation in plant defence alters herbivore- and pollinator-mediated selection on plant reproductive traits.

In this study, we experimentally test the hypothesis that plant defences alter herbivore- and pollinator-mediated selection on reproductive traits. We conducted a common garden field experiment using clonal genotypes of white clover (*T. repens*) that vary in a Mendelian-inherited defensive phenotype – cyanogenic (HCN+) or acyanogenic (HCN-) phenotypes (i.e. discrete variation in the production of HCN due to a Mendelian polymorphism). In this experiment, we factorially crossed the defence phenotype, herbivory and pollination environment. While plant chemical defences and levels of herbivory and pollination vary continuously in many plant species, the discrete nature of the cyanogenesis polymorphism and the manipulations imposed in our experiment make this an elegant system for assessing how plant defences may alter herbivore- and pollinator-mediated selection on reproductive traits. In addition, although plant defences and our

herbivory manipulation both have the effect of reducing levels of herbivore damage, defence is a property of the plant, and herbivory is imposed by invertebrate and vertebrate herbivores. Moreover, the presence/absence of HCN explains <8% of the variation in herbivory in field experiments (Thompson & Johnson, 2016). Therefore, independent manipulation of plant defence and herbivory is needed to determine whether altered patterns of selection are being driven by the contemporary herbivore community or by the plant's investment in chemical defences, filling a key gap in our understanding of the agents responsible for imposing selection on plant traits.

Our study addresses the following specific questions: (1) Do chemical plant defences, herbivores and/or pollinators alter the expression of plant reproductive traits? (2) Do herbivores and/or pollinators impose selection on plant reproductive traits? (3) Does selection on reproductive traits differ between defended and undefended plant genotypes? (4) Does the strength or direction of selection on reproductive traits imposed by herbivores or pollinators differ between defended and undefended plant genotypes? (5) What is the overall direction and magnitude of selection on plant reproductive traits imposed by plant defences, herbivores and pollinators? Answering these questions will allow us to better quantify the interdependence of plants defences and reproductive traits and the agents most responsible for their diversification.

## Materials and methods

### Study system

*Trifolium repens* (Fabaceae) is a perennial legume that reproduces clonally through the production of horizontal stems along the soil surface (i.e. stolons). Native to Eurasia, it was introduced to temperate regions worldwide as a forage and nitrogen-fixing crop (Burdon, 1983; Kjærgaard, 2003). Plants are typically found in grazed or mowed pastures, lawns and meadows where they maintain large populations (Burdon, 1983). Individuals reproduce sexually by producing inflorescences (e.g. range: 0–374 in our experiment) that arise from stolons, each consisting of multiple flowers (mean = 61 flowers/inflorescence) visited by a variety of bee species (Kakes, 1997). Flowers are hermaphroditic and plants are outcrossing because of a gametophytic self-incompatibility system (Burdon, 1983).

Many populations of *T. repens* are polymorphic for the production of HCN, an antiherbivore defence, with cyanogenic (HCN present) and acyanogenic (HCN absent) cyanotypes co-occurring in most temperate environments (Daday, 1958). The cyanogenesis polymorphism is caused by two independently segregating Mendelian loci, the molecular basis of which was recently characterized (Olsen *et al.*, 2007, 2008, 2013;

Olsen & Small, 2018). The *Ac/ac* polymorphism is caused by gene deletions at the *CYP79D15* locus which encodes the cytochrome P450 subunit involved in the synthesis of cyanogenic glycosides (linamarin and lotaustralin), which are stored in the cell vacuole (Olsen *et al.*, 2008, 2013). Plants require a dominant allele (i.e. *Ac-*) at this locus to produce cyanogenic glycosides. The *Li/li* polymorphism results from a gene deletion at the *Li* locus encoding the hydrolysing enzyme, linamarase, which is stored in the cell wall (Kakes, 1985). Plants also require a dominant allele (i.e. *Li-*) at this locus to produce linamarase. Thus, only plants with dominant alleles at both loci produce HCN (i.e. cyanotype *Ac- Li-*), which is released when cell damage causes cyanogenic glycosides and linamarase to interact (Hughes, 1991). If either locus is homozygous recessive, then a plant will lack HCN (i.e. cyanotypes *Ac- lili*, *acac Li-*, *acac lili*). Molluscs (e.g. snails and slugs), insects (e.g. lepidopteran caterpillars) and mammals (e.g. voles) all feed on *T. repens*, and generally show reduced feeding on HCN+ plants (Angseesing & Angseesing, 1973; Dirzo & Harper, 1982a,b; Burgess & Ennos, 1987; Saucy *et al.*, 1999; Thompson & Johnson, 2016).

### Plant material and genotyping

We obtained 50 clonal plant genotypes for use in our experiment, consisting of both cyanogenic and acyanogenic cyanotypes. Throughout the text, 'genotype' is used to refer to the 50 clonal genotypes collected for our experiment, 'cyanotype' is used to refer to the four possible combinations of alleles at the loci underlying cyanogenesis (i.e. *Ac- Li-*, *acac Li-*, *Ac- lili*, *acac lili*), and 'defensive phenotype' as the presence (i.e. cyanogenic, cyanotype *Ac- Li-*) or absence (i.e. acyanogenic, cyanotypes *Ac- lili*, *acac Li-*, *acac lili*) of HCN. To obtain plants for use in our experiment, we first grew plants from seed that were taken from a previous experiment (Thompson & Johnson, 2016). The maternal plants from which our seeds were collected originated from a single population located on the University of Toronto Mississauga campus (Thompson & Johnson, 2016). We germinated seeds in potting soil (Sunshine<sup>®</sup> mix #1; Sun Gro Horticulture, Agawam, MA, USA) and maintained them in a growth chamber with 16 h day<sup>-1</sup> of 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light set to 25 °C and 55% humidity. To determine the defensive phenotype (i.e. cyanogenic or acyanogenic) of these plants, we used Feigl–Anger assays (Feigl & Anger, 1966) with test papers prepared according to the procedure of Gleadow *et al.* (2011). Feigl–Anger assays are colour-change tests that allow for rapid and reliable identification of plants containing HCN and thus the presence/absence of the dominant allele at both the *CYP79D15* and *Li* loci (see online supplementary methods: 'Determination of plant defensive phenotype').

## Experimental design

To examine how plant defences, herbivores and pollinators interact to exert selection on plant reproductive traits, we conducted a fully factorial common garden experiment at the Koffler Scientific Reserve near Newmarket, Ontario, Canada (44.0296°N, 79.5316°W, 297 m a.s.l., <http://ksr.utoronto.ca/>), where *T. repens* is naturally in high abundance at the study site. We factorially crossed defensive phenotype (cyanogenic or acyanogenic), herbivory (ambient or reduced) and pollination (open or supplemental). From each of 25 cyanogenic and 25 acyanogenic genotypes, we generated 16 stolon cuttings (i.e. genetically identical ramets) and randomly assigned four stolons per genotype into one of four herbivory × pollination treatment combinations: (1) ambient herbivory and open pollination; (2) ambient herbivory and supplemental pollination; (3) reduced herbivory and open pollination; and (4) reduced herbivory and supplemental pollination. Thus, our experiment consisted of 800 plants (50 genotypes [25 HCN+ and 25 HCN−] × two herbivory treatments × two pollination treatments × four replicates per herbivory–pollination treatment combination). To detect selection on individual loci involved in cyanogenesis (i.e. *Ac* and *Li*), and to avoid confounding defensive phenotype with cyanotype, the 25 acyanogenic clonal genotypes were chosen according to the following proportions: seven genotypes were *acac Li* (i.e. linamarase only), seven were *Ac− lili* (i.e. cyanogenic glycosides only), and 11 were *acac lili* (i.e. neither glycosides nor linamarase).

