Interactions between two herbivores introduced as biocontrol agents against invasive purple loosestrife

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ABSTRACT

Theory predicts that, upon release from natural enemies in the new range, invasive species will evolve to allocate more resources towards traits that improve their ability to compete with native species. The motivation for most biological control programs is to re-introduce native enemies in order to oppose this effect and reduce or even reverse some of the negative impacts of invasive species on native and economically important ecosystems. In many cases, multiple biocontrol agents are introduced, often under the assumption that their impacts will be complementary. However, studies that attempt to quantify the nature of interactions among biocontrol agents tend to find unique outcomes: as yet, no general patterns have emerged. Here I describe the findings of a study of the impacts of the introduction of one species of biocontrol agent of invasive purple loosestrife, the leaf beetle *Neogalerucella calmariensis*, on the reproductive success of a second biocontrol agent, the flower-feeding weevil *Nanophyes marmoratus*. Somewhat surprisingly, I found that more adult weevils tended to emerge from plants that were simultaneously infested with both types of beetles. This was true even when differences in flower number among plants were taken into account. Moreover, there were more aborted flowers on an inflorescence where both biocontrol agents were present. Finally, more pollinators visited the plants that were infested by both biocontrol agents. The observational data also show a positive correlation between the presence of the leaf beetle larvae and the number of adult flower weevils found on an inflorescence. I discuss various explanations, including the possibility that biocontrol-induced changes in flowering phenology and plant nutritional compounds could be, at least partially, responsible for these findings.

RÉSUMÉ

D'après la théorie, les espèces invasives exemptes de leurs ennemis naturels évoluent de sorte à allouer plus de ressources envers les traits leur concédant une capacité compétitive accrue contre les espèces natives. C'est en suivant cette logique que les programmes de contrôles biologiques sont mis en place, afin de réintroduire un ennemi naturel capable de réduire ou même d'annuler les impacts négatifs des espèces invasives sur les écosystèmes natifs ayant souvent une valeur économique importante. Selon l'hypothèse que les impacts seront cumulatifs et complémentaires, plusieurs agents de contrôle biologique sont fréquemment introduits simultanément. Cependant, plusieurs études qui tentent de quantifier la nature des interactions entre les différents agents de contrôle biologique utilisés observent des résultats uniques : une tendance généralisée n'a donc pas encore été déterminée. Dans le texte qui suit, je décrirai les résultats d'une étude sur les impacts de l'introduction d'une espèce utilisée comme agent de contrôle biologique, la chrysomèle *Neogalerucella calmariensis*, sur le succès reproducteur d'un second agent, le charançon *Nanophyes marmoratus*. Surprenamment, j'ai observé plus de charançons adultes émergeant des plants infestés simultanément des deux agents de contrôle biologique. Ce fait s'est avéré exact malgré les différences considérables dans le nombre de fleurs par inflorescence. De plus, il y avait plus de fleurs avortées sur une inflorescence donnée lorsque les deux agents de contrôle biologique étaient présents. Finalement, plus de pollinisateurs ont visité les plantes étant infestées par les deux agents de contrôle biologique. Les données observationnelles ont également démontré une corrélation positive quant à la présence de larves de chrysomèle sur le nombre de charançons adultes retrouvés sur une inflorescence. Je discuterai donc de différentes explications, incluant la possibilité que la phytophagie produise des différences quant à la phénologie de la floraison et le contenu nutritionnel de la plante, ce qui pourrait, au moins partiellement, expliquer les résultats.

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CHAPTER 1: Project Overview

1.1 Competition Between Herbivores: A Long Theoretical Debate

Competition is currently accepted as a major force shaping phytophagous insect communities (Denno and Kaplan 2007), but the concept has had a controversial history (Schoener 1982, Denno et al. 1995). In the 1960s and 1970s, insect ecologists generally agreed that the population dynamics of herbivores were largely driven by competitive interactions (Denno et al. 1995). Theoreticians postulated that competitive interactions among herbivores typically led either to resource partitioning that allowed coexistence, or to local extinction of the weaker competitor (Schoener 1982). However, during this period, Hairston and colleagues' theory (Hairston et al. 1960) on 'the green world' was in direct opposition to these competition-based concepts. Hairston et al. criticized competition as a modulating force and stipulated that in a world where resources (i.e., plants) are essentially unlimited, predation, parasitism or pathogens are more likely to influence insect population dynamics. In their view, the presence of enemies effectively maintained populations at levels too low for competition to occur. Then, in the early 1980s, the finding that herbivore ranges frequently overlapped, that niches were frequently vacant (Lawton 1982) and that co-occurring herbivores could interact in a positive manner (Strong 1981, 1982), seemed to support the theory that interspecific competition was not that important, and the controversy continued (e.g., Lawton and Strong 1981, Schoener 1982, Denno et al. 1995). A lack of experimental studies in this area slowly ameliorated during the 1980's through an increase in field manipulations (Connell 1983), which provided evidence that competition did indeed exist among herbivores (Denno et al. 1995).

In the 1990s, studies of plant-mediated interactions between herbivores revealed that indirect competition via induced resistance or altered predation risk could have detectable impacts

on population dynamics (e.g., Tallamy and Raupp 1991, Denno et al. 1995, Karban and Baldwin 1997, Agrawal et al. 1999, Karban and Agrawal 2002, Denno and Kaplan 2007). In addition, these studies revealed positive interactions, or 'induced susceptibility,' such as feeding facilitation (i.e., enhanced nutritional compounds for subsequent herbivore). For example, Nykanen and Koricheva (2004) discovered that favourable changes in plant nutrients and over-compensatory plant responses to herbivory could provide more food and shelter to herbivores. Overall, however, identifying the nature of interactions among insect herbivores, whether positive, neutral or negative and plant-mediated or not, remains a significant challenge. In a review of 193 species interactions Denno et al. (1995) concluded that competition occurred in 76% of the interactions, with increased frequency when insects were closely related, introduced or aggregative. Despite the existence of general trends, each system is unique, and the nature of these interactions can even differ from one site to another, highlighting the importance of continuing to study interactions in individual systems (Denno and Kaplan 2007).

1.2 In the Context of Classical Biological Control

Classical biological control involves the use of a target organism's natural enemies in order to control and/or eradicate an undesired population found outside of its natural range (Müller-Schärer et al. 2004). Risk assessment for native species and host specificity of candidate agents are mandatory points to look at prior to choosing the best biocontrol agent(s) (Blossey 1995, Wheeler and Schaffner 2013). Typically, more than one insect is chosen for release. The justification for multi-release is based on two concepts: the Cumulative Stress Hypothesis and the Lottery Model (Myers 1985, Denoth et al. 2002, Myers and Bazely 2003). The Cumulative Stress Hypothesis theorizes that introducing multiple biocontrol agents that each target different parts of the invasive plant should have a cumulative impact on the plant and therefore enhance the overall control of

the invasive population (Denoth et al. 2002). The Lottery Model, on the other hand, is based on the theory that introducing more than one biocontrol agent improves the chances that at least one of them will be effective in harming the invasive plants (Myers 1985, Stephens et al. 2013). In a review published in 2002, Denoth et al. examined multiple studies on the control of invasive plants and found that, indeed, the effectiveness of the control increased proportionally with the number of biocontrol agents released. The literature provides evidence supporting (Hoffmann and Moran 1998) and refuting (Cilliers and Neser 1991, Milbrath and Nechols 2004a) the efficacy of several combined biocontrol agents in invasive plant populations (Denoth et al. 2002).

One of the earliest cases of 'successful' biological control occurred in Australia in the 1920s with the invasive prickly-pear, *Opuntia inermis* DC and *O. stricta* (Haw.) Haw. (Cactaceae) (Dodd 1936). The control was achieved with only one biocontrol agent, the cactus moth *Cactoblastis cactorum* (Berg, 1885) (Lepidoptera: Pyralidae) from Argentina, even though approximately 18 insect species had originally been introduced (Dodd 1936). It is thought that the other insects that were well established prior to the introduction of the *C. cactorum* were not able to compete when the invasive *Opuntia* spp. became scarce (Dodd 1936). The ease of rearing *C. cactorum* in great numbers allowed the release of approximately 3,000,000,000 insects; this high number apparently contributed to the efficacy of the control (Dodd 1936).

Studying past biological control releases can provide insight into how invasive plants adapt to new environmental conditions (e.g., Stastny and Sargent 2017). Moreover, understanding the interaction between phytophagous insects in a new environment could determine whether multiple biocontrol agents will be in competition. In this case, their effect on the target invasive species might be redundant and release of more than one biocontrol agent could be unnecessary.

The success of a biological control program may be higher if the biocontrol agents negatively impact pollination (Swope and Parker 2012). For example, because floral display can be affected by foliar and/or floral herbivory, biocontrol may reduce the pollinator visitation rate to an invasive, leading to a decline in seed production (Strauss et al. 1981, Lehtilä and Strauss 1997, Swope and Parker 2012, Sletvold et al. 2015). However, this would only be true in cases where the invasive plant's spread is seed-limited, which may not always be the case (Crawley 1989, Garren and Strauss 2009). A better understanding of the effect of a particular agent on pollinator visitation may help to expand our ability to estimate the efficacy of biocontrol agents at reducing the target plant's population.

1.3 Invasive Plants

An exotic plant species has the potential to become invasive due to its ability to outcompete native plants through an absence of natural enemies in the introduced range (i.e., 'Natural Enemies hypothesis', see Zangerl and Berenbaum 2005). A related theory, the Evolution of Increased Competitive Ability (EICA) hypothesis, stipulates that in the absence of its natural enemies, a plant will reallocate its resources from defense to increased reproduction (Blossey and Notzold 1995, Keane and Crawley 2002). This allows a plant species outside its native range to outcompete the members of its new community, who must still invest in defense against natural enemies. Experimental studies show support for the EICA hypothesis (Edwards et al. 1998, Willis et al. 1999), although there is also evidence to the contrary (Agrawal and Kotanen 2003, Bossdorf et al. 2004).

Tolerance (i.e., a plant's ability to maintain its fitness after herbivory event) to a natural enemy released as a biocontrol has also been observed (Rosenthal and Kotanen 1994, Aarssen 1995, Willis et al. 1999, Agrawal 2000, Jogesh et al. 2014). If the introduced insects are not able

to rapidly adapt to evolved host plants, tolerance can reduce the efficacy of the biocontrol (Müller-Schärer et al. 2004, Jogesh et al. 2014). The ability of an invasive plant to outcompete natives and/or tolerate herbivory may be restricted to favourable environments : nutrient-rich soil and drought-free areas, for example (Edwards et al. 1998, Jogesh et al. 2014). Furthermore, in some cases, invasive plants have been found to build up greater biomass (relative to the native range) only where soil conditions are favourable (Edwards et al. 1998, Willis et al. 1999). Top-down effects tend to dominate when the soil is less fertile, thereby improving the success of biocontrol programs (Hovick and Carson 2015).

