The relative roles of herbivore- and pollinator-mediated selection on the evolution of floral display in the invasive plant, *Lythrum salicaria*

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ABSTRACT

Studies assessing the evolution of plant traits frequently focus on pollinators as the primary drivers of floral trait evolution. However, herbivores can also play a role, and, under some circumstances, may even impose stronger selection on floral display than pollinators. This is especially true when the traits under selection are linked to anti-herbivore defense. Here I describe a study in which I quantified the relative role of herbivores and pollinators in selection for floral traits in the North American invasive plant, purple loosestrife (*Lythrum salicaria*). Because *L. salicaria* responds to leaf-chewing herbivores by producing compensatory tissue growth, and this in turn alters the architecture of the floral display, I further tested whether herbivores can indirectly modify pollinator-mediated selection through this pathway. Using a split-plot design, I measured pollen limitation and reproductive output in experimentally manipulated plants in the presence and absence of simulated herbivore damage in order to quantify the effects of damage and pollination on natural selection for floral display. My results showed that damage significantly increased direct selection (β_i) for earlier flowering time and decreased selection on the number of inflorescences, even more than pollinators did. Because damage did not modify pollinator-mediated selection for floral display traits, the selection imposed by herbivores is likely only having direct effects. My findings demonstrate the importance of considering multiple agents of selection and their potential interactions when quantifying natural selection in a study system. In particular, it is important to consider that the agent most frequently studied may not be imposing the brunt of selection.

RÉSUMÉ

Les études évaluant l’évolution des traits végétaux se concentrent fréquemment sur les pollinisateurs en tant que déterminants principaux de l’adaptation des traits floraux. Par contre, les herbivores peuvent aussi jouer un rôle, voire même imposer une sélection plus forte sur la composition florale que les pollinisateurs, spécialement lorsque les traits en question sont liés aux défenses anti-herbivores. Dans l’étude présentée, j’ai quantifié le rôle relatif qu’ont les herbivores et les pollinisateurs dans l’altération *directe* de la sélection naturelle des traits floraux chez une plante envahissante en Amérique du nord, la salicaire commune (*Lythrum salicaria*). Cette plante réagit aux herbivores en produisant une croissance tissulaire compensatoire, ce qui a pour effet de modifier l’architecture de la composition florale; j’ai donc testé si les herbivores peuvent *indirectement* modifier la sélection par entremise de pollinisateurs à l’aide de ce phénomène. En utilisant un dispositif expérimental en tiroirs (« split-plot design »), j’ai mesuré la limitation pollinique et le rendement reproductif entre des plantes expérimentalement manipulées en présence et en absence de dommages d’herbivorie simulée. Ce dispositif m’a permis de quantifier les effets des herbivores et des pollinisateurs sur la sélection naturelle de la composition florale. Mes résultats ont démontré que l’herbivorie simulée a augmenté significativement la sélection *directe* (b_i) pour un temps de floraison plus tôt et a diminué la sélection pour le nombre d’inflorescences, encore plus que la limitation pollinique. Puisque l’herbivorie simulée n’a pas modifié *indirectement* la sélection par entremise de pollinisateurs pour les traits de composition florale, l’herbivorie naturelle entraîne vraisemblablement des effets *directs* seulement. Mes découvertes démontrent l’importance de prendre en considération plusieurs agents de sélection naturelle et leurs interactions potentielles lorsqu’on quantifie la sélection naturelle dans un système. Particulièrement, il est important de considérer que l’agent le plus fréquemment étudié n’est pas nécessairement celui qui influence le plus la sélection naturelle.

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CHAPTER 1: Project Overview

1.1 Natural Selection for Floral Traits

Plants, like all organisms, evolve in response to the positive and negative selection pressures encountered within their environment. Aspects of the environment that contribute to plant adaptations include interspecific and intraspecific competitors, mutualists (e.g., pollinators, seed dispersers), antagonists (e.g., herbivores, seed predators), and abiotic factors (e.g., temperature, altitude, light and nutrient availability). Together these factors drive natural selection and have been implicated in the evolution of a suite of plant traits. For example, the expansion of a plant species in a new geographic niche can impose selection for increased competitiveness (Leger 2008), while water availability can impose selection on leaf size and water-use efficiency (Dudley 1996). There are numerous studies identifying the agents of selection that prompt adaptations in all kinds of plant traits, but especially in the context of complex and intricate floral display traits, selection has been particularly well explored.

Pollinators, the main vehicles through which pollen is transferred among plants, have long been suspected to be key drivers of floral trait evolution (Darwin 1877, Pijl 1960). This is unsurprising considering that pollinators show preferences for flower size (e.g., Campbell 1989, Conner and Rush 1996), flower number (e.g., Vaughton and Ramsey 1998, Thompson 2001), as well as floral nectar and pollen rewards (e.g., Neiland and Wilcock 1998). Even the height of a floral display can affect the pollinator visitation rate (Cayenne Engel and Irwin 2003). Contributing to our knowledge of plant-pollinator interactions is a growing body of literature that has explicitly quantified pollinator-mediated selection on flower size (Nattero et al. 2010, Sletvold and Ägren 2010), flower number (Sandring and Ägren 2009, Parachnowitsch and Kessler 2010, Sletvold and Ägren 2010, Sletvold et al. 2010), floral height (Sletvold and Ägren 2010, Sletvold et al. 2010, Sletvold et al. 2013), and phenology (Sandring and Ägren 2009). This body of research has amply demonstrated that pollinators likely drive floral trait evolution. Recently, our understanding of plant-pollinator interactions has been further enhanced by research indicating that pollinators are not simply selecting for large, visible flowers with high nectar availability. Indeed, it has been shown that pollinators can select for flower shape (e.g., petal width) (Gómez et al. 2008), and flower colour, enough to drive rapid reproductive isolation between closely related plants (Schemske and Bradshaw 1999). Because not all pollinators share the same preferences, different pollinator species can impose differential selection on floral traits. This phenomenon is illustrated by pollination syndromes, in which pollinators of similar functional capacity tend to drive selection towards similar floral traits (Herrera 1996, Fenster et al. 2004, Ollerton et al. 2009).

While we know that pollinators play an important role in the evolution of floral traits, more recent research has begun to focus on whether we can detect other agents of selection (e.g., Galen and Cuba 2001, Caruso et al. 2010, Kolb and Ehrlén 2010, Samis et al. 2012, Sletvold et al. 2014). For example, winter temperature and precipitation varies sufficiently with longitude that it is suspected to drive genetic variation for flowering time along longitudinal clines (Samis et al. 2012). In addition, seed predators have been implicated as drivers for selection of floral traits like inflorescence height and size (Kolb and Ehrlén 2010). Herbivores, too, can drive floral trait evolution, particularly those floral traits that allow the targeted plant to defend itself. Florivores (i.e., herbivores that consume floral tissues), for instance, have been shown to impose selection for earlier flowering time (Sletvold et al. 2014), as well as a reduced number of open flowers (Lay et al. 2011). Through studies such as those described above, we are beginning to see evidence for the existence of multiple agents of selection acting on floral traits.

1.2 Interspecies Selection Dynamics

A major goal of evolutionary ecology has been to try to understand through what direct and indirect pathways species may be influencing one another. The interactions between plants and their competitors, mutualists, and antagonists provide the foundation for the evolution of a number of plant traits. Furthermore, a single plant trait often experiences selection from a number of agents and therefore the evolution of such traits may come from the culmination of conflicting or reinforcing pressures imposed by several forces (Strauss and Whittall 2006).

Numerous examples suggest that more than one agent can act on a single trait, and often their combined effects can vary considerably across geographic and temporal scales (Rey et al. 2006). Of the many agents responsible for floral trait evolution, pollinators and herbivores best exemplify how natural selection on plant traits is a dynamic process. Because herbivore damage can often decrease flower size and number, its effects can correspond to decreased pollinator visitation (Lehtilä and Strauss 1997, Mothershead and Marquis 2000, Barber et al. 2012). In studies where pollinators and insect herbivores both prefer large flowers or a high quantity of flowers, conflicting selection often arises as plants aim to attract pollinators and deter herbivores (Cunningham 1995, Ashman et al. 2004a). Gómez (2003) showed that a preference for tall plants by both pollinators and ungulate herbivores creates a similar situation where the evolution of a plant trait could result in a compromise between competing pressures. This antagonistic selection is even more apparent when the associated pollinator and herbivore are the same species (Adler and Bronstein 2004). For example, Adler and Bronstein (2004) suggest that selection for moth-attracting floral nectar in *Datura stramonium* is likely conflicting because these moths both pollinate and deposit their herbivorous offspring on the plant.

Although the pollinator-herbivore selection dynamic is often seen as conflicting, this is not always the case; Sletvold et al. (2014) found that while selection for flowering time can be conflicting, it can also be reinforcing depending on the timing of peak activity of pollinators and herbivores. Armbruster et al. (1997) provide an additional exception to this trend, finding that herbivores impose selection for a defensive chemical that also attracts pollinators. Thus, whether selection imposed by herbivores and pollinators is reinforcing or not can depend on whether a plant defense conveys some benefit to pollination. It is of value to consider through which processes (e.g., plant defenses) each agent may influence a plant, since these will precisely determine how selection materializes.

1.3 Consequences of Herbivory

By evaluating the effects that herbivores have on plant appearance and/or physiology, we can resolve how they impose selection. Immediate consequences of leaf, flower, fruit or root tissue damage are frequently cited as decreased plant biomass, number and size of flowers, as well as reduced fruit or seed production. The net effect of herbivory is therefore negative, and can reduce the chance of survival and fitness of the target plant (e.g., Marquis 1984). As discussed previously, these immediate short-term consequences can cause plants to evolve long-term defensive strategies. If these defenses are associated with, or alter floral display in any way, they may further alter pollinator interactions as well.

A common adaptive response of plants to herbivory is the evolution of increased defensive capacity. Defensive strategies can take the form of both *resistance* (i.e., traits that deter or reduce herbivory) and *tolerance* (i.e., traits that reduce the fitness impacts of herbivory) (Rosenthal and Kotanen 1994). Because both resistance and tolerance traits can alter the physical or physiological properties of a plant, they can also affect how pollinators interact with it. Resistance traits have been increasingly linked to pollinator behaviour. In particular, toxic nectar is an often-cited example of how chemical anti-herbivory defences could pose an ecological cost by contaminating nectar and deterring pollinators (Strauss et al. 1999, Adler and Irwin 2005).