Plants were grown in a common garden in the field (Fig. S1). Before moving plants to the field, we allowed each plant to establish in 530-mL square pots filled with potting soil (Sunshine<sup>®</sup> mix #1; Sun Gro Horticulture, Agawam, MA, USA). Plants were grown for 14 days in a growth chamber set to 25 °C daytime/20 °C night-time temperatures with 16-h light:8-h dark and 700  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of light before planting them in a random order into our common garden, on 1 June 2015. The garden was arranged into 23 rows and 36 columns, with 1-m spacing between rows and columns. We transplanted plants into holes (approx. 14 cm<sup>2</sup>) cut out of landscape fabric (Quest<sup>®</sup> Better Barriers<sup>™</sup>, Brampton, ON, USA), which was spread across the entire garden and served to reduce the growth of non-focal species around transplanted *T. repens* stolons (Fig. S1). We then allowed plants to grow under natural conditions (e.g. unfertilized, natural water) for the duration of the experiment.

To experimentally reduce herbivory, we applied both molluscicide and insecticide to individual plants. We applied 800 mg of molluscicide (Safer<sup>®</sup> Slug and Snail Bait, Litiitz, PA, USA) biweekly to reduce the abundance of snails and slugs – common herbivores of *T. repens* – and applied 0.0092% insecticide weekly

(Coragen<sup>®</sup>; Dupont Chemical, Mississauga, ON, USA) to reduce the abundance of chewing insects (e.g. lepidopteran larvae and beetles). For plants that did not receive insecticide, we sprayed them with an equivalent amount of water as a control. Control plants experienced approximately half of the herbivore damage as *T. repens* plants from a previous study at the same field site (13% vs. ~29%; Thompson & Johnson, 2016), likely owing to the presence of landscape fabric and absence of co-occurring plant species in the present study. This difference in herbivory makes our estimates of the effects of herbivores as agents of natural selection conservative. A pilot experiment confirmed that the combination of molluscicide and insecticide used to reduce herbivory in our experiment had no direct effects on plant growth (see online supplementary results: ‘Effects of pesticides on plant growth and fitness’; Fig. S2). Pollinators were more frequently encountered on pesticide-treated plants, potentially because of indirect positive effects of reduced herbivory on plant growth or reduced floral damage among treated plants (see online supplementary results: ‘Effects of pesticides on pollinator visitation’).

To manipulate the amount of pollen received by plants, we provided plants with supplemental pollen every 2 days – weather permitting – throughout the flowering period. Supplemental pollen provides an effective means of quantifying pollen limitation (Ashman *et al.*, 2004), and it is commonly used in studies of pollinator-mediated selection (e.g. Parachnowitsch & Kessler, 2010; Sletvold *et al.*, 2015). We used pollen supplementation rather than pollinator exclusion to quantify pollinator-mediated selection because *T. repens* requires outcrossed pollen for successful seed set; pollinator exclusion (e.g. using mesh bags) would have rendered treated plants with zero fitness thereby complicating our ability to quantify selection and would also have prevented herbivores from damaging floral structures. We pollinated plants by rubbing an acrylic paintbrush onto the anthers of donor plants and then brushing the stigma of each open flower of every focal plant. A concurrent experiment confirmed that supplemental pollination is an effective means of fertilizing ovules in *T. repens* (see online supplementary methods: ‘Efficacy of hand pollinations’). Donor pollen was collected randomly from both naturally occurring and experimental plants; by contacting a plants’ stigma during pollination, we inevitably collected pollen from that same plant. Furthermore, since plants produced multiple inflorescences, each with multiple flowers, all plants received pollen from many different donors throughout the flowering season.

## Herbivore damage

Throughout the experiment, we quantified invertebrate herbivore damage to leaves and flowers, in addition to

grazing by vertebrates. To estimate the amount of invertebrate herbivore damage to plant leaves, we conducted three surveys over the course of the experiment at monthly intervals (beginning June 23, July 23 and August 24) following planting. For each survey, we estimated the percentage leaf area lost from each of five leaves per plant and took the mean of these measurements as our estimate of plant-level herbivore damage. Importantly, plant cyanotype was unknown to the surveyor (JSS) during all herbivore damage surveys. We surveyed older nonsenescent (i.e. still completely green) leaves to ensure enough time had passed for herbivore damage to accumulate and to provide leaf age standardized estimates of herbivory across plants. Visual estimates of percentage leaf area lost provide results consistent with those derived from image software analyses (Johnson *et al.*, 2016) and are sensitive enough to detect defence-mediated differences in herbivore damage in this system (Thompson & Johnson, 2016; Thompson *et al.*, 2016). We also quantified the amount of floral damage by visually estimating the amount of area removed on each of five randomly selected banner petals (i.e. standard petal) from the middle whorl of a single, randomly selected inflorescence per plant; the mean of these measurements was used as an estimate of plant-level floral damage. Floral damage was estimated once per plant and was performed opportunistically throughout the experiment as only plants that began flowering could be measured. We only quantified floral damage on the oldest nonsenescent whorl of flowers on the inflorescence to ensure that all flowers had been exposed to florivores for the longest period possible. Finally, we recorded the presence/absence of grazing by meadow voles (*Microtus pennsylvanicus*), which damaged plants throughout the experiment by removing entire stolons with leaves and inflorescences (if any). By the conclusion of the experiment, voles had damaged 41% of plants across all treatments, although most (98%) plants that were damaged by voles survived.

### Trait measurements

We measured six traits during the experiment, many of which showed significant genetic correlations (see online supplementary results: 'Total selection and trait correlations'; Table S1) and had previously been shown to be under selection in this system at the same study site (Thompson & Johnson, 2016). These traits included (1) number of days to first flower; (2) banner petal width (mm); (3) banner petal length (mm); (4) number of inflorescences; (5) number of flowers within inflorescences; and (6) aboveground dry vegetative biomass (g). Flowering time was measured as the number of days from planting to first flower, where plants were considered to have begun flowering when both wing petals had fully opened. Next, we used digital callipers

to measure the width and height of undamaged banner petals to the nearest 0.01 mm from three flowers on each of two separate inflorescences per plant. We took the mean of all banner petal width and length measurements and recorded these as separate traits since they are only moderately correlated ( $r = -0.65$ ). Banner petals are major contributors to pollinator attraction in legumes (Schaal Barbara & Leverich, 1980) and show evidence of strong selection in this system (Thompson & Johnson, 2016). We also counted the total number of inflorescences produced by each plant over the course of the entire experiment. We counted the number of flowers on each of two dried, completely intact and randomly selected inflorescences per plant; the mean of these two measurements is our estimate of the number of flowers per inflorescence. At the end of the experiment, we harvested the aboveground biomass and reproductive biomass (i.e. inflorescences) of all plants, dried them at 60 °C for 72 h and weighed them to the nearest 0.001 g. At the time of harvest on 11 September 2015, 792 plants survived (99% survival across all plants) 102 days of growth. We extracted seed from inflorescences by macerating them with a thresher (110V Wheat Head Thresher; Precision Machine Company, Inc., Lincoln, NE, USA) and then separated the seeds from the chaff using a wind tunnel. We used the total mass of seeds produced by plants as our measure of maternal fitness. Seed mass is highly correlated with seed number in this species ( $r = 0.97$ ,  $n = 35$ ; Thompson & Johnson, 2016) and is thus an effective proxy of fecundity.

### Statistical analyses

#### *Assumptions of statistical models*

Prior to conducting analyses, we assessed whether the distribution of traits and residuals from statistical models met assumptions of normality and homogeneity of variance. Some traits were transformed to better meet these assumptions (see data transformations, Table S2), although relative fitness was left untransformed to preserve the interpretation of estimated selection gradients (Lande & Arnold, 1983; Mitchell-Olds & Shaw, 1987). We also assigned each plant to one of six equally sized spatial blocks to partition spatial variance across the common garden. With the exception of date to first flower, spatial block was a significant predictor of variation in all reproductive traits and plant fitness (all  $P \leq 0.001$ ). All analyses were performed in R version 3.4.3 (R Core Team, 2016) in the RStudio™ environment (RStudio, 2016). All data and R code are archived on Dryad.