1.4 Thesis Objectives

Lythrum salicaria Linnaeus (Lythraceae) is a well-studied wetland invasive in North America. It was once thought to pose a severe threat to native ecosystems (Thompson et al. 1987, Malecki et al. 1993), but the evidence for this is conflicting (Blossey et al. 2001, Farnsworth and Ellis 2001). Indeed, some studies have shown a positive effect of the presence of *L. salicaria* on soil nitrogen content (e.g., Fickbohm and Zhu 2006), and on seed set in a co-occurring native plant via decreased pollen limitation (da Silva et al. 2013). *Lythrum salicaria* populations can reach very high stand numbers, completely changing the original landscape. Due to public concern, biological control agents were released by government-sponsored programs across North America. Releases began in 1992, after extensive research on potential agents (Blossey 1992, 1995, Malecki et al. 1993, Blossey and Schroeder 1995). The host specificity and control effectiveness of four biocontrol agents were sufficient to start their introduction from German-sourced populations (Blossey 1995, Winston et al. 2014). Mortality, seed set reduction to less than 1%, suppressed flowering and reduction in shoot growth were the reported effects of the phytophagous leaf beetles *Neogalerucella calmariensis* Linnaeus (Coleoptera: Chrysomelidae) and *N. pusilla* Duftschmid

(Coleoptera: Chrysomelidae) on *L. salicaria* (Blossey 1992). Following the Cumulative Stress Hypothesis, the introduction of two more biocontrol agents that attacked different parts of the plants was approved: the florivorous weevil *Nanophyes marmoratus* Goeze (Coleopetra: Curculionidae), and the root-eater weevil *Hylobius transversovittatus* Goeze (Coleoptera: Curculionidae) (Blossey and Schroeder 1995, Wilson et al. 2009, Winston et al. 2014). The root-eating weevil *H. transversovittatus* was released in Canada in 1992 from German and Finnish source populations (Winston et al. 2014). The florivorous weevil *N. marmoratus* was first released in Canada only in 1997, following its introduction to the U.S., which introduced this species in 1994, from German and French source populations (Winston et al. 2014). The combined effect of all biocontrol agents seems to be more effective in Canada than in the U.S.A. (Wilson et al. 2009, Winston et al. 2014). Overall, the leaf beetles *Neogalerucella* spp. seem to have a greater effect on *L. salicaria* than the other biocontrol agents (Winston et al. 2014). All agents are quite mobile and were able to colonize all Canadian provinces, even spreading to the northern limit of *L. salicaria* (Dech and Nosko 2002, Grevstad 2006, Boag and Eckert 2013, Winston et al. 2014).

Neogalerucella calmariensis and *N. pusilla* are closely related, and their coexistence in very similar, if not the same niche, was puzzling. It seems that their coexistence is possible when resources are not limited, but *N. pusilla* appeared to have a competitive advantage when resources become scarce in a laboratory experiment (McAvoy and Kok 2007). Moreover, their fecundity seems to vary according to the ambient temperature: *N. pusilla* lays more eggs at temperatures lower than 25°C, whereas *N. calmariensis* performs better at temperature above 25°C (McAvoy and Kok 2007). These very small differences appear to allow the two leaf-beetles to coexist on the same host during overlapping periods of time, since resources are generally not a limiting factor (McAvoy and Kok 2007). The interaction between the root weevil *H. transversovittatus* and the

two leaf beetles in the genus *Neogalerucella* has been examined in order to study their combined effects on *L. salicaria*. The presence of the leaf beetles appears to affect root weevil larval survival, but not its oviposition (Hunt-Joshi and Blossey 2005). Unless heavy herbivory by leaf beetles kills the host plant, the negative interaction seems to be marginal over a long period of time (Hunt-Joshi and Blossey 2005). Because *N. marmoratus* and *H. transversovittatus* are apparently never found on their own at a site (Hunt-Joshi and Blossey 2005), only one interaction remains undefined: the interaction between the co-occurring leaf beetles *Neogalerucella* spp. and the florivorous weevil *N. marmoratus*. My thesis research addresses this gap in the literature.

CHAPTER 2: Interactions between two herbivores introduced as biocontrol agents against invasive purple loosestrife

2.1 Introduction

Interspecific competition is an important force shaping ecological communities (Denno and Kaplan 2007). Indeed, competition is a major limiting factor for some herbivores (Denno et al. 1995). Competitive interactions among species are likely to be more pronounced for herbivores that specialize on a specific part of a plant, such as florivores, seed predators, or frugivores. This is due to the smaller amount of resource available, relative to a generalist herbivore that eats all or most plant parts (Denno et al. 1995). One of the main effects of interspecific competition on herbivore growth and survival is resource deprivation through the consumption of the host plant by other individuals (Denno et al. 1995, Denno and Kaplan 2007). Equally important are indirect impacts, which include the alteration of the nutritional or phytochemical composition of the host plant by other herbivores (White 1978, Valentine et al. 1983, Pare and Tumlinson 1999, Denno and Kaplan 2007). Additionally, olfactory cues produced by other insects can attract or deter other species of herbivores, and may even attract predators (Pare and Tumlinson 1999, Denno and Kaplan 2007). Finally, a change in host plant morphology caused by herbivory, such as a reduction in the number or timing of flowers or leaves, can adversely affect herbivores arriving later in the season (Denno et al. 1995, Milbrath and Nechols 2004b).

In spite of the clear potential for interspecific competition, biological control programs (hereafter, biocontrol) frequently introduce multiple species of herbivores in order to control a single invasive plant species (Myers 1985, Denoth et al. 2002, Stephens et al. 2013). According to the Cumulative Stress Hypothesis (Denoth et al. 2002), each added herbivore species contributes to an increase in total host plant stress. Moreover, stress on a host plant tends to augment when

multiple parts of the plant are attacked (Blossey and Schroeder 1995, Denoth et al. 2002, Myers and Bazely 2003). For instance, Hoffmann and Moran (1998) found a significant decline in the density of mature *Sesbania punicea*, an invasive plant in South Africa, when three biocontrol agents were present, compared to only one. Resource partitioning appears to allow the flower weevil *Trichapion lativentre*, the seed weevil *Rhyssomatus marginatus*, and the trunk and stem weevil *Neodiplogrammus quadrivittatus*, to coexist on the same host at the same time, apparently without negative effects on each other. Their combined impact on the plant contributed directly to successful population control of invasive *S. punicea* (Hoffmann and Moran 1998). Moreover, total plant fitness may decrease if the joint effect of multiple species of herbivores contributes to a reduction in reproduction through a decrease in investment in floral display, which can negatively impact pollinator attraction (Lehtilä and Strauss 1997, Mothershead and Marquis 2000, Denoth et al. 2002, Swope 2014).

On the other hand, the utility of introducing several species of biocontrol has been challenged on several fronts. First, competition among different species of biocontrol agents has often been observed, and may reduce the success of either or both (Denno et al. 1995, Denoth et al. 2002, Milbrath and Nechols 2004b, Hunt-Joshi and Blossey 2005). In fact, the literature examining interactions among biocontrol agents provides conflicting results (Myers 1985, Crawley 1989, Hoffmann and Moran 1998, Denoth et al. 2002, Keane and Crawley 2002, Milbrath and Nechols 2004b). For example, Milbrath and Nechols (2004) found evidence of competition between two biocontrol agents introduced in North America to control the musk thistle *Carduus nutans*. Early feeding by the leaf weevil *Trichosirocalus horridus* negatively impacted the seed weevil *Rhinocyllus conicus* through the induction of changes to floral morphology, including shorter flower stems, fewer flower heads, and delayed flowering. Resource availability was

reduced, which impacted the population density of the later emerging *R. conicus*. Hence, to maximize the chance of population control success, the authors suggested avoiding the simultaneous introduction of these two biocontrol agents at a given site (Milbrath and Nechols 2004b). Overall, it appears that the introduction of multiple biocontrol agents might be beneficial in some cases, while the efficacy of such an approach is unclear or even detrimental in others. Consequently, biocontrol programs should address whether the introduction of more than one species for biocontrol is truly beneficial (Myers 1985, Denoth et al. 2002, Stephens et al. 2013).

As previously mentioned, a total of four different species of biocontrol agents have been introduced in North America to control the invasive plant species *L. salicaria*, also known as purple loosestrife (Malecki et al. 1993, Blossey and Schroeder 1995, Wilson et al. 2009). The first biocontrol agents introduced to North America in 1992 were two species of leaf eating beetles, *Neogalerucella calmariensis* and *Neogalerucella pusilla* (hereafter, *Neogalerucella*). The flower weevil *Nanophyes marmoratus* and the root weevil *Hylobius transversovittatus* were introduced a few years later. All four biocontrol agents were chosen for their host specificity, their wide geographic spread in their native range, their tolerance to cold temperatures and seasonality, and their ability to effectively harm *L. salicaria* in its native range (Malecki et al. 1993, Blossey and Schroeder 1995, Landis et al. 2003, Wilson et al. 2009, Winston et al. 2014). Because *N. marmoratus* larvae develop in the flower buds and consume the ovary, it has been suggested that the presence of *Neogalerucella* could generate negative effects such as food deprivation, a reduction in the number of oviposition sites, and delayed reproduction for the flower weevil *N. marmoratus* (Wilson et al. 2009, Winston et al. 2014). In Southeastern Ontario, the second generation of *Neogalerucella* coincides with the emergence of *L. salicaria* flower buds, and, at least at my field site, the larvae appeared to prefer to eat the flower buds over foliage (M.

Torreblanca, pers. obs.). Therefore, competition for flower buds as a resource may be a limiting factor in the success of *N. marmoratus* where it co-occurs with *Neogalerucella* (Winston et al. 2014). Indeed, the development and reproduction of *N. marmoratus* could be impacted by the presence of *Neogalerucella* due to utilization of the same resource during an overlapping period. If this is true, it is also possible that the presence of *Neogalerucella* could act as an oviposition deterrent for *N. marmoratus*. I therefore hypothesized that, because of its ability to alter key resources such as food and oviposition sites, the presence of *Neogalerucella* larvae would negatively impact *N. marmoratus* reproductive success. I also hypothesized that the presence of two different biocontrol agents (i.e., *Neogalerucella* and *N. marmoratus*, as opposed to *N. marmoratus* alone), would lead to lower reproductive success for *L. salicaria* plants. Indeed, because these biocontrol agents were introduced according to the Cumulative Stress hypothesis (Malecki et al. 1993), I expect complementarity in resource use between the two beetles.

While biocontrol agents are generally chosen based on their ability to reduce plant fitness directly through the consumption of reproductive parts and indirectly through foliar herbivory, far less is known about their potential negative effects on pollination, and the possible pollinator-mediated fitness consequences of herbivory (Wilson et al. 2009, Russell-Mercier and Sargent 2015). The activities of biocontrol agents may operate to increase pollen limitation, limiting seed production in the target plant (Mothershead and Marquis 2000, Swope 2014). Reduced pollinator visitation to smaller and sparser floral displays is a known consequence of foliar herbivory or florivory in several plant species (e.g., Lehtilä and Strauss 1997, Mothershead and Marquis 2000, Swope and Parker 2012, Swope 2014). Another possible effect is the deterrence of pollinators due to the presence of herbivory through its influence on the attractiveness of the inflorescence (Swope 2014). The presence of multiple biocontrol agents may act to reduce pollinator visitation and plant

reproductive fitness even further, especially if their effects are additive. In the interest of determining the impact of the two species of biocontrol on the pollinator-mediated fitness of purple loosestrife, I also performed observations of pollinator visitation. I hypothesized that, due to the potential impacts of florivory on floral display, pollinator visitation would be lower to plants hosting both *N. marmoratus* and *Neogalerucella*, compared to those hosting *Neogalerucella* alone.

In a purple loosestrife-infested abandoned agricultural field near Ashton, Ontario, I quantified interactions between these two biocontrol agents of purple loosestrife by examining the developmental success of *N. marmoratus* in the presence and absence of *Neogalerucella*. In addition, I explored the effects of the presence of the two biocontrol agents on reproductive success of *L. salicaria* by counting the number of seed capsules, the number of aborted buds, the number of aborted flowers and the total inflorescence length on plants attacked either by both *Neogalerucella* and *N. marmoratus*, or by *Neogalerucella* alone. Finally, I observed pollinator visitation to plants hosting *N. marmoratus* in the presence and absence of herbivory by *Neogalerucella*. I predicted that *N. marmoratus*' reproductive success would be negatively affected by the presence of *Neogalerucella* as a result of competition for food and oviposition sites (Winston et al. 2014). I also predicted that the presence of two biological control agents would have a greater effect on the reproductive success of *L. salicaria* than the presence of one alone (i.e., *Neogalerucella* only). Lastly, I predicted that the presence of these two biocontrol agents would lead to changes in pollinator visitation, due to the modification of floral display traits through delayed flowering, impaired floral display, and florivory.