In addition to resistance traits, the effect of tolerance traits on pollinator-plant interactions is an interesting avenue of research that requires more exploration. Some examples of tolerance traits include compensatory growth, flowering phenology, photosynthetic rate, and growth rate (Trumble et al. 1993, Strauss and Agrawal 1999, Stowe et al. 2000). All of these traits have the capacity to modify plant-pollinator relationships because they can alter when pollinators visit and how attractive plants are to them. Compensatory growth is a tolerance response of particular interest because of the way it can alter floral architecture. As apical dominance is lost, compensatory growth can lead to increased branching of inflorescences, and overall alterations in the structure of the floral display (McNaughton 1983, Trumble et al. 1993, e.g., Sadras 1996, Schat and Blossey 2005). Because this tolerance response has been linked to a shift in pollinator visitation (Sharaf and Price 2004, Russell-Mercier 2013), it is worthwhile to determine whether pollinator-mediated selection is consequently altered.

1.4 Thesis Objectives

The goal of my thesis is to explore the selection dynamic between herbivores and pollinators as it relates to the evolution of plant floral display. I used a multi-tiered approach that combines the methods of Lande and Arnold (1983) with experimental manipulations in order to determine the relative role of pollinators and herbivores on selection for floral traits. In doing so, I elucidated whether herbivores alter those floral traits that are typically the target of pollinator-mediated selection. Given that the architecture of plant floral display can vary considerably following herbivory, particularly through compensatory growth, I further explored whether herbivores can modify pollinator-mediated selection for floral traits by altering pollinator behaviour. Because selection has only rarely been quantified in my study system, *Lythrum* *salicaria* (i.e., O'Neil 1997), my thesis expands our understanding of selective agents in this system.

Chapter 1 is a literature review of the agents of selection that drive the evolution of floral traits, and how these agents may interact with one another. In Chapter 2 I present my thesis research, in which I specifically address the role that herbivores and pollinators have in imposing selection for floral traits in my study system. Finally, Chapter 3 serves to tie my thesis together with suggestions on how to design future studies so that we can mindfully approach some of the prevailing problems in evolutionary ecology.

CHAPTER 2: Thesis

2.1 Introduction

Identifying the factors that drive selection for floral display traits remains a critical challenge in evolutionary ecology (Ashman and Morgan 2004, Strauss and Whittall 2006). Because pollinator attraction is closely tied to fitness in animal-pollinated plants, it is often reasoned that pollinators are the primary drivers of floral adaptations (Darwin 1877). Indeed, pollinator-mediated selection has been well documented in a number of species (e.g., Benitez-Vieyra et al. 2006, Fishman and Willis 2008, Parachnowitsch and Kessler 2010, Sletvold and Ägren 2010). Yet, focusing solely on pollinators can be problematic when selection on floral display is driven by a number of other factors, including abiotic resources and herbivores (Strauss 1997, Strauss and Irwin 2004, Strauss and Whittall 2006). In order to more accurately predict the evolutionary trajectory of floral display, particularly under changing environments, multiple agents and their interactions should be taken into consideration.

When multiple agents act on the same trait, this gives rise to conflicting or reinforcing selection that determines the strength, direction, and extent to which that trait evolves (Herrera 2000, Gómez 2003, Strauss and Irwin 2004). Examples of how multiple agents can influence the same floral traits are often apparent through pollinator-herbivore dynamics. Selection by herbivores is frequently thought to oppose pollinator-mediated selection, especially in situations where flowers or seeds are consumed (Galen 1999, Ashman et al. 2004a, Cariveau et al. 2004, Parachnowitsch and Caruso 2008). Yet, selection by herbivores on floral traits has rarely been quantified concurrently with pollinator-mediated selection (Gómez 2003, Gómez 2008, Bartkowska and Johnston 2012). Doing so would allow us to determine the relative role of herbivores and pollinators on the evolution of floral traits, while also elucidating whether these agents are indirectly interacting with one another.

It seems clear that herbivory should indirectly affect pollinator-mediated selection in systems where herbivore-induced damage changes floral attractiveness. This indirect effect may arise when there is immediate damage to flowers, but may additionally arise depending on how the plant responds to damage. Tolerance traits, i.e., defensive traits that reduce the negative fitness or survival consequences in response to damage (Rosenthal and Kotanen 1994, Mauricio et al. 1997), can include shifts in flowering phenology, nutrient uptake, photosynthetic rate, or compensatory growth (Trumble et al. 1993, Strauss and Agrawal 1999, Stowe et al. 2000). Compensatory growth, which arises when leaf damage to apical meristem tissue triggers the growth of dormant axial buds, is particularly relevant to the expression of floral display because it tends to alter floral architecture by increasing inflorescence branching (Inouye 1982, McNaughton 1983, Paige and Whitham 1987, Trumble et al. 1993). This may mean that herbivores can impose selection on floral display (Marquis 1996), but it may also implicate them in having important indirect effects as well, by altering pollinator visitation to plants (Russell-Mercier 2013). Here I describe a study designed to ask: what are the relative roles of herbivores and pollinators in selection for floral display, and do these agents indirectly interact through plant compensatory growth?

The relative influence of multiple agents can be quantified using the phenotypic selection method (Lande and Arnold 1983) to compare selection strength between experimentally modified and control groups (Wade and Kalisz 1990). A few studies have compared phenotypic selection in pollen-supplemented plants to that of un-supplemented controls in order to estimate the strength of pollinator-mediated selection on a variety of floral traits (e.g., Fishman and Willis 2008, Parachnowitsch and Kessler 2010, Sletvold and Ägren 2010, Bartkowska and Johnston 2012). If supplemented plants achieve a higher seed output than control plants, this suggests that there is insufficient quantity or quality of pollen being received in the plant population, i.e., pollen limitation (Ashman et al. 2004b). The presence of pollen limitation suggests that any preferences pollinators may have for particular floral display traits should generate pollinator-mediated selection (Ashman and Morgan 2004). If compensatory growth alters floral display in a way that makes a phenotype robust to pollen limitation, then selection should favour that phenotype.

Using a split-plot design combining a pollen supplementation experiment with experimental manipulations of simulated herbivory, I attempted to quantify the relative role of pollinators and herbivores, while assessing the effects of herbivory on phenotypic selection for floral display traits in purple loosestrife (*Lythrum salicaria*). *Lythrum salicaria* serves as a good system to address this question because it uses compensatory growth to respond to apical meristem damage by specialist leaf-chewing herbivores, *Neogalerucella* spp. (Schat and Blossey 2005, Russell-Mercier 2013). Because *Neogalerucella* typically inflicts a single bout of early-season feeding, this allows sufficient time for the compensatory growth strategy to manifest prior to usual flowering (Maschinski and Whitham 1989, Strauss and Agrawal 1999, Agrawal 2000). Further, because *Lythrum salicaria* is an invasive plant in North America, with an established biological control agent (*Neogalerucella* spp.), it also serves as a relevant system to understand how a plant's evolutionary response to herbivore exposure may feed back to its interactions with pollinators, and ultimately influence the success of the biological control program. Using phenotypic selection analysis, I quantify selection imposed by herbivores and pollinators on floral display traits in the context of compensatory growth. In particular, I ask: **(1)** does the change in floral display following herbivory (compensatory growth) alter pollen limitation? **(2)** what is the relative role of herbivory and pollination in imposing selection for floral display traits? and **(3)** does herbivory modify pollinator-mediated selection for floral display traits?

2.2 Methods

Study System

Lythrum salicaria L. (Lythraceae), or purple loosestrife, is a wetland perennial plant native to Europe, but has been found in North America since the early 1800s (Stuckey 1980, Thompson et al. 1987). Although *L. salicaria* can spread clonally by rootstock, its expansion throughout most of mainland U.S. and into southern Canada is mainly attributed to its ability to produce up to 2-3 million seeds per plant that disperse easily along waterways and roadsides (Thompson et al. 1987). In southeastern Ontario, where my study was located, *L. salicaria* produces showy purple flowers, arranged on one or more racemes, from mid-July through August

(Venecz and Aarssen 1998). Optimally, individual flowers are pollinated on the first day of opening (Waites and Ägren 2006), primarily by bumblebees (O'Neil 1999). *Lythrum salicaria* is self-incompatible, and it promotes outcrossing through tristyly, a reproductive system in which an individual plant has one of three style morphs (long-, mid- or short-styled). Pollination is maximized when the stigma of each style morph receives pollen from a size-matched anther (Darwin 1877, O'Neil 1994).

In Europe, two of *L. salicaria*’s most effective herbivores are the host-specific leaf-chewing beetles, *Neogalerucella calmariensis* and *Neogalerucella pusilla* (Blossey 1995b, Blossey 1995a). As plants actively grow in the spring, *Neogalerucella* spp. adults deposit their eggs and the resulting larvae feed primarily upon apical and axillary meristem tissue. Loss of apical dominance induces compensatory regrowth through axial branching which changes the floral architecture of the maturing plant (Schat and Blossey 2005). Due to the host-specificity and effectiveness of these herbivores in their native range, *Neogalerucella* spp., in addition to a root-feeding weevil (*Hylobius transversovittatus*) and flower-feeding weevil (*Nanophyes marmoratus*), were released within Ontario in the early to mid 1990s as part of a purple loosestrife biological control program (Lindgren et al. 2002).

Field Site

I conducted my experiment in a lakeside marshland located at Queens’ University Biological Station in Elgin, Ontario, Canada (44°34’08.7”N, 76°19’13.6”W). No known biological control releases have occurred at this site, however, two of the specialist beetles released, *N. calmariensis* and *N. marmoratus*, have successfully colonized this location (pers. obs.), presumably through self-dispersal. This study population of *L. salicaria* covers approximately 7520 m^2 in area and has a density of approximately 8 plants per metre. The frequency of long-, mid-, and short-styled morphs within this population is approximately equal (34 ± 4% long, 32 ± 4% mid, 34 ± 4% short).