#### *Pollen limitation and efficacy of HCN and herbivory treatment*

Prior to examining the phenotypic effects of our treatments and defences, it was necessary for us to evaluate

whether our treatments and HCN worked as intended. To determine whether the presence of HCN and pesticides reduced herbivory, we fit percentage leaf area lost to the following linear mixed-effects model using maximum-likelihood estimation implemented in the 'lme4' (Bates *et al.*, 2015) and 'lmerTest' (Kuznetsova *et al.*, 2017) R packages: % Leaf area lost = Intercept + defence [D] + herbivory treatment [H] + D×H + *Genotype [G] + Spatial block + G×H + Error*. All effects in italics were treated as random effects with fixed intercepts, and all other terms were treated as fixed effects. We fit this model to data collected on individual plants, and we ran a separate model for each of the three herbivory surveys. Unlike analyses described below, we did not perform any backward model selection procedures as this model is relatively simple and contains only one two-way interaction. Rather, we determined the significance of fixed effects directly from the full model using *F*-tests fit with type-III sums-of-squares and adjusted the denominator degrees of freedom using the Kenward-Roger approximation (Kenward & Roger, 1997).

To assess the extent of pollen limitation in our experiment, we fit seed mass and number of seeds per inflorescence (analysed separately) to the following linear mixed-effects models: Total seed mass or # seeds/inflorescence = Intercept + Pollination [P] + *Genotype [G] + Spatial block + G×P + Error*. Pollination was treated as a fixed effect; random effects are shown in italics; and the significance was tested as described above.

*Question 1: Do chemical plant defences, herbivores and/or pollinators alter the expression of plant reproductive traits?*

To answer question 1, we first fit traits to the following mixed-effects models: *Trait* = Intercept + defence [D] + herbivory treatment [H] + pollination treatment [P] + D×H + D×P + H×P + D×H×P + *Genotype [G] + Spatial block + G×H + G×P + Error*. All effects in italics were treated as random effects with fixed intercepts, and all other terms were treated as fixed effects.

We then performed a backward model selection procedure to optimize the full model and remove higher-order interactions using the 'step' function in the 'lmerTest' R package (Kuznetsova *et al.*, 2017) while maintaining all main effects in the model. This function starts with the full model and then optimizes the random effects structure, followed by the fixed effects structure, to return the final reduced model as recommended by Zuur *et al.* (2009). We used a threshold of  $P = 0.05$  to drop terms during model selection. We determined the significance of fixed effects using the same method described above.

*Genotypic selection analyses*

To address questions 2–5, we used both univariate and multivariate genotypic selection analyses (Price, 1970; Lande & Arnold, 1983; Rausher, 1992). To determine

whether the biological assumption of selection analyses was met, we first estimated measures of clonal genetic variance, broad-sense heritability and coefficients of genotypic variance for all traits using untransformed values as per Houle (1992) (see online supplementary methods: 'Testing assumptions of selection analyses'). All analyses were performed using genotype means to avoid environmentally induced biases in the estimates of selection (Rausher, 1992; Stinchcombe *et al.*, 2002). Some genotypic trait means were transformed to better meet assumptions of normality and homogeneity of variance (genotypic transformations, Table S2). We standardized trait values to a mean of 0 and standard deviation of 1. We first performed univariate selection analyses to quantify the total selection acting on each trait, estimated as the genetic covariance between relative fitness ( $w$ ) and genotypic variance in a trait, which is equivalent to the selection differential (Price, 1970). Relative fitness of a genotype was calculated as within-treatment genotype-mean seed mass divided by the mean seed mass of all genotypes across all treatment combinations. We then conducted a multivariate selection analysis that included standardized trait values, treatments, and all two-, three-, and four-way interactions between a trait, defence (HCN+/HCN-; *CYP79D15+/ CYP79D15-*; *Li+/ Li-*), and treatments as fixed-effect predictors of relative fitness. We standardized traits and relativized fitness across, rather than within, treatments because this better allowed us to determine the direct effects of treatments on plant fitness and to estimate the role of indirect ecological effects in altering selection (TerHorst *et al.*, 2015). Second-order terms and interactions among reproductive traits (e.g. number of flowers × banner petal length) were not included in the model. Since each plant genotype had multiple trait means (i.e. one mean value for each level of the herbivory × pollination treatment combination), we included genotype as a random effect to avoid pseudoreplication. The full genotypic selection model was as follows (random effect in italics):

$$w = \text{Intercept} + \text{defence} + \text{pollination treatment} + \text{herbivory treatment} + \text{days to first flower} + \text{banner petal length} + \text{banner petal width} + \text{number of flowers} + \text{number of inflorescences} + \text{biomass} + \text{two-way interactions between each trait and treatments} + \text{three-way interactions between each trait and treatments} + \text{four-way interactions between each trait and treatments} + \textit{Genotype} + \textit{error}$$

Because selection analyses are sensitive to missing data, we only included genotypes for which we had measurements of all traits. This resulted in two genotypes being excluded from the genotype mean data set (final  $n = 48$ ). From the full model shown above, we generated an optimized model (using the approach described under 'Question 1' above), which reduced the number of fixed-effect predictors from 55 in the full

model to 20 in the optimized model. We maintained genotype as a random effect in all models. With the exception of banner width and banner length ( $r = -0.65$ ), none of the traits in the optimized model were strongly correlated (all  $|r| < 0.33$ ). We obtained selection gradients directly from this optimized model and assessed the significance of selection gradients using the Kenward–Roger method described above. We additionally confirmed whether our model was robust to type II errors by sequentially removing the most significant interactions, each time re-running the model and interpreting its output. Nonsignificant interactions did not become statistically significant when significant interactions were removed from the model, indicating that our results are robust and have adequate statistical power.

The selection gradients and significance of fixed-effect predictors in the optimized model allowed us to address questions 2–5. Specifically, a significant trait  $\times$  pollinator or trait  $\times$  herbivory treatment interaction indicated pollinator- or herbivore-mediated selection on that trait, respectively (question 2). Similarly, a significant trait  $\times$  defence treatment interaction indicated that selection on that trait differed between defended and undefended plant genotypes (question 3). Finally, a significant trait  $\times$  defence  $\times$  pollination treatment or trait  $\times$  defence  $\times$  herbivory treatment interaction indicated that the strength or direction of pollinator- or herbivore-mediated selection on the trait differed between defended and undefended plant genotypes (question 4). We verified that all observed differences in selection between acyanogenic and cyanogenic genotypes were due to HCN *per se* and not the underlying metabolic components involved in the production of HCN (i.e. *CYP79D15*, or *Li*, see online supplementary methods: ‘Effects of underlying genes on selection’).