2.2 Methods

2.2.1 Study Species

Plant (host) species - Lythrum salicaria, or purple loosestrife, is an herbaceous perennial plant that

was first introduced to eastern North America in the early 19th century from Europe (Wilson et al. 2009). Since then, multiple introductions have increased its genetic variation in North America (Thompson et al. 1987, Blossey et al. 2001). *Lythrum salicaria* is now present in nine Canadian provinces and throughout the United States with the exception of Florida and Hawaii (Wilson et al. 2009). In southern Ontario, *L. salicaria* flowers from mid-July to August. Its invasiveness can be partly attributed to its ability to produce over 2.5 million seeds per mature plant, which are then dispersed by water, wildlife, livestock, and humans (Malecki et al. 1993, Wilson et al. 2009).

Herbivore (biocontrol) species - The leaf-chewing beetles, *N. calmariensis* and *N. pusilla* are specialist herbivores of *L. salicaria* in its native range (Blossey 1995). In 1992, both species were introduced to North America from a small number of German source populations. Beetle populations, descendants of these early introductions, are now established in thirty states and eight provinces (Blossey 1995, Blossey et al. 2001, Wilson et al. 2009). Throughout the thesis, I refer to the two species collectively as *Neogalerucella,* because distinguishing them is unnecessary for the purpose of this study and because they are very difficult to tell apart in the field. Adult *Neogalerucella* beetles overwinter in plant litter, emerge in the spring, and begin feeding on the developing plant (Figure 2.1). In southeastern Ontario, where this study took place, mating occurs from mid-May through early June, after which females oviposit throughout the month of June, leaving clusters of eggs on leaves or stems. In many populations in this region, *Neogalerucella* will produce two generations over the course of one summer, with a second flush of adult beetles emerging in late July and mating until the end of August (Wilson et al. 2009). Because the second generation of larvae coincides with flower bud appearance in southeastern Ontario (Figure 1), they tend to migrate to the top of the apical meristem in mid-July to chew on the developing flower buds. When flowers open, they also eat the ovary inside the flower (M. Torreblanca, pers. obs.).

Heavy defoliation can suppress flowering in *L. salicaria* (Blossey 1992); this, paired with the consumption of flowering parts, has led to the conclusion that *Neogalerucella* control of purple loosestrife populations has been a success in Canada (Winston et al. 2014).

Nanophyes marmoratus is a small flower-feeding weevil whose overwintering adults feed on young purple loosestrife leaves until flower buds become available, starting around mid-July in southeastern Ontario (Figure 2.1). Mating occurs as soon as flower buds become available. A single egg is laid into the tip of a developing flower bud. The larva feeds on the ovary, and, as a result, the bud never produces a mature flower. As they emerge, adult weevils leave a clear circular hole in the immature bud, but buds usually fall off the plant before adult emergence occurs (Wilson et al. 2009). By consuming flower buds, *N. marmoratus* effectively reduces seed production in *L. salicaria* (Blossey 1994; Winston et al. 2014). It has been previously suggested that competition for flower buds as a resource may limit the success of *N. marmoratus* when it co-occurs with *Neogalerucella* (Winston et al. 2014).

2.2.2 Field Site

I conducted my observations and experiment in an abandoned field located near Ashton, Ontario, Canada (45°11'35.6''N, 76°01'32.3''W). The site is characterized by the presence of *L. salicaria* in varying densities (approximate total population size is 4820 m^2). Although no official biocontrol agent release has occurred at this site, both *Neogalerucella* and *N. marmoratus* are present (M. Stastny, unpublished data). *Neogalerucella* occurs at varying densities throughout the field site (M. Torreblanca, pers. obs.). *Nanophyes marmoratus* appears to occur uniformly throughout the field site (M. Torreblanca pers. obs.).

2.2.3 Experimental Design

On July 28 and July 29, 2015, using randomly drawn coordinates set along transect lines (see below), I selected 160 purple loosestrife study plants of similar phenology from a subset of the total population for the experiment. The sample population had two distinct areas delimited by a linear area of *Salix* spp. of approximately five meters in width. Hereafter I refer to these sections of the populations as the 'west plot' and 'east plot'. To select the experimental plants, I set up 10 transects of 16 meters each, set 2 meters apart, and randomly selected a number between 1.1 and 16.0. Each random number obtained corresponded to the distance in meters from the beginning of the transect. The closest plant to the random distance obtained in the transect was selected, and tagged, as an experimental plant. The procedure was repeated if the distance generated was less than 20 cm from another selected plant. Plants were selected at a point in time where inflorescences were forming but had not yet experienced any oviposition or herbivory. Plants were chosen according to similarity in height, and each plant was inspected to confirm the absence of *Neogalerucella* egg masses or oviposition or feeding activity. Only main stems (i.e., apical, not axillary meristems) were selected for study.

In order to collect the fallen flower buds and to confine *Neogalerucella* larvae, an inverted cone made of non-woven polypropylene fabric (Select, Weed Barrier™) was secured to the base of the main inflorescence on each stem using plant stakes. *Neogalerucella* larvae were collected from non-experimental plants on-site and stored in petri-dishes with food (*L. salicaria* leaves) for up to one day in an insulated cooler (Wilson et al. 2009). Half of the plants ($N = 80$) were randomly selected as controls, to which no *Neogalerucella* larvae were added. Treatment plants ($N = 80$) each received three second-instar *Neogalerucella* larvae, applied haphazardly to the inflorescence using tweezers. Only three larvae were added in order to simulate light to mild herbivory, as heavy

herbivory could have killed the experimental plants or prevented them from flowering. All inflorescences were open to ambient levels of *N. marmoratus* attack, as well as pollinator visitation, which is necessary for fruit production in this obligate outcrossing species (Wilson et al. 2009). Because *N. marmoratus* are extensively mobile and are hard to detect due to their small size, a treatment excluding *N. marmoratus* was not feasible for the time frame and setup of this study. A fully factorial design was therefore not possible for this study.

In addition to the ambient level of attack by *N. marmoratus* on experimental plants, I supplemented each study plant (both treatment and control) with 10 adult *N. marmoratus* to ensure all plants were exposed to *N. marmoratus* oviposition. Supplementing larvae would not have been feasible as they are found in flower buds and I would have need to open and risking to kill the larvae to confirm their presence. *Nanophyes marmoratus* adults were collected on non-experimental plants on-site by gently shaking the inflorescence into a plastic container (Wilson et al. 2009). The adult weevils were then immediately deposited by hand on each experimental plant. Because the sampling population was identified as a 'coldspot' for *Neogalerucella* (i.e., they did not aggregate at this particular region of the site) (M. Torreblanca *pers. obs.*), the experimental plants did not need to be covered to prevent feeding and oviposition on non-experimental *Neogalerucella*. All study plants were nonetheless checked regularly for egg masses and adult or larvae presence. Only one adult beetle was observed on these plants during the experimental period, and it was removed. Between August 18 and 19, 2015, I collected all flower buds that had dropped into the cones. I recorded final inflorescence length for each plant in the study population as a proxy for floral display size (Winston et al. 2014). On September 8, 2015, when flowering was completed at the site, I recorded final infructescence length. I also bagged and collected each infructescence in order to count the number of seed capsules and bud scars.

Back in the laboratory at the University of Ottawa, each collected flower bud was dissected and the contents (i.e., larvae, pupae, and non-emerged adult *N. marmoratus*) were counted and recorded. Aborted buds were identified based on their size (Wilson et al. 2009), while aborted flowers were recognizable because they still had their ovaries intact and were therefore recorded as not-attacked. Buds that exhibited either a clear circular hole or contained a pupation chamber were categorized as attacked.

2.2.4 Observational Data

Between August 13 and 14, 2015, I collected inflorescences from 166 randomly selected plants at the same site (i.e., plants that were not part of the study population), in order to collect information about the natural distribution of the two species of biocontrol agents. In addition, I collected 84 inflorescences on which the presence of *Neogalerucella* larvae was visually confirmed, in order to examine how frequently the two species are found together on the same plant in this population. The two set of inflorescence are combined for the analyses. For each inflorescence, I recorded the number of adult *N. marmoratus,* the number of *Neogalerucella* larvae and adults, and the length of the inflorescence.

2.2.5 Pollination Observations

Pollinator visitation was recorded by two observers working simultaneously between 9 AM and 3 PM on sunny days between August 19 and August 23, 2015 ($N = 5$ days) on treatment and control plants from the original experimental set up. It was not possible to experimentally exclude *N. marmoratus* without also influencing pollination in the field, making a fully factorial design impossible. Each day, a maximum of 20 plants from the original set up were randomly chosen for pollinator observation, without replacement (i.e., no plant was used more than once). If a randomly selected plant was not in flower, a new plant was selected. Prior to each 20-minute observation

period, we recorded the number of open flowers on the inflorescence. At this site, pollinator visitation rates were lower than what has been reported for other similar sites in the region (e.g., Russell-Mercier and Sargent 2015), hence a small group of randomly selected experimental plants (i.e., 1 - 4), could be observed by one person simultaneously. The observer recorded the number and functional group (i.e., bumble bees, honeybees, solitary bees, hoverfly or wasps) of all floral visitors, and the number of flowers probed per pollinator per plant during the 20-minutes period (following Russell-Mercier and Sargent 2015). Over the five-day period, 40 observations were conducted on a total of 84 different plants. Because the flowering period had ended for some of the study plants before pollinator observations were performed, not all plants in the study could be observed.

2.3 Statistical Analysis

2.3.1 Observational data

To evaluate whether the presence of *Neogalerucella* larvae on an inflorescence was associated with host use by *N. marmoratus*, the ambient level of adult *N. marmoratus* on non-experimental plants collected from a site near, but separate from, the experimental population was examined. The possibility of an association between the presence of *Neogalerucella* and the number of adult *N. marmoratus* on a plant was evaluated using ANCOVA with inflorescence length included as a covariate. The presence or absence of *Neogalerucella* was considered in the analyses rather than density, as the number of larva found on each inflorescence was rarely higher than one. Only one sample had more than ten *Neogalerucella* larvae. The Shapiro-Wilk normality test and the studentized Breusch-Pagan test (Shapiro and Wilk 1965, Breusch and Pagan 1979) were used to evaluate the normality and homoscedasticity of the residuals. Because the data were not normal and the residuals exhibited heteroscedasticity, a randomization test known as permutation, a

technique based on sum of squares, was used to estimate the parameters of the ANCOVA, using the lmPerm package in R (Wheeler and Torchiano 2016). Permutation relies on the assumption that the sequence of random samples from the same population can be exchangeable; therefore this test can be used with abnormal data and heteroscedastistic residuals (Wheeler and Torchiano 2016). Because randomization tests rely on resampling the data every time the analysis is performed, the permutation technique also corrects for the multiplicity of tests where appropriate (e.g., the evaluation of the different response variables for the number of buds attacked by *N. marmoratus*). Based on recommendations of the authors of the package, the maximum number of iterations for each test was set to 100 000, and the threshold for the standard error of the estimated p-value (critical p) was set to 0.00001. The homogeneity of slopes (i.e., absence of interaction) was evaluated first, followed by the intercept, in order to determine whether the presence of *Neogalerucella* larvae had an effect. The slopes were then analyzed to see if inflorescence length was correlated with the number of adult *N. marmoratus* found on a plant.

2.3.2 Experimental data: *Nanophyes marmoratus* emergence success

To test whether the experimental addition of *Neogalerucella* larvae to treatment plants had an effect on the emergence of *N. marmoratus*, a series of ANCOVA were performed. As a proxy for success, I used the number of flower buds with an exit hole, since this indicates completed development and adult emergence in *N. marmoratus*. The total number of flower buds used by *N. marmoratus* was divided into two main categories: the number of *N. marmoratus* that successfully emerged as adults (flower buds with clear circular hole) and the number of *N. marmoratus* that did not complete their development, but could still be found inside the flower buds through dissection. Incompletely developed *N. marmoratus* found in the dissected buds were categorized as adults, pupae or larvae, and are all considered unsuccessful. The effect of the treatment (presence or

absence of *Neogalerucella* larvae) on the development of *N. marmoratus* was analyzed; inflorescence length was included as covariate. As before, the Shapiro-Wilk normality test and the studentized Breusch-Pagan test were used to evaluate the normality and homoscedasicity assumptions of ANCOVA. To account for abnormality and heteroscedasticity of data, the randomization technique described above was applied using the lmPerm package in R (Wheeler and Torchiano 2016). The following steps were performed in order to evaluate the ANCOVA models. First, the best model was identified with a simple ANOVA test, where the models with or without interactions were compared. The terms that were not significant, except for the treatment and the covariate, were removed. Second, the homogeneity of slopes (i.e., absence of an interaction between the treatment and the inflorescence length) was evaluated, followed by the intercept, to see if the treatment effect was significant. The slopes were then analyzed to see if the inflorescence length covariate was correlated with the developmental success of *N. marmoratus*. Finally, the location was evaluated to verify if the results were different between the two plots.

