

Evidence that a herbivore tolerance response affects selection on floral traits and inflorescence architecture in purple loosestrife (*Lythrum salicaria*)

Christina J. M. Thomsen* and Risa D. Sargent

Department of Biology, University of Ottawa, 30 Marie Curie, Ottawa, Ontario, K1N 6N5, Canada

*For correspondence. E-mail christina.jmt@gmail.com

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• **Background and Aims** The study of the evolution of floral traits has generally focused on pollination as the primary driver of selection. However, herbivores can also impose selection on floral traits through a variety of mechanisms, including florivory and parasitism. Less well understood is whether floral and inflorescence architecture traits that influence a plant's tolerance to herbivory, such as compensatory regrowth, alter pollinator-mediated selection.

• **Methods** Because herbivore damage to *Lythrum salicaria* meristems typically leads to an increase in the number of inflorescences and the size of the floral display, an experiment was conducted to test whether simulated herbivory (i.e. clipping the developing meristem) could alter the magnitude or direction of pollinator-mediated selection on a suite of floral and inflorescence architecture traits. Using a pollen supplementation protocol, pollen limitation was compared in the presence and absence of meristem damage in order to quantify any interaction between pollinator and herbivore-mediated selection on floral traits.

• **Key Results** Surprisingly, in spite of an obvious impact on floral display and architecture, with clipped plants producing more inflorescences and more flowers, there was no difference in pollen limitation between clipped and unclipped plants. Correspondingly, there was no evidence that imposing herbivore damage altered pollinator-mediated selection in this system. Rather, the herbivory treatment alone was found to alter direct selection on floral display, with clipped plants experiencing greater selection for earlier flowering and weaker selection for number of inflorescences when compared with unclipped plants.

• **Conclusions** These findings imply that herbivory on its own can drive selection on plant floral traits and inflorescence architecture in this species, even more so than pollinators. Specifically, herbivory can impose selection on floral traits if such traits influence a plant's tolerance to herbivory, such as through the timing of flowering and/or the compensatory regrowth response.

Key words: Flowering phenology, floral display, herbivory, pollination, pollen limitation, selection experiment, compensatory regrowth, plant defence.

INTRODUCTION

Our understanding of how interactions within ecological communities shape floral trait evolution is undergoing a significant shift (Ashman and Morgan, 2004; Strauss and Whittall, 2006). Because pollinator attraction is closely tied to fitness in most plant species, pollinators were historically assumed to be the primary drivers of selection on plant floral traits (Darwin, 1877). Indeed, pollinator-mediated selection on floral traits is well documented (Fishman and Willis, 2008; Sandring and Ågren, 2009; Parachnowitsch and Kessler, 2010; Bartkowska and Johnston, 2012). However, mounting evidence indicates that floral traits are in fact driven by an array of factors, including some unexpected players, such as herbivores (reviewed in Strauss and Whittall, 2006; e.g. Kessler *et al.*, 2013).

When multiple agents of selection act on a single trait, they can give rise to conflicting or reinforcing selection (Strauss and Whittall, 2006). For example, conflicting selection can readily be seen with pollinators and herbivores because they often share preferences for the same floral phenotypes (e.g. Cariveau *et al.*, 2004; Parachnowitsch and Caruso, 2008). Indeed, herbivores probably play a greater role in the evolution of

floral traits than previously suspected; models predict that, in circumstances where multiple agents act on the same trait, antagonists such as herbivores tend to impose even greater selection than do mutualists (e.g. pollinators) (Benkman, 2013; Vanhoenacker *et al.*, 2013).

Although several recent studies have examined how herbivores and pollinators together drive selection on floral traits (e.g. Gómez, 2003; Parachnowitsch and Caruso, 2008; Sletvold *et al.*, 2010; Bartkowska and Johnston, 2012), most were carried out in systems where herbivores, generally seed predators, destroy reproductive rather than vegetative tissue. In such systems, pollinators and herbivores tend to have an additive influence on selection (e.g. Sletvold *et al.*, 2015). There is, however, an alternative possibility: herbivores and pollinators may instead have a conditional influence on selection, where the action of one agent depends on the action of another (Johnson *et al.*, 2015). For example, when herbivore damage results in a decrease in corolla size, pollinators may be less attracted to the damaged plant (Mothershead and Marquis, 2000). Conversely, herbivore damage can sometimes stimulate an increase in floral production, such as through compensatory regrowth (e.g. Juenger and Bergelson, 2000). If the action of herbivores conditionally

impacts the attraction of pollinators to plants, it could in turn alter the strength and/or direction of pollinator-mediated selection.

Compensatory regrowth provides an important avenue through which we can observe the interaction of herbivores and pollinators. As a plant defence used by many plant species, compensatory regrowth occurs when damage to meristem leaf tissue activates dormant axial buds to grow in order to safeguard plant survival and reproduction (Strauss and Agrawal, 1999; Stowe *et al.*, 2000). This ‘tolerance’ trait is of particular interest to the study of pollinator-mediated selection because it may significantly alter the architecture of the floral display through increased inflorescence branching and flowering (reviewed in Trumble *et al.*, 1993). If compensatory regrowth positively changes the attractiveness of the floral display (e.g. Russell-Mercier and Sargent, 2015), pollinators may then preferentially visit plants that have been damaged by herbivores. Thus, herbivores may not only drive selection for floral traits that give rise to plant tolerance, but may indirectly impose selection on floral traits through the modification of pollinator preferences.

