

OLFACTORY PREFERENCE AND REPRODUCTIVE ISOLATION OF TWO
MECINUS SPECIES (COLEOPTERA: CURCULIONIDAE): IMPLICATIONS
FOR BIOLOGICAL CONTROL OF DALMATIAN, YELLOW, AND
HYBRID POPULATIONS OF TOADFLAX, *LINARIA* SPECIES

by

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DEDICATION

I would like to dedicate this to my father Charles Neil Hubbard who passed away during the spring of 2013. At times when I questioned if this was something I could do, you were the one who counseled me to never quit something I start. And if nothing else, I have done just that. Thank you for being there when I needed it and for teaching me to never quit. Thank you!

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ABSTRACT

Classical biological control of the exotic, invasive toadflaxes *Linaria vulgaris* (L.) Mill. and *Linaria dalmatica* Mill. has had both successes and failures. One of the new challenges land managers face is the apparent increase in vigor shown by naturally occurring hybrid populations of the two toadflax species. This has presented practical problems because managers now are unable to decide which weevil species to use on these hybrids: *Mecinus janthinus*, which is found on *L. vulgaris*, or *M. janthiniformis* which has preference for *L. dalmatica*. This key question was addressed using olfactometer experiments to determine if the volatile profile for each plant establishes host fidelity for the naturally-occurring associated *Mecinus* species. Adults of both insect species were paired in cages on clones of naturally occurring and synthetic reciprocal cross hybrids and the parent toadflax species to quantify mating events and to determine the number of offspring produced on clones of each plant type of plant in incomplete randomized blocks. We did this using both intraspecific and interspecific pairs of *Mecinus* species to determine how many offspring are produced by intraspecific adults and also to explore the possibility of establishing a hybrid weevil population using clones of each plant type.

Host plant preference for both *Mecinus* species is influenced by olfactory responses, but this was only evident for adult females. Our first series of no-choice experiments with intraspecific mating pairs indicated that *M. janthiniformis* is more successful in terms of offspring produced for all types of hybrid toadflax tested. The results also showed that *M. janthinus* had a higher percentage of survival on all types of hybrids. As expected each *Mecinus* species performed best on its natural host plant. The results of our second series of no-choice experiments suggest that these two weevil species can produce viable interspecific offspring on clones of most of the plant types evaluated. This suggests that the newly described *M. janthiniformis* is very similar to *M. janthinus* and the separation between the two species is controlled at least in part, by olfactory cues from the favored host. In the future, land managers can better decide which *Mecinus* species to use based on whether the hybrid weed species is influenced more by *L. vulgaris*, or *L. dalmatica*.

CHAPTER ONE

BIOLOGY AND BIOLOGICAL CONTROL OF INVASIVE TOADFLAX

Introduction

Dalmatian toadflax, *Linaria dalmatica* (L.) Mill., and yellow or common toadflax, *Linaria vulgaris* Mill. , are highly invasive Eurasian weeds established throughout continental North America (Mitich 1993; Saner et al. 1995; Vujnovic and Wein 1997). Classical biological control with a toadflax stem-mining weevil identified as *Mecinus janthinus* Germar (Coleoptera: Curculionidae), initially released against North American infestations of both toadflax species, has been inconsistent but has yielded the best results to date (De Clerck-Floate and Miller 2002; McClay and Hughes 2007; Van Hezewijk et al. 2010).

Biological control of Dalmatian and yellow toadflax has been problematic but inherent challenges can in part be explained by recent revelations. Fertile and persistent hybrid toadflax populations can result from the cross pollination of *Linaria dalmatica* and *L. vulgaris*. The first confirmed naturally occurring North American hybrids of these species were discovered at two sites in Montana (Ward et al. 2009). Hybrid toadflax has since been confirmed on a growing number of sites in Washington, Idaho, Wyoming and Colorado (S. Ward, unpublished data).

Weevils released in North America initially identified as *Mecinus janthinus* with Dalmatian toadflax or yellow toadflax host-associated biotypes have been molecularly confirmed to be two morphologically similar species that are ecologically segregated by

host species in the native range (Toševski et al. 2011). Independent molecular and morphological analyses of weevils field-collected in Montana confirmed for the first time in North America that weevils established on yellow toadflax were *M. janthinus* Germar, 1821, genetically distinct from *Mecinus janthiniformis* Toševski & Caldara, weevils collected on a Dalmatian toadflax infestation situated less than 60 miles away (P. Bouchard, personal correspondence; I. Toševski, personal correspondence).

These developments raise multiple conditional management questions, including:

1. Which agent should be selected for deployment – should we always release *M. janthiniformis* against Dalmatian toadflax and *M. janthinus* to control yellow toadflax?;
2. Are the two *Mecinus* species behaviorally and reproductively isolated?;
3. How effective is either agent against hybrid toadflax?;
- and 4. How can classical biological control of toadflax be further improved with this knowledge?

Dalmatian Toadflax

Origin and Invasive History

Linaria dalmatica (L.) Mill. (Plantaginaceae) (ITIS 2015) is most frequently known by the common name Dalmatian toadflax (Lindley 1835; Kelsey and Dayton 1942; Fernald 1950 and Gilkey 1957, as cited in Alex 1959; 1962 and Vujnovic and Wein 1997), but is also colloquially referred to as broad-leaved toadflax and wild snapdragon (Alex 1959). Dalmatian toadflax is an Old World short-lived perennial forb with a native range extending from southeastern Europe – encompassing historic Dalmatia (Adriatic Sea islands to Dinaric Alps locations within present-day Serbia,

Montenegro, Bosnia and Herzegovina, Slovenia, Croatia and Albania), Greece, Romania, Bulgaria and Crete - to southwestern Asia (Turkey, Azerbaijan, Armenia, and northern Syria, Iraq and Iran) (Alex 1962; Vujnovic and Wein 1997).

Alex (1962) provides an interesting putative history of Dalmatian toadflax cultivation, crediting Jodocus de Goethuysen, the Medici gardener known as Casabona, as a likely early source of seeds used to establish this species in Europe, in the university garden at Montpellier, France and possibly also at Leyden University in the Netherlands. Under the patronage of the Medicis, Casabona made an expedition to Crete in 1590 to collect plants cited in classical medical and botanical texts, which could explain how he was able to supply Dalmatian toadflax seeds from plants growing in Florence, Italy to Montpellier. Notations in the second edition (1671) of famed Swiss botanist Caspar Bauhin's *Pinax Theatric Botanici* indicate that he had collected specimens of Dalmatian toadflax from the Montpellier garden in 1594 (Alex 1959). Multiple introductions were reportedly required to establish Dalmatian toadflax in England as the first recorded introduction in 1731 failed; a subsequent introduction originating from seeds "collected in Persia" succeeded in producing a plant for the Horticultural Society in 1834 (Lindley 1835, as cited in Alex 1962).

Considered by many to be an attractive plant, Dalmatian toadflax was also originally introduced to North America as an ornamental. The first record of New World cultivation was anecdotally reported by T.D. Hatfield, a gardener at Hunnewell Estate, Wellesley, Massachusetts in *Garden and Forest*, a weekly gardening and landscaping publication (Hatfield 1894, -1897, as cited in Alex 1962). Anecdotal reports indicate that

Dalmatian toadflax was similarly used as an ornamental plant in Ottawa, Ontario, as early as 1901 (Macoun 1908). The earliest authenticated North American specimen of Dalmatian toadflax was collected in California in 1920 (Davidson and Moxley 1923, cited in Alex 1962). The first Canadian herbarium specimen of Dalmatian toadflax was collected in Edmonton, Alberta in 1933 (Macoun 1908, cited in Alex 1962).

Dalmatian toadflax has repeatedly escaped cultivation, becoming naturalized and widely distributed, and now infests rangelands, open forests, and transportation corridors throughout North America (Lange 1958; Robocker et al. 1972; De Clerck-Floate and Miller 2002). Dalmatian toadflax is currently established across much of the continental United States, excluding some parts of the South Atlantic states, and all of Canada, other than in Labrador and the three northern territories, and is classified as a noxious weed or as noxious weed seed in three Canadian provinces and 12 U.S. states (USDA, NRCS 2015).

Biology and Life Cycle

The leaves of Dalmatian toadflax are broad, alternate, 1.2-6.0 cm long and typically less wide than long (Vujnovic and Wein 1997). The lower leaves are heart-shaped and clasp the stem while the upper leaves are smaller and ovate-lanceolate in shape; the surface of the leaves has a waxy or hazy appearance (Lajeunesse 1999; Wilson et al. 2005; Sing et al. 2008). Dalmatian toadflax produces two types of stem: upright-growing floral stems that produce flowers, seed capsules and seeds then die at the end of the current growing season, and laterally-growing prostrate stems predominantly associated with vegetative growth in the overwintering rosettes (Robocker 1974). A

single plant can produce up to 24 vertical, leafy stems that are thick-walled, fibrous, and waxy in appearance, each capable of attaining a maximum height of 120 cm (Robocker 1974). Dalmatian toadflax flowers are a bright yellow, two-lipped, snapdragon-like blossom with an orange throat and a long spur at the base (Alex 1959). The flowers develop on short peduncles that form in the axils of upper stem leaves and are 20-40-mm long (Wilson et al. 2005).

Seedlings produced from seeds which can germinate in fall or spring grow quickly, establishing a deep taproot within eight weeks and producing a combination of 2-5 vertical flowering, and prostrate, nonflowering stems in the first season (Alex 1962; Robocker et al. 1972). Seasonal growth of stems can begin as early as February in warm areas, or as late as April in cooler, more northerly latitudes or higher elevation locales (Robocker 1974). In the second and subsequent growing seasons, reproductively mature plants may produce up to 25 flowering stems and 40 nonflowering stems (Harris and De Clerck-Floate 2000). Flowering is dictated by local conditions, potentially occurring from May to October, and ending when a hard frost kills reproductive stem tissues (S. Sing, personal communication).

Dalmatian toadflax is self-incompatible and therefore an obligate out-crosser, requiring the services of robust or morphologically specialized pollinators to produce viable seed (Vujnovic and Wein 1997; Wilson et al. 2005). Seeds are produced from late June to December (Robocker 1970). A mature plant can produce up to 500,000 seeds annually, which are gradually released from the seed capsules through the fall and winter (Robocker 1970). Grieshop and Nowierski (2002) found that seed recruitment of

Dalmatian toadflax may be limited more by interspecific competition than seed availability. Many of these seeds are retained in the seed bank, remaining dormant but viable in the soil for up to 10 years (Lange 1958; Robocker 1968; Wilson et al. 2005).

In addition to sexual reproduction, Dalmatian toadflax perenniates asexually by ‘daughter’ shoots that arise from the roots of both primary and secondary shoots. Vegetative propagation, via adventitious buds produced at the base of the hypocotyl and on the upper portion of the primary root, can occur as early as 22 days after seedling emergence (Alex 1959). New shoots can also develop from root and stem fragments as short as 10 mm in length; over time these form viable roots, eventually allowing them to become independent plants (Wilson et al. 2005). Vegetative reproduction also makes this plant relatively easy to clone under greenhouse conditions (Turner 2012).

Distribution and Habitat

The North American latitudinal range for Dalmatian toadflax extends from 33-56° N (Figure 1), compared to a more restricted estimated Old World range of 35-47° N (Alex 1962). Dalmatian toadflax is typically found at elevations ranging from sea level to 2,800 m in open, sunny, rocky locations such as uncultivated fields, mountains meadows, sand hills, and limestone mountains (Alex 1962). Optimal growing conditions for Dalmatian toadflax are found in cool, semi-arid locales with sandy to coarse gravel soils that have a neutral to slightly alkaline pH (Robocker 1968). Outside its native range, *Linaria dalmatica* has readily adapted to a broad range of soil types, water, and light conditions (Allen and Hansen 1999).

Yellow Toadflax

Origin and Invasive History

Yellow toadflax (*Linaria vulgaris* Mill.) (ITIS 2015), also known by the common names butter and eggs, common toadflax and wild snapdragon, is a short-lived perennial herb with an extensive native range which includes most of Europe and Northern Asia (Chater 1972; USDA, NRCS 2015). Imported for ornamental and medicinal purposes by early American settlers, yellow toadflax had reportedly become naturalized in some eastern American colonies by 1671 (Josselyn 1672, as cited in Mack 2003) and was considered a significant and fairly common agricultural weed as early as 1849 (Darlington 1849 and Leighton 1970, as cited in Mack 2003). Unintentional dissemination, e.g., as a crop seed contaminant, in baled hay, along railway corridors, and in ships' ballasts, has contributed to the comprehensive spread of this weed (Mitich 1993; Saner et al. 1995). Yellow toadflax was first collected in Canada in the early 1800s in southern Quebec; its rapid and comprehensive invasion of the Canadian prairie provinces followed in the early- to mid-1900s (Rousseau 1968; Saner et al. 1995). Yellow toadflax's extensive North American distribution currently includes all continental U.S. states (USDA, NRCS 2015).

Biology and Life Cycle

Yellow toadflax leaves are alternately arranged, linear in form, 20-60 mm in length, typically with one main (central) vein (Sutton 1988). Leaf color varies from pale to deeper green, with isolated populations exhibiting silver-blue foliage (S. Sing and S.

Ward, personal communication). Yellow toadflax stems are generally unbranched, ranging in height from 31-90 cm tall (Wilson et al. 2005). The woody stems produced by yellow toadflax are broader and can have a reddish color at the base, becoming more slender and green closer to the apex (Wilson et al. 2005). The flowers of yellow toadflax are generically similar to Dalmatian toadflax, snapdragon-like and 20-40 mm long, except that the bearded throat of yellow toadflax flowers tends to display a brighter orange than what is typically observed in Dalmatian toadflax blossoms (S. Ward, personal communication). Flowers are usually clustered at the tip of the stem, but not in the spike-like arrangement presented by many Dalmatian toadflax plants (Wilson et al. 2005).

Yellow toadflax seeds are discoid, consisting of a 1.5-2.5 mm wide x 2.0-3.0 mm long flattened oblong central body encircled by a broad wing (Olsson 1975). Produced in a two-celled seed capsule, the seeds can number up to 250 in each capsule, with each stem having the capability of producing up to 30 capsules (Arnold 1982). Yellow toadflax seeds germinate in early to mid-May (Beck 2009). Mature plants have the potential to produce 1,500-30,000 seeds annually (Saner et al. 1995; Wilson et al. 2005). Like Dalmatian toadflax, yellow toadflax also is self-incompatible and reproduces sexually via insect pollination (Arnold 1982; Docherty 1982).

