DISSERTATION

PLANT-MEDIATED INTERACTIONS AMONG GALL FORMING INSECTS

Submitted by

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ABSTRACT

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Russian knapweed (*Rhaponticum (=Acroptilon) repens* (L.) Hidalgo) is one of the most troubling exotic weeds throughout the western United States invading many public and private lands. A classical biological control agents, the gall midge *(Jaapiella ivannikovi* Fedotova*)*, is a parasite on Russian knapweed, forming galls on the plant, inside which *J. ivannikovi* broods feed and develop. This system provides an opportunity to consider plant-mediated interactions between midge individuals and considered the merits of integrating other weed management techniques (grazing, mowing, and chemical control before insect release) with biological control. To accomplish this, I conducted releases of gall midge agents at replicated sites throughout Colorado, field cage experiments with simulated grazing, and greenhouse studies. We found that *J. ivannikovi* initially established across Colorado, however, failed to maintain populations in subsequent years. Interestingly, grazing increases *J. ivannikovi* establishment. Of further note, *J.ivannikovi* broods compete with one another even when feeding on different parts of the plant. The results of these experiments indicate that integrating management techniques can increase biocontrol agent establishment. I also question how effective this midge is at reducing Russian knapweed flowering and vegetative growth in the field. This research resulted in recommendations regarding the most judicious use of *J. ivannikovi* biocontrol agents in terms of where and when agents are most effective for management, and practitioners at the Colorado Department of Agriculture Palisade Insectary have adjusted their strategies accordingly.

ACKNOWLEDGEMENTS

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Chapter 1 - INTRODUCTION

Integrated Pest Management

When conducting biological control agent releases, managers often avoid areas that have already experienced an agent release, recent grazing/mowing, or recent spraying with chemical controls (personal communication Dan Bean). In theory, previously unmanaged sites are more likely to have vigorously growing plant tissue that should be favorable for agent establishment. However, previous management regimes such as grazing may enhance insect agent establishment by stimulating regrowth suggesting that combinations of management approaches may result in improved control of invasive plants.

Whether the prospects for successful biological control of a pest species is enhanced by the presence of other organisms (such as cattle) remains one of the most enduring questions in 'classical' (introduction) biological control (Myers et al., 1989; Denoth et al., 2002; Stephens et al., 2013). Conducting multiple intra- or interspecific releases of biocontrol agents and/or using mammalian grazers alongside insects may increase the likelihood of successful control of a weed when herbivores act in a complementary fashion. For instance, herbivores may attack different parts or growth stages of the pest (Paredes et al., 2015), are active at different times of the year, or are active in different parts of the pest's geographic range (Letourneau et al., 2009). Alternatively, using multiple management techniques may provide some level of redundancy, safeguarding against the possibility that a management technique fails due to local or regional environmental factors.

Employing multiple management techniques increases the odds that at least one will be successful. Such a "lottery" approach to management is no longer feasible given the potential for negative interactions between techniques that may reduce the overall efficacy of management

efforts (Ehler & Hall, 1982; Denoth et al., 2002). In most systems, far too little is known about how individuals interact in the field (Strauss & Irwin, 2004; Shea et al., 2005; Stephens et al., 2013; Stephens & Meyers, 2014; Milbrath & Nechols, 2014), making it difficult to assess the contribution of each management technique to the control of the pest population. In particular, understanding the relationship between plant traits and biocontrol agent interactions is especially relevant for biocontrol agents of weeds. Use of multiple management techniques may indeed be the best course of action, but careful experimental studies must be conducted to examine how management techniques interact in the field to ensure that releases of biological control agents are both safe and effective.

Most studies of IPM, particularly those integrating mammalian grazing with biocontrol releases, fail to acknowledge the potential for herbivores to interact with one another even though interactions such as competition or facilitation are likely (Stephens et al., 2013). Two or more individuals or species may directly (e.g., intraguild predation; Rosenheim et al., 1995) or indirectly interact with each other by altering the quality of the host for the other species or individual. For instance, indirect competition may occur if attack by one species may induce expression of defenses by the host (the target pest), which makes it more difficult for a second species to survive on the host (Bezemer et al., 2003). Plant responses to herbivory depend on the evolutionary context of what initial herbivory indicates for the plant's risk of subsequent herbivory (Karban 1999). Such plant-mediated interactions may be widespread (Milbrath & Nechols, 2014).

Through my dissertation, I examine the strength and direction (negative, positive, or neutral) of intraspecific interactions between insect individuals, along with mammalian grazer-insect interactions, and the consequences for effective control of an invasive weed. I did this with

the goal of answering the question of whether the use of multiple management techniques results in more effective control of the target invasive plant. My study system involves a gall-forming midge, *Jaapiella ivannikovi* Fedotova (Diptera: Cecidomyiidae), that was recently approved for release against Russian knapweed (*Rhaponticum (=Acroptilon) repens* (L.) Hidalgo) (Asteraceae, Asterales), one of the most serious invasive weeds in North America. *Jaapiella ivannikovi* is a gall forming insect that attacks the apical meristems that would otherwise develop into flowers, resulting in a strong potential for herbivore interaction on the same host plant. Because the midge has only been relatively recently approved for release (USDA APHIS, 2009), I was in an excellent position to manipulate the release of gall-forming biological control agents with other management techniques.

Study System

Russian knapweed *R. repens* is a widespread invasive weed throughout North America. It can grow on a wide range of soil types and moisture conditions and does particularly well in recently disturbed soils; it generally does not invade healthy, intact, native habitats (Zouhar, 2001). Patches of Russian knapweed that do occur in otherwise diverse, native vegetation tend to be much smaller with lower germination rates in comparison to clones in barren, disturbed soils (Barosh & Ode, unpublished data). This plant is impractical to control because of its extensive root system with which it can reproduce asexually. Furthermore, Russian knapweed is considered to be allelopathic (Stermitz et al. 2003), contributing to its ability to grow into large infestations crowding out other vegetation. Similar to many invasive weeds, Russian knapweed stands are denser in invaded North America compared to its native habitat in western Asia (e.g., Turkey; unpublished data cited in Djamankulova et al., 2008). Consequently, Russian knapweed

is a stronger competitor than many native North American plants (Ni et al., 2010; Callaway et al., 2012). Russian knapweed infestations in wheat can result in dramatic yield reductions (50-90% depending on density of *R. repens*; Streibig et al., 1989). While seedling establishment appears to play a minor role in established clones, it is likely the primary means of colonizing new sites or sites at the periphery of an established patch (Djamankulova et al., 2008). Each ramet can produce upwards of 1200 seeds, which can remain viable in the seed bank for up to 5 years (Anderson, 1993). Controlling seed production may prove to be crucial in slowing the spread of this noxious weed. Mechanical removal and use of herbicides to control large Russian knapweed infestations impractical and unsustainable (Jones & Evans, 1973; DiTomaso, 2000).

Two biocontrol agents have been recently approved for release against this weed: the gall midge *Jaapiella ivannikovi* Fedotova (Diptera: Cecidomyiidae) and the stem galling wasp *Aulacidea acroptilonica* V.Bel. (Hymenoptera: Cynipidae) (USDA APHIS, 2008 & 2009). Throughout this dissertation, I focus on *J. ivannikovi*. While I conducted pilot studies on both insects, future work will further address *A. acroptilonica*. Both agents are highly restricted to Russian knapweed*,* as they require this specific host plant to form the bloated plant tissue (galls) in which larvae develop. *Jaapiella ivannikovi* adults are short-lived (2-7 days). Galls contain on average 14 larvae with a 1:1 offspring sex ratio within individual galls (suggesting panmixis), which are formed in the apical and lateral meristems (Djamankulova et al., 2008). However, Colorado Department of Agriculture employees report female biased sex ratios and up to 50 individuals per gall (Price, personal communication). *Jaapiella ivannikovi* has an average of four generations per year (Djamankulova et al., 2008). Because the midge is multivoltine and active through much of the summer, young knapweed shoots are susceptible to attack throughout the growing season. Both gall-forming insect species have been shown to reduce growth,

aboveground biomass, and seed output of Russian knapweed (Djamankulova et al., 2008). In a field experiment conducted in its native range in Uzbekistan, *J. ivannikovi* was found to reduce shoot length by 10-15%, aboveground biomass by 20-25%, and seed output by 90-95% (Djamankulova et al., 2008). Similar results have been documented in Wyoming (Collier et al. 2006 & 2007). Maximum *J. ivannikovi* densities in the field are up to 15 galls per shoot but less than 10% of shoots harbor galls in the most heavily attacked populations (Djamankulova et al., 2008). In turn, gall-forming herbivores occasionally can suffer substantial rates of parasitism by a suite of eupelmid, eurytomid, and torymid wasp parasitoids (unpubl. data cited in Djamankulova et al., 2008; Collier et al., 2006). Parasitism rates approach 70% for *J. ivannikovi* and 85% for *A. acroptilonica* in the native range (Djamankulova et al., 2008). Whereas similar parasitoids have been found on *A. acroptilonica* in the US, albeit at much lower parasitism rates (Tim Collier, personal communication), no parasitoids have been found on *J. ivannikovi* in the US to date.

Plants Mediate Insect Interactions

Plant-mediated indirect interactions among herbivores are widely appreciated to be an important force in many plant–insect herbivore systems (Denno et al., 1995; van Veen et al., 2006; Denno & Kaplan, 2007). Unfortunately, this phenomenon is poorly documented for weed biological control programs (Milbrath & Nechols, 2014). Virtually nothing is known about how biological control agents, such as individual *J. ivannikovi*, interact with one another as well as how establishment success is affected by other management practices, either directly or indirectly. Yet, such information is vital to predict the outcome of releasing agents in the field.

Gall forming insects can interact with each other or large grazing herbivores indirectly through plant mediated interactions. For example, herbivory can induce or suppress chemical defenses in ways that affect feeding by subsequently visiting herbivores. Further, galls are well known to influence physiological and morphological aspects of their host plants (McKone et al., 2001; Gagné & Hibbard, 2008; Hall et al. 2012). Two gall-forming insects attacking the same plant may interact with each another through modifying their host plant's physiology and growth. Gall insects commonly increase auxin and cytokynin levels, hormones which are involved in plant metabolic sinks (Erb et al., 2012). In this way, insect galls act as metabolic sinks and compete with other sinks such as developing fruits, meristematic tissues, or even other galls. Multi-sink competition may occur most powerfully when galls occupy nearby and similar plant tissue (Inbar et al., 1995; Kaplan et al., 2011). Plant metabolic sinks can provide high quality nutrients and tissues for herbivorous insects. For instance, sap-feeders such as aphids can benefit from occurring near plant metabolic sinks, including galls (Forrest, 1971; Kidd et al., 1985). Similarly, many gall-forming insects attack near or at plant metabolic sinks, such as rapidly growing, meristematic plant material (*J. ivannikovi* and *A. acroptilonica:* Djamankulova et al., 2008) or reproductive nodes (Gagné, 2014). Despite these patterns, no studies of which I am aware have demonstrated that a gall-forming insect may facilitate another galling insect of the same or of different species when attacking a mutual host plant.

Rationale and Significance

Integrated pest management would be justified if grazing, mowing, and/or spraying alongside the use of biological control reduces Russian knapweed growth and/or reproduction. If only one management technique is primarily responsible for Russian knapweed control, then it is

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prudent to only use that method. This assessment depends on a thorough understanding of how individual *J. ivannikovi* midges interact with their host plant as well as how they directly or indirectly interact with other management practices, such as grazing. Therefore, the central aim guiding our research is to explore the mechanisms underlying the interactions among gall-forming herbivore conspecifics, other management techniques, and Russian knapweed.

A deeper understanding of the *J. ivannikovi*–Russian knapweed system could lead to more effective and responsible biological control practices. Determining judicious use of biological control agents could result in decreased use of chemical controls. Reducing chemical run-off and pollution would aid in protecting natural resources, including native plants and water sources. This research will lead to recommendations regarding the most judicious use of this biocontrol agent in terms of whether agent releases can be combined with other management techniques, including the use of other biocontrol agents such as *A. acroptilonica* against Russian knapweed. Sharing findings with landowners and weed county managers will allow for more informed decisions on Russian knapweed management.

Chapter 2 - INSECT BIOLOGICAL CONTROL AGENT FORMS GALLS ACROSS

HABITATS, BUT FAILS TO ESTABLISH LONG TERM

Summary

Russian knapweed, *Rhaponticum (=Acroptilon) repens* (L.) Hidalgo, (Asteraceae: Asterales), is a widespread, invasive plant found throughout crop and rangelands as well as many riparian corridors throughout the western United States. Russian knapweed invaded the US over 130 years ago, yet biological control of this troublesome weed has received little attention until recently. One biological control agent, the gall midge *Jaapiella ivannikovi* Fedotova (Cecidomyiidae: Diptera), was approved for release in 2009. I released midges at 23 sites throughout Colorado in the spring of 2015. By the end of summer 2015, I found that midges successfully galled plants at nearly half of the sites (11/23). Gall formation was particularly successful at sites that had been recently grazed (8/10), presumably because grazing stimulates the production of meristematic growth favorable to the production of midge galls. Insect initial establishment was also high at moist sites along flood zones and edges of wetlands. However, midges successfully overwintered and galled new plants the second year in only 25% of sites (6/23); four years after the initial releases, midges were present at less than 10% of sites (2/23). Sites where midges persisted one or more years tended to be wet or regularly grazed. Releases on cool days and in the densest areas of Russian knapweed also resulted in greater initial midge establishment. Given low establishment rates and minimal impacts on Russian knapweed, repeated releases of *J. ivannikovi* may be necessary to enhance long-term establishment success.

Introduction

Understanding the nature of interactions between biological control agents and their hosts across variable landscapes, and how these interactions ultimately affect the success of classical weed biological control programs have been long-standing issues in biological control (Smith 1929). Russian knapweed, *Rhaponticum (=Acroptilon) repens* (L.) Hidalgo, (Asteraceae: Asterales), is one of the most persistent invasive weeds in North America, infesting farms and rangelands throughout western North America. Russian knapweed is found in dry to wet habitats, as well as managed and natural areas. Russian knapweed can grow on a wide range of soil types and moisture conditions and does particularly well in recently disturbed soils. Russian knapweed generally does not invade intact, native habitats (Zouhar, 2001). Therefore, determining effectiveness of weed management strategies under a range of environmental conditions is important. In part, this plant is difficult to manage because it has an extensive root system through which it can propagate vegetatively. Each aboveground ramet can produce upwards of 1200 seeds, which can remain viable in the seed bank for up to five years (Anderson, 1993). Controlling Russian knapweed seed production and vegetative reproduction across the western United States provides a significant challenge.

Mechanical removal and use of herbicides over vast areas of infestation are impractical and unsustainable (Jones & Evans, 1973; DiTomaso, 2000). Herbicides are expensive, can contaminate groundwater, and can have non-target impacts on native forbs and grasses (Benz et al., 1999; Sheley et al., 2007). Furthermore, chemical treatment of Russian knapweed can result in bare ground or other weeds expanding into the area (Benz et al., 1999). Mechanical removal is difficult and rarely effective due to the extensive root system of Russian knapweed and its potential for quick regrowth (Duncan et al., 2003). An alternative to chemical and mechanical

treatments, biological control is the use of a specialist insect herbivore to control the target weed. The judicious use of biological control agents could result in decreased use of chemical controls and intensive mechanical removal. Reducing chemical run-off and disturbance would aid in protecting natural resources, including native plants and water sources.

Gall-forming insects are increasingly selected as biological control agents because they tend to be very host-specific and have relatively few non-target impacts. These insects induce their host plants to form the bloated plant material called a gall, where the immature insects develop and feed. The gall midge *Jaapiella ivannikovi* Fedotova (Diptera: Cecidomyiidae) is a recently approved biological control agent for Russian knapweed (USDA APHIS 2009). The midge is multivoltine and active through much of the summer attacking meristems on young knapweed shoots throughout the growing season. In its native range in Uzbekistan, the gall midge reduces shoot length by 10-15 percent, aboveground biomass by 20-25 percent, and seed output by 90-95 percent (Djamankulova et al., 2008). Maximum midge gall densities in the field in their native range are up to 15 galls per aboveground shoot but less than 10 percent of shoots harbor *J. ivannikovi* even in the most heavily attacked populations (Djamankulova et al., 2008). Similar observations have been documented in Wyoming where *J. ivannikovi* has been released (Meyers et al., 2015). Rainfall and grazing or mowing a site before release may increase meristematic growth (DiTomaso, 2000) and, therefore, midge insect establishment because *J. ivannikovi* needs meristematic tissue for gall formation. Targeted grazing has been shown to increase biological control agent establishment in other systems (Lym, 2005; Wilson et al., 2008).