The strength of selection imposed by multiple agents can be quantified by combining the phenotypic selection method (Lande and Arnold, 1983) with the use of experimental treatments (Wade and Kalisz, 1990). By comparing phenotypic selection in pollen-supplemented plants with that of controls, the strength of pollinator-mediated selection can be estimated (Fishman and Willis, 2008; Sandring and Ågren, 2009; Parachnowitsch and Kessler, 2010; Bartkowska and Johnston, 2012). If supplemented plants produce more seeds than control plants, this suggests that an insufficient quantity or quality of pollen is being received under natural conditions, i.e. pollen limitation. The presence of pollen limitation implies that any preferences pollinators have for particular traits should generate pollinator-mediated selection (Ashman and Morgan, 2004). Indeed, decreased strength of mutualistic interactions (e.g. pollinator interactions with plants) has been shown to correspond to stronger selection intensity (Benkman, 2013; Vanhoenacker *et al.*, 2013).

Using a split-plot design combining a pollen supplementation experiment with experimental manipulations of artificial herbivore damage, we sought to quantify the relative roles of pollination and herbivory, as well as the indirect effects of herbivory (i.e. compensatory regrowth), on phenotypic selection for a suite of floral and architectural traits in *Lythrum salicaria* L. (Lythraceae). *Lythrum salicaria*, or purple loosestrife, is an invasive wetland perennial native to Europe, which has been found in North America since the early 1800s (Thompson *et al.*, 1987). Its expansion throughout most of mainland USA and southern Canada is largely attributed to its ability to produce up to 2–3 million seeds per plant (Thompson *et al.*, 1987). *Lythrum salicaria* provides a good opportunity to address our research questions because apical meristem damage by its main herbivore (introduced as a biological control agent), two species of beetles in the genus *Neogalerucella*, can induce changes in floral display via compensatory regrowth (Schat and Blossey, 2005; Russell-Mercier and Sargent, 2015). *Neogalerucella* beetles typically inflict a single bout of early-season feeding, allowing sufficient time for compensatory regrowth effects to manifest prior to flowering. Studying selection on an invasive plant such as *L. salicaria* can also help us understand how a plant’s evolutionary response to herbivory may feed back to its

interactions with pollinators, which could influence the success of biological control programmes.

Using phenotypic selection analysis, we quantify selection imposed by artificial herbivory and pollinators on floral traits associated with compensatory regrowth. In particular, we ask the following questions. (1) Do pollinators and herbivores independently mediate selection on floral traits and inflorescence architecture? (2) Does the effect of herbivory on floral display alter pollinator-mediated selection on floral traits and inflorescence architecture (i.e. is there an interaction)?

MATERIALS AND METHODS

Study system

We studied a population of *L. salicaria* in a marshland near Elgin, ON, Canada (44°34′08.7″N, 76°19′13.6″W). Plants in our study population produce showy purple flowers, arranged on one or more racemes, from mid-July through August (Venecz and Aarssen, 1998). *Lythrum salicaria* is self-incompatible and promotes outcrossing through tristylly, a reproductive system in which an individual plant is one of three style morphs. The frequency of long-, mid- and short-styled morphs in our study population is approximately equal ($34 \pm 4\%$ long, $32 \pm 4\%$ mid, $34 \pm 4\%$ short).

As part of a programme to control the spread of *L. salicaria*, four beetle species were released in Ontario during the early to mid 1990s (Lindgren *et al.*, 2002), of which *Neogalerucella californiensis* and *N. pusilla* have been the most successful (Blossey, 1995). As plants grow actively in the spring, beetles deposit their eggs on leaves, and the resulting larvae feed on apical and axillary meristem tissue. Loss of apical dominance induces compensatory regrowth through axial branching, dramatically changing the floral architecture of the maturing plant (Schat and Blossey, 2005). No documented biocontrol releases occurred at our study site; however, *N. californiensis* has successfully colonized this location (C. J. M. Thomsen, pers. obs.), presumably through dispersal from nearby release sites.

Artificial herbivory treatment

In May 2013, a total of 178 stems, located at least 1 m apart to ensure independent genotypes (Haldane, 1936), were randomly selected to be part of our study. Selected stems were considered to represent an entire plant. Because *N. californiensis* were just beginning to emerge at the time of plant selection, there was evidence of adult feeding on some plants (no greater than 5% standing leaf damage), but no evidence of oviposition. Only plants that lacked apical meristem damage were selected, and any herbivores, if present, were removed. To prevent natural herbivory further, each stem was covered with a sleeve composed of fine mesh (‘no see-um’ netting, Rose e Dee Ltd) and was re-evaluated for damage and herbivore presence during subsequent visits. Because all stems in the study were covered, any potential effects of the covers on plant traits were controlled for.