Also capable of asexual or vegetative reproduction, yellow toadflax does not produce adventitious buds on the hypocotyl or stem like many other *Linaria* species; all vegetative reproduction occurs through adventitious buds on the roots (Bakshi and Coupland 1960; Charlton 1966; Hellström et al. 2006). Nadeau et al. (1992) found that

single ramets or genets of yellow toadflax were equally capable of initiating new infestation. Established seedlings produce a taproot within two to three weeks post-germination, and soon thereafter begin to produce horizontal vegetative roots (Nadeau et al. 1992). Adventitious roots can produce up to 100 shoots during the first season of growth (Saner et al. 1995). At northern North American latitudes vegetative growth begins in early to mid-April after soil temperature reach 5-10°C; the onset of flowering occurs in late June or early July, and peaks in late July (Saner et al. 1995). After pollination and seed maturation, seeds are released from the seed capsules beginning in August and continuing even during the winter (S. Sing, personal observation; Clements and Cavers 1990). The spread of yellow toadflax from established infestations is the subject of debate, with some studies concluding that it is mostly vegetative, from so-called ‘daughter’ shoots produced by a large network of clonal creeping roots (Wilson et al. 2005), while others concluded that seeds were primarily responsible for new infestations (Carder 1963; Lehnhoff 2008).

Distribution and Habitat

In its native range of southeastern Europe and southwestern Asia, yellow toadflax evolved in floral communities grazed by sheep, goats, and cattle. Chronic exposure to agricultural practices such as grazing and land cultivation necessitated yellow toadflax’s adaptation to frequent disturbance (Lajeunesse 1999). Due to its marked genetic diversity, yellow toadflax has the ability to successfully adapt to new environments and challenging growing conditions, evidenced by its continuously expanding invasion of the U.S. intermountain West and Alaska (Markin 2002; Ward et al. 2008). Yellow toadflax is

now considered to be naturalized throughout North America, recorded in all U.S. states except Hawaii (Figure 2). The North American latitudinal range for yellow toadflax reaches as far north as 55°-65° N (Saner et al. 1995; USDA, NRCS 2015). Yellow toadflax is frequently found in moist sandy loam soils in disturbed areas such as roadsides, rail beds, gardens, pastures and river banks (Lajeunesse 1999).

Toadflax Infestations

Toadflax Impacts

Invasive toadflaxes have the potential to exert significant and often lasting negative environmental impacts on North American ecosystems (D'Antonio et al. 2004; Dodson and Fiedler 2006). Many of their shared biological traits facilitate invasiveness (Drenovsky et al. 2012; Lehnhoff et al. 2008; Nadeau and King 1991). Although they are self-incompatible and require insect-mediated cross pollination to produce fertile seeds, both of these species are also capable of reproducing asexually via buds on lateral roots that generate daughter shoots with their own independent root systems (Vujanovic and Wein 1997). Dual reproductive modes allow both species to rapidly colonize and dominate recently disturbed sites. Tap roots also confer drought tolerant competitive advantages to established toadflax plants. Dalmatian toadflax seedlings can produce tap roots 50 cm in length within 8 weeks; yellow toadflax taproots attain a lifetime length of up to 100 cm (Alex 1962; Robocker. et al. 1972; Saner et al. 1995). Yellow toadflax species persistence has been correlated to its well-developed taproot which facilitates increased patch size even in drought years (Coupland et al. 1963). These characteristics,

in addition to high seed production and long term persistence of viable seed in the soil seedbank, make Dalmatian and yellow toadflax opportunistic, competitive ruderal species with high colonization potential.

Yellow toadflax differs from Dalmatian toadflax in that in addition to being invasive in rangeland and undeveloped, uncultivated ‘wild’ lands, it is also recognized as an economically important cropland weed (McClay and De Clerck-Floate 2002). In Canada, yellow toadflax is considered a serious invasive plant in pastures and crops (small fruits, grain and oilseed), particularly on the Prairies (Coupland et al. 1963; Harker et al 1995; Baig et al. 1999). Yellow toadflax infestations became widespread and intense enough to affect western Canadian grain crop yields on a regional basis by the early to mid-1900s (Saner et al. 1995). Yellow toadflax infestations have also caused economically significant losses to peppermint producers in Wisconsin (Volenberg et al. 1999). Currently, yellow toadflax is classified as a noxious weed or weed seed in four Canadian provinces and in 10 U.S. states (Rice 2013; USDA, NRCS 2015).

An environmental risk assessment of these two weeds identified risks associated with toadflax infestations as diverse as its ability to act as an alternate host for Cucumber Mosaic virus, a serious pathogen of crop and ornamental plant species, and as a yield-reducing crop weed (Sing and Peterson 2011). Yellow toadflax is problematic in perennial forage crops, annual crops and summer fallow, can be unpalatable to livestock, competes with strawberries, raspberries, alfalfa, grain, and has invaded wilderness areas (Darwent et al. 1975; McClay and Hughes 2007; Sutton et al. 2007). Dalmatian toadflax is believed to be especially competitive with winter annuals and shallow-rooted

perennials, decreasing cattle carrying capacity and overall rangeland value (Jacobs and Sheley 2003). It has been reported that both toadflax species are toxic to livestock; however, most species do not feed extensively or exclusively enough on toadflax to reach adverse effect ingestion exposures (Sing and Peterson 2011). Competitive displacement of desired plant species was ultimately identified as the most significant risk associated with infestations of either of these toadflax species (Jeanneret and Schroeder 1992; Sing and Peterson 2011).

Hybrid Toadflax

Hybridization may contribute to the evolution of increased invasiveness of yellow and Dalmatian toadflax in North America (Ward et al. 2009; Turner 2013; Boswell 2014). Through hybridization, the interplay of factors such as range expansion (e.g. , Ellstrand and Schierenbeck 2000), increased genetic variation and the creation of novel gene combinations (Anderson 1949; Stebbins 1959; Lewontin and Birch 1966) ramp up opportunities for natural selection to enhance invasiveness (Hovick and Whitney 2014).

Naturally occurring hybrids of Dalmatian and yellow toadflax were not historically recorded, probably due to the allopatric distribution of their sites of origin. Field observations suggest that hybridization may not be uncommon either in their intermediate European range, or in their North American invaded range, particularly in western United States where their distributions are frequently sympatric (Ward et al. 2009). Hybrid populations have now been confirmed at multiple field sites in Montana, Colorado, Idaho, Wyoming and Washington via molecular diagnostic techniques (S. Ward, personal communication; Boswell 2014). Controlled or manipulated breeding to

generate specific crosses and back-crosses in the greenhouse has also produced viable offspring between *L. vulgaris* and *L. repense* (Olsson 1974, 1975; Ward et al. 2009).

Preliminary data indicate that heterosis (hybrid vigor) could result in hybrid populations displacing one or both parent species (S. Sing and S. Ward, personal communication). Hybrids could also move into new habitats not currently invaded by *Linaria* species (Ward et al. 2009). An example of hybridization enhancing the success of an invading plant in North America is illustrated by *Tamarix* species (Gaskin and Schaal 2002). This development could greatly complicate the management of both toadflax species, generating additional economic and ecological issues.

Toadflax Management

Methods to control established infestations of Dalmatian toadflax and yellow toadflax include chemical treatments (herbicides), mechanical treatments (mowing, grazing and cultivation), and classical biological control with approved agents (Jacobs and Sing 2007a, 2007b; De Clerck-Floate and Turner 2013; De Clerck-Floate and McClay 2013). According to Saner et al. (1995), the key to controlling yellow toadflax is infestation prevention and root starvation. Few herbicides adequately control this weed through a single application; long-term control is therefore seldom achieved by herbicides due to both species' deep and spreading root systems, waxy leaf surfaces and ability to generate abundant and persistent seed banks (Lajeunesse 1999; Sing et al. 2008). Chemical control of yellow toadflax is best characterized as unpredictable, meeting with mixed success, and proper application requires a good understanding of the target weed's local phenology (Krick 2011). Mowing and grazing might reduce treatment

year seed production but toadflax's superior regrowth ability indicate that mechanical control is fundamentally insufficient, unable to eliminate established stands or deal with seeds already deposited in the soil seedbank (Olliff et al. 2001). Fire typically serves only to increase toadflax density and dominance (Jacobs and Sheley 2003). Volenberg et al. (1999) found that through two years of 'clean cultivation' it was possible to eliminate yellow toadflax in Wisconsin mint fields; however, this approach increased soil erosion and was very labor intensive and expensive.

Toadflax Biological Control

Overview

Biological control of weeds is the deliberate use of natural enemies to limit the distribution and abundance of a target weed (McFadyen 1998). Classical biological control uses agents extensively tested for host specificity and approved by appropriate regulatory entities before being intentionally released against a specific target weed (Harris 1991); this is the type of biological control currently deployed against invasive toadflax. This management approach uses host specific natural enemies from the target weed's native range to cause damage that will limit the reproduction of the weed, erode the competitive ability of the weed, or facilitate secondary infection from pathogens (Nordlund 1996). Classical biological control's goals are not to eradicate but to suppress invasive species populations below ecologically or economically damaging levels (Eilenberg et al. 2001). This approach restores at least a part of the ecological balance that is thought to limit the competitive ability of a target weed species to become

dominant within vegetation communities in its native range (Keane and Crawley 2002). Advantages of using classical biocontrol include sustainability, reducing supply and labor costs of repeated mechanical and chemical treatments while maintaining longer term control, reducing unintended and undesirable nontarget impacts (Wilson et al. 2005). Risks associated with classical biocontrol can be minimized through taxonomic certainty, and following proper host screening processes (Sing et al. 2005).

Infestations of western Canadian yellow toadflax noticeably decreased in the 1950s, possibly correlated with the first time that populations of two unintentionally introduced toadflax feeding insects, the flower-feeding beetle *Brachyterolus pulicarius* and the seed feeding weevil *Rhinusa* (formerly *Gymnaetron*) *antirrhinii*, had attained high enough densities to exert population level impacts on their host weed (Darwent et al. 1975; Harris 1991).

Mecinus janthinus and *M. janthiniformis*

Origin and History. *Mecinus janthinus* Germar, 1821 (Coleoptera: Curculionidae) is an oligophagous, univoltine stem mining weevil approved for release in Canada and the United States for classical biological control of both yellow toadflax and Dalmatian toadflax (McClay and De Clerck-Floate 2002; De Clerck-Floate and Harris 2002). This agent was originally identified as a single species non-preferentially attacking both yellow and Dalmatian toadflax (Jeanneret and Schroeder 1992). Morphological, molecular and biological evidence has since shown that in its native range, the agent known as *M. janthinus* is actually a complex of multiple closely related weevil species,

including *M. janthinus* and a newly described species, *Mecinus janthiniformis* Toševski and Caldara (Toševski et al. 2011). Molecular and morphological analyses have confirmed that weevils currently established on North American toadflax are pure or mixed populations of *M. janthinus* and *M. janthiniformis* (I. Toševski, unpublished data; S. Sing and D. Weaver, personal communication).

The native ranges of *M. janthinus* and *M. janthiniformis* are allopatric, following the disjunct distributions of their respective natural hosts in central and southern Europe, southern Russia and southwestern Asia (Jeanneret and Schroeder 1992). *Mecinus* species was initially selected from a pool of candidate agents because no native North American stem miner was known to attack the introduced weedy toadflax species (Jeanneret and Schroeder 1992). Stem mining agents were believed to have greater potential for impacting toadflax than the defoliators and seed feeders already widely established on North American toadflax (Jeanneret and Schroeder 1992; Saner et al. 1994; Egan and Irwin 2008). Of the eight total biocontrol agents that have been approved for release to control toadflax, *Mecinus* species are proving to be the best option for control (Peterson et al. 2005; Sing et al. 2008). The biological control program for invasive toadflax in North America was initiated in 1987 and the first release of *Mecinus* species took place in Canada in 1991 (De Clerck-Floate and Miller 2002).

Since its initial release, *M. janthiniformis* has become widely distributed throughout the southern parts of the western provinces of Canada and the northwest of the U.S. through natural dispersion and active redistribution (De Clerck-Floate and Miller 2002; Carney 2003; Schat 2007). The majority of all weevils imported from Europe for

release in North America were *M. janthinus* collected from yellow toadflax populations in western Europe. Records indicate that in 1997, 200 specimens of *M. janthiniformis* collected from Dalmatian toadflax plants in Macedonia were shipped for release in Canada (Toševski et al. unpublished data). This release is thought to have led to outbreak level populations on Dalmatian toadflax in North America (Toševski et al. 2011).

Mecinus janthinus, conversely, has remained rare and generally present in low densities in Canadian and U.S. populations (Toševski, unpublished data). This may be due to the high levels of genetic diversity in yellow toadflax populations (Ward et al. 2008). Based on regional dispersal of the introduced agent, significant decline in plant size and density, and widespread fragmentation of patches, it has been said that *M. janthiniformis* on Dalmatian toadflax is a successful biocontrol program (Van Hezewijk et al. 2010).

Biology and Life Cycle. Host specificity tests have shown that *Mecinus* species only will develop on a few related *Linaria* species (Jeanneret and Schroeder 1992). After copulation, *M. janthinus* and *M. janthiniformis* females chew holes into the toadflax shoots and insert a single, white, oval egg in each hole (Jeanneret and Schroeder 1992). Each oviposition hole is then covered with a lid of plant tissue to protect the egg from desiccation and predation (Jeanneret and Schroeder 1992). Individual females lay an average of 45 eggs, laying about one egg a day (Wilson et al. 2005).

After hatching, the larvae tunnel within the stem as they feed, moving no further than 3 cm from the oviposition site (Jeanneret and Schroeder 1992). Development through three larval instars within the shoots takes four to five weeks and occurs in stems

that are typically larger than 0.9 mm in diameter (Jeanneret and Schroeder 1992; Wilson et al. 2005).

Pupation occurs during the late summer within an oval chamber formed within the mined toadflax stem by the last larval instar (Toševski et al. 2011). Pupae are 3.0-4.5 mm in length and white initially, turning black as development proceeds (De Clerck-Floate and Miller 2002). The adults then remain in the stem throughout the winter and emerge the following spring by chewing their way out of the stem (Wilson et al. 2005; McClay and Hughes 2007). Once emergence has occurred, the adults copulate following a short period of feeding. Oviposition occurs at the end of May or the beginning of June and lasts until mid-July (Jeanneret and Schroeder 1992). Larval feeding causes premature wilting of shoots and suppression of flower formation for an overall effect of reducing plant vigor and reproductive output (Jeanneret and Schroeder 1992; Saner et al. 1995).