2.3.3 Experimental data: *Lythrum salicaria* reproductive success

I also examined whether there was an effect of the addition of *Neogalerucella* on the reproductive success of *L. salicaria*. Using ANCOVA, the effect of treatment (presence or absence of *Neogalerucella* larvae) on several different measures of reproductive success (i.e., the number of aborted flowers, the number of seed capsules, the number of scars and the number of aborted buds) was analyzed, including inflorescence length as a covariate. The Shapiro-Wilk normality test and the studentized Breusch-Pagan test were used to evaluate the normality and homoscedasticity assumptions. Again, the randomization technique described above was used to account for abnormality and heteroscedasticity of data. The homogeneity of slopes (absence of interaction) was evaluated first, followed by the intercept to see if the treatment had an effect.

2.3.4 Experimental data: Pollinator observations

Finally, to determine whether the addition of *Neogalerucella* influenced plant-pollinator interactions, pollinator visitation was observed in the control and treatment groups and analyzed using analysis of covariance (ANCOVA). The effect of the presence of *Neogalerucella* larvae on the number of visitors and the number of flowers probed per visit was examined, with the number of open flowers included as a covariate. The number the observer (two in total) and the day of observation (fixed, categorical variable) were also included in the model. The Shapiro-Wilk normality test and the studentized Breusch-Pagan test were used to examined the assumptions of ANCOVA. The permutation technique previously described was used to account for abnormality and heteroscedasticity of data. Because of the increased number of variables included in the analyses, model fitting was used to determine which model best explained the observed variation. The homogeneity of slopes (absence of interaction) was evaluated first, followed by the intercept to see if the presence of *Neogalerucella* larvae had an effect on pollinator visitation. The slopes were then analyzed to see if the covariate (number of open flowers) and the other error variables (observer and day) had an effect on the number of visits per inflorescence. All statistical analyses were performed using R (R version 3.3.1, R Core Team 2016).

2.4 Results

2.4.1 Observational data

For the site observational data, there were significantly more adult *N. marmoratus* on plants that were also hosting *Neogalerucella* larvae (Figure 2.2; Total Sum of Squares = 103, p = 0.0119). On average, 35.8 (± 15.6) % more adult *N. marmoratus* were found on inflorescences where *Neogalerucella* larvae were also present (average number of *N. marmoratus* in the presence of *Neogalerucella* (± standard error): 5.65 (±1.17); average number of *N. marmoratus* in the absence

of *Neogalerucella* (± standard error): 3.63 (±0.290)). The data residuals were abnormal (Shapiro-Wilk, $W = 0.876$, $p < 0.0001$) and the residuals heteroscedastistic (Breusch-Pagan, $BP = 11.4$, $p = 0.00327$). The number of *N. marmoratus* was significantly influenced by the inflorescence length ($SS_T = 312$, $p = 0.0006$) and there was a significant interaction between the presence of *Neogalerucella* and inflorescence length ($SS_T = 77.7$, $p = 0.0404$).

2.4.2 Experimental data: *Nanophyes marmoratus* emergence success

The emergence success of *N. marmoratus*, assessed as the number of flower buds with a clear circular hole on the side, was affected by the treatment. On average, I detected 21.5 (± 0.730) % more adult *N. marmoratus* that had successfully completed their lifecycle and emerged as adults on plants experimentally infested with *Neogalerucella* larvae, compared to those developing in the absence of *Neogalerucella* larvae (Table 2.1; Figure 2.3). The number of successfully emerged *N. marmoratus* was positively correlated with inflorescence length (Figure 2.4a). The residuals were abnormal (Shapiro-Wilk, $W = 0.893$, $p < 0.0001$) but homoscedastistic (Breusch-Pagan statistic, $BP = 6.64$, $p = 0.0844$). Finally, inflorescence length did not vary between the two treatments (Table 2.2).

The total number of flower buds on plants infested with *N. marmoratus* did not vary among treatments (Table 2.1), although it was correlated with inflorescence length (Figure 2.4b). The residuals were abnormal (Shapiro-Wilk, $W = 0.917$, $p < 0.0001$) and heteroscedastistic (Breusch-Pagan statistic, $BP = 8.15$, $p = 0.0430$). Meanwhile, the total number of *N. marmoratus* that did not complete their development did not differ between treatments (Table 2.1), but was correlated with inflorescence length (Figure 2.4c). The residuals were abnormal ($W = 0.953$, $p < 0.0001$) and heteroscedastistic ($BP = 15.7$, $p = 0.00128$). The number of *N. marmoratus* that did not complete their development was divided into three categories corresponding to their lifecycle stages (i.e.,

non-emerged adults, pupae and larvae). There was no difference among treatments in terms of the number of non-emerged *N. marmoratus* per plant at each lifecycle stage (Table 2.1). Once again, inflorescence length was a significant predictor of the number of *N. marmoratus* that did not complete their development (Figure 2.4d; Figure 2.4e; Figure 2.4f). The residuals for the larvae data were abnormal ($W = 0.834$, $p < 0.0001$) and heteroscedastic ($BP = 13.0$, $p = 0.00457$). The residuals for the pupae data were abnormal ($W = 0.867$, $p < 0.0001$) but homoscedastic ($BP = 4.78$, $p = 0.189$). Finally, the residuals for the non-emerged adults' data were abnormal ($W = 0.941$, $p < 0.0001$) but homoscedastic ($BP = 2.69$, $p = 0.442$).

2.4.3 Experimental data: *Lythrum salicaria* reproductive success

There were significantly more aborted flowers on treatment (i.e., those to which *Neogalerucella* larvae were added), than control plants (Table 2.2; Figure 2.5). Indeed, inflorescences with *Neogalerucella* larvae had an average of 26.3 (± 4.07) % more aborted flowers than inflorescences without *Neogalerucella* larvae. The number of aborted flowers was also significantly correlated with inflorescence length (+ 0.77 aborted flowers/cm), which was accounted for statistically by including inflorescence length as a covariate in the models. The residuals were abnormal (Shapiro-Wilk, $W = 0.800$, $p < 0.0001$) but homoscedastistic (Breusch-Pagan statistic, $BP = 3.23$, $p = 0.199$). There was no difference in the number of seed capsules among treatment and control plants (Table 2.2). The residuals for the seed capsule data were abnormal ($W = 0.982$, $p = 0.0334$) but homoscedastic ($BP = 2.65$, $p = 0.266$). The number of seed capsules produced per inflorescence was significantly correlated with inflorescence length (Table 2.2). The number of aborted buds and bud scars was similar across the treatments (Table 2.2). The residuals for the aborted bud data were abnormal ($W = 0.907$, $p < 0.0001$) but homoscedastic ($BP = 0.583$, $p = 0.747$). The residuals for the bud scar data were normal ($W = 0.991$, $p = 0.423$) and homoscedastic ($BP = 2.76$, p =

0.252). The permutation technique was not necessary for analyzing the bud scars data and the results from the ANCOVA model are reported. As previously mentioned, inflorescence length did not vary between the two treatments (Table 2.2).

2.4.4 Experimental data: Pollinator Observations

Plants to which *Neogalerucella* larvae were added as a treatment received significantly more pollinator visits per observation period than control plants (Table 2.3; Figure 2.6). The residuals were abnormal (Shapiro-Wilk, $W = 0.937$, $p = 0.0005$) and heteroscedastic (Breusch-Pagan statistic, $BP = 5.01$, $p = 0.0252$). In addition, there were significantly more visitors to inflorescences with more open flowers (Table 2.3), regardless of treatment. The number of pollinators observed per period differed significantly between observers, but did not differ across the five days of observation (Table 2.3). While the number of visitors per plant differed significantly, the average number of flowers probed per visit by pollinators per inflorescence did not vary between treatments, or by day or observer (Table 2.3). The residuals were abnormal ($W = 0.955$, $p = 0.00483$) and heteroscedastic ($BP = 6.34$, $p = 0.0118$).

2.4.5 Effect of location in the experimental data

As described earlier, the sample population was somewhat split into two sections by a large patch of willows. Although *a priori* I did not suspect that location in the field would have biological significance for the purpose of this study, it was added in the ANCOVA models as a random cofactor to control for the possibility of variation between plots. The overall trends were consistent across the two plots, although there were some differences. Plants in the east plot had 29 (± 0.21)% more buds attacked by *N. marmoratus* than plants in the west plot (Table 2.4). Moreover, plants in the east plot had 40 (±8.71)% more emerged adults than plants in the west plot (Table 2.4). There were 22 (±8.11)% more non-emerged *N. marmoratus* in the east plot compared

to the west plot (Table 2.4). Finally, there were more *N. marmoratus* in the east plot than the west plot for all the non-emerged categories (except pupae, where no significant difference was observed) (Table 2.4). The most noticeable difference was in the larvae: 29 (±11.7)% more larvae were found inside the flowers buds from the east plot, while there were 17 (±8.40)% more non-emerged adults in the east plot than the west plot (Table 2.4).

2.5 Discussion

Contrary to expectation, I found that when the invasive plant *L. salicaria* was treated with mild to moderate herbivory by *Neogalerucella*, more adults of a second biocontrol agent, the florivorous weevil *N. marmoratus*, had emerged at the time of plant collection. Although there was no overall difference in the number of attacked buds in the treatment and control groups, the increased adult emergence suggests that the development of *N. marmoratus* is impacted by the presence of *Neogalerucella*. In addition, the number of flowers aborted by *L. salicaria* plants was higher in the presence of both *Neogalerucella* and *N. marmoratus* than in the presence of *N. marmoratus* alone. Finally, a pollinator observation survey of the experimental plants revealed that plants without *Neogalerucella* larvae (control) received fewer pollinator visits than the plants with both *Neogalerucella* and *N. marmoratus* present in the inflorescence (treatment), although the effect was small.

2.5.1 Observational Data

Since *Neogalerucella* larvae consume new foliage and and flowers, it seems logical that *N. marmoratus*, which uses inflorescences as oviposition sites, would avoid plants infested by the leaf beetle. However, my results suggest the opposite: in a survey of their naturally occurring distributions, I found more adult *N. marmoratus* on inflorescences where *Neogalerucella* larvae were also present. There are two possible explanations for these results. First, it is conceivable that

these findings are the result of mismatching among these two common herbivores. If the presence of one species on a host plant leads to lower fitness in another, as I had originally predicted for these two herbivores, one would predict they might evolve mechanisms to avoid co-occurrence. In the case of two newly introduced organisms, sufficient time (and genetic variation) must exist in order for one or the other (or both) to evolve avoidance behaviour. Indeed, according to the Geographic Mosaic Theory of Coevolution (GMTC; Thompson and Cunningham 2002), when interacting species vary in their historical geographical range overlap, they should exhibit varying degrees of co-evolutionary trait matching, ranging from well-matched to mis-matched. If *Neogalerucella* and *N. marmoratus* do not have a long history of co-occurrence, they are unlikely to have evolved an adaptive avoidance response. On the other hand, if they tend to use the same cues to find plants (e.g., large floral displays, plant height, etc.), a positive association would be expected in the observational data. Unfortunately, historical site-by-site co-occurrence data for *Neogalerucella* and *N. marmoratus* from their native ranges are not currently available. No avoidance behaviour by *N. marmoratus* has been noted in presence of *Neogalerucella,* suggesting not only the absence of detrimental effects but also possibly the use of similar cues to find their host.