Because plant defences, herbivores and pollinators may all impose selection on plant reproductive traits, we were interested in assessing the overall direction and magnitude of selection imposed by each of these agents, when averaged across all traits in our study (question 5). This is possible because selection gradients reflect the standardized relationship between relative fitness and trait variation in standard deviation units (Lande & Arnold, 1983), which facilitates comparisons across traits and, importantly, across studies (Kingsolver *et al.*, 2001; Siepielski *et al.*, 2013). We first calculated selection gradients for each trait under each level of the treatments we imposed (i.e. defended vs. undefended; ambient herbivory vs. reduced herbivory; open pollination vs. supplemental pollination). We then quantified defence-mediated selection ( $\Delta\beta_{\text{Defence}}$ ) on each trait as the difference in selection gradients between defended ( $\beta_{\text{HCN+}}$ ) and undefended ( $\beta_{\text{HCN-}}$ ) plant genotypes ( $\Delta\beta_{\text{Defence}} = \beta_{\text{HCN+}} - \beta_{\text{HCN-}}$ ). We quantified herbivore-mediated selection

( $\Delta\beta_{\text{Herb}}$ ) as the difference in the selection gradient under ambient herbivory ( $\beta_{\text{Ambient}}$ ) and reduced herbivory (i.e.  $\Delta\beta_{\text{Herb}} = \beta_{\text{Ambient}} - \beta_{\text{Reduced}}$ ). Similarly, pollinator-mediated selection ( $\Delta\beta_{\text{Poll}}$ ) was quantified as the selection gradient under open pollination ( $\beta_{\text{Open}}$ ) minus the selection gradient under supplemental pollination ( $\Delta\beta_{\text{Poll}} = \beta_{\text{Open}} - \beta_{\text{Supp}}$ ; Sletvold *et al.*, 2015). Differences in the direction of selection imposed by the three agents were assessed by examining differences in the raw values of defence-, herbivore- or pollinator-mediated selection on reproductive traits, whereas differences in the strength of selection imposed by the three agents were evaluated by examining variation in the absolute values of  $\Delta\beta_{\text{Defence}}$ ,  $\Delta\beta_{\text{Herb}}$  and  $\Delta\beta_{\text{Poll}}$ .

Vole damage was not sufficiently balanced across genotypes and treatments to include in the genotypic selection analyses described above; its inclusion would have resulted in missing data, to which model selection procedures are sensitive (Nakagawa & Freckleton, 2011). We therefore ran a separate genotypic selection analysis to examine whether unanticipated damage by meadow voles and plant defences interacted to alter selection on plant reproductive traits. This model included vole damage (presence or absence), defence (HCN+ or HCN-), reproductive traits, and all possible interactions except those among reproductive traits (e.g. flower number  $\times$  banner width) as fixed-effect predictors of relative fitness. We included genotype as a random effect to avoid pseudoreplication. We obtained selection gradients and *P*-values using the same approach outlined above (i.e. backward model selection followed by type-III ANOVA with Kenward–Roger denominator degrees of freedom).

In cases where significant interactions were found in the multivariate selection analyses outlined above, we ran simple linear models on subsets of our data (e.g. ambient herbivory only, cyanogenic plants only) to examine changes in the strength and direction of selection on traits for the different levels of the interacting factor. Linear models corresponded to the reduced model following backward model selection.

## Results

### Pollen limitation and herbivore damage

Our pollination treatment was effective at fertilizing plants, yet there was no pollen limitation (see online supplementary results: ‘Pollen limitation and efficacy of hand pollinations’; Fig. S3). Similarly, HCN defences and our pesticide treatment both reduced herbivore damage on *T. repens* plants across all sampling dates (see online supplementary results: ‘Herbivore damage in response to pesticide treatment and cyanogenesis’; Figs S4 and S5).

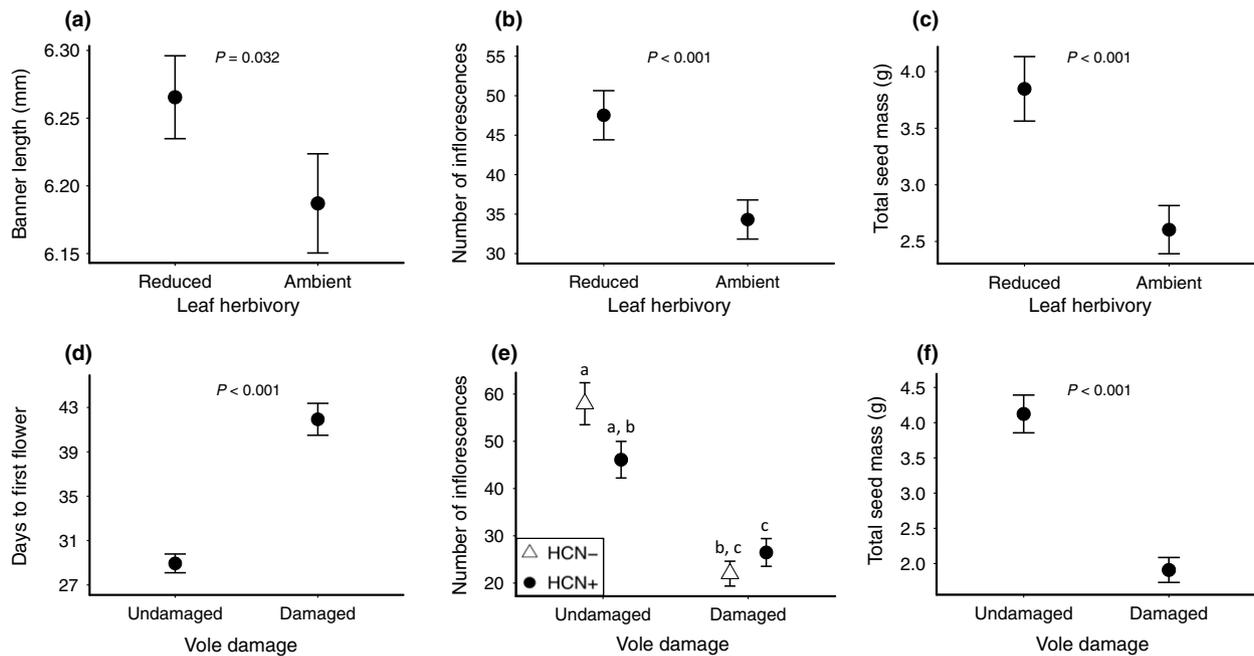
### Question 1: Do chemical plant defences, herbivores and/or pollinators alter the expression of plant reproductive traits?

Cyanogenesis and our treatments significantly influenced the expression of several plant traits. Relative to plants with experimentally reduced invertebrate herbivory, plants experiencing ambient invertebrate herbivory had slightly reduced banner petal length ( $F_{1,46} = 4.92$ ,  $P = 0.032$ ; Fig. 1a) and width ( $F_{1,541} = 5.76$ ,  $P = 0.017$ ; Fig. S6) (~1% reduction), 28% fewer inflorescences ( $F_{1,49} = 26.04$ ,  $P < 0.001$ ; Fig. 1b), 32% lower total seed mass ( $F_{1,49} = 39.98$ ,  $P < 0.001$ ; Fig. 1c) and flowered on average 6 days earlier ( $F_{1,647} = 6.77$ ,  $P = 0.009$ ; not shown). Supplemental pollination reduced banner petal width and length by ~2%, but only for acyanogenic plants (HCN  $\times$  Pollination interaction, width:  $F_{1,541} = 4.17$ ,  $P = 0.041$ ; Fig. S7a, length:  $F_{1,513} = 4.60$ ,  $P = 0.032$ ; Fig. S7b). Furthermore, cyanogenic plants produced more biomass than acyanogenic plants, but only for open pollinated plants with experimentally reduced herbivory (HCN  $\times$  Herbivory  $\times$  Pollination interaction,  $F_{1,726} = 4.08$ ,  $P = 0.044$ ; Fig. S8a).

In addition to invertebrate herbivores, damage by meadow voles influenced the expression of plant traits. Meadow voles delayed flowering by an average of 13 days ( $F_{1,668} = 56.95$ ,  $P < 0.001$ ; Fig. 1d) and reduced aboveground biomass by 44% ( $F_{1,743} = 146.14$ ,  $P < 0.001$ ; Fig. S9). In addition, plants damaged by voles produced 53% fewer inflorescences overall ( $F_{1,742} = 107.84$ ,  $P < 0.001$ ; Fig. 1e), although this effect was stronger for acyanogenic plants (Vole damage  $\times$  HCN interaction,  $F_{1,746} = 4.61$ ,  $P = 0.032$ ; Fig. 1e) and produced 54% lower seed mass ( $F_{1,738} = 112.04$ ,  $P < 0.001$ ; Fig. 1f).