Insect Hybridization. Research conducted on the hybridization of weed biological control agents is limited. Hoffman (2002) showed that two biotypes of *Dactylopius opuntiae*, a biological control agent for cactus weeds (*Opuntia* species) in South Africa that attacks *Opuntia ficus-indica* and *O. stricta*, can produce viable hybrid offspring that show no specificity to either host plant in the F1 generation. This has great management potential as the offspring can develop normally on either plant species. Will crossing of *M. janthinus* and *M. janthiniformis* produce similar results? The concept of speciation and biotypes is not new to Curculionidae. The rice weevil (*Sitophilus oryzae* L.), and the alfalfa weevil (*Hypera postica* Gyllenhal) are two of the best examples of differentiated biotypes found in the weevil family (Hernandez-Vera et al. 2010).

The seed parasitic weevil *Rhinusa antirrhini* (Coleoptera: Curculionidae), similar to *Mecinus* species, also shows differentiation via association with host toadflax species. Hybridization experiments between these biotypes showed fewer mating events and fewer offspring from mating (Hernandez-Vera et al. 2010). They further argued that maternal oviposition choice drove genetic differentiation between strains even where both host species occur sympatrically. In our system *M. janthiniformis* and *M. janthinus* are not found naturally co-occurring and forced sympatry has not resulted in hybrid population of the two *Mecinus* species (Toševski, Sing, Weaver and Ward, unpublished data). However, recently a population of *M. janthiniformis* has been found on a population of toadflax hybrids located in Idaho. This species has apparently overcome the hybrid barrier (Toševski, Sing, Weaver, unpublished data).

Summary

Factors complicating effective and consistent biological control of Dalmatian and yellow toadflax include: 1) naturally occurring hybrid toadflax populations resulting from the cross pollination of yellow and Dalmatian toadflax located at multiple sites in Montana ,Colorado, Washington and Idaho; 2) European field populations of this biological control agent have recently been confirmed as consisting of two fairly host-specific species, one associated with yellow toadflax (*Mecinus janthinus*) and one associated with Dalmatian toadflax (*Mecinus janthiniformis*) (Toševski et al. 2011); and 3) preliminary molecular diagnostics indicate that both of these weevil species have been released and are now established in North American, and follow the same host

association patterns reported in European populations (I. Toševski, unpublished data).

These three factors raise management questions, such as: Which *Mecinus* species should be selected for deployment? Are the two *Mecinus* species behaviorally *and* reproductively isolated? How effective is either weevil species against hybrid toadflax? How can we improve the overall exotic toadflax biological control program?

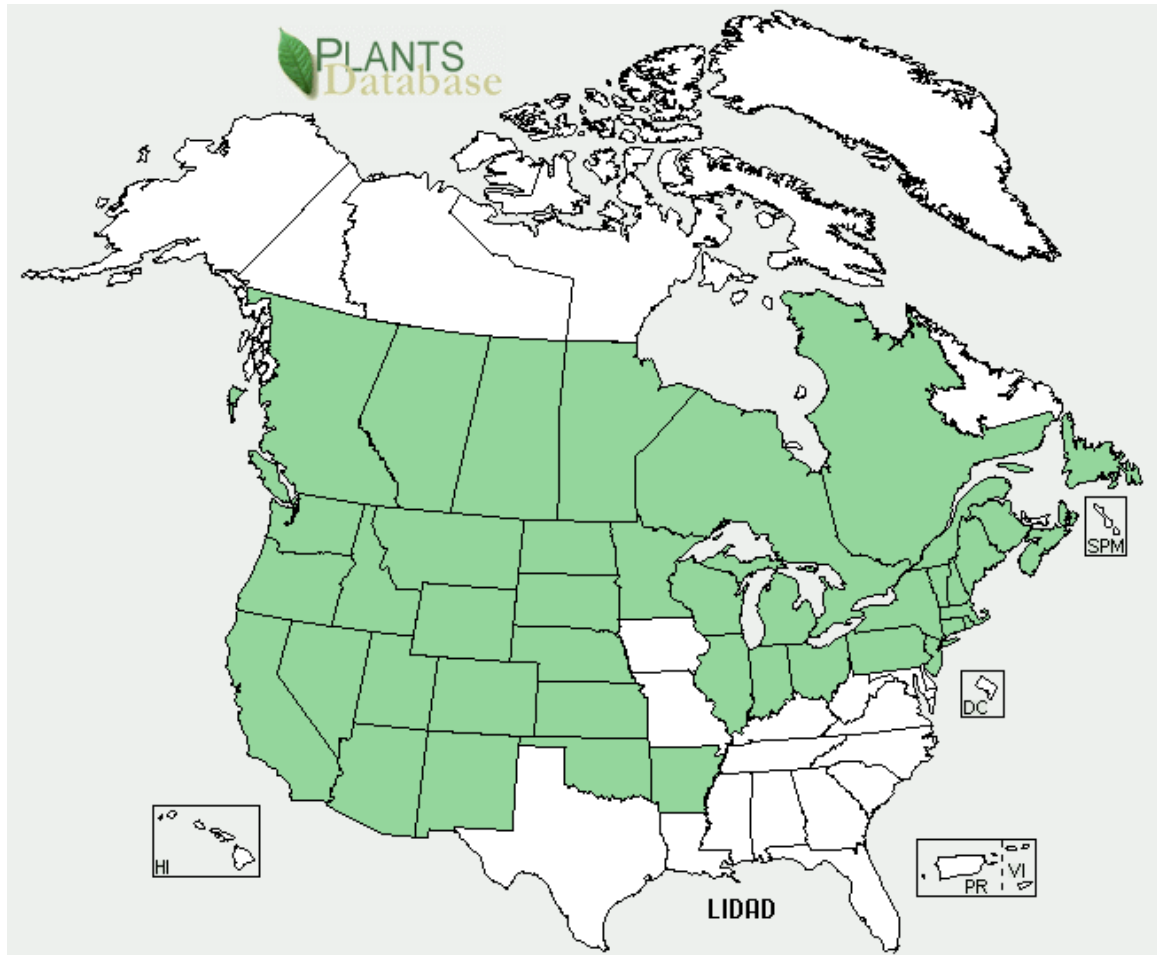
Figures

Figure 1. Distribution of Dalmatian toadflax in North America. (USDA PLANTS Database, <http://plants.usda.gov/core/profile?symbol=LIDAD>)

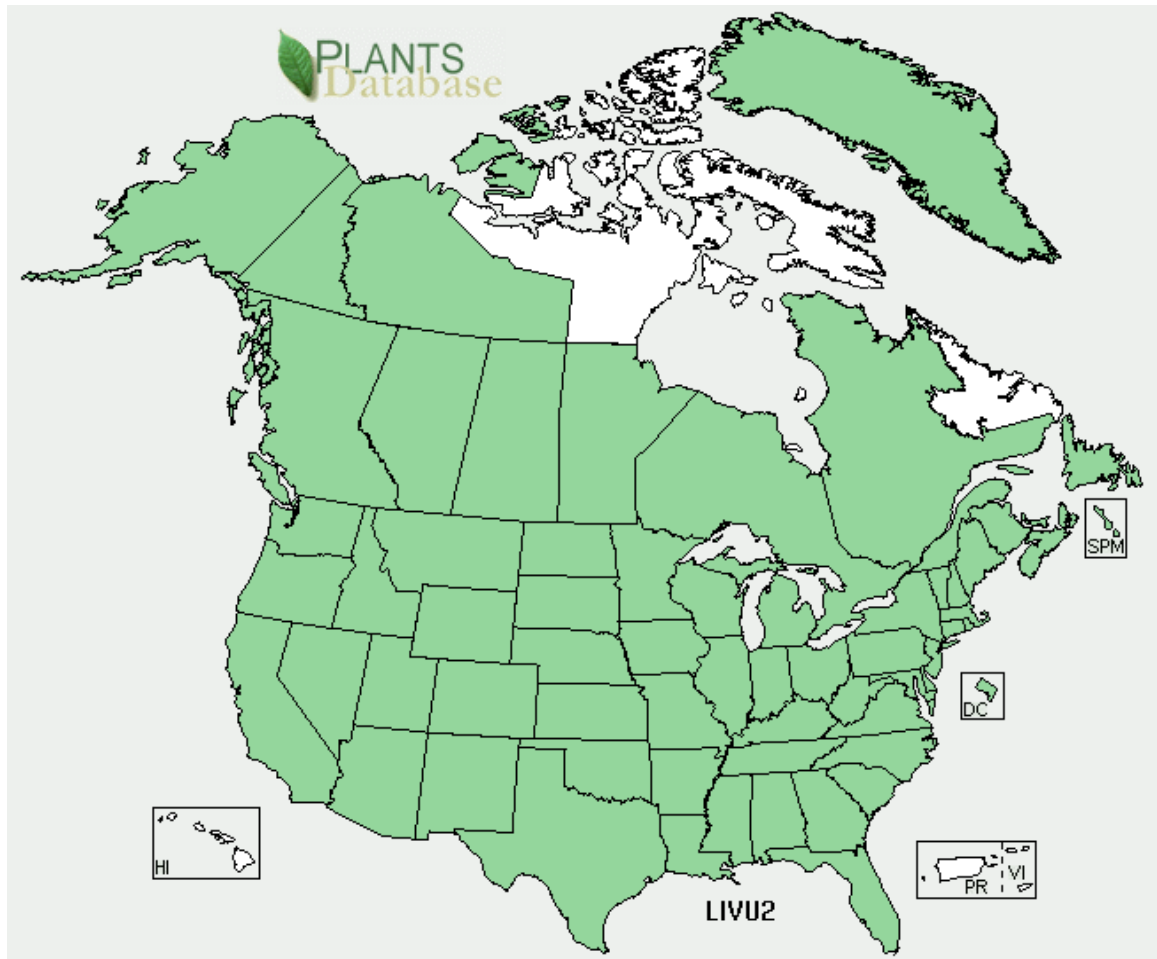


Figure 2. Distribution of yellow toadflax in North America. (USDA PLANTS Database, <http://plants.usda.gov/core/profile?symbol=LIVU2>).

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CHAPTER 2

OLFACTORY HOST PREFERENCE OF *MECINUS* SPECIES.Introduction

Biological control is a method that has been used to control invasive and noxious weeds for many years. Classical biological control of weeds involves the importation and release of exotic biological control agents (i.e. , insects or pathogens) to reduce the abundance of target weeds to acceptable threshold levels (Clerck-Floate and Bouchier 2000). Once established, classical biocontrol can provide continued and cost-effective control of target weeds, including exotic toadflax species (Wilson et al. 2005).

Hybridization of the target weed(s) in the invaded range can impede successful classical biological control. Hybridization is a mechanism that contributes to the evolution of invasiveness; Ellstrand and Schierenbeck (2000) cite 35 examples of plant hybridization leading to range expansion. Hybridization may increase genetic variation, creating novel gene combinations which undergo natural selection (Anderson 1949; Stebbins 1959; Lewontin and Birch 1966). Host specificity is a critical aspect of classical biocontrol (Sing et al. 2005). Hybrid combinations potentially create new phenotypes that inhibit mechanisms underlying a biocontrol agent's specificity for its target host. This could nullify the agent's ability to find and infest the 'natural' host. Here we address the problem that hybridization poses in the control of Dalmatian toadflax (*Linaria dalmatica* (L.) Mill. and yellow toadflax (*Linaria vulgaris* (L.) Mill. by the biocontrol agents *M. janthinus* Germar, 1821, and *M. janthiniformis* Toševski & Caldara (Coleoptera:

Curculionidae), by identifying a mechanism underlying host specificity to their natural hosts and to hybrid taxa.

Dalmatian toadflax (*Linaria dalmatica* (L.) Mill.), and yellow toadflax (*Linaria vulgaris* Mill.) (Plantaginaceae) (ITIS 2015), are highly invasive Eurasian weeds in the western U.S. and Canada (Mitich 1993; Saner et al. 1995; Vujnovic and Wein 1997). Originating in southeastern Europe, Dalmatian toadflax was introduced into North America as a garden ornamental in the late 1800s (Lange 1958; Alex 1962). It has since become classified as noxious weed or as noxious weed seed in three Canadian provinces and 12 U.S. states (USDA, NRCS 2015). According to anecdotal reports, yellow toadflax was first introduced to North America in New England in the 1600s as an ornamental plant with medicinal and textile properties. This facilitated its subsequent spread throughout North America (Mitich 1993). Yellow toadflax is currently classified as a noxious weed or weed seed in four Canadian provinces and in 10 U.S. states (USDA, NRCS 2015). Both of *Linaria* species are perennial weeds that reproduce asexually via vegetative root buds and sexually through insect mediated pollination (Vujnovic and Wein 1997; Wilson et al. 2005). Large tap roots also enable both species to have drought resistant competitive advantages (Robocker et al. 1972).

Yellow toadflax can act as an alternate host for cucumber mosaic virus, a serious pathogen of crop and ornamental plant species (Darwent et al. 1975). It has also gained status as an important crop pest in Alberta, Canada (Darwent et al. 1975). Dalmatian toadflax infestations can decrease cattle carrying capacity and overall rangeland value as

it is especially competitive with desirable winter annuals and shallow-rooted perennials (Jacobs and Sheley 2003).

The biological control agent *Mecinus janthinus* was first released in Canada in 1991 as part of a classical biological control program initiated in 1987 to control both *Linaria* species (De Clerck-Floate and Miller 2002). Recent evidence has shown that two closely related stem mining weevil species have become established on infestations of yellow and Dalmatian toadflax in North America. These weevil species are now known as *M. janthinformis*, with Dalmatian toadflax as its host plant, and *M. janthinus*, with yellow toadflax as its host plant (Toševski et al. 2011). Both species are oligophagous, univoltine stem mining weevils that occur natively in central and southern Europe and southern Russia on *L. dalmatica*, *L. vulgaris*, and/or *L. genistifolia* (Jeanneret and Schroeder 1992).

Adults copulate in the spring and oviposit in the stem of their host plant. Larvae mine the stem as they develop then pupate within the stem mid- to late summer. They complete metamorphosis and remain as adults inside the natal stem throughout the winter and emerge the following spring (Jeanneret and Schroeder 1992; Wilson et al. 2005; McClay and Hughes 2007).