A second possible explanation for the observation that more *N. marmoratus* occur in the presence of *Neogalerucella* is the use of similar cues to find their host plant. Previous studies have suggested that *N. marmoratus* may use olfactory cues to find *L. salicaria* (Hambäck et al. 2003). As for *Neogalerucella*, its inability to colonize *L. salicaria* in populations where it is well hidden behind *Myrica gale* plants has led investigators to conclude that it uses mainly visual cues to find hosts (Hambäck et al. 2003). On the other hand, *Neogalerucella* males that are actively feeding are also known to produce a pheromone, dimethylfuran lactone, that acts as an olfactory cue to

attract conspecific leaf beetles and is responsible for the beetle's aggregative behaviour (Hambäck 2010, Fors et al. 2015). It has been shown that both males and females are attracted to the pheromone and that it is only emitted by feeding males (Fors et al. 2015). This could explain the presence of *Neogalerucella* 'hotspots' at my field site and the very unequal sample sizes (plants with *Neogalerucella* averaged $N = 20$ larvae, average adult *N. marmoratus* per plant: 5.65 (±1.17)*;* plants without *Neogalerucella* averaged $N = 180$ larvae, average adult *N. marmoratus* per plant: 3.63 (±0.29)) obtained from the observational data. *Nanophyes marmoratus* could also be using *Neogalerucella* pheromones to find oviposition sites, but I cannot infer this with the current experiment as only larval *Neogalerucella* were placed on the experimental plants.

Finally, it is possible that there is no fitness cost to overlapping host use in these two species, as is suggested by the experimental results. In the absence of fitness costs, selection would not favour avoidance behaviour, and shared host use (especially of large plants, or those with large flowering displays) would be expected.

2.5.2 Experimental data: *Nanophyes marmoratus* emergence success

There are several possible explanations for the unexpected finding that more *N. marmoratus* successfully emerged from plants where *Neogalerucella* was experimentally added. Herbivory has multiple effects on the development and physiology of the host plant (Crawley 1989, Denno et al. 1995, Pare and Tumlinson 1999, Denno and Kaplan 2007, Lehndal et al. 2016). For example, plant phenology can be modified by herbivory, which can affect subsequent herbivores (Crawley 1989, Denno et al. 1995, Milbrath and Nechols 2004b, Denno and Kaplan 2007). Food quality can also be altered after herbivory (White 1978, Awmack and Leather 2002). Phytochemical changes operating in an attacked plant following herbivory can influence the other herbivores in their host

choice (Pare and Tumlinson 1999, Denno and Kaplan 2007). In the following, I will explore these ideas in the context of my present study.

First, the impact of the presence of *Neogalerucella* herbivory on the developmental success of *N. marmoratus* might be related to the phenology of the host plant. Herbivory delays flowering in many host plant species (Crawley 1989, Notzold et al. 1998, Milbrath and Nechols 2004b, Denno and Kaplan 2007), which can impact herbivore population dynamics (Milbrath and Nechols 2004b). In *L. salicaria*, previous studies have found a delay in the timing of the first flower in the presence of herbivory by *N. calmariensis* (Schat and Blossey 2005, Colautti et al. 2010, Russell-Mercier and Sargent 2015, Thomsen 2015). In the present study, significantly more *N. marmoratus* completed their development and emerged by the time of plant collection. The overall number of flower buds used by *N. marmoratus*, including those that contained *N. marmoratus* that were not able to complete development, was also determined. There was no significant difference in the overall number of buds used by *N. marmoratus* between the treatment and control plants. This might be due to the increased variability in bud use when all flower buds were included in the analysis (see Table 2.2).

It is also possible that the presence of *Neogalerucella* leads to accelerated development in *N. marmoratus*, resulting in an earlier emergence of adult weevils. Faster development may be attributed to enhanced food quality through resource reallocation by the plant (White 1978, Awmack and Leather 2002), which could explain why more *N. marmoratus* successfully emerged as adults despite the presence of another herbivore. An additional potential impact of herbivory on host plant physiology is a change in nutritional quality, which could impact herbivore development. If there is an increase in diet quality, this might provoke an acceleration in development that favours larvae later during their adulthood (Pijpe et al. 2006, Tigreros 2013).

Interestingly, Williams and Myers (1984) found a positive effect of early feeding on the red alder *Alnus rubra* by the herbivore *Malacasoma californicum pluviale* on the development of a subsequent herbivore, *Hyphantria eunea*, due to an improvement in food quality. Indeed, *H. eunea* developed faster in the fall if moderate herbivory from *M. californicum* had occurred the previous spring (Williams and Myers 1984). They argued that mild herbivory benefitted young larvae through an increase in nitrogen availability (White 1978) and an increase in net photosynthesis in the red alder tree (Heichel and Turner 1983, Williams and Myers 1984). In herbivorous insects, host plant quality is characterized by the amount of carbohydrates, nitrogen and defensive metabolites found in the consumed tissue (Awmack and Leather 2002). As previously stated, *Neogalerucella* typically aggregates following plant damage, a behaviour not only attributed to males' pheromone but also to enhanced food quality after herbivory because of increased availability of soluble carbohydrates, which is easier for the insect to assimilate (Hambäck 2010, Ferrarese and Garono 2011). Thus, it's possible that *L. salicaria* reallocates resources once attacked by herbivores (Hambäck 2010), which could benefit subsequent herbivores. Plants typically reallocate resources into reproductive parts after herbivory events or in the absence of their natural enemies (Valentine et al. 1983, Masters et al. 1993, Notzold et al. 1998). According to this concept, resource reallocation could potentially increase a flower bud's nutrient quality. If this mechanism occurs in *L. salicaria*, earlier *N. marmoratus* emergence could be favoured in the presence of *Neogalerucella* through improved larval food quality. Because I did not gather data on the lifetime reproductive success of emerging *N. marmoratus*, it is unclear whether or not it actually benefits from faster development in the presence of *Neogalerucella*.

Finally, it's important to note that, in insects, individuals with accelerated development may incur fitness costs during adulthood, such as smaller adult size, shorter lifespan and decreased

mating opportunities (Day et al. 2002, Gurney et al. 2003, Dmitriew 2011). For example, male *Telostylinus angusticollis* larvae who grew faster also reached sexual maturity quicker and had a shorter lifespan, preventing late-life reproduction and decreasing long-term survivorship (Hooper et al. 2017). Thus, the earlier emergence of *N. marmoratus* in this study is not necessarily an indication of a positive effect. Whether *N. marmoratus* developed faster due to phenology or to nutritional effects, a full study on lifetime success would be necessary to confirm if this acceleration translates into positive or negative impacts on overall fitness.

2.5.3 Experimental data: *Lythrum salicaria* reproductive success

Lythrum salicaria plants experimentally infested with *Neogalerucella* aborted significantly more flowers than control plants, which is consistent with previous findings (Blossey 1992, Notzold et al. 1998, Hunt-Joshi et al. 2004). Previous studies have speculated that damage by *Neogalerucella* should lead to an increased number of aborted flowers because of a reduction in resources available for seed maturation (Notzold et al. 1998, Hambäck 2010). Aborted flowers are flowers that did open, therefore were not attacked by *N. marmoratus*, but that did not produce any fruit. The number of seed capsules did not vary significantly between treatments but there was a trend towards fewer seed capsules in the presence of herbivory by *Neogalerucella*. The lack of significance can be attributed, once again, to the large variation in the number of seed capsules (Lindgren et al. 2001, Wilson et al. 2009) (see Table 2.3).

2.5.4 Experimental data: Pollinator observations

Herbivory can affect a plant's flowering phenology and floral display, which has implications for pollination (Swope and Parker 2012, Swope 2014, Russell-Mercier and Sargent 2015). Pollinator observations revealed significantly more pollinator visits to treatment plants (i.e., those exposed to feeding by *Neogalerucella*) relative to control plants (Table 2.4). This was true

even when the number of open flowers, which is often positively correlated with pollinator visitation (Swope and Parker 2012), was included as a covariate. The presence of *Neogalerucella* did not seem to affect the number of flowers produced by plants in this study, and the increase in pollinator visits was not accompanied by any increase in seed capsules. Because of my design, I was unable to determine whether *N. marmoratus* alone had any impact on pollinator visitation. However, *Neogalerucella*'s presence did appear to influence pollination, suggesting that *Neogalerucella* may influence pollinator foraging decisions. As previously mentioned, herbivory might cause the plant to reallocate resource into flower buds and possibly flowers, maybe even enhancing the reward provided to pollinators. Similarly, a different study found that herbivory had a positive impact on floral display and the number of flowers probed per observation period in *L. salicaria*, although again, this was not correlated with any change in fruit set (Russell-Mercier and Sargent 2015).

2.5.3 Conclusions

It has previously been speculated that *N. marmoratus* fitness would be negatively affected by the presence of *Neogalerucella* on the same host plant (Wilson et al. 2009, Winston et al. 2014). On the contrary, my results suggest that mild herbivory by *Neogalerucella* seems to accelerate the developmental phenology of *N. marmoratus*, leading to more adult emergence over the same time period. Unless there are detrimental effects related to faster development for *N. marmoratus*, it appears that the effect of *Neogalerucella*'s presence on *N. marmoratus* is neutral, or even beneficial. Although the goal of my study was to define the impacts of *Neogalerucella* on *N. marmoratus*, it is interesting to note that the presence of *Neogalerucella* also had few detectable negative consequences (i.e., more aborted flowers in the presence of *Neogalerucella* larvae) for the reproductive success of *L. salicaria*. On the contrary, mild herbivory appeared to positively

influence pollinator visitation, although there was no difference among treatments in terms of seed capsule number. It appears likely that, although the application of multiple species of biocontrol in this system does not seem to negatively impact the reproductive success of the individual biocontrol agents, the presence of multiple species may not add much in terms of actual biocontrol. It is important to note that each additional introduction of a biocontrol agent poses a potential risk to the health of native plant and insect communities, and that additional species should only be used in cases where the benefits are clear.

Table 2.1. Averages (±SE) of response variables representing the developmental success of *N. marmoratus* in the presence (treatment) or absence (control) of *Neogalerucella* larvae. Analyses were performed using ANCOVA models (Response ~ Treatment * Length * Plot). The total sum of squares (SS_T) and p-values are presented and were obtained from permutation tests (Critical p = 0.00001, maximum iterations = 100 000). Significant values are in bold.

Response (# of *N. marmoratus*)		**Presence of *Neogalerucella***	**Absence of *Neogalerucella***	**Effect of**		
				Treatment	**Length**	**Plot**
Total # of flower buds used		15.7 (±1.13)	14.7 (±1.33)	SS_T = 133 p = 0.208	SS_T = 4.78 x10^3 **p < 0.0001**	SS_T = 1.41 x10^3 **p < 0.0001**
Total # of emerged		5.48 (±0.54)	4.30 (±0.50)	SS_T = 87.9 **p = 0.0245**	SS_T = 431 **p = 0.00021**	SS_T = 279 **p = 0.00014**
Total # of non-emerged		10.2 (±0.83)	10.4 (±0.98)	SS_T = 4.54 p = 0.764	SS_T = 1.34 x10^3 **p < 0.0001**	SS_T = 438 **p = 0.00322**
Non-emerged	- # of Adults	5.51 (±0.50)	5.17 (±0.48)	SS_T = 12.6 p =0.380	SS_T = 548 **p < 0.0001**	SS_T = 70.3 **p = 0.0343**
	- # of Pupae	1.21 (±0.16)	1.42 (±0.22)	SS_T = 0.940 p = 0.562	SS_T = 34.9 **p < 0.0001**	SS_T = 4.67 p = 0.174
	- # of Larvae	3.46 (±0.42)	3.89 (±0.52)	SS_T = 0.920 p = 8.15 x10^3	SS_T = 423 **p < 0.0001**	SS_T = 97.3 **p = 0.0121**

Table 2.2. Averages (±SE) of response variables representing the reproductive success of *L. salicaria* in the presence (treatment) or absence (control) of *Neogalerucella* larvae and exposed to ambient level of *N. marmoratus* attacks. Analyses were performed using ANCOVA models (Response ~ Treatment * Length). The total sum of squares (SS_T) and p-values are presented and were obtained from permutation tests (Critical p = 0.00001, maximum iterations = 100 000). T-values, degrees of freedom (df) and their associated p-values are presented where permutation tests were not used. Significant results are in bold.