Plants were randomly assigned to one of two herbivory levels: clipped (simulated damage to the apical meristem, $n = 87$) and control (unclipped, $n = 86$). Because some plants were lost at each stage of the study, the final sample size ($n = 173$) is

lower than the original ($n = 178$). Clipping simulates two common types of herbivory in this system: meristem feeding by *Neogalerucella*, which has been shown to be adequately mimicked by clipping (Russell-Mercier and Sargent, 2015), and feeding by deer, which commonly browse the tops of purple loosestrife plants in this and other populations (Rachich and Reader, 1999). We chose to simulate herbivory because estimates of plant tolerance under natural herbivory can be noisy since they do not account for individual variation in plant resistance and environmental microsite differences, whereas simulated herbivory standardizes damage independently of these factors (Stowe *et al.*, 2000; Tiffin and Inouye, 2000). Previous research suggests that in *L. salicaria*, simulated herbivory can be especially similar to natural herbivory when quantifying tolerance, regrowth and reproductive traits (Lehtilä and Boalt, 2004; Russell-Mercier and Sargent, 2015).

Herbivory treatments were applied in early June when *N. californiensis* larval activity typically peaks at this location. To mimic larval feeding, we used microdermal scissors to remove 2 cm from the apical meristem of plants in the clipped treatment. Both clipped and control plants were visited weekly to confirm that the sleeves were secure; in rare situations, when herbivores circumvented the sleeves and caused apical damage to control plants, plants were reassigned to the clipped treatment ($n = 8$). By the end of July, beetle activity was vastly reduced, and sleeves were removed for pollen supplementation.

Pollen supplementation experiment

Pollen supplementation experiments compare natural levels of pollination with artificially saturated levels to determine the extent of pollen limitation (Ashman and Morgan, 2004). Because it was impractical to supplement all flowers on every plant (average of 980 flowers per plant), supplementation was done at the flower level, where pairs of flowers on study plants were selected to be part of the experiment. Pairs consisted of two flowers on adjacent nodes at the same height (± 1 cm) of a given inflorescence. Each flower was randomly assigned to receive either open pollination (OP) or hand pollination (HP). Up to four pairs of flowers were selected per study plant, depending on the availability of open flowers, and tagged with coloured polyester thread for later identification ($n = 429$ pairs across all plants). Because plants may shunt resources towards HP flowers, reducing seed production in OP flowers (Knight *et al.*, 2006), we tagged control flowers on untreated inflorescences on a sub-set of experimental plants. Consistent with a previous study of *L. salicaria* (Ägren and Ericson, 1996), there was no difference in seed production between control and OP flowers ($t = -1.37$, d.f. = 69, $P = 0.1747$).

To supplement HP flowers, we applied pollen from 2–5 non-experimental donor plants that were of optimal morph compatibility and no more than 10 m from the experimental plant. Because *L. salicaria* flowers only remain open for a single day, they did not require subsequent hand pollinations. To apply pollen, stamens were removed from donor flowers and the recently dehiscent anthers were rubbed directly on the recipient stigma until it was visibly saturated. Because it is difficult to access short-morph stigmas, tweezers were used to slice the side of the corolla tube (as described by O'Neil, 1992). Application of

pollination treatments spanned a 2 week period from late July to early August, corresponding to peak flowering time. Plants that did not flower in time were excluded from the pollination treatment ($n = 14$).

By the end of the season, 35 study plants from the initial sample were excluded because of beaver-, deer- or weather-related damage. In total, 49 flower pairs were excluded because of damage to either the plant or fruits. In addition, some fruit from the pollen supplementation experiment were lost, probably to fruit abortion; in total, 90 HP fruit and 124 OP fruit were aborted. Seed counts were successfully retrieved for 135 plants (70 clipped, 65 control) from the original study population.

Trait measurements

To quantify pollinator-mediated selection, a series of measurements were collected at the time of pollen supplementation: inflorescence number and length, number of open flowers, inflorescence height (i.e. plant height) and petal length (measured from flowers in supplemental pairs). Once flowers from the supplementation experiment matured, they were collected and seeds were counted using a digital seed counting program (MathWorks, 2012) in order to calculate pollen limitation.

End-of-season trait values were measured to determine overall differences between clipped and control plants: number of flowers (number of fruit + number of abortion scars), number of fruit, percentage of aborted fruit (number of abortion scars \div number of flowers $\times 100$), number of axial inflorescences, total inflorescence length (sum of all inflorescence lengths), flower density (number of flowers per total inflorescence length), inflorescence height and flowering phenology (the start and end of plant flowering, estimated within 3 d).

Statistical analyses

To test whether simulated herbivory altered floral traits and inflorescence architecture, we compared trait values between treatments using one-way analysis of variance (ANOVA) or, when appropriate, the Mann–Whitney U-test.

To measure pollen limitation, we calculated the difference in seed set (the number of seeds per flower) between each flower pair, $\Delta\text{Seed Set} = \text{Seed Set}_{\text{HP}} - \text{Seed Set}_{\text{OP}}$; a difference greater than zero indicates pollen limitation. Aborted fruit were assigned a seed set of zero, rather than excluded from the analysis, because fruit abortion can reflect the quality or quantity of pollen received (e.g. Pflugshaupt *et al.*, 2002). To assess whether plant damage influenced seed set, a linear mixed-effects model (LMM), with plant nested within the damage treatment as a random effect, was fit via restricted maximum likelihood (REML). Because previous studies on *L. salicaria* have detected differences in pollen limitation among style morphs (O'Neil, 1992; Ägren, 1996; Ägren and Ericson, 1996), we further tested morph as a fixed effect within the same model.

