

# Pollinators and herbivores interactively shape selection on strawberry defence and attraction

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## Abstract

Plant-herbivore-pollinator interactions are of ecological relevance for most angiosperms. However little is known on how plants evolve in response – and in particular how these tripartite interactions influence phenotypic selection on traits that link pollination and herbivory. We here conducted a common garden experiment with woodland strawberry (*Fragaria vesca* L.) to quantify pollinator- and herbivore-mediated selection on nine traits related to plant defence and attraction. Our results showed that pollinators imposed stronger selection than herbivores on both direct and indirect (i.e., tritrophic-related) defence traits – whereas conflicting selection was imposed on inflorescence density. However, in all cases, selection imposed by one agent was context-dependant on the other, suggesting that dynamic patterns of selection are likely a prevalent feature of these interactions. Our findings highlight the significance of plant-herbivore-pollinator interactions as potential drivers of evolutionary change, and reveal that pollinators may play a generally underappreciated role as selective agents on plant defence.

## INTRODUCTION

To maximise reproductive success, most angiosperms are tasked with attracting pollinating mutualists whilst also evading herbivore antagonists (Strauss 1997; Lucas-Barbosa 2016; Kessler & Chautá 2020). However, plant interactions with pollinators and herbivores, and the traits that mediate these interactions, are often inter-linked (Theis *et al.* 2007; González-Teuber & Heil 2009; Galen *et al.* 2011; Kessler *et al.* 2019; Rusman *et al.* 2019). Such linkages typically lead to ecological trade-offs for plants. For instance, pollinators often preferentially forage on larger, more apparent flowers and inflorescence displays (Conner & Rush 1996; Parachnowitsch & Kessler 2010). Yet greater visual or olfactory apparency can also come at the cost of increased herbivore damage when co-opted as shared signals (Halitschke *et al.* 2008; Sletvold & Grindeland 2008; Theis & Adler 2012; Knauer & Schiestl 2017; Santangelo *et al.* 2019). Plant-herbivore interactions can likewise bear large consequences for pollination success (Kessler *et al.* 2011; Muola *et al.* 2017). The presence and action of herbivores, and in particular their damage to leaves (folivory) and flowers (florivory), can deter pollinators via a range of direct and indirect mechanisms (Jacobsen & Raguso 2018; Moreira *et al.* 2019; Haas & Lortie 2020). These include visually (e.g. modification of floral aesthetics; reduced resource allocation to floral rewards and displays (McCall & Irwin 2006)), olfactorily (e.g. release of herbivore-induced plant volatiles (Kessler & Chautá 2020)), and gustatorily (e.g. upregulated expression of unpalatable defensive compounds in floral nectar (Adler *et al.* 2006)). Hence given the multiple routes by which these ecological effects can manifest for plants, the net outcome for fitness is thereby expected to be highly context-specific e.g. (Gegean *et al.* 2007).

Yet despite growing appreciation of the ecology of plant-herbivore-pollinator systems, it remains poorly understood how these interactions ultimately affect phenotypic selection on traits at the microevolutionary

scale. The high degree of ecological linkage and specificity inherent in these systems suggest that pollinator and herbivore selection may often be conflicting (i.e., exerted in opposing directions) and diffuse (i.e., context-dependant on the presence or ecological effects of the other) (Strauss *et al.* 2005; Sletvold *et al.* 2015; Knauer & Schiestl 2017; Ramos & Schiestl 2019; Sletvold 2019). However, although herbivory is known to potentially constrain floral evolution (Johnson *et al.* 2015; Jogesh *et al.* 2017; Ramos & Schiestl 2019; Santangelo *et al.* 2019), a recent meta-analysis by Caruso *et al.* (2019) revealed an almost categorical lack of studies which simultaneously quantify pollinator- and herbivore-mediated selection on the same trait. Further studies are hence required to examine the relative importance and dynamics of pollinator and herbivore selection on traits that link pollination and herbivory. Beyond floral traits, these should also include direct and indirect (i.e., natural enemy-mediated) defences. Few studies to date have examined the potential for pollinators to select on defence traits related to herbivory (Kessler & Halitschke 2009; Egan 2015; Ramos & Schiestl 2019). Yet such selection may in fact be commonplace in plants (Egan *et al.* 2016), especially as an adaptation to mitigate herbivore deterrence of pollinators.