Previous management or integrative management techniques likely impact biological control. Further, the establishment rate of the Russian knapweed gall midge when released at

field sites in North America is unknown. Therefore, I conducted releases at sites across Colorado to address how previous management strategies, release methods, and abiotic site characteristics impact Russian knapweed gall midge initial establishment. I also considered rates of midge overwintering and population persistence after three years.

Methods

Identifying Knapweed Sites

Rhaponticum repens occurs throughout much of Colorado, providing the opportunity to study the colonization capacity of *J. ivannikovi*. In collaboration with the Colorado Department of Agriculture, county weed managers, private landowners, and federal land managers, I chose 23 knapweed infestations across Colorado (Figure 2.1) occurring in the Front Range (Weld County and Adams County), Grand Junction (Mesa County), the San Louis Valley (Alamosa County), Archuleta County, and the Arkansas Valley (Las Animas County). I selected release sites such that each have a minimum infestation size of 15 m across and are at least two kilometers away from one another to reduce potential for insect travel between sites.

Releasing Agents

Because midges are short lived as adults (typically 2-7 days under field conditions; Djamankulova et al. 2008), the typical release method is to place a bouquet of galls in the center of the release site so that adults can emerge, mate, and attack apical and lateral meristems in the field. Emerging midges have a reported 1:1 sex ratio with up to 14 larvae per gall (Djamankulova et al. 2008). I placed 25 galls in the center of the infestation at each of the 32 release sites. The midge has approximately four generations per year starting in the early spring.

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Therefore, I conducted releases between mid-May and mid-June when emergine midges could be placed in the field alongside young knapweed shoots with plenty of meristematic material.

Site Monitoring

To determine the impacts of midges on knapweed infestations, I released midges at 23 sites in Colorado in the spring of 2015. At the time of release, I measured the elevation (m) and the area of the infestation (m^2). I recorded the time of day of each release and used data from the nearest weather station to determine the maximum temperature on the release day. National Oceanic and Atmospheric Administration (NOAA) weather stations were up to 10 km from a field site. I used the NOAA National Centers for Environmental Information site to access weather data.

I released midges at the center of two intersecting, perpendicular transects, each 16 meters long (Figure 2.2). Along these transects, I measured 21 plots per site (each 0.25 m^2), including the plot at the central release point, and plots at 1m, 2m, 4m, 6m, and 8m from the release plot in all four directions. I established permanent transect markers and measured, at the time of release, height and number of Russian knapweed ramets, number of reproductive nodes including buds and flowers, and gall counts per plot. For each plot I also estimated percent cover of knapweed, other noxious weeds, forbs, grasses, and bare ground.

I revisited sites in 2015 between mid-August and mid-September to determine if midges initially established (formed galls), a time period approximately two to three generations after release, and to take plot measurements again. I also visited sites between mid-May and mid-June of 2016 to determine if midges overwintered and successfully galled new Russian knapweed

growth. I re-visited sites in August of 2016, 2017, and 2018 to monitor populations. No subsequent releases were made after the initial releases made in 2015.

At one site in 2016, I tracked aboveground height of 36 ramets that had been galled and their closest ungalled neighbor to determine how midges impact plant growth. I used a roadside site in Alamosa County because of ease of access and plenty of galled ramets (N 37.39827, W 105.94717). I marked and measured galled ramets and their closest neighbors on June 5, 2016 and August 21, 2016. Note that these neighbors were very likely connected via underground roots.

Statistical Methods

I used logistic regressions in SAS University Edition to determine which factors impact initial midge establishment (0 or 1) at sites, including the predictive variables of time of release, region, moisture, grazing history, elevation, and size of infestation. Plot level data included knapweed height, knapweed percent cover, and aboveground ramet density. Plot was nested in site for statistical analyses. I ran each predictive variable separately without model selection because of limited statistical power, largely due to a relatively small sample size of sites that could be used in the models (N=21). Moist sites (yes/no) were categorized by irrigation or being within two meters of a natural water source. Grazing history (yes/no) indicates that sheep, goats, cattle, or alpaca regularly have access to the site. Historical grazing pressure could not be quantified.

Results

Midges initially established at 11 of the 23 release sites across Colorado, as measured by the presence of new galls in August/September of 2015. Midges were more likely to initially establish at wetter sites (nearby irrigation or water sources) or sites that had been previously grazed or mowed over a long term regime. Within grazed sites, midges established at 8 of the 10 sites ($\chi^2 = 0.48$, df =1, P=0.71). Releases conducted on days with lower maximum temperatures were 20% more likely to experience initial establishment (Figure 2.3), defined here as the successful production of new galls after release. The time of day releases were conducted did not affect initial establishment significantly (N=21; Wald χ^2=2.32, df = 1, P=0.13). Elevation (m) and infestation size also did not significantly determine initial establishment (respectively; Wald $\chi^2 = 1.11$, df =1, P=0.29; Wald $\chi^2 = 1.68$, df =1, P=0.19). Nor was average height of the plant correlated with establishment success (Wald $\chi^2 = 0.039$, df =1, P=0.99). Though not statistically significant, midges initially established at sites with less dense plots, measured both by total stems (Wald $\chi^2 = 0.25$, df =1, P=0.12) and knapweed percent (Wald $\chi^2 = 3.19$, df =1, P=0.07).

In the summer of 2016, galled plants grew by 50% whereas ungalled plants grew to 100% of their initial height (Figure 2.4: 37.516596, -105.882752). In spring of 2016, one year after initial insect release, 30.4% of release sites still had *J. ivannikovi* galls present (7/23). In fall of 2018, less than 10% of initial release sites still had galls present (2/23), indicating that long-term establishment success declined over time. These two sites are heavily grazed, with high water availability due to irrigation.

In fall of 2015, even at the sites with the highest initial midge establishment rates, less than 20% of aboveground ramets were attacked with most of those having one gall. Overall 2.4% of field plots within a site were galled. Within galled plots, 82% of plots have more than one

gall. Half of surveyed galls occurred on meristematic tissues of the main aboveground ramet, while the other half occurred on aerial branches.

In 2015, I observed midges after an evening release at 10:00 p.m. at one of the sites in the San Luis Valley (N 37.460766, W -105.833647), returning to the release point every hour until midnight. Midges emerged from galls quickly, and I observed females extending their ovipositors and waving them, which is presumed to be a part of pheromone emission and a calling behavior to attract mates based on studies of other cecidomyiid species (Pivnik & Labbe 1992), within an hour of setting out the bouquet of galls. After an hour 11:00 p.m., midges were observed mating nearby the release point. Near midnight, I observed a female midge ovipositing on meristematic materials of Russian knapweed.

Discussion

Jaapiella ivannikovi initially established at nearly half of the release sites across Colorado during the first season. This compares favorably with a meta-analysis of 59 weed biocontrol projects that found establishment rates of biological control agents ranging from about 40-80% (Denoth et al., 2002). While 48% for initial establishment of *J. ivannikovi* is on the low end of that range, the following recommendations for releases based on our findings may result in higher *J. ivannikovi* establishment rates. My recommendations include making releases on cool days, later in the evenings, and a few days after mowing or grazing. While *J. ivannikovi* initially established in locations with variable abiotic characteristics, populations die out quickly in the following years. A single release does not appear to be sufficient to build a strong population of biological control agents in this case and others (Denoth et al., 2002). Supplemental releases after the initial release may further support agent populations.

Releases after 6:00 pm resulting in better initial *J. ivannikovi* establishment may be due to the high activity levels of midges in the cool, nighttime hours. Midday temperatures can get above 30°C, resulting in desiccation of insect and plant materials. Avoiding releases on particularly hot days (25°C or above) could enhance establishment. Cecidomyiids have been shown to display circadian behavioral rhythms, with females exhibiting mating behaviors at higher rates in the nighttime (Pivnik & Labbe, 1992). Another study found that the cecidomyiid orange wheat blossom midge tends to oviposit most near the evening hours around sunset (Pivnik & Labbe, 1993). *Jaapiella ivannikovi* appears to exhibit similar behaviors, so releasing them in the field in the late afternoon or evening may facilitate mating and oviposition behavior.

I did not detect strong regional or habitat patterns of midge biological control agent establishment. I anticipated that large scale spatial patterns in plant host genetics (Gaskin & Littlefield, 2017), weather patterns, or other regional characteristics may determine which parts of Colorado the midge establishes in. Because Russian knapweed occurs in variable habitats with a range of genetic variability (Goslee et al., 2003), I expected some regional incompatibility between knapweed-midge interactions. However, I found *J. ivannikovi* forming galls within each region of Colorado sampled within this study. Further considering spatial factors, regions within Colorado can vary greatly in precipitation and weather patterns especially contrasting the eastern plains region to the mountainous regions. Insects can have greatly different responses with varying photoperiods found across a latitudinal gradient (McEvoy et al., 2012). However, across Colorado, latitude did not have a strong impact on establishment. There were few commonalities between the two sites with *J. ivannikovi* midges still present after three years. One of these sites is along the Front Range of Colorado (Weld County), while the other is in the San Luis Valley (Alamosa County) over 250 kilometers southwest. The Front Range site has multiple fence lines,

with regular grazing and mowing occurring at different times in each fenced area. There are also

bushes that lend protection to galled knapweed stems. This combination of disturbance with

small havens promotes *J. ivannikovi* population growth and subsistence.

Management histories and strategies after agent release clearly determine agent

establishment and population maintenance, suggesting potential for integrative weed

management strategies. Integrating weed management strategies of grazing or mowing before

and after releasing the biological control agents will increase agent retention at sites.
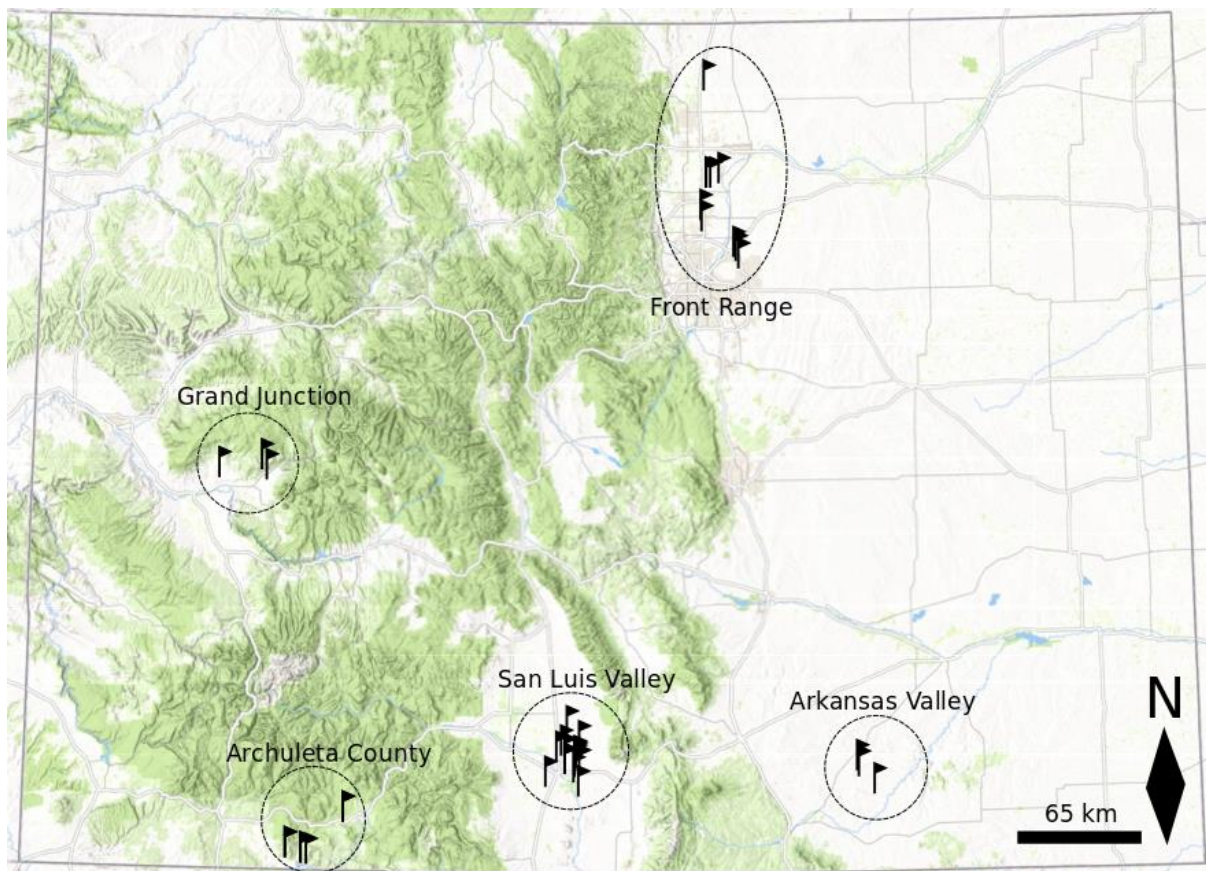
Figure 2.1. Map of Colorado with 32 release sites marked by black flags. Midges experienced initial establishment in each region. No midges overwintered in the Arkansas Valley (Las Animas County), though they did in other regions.
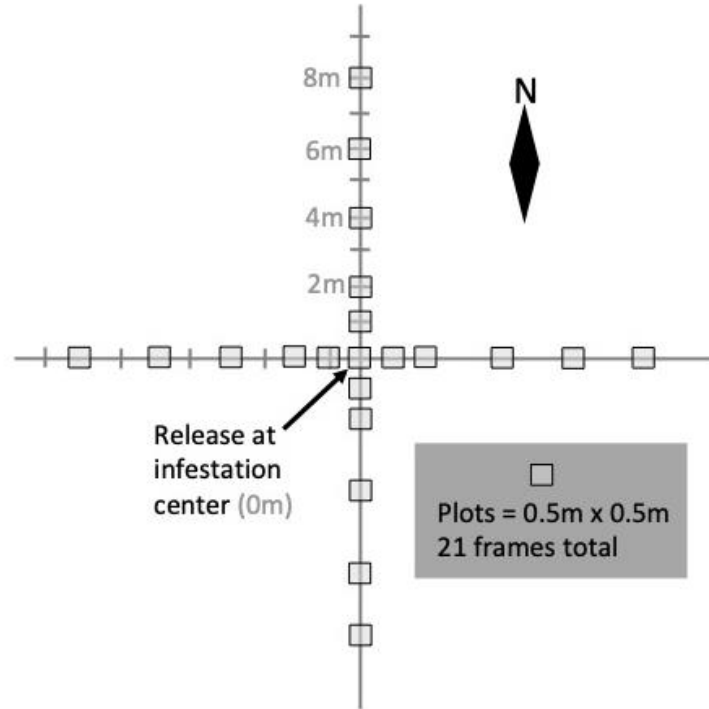
Figure 2.2. Each of the 32 release sites had 25 *Jaapiella ivannikovi* midge galls set in the center of the infestation and 21 plots were measured in crossed transects.
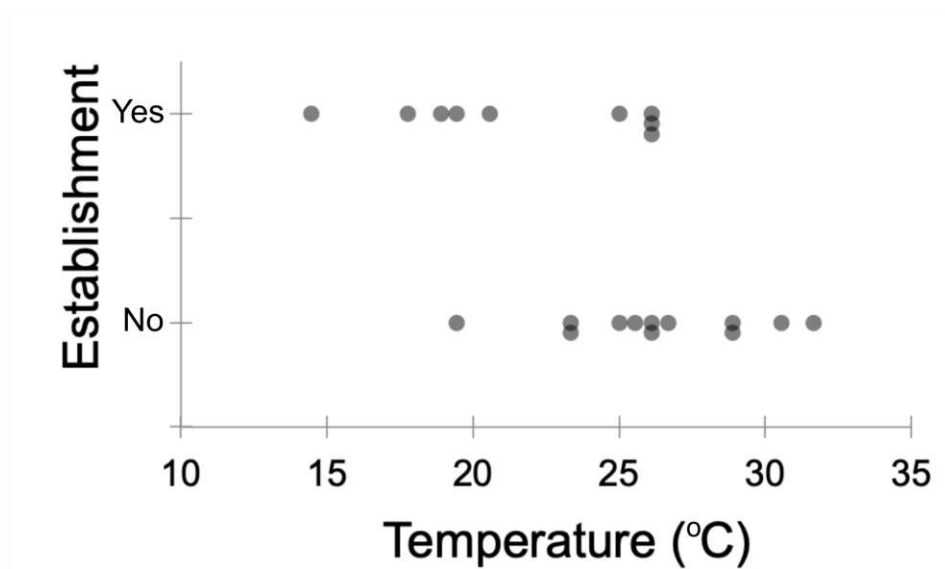
Figure 2.3. When *Jaapiella ivannikovi* midges were released on cooler days, they were more likely to establish. Temperature is the highest temperature reached on the day of release. Lower temperature days experienced higher establishment (0 or 1 establish response) according to a logistic regression (Wald χ^2= 4.53, df=1, *P*=0.033). Maximum day temperatures were obtained from the nearest NOAA weather stations.
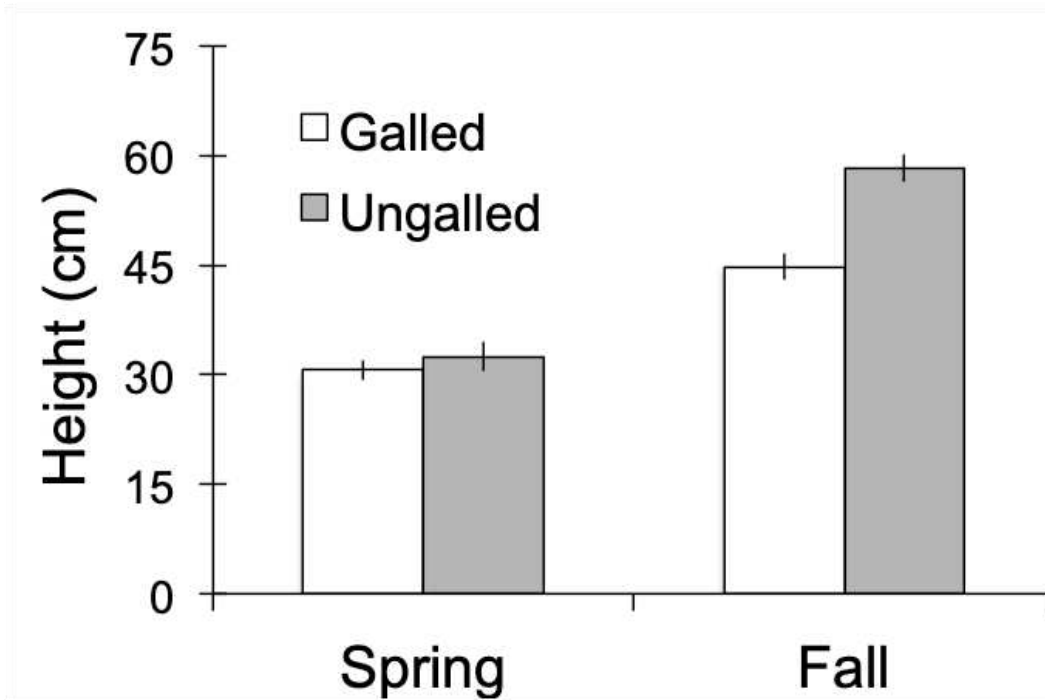
Figure 2.4. 2016 Tracked aboveground field ramets of Russian knapweed are shorter than their closest neighbors at the end of the season ($t = 2.9974$, $P = 0.0055$) despite starting out at similar heights ($t = 0.6004$, $P = 0.5526$).