Using the difference in seed set, as described above, allows for a more simplistic LMM because it removes the need for a zero-inflated model to account for the high quantity of aborted fruit; however, we used a zero-inflated generalized linear mixed

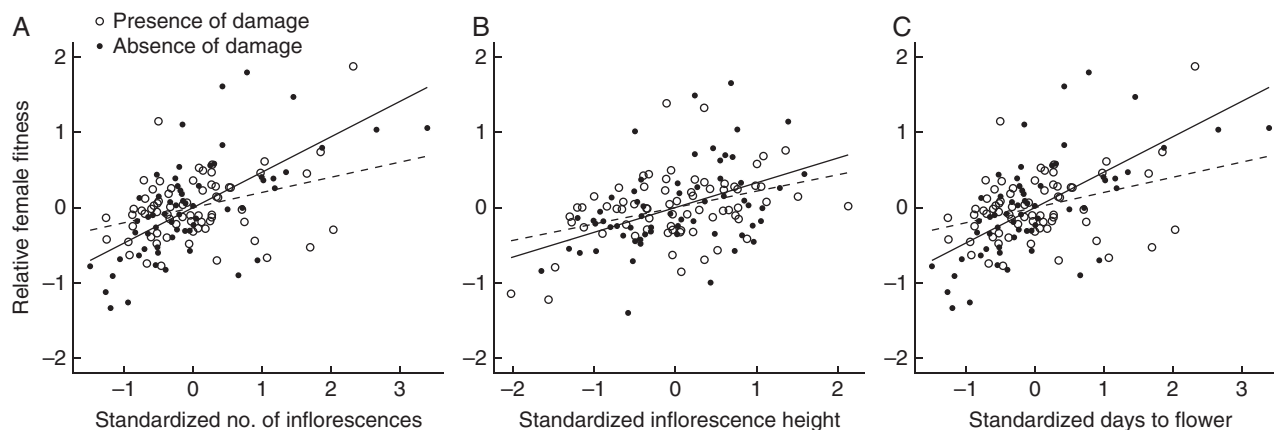


Fig. 1. Added-variable plot displaying phenotypic selection gradients for (A) number of inflorescences, (B) inflorescence height and (C) flowering start time in *Lythrum salicaria*. Linear selection gradients for plants in the presence and absence of artificial herbivore damage are illustrated using added-variable plots, where the residuals from a regression of relative fitness against all predictors except the target trait are plotted against the residuals from a regression of the target trait against all other predictors.

model (GLMM) with negative binomial distribution to compare raw seed counts between HP and OP flowers and found the same results. Further, two separate models: an LMM for seed set (excluding 'zero' values) and a binomial regression GLMM for fruit set, yielded the same conclusions.

Selection analyses

Phenotypic selection analyses estimate selection by determining the relationship between a range of possible trait values and fitness; the coefficient extracted from this relationship determines the strength and direction of selection. Using the methods described by Lande and Arnold (1983), we regressed relative fitness (individual fitness value \div treatment mean fitness) with standardized trait values [(individual trait value – treatment mean value) \div treatment standard deviation]. Direct selection coefficients were estimated using a multitrait model to quantify linear selection gradients (β_i). By including multiple traits in the model, we can identify direct selection on a single trait by excluding its correlation with other traits in the model. In addition to including the linear term for each trait, quadratic terms were also included to estimate non-linear selection (γ_{ii}). Although a marginally significant quadratic term was detected for the number of inflorescences, quadratic terms did not improve model fit and did not show any clear fitness optima; therefore, we only provide linear selection gradients. Cross-product terms (γ_{ij}) were also excluded in all models to reduce model complexity; however, raw correlations were calculated separately (Supplementary Data Table S1).

We estimated the linear selection coefficients for the simulated herbivory and control groups using seed production as the measure of relative fitness. Total seed production was estimated per plant as total fruit count \times seed set, averaged across OP fruit from each plant. Because total fruit count already takes fruit abortion into account, we excluded aborted OP fruit (i.e. fruits with '0' seed count) from the analysis and replaced them with additional OP fruit marked during pollination treatments. Selection coefficients were quantified for a sub-set of the measured traits to limit the influence of among-trait correlations. We

included: the number of inflorescences, inflorescence height and flowering start time because (a) they are the traits most closely associated with compensatory regrowth, and (b) there is evidence suggesting that (at least) the latter two have been under selection in *L. salicaria* (O'Neil, 1997). Calculating the variance inflation factor revealed that multicollinearity between the target traits was not an issue (< 2.0) (Quinn and Keough, 2002).

We used analysis of covariance (ANCOVA) to detect differences in the selection coefficients between treatments. The effect of simulated herbivory on selection coefficients was tested using relative fitness (total seed production) as the response variable, and each of the standardized traits, as well as the damage treatment \times trait interaction, as predictor variables. Calculations for relative fitness and standardization of traits were computed within treatment groups.