Simulated Herbivory

In May 2013, 178 stems were randomly selected from mature rootstocks that were at least 1 m apart to ensure independent genotypes (Haldane 1936). In some circumstances, a single rootstock may produce more than one flowering stem; due to the difficulty of defining which stems belong to a single genotype, the selected stem was chosen to represent an entire plant. During the period of selection, plants were in their active growth phase and averaged a height of 27.8 ± 1 cm (mean ± SE), approximately 24% of the average end-of-season maximum height.

Each plant was covered with a purpose-built sleeve composed of fine, white mesh (Rose e Dee Ltd. ‘no see-um’ netting) that prevented intense feeding and egg deposition by herbivores and so that damage levels could be standardized. Prior to covering, each experimental plant was carefully inspected for herbivores. Although herbivores were not fully active at this time, there was evidence of feeding on some plants (no greater than 5% standing leaf damage); therefore, only plants that lacked apical meristem tissue damage were chosen and any herbivores were removed. After bagging, natural herbivory levels were monitored in the population so that the application of the simulated herbivory treatment could be timed with the emergence and peak feeding time of *N. calmariensis* larvae.

In early June, sleeves from all plants were opened, and each plant was randomly assigned to one of two herbivory treatments reflecting two levels of herbivory: damaged (simulated damage to the apical meristem) and control (no simulated damage). Simulated herbivory was chosen because it allows the plant to express a tolerance phenotype that is more representative of its genotype (Tiffin and Inouye 2000). Estimates of tolerance under natural herbivory can be noisy because they cannot account for individual variation in plant resistance traits (e.g., chemical toxins), whereas simulated treatments standardize damage independently of resistance level (Stowe et al. 2000, Tiffin and Inouye 2000). While there is some question of whether simulated damage can truly mimic natural herbivory, research suggests they are especially similar when quantifying tolerance, growth and reproductive traits (Lehtilä and Boalt 2004). In some species, including *L. salicaria*, comparing natural and simulated apical meristem damage resulted in no detectable difference in traits associated with compensatory growth (Gavloski and Lamb 2000, Russell-Mercier 2013).

To simulate larval feeding of the apical new growth tissue I used microdermal scissors to cut approximately 2 cm off the tip of the apical meristem from those plants assigned to the damage treatment. Following treatment application, sleeves were replaced on both damaged and control plants and damaged plants were checked a week later to verify that the apical damage treatment was successful (i.e., growth of the apical meristem was discontinued). All plants were subsequently visited each week to confirm that sleeves were secure; in rare situations, when herbivores managed to circumvent the sleeves and cause apical damage to control treatment plants, the plant was reassigned to the damage treatment (N = 8). In July, all experimental plants were reassessed for general leaf damage and determined to have an insubstantial amount (less than 5%).

Each week, plants were assessed for start and end of flowering to estimate flowering phenology. Once flowering began, sleeves were removed to facilitate the application of pollen supplementation treatments. Fortunately, this coincided with a period of reduced *N. calmariensis* activity because most beetles were pupating.

Pollen Limitation

Pollen supplementation experiments compare natural levels of pollination to artificially saturated levels in order to determine if pollen limitation is occurring (i.e., whether pollinators are providing a sufficient quantity or quality of pollen to their mutualist host) (Ashman and Morgan 2004). Application of open pollination (OP) and hand pollination (HP) treatments (described below) spanned a two-week period from late July to early August, corresponding to peak *L. salicaria* flowering time. Fourteen plants from the initial sample were excluded from pollen supplementation because they did not flower in time.

During the supplemental pollination treatment period, a random subset of treatment plants were selected and supplemented each day. On average, *L. salicaria* plants in this population produced over 980 flowers across the season, making it impractical to supplement all flowers on every plant. Instead, pairs of open flowers on study plants were randomly selected to be part of the experiment. Flower pairs consisted of two flowers on adjacent nodes at the same height (±1 cm) of a given inflorescence. Each flower was tagged with coloured polyester thread for later identification. One flower per pair was then randomly chosen to be the supplemented flower, while the other flower was left for open pollination. Up to four pairs of flowers were selected per plant (N = 429 pairs across all plants), depending on the availability of open flowers on each pollination day. Because previous research suggests that shunting of resources towards HP flowers could minimize fruit set in OP flowers, I controlled for this by simultaneously tagging open flowers on untreated inflorescences for a subset of all experimental plants. Comparing the number of seeds per fruit (i.e., seed set) between the controls and OP flowers revealed no significant difference ($t = -1.37$, $df = 69$, $P = 0.1747$), which is consistent with previous findings in *L. salicaria*. (Ägren and Ericson 1996)

Flowers in the OP treatment were not manipulated and therefore exposed to the natural pollination environment while those in the HP treatment received additional pollen from non-experimental donor plants in the study population. Flowers were removed from 2-5 donor plants that were (a) of optimal morph compatibility (possessed stamen that were size-matched to the recipient style) and (b) within 10 m of the experimental plant. To apply pollen, stamens were removed from donor flowers and the recently dehisced anthers were rubbed directly on the recipient stigma until it was visibly saturated. Because it is difficult to access short-morph stigmas using this method, tweezers were used to slice the side of the corolla tube (as described by O'Neil 1992).

On the day of pollen supplementation, a series of floral display measurements were collected from the study plant as the target traits for quantifying pollinator-mediated selection. To characterize attractiveness, the number of flowering inflorescences, inflorescence length, total number of recently opened flowers, and inflorescence height (tallest point of the plant) were measured.

Approximately 6-8 weeks following the application of pollen supplementation treatments, mature fruits were collected. In order to gather final measurements of floral display architecture, and fruit set, all study plants were cut to ground level and bagged at the end of September. Because of the difficulties associated with counting the large number of seeds per fruit (up to 150 seeds), I used a seed counting program implemented in MATLAB 8.0 (The MathWorks Inc. 2012) to automatically detect and count seeds within photographs.

Across the field season, 19.7% (35 plants) of the initial study population was eliminated as a result of beaver, deer, or weather-related damage. In all, 49 of 429 target flower pairs were lost through plant or fruit damage. Missing fruit, which were considered a result of fruit abortion, were also common; in total 28.1% of fruit were aborted in both treatments (90 HP fruit lost, 124 OP fruit lost). Aborted fruit were retained in the data analysis as a component of pollen limitation (see section *2.3 Statistical Analysis*). Overall, fruit count and seed set were successfully retrieved for 147 plants (78 damaged, 69 control) from the initial 178 plants that were selected.

2.3 Statistical Analysis

Effects of Plant Damage

To ensure that simulated herbivory successfully altered floral display attractiveness, floral phenology and reproduction, I compared final plant measurements of various traits between damage treatments using a one-way analysis of variance (ANOVA), or, when appropriate, the Mann-Whitney U test, which is the non-parametric alternative. Traits associated with floral display included: number of flowers produced (number of fruit + number of abortion scars), number of open flowers (measured at the time of pollen supplementation), number of axial inflorescences produced, total inflorescence length (sum of infructescence lengths per plant), petal length (measured at pollen supplementation), flower density (number of flowers produced per total inflorescence length), and inflorescence height. Phenology traits included start and end of flowering. Reproductive traits included the number of seeds per fruit (average of OP-appointed flowers), number of fruit per plant, and the percentage of aborted fruit (number of abortion scars ÷ number of flowers x 100). Applicable transformations for ANOVA-tested traits are found in Table 1 of the *Results*.

Pollen Limitation

To assess pollen limitation, the difference in seed set (number of seeds per fruit) was calculated for each pair of flowers as $\Delta \mathit{Seed\ Set} = \mathit{Seed\ Set}_{\mathrm{HP}} - \mathit{Seed\ Set}_{\mathrm{OP}}$. A difference significantly greater than zero indicates pollen limitation, while a difference of zero indicates an absence of pollen limitation. HP or OP fruit that were aborted were assigned a seed count of zero rather than excluded from the analysis because fruit abortion is generally considered to be a reflection of the quality or quantity of pollen received (Stephenson 1981, Guittian 1993, Pflugshaupt et al. 2002). To assess the difference in pollen limitation between damage and control treatments, a linear mixed-effects model (LMM), with plant as a random-effect, was fit via restricted maximum likelihood (REML). Because previous studies on *L. salicaria* have found evidence for differences in pollen limitation among style morphs (O'Neil 1992, Ägren 1996, Ägren and Ericson 1996), a post-hoc test that included morph in the fixed effects was run to determine potential sources of variation in pollen limitation:

seed difference = damage treatment + morph + pair (plant (damage treatment))

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, I also confirmed that a zero-inflated generalized linear mixed model (GLMM) comparing raw seed counts between HP and OP flowers yields the same qualitative results as the LMM. Further, using two separate models: an LMM for seed counts (excluding ‘zero’ values), as well as a binomial regression GLMM for fruit set (1/0 binary of target fruit set vs target fruit aborted), yielded the same conclusions.

Selection Analyses

Phenotypic selection analyses were conducted using the estimation methods described in Lande and Arnold (1983), in which selection coefficients are extracted from a regression of relative fitness (individual fitness value ÷ treatment mean fitness) versus standardized trait values ([individual trait value – treatment mean value] ÷ treatment standard deviation). Both total selection and direct selection were estimated. Total linear selection, which quantifies selection differentials (s_i) through a univariate (single-trait) model, determines the overall selection experienced by the target trait without taking into account its correlation with other traits. In contrast, direct linear selection, which uses a multivariate (multi-trait) model to calculated selection gradients (β_i), identifies the level of selection by excluding between-trait correlations between those traits included in the model. Direct selection is therefore a more accurate estimate of how much selection is specifically acting upon a target trait. In addition to determining the linear selection gradient and selection differentials for each target trait, I further included a quadratic term to estimate nonlinear selection (γ_{ii}); selection is calculated by doubling the regression coefficients from the quadratic terms (Stinchcombe et al. 2008). Cross-product terms (γ_{ij}) were excluded in all models to reduce model complexity, however, raw correlations between traits were calculated separately.