### Questions 2: Do herbivores and/or pollinators impose selection on plant reproductive traits?

Both invertebrate and vertebrate herbivore damage altered genotypic selection on reproductive traits. Invertebrate herbivore damage weakened selection for more inflorescences by 32% (ambient herbivory:  $\beta = 0.91$ ,  $F_{1,69} = 133.38$ ,  $P < 0.001$ , reduced herbivory:  $\beta = 1.34$ ,  $F_{1,77} = 267.10$ ,  $P < 0.001$ ; # inflorescences  $\times$  herbivory treatment:  $P < 0.001$ ; Table 1, Fig. 2a). Similarly, meadow vole damage weakened selection for more



**Fig. 1** Effects of invertebrate (upper; panels a, b, c) and vertebrate (lower; panels d, e, f) herbivores on the expression of plant traits. (a) Plants exposed to ambient levels of herbivore damage had shorter banner petals. Note that invertebrate herbivore damage also resulted in narrower banner petals (not shown, see Fig. S6). (b) Plants damaged by invertebrate herbivores produced fewer inflorescences and (c) lower seed mass. (d) Plants damaged by meadow voles (*Microtus pennsylvanicus*) flowered an average of 13 days later and (e) produced fewer inflorescences, although this effect was stronger for acyanogenic plants (hydrogen cyanide  $\times$  vole damage interaction,  $P = 0.032$ ). Note that invertebrate herbivores also delayed flowering by an average of 6 days (not shown). (f) Meadow vole damage reduced plant total seed mass ( $P < 0.001$ ). All points represent means  $\pm$  1 SE. Asterisks denote significant differences at  $P = 0.05$ . Groups with different letters in (e) are significantly different at  $P = 0.05$  based on *post hoc* HSD tests.

inflorescences by 44% (damaged by voles:  $\beta = 0.68$ ,  $F_{1,39} = 110.11$ ,  $P < 0.001$ ; undamaged by voles:  $\beta = 1.21$ ,  $F_{1,41} = 166.59$ ,  $P < 0.001$ ; # inflorescences  $\times$  vole damage:  $P < 0.001$ ; Table S5, Fig. 2b) and eliminated selection for more flowers per inflorescence (damaged by voles:  $\beta = 0.23$ ,  $F_{1,41} = 6.13$ ,  $P = 0.02$ , undamaged by voles:  $\beta = 0.06$ ,  $F_{1,39} = 2.07$ ,  $P = 0.16$ ; # flowers per inflorescence  $\times$  vole damage:  $P = 0.02$ ; Table S5, Fig. 2c). In contrast to invertebrate and vertebrate herbivores, pollinators on their own did not significantly influence selection on any plant reproductive trait.

### Question 3: Does selection on reproductive traits differ between defended and undefended plant genotypes?

Cyanogenesis altered genotypic selection on reproductive traits. Both cyanogenic and acyanogenic plants

**Table 1** Results from multivariate genotypic selection analysis examining the interactive effects of hydrogen cyanide (HCN), invertebrate herbivores and pollinators on selection on reproductive traits. For each trait or factor, we show the coefficient of directional selection ( $\beta$ ), which represents the slope of the line of standardized traits (predictor variables) against relative fitness (response variables). We additionally show one standard error of the coefficient,  $F$ -statistics, denominator degrees of freedom (dd.f., rounded), and  $P$ -values obtained from an  $F$ -test using type-III sums-of-square and the Kenward–Roger corrected denominator degrees of freedom. Shown are the fixed-effect parameters retained following backward model selection. Bolded terms are significantly different at  $P < 0.05$ .

Trait/factor	$\beta$	SE	$F$	dd.f.	$P$
HCN	0.220	0.145	1.017	45	0.319
Pollination	0.115	0.084	0.002	121	0.964
Herbivory	0.058	0.095	0.941	135	0.334
Date to first flower (FF)	0.121	0.074	0.689	154	0.408
Banner width (BW)	-0.077	0.127	1.132	149	0.289
Banner length (BL)	0.061	0.112	0.070	107	0.791
<b># of inflorescences</b>	<b>0.844</b>	<b>0.090</b>	<b>405.770</b>	<b>154</b>	<b>&lt; 0.001</b>
HCN $\times$ Pollination	-0.224	0.119	3.556	121	0.062
HCN $\times$ Herbivory	0.015	0.127	0.013	129	0.909
FF $\times$ HCN	-0.154	0.107	0.002	154	0.967
FF $\times$ Pollination	-0.171	0.082	0.042	127	0.838
BW $\times$ HCN	-0.086	0.155	0.725	104	0.396
<b>BL <math>\times</math> HCN</b>	<b>0.086</b>	<b>0.151</b>	<b>4.112</b>	<b>149</b>	<b>0.044</b>
BW $\times$ Herbivory	-0.267	0.113	0.765	126	0.384
BL $\times$ Herbivory	-0.252	0.146	0.582	126	0.447
<b># Inflorescences <math>\times</math> HCN</b>	<b>0.309</b>	<b>0.113</b>	<b>7.224</b>	<b>151</b>	<b>0.008</b>
<b># Inflorescences <math>\times</math> Herbivory</b>	<b>0.351</b>	<b>0.087</b>	<b>16.001</b>	<b>137</b>	<b>&lt; 0.001</b>
<b>FF <math>\times</math> HCN <math>\times</math> Pollination</b>	<b>0.316</b>	<b>0.125</b>	<b>6.324</b>	<b>126</b>	<b>0.013</b>
<b>BW <math>\times</math> HCN <math>\times</math> Herbivory</b>	<b>0.392</b>	<b>0.156</b>	<b>6.256</b>	<b>122</b>	<b>0.014</b>
<b>BL <math>\times</math> HCN <math>\times</math> Herbivory</b>	<b>0.358</b>	<b>0.177</b>	<b>4.046</b>	<b>124</b>	<b>0.046</b>

All traits were standardized to a mean of 0 and standard deviation of 1 prior to analyses, such that slopes are in standard deviation units. Acronyms: date to first flower (FF); banner length (BL); banner width (BW).

experienced directional selection for more inflorescences, but selection was 29% weaker for acyanogenic ( $\beta = 0.98$ ,  $F_{1,70} = 176.71$ ,  $P < 0.001$ ; Fig. 2d) than for cyanogenic genotypes ( $\beta = 1.39$ ,  $F_{1,75} = 24.46$ ,  $P < 0.001$ ; # inflorescences  $\times$  defence:  $P = 0.008$ ; Table 1, Fig. 2d).