Chapter 3 - SIMULATED GRAZING LEADS TO GREATER GALL INSECT

ESTABLISHMENT ON A RHIZOMATOUS PLANT

Summary

Using multiple weed management strategies in tandem may result in greater suppression

and control of rangeland weeds than use of any single strategy. Grazing or mowing can

significantly reduce plant growth by removing meristematic tissues. Alternatively, grazing may

stimulate new growth in neighboring undamaged meristems. Therefore, grazing may either

interfere with biological control agent establishment and effectiveness by reducing the amount of

tissue available for herbivores or enhance it if growth is stimulated in undamaged portions of the

plant. I explore the interaction between grazing and biocontrol by comparing the combined

effects of aboveground plant tissue removal (simulated grazing) and a gall midge (*Jaapiella

ivannikovi* Fedotova; Cecidomyiidae, Diptera) on the clonal plant Russian knapweed

(*Rhaponticum I=Acroptilon) repens* (L.) Hidalgo; Asteraceae, Asterales) with a two by two

factorial field cage experiment. I asked whether simulated grazing that removed 50% of

aboveground stems (ramets) affected the likelihood of midge establishment and how the two

treatments, alone and together, altered knapweed aboveground growth and reproductive traits. I

found that ramet removal in caged plots resulted in higher midge establishment on remaining

cage aboveground ramets. Many gall-forming insects require fresh, growing plant meristems, so

the allocation of resources toward younger ramets is a likely explanation for an increase in

gall midge establishment within the ramet removal treatment. Over the one-month period of

the study, I determined that insect attack or ramet removal alone resulted in increased growth of

remaining knapweed ramets. However, when herbivory and ramet removal occurred together,

growth of the undamaged knapweed ramets in the cage decreased compared to control cages

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where neither treatment was applied. Stems in the fringe plots surrounding the treatment cages responded similarly to stems inside the treatment cages, likely because they were connected via rhizomes. Knapweed may differentially allocate resources to intact, younger ramets when stressed, explaining observations of increased knapweed growth under conditions of either grazing alone or herbivory alone. Results suggest that releasing biological control agents on Russian knapweed shortly after grazing will likely increase midge establishment. Plant-mediated interactions among herbivores are widespread in ecological communities and especially important to consider when managing plant community composition.

Introduction

Plant-mediated interactions among mammalian and insect herbivores are widespread (Denno et al., 1995; van Veen et al., 2006; Kaplan & Denno, 2007; Ohgushi, 2007; Stokes & Stilling, 2015; Cunan et al., 2015). Such interactions occur when one herbivore alters the quality and/or quantity of a host plant shared by another herbivore species. Generally, reductions in plant quantity result in exploitative competition, as fewer resources are available to competitors. Exploitative competition negatively affects herbivores feeding on the same plant. Changes in plant quality, on the other hand, can be either harmful or beneficial to insects sharing a host plant depending on how the herbivore-induced plant traits, including induced chemical defenses and changes in patterns of resource allocation, affect the other herbivore. Grazing by mammalian herbivores reduce aboveground plant biomass for insect herbivores. However, grazing can also alter plant regrowth traits, such as branching architecture (quality of resources), which may result in the production of increased meristematic growth that are highly nutritious to many insect

herbivores (DiTomaso, 2000; Limb et al., 2018). Mammalian and insect herbivores use extrememly different strategies to feed and impact plants in very different, yet significant, ways.

Use of integrative weed management strategies, such as combinations of mammalian grazing and biological control, often result in greater suppression and control than simply using one management strategy (Lym, 2005; Davis et al., 2018; Minteer et al., 2018). Grazing can change plant communities in favor of native species (Limb et al., 2018), decrease aboveground plant biomass, and significantly alter subsequent plant traits, such as how tall or bushy a plant grows after tissue loss (DiTomaso, 2000). Changes in plant traits due to grazing can increase biological control agent establishment and effectiveness, especially with targeted grazing (Lym, 2005; Wilson et al., 2008). Large mammal grazers will likely affect insect biological control agents indirectly through plant-mediated interactions. For example, grazing may lead to shorter, more highly branched plants with more actively growing meristematic tissues (DiTomaso, 2000) that can be highly nutritious to insects. This suggests that the two management approaches of grazing and biological control may be more effective when implemented together than use of either strategy alone. Mammalian grazers and insect biological control agents likely experience strong interactions, though it is unclear how they interact, making the study of the combined effects of mammalian grazing and insect herbivory imperative for developing effective and efficient biological control methods.

Gall-forming insects are commonly approved as biological control agents due to their high degree of host plant specificity and minimal non-target effects. Such insects provide a convenient opportunity to explore plant-trait mediated interactions and competition between herbivores for plant-resources because gall-forming insects are easy to manipulate and remain within the gall while feeding on the plant. Galls are bloated or otherwise expanded plant tissues

formed in response to oviposition or the resulting developing insect larvae (Giron et al., 2006).

Galling insects are typically limited to specific parts of the plant, often meristematic tissues,

which can reduce flower and fruit production (Djamankulova et al., 2008; Gangé, 2014). Gall

insects commonly increase auxin and cytokinin levels in galled plant material, hormones which

characterize plant metabolic sinks (Erb et al., 2012; Giron, 2016). In this way, insect galls act as

metabolic sinks, which may compete with other plant metabolic sinks such as developing fruits

or meristematic tissues (Forrest, 1971; Inbar et al., 1995; Harris & Shorthouse, 1996; Larson &

Whitham, 1997; Dorchin et al., 2006; Kaplan et al., 2011; Giron, 2016). Galling influences

chemical, physiological, and morphological aspects of host plants, such as branching (McKone

et al., 2001; Gagné & Hibbard, 2008; Hall et al., 2012). Gall insects are restricted to galled plant

material, making them unable to feed on other parts of the plant, unlike mobile chewing insects

or mammalian grazers which can remove parts of the plant to varying degrees. As feeding gall

insects are restricted to their gall, they are at the mercy of plant mediated interactions with other

herbivores, such as grazers, utilizing the host plant.

Nutrient allocation between different parts of a plant represents one way that plants can

mediate interactions between herbivores, and natural selection likely acts upon nutrient

allocation traits. Asexual reproduction through rhizomatous growth results in a genet or clone

made of many ramets (aboveground individual stems/shoots), allowing resources to be allocated

between ramets within the clone (Cain, 1990). For instance, resource allocation in insect galled

goldenrod plants depends heavily on rhizome age and connectivity of ramets, with older ramets

supplying younger ramets with photosynthates (Hartnett & Abrahamson, 1979; Abrahamson and

McCrea, 1986). Therefore, herbivores attacking different aboveground ramets within a plant

clone may still compete for plant resources. Alternatively, if damage by one herbivore redirects

plant nutrients toward plant organs used by another herbivore, growth of the second herbivore may be facilitated. Movement of photosynthates from older to younger ramets may lead to asymmetrical intraspecific resource competition among herbivores (Shea and Watson, 1989; Cain, 1990; Marquis, 1996). There are likely many trade-offs between attacking different parts of a clone, especially when considering the clone's history of herbivory. Ramets sharing resources within a genet could enable resource competition between herbivores feeding on different parts of a plant host, determining the compatibility of grazing and biological control.

While some studies on integrated pest management have considered grazing alongside chewing herbivore biological control agents (Lym, 2005; Wilson et al., 2008), little is known about how and if mammalian grazers interact with gall insects. Herbivory tends to stress a plant and reduce its resources, and multiple types of herbivory may increase stress. Grazers can remove aboveground resources for galling insects, decreasing meristematic tissues available to insects. Alternatively, moderate to low grazing may induce new meristematic growth resulting in more locations for insects to gall and establish. Thus, aboveground ramet removal through grazing may induce changes in plant traits that benefit galling insects, such as formation of more meristematic tissues, or impede establishment of galling insects, as grazers remove or reduce the quality of meristematic tissues (Olofsson and Strengbom, 2000; Martinez and Wool, 2003). Factors associated with mammalian grazers other than removal of aboveground plant material, such as feces deposition, can also alter nutrient availability and resulting plant traits in ways that impact gall insects (Olofsson and Strengbom, 2000). This makes it hard to pinpoint the mechanisms for how grazing influences other community members and indicates a need to test components of grazing and their influence on galling insects in manipulative experiments.

Russian knapweed, *Rhaponticum (=Acroptilon) repens* (L.) Hidalgo (Asterales:
Asteracea), is a rhizomatous plant with a gall insect biological control agent, *Jaapiella
ivannikovi* Fedotova (Diptera: Cecidomyiidae), which has been introduced and established in the
western US (Meyers et al., 2015). This midge induces gall formation by the plant by laying eggs
in the apical meristems of Russian knapweed plants. In this study, I manipulated aboveground
ramet density with simulated grazing to determine impacts on midge establishment and
aboveground plant traits. Treatments of ramet removal (simulated grazing by removing 50% of
aboveground ramets) and insect presence occurred in a two by two factorial design, implemented
on the same day. I expected aboveground ramet removal to result in lower midge establishment,
as insects would have half as many meristematic tips left in the cages to attack. I predicted fewer
galls per ramet and fewer total insects associated with aboveground ramet removal due to
reduced resources. Furthermore, I expected the most effective weed management when
treatments co-occurred due to the plant experiencing multiple stressors that restrict growth. As a
rhizomatous weed, I predicted that treatments would also decrease growth in nearby, untreated
knapweed plants due to clonal resource sharing.

Methods

Study System

Russian knapweed reproduces sexually and asexually (Gaskin & Littlefield, 2017). This
plant is native to Eurasia and is categorized as a noxious weed in rangeland throughout much of
the western US. Similar to many invasive weeds, Russian knapweed stands are denser in invaded
North America compared to their native range in western Asia (e.g., Turkey; unpublished data
cited in Djamankulova et al., 2008). Russian knapweed is a stronger competitor than many native

North American plants (Ni et al., 2010; Callaway et al., 2012). Russian knapweed infestations in wheat can result in dramatic yield reductions (50-90% depending on density of Russian knapweed; Streibig et al., 1989). With an extensive root system, knapweed has the ability to store large amounts of nutrients in root organs. Within populations, ramets, or aboveground stems, tend to be the same genetically, suggesting that stands of knapweed grow through the spread of rhizomes and that establishment of new populations occurs primarily through dispersal of sexually produced seeds (Gaskin & Littlefield, 2017).

The knapweed midge *J. ivannikovi* was recently approved as a specialist biocontrol against Russian knapweed (USDA APHIS, 2009). Midges need young, meristematic plant material to induce a gall. The knapweed midge can lay upwards of 15 eggs that stimulate production of a meristematic gall (Djamankulova et al., 2008). Gall formation is visible within a week of midge oviposition. Typical generation times occur within approximately four weeks and, if plant growth conditions are favorable, four generations can occur per season (Djamankulova et al., 2008). The midge has been found to reduce plant reproductive success as measured by reduced floral production (Djamankulova et al., 2008); however, impacts on clonal growth and ramet production are unknown.

Experimental Design

In order to test the effects of simulated grazing (50% aboveground ramet removal) and attack by gall midges (*J. ivannikovi* presence), I used a two by two factorial design. I randomly assigned each of 28 cages to one of the following four treatments: control, *J. ivannikovi* release, ramet removal, or a combination of *J. ivannikovi* release and ramet removal with seven replicates per treatment. Each one-m^3 cage was enclosed in fine "No-See-Um" mesh made of nylon with a

weight of 0.0325 kg/m^2 and 0.2286 mm thickness purchased from Rockywood Fabrics

(Loveland, Colorado). Each cage had four smaller ¼ m^2 inner plots and eight ¼ m^2 fringe plots

along the outside of the cage (Figure 3.1). I used multiple plots per cage in order to control for

microhabitat differences and maximized measurement consistency across time points and

between different observers. With a pilot study, I found that observers are more consistent in

measuring ¼ m^2 plots than 1 m^2 plots. The 14 *J. ivannikovi* release treatment cages (seven *J.*

ivannikovi release and seven combination cages) were each established by placing a bouquet of

three mature midge galls, yielding a total of approximately 30 adult midges within a week, in a

vial that was placed at the center of the cage. Vials contained water so that galled-stems could

remain fresh as midges emerged over the next few days. I put tape around stems at the top of the

vial to keep stems secure in wind as well as to reduce evaporation. The 14 ramet removal

treatment cages had exactly half of the knapweed ramets within the cage trimmed to a stubble

height of approximately 10 cm, which is typical of sheep and cow grazing on Russian knapweed

(Barosh & Ode, personal observation).