Due to the nature of the experimental design, two separate selection analyses were needed to quantify pollinator and damage effects. Because a subset of flowers were pollinated during a snapshot of the total flowering period, the selection analysis used to quantify pollinator-mediated selection used separate relative fitness values (seed set averaged per plant) than the selection analysis used to quantify selection imposed by damage (fruit count; square-root transformed). Because the analysis using seed set does not include total fruit count for a given plant, the raw selection differentials (s_i) and gradients (β_i) extracted from this analysis are not interpretable as true selection measures; however, pollinator-mediated selection (Δs_i and $\Delta\beta_i$) are interpretable because they strictly represent a comparison of differences between HP and OP seed sets. To resolve this issue, I ran a separate selection model testing pollinator-mediated selection that used an estimate of seed count for the total plant ($\textit{Seed Set}_{OP}$ x $\textit{Fruit Set}_{OP}$ x *Fruit Count Per Plant*). This analysis produced the same conclusions; I therefore only show results from the seed set model.

Of the plant traits measured (see section *2.3 Statistical Analysis: Effects of Plant Damage*), only a subset were chosen for the analyses in order to limit the influence of correlation among traits on the results (Lande and Arnold 1983, Endler 1986, Mitchell-Olds and Shaw 1987). In particular, I chose to include the number of inflorescences, inflorescence height, and flowering start date because (a) they are closely associated with compensatory growth, and (b) there has been some evidence suggesting that the latter two are experiencing selection in *L. salicaria* (O'Neil 1997). Further, multicollinearity was assessed by quantifying the variance inflation factor (VIF); because VIF values were all below 2, collinearity between the selected traits were not considered to be an issue (Quinn and Keough 2002).

Because the two selection analyses used separate measures of relative female fitness (see above), it made sense to then use separate trait measures. In the analysis measuring herbivore-mediated selection, trait values for number of inflorescences and floral height were taken from end-of-season measurements. In contrast, because the pollen supplementation treatment was conducted during a single snapshot of the entire flowering period, and therefore represents a snapshot of total seed production, the analysis of pollinator-mediated selection incorporated measurements that occurred at the time of supplementation, rather than at the end of the season. Consequently, the pollinator-mediated effects of herbivory on flowering phenology could not be accurately assessed and were therefore omitted from the pollinator-mediated selection model.

I used analysis of covariance (ANCOVA) to assess pollinator-mediated selection and the indirect effect of damage on pollinator-mediated selection. The dependent variable, relative fitness (seed set), was modeled with the independent variables: pollination, damage, each standardized trait (number of inflorescences, plant height), pollination x trait, damage x trait, and pollination x damage x trait. Plant was included as a random effect.

Similarly, to test the effect of herbivory on selection gradients, a separate ANCOVA was used. The dependent variable, relative fitness (fruit count), was modeled with the independent variables: damage, each standardized trait (number of inflorescences, plant height, flowering time), and damage x trait. Plant was included as a random effect. Selection gradients in this model are illustrated for each target trait with 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 (Figure 3). Because trait values were standardized within each treatment, it is difficult to gauge from Figure 3 whether the phenotypic trait distributions are sufficiently overlapping to provide a biologically meaningful comparison (Chenoweth et al. 2013). Phenotypic distributions for each of my target traits were determined to be almost entirely overlapping and therefore is not considered an issue.

Because morph was included in a post-hoc model testing pollen limitation (see *2.3 Statistical Analysis, Pollen Limitation*), additional ANCOVAs were also used to test whether there was an interaction effect of the herbivore and pollination treatments with morph; because morph as a covariate did not improve model fit, it was excluded.

All statistical analyses were performed with R version 3.0.2 (R Foundation for Statistical Computing 2013). All selection analysis and pollen limitation models met the criterion of homoscedasticity, and possessed unimodal distributions, lacking skew (Quinn and Keough 2002). Selection models were fit via maximum likelihood (ML) in order to test the significance of the effects using likelihood ratio tests.

2.4 Results

Effects of Plant Damage

Simulated herbivory had notable and significant effects on the floral display of the damaged plants (Table 1). Overall, damaged plants produced 29.2% more flowers and 48.1% more axial inflorescences, resulting in overall greater total inflorescence length than the control plants. Although damaged plants also produced significantly shorter petals, this difference was not large (5.7% shorter). Damaged plants began flowering approximately 4 days later than control plants and ended up flowering for fewer days than control plants, thereby reducing their total flowering period.

Damaged plants were also 8.4% shorter than controls, though this result was not due to slower inherent growth rates since there was no significant difference in growth per day between treatments prior to damage (one-way ANOVA, $F = 0.0049$, $df = 1, 128$, $P = 0.9445$).

Given *L. salicaria*'s perennial life cycle and the associated difficulties of assessing total lifetime fitness, it was only reasonable to assess proxies for lifetime female fitness. In terms of reproductive output in the given sampling year, damaged plants produced 32.8% more fruits per plant, but aborted the same proportion of fruit and set a similar number of seeds as control plants (Table 1). Although damaged plants produced an average of 10.4% more seed per fruit than control plants, this difference was not significant (Table 1).

In both the damage and control treatments, the number of inflorescences, inflorescence length and number of flowers were each strongly and positively correlated ($r = 0.73 – 0.75$; Table 2). Inflorescence height was also moderately and positively correlated with these floral display traits, but exhibited a slightly stronger correlation with the number of inflorescences in damaged ($r = 0.59$) versus control plants ($r = 0.39$; Table 2). In damaged plants, flowering start time was moderately and negatively correlated with all floral traits ($r = -0.52$ to -0.62) but slightly more weakly correlated in control plants for these same traits ($r = -0.12$ to -0.40; Table 2). Flowering start time also exhibited a moderately negative correlation with inflorescence height in both the damage treatment and the control (Table 2). Petal length was not significantly correlated with any other trait except flowering start time, and only weakly so in control plants ($r = 0.28$, Table 2).

Pollen Limitation

Pollen supplemented flowers produced significantly more seeds (17.5%) than those in the open pollinated group (average seed set ± SE was 41.7 ± 2.0 in OP fruit vs. 49.0 ± 2.0 in HP fruit), suggesting that this population of *L. salicaria* was pollen limited in the study year. Because the average difference in seed set was positive in both damaged (+5.3 seeds) and control plants (+9.3 seeds), pollen limitation was apparent in both treatments, although the difference between treatments was not significant (Figure 1).

Assessing among-morph differences revealed that morph explains some of the variation in pollen limitation. In particular, pollen limitation in the long-styled morph (+13.7 seeds) was significantly greater than in the short-styled morph (+2.1 seeds; t = -2.68, df = 135, P = 0.008), but not greater than the mid-styled morph plants (+6.0 seeds; t = -1.81, df = 135, P = 0.073; Figure 2).

Selection Analysis: Direct Effects of Selective Agents

In both the damage and control groups, the total linear selection differentials (s_i) for number of inflorescences and inflorescence height were significantly positive while for flowering start time they were significant, but moderately negative (Table 3). Since the total nonlinear selection differential (γ_{ii}) of the number of inflorescences provides a better model fit than does the linear selection differential in both the damage and control models, the quadratic term was retained (Table 3). Although total selection was significant for all measured traits, the ANCOVA testing the trait by damage interaction revealed that selection did not vary between the damage and control treatment groups (Table 3).

As with total linear selection, the direct linear selection gradients (β_i) were also significantly positive for number of inflorescences and inflorescence height, and significantly negative for flowering start time in both damage treatments (Table 4; Figure 3). The number of inflorescences in the control group was identified as being under stabilizing (quadratic) selection (Table 4; Figure 3). In comparison to control plants, damaged plants exhibited significantly stronger selection for early flowering start time (linear selection) and significantly weaker positive selection for the number of inflorescences (linear and stabilizing quadratic selection) as illustrated in the ANCOVA results (Table 4; Figure 3).

In comparing the selection differentials and gradients, I observed few distinct patterns (Table 3; Table 4). Because direct linear selection for the number of inflorescences was nearly equivalent to total linear selection in the control group, selection on this trait in the control group was unaffected by correlation with other traits ($s_i = 0.28$, $\beta_i = 0.32$). In contrast, direct selection only partly explains total selection in the damage group ($s_i = 0.27$, $\beta_i = 0.10$). Direct selection on inflorescence height in both the damage and control groups was lower than total linear selection ($s_i = 0.24$, $\beta_i = 0.10$), suggesting that other correlated traits were increasing selection for inflorescence height. Consequently, such correlations may also explain why direct selection on flowering start time could only explain part of the total selection experienced in both the damage ($s_i = -0.25$, $\beta_i = -0.15$) and control groups ($s_i = -0.17$, $\beta_i = -0.06$).

Selection Analysis: Indirect Effects of Selective Agents

There was no significant difference in total linear selection (s_i) or direct linear selection (β_i) between hand-pollinated and open-pollinated treatments, indicating a lack of pollinator-mediated selection (Table 5; Table 6). When comparing pollinator-mediated selection between the damage and control groups, no statistically significant difference was evident (Table 6). A post-hoc ANCOVA that excluded damage treatment, but included pollination treatment x trait interactions for floral height, number of inflorescences, number of flowers and petal length was run. Because petal length and floral height produced marginally significant results, a lack of significance in the other models indicates power was likely not an issue.

2.5 Discussion

Pollinators are assumed to be the primary drivers of selection on floral display in animal-pollinated plants because of the direct relationship between floral display and plant reproduction (Fenster et al. 2004). My results highlight the importance of concurrently studying other possible agents of selection, as well as understanding whether the interactions among agents of selection can lead to variation in the selection pressures experienced by plants. This study provides evidence that indeed, herbivores have the capacity to drive selection for the number of inflorescences and flowering time, and, in this study, even more so than pollinators.

Direct Effects of Selective Agents

Herbivory can have an immediate direct effect on floral display that is frequently associated with florivory (Krupnick and Weis 1999, Mothershead and Marquis 2000, Strauss and Whittall 2006), but that can arise in a number of other ways. Plant species that experience apical meristem damage typically respond with compensatory growth, which often leads to changes in floral architecture through increased inflorescence branching (Inouye 1982, McNaughton 1983, Paige and Whitham 1987, Trumble et al. 1993). Associated changes related to compensatory growth include reduced plant height (Wise and Abrahamson 2008), and delayed flowering time (Brody and Irwin 2012). In this study, I found that damage reduced inflorescence height and delayed flowering time, but additionally altered overall floral display size by increasing the number of flowers and inflorescences (Table 1). This lies in direct contrast to previous studies in *L. salicaria* that found that, although damage reduced inflorescence height, delayed flowering and increased the number of inflorescences, the overall quantity of flowers and the resulting fruit production were no greater (Venecz and Aarssen 1998, Russell-Mercier 2013).