Lythrum salicaria reproductive success responses	**Presence of *Neogalerucella***	**Absence of *Neogalerucella***	**Effect of Treatment**	**Effect of Inflorescence Length**
Aborted Flowers	21.1 (±2.09)	15.6 (±1.23)	$SS_T = 1.31 \times 10^3$ **p = 0.0170**	$SS_T = 2.02 \times 10^3$ **p = 0.00287**
Seed capsules	86.1 (±6.11)	97.2 (±6.34)	$SS_T = 2.46 \times 10^3$ p = 0.171	$SS_T = 2.67 \times 10^5$ **p < 0.0001**
Aborted buds	35.7 (±2.38)	35.6 (±2.26)	$SS_T = 0.240$ p = 1.00	$SS_T = 408$ p = 0.327
Scars	96.5 (±4.45)	96.9 (±5.25)	t = 0.273 (df = 154) p = 0.785	t = 11.6 (df = 154) **p < 0.0001**
Inflorescence Length	11.5 (±0.48)	12.0 (±0.58)	t = 11.6 (df = 155) p = 0.507	N/A

Table 2.3. Effects of *Neogalerucella* presence (treatment) or absence (control) and ambient *Nanophyes marmoratus* on *Lythrum salicaria* pollinator visitation during 20-minute observation periods. Analyses were performed using ANCOVA models (Response ~ Treatment * Open * Day *Observer). Interactions were only kept in the model if they were significant. The total sums of squares (SS_T) and p-values are presented and were obtained from permutation tests (maximum iterations = 100 000, critical p = 0.00001). Averages (±SE) are presented for each treatment (presence or absence of *Neogalerucella* larvae). Significant values are in bold. Significant interactions were only present in the model for the number of visitors and are presented at the bottom.

Response	Presence of *Neogalerucella*	Absence of *Neogalerucella*	Treatment	Open Flowers	Day	Observer
Number of Visitors	1.85 (±0.28)	1.49 (±0.28)	SS_T = 9.60 **p = 0.0185**	SS_T = 74.9 **p < 0.0001**	SS_T = 0.0210 p = 1	SS_T = 22.3 **p = 0.00038**
Average flowers probed per visit	1.86 (±0.27)	1.39 (±0.28)	SS_T = 4.42 p = 0.139	SS_T = 92.1 **p < 0.0001**	SS_T = 0.435 p = 0.650	SS_T = 6.96 p = 0.650
Number of open flowers at observation time	12.7 (±1.74)	12.9 (±1.44)	SS_T = 0 p = 1	-	SS_T = 431 **p = 0.0393**	SS_T = 46.2 p = 0.50

Day*Observer (SS_T = 11.7; **p = 0.00745**)

Treatment*Day (SS_T = 11.8; **p = 0.00859**)

Treatment*Observer (SS_T = 8.52; **p = 0.0249**)

Open Flowers*Observer (SS_T = 7.48; **p = 0.0286**)

Table 2.4. Effect of location (east plot or west plot) on *N. marmoratus* developmental success. Average (±SE) response variables representing the developmental success of *N. marmoratus* in the two plots are presented. Analyses were performed using two-way ANCOVA models (Response ~ Treatment * Length * Plot). The total sums of squares (SS_T) and p-values are presented and were obtained from permutation tests (Critical p = 0.0001, maximum iterations = 100 000) in order to account for abnormal and heteroscedastic data. Significant values are in bold.

Response (# of *N. marmoratus*)		West plot	East plot	Effect of Plot
Total # of flower buds used		12.2 (±1.00)	17.0 (±1.22)	$SS_T = 1.42 \times 10^3$ **p < 0.0001**
Total # of emerged		3.48 (±0.49)	5.74 (±0.50)	$SS_T = 279$ **p = 0.00014**
Total # of non-emerged		8.71 (±0.74)	11.2 (±0.91)	$SS_T = 438$ **p = 0.00322**
Non-emerged	- # of Adults	4.73 (±0.46)	5.71 (±0.48)	$SS_T = 70.3$ **p = 0.0343**
	- # of Pupae	1.15 (±0.18)	1.42 (±0.18)	$SS_T = 4.67$ p = 0.174
	- # of Larvae	2.92 (±0.38)	4.12 (±0.48)	$SS_T = 97.3$ **p = 0.0121**

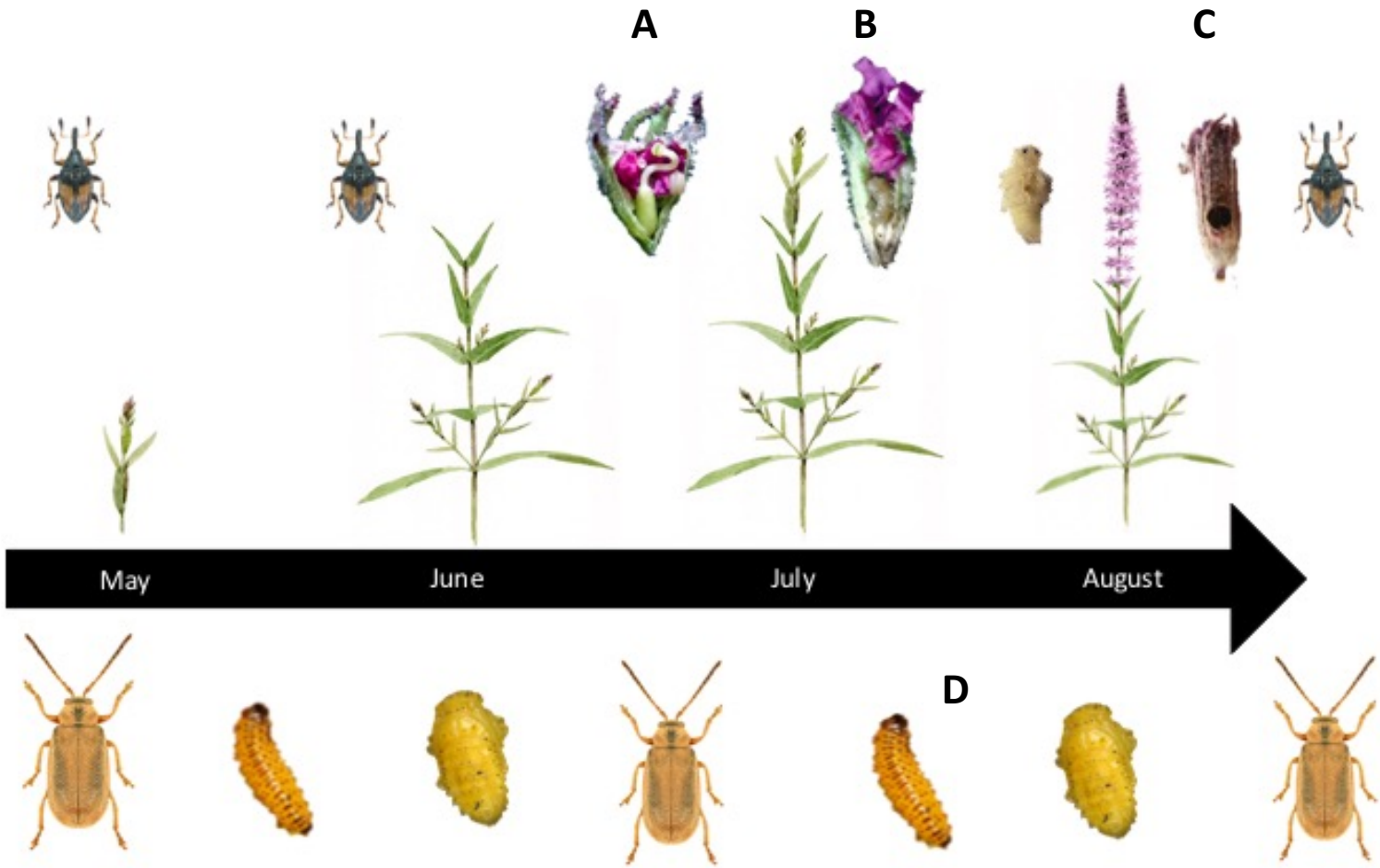

Figure 2.1. Timeline showing *N. marmoratus* (top) and *Neogalerucella* (bottom) lifecycles in Southeastern Ontario. *N. marmoratus* emerges as adult in May and feeds on foliage until flower buds are available for oviposition (a) from mid-June until mid-July (before *L. salicaria* flowering period). The larvae develop in the ovary (b), pupates and emerges as adult between mid-July until end of August, leaving a circular hole in the used bud when emergence was successful (c). In Southern Ontario, *Neogalerucella* will typically have two generations, where the second generation larvae (d) feeds on the developing flower buds, overlapping the oviposition and larvae development of *N. marmoratus*.

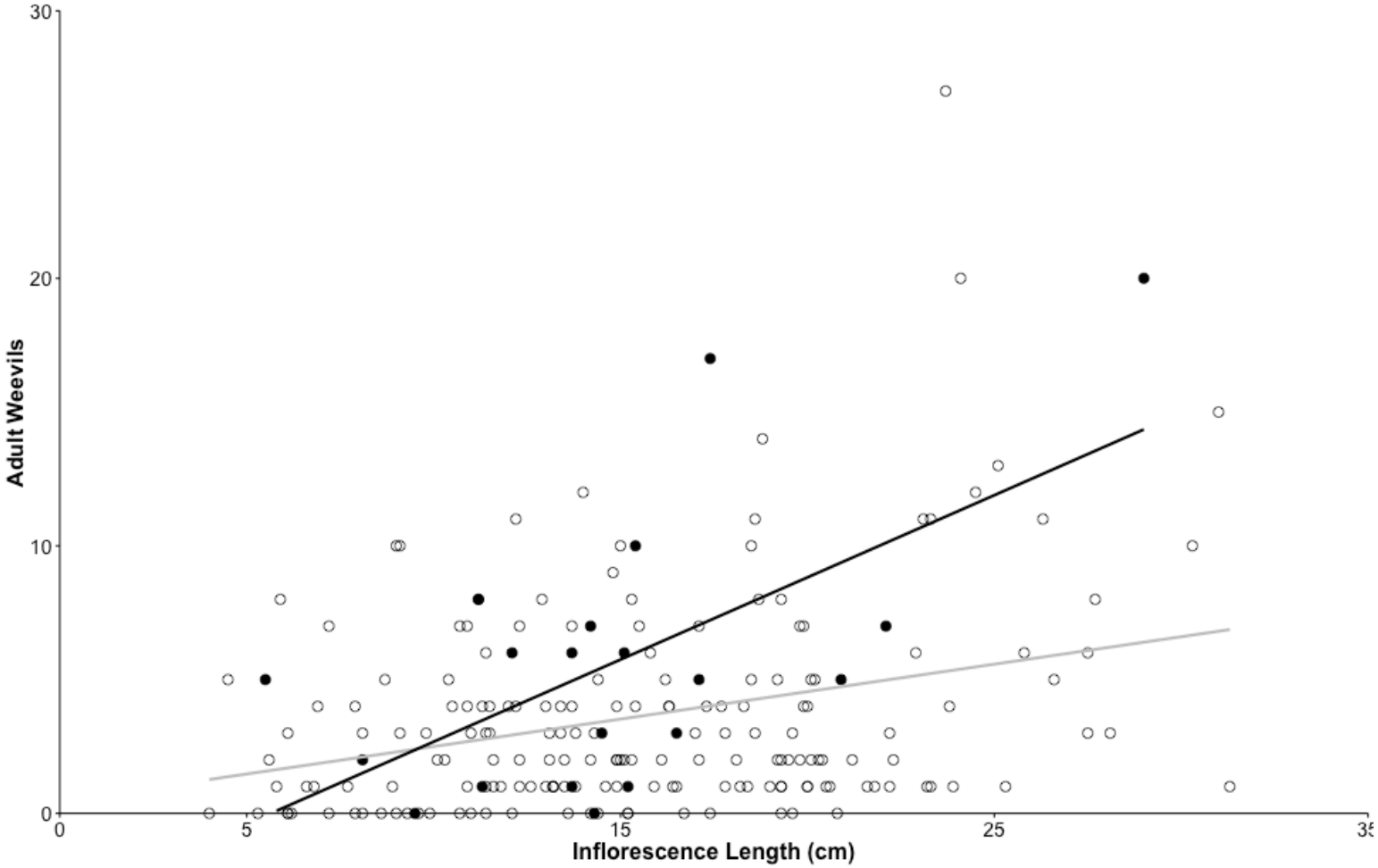

Figure 2.2. Observational data. The number of adult *N. marmoratus* in relation to inflorescence length. Significantly more *N. marmoratus* emerged in the presence of *Neogalerucella* larvae (filled circles, black line) ($N_{present}$ = 20; p = 0.0119), while fewer adult *N. marmoratus* were recorded in the absence of *Neogalerucella* larvae (N_{absent} = 180; open circles, grey line). Both regressions show a significant correlation between the number of *N. marmoratus* and inflorescence length (p = 0.0006). The interaction between the presence of *Neogalerucella* and inflorescence length was also significant (p = 0.0404).