I conducted the experiment during a four-week period, from May 24, 2016, to June 20,

2016, on a field site near the Denver International Airport, managed by the Parks and Recreation

Department of the City of Denver. This uncultivated tract of land contained multiple patches of

Russian knapweed along Secondhand Creek. In one large patch of knapweed covering

approximately 1,500 m^2, I placed 28 one-m^3 cage frames made of rebar and PVC pipes over one-

m^2 patches of land. Cage edges were at least two meters apart from one another on an east

sloping hillside in an approximate seven by four array. Treatments for each cage were

completely randomized. Plots had a minimum of at least 20% knapweed groundcover. At the

start of the experiment, I measured the number of flower buds, the height of the tallest ramet, and

the total number of aboveground ramets in all 12 plots (four internal plus eight fringe) for each of the 28 cages. In three of the seven cages within each treatment, 15 stems were randomly selected from within the cage and marked with flagging tape. The following measurements were recorded for each marked stem at the beginning and termination of the experiment: plot location within each cage, height of stem, number of branches, number of leaves, number of flower buds, and number of galls. I harvested and measured aboveground wet mass of these marked ramets at the end of the experiment.

I visited the field site once a week for three weeks (May 30, June 7, and June 14, 2016) to check that cages were secure and to repeat the fringe plot measurements (the same measurements as the inner plots). On June 20, I repeated the measurements taken at the start of the experiment, in addition to counting the number of galls per plot, and deconstructed the cages. In each cage whith gall midges release, all knapweed ramets were evaluated for gall formation, and all galled stems as well as stems that I marked and tracked throughout the study were harvested and taken to the lab, where I measured stem height (cm) and aboveground wet biomass (g), along with the number of leaves, buds, and galls. I measured wet mass, rather than dry mass, of collected ramets so that I could subsequently remove and dissect galls for larval counts. Gall height and width (mm) were measured for all galls. Galls were subsequently placed in cold storage and were subsequently dissected to count midge eggs, larvae, and pupae. Gall size was an estimate of resource quality of the gall as larger galls had more available food for the feeding larvae. I tallied larvae and pupae separately. During this study, no adults or evidence of adult emergence was observed from the galls formed in the field.

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Statistical Methods

All data analyses were conducted with SAS© University Edition 9.4 software. Tests were conducted after checking residuals, variance, and distribution for model fit assumptions. I compared midge traits between the treatments of no ramet removal and ramet removal, controlling for cage and plot. I used t-tests to compare gall width and height from cages with and without ramet removal. Further, total number of galls per treatment was considered alongside total number of galled ramets and total number of larvae per treatment, with a null hypothesis of equal likelihood of galling between the treatments of no ramet removal and ramet removal.

The effects of insect release and ramet removal on plant height, number of branches, number of buds, and change in number of leaves were analyzed with a series of two-way ANOVAs. Insect release and ramet removal were considered as fixed factors, and the plots nested in cage replicates were treated as random effects. Cage and plot were left in the model to control for microhabitat variation. I incorporated an interaction term between treatments of midge release and ramet removal. I compared the ramets that I marked and tracked throughout the study from insect exposed cages to galled ramets (none of the marked ramets happened to be galled). These comparisons include change in height (cm), number of branches, number of buds, and the number of leaves. Tracked ramets were measured at the beginning and end of the experiment to compare the change in plant ramet traits including height (cm), number of branches, number of buds, and number of leaves (end minus the start values) between treatments. Considering treated plots within cages included models for height of the tallest aboveground ramet, total number of stems per plot, and number of buds per plot. Fringe plot measures were conducted weekly throughout the experiment for a total of four times, with repeated measures. Some fringe plot data at the end of the experiment was not included in the analysis due to

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disturbances to the plots, likely from deer walking on the plots. Fringe plot models were created for plant traits including height of the tallest ramet, total number of stems per plot, and total buds per plot. I report type 3 tests of fixed effects for all ANOVAs. Response variables of branch numbers, bud numbers, and leaf numbers had a Poisson distribution. Plots were nested within cage. When applicable, ramets were nested within plot.

Results

Insects establish better with grazing

Ramets in cages with ramet removal were more than five times more likely to be galled than ramets in cages without ramet removal taking into account how many available aboveground shoots were present at the time of galling (Odds ratio Y:N 5.24 (95% CI 3.09-8.85), Log-Likelihood Chi-square=37.82 df=1, p<0.001). In ramet removal cages where midges established (at least one gall formed), midge galls occurred in higher numbers than cages without ramet removal (no ramet removal = 31 total galls in five cages, ramet removal = 58 total galls in three cages). Furthermore, dissections of those galls showed that cages with no ramet removal had a total of 130 midges versus 237 midges in ramet removal cages (Chi-square=30.62, df=1, *P*=0.01), and gall height (cm) was greater in cages with ramet removal suggesting greater resource allocation to galls with simulated grazing (Satterthwaite *t*-test, *T*=2.23, *P*=0.056). Gall width (cm) and the number of insects per gall (average 4.1-4.2) did not differ significantly with ramet removal (respectively, *t*-tests: *T*=0.97, *P*=0.34 and *T*=-0.07, *P*=0.95).

Galling and ramet removal change aboveground stem growth

To further consider the impacts of galling on ramets, I compared galled and ungalled ramets from cages with and without ramet removal, only including ramets from cages with insect releases. There were significant interactions between treatments in all models except for ramet wet mass (Figure 3.2 A-D). Galled aboveground ramets produced an average of six fewer branches than ungalled ramets (Figure 3.2A: insects, $F_{1,187}=37.68$, $P<0.001$; Ramet Removal, $F_{1,187}=3.30$, $P=0.070$; insects*Ramet Removal, $F_{1,187}=9.45$, p=0.0024). There were on average a few more flower buds on ungalled ramets compared to galled ramets (Figure 3.2B: midges, $F_{1,187}=40.28$, $P<0.001$; Ramet Removal, $F_{1,187}=2.50$, $P=0.12$; midges*Ramet Removal, $F_{1,187}=4.54$, p=0.035). The influence of galling on leaf number was stronger without ramet removal, decreasing average buds by 2.7 leaves compared to a decrease of 1.1 leaves with ramet removal (Figure 3.2C: midges, $F_{1,187}=133.40$ $P<0.001$; Ramet Removal, $F_{1,187}=3.28$, $P=0.072$; midges*Ramet Removal, $F_{1,187}=38.98$, p<0.001). Ungalled ramets grew on average 8.8cm taller than galled ramets when no ramet removal occurred, but in ramet removal cages galled and ungalled plants grew to similar heights (Figure 3.2D: midges, $F_{1,187}=16.82$, p<0.001; Ramet Removal, $F_{1,187}=0.38$, p=0.54; midge*Ramet Removal, $F_{1,187}=5.13$, p=0.025). Galling and ramet removal did not significantly affect aboveground wet ramet mass (midges, $F_{1,187}=1.40$, $P=0.23$; Ramet Removal, $F_{1,187}=0.23$, $P=0.63$; midges*Ramet Removal, $F_{1,187}=1.17$, p=0.28).

Marked ramets respond to treatments

Ramets randomly selected at the beginning of the experiment differed little in measured traits from the beginning to the end of the experiment. None of the preselected, marked ramets were directly galled. Nevertheless, the presence of midges resulted in production of an average

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of six more branches on the marked ramets (Insects, $F_{1,8}=6.63$, p=0.0329; Ramet Removal, $F_{1,8}=0.001$, p=0.9542 insect*Ramet Removal, $F_{1,8}=1.20$, p=0.3049; Time, $F_{1,310}=99.71$, p<0.0001). The treatment of ramet removal increased the tracked remaining ramet's production of buds by less than five compared to ramets from cages without ramet removal (Insects, $F_{1,8}=0.3909$, p=0.3909; Ramet Removal, $F_{1,8}=0.01$, p=0.9072; Insect*Ramet Removal, $F_{1,8}=0.11$, p=0<0.0001; Time, $F_{1,310}=89.74$, p<0.0001). Ramet removal and insect exposure did not appear to affect the change in tracked ramet height over the experiment duration compared to the growth in control plots (Insects, $F_{1,8}=3.47$, p=0.0995; Ramet Removal, $F_{1,8}=0.49$, p=0.5054; insect*Ramet Removal, $F_{1,8}=0.49$, p=0.5048; Time, $F_{1,306}=629.49$, p<0.0001). Insect release increased the production of leaves by nearly 40 in the cages with ramet removal (Insects, $F_{1,8}=5.24$, p=0.0515; Ramet Removal, $F_{1,8}=0.08$, p=0.7822; insect*Ramet Removal, $F_{1,8}=1.61$, p=0.2400; Time, $F_{1,311}=182.07$, p<0.0001). Exposure to *J. ivannikovi* and ramet removal did not significantly impact ramet aboveground wet mass (g), though the combination of *J. ivannikovi* and ramet removal did reduce knapweed mass (Insects, $F_{1,28}=1.28$, p=0.2684; Ramet Removal, $F_{1,15}=0.19$, p=0.67; insect*Ramet Removal, $F_{1,28}=1.36$, p=0.2530).

Inner plots

The number of buds/plot inside of cages did not differ significantly between treatments (Figure 3.3A: Insects, $F_{1,24}=0.38$, p=0.54; Ramet Removal, $F_{1,24}=0.009$, p=0.98; Ramet Removal*Insects, $F_{1,24}=1.08$, p=0.30). The shortest plants occurred in the combination treatment cages (insect exposure and ramet removal) (Figure 3.3B: Insects, $F_{1,24}=1.52$, p=0.2293; Ramet Removal, $F_{1,24}=0.07$, p=0.7916; Ramet Removal*Insects, $F_{1,24}=1.69$, p=0.2053). The number of stems per plot did not vary as a function of whether or not midges were present (Insects,

$F_{1,24}$=0.01, p=0.9107) or whether ramets were removed (Ramet Removal, $F_{1,24}$=0.001, p=0.9756), and, though not significant, the combination of midges and ramet removal resulted in the least number of knapweed stems present (Figure 3.3C: Ramet Removal*Insects, $F_{1,24}$=2.05, p=0.1648). On the last measurement date, without ramet removal, *J. ivannikovi* increased average number of ramets/plot by 4.6 ramets, while with ramet removal, insect presence decreased average ramets/plot by 5.4 ramets (Figure 3.3C).

Fringe plots

There were treatment impacts on fringe plots occurring outside of the treatment area. Fringe plots next to cages with ramet removal experienced a greater decrease in buds compared to plots without ramet removal when next to cages with insect release at 7.3 average buds compared to cages without ramet removal at 1.2 average buds (Figure 3.4A); buds were significantly affected by the treatments, with an interaction between ramet removal and insect presence (Insects, $F_{1,24}$=1.96, p=0.1741; Ramet Removal, $F_{1,24}$=0.03, p=0.8721; insect*Ramet Removal, $F_{1,24}$=0.59, p=0.4506; Time, $F_{1,24}$=144.66, p<0.001). Fringe plots experienced a strong interaction between treatments, with insect presence increasing average plant height by 1.4 cm in plots without ramet removal, yet decreasing average height by 4.8 cm in cages with ramet removal on the last date of measurement (Figure 3.4B: Midges, $F_{1,24}$=0.27, p=0.6085; Ramet Removal, $F_{1,24}$=0.15, p=0.7030; Midges*Ramet Removal, $F_{1,24}$=3.61, p=0.0696; Time, $F_{1,653}$=273.62, p<0.0001). Ramets per plot experienced similar patterns with either treatment alone increasing ramet density, yet the combination of treatments reduced ramet density (Figure 3.4C: Midges, $F_{1,24}$=4.77, p=0.0391; Ramet Removal, $F_{1,24}$=2.21, p=0.1499; Midges*Ramet Removal, $F_{1,24}$=4.87, p=0.0371; Time, $F_{1,653}$=58.13, p<0.0001).

Discussion

Simulated grazing (50% aboveground ramet removal) increased gall midge establishment, likely due to changes in clonal plant resource allocation and availability. Ramet removal led to fewer aboveground ramets using belowground resources, increasing growth of the remaining ramets and resulting in better oviposition sites for gall insects. Gall size was also greater in cages with ramet removal, further suggesting that aboveground ramet removal resulted in nutrient allocation towards remaining meristematic tissues, increasing the quality of resources for developing galls. Additionally, ramet removal may stimulate new ramet growth, providing *J. ivannikovi* with preferred younger plant material to gall. This is unlikely in my study, as ramet removal and insect release were implemented on the same day with *J. ivannikovi* emerging from galls and attacking plants within days after aboveground ramet removal, not leaving enough time for new ramets to appear. Therefore, gall insects only had access to the aboveground ramets that remained after simulated grazing, and increased gall formation occurred because of a benefit to the insect achieved from attacking those remaining ramets.

Jaapiella ivannikovi attack limited ramet height but not overall ramet mass when comparing galled versus ungalled ramets from within insect release cages (Figure 3.2). This is likely due to midge induced changes in plant branching architecture, as galling on Russian knapweed tends to restrict further growth at the galled meristem while other meristems can continue growing and result in a branched, bushy plant. When comparing ungalled, tracked ramets, branches and leaves were decreased by *J. ivannikovi* release, suggesting that resources are diverted toward other processes, such as gall formation. The location of a gall within a plant can enormously alter the cost to the plant and determine insect fitness: even within a single leaf, strategic gall placement can confer a significant benefit to the insect due to resource availability

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(Larson & Whitham, 1997). *Jaapiella ivannikovi* selection of a vigorously growing ramet likely also results in benefits to its offspring and explains the larger gall sizes seen in cages with ramet removal as plants allocate resources toward ramets that did not experience mechanical damage. Altered plant growth patterns in response to herbivory can determine resource availability to insects and ramets within a clonal plant; Russian knapweed is much like other plants that tolerate herbivory with regrowth and resource reallocation (Tiffin, 2000; Stowe et al., 2000; Karban et al., 1999).

Chemical defenses and other induced plant defenses also play a role in midge female oviposition, gall formation, and growth (Rostás et al., 2013; Heath et al., 2014; Rand et al., 2014). However, if induced chemical defense played a large role in this experiment, I might expect tissue damage from aboveground ramet removal to result in increased plant defenses and decreased number of galls contrary to our results. If tissue damage reduces resources so greatly that plants experience reduced defenses, I expected to see reduced gall sizes with plant damage, which is also contrary to our results. Plants often experience a trade off between defense against insects and allocating resources toward growth (Herms & Mattson, 1992), in which case insect presence should have reduced plant growth. Perhaps, this is occurring at the scale of the individual ramet where galling appears to limit plant growth (Figure 3.2), however insect presence resulted in higher ramet density per plot (Figure 3.3&4). Therefore, nutrient allocation among ramets likely drives the observed interactions between Russian knapweed and its galling midge more than induced defenses.

Galling and simulated grazing impact plants in different ways. Mammalian grazing and attack from gall-forming insects represent extremely different strategies of feeding, so I expected knapweed to respond differently to each treatment. While density responses

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were similar in ramet removal and insect release treatments (Figure 3.3C), plants responded differently in bud production and height (Figure 3.3A&B and 3.4).