It is not surprising that reproductive output is higher following herbivory. Overcompensation, the process by which post-herbivory plant growth leads to greater reproductive output than in the absence of herbivory, has been documented numerous times (e.g., Paige and Whitham 1987, Lennartsson et al. 1998, Paige 1999, Olejniczak 2011). The theory that herbivores and plants may have evolved a mutualistic relationship is not without criticism; studies revealing overcompensation due to herbivory often fail to consider total lifetime fitness (Belsky et al. 1993, Aarssen 1995, Simons and Johnston 1999). Although a perennial plant may be able to overcompensate in the first year, a drain of resources may cause it to suffer in subsequent years. This is not likely an issue in *L. salicaria* because previous research shows that plants that compensated for apical clipping damage in one year, still produced as much fruit as undamaged plants in the subsequent year (Venecz and Aarssen 1998). Overcompensation may be more likely to occur in *L. salicaria* specifically, since it is easier to achieve if plants experience early- rather than late-season herbivory (Maschinski and Whitham 1989, Paige 1994, Strauss and Armbruster 1997). Further, because the experimental plants in my study were from established rootstocks, a higher nutrient availability may have lent itself to stronger compensation (Maschinski and Whitham 1989, Whitham 1991, Sadras 1996). Thus, evolution of overcompensation is possible if the associated benefits are not being outweighed by the costs of regrowth and the herbivore damage itself (Agrawal 2000), or if apical dominance is limiting fitness potential (Harris 1974). The findings from this study, in conjunction with similar studies on *L. salicaria* (Venecz and Aarssen 1998, Russell-Mercier 2013), reveal that the probability of overcompensation is highly variable among plants and therefore likely dependent on plant maturity and environmental conditions.

Proximate changes to floral display traits following herbivory are ultimately a consequence of the evolutionary pathway experienced by *L. salicaria*. To test current selection pathways, I quantified the selection imposed by damage for: number of inflorescences, plant height, and flowering start time. Of the traits measured, selection for the number of inflorescences varied the most between the damage and control groups. The simulated damage treatment caused significant direct selection (gradient, β_i; Table 4) on the number of inflorescences, although it did not have an effect on total selection (differential, s_i; Table 3). This finding may be a result of between-trait correlations being strongly present in the damage group but not in the control group. In Table 2, the correlation between the number of inflorescences and height, and specifically flowering time, was weaker for control plants. Because total selection does not parse out correlations, it is likely that between-trait correlations were at least partly driving up total selection in plants in the damage group but not the control group and therefore masking the direct selection effects of damage.

Consistent with previous findings (Hakes and Cronin 2011), direct selection for the number of inflorescences in my experimental plants was significantly positive in both the damage and control groups; however, selection was significantly positive but weaker in the damage group (Figure 3; Table 4). This finding suggests that, even though fruit production tended to be greater in damaged plants (Table 1), herbivores may still be negatively influencing the benefits of increased inflorescence branching. My results show high variability in fruit production by damaged plants, suggesting that not all plants have a strong capacity to compensate for herbivory, perhaps because shifting resources to the production of branches limits the resources available for fruit production. This may explain why the ability to produce fruit far beyond the mean, irrespective of the number of inflorescences, was more limited in damaged plants than control plants (Figure 3a). Further, because I found significant stabilizing selection in the control group, but not in the damage group

(Table 4), this may indicate a cap or optimum created by a trade-off between the number of inflorescences and fruit production. In *Ipomopsis aggregata*, Juenger and Bergelson (2000) detected significant positive selection for inflorescence branching in damaged plants but significant negative selection in undamaged plants. Juenger and Bergelson (1997) also found significant positive selection for branching in undamaged plants, but no selection in damaged plants. These findings, in conjunction with my own results, indicate that under herbivory, increased inflorescence branching is typically favoured while in the absence of herbivory, it can vary greatly depending on the benefits of apical dominance (Juenger and Bergelson 2000).

The damage treatment had no direct effect on total or direct selection for inflorescence height (Table 3, Table 4). Although I found evidence that all treatment groups experienced positive selection for height, consistent with previous findings in *L. salicaria* (O'Neil 1997), there was no indication that herbivory could be mediating selection for it since there was no difference between the damage and control groups. Juenger and Bergelson (1997) also found evidence for positive selection on plant height in both damaged and control *I. aggregata* plants, although they could not determine between-group differences. In contrast, herbivores have been shown to impose selection for plant height in *Erysimum mediohispanicum* because of a preference for larger plants (Gómez 2003). Herbivores of *L. salicaria* feed on apical meristem tissue early in the growing season, when plants are still relatively small in stature, and are therefore unlikely to exhibit any height preferences or reduce selection for inflorescence height. In my study, an overall lack of selection imposed by damage for floral height is perhaps an indication that damaged plants were capable of compensating sufficiently such that height differences between treatment groups were not biologically relevant enough to cause differences in selection (Table 1).

Although total and direct selection for earlier flowering time was present in both the damage and control groups, only direct selection differed between treatments, indicating that herbivores have a specific effect on selection for flowering phenology (Table 3, Table 4). Because flowering time is correlated with the number of inflorescences more strongly in the damage group than the control group, this correlation is at least partly masking the direct selection effects of damage. Although my findings suggest that herbivory mediated selection for flowering time, previous research by Sandring et al. (2007) found no evidence for this, instead suggesting a stronger effect of abiotic conditions, such as climate effects associated with altitude. However, in a system involving a seed-predating herbivore, Pilson (2000) identified selection for later flowering. Although flowering phenology can often be appreciated as a tolerance trait that allows plants to avoid early or late season herbivory, few studies have directly quantified selection for it in the context of tolerance (Juenger and Bergelson 2000). Juenger and Bergelson (2000) found that selection for earlier flowering time was stronger in apically damaged plants. The damaged plants in my study also experienced stronger selection for early flowering; however, because the simulated damage was applied at a specific time of the season to coincide with natural herbivory levels, it is unlikely that earlier flowering is preferred to avoid herbivory in either the study population or in *L. salicaria* populations experiencing natural herbivore damage. Instead, because *L. salicaria* is able to flower for most of the summer, and because damage delays flowering time (Table 1), selection for earlier flowering likely represents selection for a more rapid compensatory growth response, thereby increasing fitness by extending flowering time across a season.

Indirect Effects of Selective Agents

Despite finding evidence for increased floral display following plant damage (Table 1), I did not find evidence that herbivores are indirectly altering pollinator visitation, since pollen limitation did not differ between the damage and control groups (Figure 1). Pollen limitation reflects the effectiveness of pollinators at bringing sufficient quantity or quality of pollen to a plant; therefore, pollinator visitation behaviour may not have been altered by the damage-induced increase in floral branching. Unpublished data by Waites (2005) also found no evidence that reduced floral display size in *L. salicaria* led to increased pollen limitation, but did find increased pollinator visitation with more open flowers. Furthermore, Russell-Mercier (2013) found that pollinators tended to probe damaged *L. salicaria* plants more, potentially as a result of a higher number of open flowers. Increased pollinator visitation because of an increased number of open flowers is well supported in the literature (e.g., Conner and Rush 1996, Ohashi and Yahara 1998). Although damaged plants in this study had more open flowers (Table 1), this did not result in reduced pollen limitation. Because pollen limitation was not severe in my study, any preference pollinators may have had for larger floral displays could have been obscured by a high frequency of total pollinator visitation.

The relatively low level of pollen limitation likely explains why there was no difference in pollinator-mediated selection detected between the damage and control groups, and therefore there was no evidence that herbivores indirectly modify pollinator-mediated selection (Table 5; Table 6). Not only was there no difference between the damage and control groups, I found no evidence for pollinator-mediated selection on any of the assessed traits. If pollinator-mediated selection is occurring in *L. salicaria* it may be more likely in small populations since plant species in large populations tend to have less pollen limitation (Ägren 1996, Waites and Ägren 2004). Further, as my results confirm, there are between-morph differences in pollen limitation (Figure 2), where pollen limitation has often been found to be greatest in the long morph (Ägren 1996, Ägren and Ericson 1996, Waites and Ägren 2004) and sometimes in the short morph (O'Neil 1992). Any selection targeting more pollen-limited morphs is likely to be countered by the benefits associated with maintaining reciprocal herkogamy. Although pollinator-mediated selection is often thought to arise in situations of pollen limitation (Ashman and Morgan 2004), some studies have shown it can exist where pollen limitation is absent (Galen 1996, Caruso et al. 2010, Parachnowitsch and Kessler 2010). Thus, although little pollen limitation may contribute to the lack of detectable pollinator-mediated selection, it may not be the sole contributing factor (Sletvold and Ågren 2014).

Quantifying Agents of Selection

In this study we present evidence that selection on a plant's response to herbivory is stronger than pollinator-mediated selection for certain floral display traits. Because I did not detect that damage altered pollinator-mediated selection, selection on the response to herbivory is likely occurring through direct effects. Thus far, herbivory has only been shown to impose greater selection for floral traits than pollinators in systems involving seed predators (Cariveau et al. 2004, Rey et al. 2006, Parachnowitsch and Caruso 2008), making this the first study to demonstrate it can be imposed by leaf-feeding herbivores. The phenomenon of herbivores imposing greater selection on floral traits than pollinators may be more pervasive than previously thought, particularly in systems where biocontrols have been recently introduced. Once a plant population has successfully tapped into a specific community of pollinators, pollinator-mediated selection may no longer be as strong as the selection imposed by other agents (Strauss and Whittall 2006). Because the specialist herbivores in this study, released as a biological control, have been present in Ontario ecosystems for less than 22 years (Lindgren et al. 2002), the novelty of the selection imposed may intensify *L. salicaria*'s response. Further, if herbivore pressure is of sufficient strength to alter the trait-fitness relationship created by pollinators, it could in turn mask those pressures imposed by pollinators (Herrera 2000, Mothershead and Marquis 2000, Gómez 2003, Gómez 2008).