Further complicating the management of both Dalmatian and yellow toadflax is the discovery of naturally-occurring, persistent populations of hybrid crosses between the two weed species. Hybrids were first found in Montana with subsequent discoveries of populations in Colorado, Idaho, Wyoming and Washington (S. Ward, personal communication). These hybrid populations have been confirmed using molecular

diagnostic techniques (Ward et al. 2009). Hybrid toadflax could prove to be the source of a significant land management problem. Hybrid populations of Dalmatian and yellow toadflax have increased fitness compared to the parental species (Turner 2012). Yellow and Dalmatian toadflax seeds are gravity dispersed, thus it is possible that limited seed dispersal from maternal parents generates kinship demes of genetically similar plants located closer together (Ward et al. 2008). This also applies to the hybrids they create; for this reason, we hypothesize that the maternal parent of a hybrid cross will likely exert a greater influence on the phenotype of the hybrid plant than the paternal parent.

Little is known about the mechanisms facilitating the cryptic speciation of the two *Mecinus* species. However, we hypothesize that green leaf volatiles unique to each of the two *Linaria* species continue to reinforce ecological partitioning. If the volatile profiles of the plants are signals for host location, how does hybridization affect this process? Will hybrids attract one species over another, or will they be unattractive? An extensive infestation of hybrid toadflax (S. Ward, unpublished data) discovered in Palisades, ID (J. Milan, personal communication) was found to be successfully colonized by a vigorous population of *M. janthiniformis* (I. Toševski, S. Sing and D. Weaver, unpublished data). This suggests that some toadflax hybrids are attractive, acceptable, and suitable hosts to *Mecinus* species.

The objectives of the current study are to: 1) determine whether each *Mecinus* species locates its preferred host based on olfactory responses to the volatile profile of their respective ‘natural’ host plant; 2) establish if each weevil species shows preference for select its natural host over other plant species, based on olfactory responses; and 3)

establish if either *Mecinus* species shows a preference for a particular hybrid cross based on its lineage, i.e., with yellow toadflax or Dalmatian toadflax as the maternal parent in the hybrid cross. We hypothesize that: 1) both *Mecinus* species select their host plants based on olfactory responses to volatiles released by their natural host; 2) each species will select its natural host over other plant species; and 3) if preference for a particular hybrid is shown, it will be a cross with the natural host as the maternal parent.

Materials and Methods

Experiments were conducted during summer 2011 and 2012, with weevils collected in May 2011 and April 2012, respectively, from well-established Dalmatian or yellow toadflax biological control release sites. Because both *Mecinus* species used in these experiments overwinter in their natal stems as adults, infested toadflax stems were field-collected in spring before adult emergence. *Mecinus janthiniformis*-infested Dalmatian toadflax stems were collected from Missoula, MT (46°52'59.75" N 113°59'34.26" W) and *M. janthinus*-infested yellow toadflax stems were collected from Ovando, MT (47°02'56.24" N 113°15'47.57" W). Samples of each population were sent to Dr. Ivo Toševski for molecular confirmation of identity.

Harvested stems were placed in 20 cm x 40 cm 4-mm polyethylene soil sample bags (approximately 63 Dalmatian toadflax or 173 yellow toadflax stems bag⁻¹) and retained at 4°C in a cold, wet plant storage room in Montana State University's Plant Growth Center (Bozeman, MT). This approach allowed us to extract virgin and not yet active adult weevils for experiments as needed. All adult weevils were individually extracted from host stems before each experiment. Each extracted weevil was isolated in

a clear gelatin capsule which was placed on a laboratory chill table so gender could be determined under magnification using sexually dimorphic characters (Schat et al. 2007; Carney et al. 2004); individual weevils presenting ambiguous features were excluded from use in any experiments. Each sexed weevil was then transferred to a capped, labeled 15-dram plastic shell vial and individually retained (12 hours or less) under moderate refrigeration (7.2 - 10°C) until the beginning of the respective experiment.

Toadflax Genotype Clones

Plants used in the 2011 experiments originated from field-collected Dalmatian and yellow toadflax obtained in the spring of 2011 from Missoula, MT and Ovando, MT. These were transplanted into 50:50 (by volume) MSU mix (steam sterilized 1:1:1 ratio by volume of mineral soil, Canadian sphagnum peat moss, and washed concrete sand with 0.45 kg of Aqua-Gro 2000G (Aquatrols, Paulsboro, NJ) wetting agent added per cubic meter of mixed soil) and Sunshine Mix #1 (Sun Gro Horticulture, Bellevue, WA). Individual plants were transplanted into 17.5 x 13.3 x 13.5-cm plastic pots and grown under constant conditions in a light and temperature automated greenhouse equipped with GE Multi-Vapor MVR1000/C/U lights (GE Lighting Global, Bucyrus, OH) which was located in the MSU Plant Growth Center. The greenhouse temperature was maintained at 22°C during the day (lights on) and 20°C at night (lights off). A 16L (0600-2200): 6D photoperiod was maintained during the fall and winter months (October – March); supplemental lighting was not used during the spring and summer months (April – September). Soil moisture in each pot was evaluated daily so plants could be individually watered as needed. All pots were treated weekly with a 400 ml solution containing Jack's

Professional™ 20-20-20 (J. R. Peters Horticulture, Allentown, PA) water soluble fertilizer at a concentration of 100 ppm, which was obtained by using a Siphonex® proportioner (Hozon, Earth City, MO).

Hybrid plants used in all 2012 experiments originated from Dalmatian and yellow plants obtained in December 2010 from the same two Montana field sites discussed above. Plants were express shipped to Dr. Sarah Ward at Colorado State University (CSU) (Fort Collins, CO) where she performed controlled hand-pollinations to generate hybrid progeny with specified pedigrees. All crosses involved a single Dalmatian toadflax founder specimen and one of two yellow toadflax founder specimens in various combinations. Seeds collected from hybrid progeny were subjected to 6 weeks dry stratification in a freezer at -20°C followed by 2 weeks wet stratification (in wet paper towels) in the refrigerator at 4°C. The seeds were then germinated and grown in ProMix BX (Premier Tech Horticulture, Quakertown, PA) in a CSU greenhouse under constant conditions (25°C; 14 h light:10 h dark) for 6 weeks before being delivered by automobile to Montana in spring 2012. Plants were transplanted into larger pots and grown under the MSU conditions described above.

Both the hybrid progeny and accessions of the parental species used to generate those hybrids were used in these experiments. Cloned individuals obtained from a single potted plant (unless otherwise indicated) of seven total toadflax genotypes were used: one Dalmatian toadflax plant collected from Missoula (DT), two separate yellow toadflax plants collected from Ovando (YT1 and YT2), and four ‘manipulated’ hybrids generated by Dr. Ward through hand pollinations: (YT1 x DT), (DT x YT1), (YT2 x DT), and (DT

x YT2). Hybrid genotypes are consistently labeled with the maternal parent listed first in the cross-pollination description (Table 1). To obtain a reciprocal of the cross only one Dalmatian toadflax parent could be used for both sets of crosses.

Clones of the CSU plants were generated from 4-cm shoot tip cuttings of the parent plant. Meristematic cuttings were dipped in Clonex[®] (Hydronamics International, Lansing, MI) rooting compound then placed in low density plastic UV-stabilized Ray Leach cone-tainers (Stuewe and Sons, Tangent, OR) filled with the same MSU mix potting medium as described above. Each cone-tainer was temporarily covered with an inverted 50 ml BD Falcon[™] Blue Max (Fisher Scientific, Logan, UT) centrifuge tube to maintain temperature and humidity range until cuttings became established. Cuttings were transplanted at 3-4 weeks and grown under the same conditions as described above for plants used in the 2011 experiments.

Y-tube Behavioral Bioassays

Y-tube bioassays were conducted to test the behavioral response of individual weevils to host-plant volatiles, specifically to assess for gender and/or toadflax genotypes mediated consistency in preference when subjects were presented with a choice between volatile profiles. The Y-tube system used was similar to that previously described and illustrated in Daisy et al. (2002) and Piesik et al. (2008) (Figure 3).

Separate volatile profiles were delivered to either arm of the Y-tube in pressure regulated, charcoal-purified and humidified air streams via Teflon[®] tubing (Enflo Corporation, Bristol, CT) (outer diameter 0.64 cm) connected to glass volatile collection chambers (VCCs) (outer diameter 100 mm, length 254 mm). Each VCC (Analytical

Research Systems, Micanopy, FL) was fitted with a volatile collection port at one end and open on the other end to enclose a single potted plant. The base of each plant was enclosed by a Teflon[®] ‘guillotine’ (Analytical Research Systems, Micanopy, FL) structure (diameter 995 mm, center opening 15 mm) and fitted into the base of the VCC to prevent outside air from entering the system. A threaded-glass joint at the base of each VCC connected a 24/410 threaded air supply cap at the end of the Teflon[®] tubing.

Each volatile collection port was also fitted with a threaded-glass joint to receive the 24/410 air supply cap to carry the air to each branch of the Y- tube via Teflon[®] tubing. The tubing was connected to the Y-tube arms by a Teflon[®] liner (Analytical Research Systems, Micanopy, FL) coupled to a 0.64-cm Swagelock[®] union (Swagelock, Solon, OH) delivering the air. Airflow was set at 0.80 L min⁻¹ for experiments in 2011 and 0.35 L min⁻¹ for experiments in 2012 using a flowmeter. The Y-tube olfactometer was constructed from Corning[®] glass tubing (Corning Incorporated, Corning, NY) (outer diameter 28 mm, length 30 cm) branched at 20 cm. The interior angle of the ‘Y’ was 120°, with the two diverging arms extending 4 cm laterally before becoming parallel to the central ‘stem’ for their final 10 cm, and then terminated in a female ground-glass joint at the end of each arm. A male ground glass joint on the stimulus-delivery tube was inserted into the receiving arm of the Y-tube, yielding a consistently airtight fit.

The Y-tube was positioned lengthwise inside a black poster board box (46.0 x 32.0 x 101.5 cm) to block out ambient light. The box was open on one end and closed on the other except for two 15-mm holes allowing the Teflon tubing from the VCCs to connect with the two Y-tube arms. Weevils were introduced to the bottom of the un-

branched ‘stem’ of the Y-tube approximately 2 cm above the opening. It was necessary to place a 28-cm long wire along the bottom of the ‘stem’, extending from the introduction point to the junction between the two branching arms of the ‘Y’, to facilitate subject movement toward the test junction. Positive phototaxis was additionally employed to enhance insect movement using a 20-watt compact fluorescent bulb that was carefully located equidistant between the Y-tube arms. Plants used as volatile odor sources were randomly assigned to the Y-tube arms.

Initial 2011 Y-tube bioassays ran from early July to mid-August. Each experiment evaluated the preference of 10 female weevils and 10 male weevils for volatile profiles delivered via a VCC enclosing either one plant of a specific toadflax genotype (treatment) or a 17.5 x 13.3 x 13.5 cm plastic pot filled with the same potting soil that was used for the plants as a control (Table 2). Plants were chosen randomly for each experiment and no plant was used more than once. No flowering plants (i.e., with vegetative growth only) were used to maintain fidelity with agent-host plant phenology as typically found under native range field conditions.

Plants were placed in the VCCs and exposed to a 90-W LED plant growth light for one hour before the experiment was run to ensure the plants were photosynthetically active before the experiment began. All 10 subjects of one gender (same weevil species, sourced from overwintered stems of same host plant species collected from same field site) were tested, then all 10 subjects of the other gender (same weevil species, host plant and site). The order in which individual weevils were tested was random within each gender group. Timing began once the test weevil was placed at the base of the Y-tube,

and if a choice had not been made within 5 minutes it was counted as a “no choice” and was not used in the analysis. Experiments in 2012 ran from June to early September and only used female weevils because males failed to show olfactory preference in the experiments in 2011.

Twenty-four hours before each experiment 10 females from each site were extracted from the stems, each vial was assigned a number; numbers 1-10 were reserved for *M. janthinus* and 11-20 for *M. janthiniformis*. A random number generator was used to create an order in which the weevils would be used in the experiment. The order in which the plants were tested was also chosen randomly. No weevil or plant was used more than once. As in the previous year’s experiments, plants were placed in the VCCs and exposed to a 90-W LED plant growth light for one hour before the experiment was run to ensure the plants were photosynthetically active before the experiment began. A list of treatment choices to be used in replicated trials is provided in Table 3. A chi-square test for independence was used with an alpha level of 0.05 to test for significance in both the 2011 and 2012 experiments.

Results

2011 Experiments

The results from the experiments in 2011 are listed in Table 4. For both *Mecinus* species, only the female subjects chose their host plant significantly over the control ($p < 0.05$). The male subjects showed no preference for the control or their host plant ($p > 0.05$).

2012 Experiments

Because the 2011 Y-tube bioassay results indicated that adult males did not respond to volatile profiles in the airstream passing over their host plants, only female weevils were assessed in the 2012 experiments. The same plant choice pairings were offered to both *Mecinus* species. The results for each species are presented in separate Tables (Table 5 and Table 6).

Female *M. janthinus* significantly preferred yellow toadflax (labeled YT-O6 and YT-O2) over the control, and over Dalmatian toadflax (labeled DT) ($p < 0.05$) in all experiments (Table 5). In the trials with hybrid crosses (trial 6 and 7, Table 5), *M. janthinus* displayed significant preference only in trial 7, choosing the hybrid with yellow toadflax as the maternal parent ($p < 0.05$). In trials with parental toadflax clones, female *M. janthiniformis* significantly preferred Dalmatian toadflax only in trial 1 ($p < 0.05$), although this preference was elicited only when the other choice was the plant free control airstream (Table 6). In trials 4 and 5 female *M. janthiniformis* selected Dalmatian toadflax slightly more often than yellow toadflax, but the differences were not significant ($p < 0.05$) (Table 6). In trial 7, *M. janthiniformis* significantly preferred a hybrid with a Dalmatian toadflax maternal parent ($p < 0.05$), but in trial 6, no preference was exhibited for a hybrid with a yellow toadflax maternal parent ($p < 0.05$) (Table 6).

Discussion

The results from the experiments in 2011 support the initial hypothesis that the volatiles from green foliage are attractive for each *Mecinus* species from its respective natural host, the plant species it is associated with in the native range. We did not expect

that only female *Mecinus* species would be attracted to their host plants, and that male weevils would show no discernable or consistent preference. This could indicate another aspect of the semiochemically mediated relationship between the weevils and their host plants. Do females emerge first in the field and find their host plant? Perhaps the males are initially indifferent and subsequently attracted to a sex pheromone emitted by the female, once she has located and fed on her host plant. Because the males showed no preference for their host plants, it suggests that the females plus the host plant likely attracts the males. In this case, the volatile profile of the plant acts as a pheromone synergist or compounds from the plant are used in the synthesis of sex pheromone. This common behavior may have evolved to increase the efficiency of mating and resource allocation for females (Landolt and Phillips 1997).