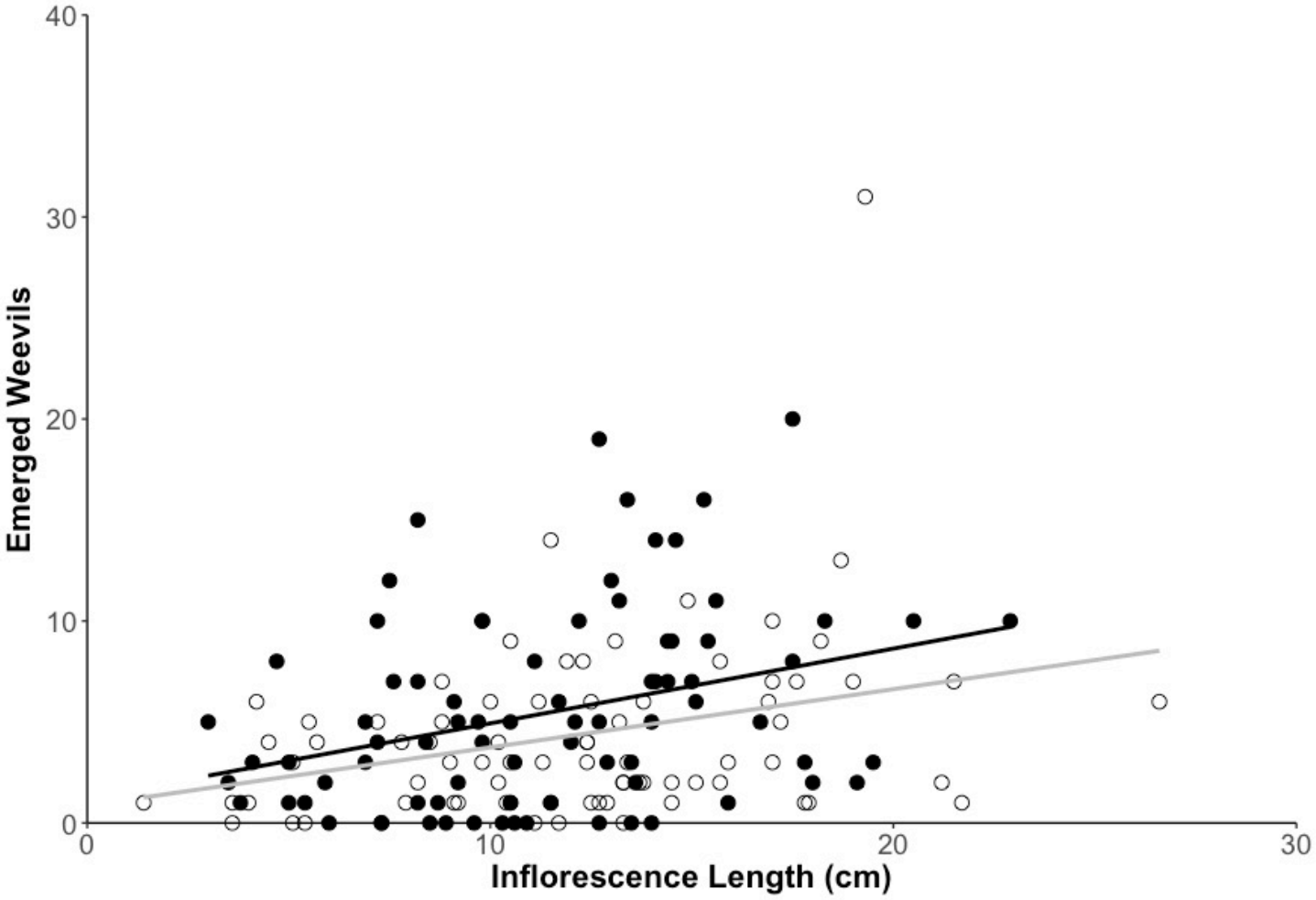

Figure 2.3. Experimental data. The number of *N. marmoratus* that successfully emerged in relation to inflorescence length (in centimeters). Significantly more *N. marmoratus* emerged in the presence of *Neogalerucella* larvae ($N = 80$; filled circles, black line; $p = 0.0245$). Less emerged *N. marmoratus* were recorded in the absence of *Neogalerucella* larvae ($N = 78$; open circles, grey line). Both regressions show a significant correlation between the number of emerged *N. marmoratus* and inflorescence length ($p = 0.00021$).

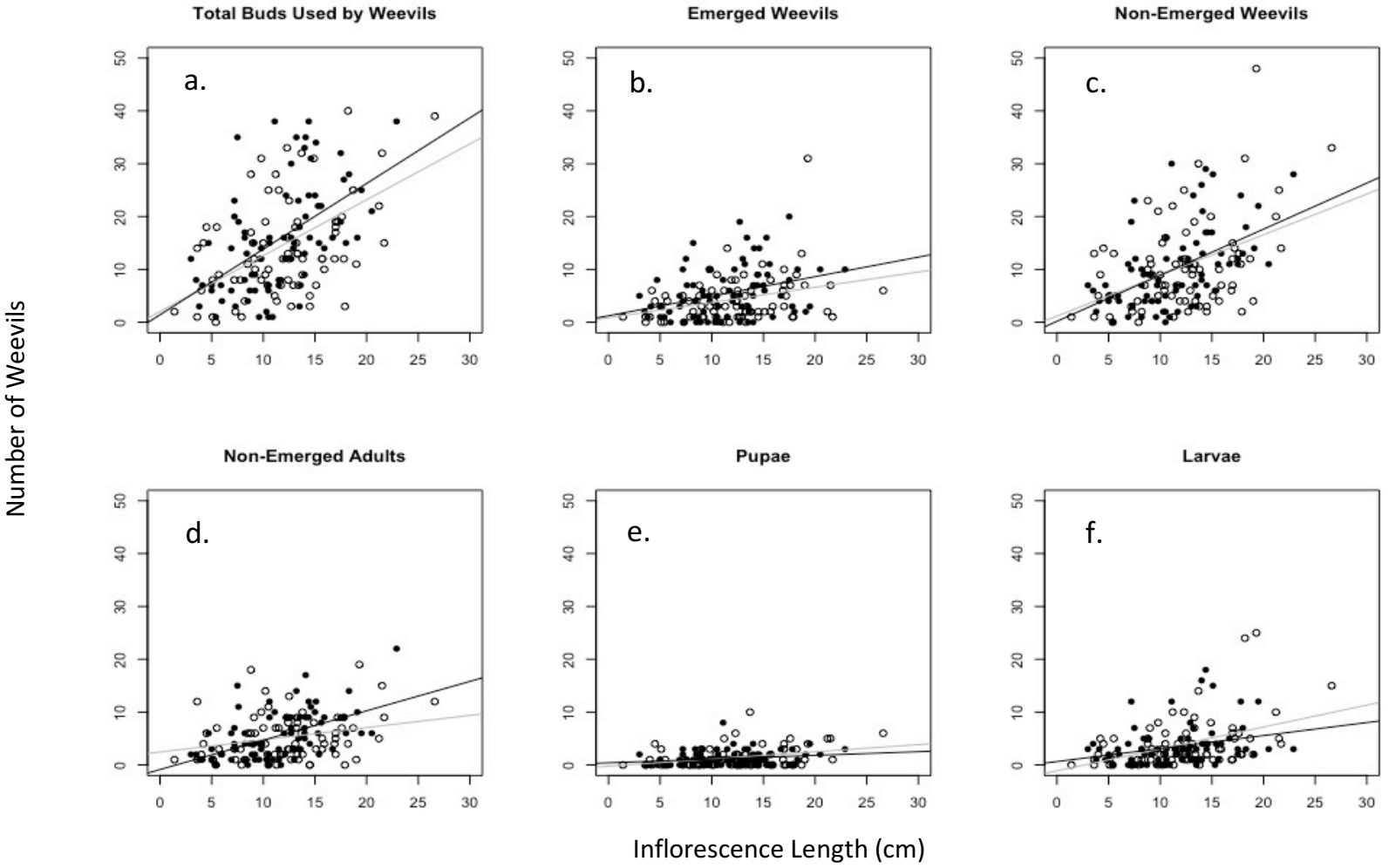

Figure 2.4. Experimental data. The correlation between the different response variables and inflorescence length were all significant. The treatment (presence of *Neogalerucella* larvae) is represented by filled circles and black lines ($N = 80$); the control is represented by open circles and grey lines ($N = 78$). The p-values presented refer to the correlation between the number of N. marmoratus and the covariate inflorescence length, regardless of treatment. a) The total number of flowers buds used by *N. marmoratus* ($p < 0.0001$). b) The number of *N. marmoratus* that successfully emerged as adults, leaving a circular hole in the flower bud ($p = 0.00021$). c) The number of *N. marmoratus* that did not complete their lifecycle, i.e., those that were found as non-emerged weevils in egg, larvae or pupal stage ($p < 0.0001$). The non-emerged weevils were divided into the following categories: d) The number of non-emerged adults ($p < 0.0001$). e) The number of pupae ($p < 0.0001$) and f) The number of larvae ($p < 0.0001$).