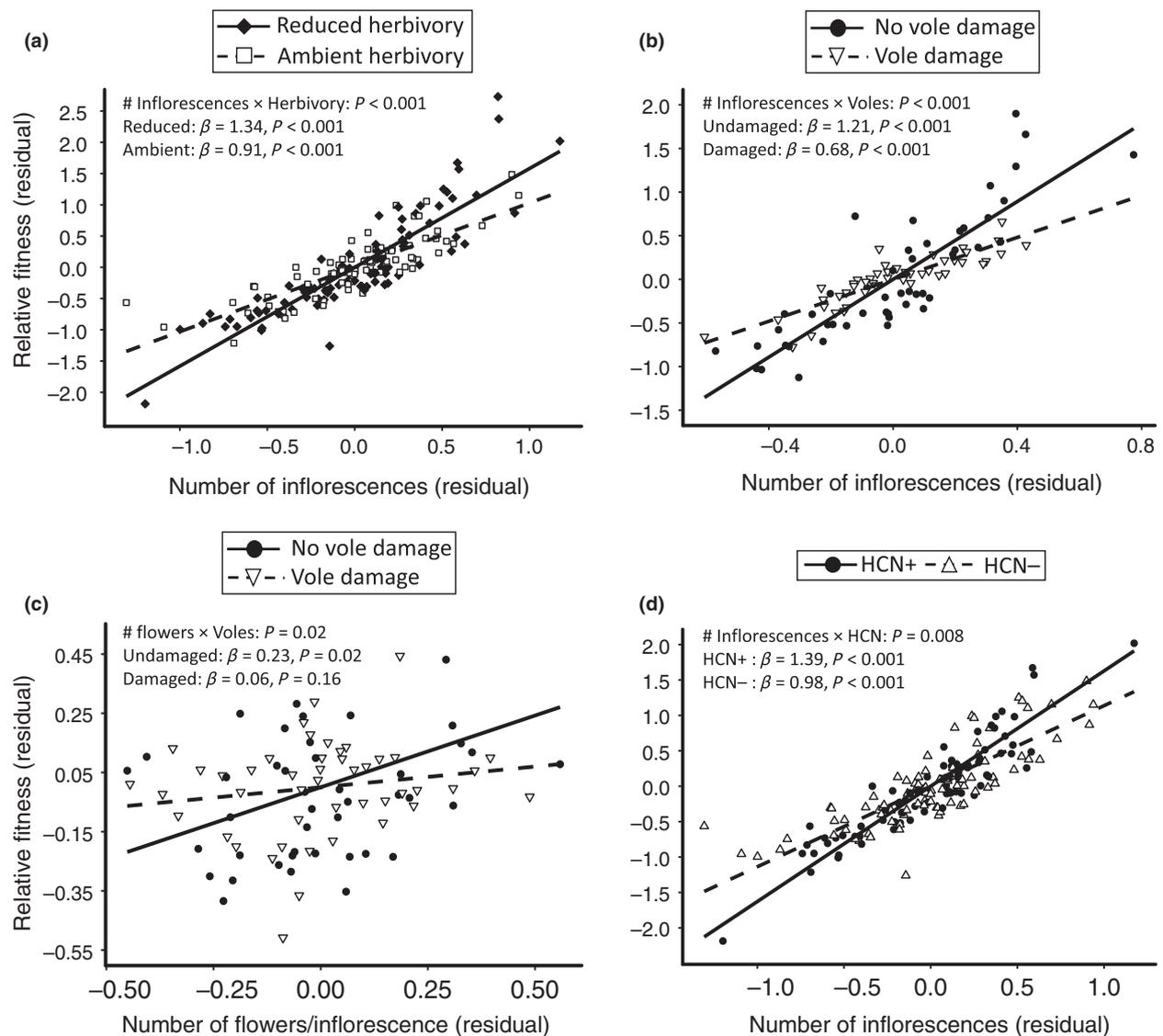
### Question 4: Does the strength or direction of selection on reproductive traits imposed by herbivores or pollinators differ between defended and undefended plant genotypes?

The strength of selection on banner petal size that was imposed by herbivores differed between cyanogenic and acyanogenic plants. Among acyanogenic plants, invertebrate herbivore damage weakened selection for narrower (ambient herbivory:  $\beta = 0.03$ ,  $F_{1,35} = 0.06$ ,  $P = 0.76$ , reduced herbivory:  $\beta = -0.27$ ,  $F_{1,27} = 7.69$ ,  $P = 0.009$ , banner petal width  $\times$  defence  $\times$  herbivory treatment interaction:  $P = 0.014$ ; Table 1, Fig. 3a) and shorter (ambient herbivory:  $\beta = -0.10$ ,  $F_{1,35} = 0.51$ ,  $P = 0.49$ , reduced herbivory:  $\beta = -0.39$ ,  $F_{1,29} = 9.50$ ,  $P = 0.004$ ; Fig. 3b; banner petal length  $\times$  defence  $\times$  herbivory treatment interaction:  $P = 0.046$ ; Table 1) banner petals, whereas herbivores did not alter selection on these traits among cyanogenic plants (Fig. 3). Similarly, meadow voles weakened selection for narrower flowers among acyanogenic plants (banner petal width  $\times$  defence  $\times$  vole damage interaction:  $P = 0.015$ ; Table S5) but did not impose selection on this trait among cyanogenic plants.

Selection imposed by pollinators on flowering phenology differed between cyanogenic and acyanogenic plants. Among cyanogenic plants, pollinators weakened selection for later flowering (open pollination:  $\beta = -0.03$ ,  $F_{1,27} = 0.09$ ,  $P = 0.77$ , supplemental pollination:  $\beta = 0.19$ ,  $F_{1,34} = 5.20$ ,  $P = 0.03$ , date to first flower  $\times$  defence  $\times$  pollination treatment interaction:  $P = 0.013$ ; Table 1, Fig. S10), whereas pollinators did not impose selection on flowering phenology among acyanogenic plants (Fig. S10). However, this effect may be driven by pleiotropic effects or allocation costs associated with the expression of cyanogenic glycosides (*CYP79D15*) rather than HCN itself; we detected a significant date to first flower  $\times$  *CYP79D15*  $\times$  pollination treatment interaction ( $P = 0.006$ ; Table S6), whereas the expression of linamarase (*Li*) did not influence selection on any reproductive traits (Table S7).

### Question 5: What is the overall direction and magnitude of selection on plant reproductive traits imposed by plant defences, herbivores and pollinators?

Plant defences, herbivores and pollinators differed in the direction and strength of selection they imposed on reproductive traits. Overall, plant defences consistently



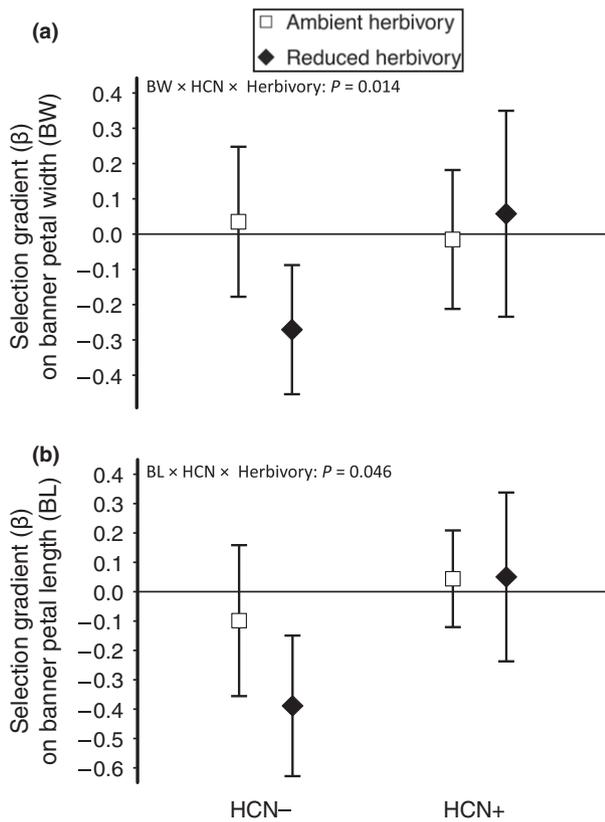
**Fig. 2** Herbivores and cyanogenesis influenced selection on plant reproductive traits. (a) Selection for increased inflorescence production was weakened by (a) invertebrate herbivory damage and (b) damage by meadow voles. In addition, (c) meadow voles weakened selection for more flowers per inflorescence among plants. (d) Selection for increased inflorescence production was also weaker among acyanogenic plants. Selection gradients and  $P$ -values shown in figure panels are from univariate selection analyses performed on subsets of our data (e.g. HCN+ only, ambient herbivory only) when significant trait  $\times$  herbivore treatment or trait  $\times$  vole damage interactions were found in the genotypic selection analyses (Tables 1 and S5). Figures and selection gradients are based on multivariate selection models where the effects shown are averaged across the other treatments in the experiment and include plant genotype as a random effect. In panel (a), two outliers with high residual relative fitness are outside of the plot area shown but are included in the analysis; three points are not shown in panels (c) and (d).

strengthened selection on reproductive traits leading to overall positive defence-mediated selection ( $0.02 < \Delta\beta_{\text{Defence}} < 0.41$ , mean  $|\Delta\beta_{\text{Defence}}| = 0.14$ ; Fig. 4). Invertebrate herbivores imposed strong negative and weaker positive directional selection depending on the trait ( $-0.41 < \Delta\beta_{\text{Herb}} < 0.14$ ) but overall imposed strong herbivore-mediated selection (mean  $|\Delta\beta_{\text{Herb}}| = 0.15$ ; Fig. 4 inset). In contrast to cyanogenesis and invertebrate herbivores, pollinators imposed only weak positive or

negative selection ( $-0.05 < \Delta\beta_{\text{Poll}} < 0.07$ ; Fig. 4) and thus weak selection overall (mean  $|\Delta\beta_{\text{Poll}}| = 0.04$ ; Fig. 4 inset).