Two additional ANCOVAs were used: one to determine the effect of pollinator-mediated selection (i.e. pollination treatment \times trait interaction) and one to determine whether pollinator-mediated selection differed between plant damage treatments (i.e. pollination treatment \times damage treatment \times trait interaction). Within each model, traits and treatment \times trait interaction terms were included as predictors of relative fitness (total seed production). Because both pollination treatments were included in the analysis, the total seed production estimate was calculated within each pollination treatment (e.g. Seed Set_{OP} \times Fruit Set_{OP} \times Flower Count Per Plant). To ensure this ANCOVA did not pose too great an extrapolation, we also tested a relative fitness measure that used raw seed set (number of seeds per flower); because this approach yielded the same conclusions, we focus on the total seed production model.

We visualized the selection gradients from our results using added-variable plots; the residuals from a regression of relative fitness against all predictors except the target trait were plotted against the residuals from a regression of the target trait against all other predictors (Fig. 1). Because trait values were standardized within each treatment, it is difficult to gauge whether the phenotypic trait distributions are sufficiently overlapping to provide a biologically meaningful comparison (Chenoweth *et al.*, 2013). Phenotypic distributions for each of

TABLE 1. Effect of herbivore-simulated damage on floral display, floral phenology and reproduction in *Lythrum salicaria*

Trait	Clipped	Control	Test statistic	Effect
Number of open flowers	48.3 ± 3.78	34.4 ± 3.45	$W = 3035$	$P = 0.0029$
Number of flowers	1048 ± 83.4	825 ± 80.6	$W = 2943$	$P = 0.0244$
Number of inflorescences*	8 ± 0.51	5.4 ± 0.72	$F = 17.65$	$P < 0.0001$
Total inflorescence length (cm) [†]	136.1 ± 10.7	107.0 ± 10.6	$F = 4.355$	$P = 0.0389$
Petal length (mm)	7.3 ± 0.15	7.8 ± 0.17	$W = 1616$	$P = 0.0226$
Flower density (per cm) [†]	7.8 ± 0.15	7.9 ± 0.18	$F = 0.029$	$P = 0.8649$
Flowering start time (Julian days)	196 ± 1.06	191 ± 0.98	$W = 3497$	$P < 0.0001$
Flowering end time (Julian days)	236 ± 0.75	236 ± 0.68	$W = 2187$	$P = 0.4159$
Inflorescence height (cm)	111.6 ± 3.18	121.8 ± 2.78	$F = 5.623$	$P = 0.0191$
Number of seeds per fruit	43.7 ± 2.65	39.6 ± 3.02	$F = 1.010$	$P = 0.3168$
Number of fruit [‡]	584 ± 46.9	445 ± 43.2	$F = 4.967$	$P = 0.0275$
Fruit abortion (%)	44.5 ± 1.22	45.1 ± 1.44	$F = 0.111$	$P = 0.7395$

Average (± s.e.) trait values for each treatment are displayed with appropriate one-way ANOVA or Wilcoxon Mann–Whitney test.

*Square root transformed.

[†]Log transformed.

TABLE 2. Direct linear selection (± s.e.) for *Lythrum salicaria* floral and inflorescence architecture traits in the presence and absence (control) of herbivore-simulated damage (clipping)

	Clipped	Control	Damage treatment × trait	Pollination treatment × trait	Pollination treatment × damage treatment × trait
	$\beta_i \pm \text{s.e.}$	$\beta_i \pm \text{s.e.}$	$\Delta\beta_i \pm \text{s.e.}$	$\Delta\beta_i \pm \text{s.e.}$	$\Delta\beta_i \pm \text{s.e.}$
No. of inflorescences	0.20 ± 0.07**	0.47 ± 0.07***	0.27 ± 0.10**	−0.01 ± 0.05	−0.12 ± 0.12
Inflorescence height	0.22 ± 0.06***	0.33 ± 0.09***	0.11 ± 0.11	0.00 ± 0.06	0.11 ± 0.13
Flowering start time	−0.28 ± 0.06***	−0.03 ± 0.08	0.25 ± 0.10*	−0.07 ± 0.06	0.01 ± 0.12

Damage treatment × trait ANCOVA tests if the presence of herbivore-associated damage can mediate selection of traits. A separate ANCOVA tests pollinator-mediated selection (pollination treatment × trait) and the effects of damage on pollinator-mediated selection (pollination treatment × damage treatment × trait).

* $P = 0.05$; ** $P < 0.01$; *** $P < 0.0001$.

the target traits were determined to be almost entirely overlapping and therefore this is not considered an issue [flowering time post-damage, 18–67 d (control), 18–70 d (clipped); inflorescence height, 69–176 cm (control), 68–169 cm (clipped); number of inflorescences, 1–22 (control), 1–21 (clipped)].

All statistical analyses were performed in R (R Core Team, 2013). All selection analysis and pollen limitation models met the criteria of homoscedasticity, unimodality and lack of skew (Quinn and Keough, 2002). Pollen limitation and all ANCOVAs were tested using the R package nlme, while GLMMs were tested with lme4. ANCOVAs included plant nested within damage treatment as random effects, and were fit via REML.

RESULTS

Effects of artificial herbivory

Clipped plants were shorter, produced more flowers and fruits, produced more axial inflorescences, produced flowers with shorter petals and began flowering later than control plants. They aborted the same proportion of fruit and set a similar number of seeds to control plants (Table 1).