Biological control gall midges, *J. ivannikovi,* have a widespread influence across the genet of Russian knapweed. Herbivory impacts the genet beyond the attacked ramets, as seen in fringe, untreated plot outcomes (Figure 3.4) that are similar to treated plots (Figure 3.3). When invasive or weedy plants reproduce asexually, they may be affected by biological control agents differently than non-rhizomatous plants, as attack can impact the whole clone. As biological control agents are being considered for effectiveness, especially with physiologically manipulative, gall-forming insects, researchers should consider how much the insect impacts plant physiology and at what scale of the plant the influences are seen (e.g., branch, ramet, nearby ramets, or the whole genet). Some research suggests that the effects of galling on ramets is restricted to the attacked ramet (e.g. Hartnett & Abrahamson, 1979; Fay & Hartnett, 1991), in contrast to our findings. However, studies on goldenrod suggest that resources travel between ramets from the older "parent" ramet to the younger ramets (Cain, 1990). A similar mechanism appears to occur in Russian knapweed given herbivory. Ramet connectedness and resource movement between ramets determine interactions between grazing mammals and insect herbivores on disparately located ramets of Russian knapweed. In management efforts of plant based communities, plant-mediated interactions play an important role in determining treatment outcomes. Mowing or grazing knapweed patches before insect biological control release could provide land managers with an effective and inexpensive way to promote agent establishment.
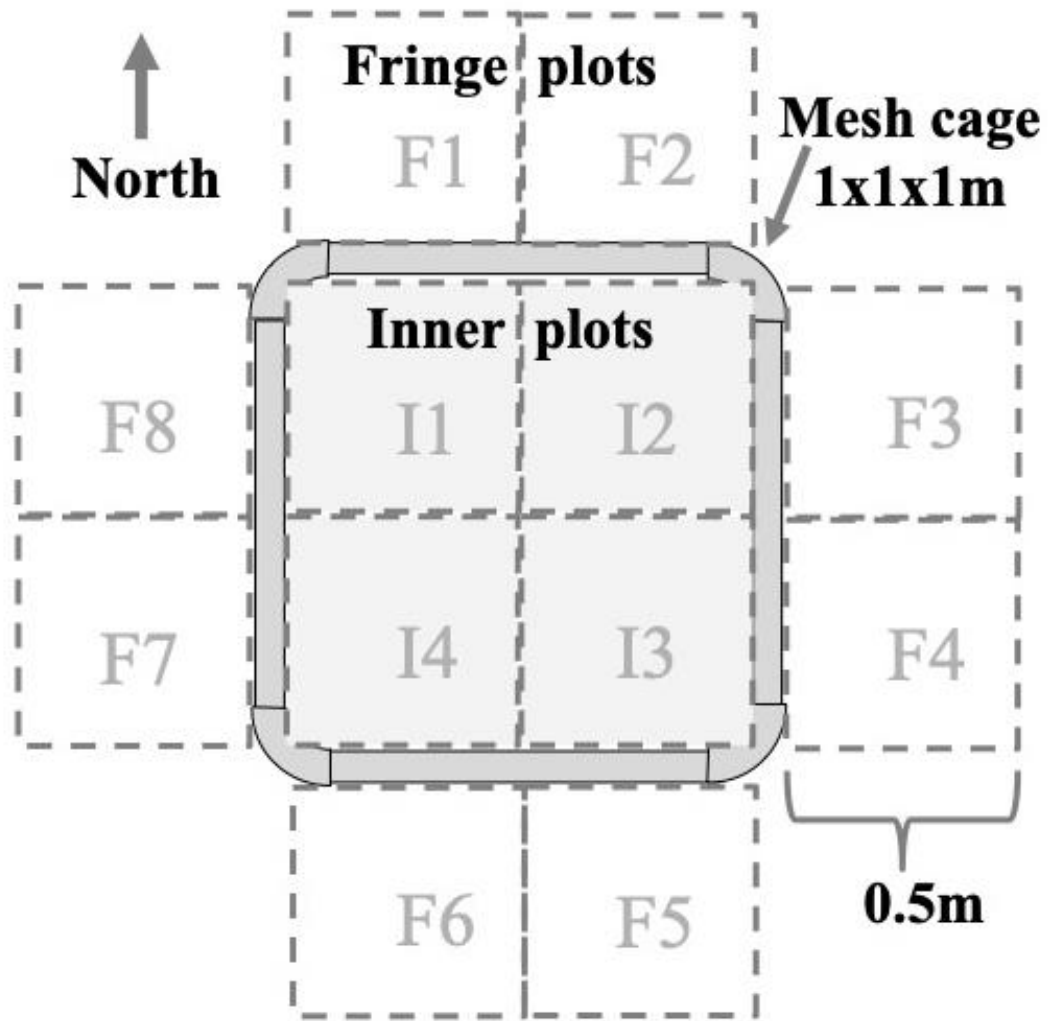
Figure 3.1. View from above of a field cage with inner and outer sampling plots. Each of 28 cages was set up with four inner plots (I1-4) and eight fringe plots (F1-8).
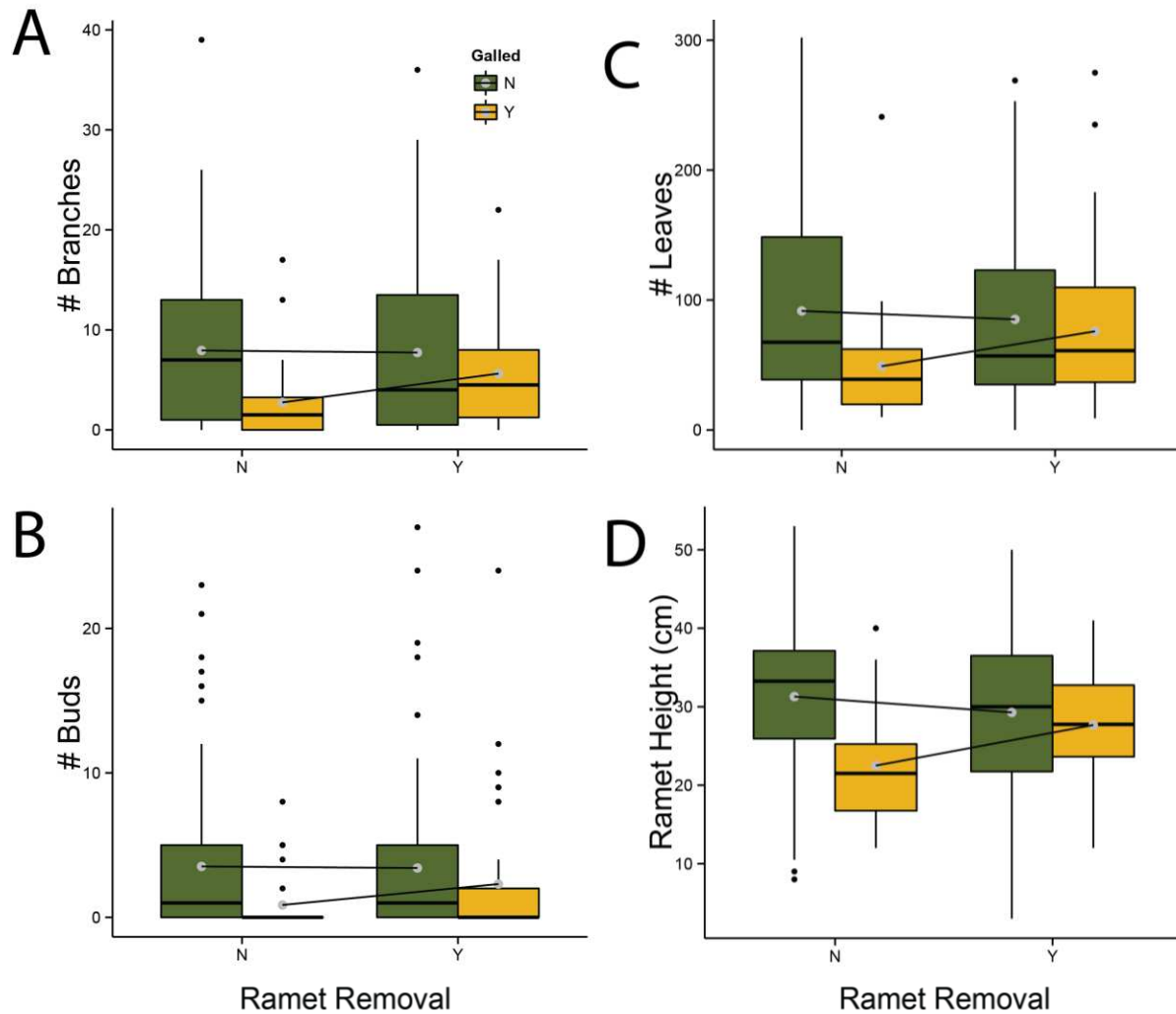
Figure 3.2. Galled ramets (N=74) from only within galled cages versus ungalled ramets (N=175). Ungalled ramets were tracked throughout the experiment, so they were present at the beginning of the experiment. These occurred in the same cages as galled ramets. Ramet Removal of "N" indicates no researcher-induced ramet damage, while a "Y" indicates that a random 50% of aboveground ramets in cages were cut. The lighter colored, yellow bars "Galled Y" indicate galling, while "N" represents no *Jaapiella ivannikovi* midge attack. Gray dots represent mean, while the boxplot depicts median, quartiles, maximum, minimum, and outliers. Graphs depict end of experiment measures: A) number of branches per ramet (Overall Model: $\chi_{61,187}$=2.15, p<0.0001), B) number of buds per ramet (Overall Model: $\chi_{61,187}$=2.21, p<0.0001), D) number of leaves, C) ramet height from ground level to the highest point (Overall Model: $F_{61,187}$=2.08, p<0.0001)
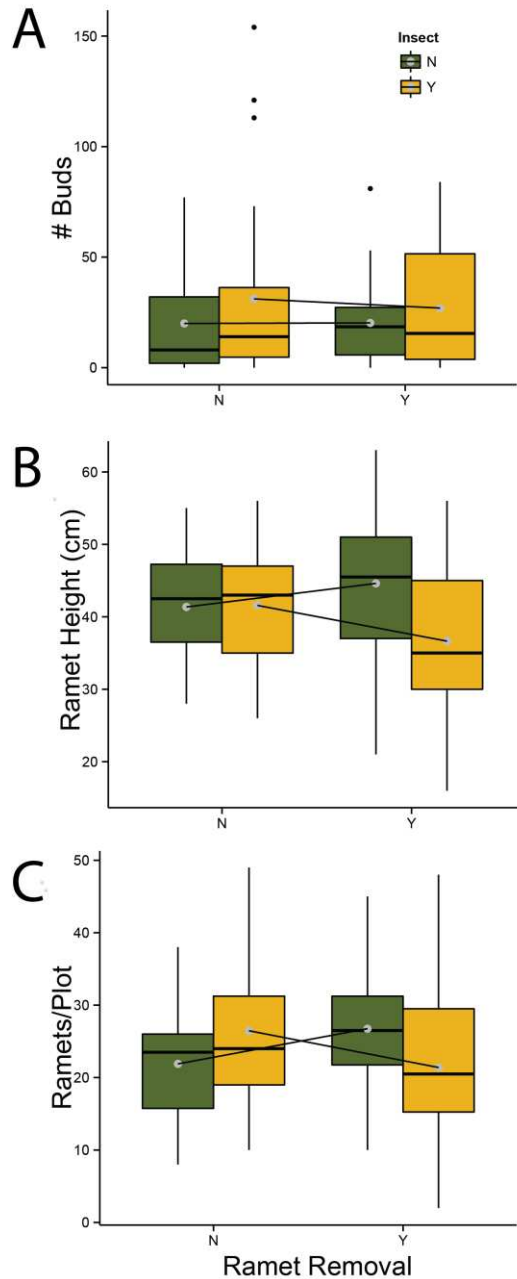
Figure 3.3. End measurement for the inner plots including four ¼ m² plots per inside of each cage (total N=28), with seven cages per treatment. Ramet Removal is indicated with a "N" for no manipulation and "Y" indicating that a random 50% of aboveground ramets in cages were cut. Lighter colored, yellow bars "Insect Y" indicate that *Jaapiella ivannikovi* midges were released in the cages, though did not necessarily establish. The "N" indicates no release. A) Depicts total number of buds per plot (Overall Model: $F_{114,42}=1.24$, p=0.2139). B) Indicates the height in cm of the tallest ramet per plot (Overall Model: $F_{115,104}=4.55$, p<0.0001). C) Displays the total number of ramets or aboveground stems per plot (Overall Model: $F_{114,41}=4.27$, p<0.0001). The boxplot depicts median, quartiles, maximum, minimum, and outliers. Gray dots represent means.
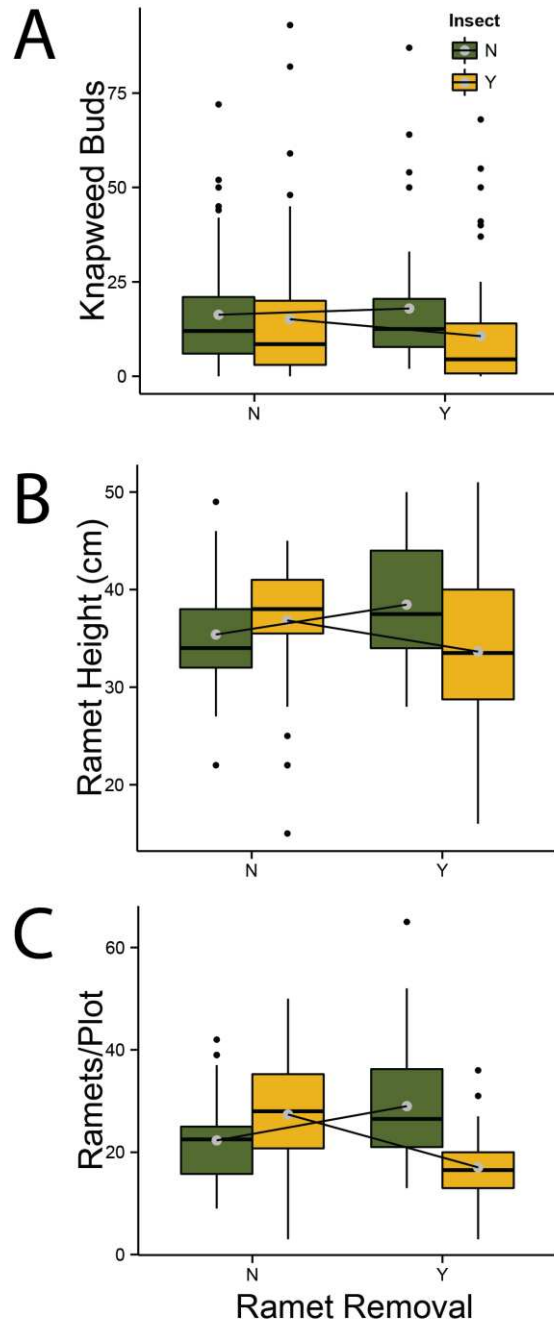
Figure 3.4. End measurement for the fringe plots including eight ¼ m² plots along the outside edges of each cage (total N=28), with seven cages per treatment. Ramet Removal is indicated with a "N" for no manipulation and "Y" to indicate that a random 50% of aboveground ramets in the cages were cut. Lighter colored, yellow "Insect Y" indicates that *Jaapiella ivannikovi* midges were released in the cages (not all established) while "N" indicates no release. A) Depicts total number of buds per plot (Overall Model: $F_{224,655}$=2.97, p<0.0001). B) Indicates the height in cm of the tallest ramet per plot (Overall Model: $F_{224,655}$=5.39, p<0.0001). C) Displays the total number of ramets or aboveground stems per plot (Overall Model: $F_{224,655}$=6.61, p<0.0001). The boxplot depicts median, quartiles, maximum, minimum, and outliers. Gray circles represent means.

Chapter 4 - GALL-FORMING HERBIVOROUS INSECTS COMPETE EVEN WHEN ON

DISTANT BRANCHES OF THE SAME PLANT

Summary

Gall insects strongly influence host plant physiology and patterns of within-plant

resource allocation. Therefore, they are likely to experience plant-mediated intraspecific

interactions when two or more insects share a host plant. It is anticipated that intraspecific

interactions mediated by plants may result in competition between insect herbivores that attack

different parts of the plant. To test the effects of plant-mediated intraspecific interactions among

gall midges *Jaapiella ivannikovi* Fedotova and its impact on the Russian knapweed *Rhaponticum*

repens (L.) Hidalgo, I conducted two experiments each involving control plants, plants exposed

to female midges once, and plants exposed twice to female midges. In the first experiment

ovipositing female insects were allowed access to the whole plant, while in the second

experiment ovipositing insects were restricted to different branches of the plants (around 10 cm

apart). Surprisingly, exposure to *J. ivannikovi* did not reduce flowering or aboveground growth.

Indeed, in the second oviposition experiment, I found plants growing larger with midge

exposure. *Jaapiella ivannikovi* galls had negative effects on one another even when galls were

located on distant parts of the plant. I anticipate that such interactions are widespread in gall-

forming insects, even when occupying different parts of a shared host plant.