In this study we manipulated pollination and herbivory in a common garden experiment with woodland strawberry (*Fragaria vesca* L.). We examined phenotypic selection on several chemical traits previously identified as markers of direct and indirect defence for this species (see below and Weber *et al.* , 2020a; Weber *et al.* , 2020b), alongside several morphological traits potentially important for attraction of pollinators and herbivores. We tested and found at least partial support for the hypotheses that: 1) pollinators and herbivores positively select on defence-related traits – which in this system are expected to aid against the direct and pollinator-mediated costs of herbivory (Muola *et al.* 2017; Muola & Stenberg 2018; Weber *et al.* 2020a); 2) pollinators and herbivores impose conflicting selection on plant attractive traits – as shared host-selection cues potentially used by both agents; and 3) that the above selection regimes are diffuse – i.e., that pollinator-mediated selection is modified (in strength, and possibly direction) when herbivory is manipulated, and vice versa. Investigations of this kind can thereby provide greater insight into the eco-evolutionary dynamics of plant-herbivore-pollinator interactions.

## MATERIAL AND METHODS

### Common garden

A common garden experiment was initiated in which pollination and herbivory were manipulated to examine phenotypic selection on nine plant attractive and defence-related traits in *F. vesca*. Four blocks were established containing 81 clonally-replicated plant genotypes each (listed in Table S1). We employed a split-plot design in which two pollination treatments (control versus hand pollination) were applied within blocks, and two herbivory treatments (addition versus removal) were applied across blocks, to afford a total of four unique treatment combinations. Details of the common garden establishment, and sourcing and propagation of plant genotypes from wild Swedish populations, are provided in Appendix 1.

### Pollination and herbivory treatments

For the pollination treatments, all flowers on a plant were either exposed to ambient pollination conditions (control) or received supplemental hand pollinations every ca. 5 days for the full flowering period. Only three plant genotypes were used as pollen donors to control for pollen quality effects. Herbivory was manipulated through the addition or removal of an ecologically important herbivore; the strawberry leaf beetle, *Galerucella tenella* L. (Coleoptera: Chrysomelidae). This oligophagous herbivore feeds on leaves and flowers of several Rosaceae plants (Stenberg & Axelsson 2008). For woodland strawberry this can reduce fitness both directly and indirectly, via pollinator limitation (Muola *et al.* 2017; Muola & Stenberg 2018). The plantation in which the herbivore treatments were applied was fenced with a 2 m fine mesh to exclude herbivory from small and large browsing vertebrates, including deer and digging rodents. For the ‘herbivore addition’ treatment, several hundred adult individuals of the strawberry leaf beetle were collected in early May soon after their emergence on their main host plant meadowsweet (*Filipendula ulmaria* (L.) Maxim.) (Stenberg & Axelsson 2008). Further details of these collection localities are provided in (Weber *et al.* 2020b). The beetles were released onto plants at a density of 0.4 individuals per plant. Adult and larval feeding damage were apparent

within one and five weeks of release respectively. Most feeding damage in the ‘herbivore addition’ treatment was hence caused by the strawberry leaf beetle. However, damage from larvae of two species of leaf- and flower-feeding Lepidoptera – *Cnephasia asseclana* Denis & Schiffermuller (Tortricidae) and *Ceramica pisi* L. (Noctuidae) – was also observed on experimental plants. The former is a pest of cultivated strawberry (Sigsgaard *et al.* 2014), whereas the latter is a generalist moth that feeds on genera including *Rubus* and *Salix* (Robinson *et al.* 2010). As a common practice for the experimental removal of insect herbivores (Siemann *et al.* 2008), the ‘herbivore removal’ treatment was made through application of low doses of an insecticide. For this we employed foliar applications of Calypso (Bayer CropScience); a systemic insecticide based on the active substance thiacloprid. All manufacturers’ recommendations were followed concerning dosage concentration and frequency of application. Three rounds of application were made every ca. 4 weeks between early May and mid-July. Spraying took place when conditions were dry and windless so as to avoid cross-contamination. Virtually no damage to plants in the ‘herbivore removal’ treatment was observed.