Traps used to catch *Rhynchophorus cruentatus* (F.) (Coleoptera: Curculionidae) are most effective when sex pheromone is combined with volatile attractants from the host (Giblin-Davis et al. 1994). Similar results have been found for species other than weevils. Males of both the corn earworm (*Helicoverpa zea*) (Boddie) and the codling moth (*Cydia pomonella*) (L.), displayed little to no response when exposed to host plant volatiles, but when those volatiles are released with the female sex pheromone, a stronger response was recorded (Ochieng et al. 2002; Yang et al. 2004). Further experiments are needed to determine how male *Mecinus* species, which do consume host plant foliage, locate their host plants in natural settings.

The results from the 2012 bioassays indicate a strong specificity for yellow toadflax, the preferred natural host, by female *M. janthinus*. They preferred both YT-O2

and YT-O6 over the control and over DT-M6 in every experiment ($p < 0.05$). Yellow toadflax is thought to have greater genetic variation than Dalmatian toadflax (Ward et al. 2008). With a high level of specificity, one may infer that any change in the volatile profile of yellow toadflax may alter *M. janthinus* success in host finding. According to the niche-variation hypothesis, genetic variation may expand a population's ecological amplitude beyond that of a monomorphic population, such as that presented by *M. janthinus* (Futuyma and Peterson 1985). With the long absence of exposure to *M. janthinus*, yellow toadflax may have evolved to fit new environments, altering its volatile profile in certain areas. With an innately higher level of specificity, this could explain the limited success *M. janthinus* has had as a biocontrol agent compared to *M. janthiniformis* (Toševski et al. 2011). Acceptance and physiological tolerance is needed to overcome such barriers and requires close proximity to the host plant to evolve with it. Disruption of herbivore-host plant association could require a high level of specificity on a set of chemically similar hosts, eventually leading to an evolutionary dead-end (Rausher 1993).

Mecinus janthiniformis displayed a decreased ability to select its natural host Dalmatian toadflax over both YT-O2 and YT-O6 ($p > 0.05$). However, still showing preference for its host plant over the control ($p < 0.05$), perhaps the fidelity of *M. janthiniformis* for its natural host is not as highly tuned as *M. janthinus* for yellow toadflax. If the specificity displayed by *M. janthiniformis* is based on a few prevalent volatile compounds, as opposed to a greater number of specific compounds, it could be attracted to more populations of Dalmatian toadflax, perhaps making it a more adaptable biological control agent facing hybrid populations of toadflax. This conjecture is

supported by the occurrence of a population of *M. janthiniformis* found on a site composed entirely of hybrid toadflax in Palisades, ID (S. Ward, unpublished data).

In trials using clones of synthetic reciprocal hybrids, both species of *Mecinus* chose a hybrid with their natural host as the maternal parent in the 7th trial pairing of reciprocal hybrid backcrosses ($p < 0.05$), supporting our hypothesis that maternal input has a greater effect on the attractiveness of hybrids to both *Mecinus* species. Obviously, this is challenged when both species showed no preference for either hybrid in the preceding trial pairing of reciprocal backcrosses. According to these results, we would expect an analysis of the hybrid toadflax in Palisades, ID to have greater genetic input from Dalmatian toadflax than from yellow toadflax. Initial results, however, indicate that these hybrid plants have yellow toadflax chloroplast DNA (and therefore cytoplasm) with more mixed nuclear genetic composition (Boswell 2013). It is important to note that additional analysis of nuclear DNA is required before it is reasonable to conclude that these plants are genetically more representative of yellow than of Dalmatian toadflax (Boswell 2013).

Conclusions

In hybrid toadflax populations, it is difficult to determine to what extent the genetic influence of each parent species has due to the large amount of backcrossing that could occur. Generally speaking, these results indicate that a population of hybrid toadflax that seems to have a larger genetic content from yellow toadflax would be more suitable for *M. janthinus*; conversely a population that appears to favor genetic inputs

from Dalmatian toadflax would be more likely to be suitable for *M. janthiniformis*, although there might be exceptions.

The results from this experiment do help to identify potential strategies that will help both *Mecinus* species overcome the hybridization barrier that threatens successful toadflax biocontrol. More study is needed in regards to the specific compounds that attract *M. janthinus* and *M. janthiniformis* to their respective host plants. Further research is also needed to establish which hybrids will attract either species and allow them to establish. Similar principles may also be identified to help overcome other hybrid barriers in other systems improving overall classical biocontrol of weeds in North America.

Tables and Figures

Plant Label	Description
DT-M6	Dalmatian toadflax (DT) - Missoula (M) location 6
YT-O2	Yellow toadflax (YT) - Ovando (O) location 2
YT-O6	Yellow toadflax - Ovando location 6
♀YT-O6 x ♂DT-M6	YT from location 6 crossed with DT from location 6
♀DT-M6 x ♂YT-O6	DT from location 6 crossed with YT from location 6
♀YT-O2 x ♂DT-M6	YT from location 2 crossed with DT from location 6
♀DT-M6 x ♂YT-O2	DT from location 6 crossed with YT from location 2

Table 1. Description of plants used in Y-tube bioassays. Plant species, collection site, and location within collection site where plant was collected.

Trial	Species and Gender	Stimulus Choices
1	<i>M. janthinus</i> ♀	YT
		Control
2	<i>M. janthinus</i> ♂	YT
		Control
3	<i>M. janthiniformis</i> ♀	YT
		Control
4	<i>M. janthiniformis</i> ♂	DT
		Control

Table 2. 2011Y-tube bioassays. Olfactory trials listed by species and gender of test weevil, and plant odor choices.

Trial	Species (all ♀)⁴⁹	Plant choices
1	<i>M. janthinus</i>	DT-M6 Control
1	<i>M. janthiniformis</i>	DT-M6 Control
2	<i>M. janthinus</i>	YT-O2 Control
2	<i>M. janthiniformis</i>	YT-O2 Control
3	<i>M. janthinus</i>	YT-O6 Control
3	<i>M. janthiniformis</i>	YT-O6 Control
4	<i>M. janthinus</i>	YT-O6 DT-M6
4	<i>M. janthiniformis</i>	YT-O6 DT-M6
5	<i>M. janthinus</i>	YT-O2 DT-M6
5	<i>M. janthiniformis</i>	YT-O2 DT-M6
6	<i>M. janthinus</i>	YT-O6 x DT-M6 DT-M6 x YT-O6
6	<i>M. janthiniformis</i>	YT-O6 x DT-M6 DT-M6 x YT-O6
7	<i>M. janthinus</i>	YT-O2 x DT-M6 DT-M6 x YT-O2
7	<i>M. janthiniformis</i>	YT-O2 x DT-M6 DT-M6 x YT-O2

Table 3. 2012 Y-tube bioassays. Olfactory trials listed by species of test weevil, and plant odor choices.

Trial	Species and Gender	Plant		Expected	p-value
		Choices	Observed		
1	<i>M. janthinus</i> ♀	YT	21	15	0.03*
		Control	9	15	
2	<i>M. janthinus</i> ♂	YT	18	16	0.48
		Control	14	16	
3	<i>M. janthiniformis</i> ♀	DT	21	14	0.01*
		Control	7	14	
4	<i>M. janthiniformis</i> ♂	DT	11	12	0.68
		Control	13	12	

Table 4. Results of 2011 Y-tube bioassays. “Observed” is the number of weevils choosing a specific plant odor. “Expected” is the one half of the total number of weevils tested that made a definitive choice. These numbers were used in the chi squared analysis. * indicates significance

Trial	Plant Choices	Observed	Expected	p-value
1	DT-M6	6	7	0.59298
	Control	8	7	
2	YTMT0209	23	17	0.0395*
	Control	11	17	
3	YTMT0609	13	8.5	0.0290*
	Control	4	8.5	
4	YTMT0609	24	17.5	0.0279*
	DT-M6	11	17.5	
5	YTMT0209	15	10.5	0.0495*
	DT-M6	6	10.5	
6	♀YT-O6 x ♂DT-M6	8	11.5	0.1444
	♀DT-M6 x ♂YT-O6	15	11.5	
7	♀YT-O2 x ♂DT-M6	15	10.5	0.0495*
	♀DT-M6 x ♂YT-O2	6	10.5	

Table 5. Results of 2012 *Mecinus janthinus* Y-tube bioassays. “Observed” is the number of weevils choosing a specific plant odor. “Expected” is the one half of the total number of weevils tested that made a definitive choice. These numbers were used in the chi squared analysis. * indicates significance

Trial	Plant Choices	Observed	Expected	p-value
1	DT-M6	15	9.5	0.0117*
	Control	4	9.5	
2	YT-O2	13	13	1.000
	Control	13	13	
3	YT-O6	9	8.5	0.8084
	Control	8	8.5	
4	YT-O6	14	17.5	0.2367
	DT-M6	21	17.5	
5	YT-O2	12	14.5	0.3532
	DT-M6	17	14.5	
6	♀YT-O6 x ♂DT-M6	14	15	0.7151
	♀DT-M6 x ♂YT-O6	16	15	
7	♀YT-O2 x ♂DT-M6	9	14.5	0.0411
	♀DT-M6 x ♂YT-O2	20	14.5	

Table 6. Results of 2012 *Mecinus janthiniformis* Y-tube bioassays. “Observed” is the number of weevils choosing a specific plant odor. “Expected” is the one half of the total number of weevils tested that made a definitive choice. These numbers were used in the chi squared analysis. *indicates significance

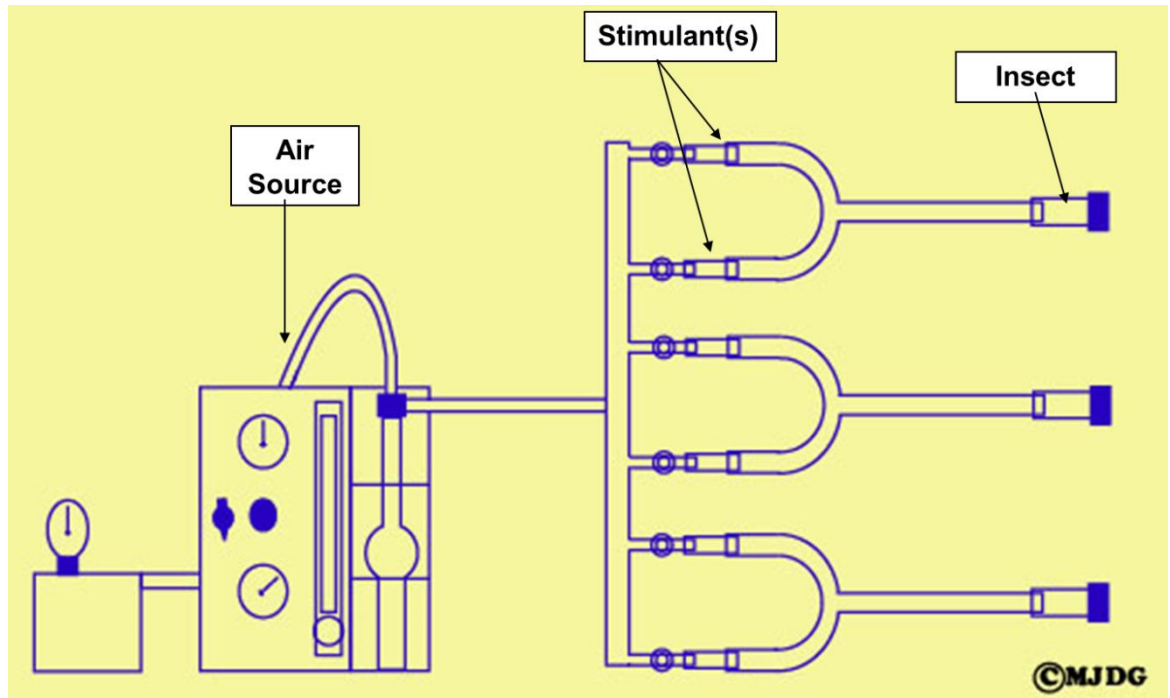


Figure 3. Schematic for Y-tube bioassays.

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CHAPTER 3

HOST-MEDIATED REPRODUCTIVE POTENTIAL AND SURVIVAL

Introduction

Classical weed biological control is a tactic used by land managers and researchers to control invasive and noxious weeds. It involves the importation and release of exotic biological control agents to reduce the abundance of target weeds to acceptable threshold levels (De Clerck-Floate and Bouchier 2000). The expectation of classical biocontrol is that these agents will establish in their new environment, making further releases unnecessary and in this way provide continued and cost-effective control of target weeds (Wilson et al. 2005). After a plant is targeted for biological control, candidate agents need to be identified and screened for an acceptable level of host-specificity before being released to avoid damage to non-target organisms (Grevstad 1999). After initial release, the agent needs to establish and the population must increase, spread throughout the weed infestation, cause plant injury and eventually control the weed by maintaining it at or below economically or ecologically acceptable levels (Crawley 1987; Syrett et al. 2000).

For a biocontrol agent to establish readily, the target weed must be a suitable host, meeting requirements for attractiveness and support of growth and development, allowing an agent to complete its life cycle. Hybridization of invasive weeds in the invaded range presents challenges for classical biocontrol because the hybrid weed may not be attract to agent(s), or support development to the reproductive stage, inhibiting

population increase. Ellstrand and Shierenbeck (2000) cite 35 examples of hybridization of plants leading to range expansion and changes in the original genotype. Hybridization increases genetic variation and creates novel gene combinations that can undergo natural selection (Anderson 1949, Stebbins 1959, Lewontin and Birch 1966). Studies indicate that control of hybrid weeds has had mixed success. Roley and Newman (2006) found that a native weevil, *Euhrychiopsis lecontei* (Dietz), had greatest survival on an introduced watermilfoil, lowest survival on the native watermilfoil, and intermediate survival on the hybrid between the two species. In contrast, Whitham (1989) found that hybrid cottonwoods were more susceptible to injurious aphid populations than either pure parent species. This study addresses the problem that hybridization poses to the development of biocontrol agents *Mecinus janthinus* and *Mecinus janthiniformis* in the control of Dalmatian and yellow toadflax.