In both the clipped and control treatments, the number of flowers and inflorescences, inflorescence length and inflorescence height were positively correlated (Supplementary Data Table S1). In the clipped group, flowering start time was

negatively correlated with all floral traits except petal length, while in the control group it was more weakly, but still negatively, correlated with these same traits.

Pollen limitation

Average seed set per plant was 17.4 % greater in hand-pollinated than open-pollinated flowers, indicating the presence of pollen limitation. Both treatment and control plants showed evidence of pollen limitation; however, there was no difference in the degree of pollen limitation between herbivore treatments ($t = 1.10$, d.f. = 135, $P = 0.2718$). Pollen limitation was greater in the long-styled than the short-styled morph ($t = -2.68$, d.f. = 135, $P = 0.008$) but not greater than the mid-styled morph ($t = -1.83$, d.f. = 135, $P = 0.070$).

Selection analysis

Direct linear selection (β_i) for more inflorescences and taller inflorescences was significant in both the clipped and control groups, while earlier flowering time was significant in only the clipped group (Table 2; Fig. 1). In comparison with control plants, clipped plants exhibited stronger selection for earlier flowering time and weaker selection for more inflorescences (ANCOVA; Table 2).

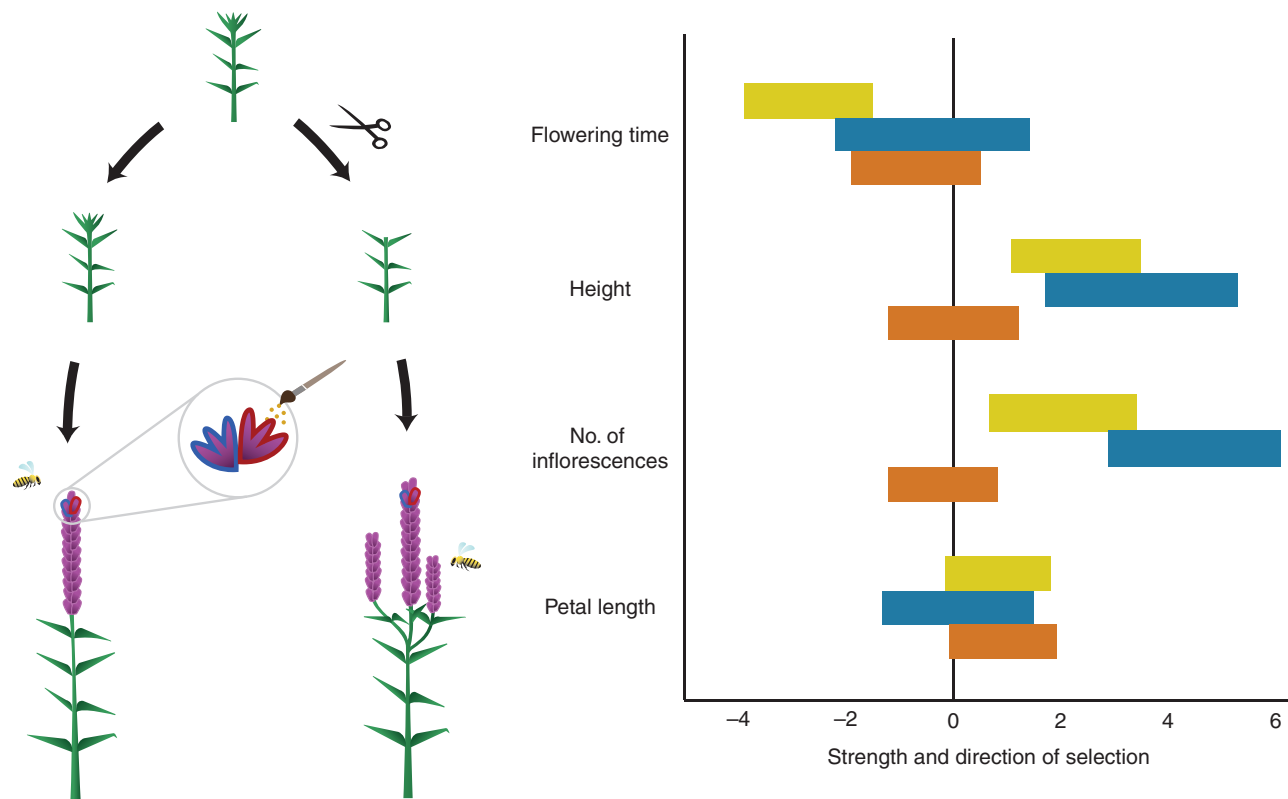


FIG. 2. Effect of pollination and herbivory treatments on the strength and direction of selection on several floral and inflorescence architecture traits in *Lythrum salicaria*. (A) Plants in the population were randomly assigned to herbivory or control treatment groups. Those in the herbivory group received artificial clipping of the developing meristem before flowering commenced. This stimulated a compensatory response in most plants that led to the production of additional inflorescences. We then applied a pollen supplementation treatment to a pair of randomly selected flowers on every plant, with one flower in the pair receiving hand pollination (red outline) and the other flower receiving no additional pollen (blue outline). (B) The strength and direction of selection (x -axis) of the two treatments on a variety of floral and inflorescence architecture traits (y -axis) were measured. We present the direct linear selection coefficients in the presence (yellow bars) and absence (blue bars) of herbivory. We also show the difference in selection coefficients between pollination treatments (orange bars). Bars that overlap with zero were not significantly different from zero. The length of the bar is proportional to the standard error of measurement ($2s.e.$). Positive selection is represented on the right and side of zero on the x -axis, negative selection on the left hand side.