Phenotypic selection analysis continues to emerge as an important tool for quantifying selection. More specifically, the Lande-Arnold approach has been recently applied in conjunction with pollen limitation experiments to further our understanding of pollinator-mediated selection (e.g., Fishman and Willis 2008, Sandring and Ägren 2009). While these 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 with plants, and even with one another. Multi-tiered experimental designs, such as the one presented in this study, may allow us to elucidate through which pathways mutualists and antagonists are altering the evolutionary trajectory of a plant species. To reduce the potential for environmental correlations between traits and fitness to bias any quantification of selection, additional studies should take this multi-tiered approach in combination with genotypic selection analysis (Rausher 1992, Stinchcombe et al. 2002). In addition, because most studies tend to focus on selection through female fitness traits (e.g, seed set, fruit set), future selection studies should consider the relevance of concurrently quantifying selection through male function (e.g., pollen removal) in order to more comprehensively estimate phenotypic selection (Ashman and Morgan 2004).

Table 1 Effect of herbivore-simulated damage on floral display, floral phenology and reproduction in *Lythrum salicaria*. Average (±SE) trait values for each treatment are displayed with appropriate one-way ANOVA or Wilcoxin Mann Whitney test.

Trait	Damaged	Control	Test Statistic	Effect
# of Open Flowers	48.3 ± 3.78	34.6 ± 3.50	W = 2983	P=0.003272 **
# of Flowers	1106 ± 90	856 ± 87.0	W = 2968	P=0.01849 *
# of Inflorescences†	8 ± 0.51	5.4 ± 0.72	F = 17.65	P<0.0001 ***
Total Inflorescence Length (cm)††	142.6 ± 11.8	111.4 ± 11.4	F = 4.35	P=0.03885 *
Petal Length (mm)	7.3 ± 0.15	7.7 ± 0.17	W = 1621	P=0.02422 *
Flower Density (per cm)††	7.9 ± 0.17	7.8 ± 0.18	F = 0.101	P=0.7508
Flowering Start Time (Julian Days)	195 ± 1.06	191 ± 0.98	W = 3496.5	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=0.01909 *
# of Seeds per Fruit	43.7 ± 2.60	39.6 ± 3.00	F = 1.01	P=0.3168
# of Fruit†	619 ± 51.0	466 ± 47.0	F = 5.222	P=0.02384 *
% Fruit Abortion	44.4 ± 1.26	44.8 ± 1.42	F = 0.061	P=0.806

† Square root transformed †† Log transformed * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

Table 2 Correlations between select floral display traits within *Lythrum salicaria* plants in the presence or absence of herbivore-simulated damage. Correlations for plants in the damaged treatment (below the diagonal) and control treatment (above the diagonal) are both displayed.

	# of Flowers	# of Inflorescences	Inflorescence Length	Petal Length	Flowering Start Time	Inflorescence Height
# of Flowers		0.73***	0.96***	0.02	-0.37**	0.60***
# of Inflorescences	0.75***		0.75***	-0.19	-0.12	0.39**
Inflorescence Length	0.96***	0.75***		-0.11	-0.40**	0.58***
Petal Length	0.02	-0.06	0.09		0.28*	-0.08
Flowering Start Time	-0.59***	-0.52***	-0.62***	-0.11		-0.56***
Inflorescence Height	0.68***	0.59***	0.67***	-0.02	-0.49***	

* $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

Table 3 Total linear and quadratic selection (± SE) for select *Lythrum salicaria* floral traits in the presence and absence (control) of herbivore-simulated damage. Trait x treatment interaction (ANCOVA) tests if the presence of herbivore-associated damage can mediate selection of traits.

	Damaged		Control		Trait x Damage Interaction	
	s_i ± SE	γ_{ii} ± SE †	s_i ± SE	γ_{ii} ± SE †	Δs_i ± SE	$\Delta\gamma_{ii}$ ± SE †
# Inflorescences	0.27 ± 0.03***	-0.11 ± 0.04*	0.28 ± 0.03***	-0.15 ± 0.04**	0.01 ± 0.04	-0.04 ± 0.06
Inflorescence Height	0.24 ± 0.03***	-0.02 ± 0.05	0.24 ± 0.04***	0.02 ± 0.06	-0.00 ± 0.05	0.04 ± 0.08
Flowering Start	-0.25 ± 0.03***	0.01 ± 0.03	-0.17 ± 0.05***	0.02 ± 0.04	0.08 ± 0.05	0.01 ± 0.06

* $P < 0.05$ ** $P < 0.01$ *** $P < 0.0001$ † nonlinear selection estimate is obtained by doubling quadratic regression coefficient

Table 4 Direct linear and quadratic selection (± SE) for select *Lythrum salicaria* floral traits in the presence and absence (control) of herbivore-simulated damage. Trait x treatment interaction term (ANCOVA) tested if the herbivore-associated damage could mediate selection of traits.

	Damaged		Control		Trait x Damage Interaction	
	β_i ± SE	γ_{ii} ± SE †	β_i ± SE	γ_{ii} ± SE †	$\Delta\beta_i$ ± SE	$\Delta\gamma_{ii}$ ± SE †
# Inflorescences	0.10 ± 0.04***	0.02 ± 0.04	0.32 ± 0.05***	-0.11 ± 0.04**	0.22 ± 0.06***	-0.13 ± 0.06*
Inflorescence Height	0.10 ± 0.03***	-0.01 ± 0.04	0.10 ± 0.03***	0.02 ± 0.04	0.00 ± 0.04	0.03 ± 0.06
Flowering Start	-0.15 ± 0.03***	0.01 ± 0.03	-0.06 ± 0.04***	0.01 ± 0.03	0.09 ± 0.05*	0.00 ± 0.03

* $P < 0.05$ ** $P < 0.01$ *** $P < 0.0001$ † nonlinear selection estimate is obtained by doubling quadratic regression coefficient

Table 5 Total linear selection (± SE) for select *Lythrum salicaria* floral traits in open- and hand-pollinated treatments. Trait x treatment interaction term (ANCOVA) tested if the presence of herbivores or supplementation of pollen can mediate selection of traits.

	Open Pollination	Hand Pollination	Trait x Pollination Interaction	Trait x Pollination x Damage Interaction
	s_i ± SE	s_i ± SE	Δs_i ± SE	Δs_i ± SE
# Inflorescences	-0.03 ± 0.05	-0.02 ± 0.04	0.01 ± 0.04	-0.02 ± 0.09
Inflorescence Height	0.01 ± 0.05	0.06 ± 0.04	0.07 ± 0.04	0.02 ± 0.09

Table 6 Direct linear selection (± SE) for select *Lythrum salicaria* floral traits in open- and hand-pollinated treatments. Trait x treatment interaction terms (ANCOVA) tested if the presence of herbivores or pollen supplementation can alter selection of traits.

	Open Pollination	Hand Pollination	Trait x Pollination Interaction	Trait x Pollination x Damage Interaction
	β_i ± SE	β_i ± SE	$\Delta\beta_i$ ± SE	$\Delta\beta_i$ ± SE
# Inflorescences	-0.03 ± 0.06	-0.02 ± 0.05	-0.03 ± 0.05	-0.03 ± 0.10
Inflorescence Height	0.02 ± 0.06	0.07 ± 0.05	0.08 ± 0.05	0.04 ± 0.10

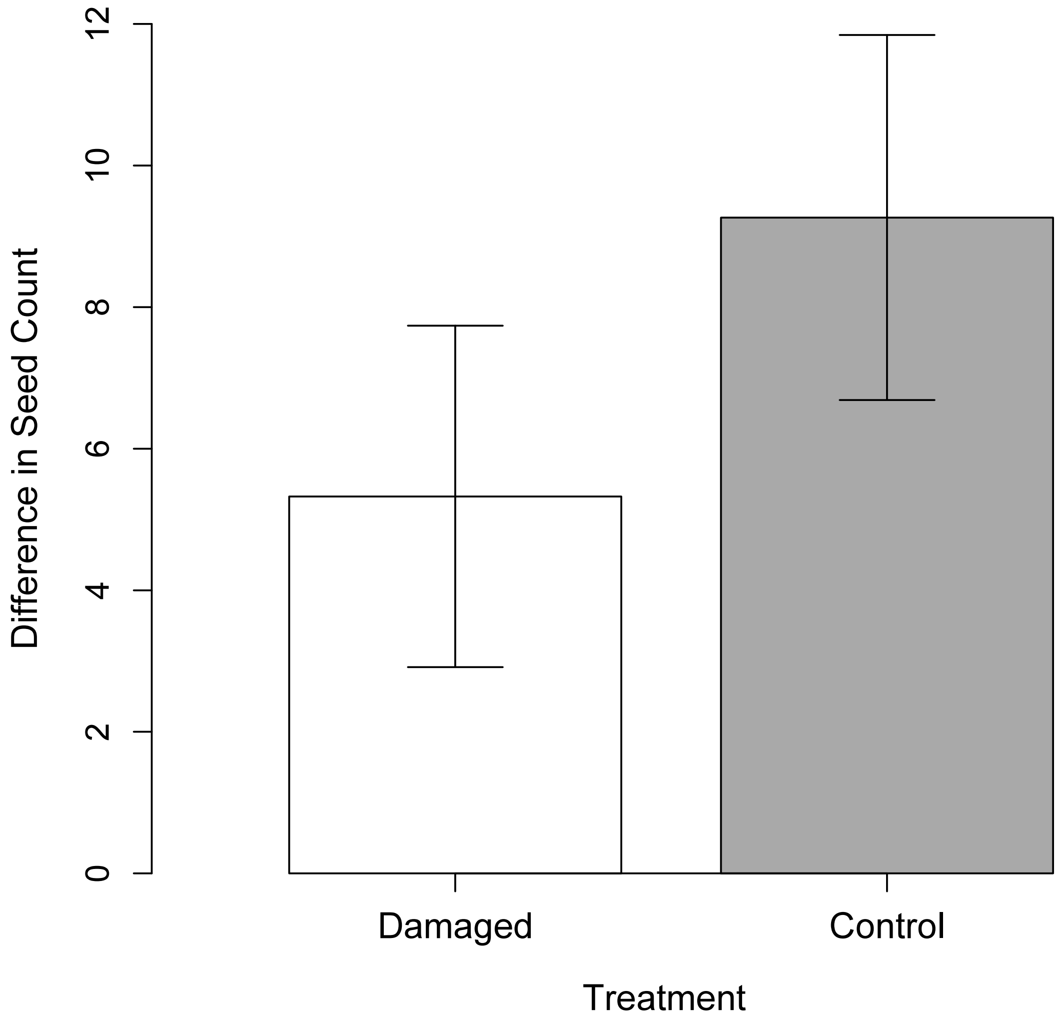


Figure 1 Comparing pollen limitation between *Lythrum salicaria* plants in the presence and absence (control) of herbivore-simulated damage. Pollen limitation is represented by the difference in seed count between open-pollinated (OP) flowers and hand-pollinated (HP) flowers. Least square means (± SE) were calculated from a linear mixed-effects model, incorporating the random effect of multiple flower pairs (HP-OP) nested within each plant.