In 1987, a management program began designed to control the spread of yellow toadflax (*Linaria vulgaris* (L.) Miller) and Dalmatian toadflax (*Linaria dalmatica* (L.) Miller) (Plantaginaceae) (ITIS 2015), Eurasian noxious weeds that have extensively invaded the western US and Canada (Mitich 1993, Saner et al. 1995, Vujnovic and Wein 1997). Both are perennial species that reproduce asexually and sexually. Dalmatian toadflax typically occupies dry, rocky, uncultivated land, becoming invasive in response to a profound disturbance, such as fire (Jacobs and Sheley 2003). Yellow toadflax occurs in a variety of habitats including cultivated and uncultivated land, favoring moister conditions than Dalmatian toadflax (Darwent et al. 1975, Volenberg et al. 1999). What complicates the management of both Dalmatian and yellow toadflax is the discovery of

naturally occurring populations of hybrid crosses between the two species found first in Montana (Ward et al. 2009) with subsequent populations located in Colorado, Idaho, and Washington. These populations have been confirmed using molecular diagnostic techniques (Ward, unpublished data). Hybrid plants are, like their parents, fertile perennials capable of asexual as well as sexual reproduction. Preliminary results indicate that hybrid populations are vigorous and could outcompete both Dalmatian and yellow toadflax (Turner 2012), creating additional challenges for invasive toadflax management.

As part of the management program, the biological control agent *Mecinus janthinus* Germar (MJ) (Coleoptera: Curculionidae), was first released in Canada in 1991 to control existing populations of both Dalmatian and yellow toadflax (De Clerck-Floate and Miller 2002). *Mecinus janthinus* is an oligophagous, univoltine stem mining weevil that occurs natively in central and southern Europe and southern Russia on Dalmatian and yellow toadflax (Jeanneret and Schroeder 1992). Recent evidence suggests that *M. janthinus* is actually two different cryptic species: *Mecinus janthinformis* Toševski and Caldara, with Dalmatian toadflax as host; and *Mecinus janthinus*, with yellow toadflax as host (Toševski et al. 2011). Adults of both species copulate in the spring and oviposit in the stems of their host plant. Larvae mine through the stem as they develop and pupate in the late summer. They remain as adults inside the stem throughout the winter and emerge again the following spring (Jeanneret and Schroeder 1992, Wilson et al. 2005, McClay and Hughes 2007).

To further complicate the control program, there has been a recent discovery of a population of *Mecinus janthinformis* located on a population of hybrid toadflax plants

located in Palisades, ID. This illustrates that in at least some cases, hybrid toadflax populations can attract and support at least one these two *Mecinus* species. Little is known about either biocontrol agent's ability to reproduce and survive on hybrid plants. The objective of this experiment is to test the reproductive potential and survivability of *M. janthiniformis* obtained from Missoula, MT, *M. janthinus* obtained from Ovando, MT, and *M. janthiniformis* obtained from the Palisades, ID site, on both Dalmatian and yellow toadflax as well as laboratory and field collected hybrids. Hybrid toadflax seed, like that from the parents, are gravity dispersed; therefore it is possible that limited seed dispersal from maternal parents generates kinship demes of genetically similar plants located closer together (Ward et al. 2008). For this reason, we hypothesize that the maternal parent of a hybrid cross has a larger effect on the phenotype of the hybrid plant than the paternal. If this is true, we would expect *M. janthiniformis* to perform better in terms of number of mating events, numbers of eggs laid, percentage of eggs surviving to adulthood and the mean number of adults stem⁻¹, on hybrid crosses with Dalmatian toadflax as the maternal parent and *M. janthinus* to perform better on hybrids with yellow toadflax as the maternal parent. We theorize that the *M. janthiniformis* found at the Palisades, ID site would perform best on hybrid plants since these are the source host plants. This population probably originated from a Dalmatian toadflax source and we expect that it would perform better on hybrids with Dalmatian toadflax as the maternal parent as well.

Materials and Methods

Collection of Insects

Experiments were conducted in the summer of 2012 with adults of all experimental organisms collected in April 2012 from well-established Dalmatian or yellow toadflax biological control release sites. Because both *Mecinus* species used in these experiments overwinter in their natal stems as adults, infested toadflax stems were field-collected in spring before adult emergence. *Mecinus janthiniformis*-infested Dalmatian toadflax stems were collected from Missoula, MT and *M. janthinus*-infested yellow toadflax stems were collected from Ovando, MT. An additional *Mecinus janthiniformis* biotype was field-collected in infested hybrid (Dalmatian x yellow) toadflax stems from Palisades, ID in May 2012. The species identity of each *Mecinus* population was confirmed using molecular diagnostics (Toševski et al. 2011). Harvested stems were placed in 25 x 40 cm 4-mm polyethylene soil sample bags and retained at 4°C in a cold, wet plant storage room in Montana State University's Plant Growth Center (Bozeman, MT). This approach allowed us to extract virgin and not yet active adult weevils for experiments. All adult weevils were individually extracted from host stems before each experiment. Each extracted weevil was isolated in a clear gelatin capsule which was placed on a laboratory chill table so gender could be determined under magnification using sexually dimorphic characters (Schat et al. 2007; Carney et al. 2004). Each successfully sexed weevil was then transferred to a capped, labeled 15-dram plastic shell vial and individually retained (12 hours or less) under moderate refrigeration (7.2-10. 0°C) until the beginning of the experiment.

Plant Material

For this experiment, six toadflax genotypes were used. These included one field-collected Dalmatian toadflax plant (DT-M6) and one field-collected yellow toadflax plant (YT-O6); two reciprocal hybrid crosses, one with a Dalmatian toadflax maternal parent (DT-M6 x YT-O6) and one with a yellow toadflax maternal parent (YT-O6 x DT-M6); and two field-collected, confirmed hybrid plants (Ward et al. 2009), the first collected near Radersburg, MT on the Helena National Forest (Radersburg), and the second collected outside Boulder, MT on the Beaverhead-Deerlodge National Forest (B.R.).

All plants used in this experiment were clones of the plants collected in the field or clones of synthetic hybrid plants. Parent Dalmatian and yellow toadflax plants were collected in 2010 in the field from Missoula MT and Ovando MT respectively. Plants were express shipped to Dr. Sarah Ward at Colorado State University (CSU) in Fort Collins, CO where she performed controlled hand-pollinations to generate hybrid progeny with specified pedigrees. All crosses involved a single Dalmatian toadflax founder specimen and a single yellow toadflax founder specimens in various combinations. Seeds collected from hybrid progeny were subjected to 6 weeks dry stratification in a freezer at -20 C followed by 2 weeks wet stratification (in wet paper towels) in the refrigerator at 4° C, the seeds were then germinated and grown in ProMix BX (Premier Tech Horticulture, Quakertown, PA) in a CSU greenhouse under constant conditions (25°C; 14 h light: 10 h dark) for 6 weeks before being delivered by automobile to Montana in spring 2012.

Clones of all toadflax genotypes were generated from 4-cm shoot tip cuttings of the parent plant. Meristematic cuttings were dipped in Clonex[®] rooting compound (Hydronamics International, Lansing, MI) then placed in low density plastic UV-stabilized Ray Leach cone-tainers (Stuewe and Sons, Tangent, OR) filled with the same MSU mix potting medium as described above. Each cone-tainer was temporarily covered with an inverted 50-ml BD Falcon[™] Blue Max centrifuge tube (Fisher Scientific, Logan, UT) to maintain temperature and humidity range until cuttings became established. These were subsequently transplanted into 50:50 (by volume) MSU mix (steam sterilized 1:1:1 ratio by volume of mineral soil, Canadian sphagnum peat moss, and washed concrete sand with 0.45 kg of Aqua-Gro 2000G wetting agent (Aquatrols, Paulsboro, NJ) added per cubic meter of mixed soil) and Sunshine Mix #1 (Sun Gro Horticulture, Bellevue, WA).

Individual plants were transplanted into 17.5 x 13.3 x 13.5 cm plastic pots and grown under constant conditions in a light and temperature automated greenhouse equipped with GE Multi-Vapor MVR1000/C/U (GE Lighting Global, Bucyrus, OH) lights which was located in the MSU Plant Growth Center. The greenhouse temperature was maintained at 22° C during the day (lights on) and 20° C at night (lights off). A 16L (0600-2200): 6D photoperiod was maintained during the fall and winter months (October – March); supplemental lighting was not used during the spring and summer months (April – September). Soil moisture in each pot was evaluated daily so plants could be individually watered as needed. All pots were treated weekly with a 400-ml solution containing Jack's Professional[™] 20-20-20 (J. R. Peters Horticulture, Allentown, PA)

water soluble fertilizer at a concentration of 100 ppm, which was obtained by using a Siphonex[®] proportioner (Hozon, Earth City, MO).

Before adding weevils, each plant was enclosed in a 7.5 x 30.5 cm cylindrical cage made from transparent polycarbonate plastic, open on the bottom and enclosed on the top with an 8-cm diameter cap of mite-proof mesh sealed along the edge with silicone caulking. In addition to the mesh cap, each cylinder had four, 4-cm diameter mite-proof, mesh-covered, silicone-sealed ports placed randomly along the sides of the cage to facilitate cross-ventilation. A 1.5-cm hole, subsequently plugged with cotton batting, was made in the side of each cage to allow for the addition of weevils after cages were secured over the test plants. Each cage was placed over the plant and pushed 1.5 cm into the soil. The base of each cage was then covered with white bunker sand (Lane Mountain Company, Valley, WA) on the outside to prevent the weevils from crawling out from the bottom of the cage, and on the inside to increase ease of observations.

This study was conducted in the Forestry Sciences Laboratory greenhouse (USDA Forest Service – Rocky Mountain Research Station, Bozeman, MT). The layout for the experiment was a complete randomized block design with light intensity as the blocking factor. Light intensity was measured using a LI-COR model LI-1400 (LI-COR Biosciences, Lincoln, NE) in every position in each block and then averaged to get the overall block light intensity. There were a total of 6 toadflax genotypes used with 3 treatments of weevil placed on them. Pairs of either *M. janthinus* (MJ-YT), *M. janthiniformis* (MJ-DT), or *M. janthiniformis* on hybrids from Palisades (MJ-HT) for a total of 18 treatments. Each treatment was replicated 3 times. One plant from each

treatment was placed in one of three blocks. Plants were randomly placed within each block and changed randomly within the block each week. Three pairs of weevils were placed on each plant on 11 July 2012. A week later, on 18 July, three more pairs of weevils were placed on each plant for a total of 12 weevils on each plant. Plants were watered daily as needed and observed daily to record the number of mating events taking place. Weevils and cages were removed on 1 August 2012 and the plants remained in the greenhouse for an additional 70 days to allow weevils to complete development. The stems from each pot were dissected and the numbers of eggs, dead and live larvae, pupa, and adults were recorded as well as the stem length and width.

Data were initially analyzed using an ANOVA treating the light intensity as a factor and also as a co-variate in an ANCOVA. The results from using light intensity as a blocking factor or a co-variate in the four ANOVA's showed there was no significant light effect. Light was removed as a factor in the model. A separate ANOVA was conducted for mean mating events day⁻¹, for total number of eggs laid, for percentage survival (the number of weevils that survived to adulthood divided by the total number of eggs laid), and for adults cm⁻¹ of stem as the dependent factor in each analysis.

Results

Mating Events

An ANOVA was conducted to determine the effect that toadflax genotype, weevil phenotype, toadflax genotype*weevil phenotype had on the number of mating events day⁻¹. There was a small toadflax genotype effect ($F = 2.32$, d.f. = 5, $p = 0.062$) and a

larger weevil genotype effect ($F=15.14$, d.f. = 2, $p < 0.0001$) (Figure 4). Both MJ-DT and MJ-HT had significantly more mating events per day than MJYT on clones of all toadflax genotypes except for those derived from the B.R. hybrid. The plant effect is driven primarily by the reduced mating events on the clones of the B.R. hybrid for MJ-HT and MJ-DT.

Number of Eggs Laid

An ANOVA was conducted to determine the effect that toadflax genotype, weevil phenotype, toadflax genotype*weevil phenotype had on the total number of eggs in each type of plant. The results indicate a strong plant effect ($F = 6.81$, d.f. = 5, $p = 0.0002$) and a strong toadflax genotype by weevil phenotype interaction ($F = 4.02$, d.f. = 10, $p = 0.0009$). Most eggs laid by the MJ-YT population were in the clones of the yellow toadflax parent and in clones of the yellow toadflax maternal hybrid, as predicted (Figure 5). Clones of the Dalmatian toadflax parent and clones of the hybrid from B.R. were least infested and only the MJ-DT population successfully infested clones of DT-M6. Clones of the hybrid with the maternal Dalmatian parent and the clones of the hybrid from Radersburg hybrid had intermediate results with all three weevil populations laying a substantial number of eggs.

Percentage Survival

Percentage survival was analyzed after the treatments with 0% survival were removed to better meet the assumptions of normality (Figure 6). This was done because one treatment had no eggs in any clones which complicated the analysis. An ANOVA

was conducted to determine the effect of toadflax genotype, weevil phenotype, toadflax genotype*weevil phenotype on the percent of surviving adults. The results indicate a small weevil effect ($F = 2.69$, d.f. = 2, $p = 0.085$). For the MJ-YT population, percent survival was greater in clones of yellow toadflax and clones of the yellow toadflax maternal hybrids, but less than the clones of the Dalmatian toadflax maternal hybrid or the Radersburg hybrid. For the MJ-DT population, clones of the Dalmatian toadflax maternal hybrids and clones of the hybrid from Radersburg hybrids yield intermediate levels of percentage survival, with greater levels of survival on clones of the parent Dalmatian plants and clones of the hybrid from B.R., while the MJ-HT population did best on clones of the Dalmatian maternal hybrid. No individuals from the MJ-YT or MJ-HT populations were able to infest clones of the Dalmatian parent DT-M6.