There was no difference in the linear selection gradients (β_i) between hand- and open-pollinated treatments, suggesting a lack of pollinator-mediated selection (ANCOVA; Table 2). When comparing pollinator-mediated selection between the clipped and control groups, no difference was detected (ANCOVA; Table 2). Therefore, there was no interaction between pollinator- and herbivore-mediated selection.

In order to assess statistical power to detect pollinator-mediated selection in our study, we ran a post-hoc analysis that excluded the damage treatment, but included pollination treatment \times trait interactions for inflorescence height, number of inflorescences, flowering start time and petal length. This analysis revealed marginally significant evidence for pollinator-mediated selection on petal length ($F = 2.92$, d.f. = 111, $P = 0.09$), suggesting that a lack of power does not explain our inability to detect pollinator-mediated selection.

DISCUSSION

Our findings do not support the hypothesis that herbivores are indirectly modifying pollinator-mediated selection to influence

the evolution of floral traits and inflorescence architecture in purple loosestrife. Instead, we found that only herbivory imposed direct selection on floral and architectural traits, with plants in the clipped group under stronger selection to flower earlier and weaker selection to produce multiple inflorescences than those in the control group (Fig. 2).

In a review of 21 published studies, Johnson *et al.* (2015) concluded that the strength of herbivore-mediated selection frequently surpasses that of pollinator-mediated selection, and that these two forces commonly oppose each other in their effects on floral traits. Consistent with our findings, Johnson *et al.* (2015) reported that herbivores commonly select for fewer flowers. Very few studies in their review experimentally manipulated herbivory, making it difficult to draw general conclusions about whether herbivores had a direct or indirect effect on floral traits; however, given what we know from these existing studies, there appears to be support for both direct and indirect effects. Our study adds critical experimental support to the conclusion of Johnson *et al.* that when at least one type of selection is present, the strength of selection by herbivores on floral traits was often as strong or stronger than the strength of selection by pollinators.

No evidence for pollinator-mediated selection

Studies of European populations of purple loosestrife have found evidence for significant pollen limitation (e.g. Ågren, 1996; Ågren and Ericson, 1996). However, as far as we can tell, only one other study has examined pollen limitation in a North American population of purple loosestrife, finding that short-, but not mid- or long-styled flowers, exhibit significant pollen limitation (O'Neil, 1992). This finding was also supported in our study population (C. J. M. Thomsen, unpubl. data). Evidence for reduced pollen limitation in North American populations supports the hypothesis that purple loosestrife's success as an invader could be at least partially driven by its ability to co-opt local pollinators (Brown *et al.*, 2002). Because of the low level of pollen limitation in our study population, any preferences pollinators may have had for particular reproductive traits may have been obscured by an overall high level of pollinator visitation. Generally, traits that influence pollinator visitation, such as floral display traits, are thought to be under stronger selection in pollen-limited populations, while populations that are not pollen limited are more probably at an evolutionary equilibrium (Knight *et al.*, 2005; although see also Parachnowitsch and Kessler, 2010).

Evidence for herbivore-mediated selection

Plants in our clipped group exhibited significantly reduced inflorescence height and delayed flowering, but, somewhat counter-intuitively, also produced a greater number of flowers and inflorescences (Fig. 2; Table 1). This contradicts the findings of two previous studies reporting that although clipping *L. salicaria* meristems led to an increase in the number of inflorescences, total flower number and fruit set were unaffected (Venecz and Aarssen, 1998; Russell-Mercier and Sargent, 2015). Compensatory regrowth, where a boost in reproductive investment occurs following herbivory, is well documented both across angiosperms (reviewed in Agrawal, 2000) and in purple loosestrife specifically (Schat and Blossey, 2005). When compensatory regrowth of damaged plants leads to greater fitness than that of undamaged plants, overcompensation occurs (e.g. Paige and Whitham, 1987). Overcompensation may be more likely in our study system because it tends to be more common in those plants experiencing early-season herbivory (Maschinski and Whitham, 1989). Indeed, the evolution of overcompensation is possible if the associated benefits are not outweighed by the costs of apical dominance or herbivory itself (Agrawal, 2000). The findings from our study, in conjunction with similar studies on *L. salicaria* (Venecz and Aarssen, 1998; Russell-Mercier and Sargent, 2015), reveal that the compensatory response is highly variable and probably depends on plant maturity as well as environmental conditions.

The ability of *L. salicaria* to overcompensate may also provide insight into why we found that clipping significantly altered selection on inflorescence number. Consistent with previous findings (Hakes and Cronin, 2011), direct selection for more inflorescences existed in both treatment groups, although it was significantly weaker in clipped plants (Figs 1A and 2; Table 2). This suggests that, even though clipped plants produced more inflorescences than the controls (Table 1), there is some cost to regrowth that negates this benefit (i.e. there is a

trade-off between inflorescence number and fruit production in clipped plants). Further, clipped plants varied widely in how much fruit they produced, indicating that not all plants are able to compensate, perhaps because when resources are limited, shifting resources to the production of branches is risky.