## Trait measurements

The plant genotypes used in this study were previously shown to harbour high genetic variation in direct and indirect defence against the strawberry leaf beetle (Weber *et al.* 2020a; Weber *et al.* 2020b), as evidenced by the differential performance and preference of both the herbivore and its specialist endoparasitoid, *Asecodes parviclava* Thompson (Hymenoptera: Eulophidae), on diets of varying genotypic composition. Several primary and secondary compounds were ultimately identified as markers associated with either direct or indirect defence (Weber *et al.* 2020a). We included here the five compounds most strongly associated with defence: two carbohydrates (dehydroascorbic acid and myo-inositol), and three phenolics (catechin, dihydroxybenzoic acid, and shikimic acid). Details of leaf sampling and GC/TOF-MS metabolomic profiling are presented in Weber *et al.* (2020a), which was conducted concurrently for the same plants used in this study. Owing to capacity limitations experienced with metabolomic profiling, defence-related traits could only be quantified for 27 of the 81 genotypes. However, selection of these 27 genotypes was made such that the full spectrum of genetic variation in direct and indirect defence was still represented (following Weber *et al.* 2020a and Weber *et al.* 2020b). The four attractive traits examined were plant size, total flower number, flower frost tolerance, and inflorescence density. Frost damaged flowers show a complete blackening of the receptacle at the centre of the flower, and in this way could be expected to impact floral attractiveness to pollinators and florivores. Details of how each trait was quantification are provided in Egan *et al.* (2018).

## Fitness measurement

Plant fitness was measured as total seed output per plant. For this, berries were picked fresh as they ripened on plants up until most fruiting had finished by mid-July. After this time berries were allowed to dry on the plant before a final picking in early August. Although birds were not excluded from the common garden, we did not observe any bird frugivory. All berries were dried in an oven at 80°C for 1.5 days. To estimate the total number of fertilised seeds per plant from dry berry weight, we established two regression equations; one for berries that were picked fresh ( $y = 0.9937x - 14.257$ ,  $R^2 > 0.99$ ), and one for berries that were picked dry ( $y = 1.0017x - 14.423$ ,  $R^2 > 0.99$ ). Berries were randomly selected across all genotypes and treatments in order to establish these equations. Only fertilised seeds were counted, which are easily visually differentiated from non-fertilised seeds (Thompson 1971). Owing to their much-reduced size, non-fertilised seeds made only a negligible contribution to dry berry weight (data not shown).

## Phenotypic selection analysis

To quantify phenotypic selection on traits, multiple regression was used to provide estimates of selection gradients, whereas individual univariate models were used to provide estimates of selection differentials. Selection gradients ( $\beta$ ) describe the strength of unique or direct selection acting on a trait (after controlling for inter-trait correlations), whereas selection differentials (S) describe ‘total’ selection (Lande & Arnold 1983). For the analysis of  $\beta$ , a multiple regression model was fitted in which relative fitness (seed number divided by its mean) was regressed on standardized traits (standardised by standard deviation) (Lande & Arnold 1983). We also added to the model the two-level factors of ‘pollination’ (control, hand pollination) and

‘herbivory’ (addition, removal), and their interaction with all traits and each other. In this way estimates of  $\beta$  were output for all traits in all four treatment combinations. Following Sletvold (2019), the calculated difference in trait  $\beta$  between treatment combinations thereby provided estimates of pollinator-mediated selection (in the presence and absence of herbivory), herbivore-mediated selection (with and without pollinator limitation), and combined pollinator- and herbivore-mediated selection. We used the ‘emtrends’ function of R package emmeans (Lenth *et al.* 2018) to calculate these differences in  $\beta$ , and to test whether the result differed significantly from zero (after adjusting p-values for multiple comparisons via Benjamini–Hochberg correction). Although the multiple regression included only 27 genotypes for which complete trait data was available, we still considered these inferences to be robust given that: 1) the full spectrum of genetic variation in defence was represented (see above), and 2) no qualitative differences were observed in univariate regressions regardless of whether some ( $n = 27$ ) or all ( $n = 81$ ) genotypes were used (Table S2). For the analysis of  $S$ , univariate regressions were fitted for each trait individually. Only data from the ‘control pollination/herbivore present’ treatment combination were used for the estimation of  $S$ , as this treatment combination is typically considered most representative of natural population conditions (Sletvold 2019).