Adults cm⁻¹ of Stem

This ANOVA determined the effect of toadflax genotype, weevil phenotype, toadflax genotype*weevil phenotype, and block to the average number of living adults cm⁻¹ of stem. Data for adults cm⁻¹ of stem were transformed with a Box-Cox transformation to better meet the assumptions of normality. The results indicate a strong toadflax genotype effect ($F = 5.49$, d.f. = 5, $p = 0.0007$), and a smaller, but significant toadflax genotype by weevil phenotype interaction ($F = 2.32$, d.f. = 10, $p = 0.0317$). Overall, these results indicate that the MJ-YT population is best suited to produce offspring on clones that were predominantly yellow toadflax genotypes rather than on clones of plants that were predominantly Dalmatian toadflax genotypes, as predicted (Figure 7). The set of weevil pairs from the MJ-HT population, as expected, produced

adults most efficiently on clones of hybrid plants, with clones of the Dalmatian maternal hybrid being most productive. Weevils from this MJ-HT population were unable to infest clones from the maternal parent DT-M6 and had were minimally effective in producing offspring on clones of the pure yellow toadflax YT-O6. For members of the MJ-DT population, offspring were most efficiently produced on clones of the hybrid from Radersburg, followed closely by their efficiency on clones of the hybrid from B.R., slightly less on clones the Dalmatian maternal hybrid, with the efficiency in offspring production being least and equal on clones of the yellow maternal parent YT-06 and clones of the Dalmatian maternal parent DT-M6.

Discussion

The number of mating events for weevil pairs from the *M. janthinus* population sets on clones of the hybrid from B.R. is not different from the number of mating events for weevil pairs on clones of the other toadflax genotype sets. However, the number of mating events for weevil pairs from sets of both the Dalmatian, and hybrid toadflax sourced *M. janthiniformis* populations was lower on clones of the hybrid from B.R. hybrid than on clones from all other toadflax genotypes. However, the number of mating events for weevil pairs from sets of both *M. janthiniformis* populations on the clones of the hybrid from B.R. hybrid was not significantly different from the number events for pairs of *M. janthinus* in the sets from this genotype only. The factor that is inducing more overall mating events for both populations of *M. janthiniformis* on clones of all other

toadflax genotypes is apparently nullified for weevil pairs on the sets of clones of the hybrid from B.R. (Figure 3).

It is possible that this is due to a compound that is utilized by *M. janthiniformis* females to make a sex pheromone is not found in the clones of the B.R. hybrid or perhaps less abundant. This would suggest behavior that requires plant compounds to synergize pheromone production that has evolved to increase the efficiency of mating and resource allocation by females (Landolt and Phillips 1997). More research is needed to establish what causes greater numbers of mating events in *M. janthiniformis* on all genotypes except clones from the B. R hybrid, while the number of mating events for *M. janthinus* does not significantly differ on clones of any toadflax genotype.

The large number of eggs deposited in clones of the parent yellow toadflax by sets of weevil pairs from the *M. janthinus* population is expected. The overall low numbers of eggs deposited in clones of Dalmatian toadflax and clones of hybrid from B.R. by sets of weevil pairs from both populations of *M. janthiniformis* is unexpected, but we did expect that overall the greatest number of eggs that would be laid on clones from these toadflax genotypes would be deposited by the sets of weevil pairs from these *M. janthiniformis* populations relative to those from the *M. janthinus* population.

The low number of eggs in the clones of pure Dalmatian plant is perplexing. A resource-based cause may be the average stem width of these plants. The average stem width for DT-M6 in this experiment was only 1.05 mm compared to that average of nearly 3.5 mm in the field (data not shown). Neither *M. janthinus* nor *M. janthiniformis* from Palisades laid any eggs on clones of this pure Dalmatian toadflax genotype. It was

expected that *M. janthinus* might not perform well on clones of Dalmatian toadflax, but we did not expect that *M. janthiniformis* from hybrid toadflax in Palisades, ID would also fail to infest this genotype. This could indicate that this particular strain of *M. janthiniformis* no longer recognizes a pure Dalmatian toadflax plant as a suitable host and has undergone a host-shifting event. An example of this is found in the cladistic analyses of papilionid butterflies and *Ophraella* leaf beetles and their host plants which revealed that, for these species, host shifts are more likely to occur among chemically similar plants (Jaenike 1990). The Dalmatian maternal hybrid and the Radersburg hybrid received eggs from all three weevil populations as might be expected on hybrid plants. The low overall infestation for the hybrid from B.R. by pairs of *M. janthiniformis* from Dalmatian toadflax, despite depositing the greatest number of eggs amongst the weevil populations, would indicate that this particular hybrid plant is genetically more similar to Dalmatian parents. This would also explain why overall experimental results for clones of the hybrid from B.R. are more similar to that found for the pure Dalmatian plants.

The percentage survival results for offspring from the *M. janthinus* population indicate that, although oviposition on the Dalmatian maternal hybrid and the Radersburg hybrid was less than on the pure Dalmatian and hybrid B.R. genotypes, hybrid toadflax is a very suitable host for *M. janthinus*. Their percentage survival was higher for these hybrid genotypes relative to the other two hybrid genotypes that were favored for oviposition. The fact that no *M. janthinus* infested Dalmatian toadflax or and that the marginal infestation on the hybrid from B.R. resulted in no survival would indicate that, over time, the preference for yellow toadflax has not allowed *M. janthinus* to develop the

capability to utilize Dalmatian toadflax. In this case, it appears that the preference of each weevil species that was shown in Chapter 2 and the ability to fully develop on their host is linked. What remains unexpected was the limited survival of *M. janthiniformis* from pure Dalmatian plants on the Dalmatian maternal hybrid plant.

Measuring the number of adults cm^{-1} of stem is an overarching illustration of the productivity of each weevil population on each toadflax genotype. *Mecinus janthinus* performed best on pure yellow toadflax and the yellow maternal hybrid and failed on pure Dalmatian and the B.R. hybrid. Although *M. janthinus* performed well on the Dalmatian maternal hybrid and the Radersburg hybrid, it must be remembered that these results are from no-choice tests and earlier preference tests (Chapter 2) indicated that *M. janthinus* are not attracted to Dalmatian plants or their maternal hybrids. *Mecinus janthiniformis* from Palisades, ID performed best on the Dalmatian maternal hybrid as we would predict, but also performed well on the yellow maternal hybrid.

This indicates that this particular strain of *M. janthiniformis* would perform well on hybrid populations of toadflax, as expected. The exception is the B.R. hybrid genotype. However, this B.R. hybrid shows an overall pattern of host attributes that favor a stronger similarity to pure Dalmatian toadflax. *M. janthiniformis* from pure Dalmatian plants was the only weevil population that showed productivity, often reduced, but on all toadflax genotypes. Overall, this population was most productive on the Radersburg hybrid and the B.R. hybrid with moderate success on the other genotypes. This supports *M. janthiniformis* as a more “general” agent and is better suited for variation in toadflax genotypes; *M. janthiniformis* from hybrid toadflax was also able to use most host

genotypes, only failing on the recalcitrant pure Dalmatian plants. Yet, *M. janthiniformis* was the only weevil to successfully infest and survive on this pure Dalmatian toadflax. As mentioned earlier, the limited suitability of this Dalmatian toadflax genotype is probably due to the poor quality of clones, which were smaller and more water sensitive than clones of all other toadflax genotypes, including clones of the synthetic hybrid with this same population as the maternal parent.

Conclusion

Potential problems that hybridization poses to the development of *M. janthinus* and *M. janthiniformis* from yellow and Dalmatian toadflax can potentially be overcome by these biocontrol agents. *Mecinus janthinus* seems to develop only on plants with some yellow toadflax genetic influence and *M. janthiniformis* develops modestly on every genotype in the experiment.

Hybridization of the two toadflax species is an apparently rare event in their native range as prevalent hybrid plants would allow for more gene flow between the two *Mecinus* species which would prevent the evident speciation that is taking place (Futuyma and Peterson 1985). Although it is unclear how genetically isolated the two *Mecinus* species are from each other, it is apparent that they prefer and develop better on the separate native hosts (Toševski et al. 2011). These results also indicate that preference and host suitability are linked in the case of *M. janthinus*. It is unclear if suitability drove preference, but it is more likely. Regardless of how host preference developed for *M. janthinus*, the ultimate conclusions are that *M. janthinus* prefers and performs better on

yellow toadflax and hybrids with a more prominent yellow toadflax genetic input while *M. janthiniformis* performs better on Dalmatian toadflax and hybrids with a more prominent genetic contribution from Dalmatian toadflax.

Land managers may decide that when challenged with hybrid populations of toadflax, *M. janthiniformis* would be the preferred agent to use when long-term establishment and impact is the goal. However, if the hybrid population strongly resembles yellow toadflax, *M. janthinus* may be a better option.

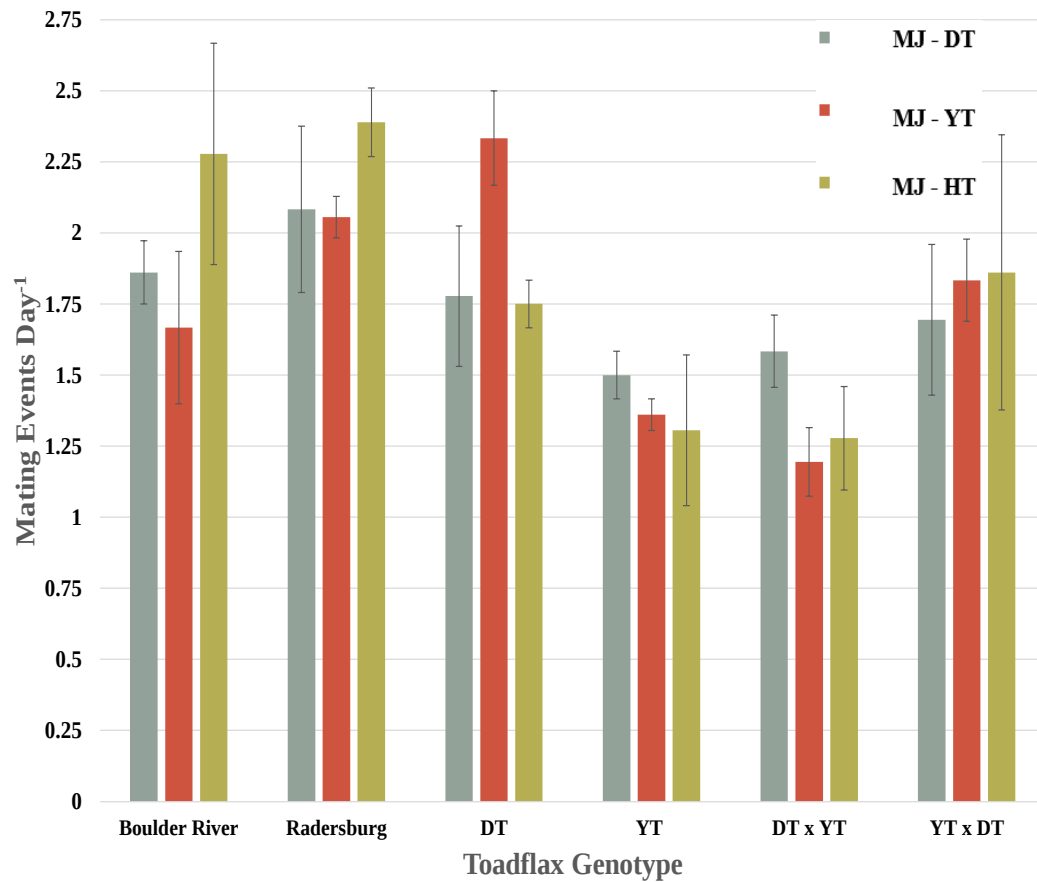
Figures

Figure 4. Mean number of mating events ($\pm$ standard error) for each set of weevil pairings. There were two populations of *M. janthiniformis* and one population of *M. janthinus* on six toadflax genotypes. There is a weak toadflax genotype effect ($p < 0.10$) and an effect of weevil species ($p < 0.05$).

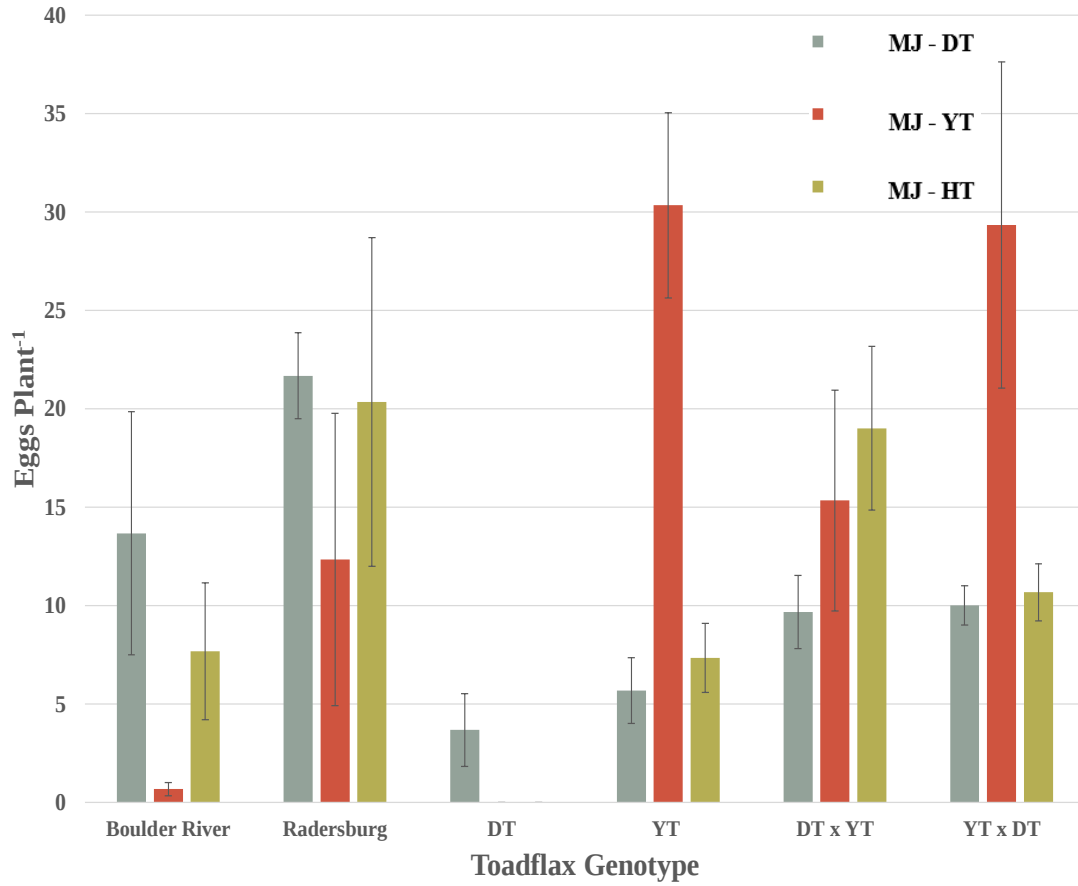


Figure 5. Mean number of eggs ($\pm$ standard error) for each set of weevil pairings. There were two populations of *M. janthiniformis* and one population of *M. janthinus* on six toadflax genotypes. There was an effect of toadflax genotype and an effect due to the interaction between toadflax genotype and weevil species ($p < 0.05$).