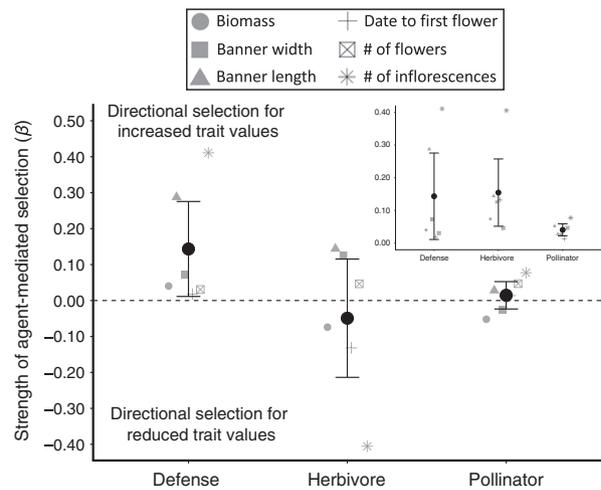
## Discussion

Our experiment tested the hypothesis that plant defences alter herbivore- and pollinator-mediated selection on reproductive traits. Several key results are



**Fig. 3** Herbivore-mediated selection on banner petal width and length depended on plant defensive phenotype. There was selection for (a) narrower banner petals and (b) shorter banner petals among acyanogenic plants exposed to reduced invertebrate herbivore damage, whereas selection did not act in any of the other hydrogen cyanide  $\times$  herbivory treatment combinations. Shown are univariate selection gradients ( $\beta$ ) acting on each trait  $\pm$  95% confidence intervals.

important in answering our research questions. Specifically, we found that herbivory directly altered banner petal width, length, and number of inflorescences, with corresponding effects on fitness, whereas antiherbivore defences did not directly influence any of the traits we measured (question 1). We found that invertebrate and vertebrate herbivores (question 2) and cyanogenic plant defences (question 3) both altered selection on the number of inflorescences. Both invertebrate and vertebrate herbivores altered selection on flower size, although the presence of herbivore-mediated selection occurred only among acyanogenic plants (question 4). Overall, herbivores imposed stronger selection on plant reproductive traits than pollinators and the presence of cyanogenic plant defences consistently strengthened selection (question 5). We discuss the implications of these results as they relate to the agents responsible for imposing selection on plant reproductive traits.



**Fig. 4** Strength of defence-mediated, herbivore-mediated and pollinator-mediated selection acting on each of the reproductive traits in our experiment. Selection gradients were obtained by performing multivariate selection analyses on subsets of our data (e.g. ambient herbivory only, cyanogenic plants only). Thus, the effects of each agent on selection are averaged across the other agents present in the experiment. Also shown is the mean strength of selection across all six traits  $\pm$  95% confidence intervals. Defence-mediated selection was calculated as the strength of selection on a trait among cyanogenic genotypes (i.e.  $\beta_{\text{HCN}+}$ ) minus the strength of selection on that trait among acyanogenic genotypes (i.e.  $\beta_{\text{HCN}-}$ ). Herbivore-mediated selection was calculated as  $\beta_{\text{Ambient}} - \beta_{\text{Reduced}}$ , and pollinator-mediated selection was calculated as  $\beta_{\text{Open}} - \beta_{\text{Supplemental}}$ . Inset shows the absolute value of the strength of defence-, herbivore- and pollinator-mediated selection. The presence of cyanogenic plant defences consistently increased the strength of positive directional selection on reproductive traits. Herbivores and pollinators both strengthened and weakened the strength of selection on reproductive traits, although herbivores tended to exert stronger selection.

### Effects of treatments on phenotypic traits and fitness

Biotic agents may alter selection by influencing patterns of resource allocation and the expression of phenotypic traits. In our experiment, both vertebrate and invertebrate herbivory were associated with changes in morphological floral traits and flowering phenology. Similarly, both invertebrate herbivores and meadow voles (*M. pennsylvanicus*) reduced plant fitness, although meadow voles induced stronger reductions in plant fitness. The smaller trait values and reduced fitness in the presence of herbivores could be due to: (1) damage resulting in fewer resources available for trait and seed production; (2) herbivores reducing trait values and seed set by directly consuming reproductive tissue, as is frequently documented in studies that quantify selection through female fitness (Conner & Rush, 1997; Sandring & Ågren, 2009; Benitez-vieyra *et al.*, 2016); or (3) a

combination of both (1) and (2). Because we only measured undamaged flowers, the reduced trait values in the presence of herbivores were likely driven by herbivore damage reducing investment in floral structures (*sensu* Strauss *et al.*, 1996, 1999; Strauss, 1997), although in our case these effects were small (~1%). However, voles also consumed entire stolons with intact inflorescences and fruit resulting in a direct reduction in fitness, thereby explaining their stronger effect at reducing fitness relative to invertebrate herbivores.

Although cyanogenesis on its own did not influence the expression of plant reproductive traits, it interacted with vole damage to affect inflorescence production. Specifically, in the absence of vole damage, acyanogenic plants produced more inflorescences, whereas this effect was reversed in the presence of damage. HCN is predicted to be costly under conditions of low herbivore pressure (Daday, 1965; Dirzo & Harper, 1982b; Kakes, 1989; Kooyers *et al.*, 2014), and our results support this prediction and show that HCN can be beneficial in the presence of vole damage, but costly in its absence. In contrast to herbivores and cyanogenesis, the effects of pollinators in influencing trait expression were minor, likely because pollinators only interact with plants once flowers have already been produced. Together, our results show that herbivores alter the expression of several reproductive traits and mean fitness, which may alone be sufficient to alter selection.

### Selection on reproductive traits imposed by plant defence, herbivores and pollinators

Cyanogenic plant defences and invertebrate and vertebrate herbivores both altered selection on floral display size. Because cyanogenic plant defences did not directly alter investment in reproductive traits, the altered selection on display size among cyanogenic and acyanogenic genotypes was likely driven by HCN's effect at reducing herbivore damage. Selection was always weakest among plants subject to higher levels of herbivore damage (i.e. HCN-, ambient herbivory treatment, vole-damaged plants). These results suggest that herbivory weakens selection on inflorescence production and provides strong evidence for a cost to large displays in the presence of high herbivore pressure. Previous work in this system also found stronger selection among HCN+ plants for increased display size through increased flower production (Thompson & Johnson, 2016), and the authors speculated this may reflect a cost of increased apparency to herbivores among undefended plants. Our results support this hypothesis as we find experimental evidence that although producing more inflorescences always increases plant fitness, this effect is weaker among plants experiencing greater herbivore damage, suggesting the cost of increased apparency to herbivores is not outweighed by the benefits of producing more inflorescences. This cost of larger displays

because of increased apparency to herbivores may be mitigated by plant chemical defences that allow plants to capitalize on the fitness benefits of producing more flowers and inflorescences.