Introduction

Plant-mediated indirect interactions among herbivores are widely appreciated as an

important force structuring communities (Denno et al., 1995; van Veen et al., 2006; Denno &

Kaplan, 2007). In a meta-analysis of interactions among herbivore species (243 observations:

Kaplan & Denno, 2007), indirect interactions made up nearly 90% of the cases. Among those

cases involving indirect interactions, herbivore fitness outcomes were largely mediated by

changes in plant quality (70% of cases). Plant quality-mediated interactions occur when one

herbivore induces changes in plant traits such as defensive chemistry, plant morphology, or plant

nutrient allocation, that affect the performance of a subsequently feeding herbivore. Of these

interactions mediated by changes in plant quality, nearly 80% represented indirect competition

between herbivores while the other 20% represented cases where facilitation between herbivores

was mediated by plant quality. Much less in known about the effect of plant quality on

intraspecific interactions among herbivores (Bird et al., 2019). However, intraspecific

competition between herbivores is expected to be even greater than interspecific interactions

according to classical niche theory because individuals of the same species are more likely to

share similar abiotic and biotic requirements. (Chesson & Grubb, 1990; Hutchinson 1959). A

recent meta-analysis found that physical proximity is associated with increased competition

between insect herbivores (Bird et al., 2019). However, competition can occur across a wide

range of spatial scales ranging from individual plant to across landscapes. Studies on

interspecific competition have found interactions between spatially separated root and leaf

feeders (Moran & Whitham, 1990; Master & Brown, 1992; Anderson et al., 2011). At the

landscape level, plant leaf functional traits affect intraspecific competition of an insect herbivore

across the whole range of *Quercus garryana* (Loughnan & Williams, 2018).Exactly how spatial

separation on the same plant and plant-mediated interactions impact intraspecific interactions

among herbivores is unclear, particularly in weed biological control systems (Milbrath &

Nechols, 2014).

The physical co-location of gall-forming insects within a shared host plant is easy to manipulate allowing the study of proximity on the strength of competitive or facilitative interactions. Immature gall-forming insects are confined to galls, bloated or otherwise expanded plant tissue that is formed in response to oviposition or the developing insect larvae. Gall-forming insects are well known to influence chemical, physiological, and morphological aspects of their host plants (McKone et al., 2001; Gagné & Hibbard, 2008; Hall et al., 2012). Gall formers alter photosynthetic rates and divert nutrients from plant growth and reproduction (Harris & Shorthouse, 1996). Many gall-forming insects oviposit near or at plant metabolic sinks, such as meristems and flower buds. Galls commonly act as metabolic sinks themselves by increasing the production of auxin and cytokinin, hormones whose production are associated with plant sinks (Erb et al., 2012). Insect galls can compete with other plant metabolic sinks, like developing fruits or meristematic tissues. Studies have suggested that gall-forming insects (e.g., Inbar et al., 1995; Larson & Whitham, 1997; Dorchin et al., 2006) and gall-forming nematodes (Kaplan et al., 2011) compete with each other as metabolic sinks when occurring on the same host plant. Multi-sink competition is expected to be strongest when galls occupy nearby and similar parts of a plant (Inbar et al., 1995; Kaplan et al., 2011). Plant metabolic sinks can provide high quality nutrients and tissues for herbivorous insects. Sap-feeders, such as aphids, can benefit from feeding near plant metabolic sinks, including galls (Forrest, 1971) and pine needles (Kidd et al., 1985). The Russian knapweed gall midge *J. ivannikovi* attacks the apical meristems before they start to form flower buds, reducing seed production (Djamankulova et al., 2008). This system provides an opportunity to explore intraspecific insect competition and its impacts on host plant fitness.

Rhaponticum repens is a perennial plant native to Eurasia ranging from Turkey to China. It has been present in the western United States for over a century and is categorized as a noxious weed in many states (Djamankulova et al., 2008). It is toxic to horses, causing a "chewing disease" (nigropallidal encephalomalacia) and brain damage, which can lead to death (Chang et al., 2012). *Rhaponticum repens* can grow on a wide range of soil types and moisture conditions and does particularly well in recently disturbed soils. Russian knapweed generally does not invade healthy, intact, native habitats (Zouhar, 2001). However, it occurs in rangelands of varying grazing intensity throughout the western United States. In part, this plant is difficult to control because it has an extensive root system through which it can propagate asexually creating large clones. In North America, aboveground ramets within a patch of *R. repens* tend to be a single genetic clone (Gaskin & Littlefield, 2017). Nutrients stored in root tissues may be allocated among ramets depending on external factors, such as rainfall (Hartnett & Abrahamson, 1979; Abrahamson and McCrea, 1986). Seedling establishment appears to play a minor role in maintaining or growing established clones, and seedling establishment is likely the primary means of colonizing new sites or sites at the periphery of an established patch (Djamankulova et al., 2008). Farming equipment may move rhizomes to new sites. Each ramet can produce upwards of 1200 seeds, which may remain viable in the seed bank for up to five years (Anderson, 1993). Controlling seed production will prove crucial in slowing the spread of this noxious weed. Mechanical removal and the use of herbicides to manage *R. repens* over vast areas of infested land, which generally has low economic value, are impractical and unsustainable (Jones & Evans, 1973; DiTomaso, 2000). Therefore, interactions between individual *J. ivannikovi* galls and their effects the knapweed plants are particularly important to quantify.

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The specialist gall midge *J. ivannikovi* was recently approved as a biocontrol agent for release against *R. repens* (USDA APHIS, 2009). These insects need young meristematic plant tissue to induce gall formation, making plant phenology and growth essential to insect survival. *Jaapiella ivannikovi* is multivoltine and active through much of the summer, making young *R. repens* shoots susceptible to attack throughout the growing season. In its native range in Uzbekistan, *J. ivannikovi* reduces shoot length by 10-15%, aboveground biomass by 20-25%, and seed output by 90-95% (Djamankulova et al., 2008). However, field observations at a release site in an invaded range in Wyoming showed that the fly had minimal impacts on Russian knapweed (Meyers et al., 2015). Impacts of herbivory by galling insects on clonal growth and aboveground ramet production are unknown, especially for *R. repens* growing in North America. Manipulative studies addressing intraspecific interactions can address abiotic and biotic factors that determine why biocontrol agent populations stay low after release and/or have minimal effects on knapweed populations.

I conducted two experiments that varied the numbers of ovipositing adult females on potted plants in the greenhouse to observe competitive interactions between resulting gall broods and the resulting impacts on knapweed plants. In the first experiment, midge females had access to the whole plant for oviposition. In the second experiment, I restricted female oviposition to specific branches of the plant, with multiple exposures occurring on spatially separated parts of the plant. I asked two main research questions about *J. ivannikovi* and *R. repens* interactions. First, do plant mediated indirect interactions occur between broods of midges in spatially-separated galls on a shared host plant? I expected competitive effects on midge offspring to increase with increased resource competition when more *J. ivannikovi* females were attacking the plant. I also anticipated that restricting female oviposition to particular plant tissues would

still allow for interbrood competition between distant parts of the plant due to plant-wide physiological changes. Second, I asked how midge gall formation affects plant traits. I expected herbivory to reduce plant size and flowering, with greater numbers of herbivores to have the largest impact on plant hosts.

Materials and Methods

Study system

Russian knapweed, *R. repens,* can reproduce both sexually and asexually, with clonal growth resulting in genetically identical stems in large dense patches (Gaskin & Littlefield, 2017). In early April 2016, Russian knapweed ramets were collected from a dense clone in northern Colorado (N 40.673672, W -104.982033) and transplanted with rooting hormone into the greenhouse for both experiments. Since stems were collected within three meters of one another, plants in the experiment were likely from one genetic clone and had experienced similar environmental conditions (Gaskin & Littlefield, 2017).

One *J. ivannikovi* generation takes more than 21 days and less than four weeks (Meyers et al., 2015), the duration of our experiments. *Jaapiella ivannikovi* adults are short-lived (2-7 days), and females often lay more than ten eggs per gall (with a reported 1:1 sex ratio), which are formed in the apical and lateral meristems (Djamankulova et al., 2008). I received *J. ivannikovi* gall midges for the experiments from a greenhouse reared colony at the Palisade Insectary (Palisade, Colorado) of the Colorado Department of Agriculture.

Experimental Design

In 2016, I conducted two experiments: 1) a Whole Plant Exposure experiment in January, and 2) an Oviposition Restriction experiment in August where I varied the number of mated females attacking a plant (Figure 4.1). Both experiments included three treatments: a) control plants with no *J. ivannikovi* midge exposure, b) one exposure to three adult females, and c) two exposures to different groups of three adult females at one week apart from one another (six midges total). Each exposure included three female *J. ivannikovi* midges (previously exposed to males for 24 hours to ensure mating) to increase the likelihood of gall formation because females are short lived with high mortality rates. In June, 167 plants were individually grown in one gallon pots in the greenhouse after transplantation for the Whole Plant Exposure experiment. After plant deaths, resulting sample sizes include; control N=13, single exposure N=13, two exposures N=34. In August I repeated this design with 92 potted plants for the Oviposition Restriction experiment (control N=16, single exposure N=48, two exposures N=28). They were watered as needed, typically four to six times a week. The greenhouse settings include a 16:8 L:D photoperiod, with temperature held between 20 and 27°C.

Plants were randomly assigned to treatments and distributed randomly across the greenhouse bench. At the time of exposure treatments, plants were similar in height, as well as number of branches and leaves for each experiment. *Jaapiella ivannikovi* were confined to plants with mesh bags made from nylon tulle. In the Whole Plant Exposure experiment, I placed mesh bags around the top of the pot covering the whole plant. In the Restricted Exposure experiment, I confined ovipositing *J. ivannikovi* females to specific meristems of a plant by first placing a mesh bag over the apical meristem with the bottom of the mesh bag sealed 5 cm below the apical meristem around the stem with a twist tie (experiment 2 in Figure 4.1). In the double exposure

treatment, I selected two meristems separated about 10 cm apart along the main stem on each plant and enclosed each in separate mesh bags. This was usually an apical meristem and a lateral meristem. I aspirated three mated female *J. ivannikovi* into the mesh bags for each exposure. I removed the bodies of females only after they died a few days after the treatment. About half of the Whole Plant Experiment exposures resulted in galls, with some plants resulting in upwards of 8 galls/plant. All of the Oviposition Restriction Experiment exposures resulted in galls, with only two extra galls forming.

Measurements of plant height, number of leaves, number of flowers (including buds), insect offspring emergence per plant, number of galls, and gall height and width were taken at the beginning and end of the month-long experiments. The mesh bags were left on all plants until the end of the experiment to catch emerging adult offspring. In the Oviposition Restriction Experiment (2), I tracked insect emergence/exposure. For the second experiment, I also measured the number of ramets/plant, the number of root buds/plant, and wet mass of aboveground (all aboveground biomass per potted plant excluding galls) and belowground tissue before preserving plant materials at 20°C, for later reference.

The mesh bags were removed from plants after four weeks and the number of emerged adult offspring was counted. *Jaapiella ivannikovi* galls are round, occur at the meristematic tissues, and are made of many leaves, and possess long trichomes compared to other leaves on Russian knapweed. I cut a random subset of the galls off where the stem ended and stored them at 1.5˚C until each gall could be dissected to count the number of larvae inside. Other galls were left to determine adult emergence. Gall dissections occurred within a week of removal. I examined resulting galls under a dissecting microscope, recording gall length (mm) from stem to farthest gall leaf, maximum gall width (mm), number of larvae, number of pupae, number of

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adults, and number of exuviae (pupal casings of emerged adults) at the time of gall dissection. Gall size, measured with hand calipers, represents resources available to insects. Resulting offspring totals from female oviposition are proxies for fitness.

Statistical Methods

Response variables of plant traits were considered separately for each experiment and compared using ANOVAs across the three treatments (control, one exposure, and two exposures). Response variables of insect traits were analyzed using t-tests comparing gall height and width from plants with one exposure to plants with two insect exposures. When data did not fit assumptions of a parametric test, I used a Kruskal-Wallis ANOVA. In the second experiment, I had plant deaths after insect exposure treatments, so I ran a log-linear chi-square test on a 2 by 3 table to determine if plant deaths were a result of treatment. All statistical methods were conducted with SAS$^©$ University Edition 9.4 software.

Results

First Experiment: Whole Plant Exposure

Gall midges experience increasing competition with an increase in the number of midge exposures. From plants that received one insect exposure I dissected and measured 20 galls, and from two insect exposures I measured 13 galls. I found an average of 4.4 more insects/gall for single exposures compared to two exposures (Figure 4.2). Galls were an average of 1.7 mm wider when formed alone compared to multiple exposures ($F_{1,24}$=0.46, P =0.0433). Gall height was not significantly different between treatments (Kruskall-Wallis Test, $\chi^2_{1,24}$=0.53, P =0.4674). After the first set of exposures, I observed adult female midges from the second set of exposures

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ovipositing on fully formed galls that resulted from the previous exposure. Though not significant, we saw an average of 1.15 galls/plant on singly exposed plants compared to an average of 3.0 galls/plant on twice exposed plants (Kruskall-Wallis Test, $\chi^2_{1,45}$=0.9570, P =0.3393). A total of 79 galls formed, with a maximum outlier of 15/plant and mode of one gall/plant. A total of 24 insects emerged from the galls that were not dissected, with a 9:2 female:male ratio, which is significantly different from an expected 1:1 ratio ($\chi^2_{1,23}$=8.909, P =0.0028).

Exposure to insects did not have strong effects on plant growth when ovipositing females had access to the whole plant. Average plant height (cm) ($F_{2,57}$=0.26, P=0.7750), average number of branches (Kruskal-Wallis: $\chi^2_{2,57}$=4.91, P =0.0859), and average number of reproductive nodes (buds and flowers) did not differ significantly across the three treatments (ANOVA: $F_{2,57}$=0.88, P=0.4186).

Second Experiment. Oviposition Restriction

Similar to the first experiment, multiple exposures to ovipositing midges has negative effects on the resulting number of offspring per gall with an average of 2.3 fewer larvae in galls for plants exposed twice versus a the palnts exposed once (Figure 4.3). There were no strong effects of exposure number on gall width (Kruskal-Wallis: $\chi^2_{1,53}$=0.01, P =0.9260) or gall height (Kruskal-Wallis: df=1, $\chi^2_{1,53}$=0.02, P =0.8794). One gall per exposure formed in all but two treatments (with two galls) because insects were limited to one potential oviposition site, with 100% successful gall formation for exposures overall in the oviposition restriction experiment. For one exposure treated plants, I measured 29 randomly-selected galls from 48 exposed plants. For two exposure treatments, I measured 26 randomly-selected galls from 56 exposures on 28

plants. The undissected galls provided 57 female and 40 male insects emerging out of 43 galls, which is not significantly different from an expected 1:1 ratio ($\chi^2_{1,98}$=1.87, P =0.0843).

In this experiment, I observed plant wilting and death after insect exposure treatment starting with 166 plants and resulting in sample sizes of control N= 43, one exposure N= 78, and two exposures N= 45. Control plants had a 79% survival rate, one exposure had a 90% survival rate, and multiple exposures resulted in a 58% survival rate (χ^2=3.36, P =0.186). Remaining analyses include only plants that were alive at the end of the experiment and, thus, measured for response variables.

Galling, when restricted to certain parts of the plant, changed the way that plants grow. Compared to the control treatments with an average of 3.5leaves, there were an average of one and two more leaves on plants with single and multiple exposures, respectively (Kruskal-Wallis: $\chi^2_{2,89}$=6.48, P =0.0392). Surprisingly, aboveground biomass of plants doubly exposed to midges were 60% greater than control plants, while those exposed once were 15% heavier than control plants (Figure 4.4). Control plants had an average of 2.06 aboveground ramets per plant, while *J. ivannikovi* exposed plants had 2.52 ramets for single exposures and 2.19 ramets for multiple exposures (Kruskal-Wallis: $\chi^2_{2,89}$=4.68, P=0.0962). Notably, single exposure and multiple exposure *J. ivannikovi* galled plants had 72% and 61% larger root mass than ungalled control plants, respectively (Figure 4.5). Control plants had an average of 3.7 root buds per plant, while *J. ivannikovi* exposed plants had 8% more for single exposures and 60% more for multiple exposures, though not quite statistically significant (Kruskal-Wallis: $\chi^2_{2,89}$=5.55, P=0.0625). I found no significant plant differences between treatments in average height (Kruskal-Wallis: $\chi^2_{2,89}$=4.50, P =0.1053), average flowers and buds per plant (Kruskal-Wallis: $\chi^2_{2,89}$=0.61, P =0.7379), average number of branches (Kruskal-Wallis: $\chi^2_{2,89}$=0.66, P =0.7197), and vegetative

regrowth as measured by the number of new ramets produced (Kruskal-Wallis: $\chi^2_{2,89}=0.02$, P =0.8794).