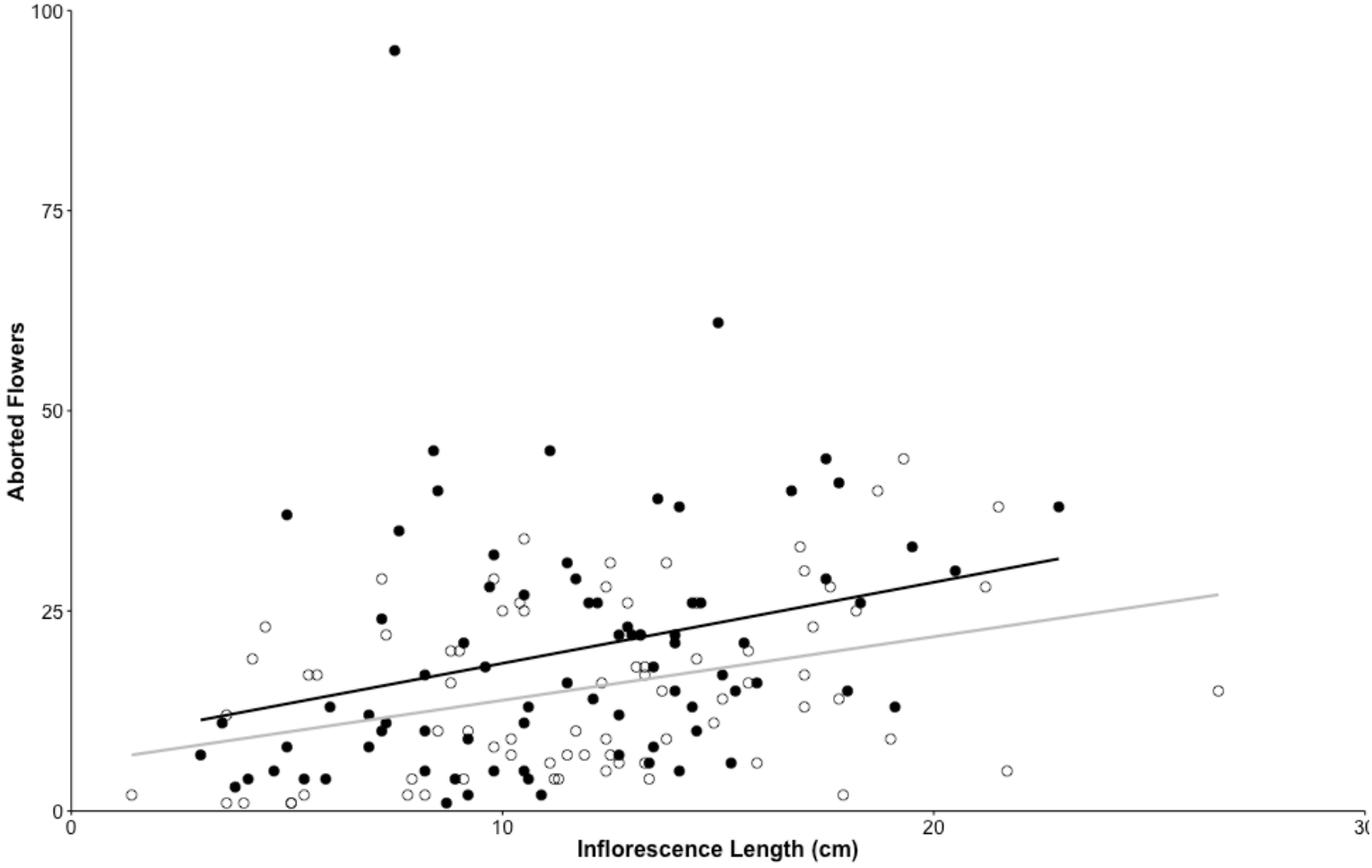

Figure 2.5. Experimental data. In the presence of *Neogalerucella* larvae (*N* = 80; filled circles, black line), significantly more flowers aborted flowers were recorded (p = 0.0170), while fewer aborted flowers were recorded in the absence of *Neogalerucella* larvae (*N* = 78; open circles, grey line). Both regressions show a significant correlation between the number of aborted flowers and inflorescence length (p = 0.00287).

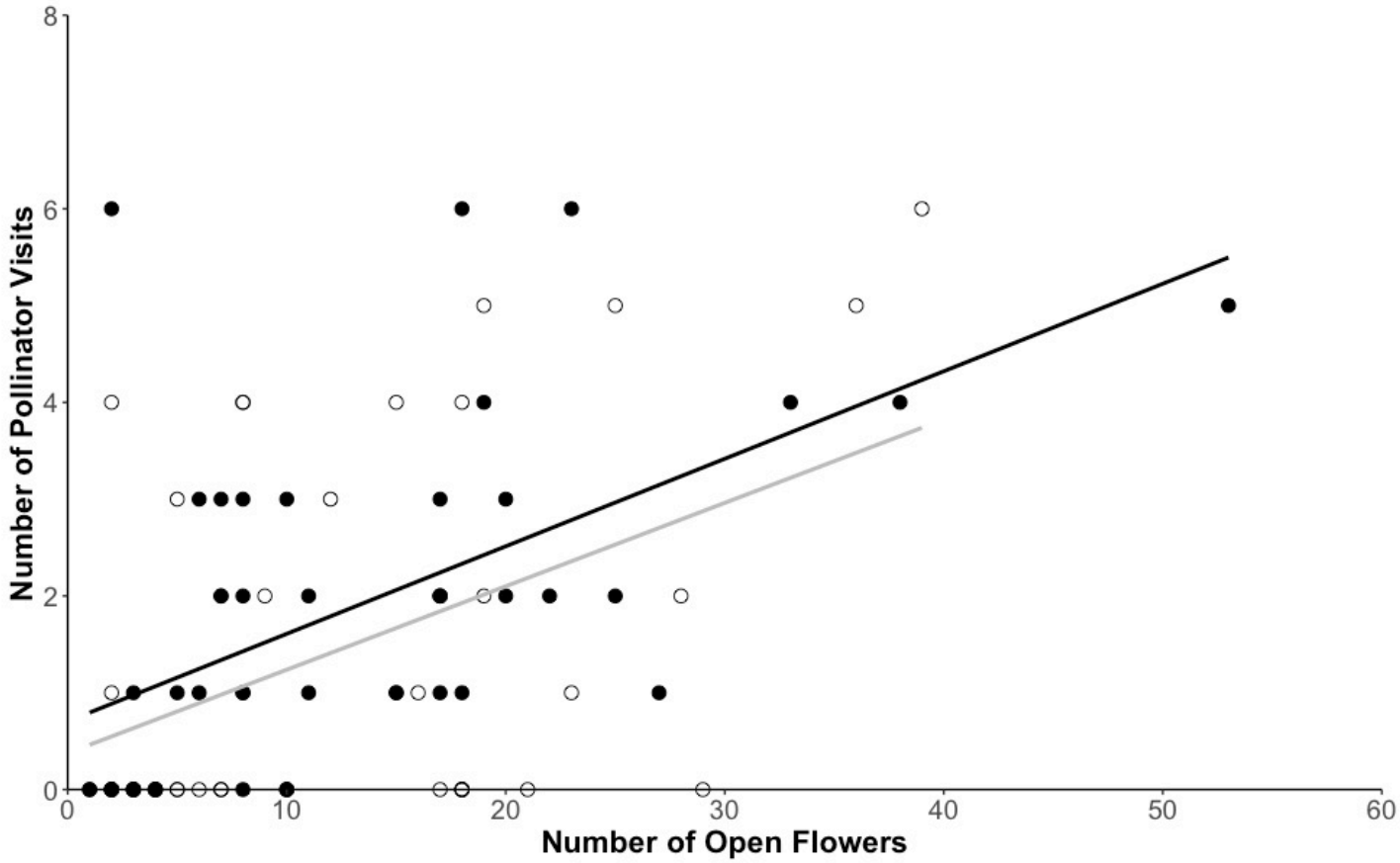

Figure 2.6. Experimental data. The number of pollinator visits per inflorescence in relation to inflorescence length, analyzed with an ANCOVA model by using a permutation technique (max iterations 100 000, critical p = 0.00001). The effect of the presence of *Neogalerucella* larvae (*N* = 41; filled circles, black line) on the number of visits was significant (p = 0.0185). Fewer visitors were recorded in the absence of *Neogalerucella* larvae (*N* = 43; open circles, grey line). Both regressions show a significant correlation between the number of pollinator visits and the number of open flowers (p < 0.0001).

CHAPTER 3: Conclusions and Future Directions

Although several sources have promoted the introduction of multiple biocontrol agents to control purple loosestrife following the 'cumulative stress hypothesis', no evidence for enhanced biocontrol with multiple agents present has been previously observed (Blossey and Schroeder 1995, Hunt-Joshi et al. 2004). Accordingly, my study does not support the idea that biocontrol is enhanced when both *Neogalerucella* and *N. marmoratus* are present, although it does suggest that *Neogalerucella* has no direct, negative impact on the fitness of *N. marmoratus*.

3.1 Future Directions

3.1.1 Experimental Design

A fully factorial design would have improved my ability to better understand the independent and combined effects of each species of biocontrol agent on each other, and their impacts on *L. salicaria*'s reproductive success. Unfortunately, covering the plants, which would have been necessary to keep them free of *N. marmoratus* attack, would have disallowed pollinator access to the experimental plants. Since pollination is critical to *N. marmoratus* reproductive success, only *Neogalerucella* could be transplanted, leaving out the treatment options of 'no herbivore' and '*Neogalerucella* alone', and hampering my ability to explore the effects of each herbivore alone, and in combination. A fully factorial design would help verify whether purple loosestrife biocontrol conforms to the Cumulative Stress hypothesis, with more plant damage observed when both beetles are present as opposed to only one. On the other hand, if damage is not cumulative, the only reason to introduce multiple biocontrol agents is if there is evidence to support the Lottery Model. My data do not support the Cumulative Stress Hypothesis, and since *Neogalerucella* is largely agreed to be the more effective biocontrol, there is no strong argument to introduce multiple agents to control purple loosestrife.

Another important factor that should be considered in future studies is that there are likely differences among sites that could impact the outcome of biological control. Indeed, differences in defence mechanisms against herbivores, including the amount of phenolics in the foliage and tolerance for browsing, have been observed to follow a latitudinal cline (Colautti and Barrett 2013, Anstett et al. 2015, Lehndal and Agren 2015). Because plant resistance often varies with latitude, competition among biocontrol agents may vary as well. Information about site fertility could help to understand whether a particular site exhibits top-down or bottom-up effects (Hovick 2015). Flowering phenology may also differ across sites, especially those at different latitudes (Colautti and Barrett 2013), but also on a smaller scale. For the purpose of this study, I selected plants exhibiting similar phenology, but surveys should be designed that examine whether flowering phenology impacts the development patterns of *N. marmoratus*.

3.1.2 Additional Information Required

Information about the chemical compounds in tissue of *L. salicaria*, especially in the flower buds, prior to and following herbivory by *Neogalerucella* could help determine whether *N. marmoratus* is more attracted to plants experiencing herbivory. Testing the attractiveness of dimethylfuran lactone (the pheromone produced by *Neogalerucella*) to *N. marmoratus* could also clarify whether *N. marmoratus* uses this pheromone to choose plants (Hambäck 2010). Choice experiments could be used to determine *N. marmoratus'* preferences. An experimental study evaluating the floral reward in the presence or in the absence of leaf beetle larvae would help verify if and why pollinators are more (or less) attracted to plants attacked.

Starting the same experiment earlier in the summer could intensify the response obtained in the present study. Indeed, early feeding by *Neogalerucella* can lead to overcompensation responses in *L. salicaria*, where more inflorescences are typically observed (Agrawal 2000). The

increased availability of flower buds per plants might attract even more *N. marmoratus*. A follow-up study about the true fitness of *N. marmoratus* could complement the experiment to understand if the development occurs faster and if this acceleration result in negative or positive impacts for the flower weevil.

3.1.3 *Nanophyes marmoratus* parasitism

In more than 20% of buds sampled, a wasp suspected of being parasitic on *N. marmoratus* was found. Plant signals, such as volatile compounds, can advertise the presence of a herbivore host to parasites (Denno et al. 1995, Pare and Tumlinson 1999, Denno and Kaplan 2007). The wasps were often found inside the flower buds where an *N. marmoratus* pupal chamber was present but where no *N. marmoratus* larvae, pupae or adult was found, suggesting the wasp ate the developing *N. marmoratus*, the latest being able to reach pupal stage only because a pupal chamber was found. Some wasps were also found among the inflorescence material that had fallen into the experimental cones. No previous evidence on the parasitism of *N. marmoratus* in North America has been reported. While I was unable to identify the specimen to species, the wasp genus has been identified as *Pteromalus* (Chalcidoidea: Pteromalidae) (Dr. Gary A.P. Gibson pers. comm.). *Pteromalus* parasitism on the genus *Nanophyes* has only been reported once in India by Sankaran and Krishna (1967). Unfortunately, because this is an incidental discovery and was not the focus of the study, the data are insufficient to clearly report whether *N. marmoratus* is more affected by the potential parasitic wasp in the presence of *Neogalerucella* or not. Evaluating the interaction between both biocontrol agents in the context of parasitism should be a goal of future studies, including studies that explore the effects on the plant and the biological control success. Moreover, several *Neogalerucella* larvae were infected by mites at the site (M. Torreblanca pers. obs.) and these larvae could not be avoided when choosing the second instar larvae that would contribute to

the experimental treatment. Finally, parasitism could alter the interaction relationship between the two biocontrol agents, changing the previous beliefs about both the effects of *Neogalerucella* on *N. marmoratus* and the efficiency of them on the biological control of *L. salicaria*.

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