## RESULTS

Of the nine traits examined in this study, pollinators were implicated as agents of selection on four (three defence-related and one attractive), and herbivores on two (one defence-related and one attractive) (Fig. 1). Pollinators generally imposed stronger selection pressures than herbivores as a whole, and only pollinator-mediated selection appeared to constitute an important component of ‘total’ selection overall (Table S2) – i.e., for dihydroxybenzoic acid (net positive selection) and shikimic acid (net negative selection). Conflicting selection – where pollinators and herbivores exerted selection in opposing directions – was observed for inflorescence density, for which these agents imposed positive and negative selection respectively. However, each of the above selection regimes was in addition seen to be diffuse – meaning that the strength of selection by one agent was context-dependent upon (or modified by) the presence or ecological effects of the other (Fig. 1).

## DISCUSSION

The findings of this study make a significant contribution to the understanding of plant-herbivore-pollinator systems in demonstrating, firstly, the relative importance of pollinators as selective agents on both plant-attractive and defence traits, which here even surpassed that of herbivores; and secondly, the extent to which net selective outcomes were dynamic, and context-specific.

Herbivores have long been considered the primary drivers of defence trait evolution in plant populations (Johnson *et al.* 2015). However, in line with our predictions, pollinators were found to impose positive selection on direct defence-related traits in one instance (for dihydroxybenzoic acid). Although negative selection was also surprisingly apparent (for shikimic acid) (Fig. 1). Dihydroxybenzoic acid is a phenolic that is strongly associated with resistance against the strawberry leaf beetle (Weber *et al.* 2020a). Given that pollinators were previously found to avoid damaged flowers of woodland strawberry (Muola *et al.* 2017), it appears logical that pollinator selection on this compound was exerted only in the presence of herbivores, and not in their absence. Hence the suggested link is that when herbivores are present, plants with higher direct defences receive less damage and are preferred by pollinators.

While this mechanism is intuitive for dihydroxybenzoic acid, the opposite pattern was found for shikimic acid – where pollinators selected against this defensive compound in the presence of herbivores. Shikimic acid is probably a common constituent of floral nectar (Hölscher *et al.* 2008), and like other nectar secondary compounds may be rapidly upregulated in response to herbivore attack (Adler *et al.* 2006; Kaczorowski *et al.* 2014). Thus, one explanation is that herbivore-induced changes in this compound in nectar (or other correlated derivatives of the shikimic acid pathway) could have led to gustatory deterrence of pollinators, similar to other nectar phenolics (see Stevenson *et al.* , 2017 and references therein). This explanation is also consistent with our finding that pollinator-mediated selection on shikimic acid was diffuse, and disappeared in the absence of herbivores (Fig. 1). However, regardless of the underlying mechanisms, these findings

nonetheless establish the capacity of pollinators to impose both positive and negative selection on direct defence traits of relevance to herbivores.

Indirect defence-related traits were in contrast selected on by both agents (Fig. 1). Herbivores selected for higher levels of dehydroascorbic acid, meaning that plants with lower levels of this compound suffered greater fitness damage by herbivores. This carbohydrate is strongly associated with indirect defence in woodland strawberry owing to its positive link with parasitoid development in the herbivore host (Weber *et al.* 2020a). Why herbivores appeared to prefer plants with lower levels of dehydroascorbic acid could relate to a natural deterrence effect of this compound (Felton & Summers 1993), or innate avoidance as a behavioural adaptation against parasitism (as per the concept of ‘enemy free space’ (Stamp 2001)). Pollinators made only a minor contribution to selection on this compound (Fig. 1), but did clearly positively select on dihydroxybenzoic acid; a phenolic that – in addition to serving as a direct defence, as discussed – is associated with strawberry leaf beetle vulnerability to parasitism (Weber *et al.* 2020a). This selection was most likely imposed via herbivore deterrence effects (Muola *et al.* 2017). These findings build on past work by Kessler and Halitschke (2009) indicating potential pollinator selection on volatile signals associated with indirect defence, and establish the potential for pollinators to also mediate selection (coincidentally or otherwise) on non-volatile chemical traits linked to tritrophic interactions.