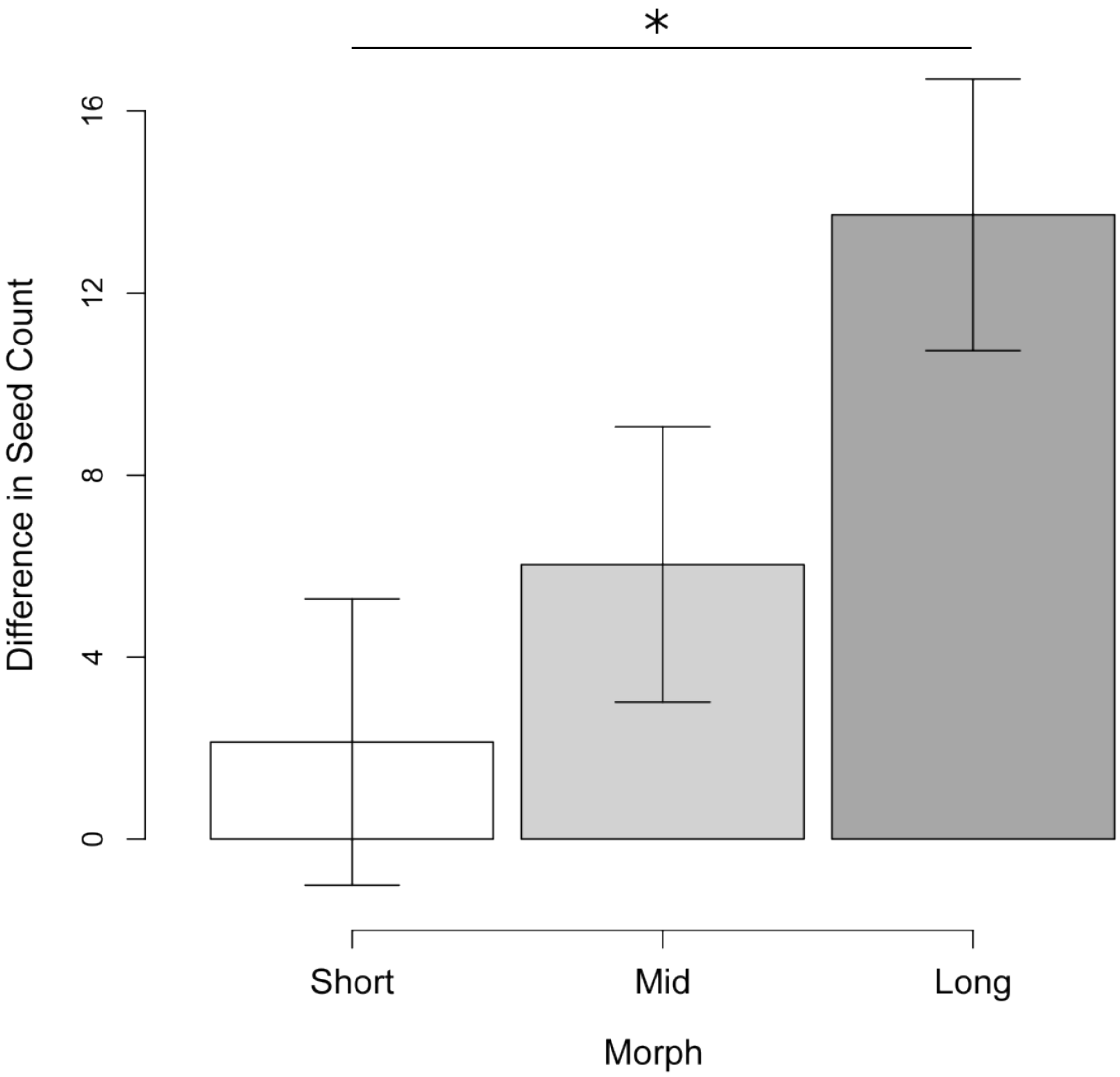
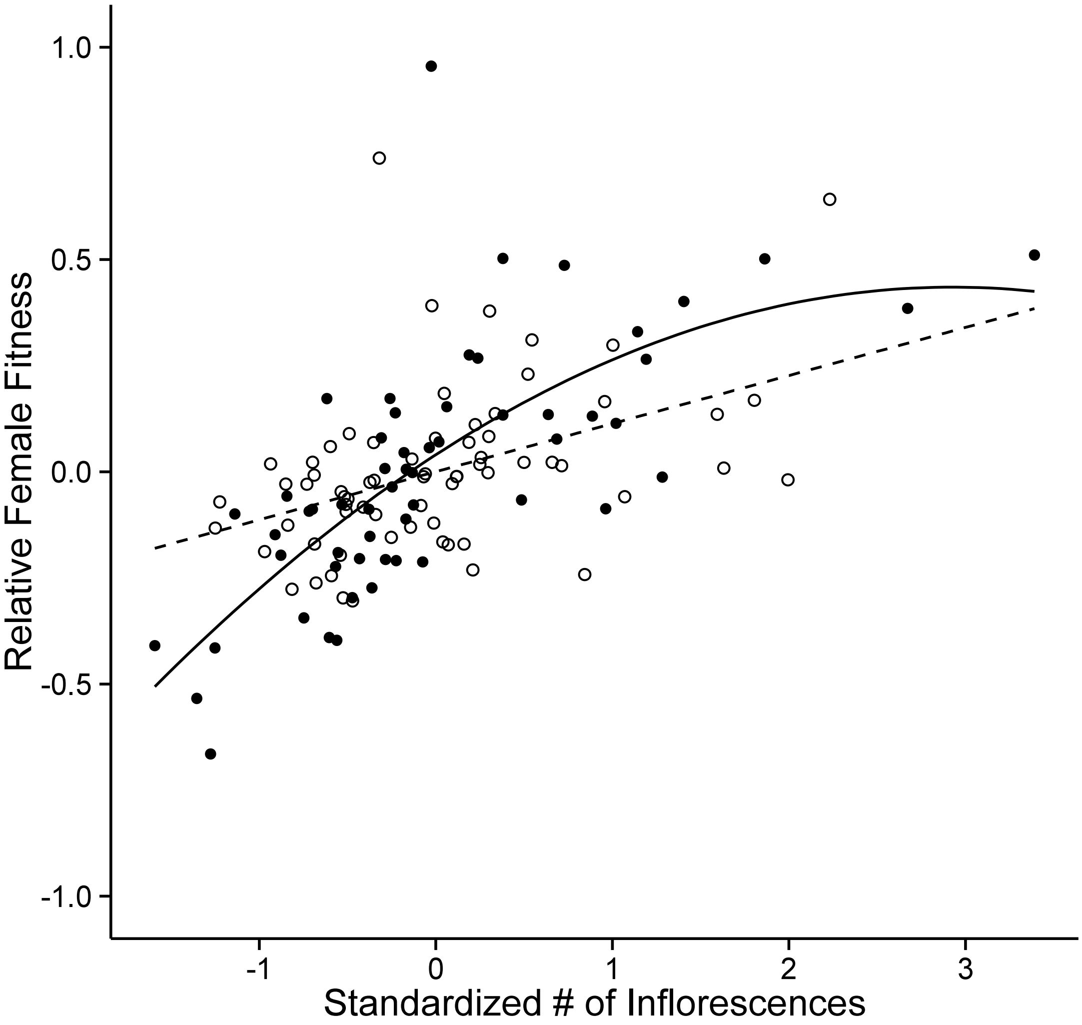


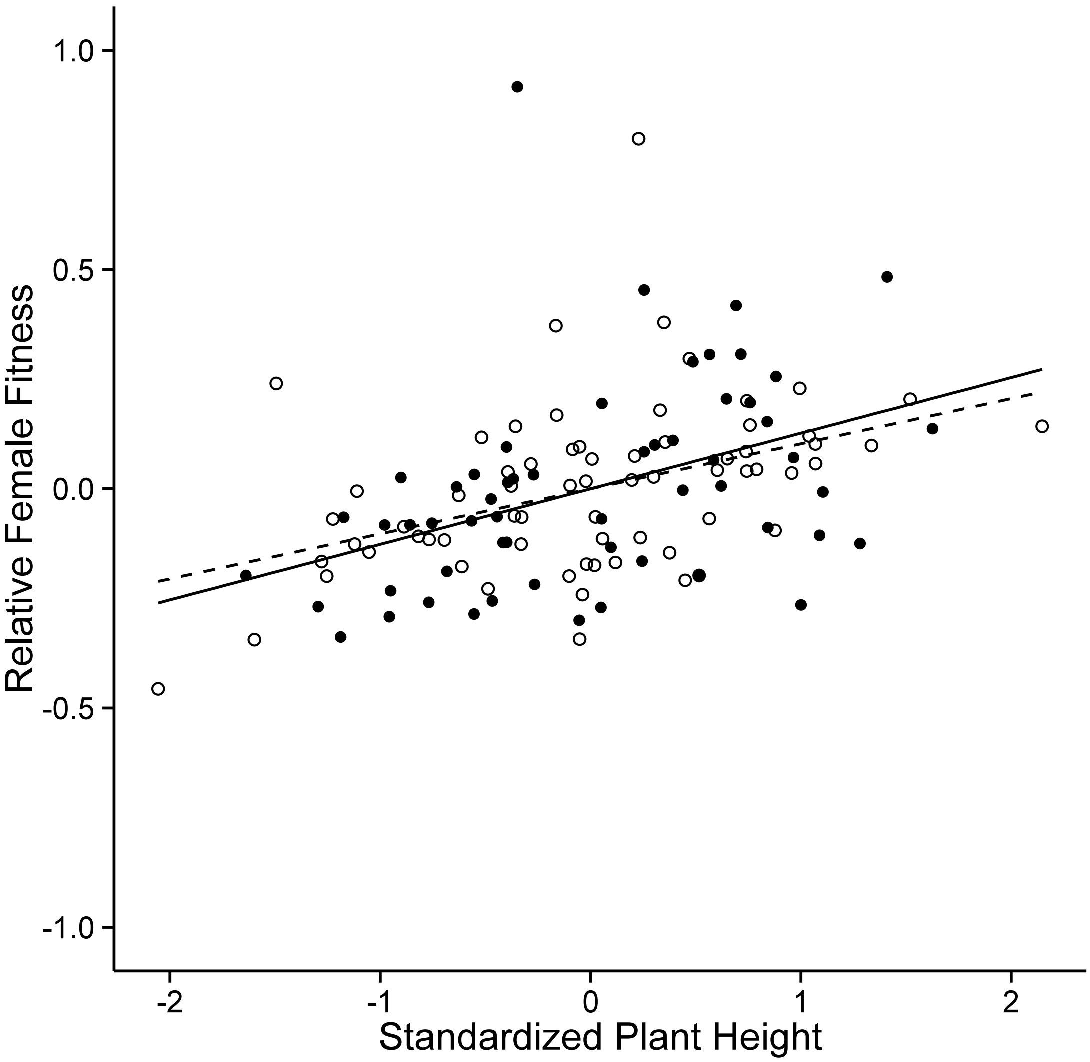
Figure 2 Comparing pollen limitation between tristyly morphs of *Lythrum salicaria*. Pollen limitation is represented by the difference in seed count between open-pollinated (OP) flowers and hand-pollinated (HP) flowers. Least square means (± SE) were calculated from a linear mixed-effects model, incorporating the random effect of multiple flower pairs (HP-OP) nested within each plant. * *P* < 0.01.