Discussion

As expected, more *J. ivannikovi* midge galls per plant led to stronger intraspecific competitive effects. This pattern held both when ovipositing midges had access to the whole plant and when they were restricted to spatially separated parts of the plant. In the treatments with multiple insect exposures restricted to different locations within a plant, there was evidence of plant-mediated competition. Perhaps, gall formation altered resource allocation and midge broods competed for resources within the plant, much like findings in goldenrod gall insect attack (Hartnett & Abrahamson, 1979; Abrahamson and McCrea, 1986).

Unexpectedly, greater midge exposures resulted in larger plants. While I expected that the formation of galls would reduce flowering and aboveground biomass as the biocontrol agent reallocated resources away from sexual reproduction, I did not find that *J. ivannikovi* reduces flowering and aboveground growth of *R. repens*. On the contrary, there were more root buds on plants exposed to multiple *J. ivannikovi* ovipositing females. Increased root growth may represent a compensation mechanism by which future ramets can be produced in the field after *J. ivannikovi* are actively ovipositing, thereby helping the plant to reduce the impacts of herbivory. However, *J. ivannikovi* is multivoltine so can attack these newly produced shoots.

These results contrast with those of *J. ivannikovi* midge attack on ramets in the field in its native range (Djamankulova et. al., 2008) that showed reduction in aboveground ramet growth in the field. I found that midge sex ratios of newly emerged adults from individual galls were strongly female biased, suggesting that midges may be haplodiploid and experience local mate

competition. Some Cecidomyiidae species are haplodiploid or reproduce with cyclic thelytoky (Normark, 2003). *Rhaponticum repens* clone sizes in the western US can be large and dense with extensive aboveground ramets (Meyers et al., 2015), and our greenhouse results suggest that *J. ivannikovi* may have minimal or even positive interactions increasing ramet production over a month. *Jaapiella ivannikovi* does not appear to have a strong impact on a *R. repens* infestation in Wyoming Meyers et al. (2015). When invasive plants are reunited with their specialist herbivores (such as with biocontrol) they can experience greater damage from these herbivores (Enemy Release Hypothesis). Reciprocal transplant and common garden experiments could address whether *R. repens* in the western US is indeed better able to tolerate *J. ivannikovi* attack than *R. repens* in the native Eurasian range.

Controlled, short-term experiments in the greenhouse such as presented in this study are likely to have different results than larger community and ecosystem level studies. Where feeding occurs (which meristem or ramet) on a plant, may determine how herbivores impact plants at larger scales across the whole clone. Studies show that both spatial and temporal separation can still allow for intraspecific interactions among insects (Barnes & Murphy, 2018). Such factors may be especially important to consider when dealing with perennial, clonal plants. Future field observations and field experiments can be paired with greenhouse experiments to address how *J. ivannikovi* functions as a biological control agent. Plant physiological traits and responses could drive larger patterns relevant to restoration, conservation, and weed management. I anticipate that temporal and spatial separation within new populations of biocontrol agents will largely determine population establishment and persistence over time.
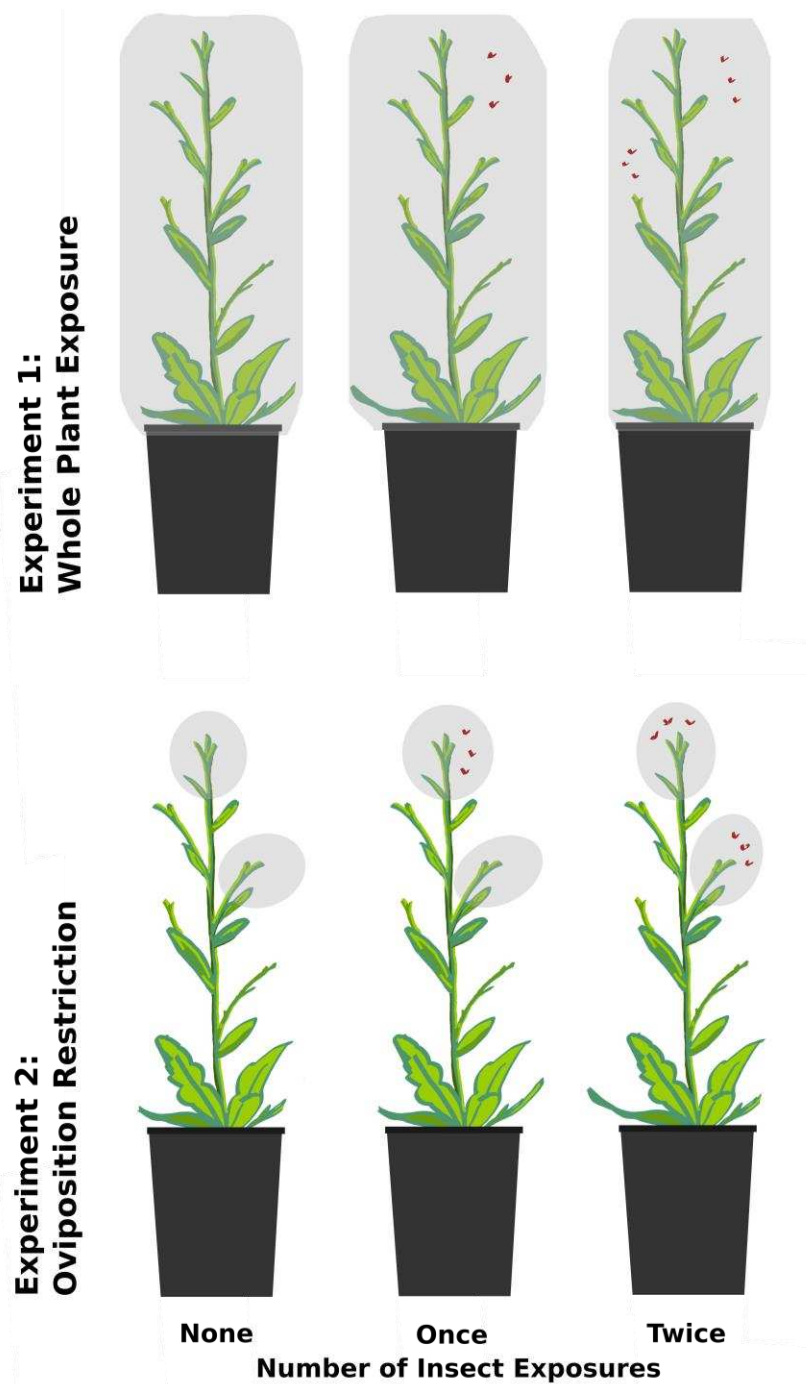
Figure 4.1. For the first experiment, I conducted whole plant exposures with mesh covering the entirety of the aboveground plant tissues. For the second experiment, I used small mesh bags to restrict oviposition to one meristematic branch of the plant. Control plants and branches also had mesh placed over them to control for shading and weight of the treatments. Small dark red marks indicate *Jaapiella ivannikovi* midges. There were three mated female midges per release. In the two exposure treatments there was a week between the first and second exposure.
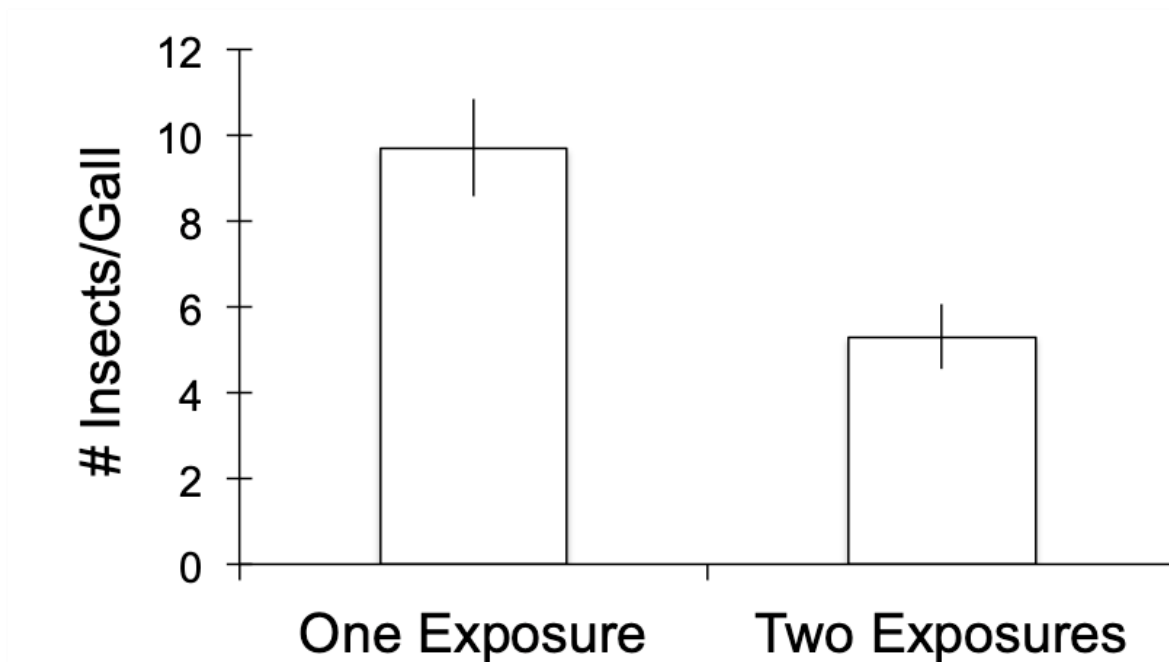
Figure 4.2. Average number of *Jaapiella ivannikovi* per gall with standard error bars for the first whole plant exposure experiment (Kruskal-Wallis Test, χ^2=0.03, df=1,24, P =0.8677). From plants that received one insect exposure I dissected and measured 20 galls, and from two insect exposures I measured 13 galls, randomly selected subsamples.
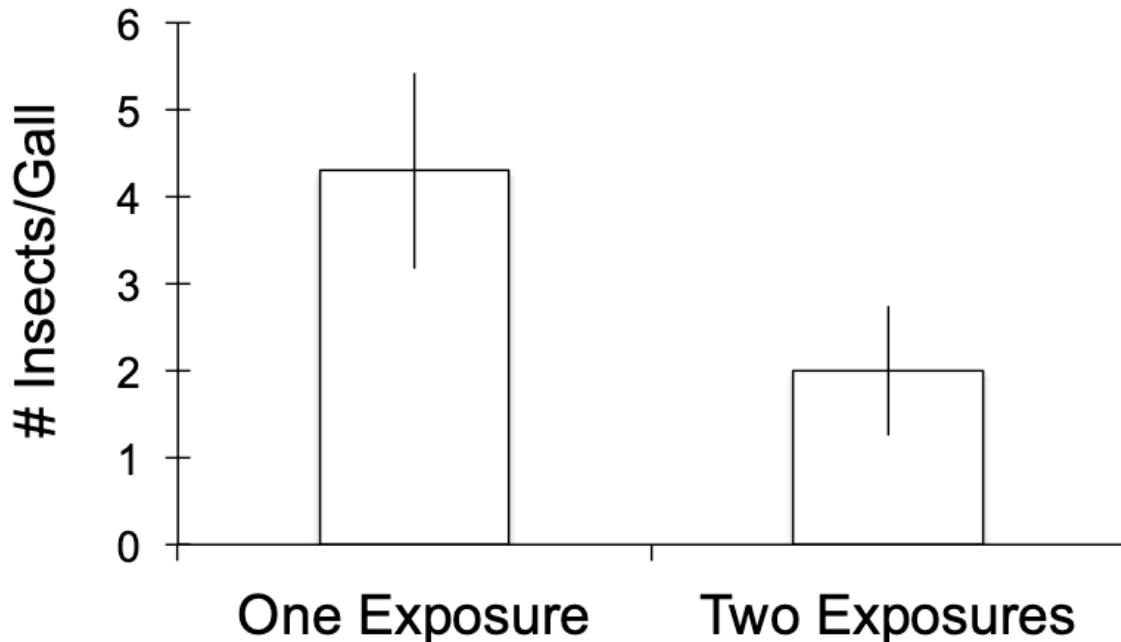

Figure 4.3. Average number of *Jaapiella ivannikovi* per dissected gall for treatments with standard error bars for the second, oviposition restriction experiment. Galls on plants exposed to multiple insects had an average of 2.3 fewer larvae than the ones from a single exposure (Kruskal-Wallis: $\chi^2_{1,53}$=1.87, P =0.1718).
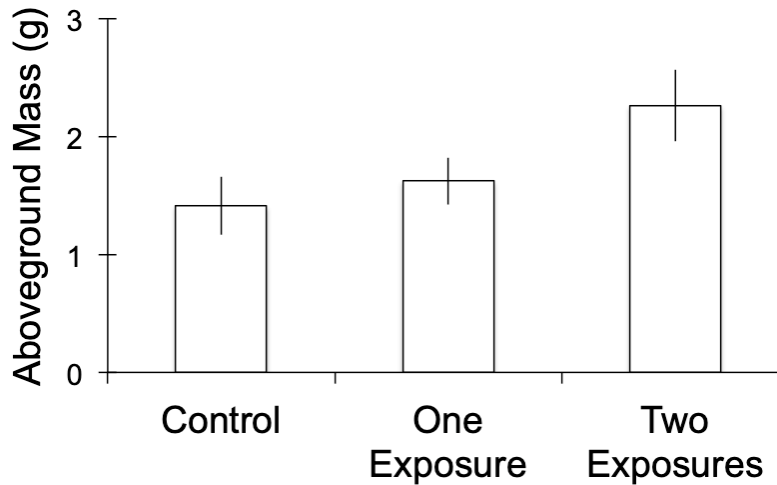
Figure 4.4. Average wet aboveground biomass (g) of Russian knapweed per treatment with standard error bars for the second, oviposition restriction experiment (Kruskal-Wallis: $\chi^2_{2,59}$=8.63, *P*=0.0134; N=34 control,70 one exposure, & 26 two exposure). This includes all aboveground biomass per potted plant except for galled material.
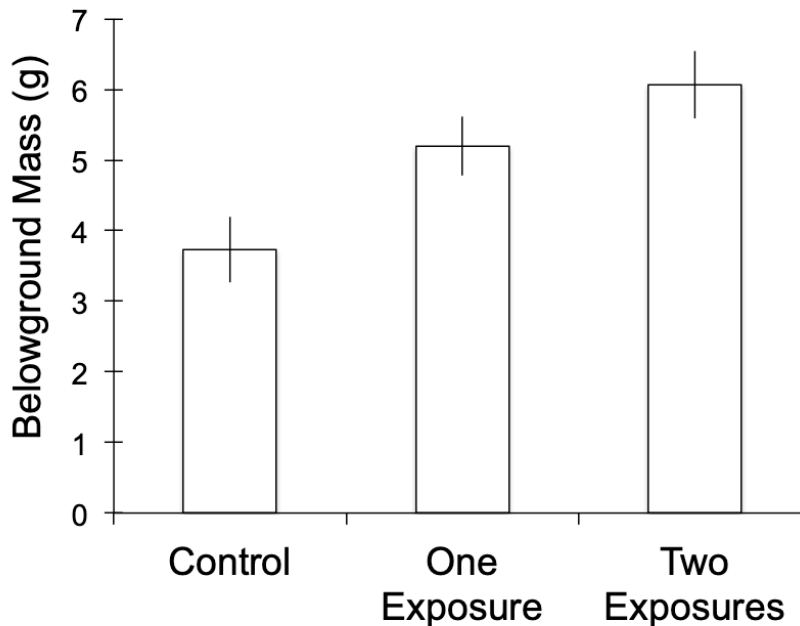
Figure 4.5. Average wet belowground biomass (g) of Russian knapweed per treatment with standard error bars for the second, oviposition restriction experiment. Notably, one exposeure and two exposure *J. ivannikovi* galled plants had larger root mass than ungalled control plants (Kruskal-Wallis: df=2 & 89, χ^2=19.95, P<0.001). Galls were less than 0.2 grams each, often much smaller. Control plants had an average of 3.7 root buds per plant, while *J. ivannikovi* exposed plants had 8% more for single exposures and 60% more for multiple exposures, though not quite statistically significant (Kruskal-Wallis: $\chi^2_{2,89}$=5.55, P=0.0625).