Of the four plant attractive traits examined in this study, significant herbivore- and pollinator-mediated selection was observed only for inflorescence density (Fig. 1). Selection on this trait was both diffuse and conflicting; a pattern that is likely to arise when a trait is shared as a positive host-plant selection cue by both pollinators and herbivores (Knauer & Schiestl 2017; Ramos & Schiestl 2019). Use of this cue by the strawberry leaf beetle and another present herbivore (see Methods) appears logical given that both are florivores, and that the former is thought to occupy dense inflorescences as a means of enemy escape in its primary host plant, meadowsweet (*Filipendula ulmaria* (L.) Maxim.) (Stenberg 2012). Shared use of this cue would hence explain why this trait was negatively selected against by herbivores only when the influence of pollinators was controlled for, and positively selected for by pollinators when herbivores were absent. Hence the combined effect of both agents was for intermediate selection, which was neither significantly negative nor positive overall (Fig. 1), nor appeared to contribute to the ‘total’ selection acting on this trait (Table S2).

As a whole, this study permits greater insight into the eco-evolutionary dynamics of plant-herbivore-pollinator interactions, and how multiple selective forces can act to shape the microevolution of traits that link pollination and herbivory. Our findings suggest that dynamic patterns of selection may be a common feature of these tripartite interactions, given that all significant selection on traits was notably context-specific. Diffuse interactions between pollinators and herbivores may thereby explain why the phenotypic optima of plant attractive and defence-related traits could be expected to fluctuate in populations across time, in accordance with changing biotic interaction strengths. Furthermore, our study demonstrates the highly significant role that pollinators can play in selecting for increased direct and indirect defence-related traits. While the evolution of increased selfing has been proposed as one way for plants to overcome herbivore-induced pollinator limitation (Kessler & Halitschke 2009; Adler *et al.* 2012; Johnson *et al.* 2015), pollinator selection for increased anti-herbivore defences could offer another potentially commonplace route (Egan 2015), and one that does not risk the disadvantages of increased inbreeding. However, ours and a previous study (Egan *et al.* 2016) also show that when defence-related traits themselves are associated with pollinator limitation, then negative selection can also be expected. Together these findings highlight the complexity of potential selection pressures that can act on plant attractive and defence-related traits. The predictions generated from this study can hence serve as valuable hypotheses to test in future studies in wild plant populations.

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## Figure legends

**Figure 1.** Direction and strength of phenotypic selection mediated by pollinators and herbivores on defence-related traits (top) and plant attractive traits (bottom) in woodland strawberry. Pollinator- and herbivore-mediated selection was quantified both under control and manipulated conditions to reveal whether selection was diffuse (context-dependent on the other agent) and/or conflicting (exerted in opposing directions). Presented are selection gradient ( $\beta$ ) means and their associated confidence intervals. Asterisks indicate that selection is significantly different from zero, following adjustment of p-values for multiple comparisons (see Methods).

## Supporting Information

**Appendix 1:** Collection of wild plant genotypes and establishment of the common garden

Table S1. The 81 plant genotypes used in this study and the coordinates of their collection locality from wild populations around Uppsala county, Sweden.

**Appendix 2:** Total Selection

Table S2. Direction and strength of total selection on defence-related traits and plant attractive traits in woodland strawberry

## Hosted file



Supporting\_information.docx available at <https://authorea.com/users/351521/articles/476102-pollinators-and-herbivores-interactively-shape-selection-on-strawberry-defence-and-attraction>

figures/Figure-1/Figure-1-eps-converted-to.pdf