Although all treatment groups experienced positive selection for plant height (Fig. 2), consistent with previous studies (O'Neil, 1997), we found no indication that damage drives selection on this trait (Figs 1B and 2; Table 2). Juenger and Bergelson (1997) also found evidence for positive selection on plant height in both clipped and control plants, with no difference between groups. In our study, the lack of additional selection imposed by herbivory on inflorescence height suggests that clipped plants were capable of compensating such that height differences between treatment groups were not biologically relevant to cause differences in selection (Fig. 2; Tables 1 and 2).

Selection for earlier flowering was evident in the clipped but not the control group (Figs 1C and 2; Table 2). Although flowering phenology is often appreciated as a tolerance trait that allows plants to recover from or avoid early- or late-season herbivory, few studies have directly quantified selection via compensatory regrowth on flowering time. Because our herbivory treatment, which coincided with the timing of natural herbivory, occurred prior to flowering, selection for earlier flowering is not a means to avoid herbivory. Instead, because *L. salicaria* is able to flower for most of the summer, and because meristem damage delays flowering time (Fig. 2; Table 1), selection for earlier flowering probably represents selection for a more rapid compensatory regrowth response, allowing for extended flowering across the season, and perhaps a greater potential for compensation.

Floral traits as a function of plant defence

We found, consistent with Johnson *et al.* (2015), that selection mediated by our herbivory treatment was stronger than pollinator-mediated selection for certain reproductive traits, including inflorescence number and flowering time (Fig. 2). We suggest that herbivores in this system are not driving floral trait selection through the direct effects of feeding, but rather through selection for plant defence causing overcompensation. Our results underscore the importance of considering the multifunctionality of floral traits, which may be simultaneously involved in both plant defence and pollinator attraction. Our conclusion, that herbivory imposes greater selection on plant floral traits than pollinators, may be especially true in populations with low or negligible pollen limitation. Once a plant population has successfully tapped into a specific community of pollinators, pollinator-mediated selection could become less important than selection imposed by other agents (Strauss and Whittall, 2006). Because *Neogalerucella* herbivores have been present in Ontario ecosystems for < 23 years (Lindgren *et al.*, 2002), selection via this relatively novel interaction is probably not yet at equilibrium, and may therefore be easier to detect. Indeed, M. Stastny and R. D. S. Sargent (unpubl. res.) recently reported on the rapid adaptation of tolerance traits in Ontario populations of purple loosestrife in response to *Neogalerucella*. Moreover, if herbivore pressure is of sufficient strength to alter the trait-fitness relationship created by pollinators, it could

mask the pressures imposed by pollinators and alter the total selection experienced by a given trait (Gómez, 2003, 2008; Vanhoenacker *et al.*, 2013). Therefore, although we did not find that imposing herbivory modified pollinator-mediated selection, this question may still be relevant in populations with greater pollen limitation (e.g. in Europe).

Furthermore, while we did not find evidence that imposing herbivory altered selection through female function, selection through male function may still be of importance. Sub-components of fitness are often correlated with whole plant fitness and can provide good estimates of selection; however, the strength of selection can sometimes vary depending on the fitness component used (Kingsolver *et al.*, 2012). Some evidence suggests that selection for large or small floral display may be stronger in male than in female fitness components (Ehlers *et al.*, 2002; Lau *et al.*, 2008), although it may be context dependent (Ashman and Morgan, 2004). While pollinator-mediated selection was not evident for the traits we studied, future studies should consider whether there is evidence for selection via male function.

A possible criticism of our design is the use of an artificial, rather than natural, herbivore treatment. This decision, commonly applied in studies of herbivory (Strauss and Agrawal, 1999), allowed us to control tightly the type of damage applied to our treatment plants (i.e. ensure uniform meristem damage). However, one concern is that plants may respond differently to artificial herbivory than they would to natural herbivory (Baldwin, 1990). Tiffin and Inouye (2000), in a review, point out that both artificial and natural herbivory treatments have benefits as well as drawbacks in terms of their suitability for experiments on herbivore defence traits. In our case, the fact that purple loosestrife has been shown to respond in a phenotypically similar way to artificial and natural clipping (Schat and Blossey, 2005) and the ability to control for possible microsite differences in environmental factors justified the use of simulated, rather than natural, herbivory. Furthermore, improving our understanding of selection imposed by artificial herbivory could have economic implications, such as in agricultural (e.g. cutting of hay) and horticultural (e.g. clipping of flowers or grass) settings.

Phenotypic selection analysis is an important tool for quantifying selection. While selection studies provide important inroads for predicting the outcomes of plant–pollinator interactions, it is also crucial to consider the underlying network of interactions that multiple agents of selection can have on plants, and even on one another. Studying selection on invasive species such as *L. salicaria* can also help us understand how a plant's evolutionary response to herbivory may feed back into its interactions with pollinators, which could influence the outcome of biological control programmes. Multitiered experimental designs such as the one presented here allow us to elucidate the pathways through which mutualists and antagonists alter the evolutionary trajectory of plant traits, either through interactions or (in)directly via individual effects.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of Table S1: correlations between

select floral display traits within *Lythrum salicaria* plants in the presence or absence of herbivore-simulated damage.

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