Chapter 5 - PLANT-MEDIATION REPRESENTS ALTERNATIVE HYPOTHESES TO THE

STRESS GRADIENT HYPORTHESIS FOR HERBIVORES

Summary

Developing theory to predict when facilitative interactions take place requires designation of clear alternative hypotheses to the Stress Gradient Hypothesis (SGH). In attempts to predict facilitation, researchers have applied inference from the SGH to intraspecific plant interactions and interspecific insect interactions even though the SGH was first proposed to predict interactions between plant species given an abiotic environmental stress gradient.

I propose assessing the limits of the SGH by conducting empirical tests on intraspecific herbivore insect interactions. The SGH provides a framework for testing the factors and interactions that shape herbivore intraspecific facilitation and competition.

If inference from the SGH applies to intraspecific herbivore interactions, researchers should find an abiotic stress gradient significantly impacting interaction outcomes. Alternatively, plant biotic factors are likely important for governing interaction outcomes.

Employing clear hypotheses with well-designed experiments and statistical methods will help determine the most important factors driving species interactions. Beyond confirming or modifying the SGH as it applies to higher trophic levels, future research on this topic will also contribute to understanding how herbivores respond to and alter plant traits. This framework should be used in future Russian knapweed biocontrol research. I anticipate future research demonstrating that biotic mediation is central across study systems, trophic levels, and ecosystems.

Inference Applied Widely

Determining when facilitation is likely to occur contributes to understanding processes at broader scales of community composition, evolutionary patterns, and ecosystem services (Bertness & Callaway, 1994; Maestre, Callaway, Valladares, & Lortie, 2009; Monroe et al., 2018). Herbivores can interact with one another positively or negatively while sharing a plant resource, and they particularly impact plant fitness, growth, and functional traits. Insect herbivores are diverse and widespread with significant impacts on ecological communities and ecosystems (Kaplan & Denno, 2007; Lau & Strauss, 2005). Researchers have observed extensive facilitation occurring between herbivorous insects (Bird, Kaczvinsky, Wilson, & Hardy, 2019), contradicting predictions based on classic competition theory (Chesson & Grubb, 1990; Denno, McClure, & Ott, 1995; Inbar, Eshel, & Wool, 1995; Kaplan, Sardanelli, Rehill, & Denno, 2011). Some progress has been made in predicting when interactions between herbivores will be facilitative as researchers applied inference from the Stress Gradient Hypothesis (SGH) to insect systems (Carbonell et al., 2017; Dangles, 2019; Dangles, Herrera, & Anthelme, 2013).

The SGH was first proposed to predict interspecific plant interactions, suggesting a greater number of facilitative interactions should occur in communities in high abiotic stress environments contrasting with more competitive interactions occurring in low stress environments (Bertness & Callaway, 1994; Lortie & Callaway, 2006). Since then, inference from the SGH has been applied to the direction and magnitude of pairwise interactions (Castro, Zamora, Hódar, Gómez, & Gómez-Aparicio, 2004), higher trophic levels (Bakker, Dobrescu, Straile, & Holmgren, 2013; Grinath, Larios, Prugh, Brashares, & Suging, 2019), and intraspecific interactions (Biswas & Wagner, 2014; Eränen & Kozlov, 2008). Most surprisingly, even though the SGH was formed around abiotic environmental stress, researchers tested the hypothesis on

interspecific insect interactions using a plant resistance trait as a biotic stress gradient and found evidence to support the hypothesis (Dangles et al., 2013).

   With mixed support and apparent a posteriori usage, the SGH may not apply to higher trophic levels (Bakker et al., 2013; Kawai & Tokeshi, 2007). Biotic factors and mechanisms function differently than abiotic environmental stress and likely result in dissimilar outcomes from those proposed by the SGH (Smit, Rietkerk, & Wassen, 2009). Abiotic and biotic factors interact with one another to create a complex system, with many potential outcomes. There are critiques on the part of the SGH focused on predicting interaction outcomes on fitness direction (facilitation, neutral, or competitive) and magnitude (strength) (Bakker et al., 2013; Castro et al., 2004; Holmgren & Scheffer, 2010; Michalet, Le Bagousse-Pinguet, Maalouf, & Lortie, 2014). Whether or not inference from the SGH concerning interactions outcomes applies to insect conspecifics sharing an individual plant resource, the concept provides an opportunity to test which factors out of many do indeed drive insect intraspecific interactions in complex biological settings.

Alternative Hypotheses to the Stress Gradient Hypothesis

   If inference from the SGH applies to intraspecific interactions at the consumer trophic level, abiotic stress may act in much the same way the SGH applies to plants (Figure 5.1A). Alternatively, stress may drive intraspecific interactions as mediated by biotic factors. This may occur from a bottom-up mode with plant traits mediating abiotic stress (Figure 5.1Bi). Otherwise, biotic stress represented by plant traits or host genetics may largely shape insect interactions (Figure 5.1Bii). Furthermore, insect interactions may be largely plant trait-mediated as a conspecific alters plant traits in a way that impacts insect fitness (Figure 1Biii). See Table

5.1 for examples of abiotic and biotic factors describing each hypothesis. If plant biotic factors play a significant role by either mediating abiotic stress or representing biotic stress (Figure 5.1B & Table 5.1B), inference from the SGH needs to be modified when applied to consumers.

Study Recommendations

To select ideal systems for testing the SGH and alternative hypotheses, a researcher must identify components represented in Figure 5.1C, including individual fitness, conspecific presence, plant traits, and abiotic factors. Using the hypotheses laid out above, researchers can clarify which factors most affect how conspecific insect presence impacts focal insect fitness and vice versa. Most importantly, hypotheses and alternative hypotheses need to be explicitly defined before conducting an experiment. I suspect the SGH is often used a posteriori when ecologists uncover facilitative interactions between consumers. If so, empirical studies testing the SGH in the broader literature represent more support for the SGH than is justified. If null results remain unpublished or do not explicitly mention the SGH, an accurate literature review and meta-analysis of the SGH would be challenging to conduct. Therefore, when setting out to test the SGH in insect interactions, I propose using the alternative hypotheses listed in Table 5.1A-B to clarify the focus of the research. Studies may address a subset of these factors.

Forethought about the biology of a given system will inform hypotheses and model building (Restif et al., 2012). When conducting empirical studies to address alternatives to the SGH, researchers must consider how abiotic factors impact plants and/or insects directly and indirectly. If abiotic factors directly impact conspecific presence and insect fitness, then the system may most represent the abiotic SGH (Figure 5.1A). However, if abiotic factors are mediated by plant traits, then we are likely to see deviations from typical SGH mechanisms and

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outcomes. Spatial and temporal relationships between tested factors are also important to consider, as the direction of causation needs to be clearly specified. For example, one needs to know whether the presence of a conspecific altered plant traits or if a given plant trait led to conspecific presence. Note the differences in Figure 5.1 hypotheses with an arrow from plant traits to conspecific presence (Bi & Bii) versus the arrow going the other direction away from conspecific presence and toward plant traits (Biii). By predefining research questions and hypotheses, ecologists can use experiments to resolve how widely the SGH applies.

Data analysis and ecological model building should reflect the hypotheses to be addressed. Methods such as model selection can allow ecologists to decide which factors to include in a model, explicitly addressing the Table 5.1 hypotheses by eliminating factors. Path analysis and Structural Equation Modeling (SEM) are common methods for determining the strength of causation between factors (Fan et al., 2016; Shipley, 2016). Figure 5.1C represents a generalized diagram of a path analysis, with arrows indicating interactions between factors. The analysis results in an understanding of the direction and magnitude of the interaction between factors. Therefore, findings of strong interactions that mirror one of the hypotheses represented in Figure 5.1 would provide support for that hypothesis. Building up evidence for the SGH or an alternative hypothesis will require a large, new body of empirical work.

Does Biotic-Mediation Shape Interaction Outcomes?

Testing to what extent inference from the SGH applies to disparate systems will shape future ecological work. Specifically, testing herbivore intraspecific interactions will represent a new application of the SGH. Given trends in how the SGH has been applied, the extension of inference to this area is warranted. Research at the forefront of recent use of inference from the

SGH (Dangles, 2019) will particularly shape theory and future use. With similar methods to thoseproposed, ecologists can employ inference to examine other trophic levels. In particular, considering interactions in soil communities may further improve ecological theory and inform plant resource acquisition. Plant interactions with micorrhizal fungi, mutualistic bacteria, and other biotic factors likely merit a re-examination of the SGH as it is used to describe plant interactions. The SGH is commonly applied to plant interactions, suggesting it will continue to guide future botanical research (Kjær, Olsen, & Klanderud, 2018; Kleinhesselink & Cushman, 2019; Lett, Wardle, Nilsson, Teuber, & Dorrepaal, 2018; Meysick et al., 2019). Even though the SGH is used widely, careful scrutiny should accompany its application to an empirical study and aid in clarifying which variables to measure and manipulate.

Determining whether the SGH applies to higher trophic levels is of particular importance to plant ecology and weed biological control. First, because the SGH was developed by plant ecologists, they should best be able to determine its restrictions. Second, plant-mediation is likely an extremely important mechanism in ecological systems (Kaplan & Denno, 2007). Plants, representing biotic stress or as mediators of stress, play a major mechanistic role in determining ecological interaction outcomes. I predict this role is extremely significant in ecological communities, and anticipate future empirical studies on insect herbivores, such as Russian knapweed's biological control agents (Djamankulova et al., 2008), will result in more support for the alternative hypotheses with plant-mediation (Figure 5.1B) than the abiotic focused SGH (Figure 5.1A). Primary producers form the foundation of food webs across ecosystems, playing an important role in governing outcomes for organisms at all trophic levels. Determining whether biotic factors play a role in facilitation across trophic levels is the first step toward establishing how widely the SGH applies.
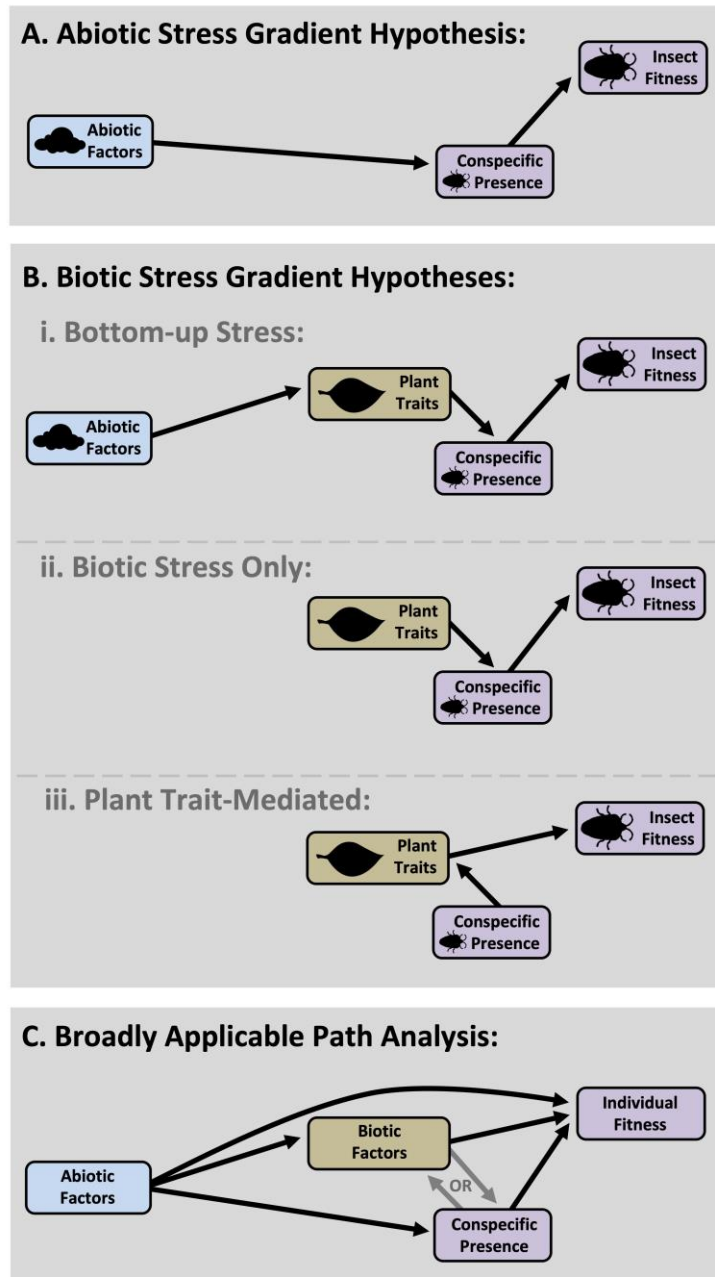
Figure 5.1. Display of which factors determine intraspecific insect interactions. A) Representation of abiotic factors driving interactions. B) These diagrams show biotic-mediation: i) displays bottom-up forces, with plant traits mediating abiotic stress impacts on insect interactions, ii) displays plant traits determining insect interactions, and iii) shows plant traits mediating conspecific impacts on insect individual fitness. C) The broadly applicable path analysis displays how to test which factors are most influential at any trophic level with a potential biotic-mediator. Such an analysis could employ multiple biotic and abiotic factors. The arrow connecting biotic factors (e.g. plant traits) to conspecific presence is only able to go in one direction, so system specific knowledge is important to determine biological relevance of the arrow direction in a given model.

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Table 5.1. Our hypotheses (A&Bi-iii) on factors that impact insect interactions are laid out with potential expectations, supporting conceptual studies and reviews, and biological examples surrounding the willow (*Salix lasiolepis* Smith) – sawfly (*Euura lasiolepis* Bentham) system.

Hypotheses		Influential Factors	Potential Expectations	Conceptual Literature	Examples: *Salix - Euura* system	Example Citation
A. Abiotic Stress Gradient		Abiotic factors	Herbivorous conspecifics experience stronger competition in lower stress environments and stronger facilitation in higher stress environments.	Bertness & Callaway, 1994; Castro et al., 2004; Lortie & Callaway, 2006	Low humidity desiccates adult sawflies and dries out eggs, while excessive humidity renders wings dysfunctional.	Perez-Mendoza & Weaver, 2006; Price & Clancy, 1986
B. Biotic Stress Gradient	**i. Bottom-up Stress**	Abiotic & biotic factors	Abiotic stress reduces biotic plant resources, resulting in greater competitive effects for the low supply of resources	Price, 1991; Staley et al., 2011; Vidal & Murphy, 2018	Drought reduces *Salix* plant quality for *Euura* gall sawflies in a bottom-up fashion.	Price & Hunter, 2005
	ii. Biotic Stress Only	Biotic factors: plant traits and/or plant host genetics	Conspecific presence mediates plant trait impacts on a focal individual's fitness.	De Bruyn, 1995; Huberty & Denno, 2004; Lenhart, Eubanks, & Behmer, 2015; Veldtman & McGeoch, 2003	Genetics of the *Salix* plant host determine amount of resources diverted toward shoot and gall growth. *Euura* sawfly preferring male *Salix* plants with higher leaf nutrients.	Boecklen, Price, & Mopper, 1990
	iii. Plant Trait-Mediated	Biotic factors: plant traits	A conspecific alters plant traits that impact insect fitness.	Denno et al., 2000; Erb et al., 2011; Kaplan & Denno, 2007; Masters & Brown, 1992; Masters, Brown, & Gange, 1993	Sawfly individuals experience facilitation when *S. lasiolepis* plants respond to galling by growing quickly as measured by shoot length and insect adult females experience competition for limited oviposition sites.	Craig, Itami, & Price, 1990

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