Given that herbivores, pollinators and plant defences can all impose selection on reproductive traits, we were interested in examining the overall direction and magnitude of selection exerted by each of these three agents. Consistent with a recent review (Johnson *et al.*, 2015), we found that herbivores imposed ~2.7× stronger selection on plant reproductive traits than pollinators and that selection could be positive or negative. By contrast, plant defences consistently strengthened selection on reproductive traits leading to overall positive defence-mediated changes in selection, suggesting that selection favours larger reproductive trait values among defended plants. In addition to experiencing less damage, defended plants may better compete for pollinators due to increased reproductive trait values.

### Variation in herbivore-mediated selection among defended and undefended plant genotypes

Herbivore-mediated selection on two traits in our experiment occurred only among undefended (i.e. acyanogenic) plant genotypes. By reducing the intensity of interactions with herbivores, plant defences are expected to reduce the strength of herbivore-mediated selection on plant traits. Our results are consistent with this hypothesis; herbivores did not impose selection on banner petal width and length among cyanogenic genotypes. By contrast, herbivores weakened the negative selection on flower size among acyanogenic plants, leading to positive herbivore-mediated selection among undefended plants. This suggests that producing small flowers is costly for undefended plants in the presence of herbivores. Both floral (Krupnick *et al.*, 1999) and foliar (Mothershead & Marquis, 2000) herbivory can reduce flower size, leading to reduced pollinator visitation and plant fitness. Damaged plants in our experiment similarly produced smaller flowers (Figs 2a and S6), which may have greater consequences for acyanogenic plants that experience greater herbivory overall (Fig. S4), thereby explaining the cost to small flowers among undefended plants. Cyanogenic plants are damaged less and may not experience as large a decrease in fitness due to reductions in the size of flowers, explaining the lack of herbivore-mediated selection on the size of flowers among defended plants.

Pollinators weakened selection for later flowering among cyanogenic plants, suggesting a cost to later flowering among defended plants through reduced pollination. However, this effect is likely driven by investment in cyanogenic glycosides rather than the defence itself because acyanogenic plants expressing *CYP79D15*, but not *Li*, similarly altered pollinator-mediated selection on flowering date. This pollinator-mediated

selection occurred despite the absence of pollen limitation. Pollen limitation is known to increase the opportunity for – and strength of – pollinator-mediated selection (Ashman & Morgan, 2004; Sletvold & Ågren, 2016; Trunschke *et al.*, 2017), although examples of selection by pollinators in the absence of pollen limitation do exist (Galen, 1996; Caruso *et al.*, 2010; Parachnowitsch & Kessler, 2010). This may occur because of environmental variation that alters pollinator behaviour (e.g. presence of co-flowering species) and increases individual variation in pollination success. In such cases, population-level pollen limitation may be a poor predictor of the strength of pollinator-mediated selection (Sletvold & Ågren, 2014). Future work in systems where pollen limitation is more common will inform the generality of this result. Such studies should take explicit consideration of underlying defensive phenotype, including the metabolic components of defence, and manipulate the herbivory environment, to enable a better understanding of the relative effects of plant defences, pollinators, and herbivores in imposing selection on reproductive traits. We speculate that the role of herbivores and defences in altering selection on plant reproductive traits will be greatest when pollen limitation is absent, and decline in importance in plant species with stronger pollen limitation.

## Conclusion

The diversification of plant reproductive traits has been driven by coevolutionary interactions between plants and their associated mutualists and antagonists. Although the roles of herbivores and pollinators in this process have typically been studied in isolation, increasing evidence suggests that the evolution of reproductive traits and plant defences are interdependent (Campbell, 2014; Carr & Eubanks, 2014; Johnson *et al.*, 2015). We have provided an experimental test of how the expression of plant defences alters herbivore- and pollinator-mediated selection on plant reproductive traits using a system with discrete variation in their defensive strategy. Our results provide the strongest evidence to date that the effects of endogenous plant defences on the evolution of plant reproductive traits are driven primarily by their role in altering interactions with herbivores, rather than allocation costs associated with the plant's investment in chemical defence or interactions with pollinators. In addition, our results support the recent conclusion that herbivores often impose stronger selection on plant reproductive traits than pollinators (Johnson *et al.*, 2015), although we stress that the absence of pollen limitation in our system likely contributed to this effect. Finally, our results highlight plant defences as important agents consistently strengthening selection on reproductive traits. Most plants contain quantitative variation in plant defensive chemistry, which may increase the opportunity for – and strength of – defence-

mediated changes in selection due to greater variation in defensive phenotype among individuals. Thus, an important avenue for future research will be to understand how continuous variation in plant defences alters biotic selection by herbivores and pollinators on plant reproductive traits in a broader range of systems.

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## Conflict of interest

The authors declare no conflict of interests.

## Author contributions

All authors contributed to the experimental design. KAT provided seeds to generate the experimental plants. JSS conducted fieldwork and collected data, with assistance from KAT and MTJJ. JSS analysed the data with input from MTJJ and KAT. JSS and MTJJ wrote the manuscript, and all authors contributed to revisions.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Appendix S1** Supplementary text, figures, and tables.

**Figure S1** Pictures of (A) our landscape fabric covered field site at the Koffler Scientific Reserve and (B) an individual *Trifolium repens* plant growing out of a hole cut through the landscape fabric.

**Figure S2** Effects of molluscicide and insecticides on (A) aboveground biomass (g) and (B) the number of seeds produced per inflorescence.

**Figure S3** Seed mass (g) of inflorescences receiving either only open pollination, only hand pollination, or both hand and open pollination (i.e. supplemental pollination).

**Figure S4** Herbivore damage (mean percent leaf area removed) associated with the pesticide treatment and cyanogenesis for all three herbivory surveys.

**Figure S5** Percent banner petal damage on plants exposed to either ambient ( $n = 245$ ) or experimentally reduced ( $n = 270$ ) invertebrate herbivory using pesticides.

**Figure S6** Banner petal width under ambient or experimentally reduced levels of invertebrate herbivore damage.

**Figure S7** Effects of cyanogenesis and supplemental pollination on (A) banner petal width (mm) and (B) banner petal length (mm). Points represent means  $\pm$  1 SE.

**Figure S8** Aboveground biomass (g) of cyanogenic (filled circle) and acyanogenic (open triangles) plants exposed to ambient or experimentally reduced invertebrate herbivory and open or supplemental pollination.

**Figure S9** Aboveground biomass (g) of plants damaged or undamaged by meadow voles (*Microtus pennsylvanicus*).

**Figure S10** Pollinator-mediated selection on date to first flower depended on plant defensive phenotype ( $P_{\text{Trait} \times \text{HCN} \times \text{Pollination}} = 0.013$ ).

**Table S1** Pairwise genetic correlations among traits measured in our experiment.

**Table S2** Data transformations used on traits to improve normality and homogeneity of variance.

**Table S3** Mean, coefficient of genotypic variation ( $CV_g$ ) and broad-sense heritability ( $H^2$ ) of all reproductive traits measured in our experiment.

**Table S4** Total selection (S) in units of standard deviation acting on each trait in all treatment combinations.

**Table S5** Results from multivariate genotypic selection analysis examining the interactive effects of HCN and unplanned meadow vole damage for selection on reproductive traits.

**Table S6** Results from multivariate genotypic selection analysis examining the interactive effects of *CYP79D15*, invertebrate herbivores, and pollinators on selection on reproductive traits.

**Table S7** Results from multivariate genotypic selection analysis examining the interactive effects of *Li*, invertebrate herbivores, and pollinators on selection on reproductive traits.

All R code, data and metadata are available on the GitHub page for J.S.S ([https://github.com/James-S-Santangelo/HPS\\_Herbivore-Pollinator-Selection](https://github.com/James-S-Santangelo/HPS_Herbivore-Pollinator-Selection)).

Data deposited at Dryad: <https://doi.org/10.5061/dryad.h6qg003>.

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