(a)



Relative Female Fitness
1.0
0.5
0.0
-0.5
-1.0
-1
0
1
2
3
Standardized # of Inflorescences

(b)


1.0
0.5
0.0
-0.5
-1.0
Relative Female Fitness
-2
-1
0
1
2
Standardized Plant Height

(c)

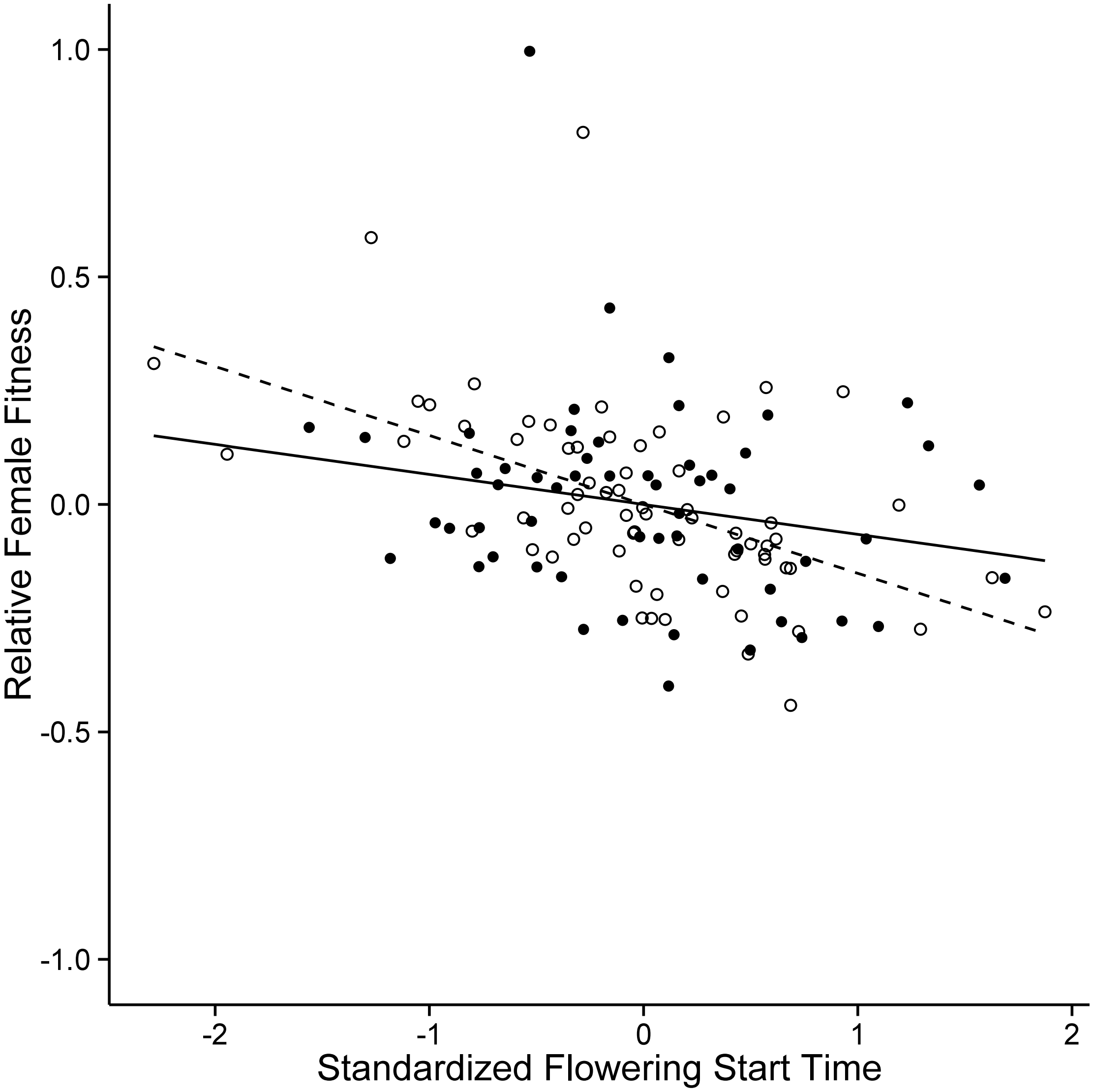


Figure 3 Added-variable plot displaying phenotypic selection gradients for (a) number of inflorescences (b) floral height and (c) flowering start time in *Lythrum salicaria*. Linear and quadratic selection gradients for plants in the presence (open circle, dashed line) and absence (closed circle, solid line) of herbivore-simulated damage are estimated using multiple regression models (**Table 4**).

CHAPTER 3: Project Summary

3.1 Future Directions

My thesis work addresses how evolutionary ecologists can study the relative importance of multiple selective agents in driving the evolution of floral display traits. This study demonstrates why it is important to not only consider how each agent can directly drive selection independently, but also how they may indirectly influence one another as a result of complex and sometimes unexpected interactions (Strauss and Irwin 2004). My results highlight the fact that it is especially important to consider multiple agents as it is not always the most obvious agent that is contributing the brunt of selection; for floral traits in particular, herbivores can sometimes cause greater selection than pollinators themselves (e.g., Parachnowitsch and Caruso 2008). By considering how plant defenses, such as compensatory growth or resistance toxins, may have integrated effects on floral traits, we can identify the role of herbivores in driving selection for these traits, as well as their potential to change pollinator behaviour (e.g., Armbruster et al. 1997, Adler and Irwin 2005, Russell-Mercier 2013). While I did not find evidence that herbivory altered pollinator-mediated selection in my study, this is likely a result of a low level of pollen limitation in the experimental population. Additional research that quantifies pollinator-mediated selection in the presence and absence of herbivores in a species that exhibits more severe pollen limitation would likely provide a clearer picture of whether herbivores can have such an indirect influence.

I have provided evidence that the damage caused by herbivory can alter female fitness and selection through female function, but it is also important to consider the other side of the coin: are herbivores also affecting male fitness? When quantifying the relative role that herbivores, pollinators, or other agents play in plant selection, it is valuable to consider multiple fitness components, and across multiple years. Single components of fitness are often correlated with whole plant fitness and can therefore provide good estimates of selection; however, strength of selection can sometimes vary depending on the fitness component used (Kingsolver et al. 2012). Some evidence suggests that selection on floral display may be stronger in male than in female fitness components (Ehlers et al. 2002, Lau et al. 2008). Whether selection for floral display traits occurs through male or female function is highly variable depending on context (Ashman and Morgan 2004), and therefore both should be considered. Although pollinator-mediated selection was not evident in the floral traits I studied, it may still be occurring through male, rather than female, function. It would be a relevant next step to consider both male and female components of fitness in future assessments, while also taking into account multi-year contributions to total lifetime fitness.

This thesis illustrates that phenotypic selection analysis is a great tool for determining the strength of selection and, when combined with experimental manipulations, can be an even greater tool for identifying selective agents. But it is important to recognize that the strength of selection is rarely static, and that geographical and temporal variation should ultimately be considered when estimating the evolutionary trajectory of traits (e.g., Rey et al. 2006). Further, when we consider the likelihood that selection pressures will contribute to that evolutionary trajectory, it is necessary to be critical of unmeasured factors that could potentially reduce the rate of evolution. In a perennial plant like *L. salicaria*, a well-established population could deter seedling establishment, reducing the speed with which selection could give rise to beneficial traits. Thus, although I find that the damage imposed by biocontrol herbivores could cause direct selection for particular traits, large, mature populations of *L. salicaria* may not show signs of rapid evolution.

This is the first study in *L. salicaria* to show that herbivory has the capacity to induce overcompensation. Since there is little evidence that *L. salicaria* employs resistance traits (Willis et al. 1999), it seems clear that tolerance, in particular compensatory growth, is the primary mode of anti-herbivore defense in this species. My thesis emphasizes the importance of considering alternative methods of defense, such as compensatory growth, when evaluating selection on floral traits. Given that compensatory growth varies in its success as a defensive strategy in *L. salicaria*, the effectiveness of *Neogalerucella* spp. as a biological control agent may be contingent on their quantity within a given mature, invasive population; if populations of a biological control are not inducing sufficient plant damage, compensation, and even overcompensation may occur. It would be beneficial to determine what environmental conditions are favourable for allowing strong compensation responses. An experiment using multiple manipulations controlling the timing of damage, level of damage, and resource availability, would provide insight into the optimal conditions needed to control *L. salicaria* and would allow us to determine if established populations of this invasive can be efficiently controlled by *Neogalerucella* spp.

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