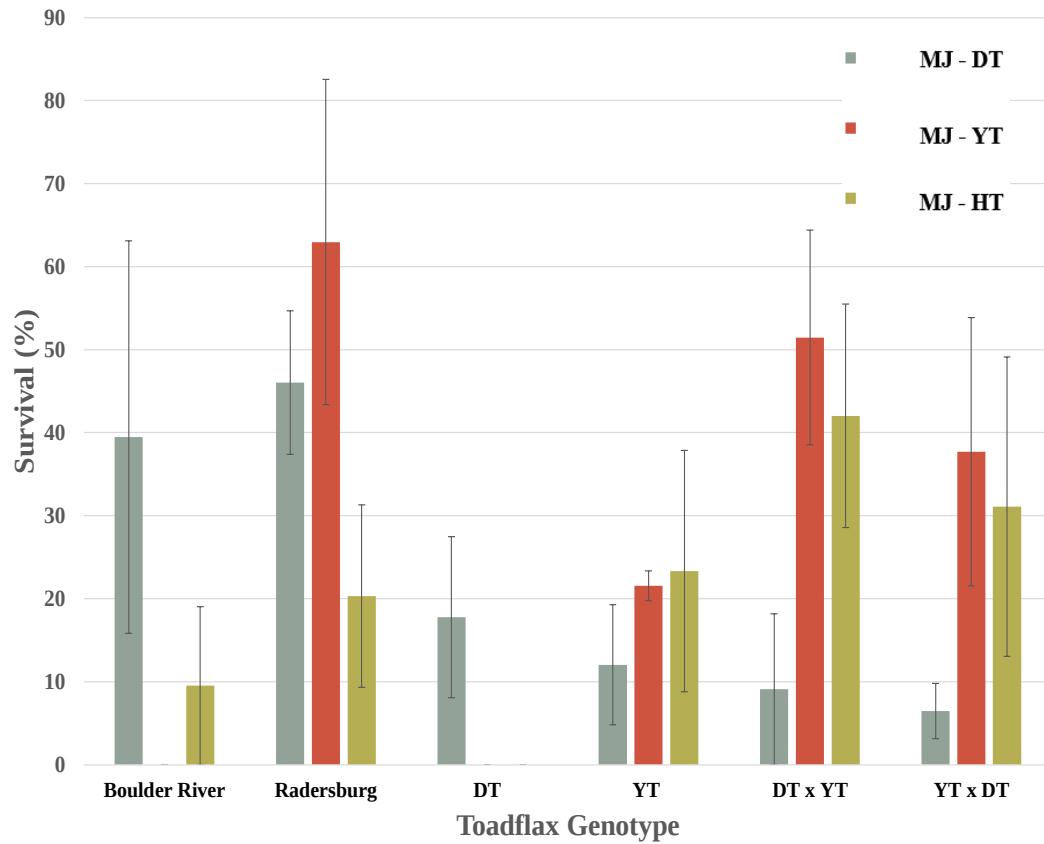


Figure 6. Mean percent survival to adulthood ($\pm$ standard error) of offspring for each set of weevil pairings. There were two populations of *M. janthiniformis* and one population of *M. janthinus* on six toadflax genotypes. There was a weak effect of weevil species ($p < 0.10$)

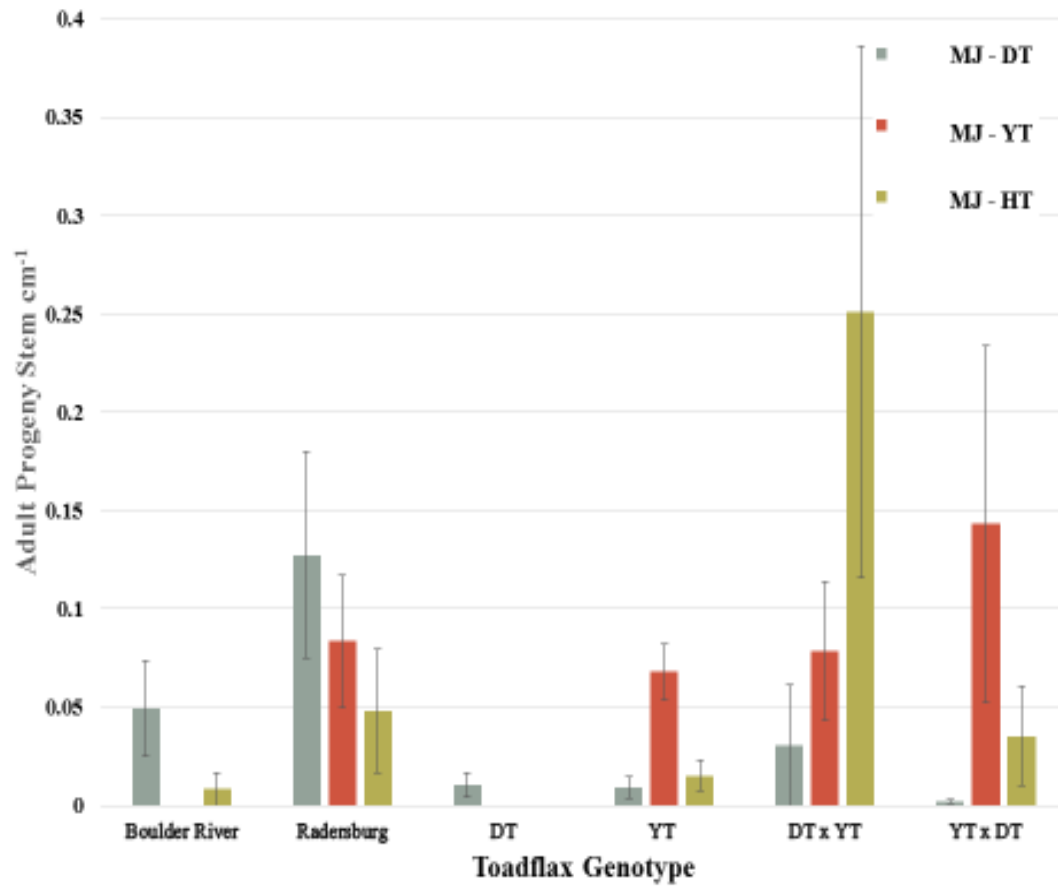


Figure 7. Mean adults cm^{-1} of stem ($\pm$ standard error) derived by dividing the total number of surviving adults by the total length of all stems for each plant. These were based on the set of weevil pairings for each of the three weevil populations on clones of the six toadflax genotypes. There was an effect of toadflax genotype and an effect due to the interaction of weevil species and toadflax genotype ($p < 0.05$).

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CHAPTER 4

REPRODUCTIVE ISOLATION OF *MECINUS* SPECIES.Introduction

Classical weed biological control involves the introduction of foreign natural enemies to control a foreign invader (Sing et al. 2005). The ultimate goal is to reduce the levels of the target species below economic and environmental thresholds (Harris 1991, McFadyen 1998). The objective is that the agent(s), once released, will become a sustained population over time, resulting in equilibrium between pest and biocontrol agent; an equilibrium like that found in the native range (McFadyen 1998). Hybridization is a mechanism that can potentially inhibit the effectiveness of classical biocontrol because it can create novel genotypes and allow for range expansion (Ellstrand and Schierenbeck 2000). An interesting example of this can be found in the extensive hybridization and expansion of *Tamarix* (Gaskin and Schaal 2002) and in their associated, introduced *Diorhabda* biological control agents (Bean et al. 2013, Michels, Jr. et al. 2013).

There is also the case of hybrid or “admixture” vigor where isolated, climatically distinct populations of the same species interbreed, as in Swiss and Italian populations of *Longitarsus jacobaeae* Waterhouse. These two populations have varying success on invasive tansy ragwort, *Jacobaea vulgaris* Gaertner in the western US, but now show hybrid vigor when interbreeding (Szűcs et al. 2012a, 2012b; 2013) that is advantageous in biological control of this invasive weed. Another complication can occur when a

biocontrol agent begins sympatric or parapatric speciation. Although under debate, it has been suggested that “host races” are non-inter-breeding sympatric populations that differ in biological characteristics but not in morphology, and are prevented from interbreeding solely by preference for different host plant species, or as a direct consequence of adaptation to different host plant species (Mathenge et al. 2010). These host races are thought to be a stepping stone in the speciation process (Marohasy 1996). Having both of these complications in a biological control program is rare but provides difficulties that need to be addressed. In these experiments, we address the problems that Dalmatian and yellow toadflax hybridization and the cryptic speciation of the biological agent *Mecinus janthinus* present to the classical biocontrol program.

In 1991, the biological control agent *Mecinus janthinus* Germar (MJ) (Coleoptera: Curculionidae) (ITIS 2013) was approved for release in Canada in order to control the spread of Dalmatian toadflax (*Linaria dalmatica* [L.] Miller) (Plantaginaceae) and yellow toadflax (*Linaria vulgaris* Miller) (ITIS 2013). Yellow toadflax was introduced into the northeastern United States sometime before 1672 as an ornamental and medicinal plant and by the 1950s had spread throughout North America (Lajeunesse 1999). It has typically been found in areas both cultivated and uncultivated, grazed areas or other forms of disturbance both in North America and in its native European range (Jacobs and Sheley 2003). Dalmatian toadflax is thought to have originated in the Mediterranean region, with a native range extending from Yugoslavia to northern Iran (Alex 1962). It has been suggested that it was first imported as an ornamental in 1894, although the earliest known authenticated specimen was collected in 1920 in California (Vujnovic and Wein

1997). Both species are an insect-pollinated out-crossing diploid woody perennial species that reproduce both sexually via seed and vegetatively by means of adventitious shoots from rhizomes. This and the large number of seeds they produce, contribute to their large success as invaders (Lajeunesse 1999). Further it is now known that these two species of toadflax can readily produce hybrid offspring populations that have been found in Montana, Colorado, Washington and Idaho. Initial studies suggest that they have the potential to outcompete their parents (Ward et al. 2009).

Since its initial release, a combined morphological, molecular and biological study shows that the weevil species *M. janthinus* is actually composed of two different cryptic species. *Mecinus janthinformis* Toševski & Caldara and *Mecinus janthinus* Germar whose host plants are Dalmatian toadflax and yellow toadflax, respectively (Toševski et al. 2011). Both species are native to central and southern Europe and southern Russia on Dalmatian and yellow toadflax (Jeanneret and Schroeder 1992). They were chosen as a biological control agent because there is no North American stem-miner that attacks any introduced toadflax species which would be more effective than defoliation or seed feeding (Jeanneret and Schroeder 1992, Saner et al. 1994, Egan and Irwin 2008). *M. janthinformis* has become widely distributed throughout the southern parts of the western provinces of Canada and the Northwest U.S. through natural dispersion and active redistribution since its initial release (De Clerck-Floate and Miller 2002, Carney 2003, Schat et al. 2007). *M. janthinus*, conversely has remained rare and at low densities in Canadian and U.S. populations. This may be due to the high levels of genetic diversity in yellow toadflax populations (Ward et al. 2008, Toševski et al. 2011).

In light of these recent findings, how little is known or understood about the extent of reproductive isolation between the two *Mecinus* species becomes obvious. The concept of cryptic speciation is not new to the family Curculionidae. One of the best known examples is the cabbage seedpod weevil *Ceutorhynchus obstrictus* (Marsham) (syn. *C. assimilis* [Paykull]) (Fumanal et al. 2004), that includes a potential biological control variant (Kuhlmann et al. 2006). Little has been done to establish if these species can still produce viable offspring. This study examined the extent to which speciation and reproductive isolation has evolved in *Mecinus*. The research objectives were to investigate (a) whether *M. janthiniformis* and *M. janthinus* will mate and produce viable genetically validated F1 offspring; and (b) whether the host toadflax genotype (Dalmatian, yellow or hybrid toadflax) used as a substrate for interbreeding affects survival of F1 generations.

Materials and Methods

Collection of Insect Populations

Because both weevil species overwinter in the stem as an adult, toadflax stems were collected from each field site in the spring and placed in 28 x 40 cm plastic bags for subsequent extraction for experiments. Weevils were collected from two separate sites in Montana, *M. janthiniformis* collected from Missoula, MT on Dalmatian toadflax and *M. janthinus* from Ovando, MT collected on yellow toadflax in April, 2012. We collected 25 bags of stems from each site. Each bag contained approximately 60 stems. These smaller bags were placed in one of three, 190-L garbage bags, one per site, and kept on separate

shelves in a cold storage facility located at the Plant Growth Center (PGC) at the Montana State University (MSU) campus at a constant temperature of 4° C until the weevil were removed from the stems for use in the experiment.

Weevils were extracted from the stems and sexed using features identified by Schat et al (2007) and Carney et. al (2004). After they were sexed, each weevil was individually placed in a 15-dram shell vial and kept in a laboratory refrigerator until placed on test plants. Each sex of weevil was paired with a member of the opposite sex from the other location i.e. all female *M. janthiniformis* were paired with male *M. janthinus* and vice versa. This created reciprocal combinations of weevil pairs that were placed on each plant variety. In addition, field-collected Dalmatian and yellow toadflax plants from Missoula and Ovando respectively had an additional *Mecinus* treatment. For this, male and female *M. janthinus* were placed on yellow toadflax and male and female *M. janthiniformis* were placed on Dalmatian toadflax to act as a control for the experiment.

Production of Toadflax Clones

Dalmatian and yellow toadflax plants were collected in 2009 in the field from Missoula, MT and Ovando, MT respectively. We used clones of one Dalmatian plant labeled “DT-M6” (Dalmatian Toadflax- located in Missoula site 6) and clones of one yellow plant “YT-O6” (Yellow Toadflax-located in Ovando site 6). Field-collected plants were sent to Dr. Sarah Ward, Colorado State University (CSU) in Fort Collins CO to be crossed in order to breed hybrid plants. Clones of two of the resulting F1 hybrid plants were used in this experiment: “♀YT-O6 x ♂DT-M6”, and “♀DT-M6 x ♂YT-O6” were

reciprocal crosses of the same parent plants with YT-O6 as the maternal parent in the first cross and DT-M6 as the maternal parent in the second cross. The other two hybrid plants used were clones of two field collected and molecularly confirmed, hybrid plants with unknown parents and uncertain levels of genetic introgression. One hybrid population was from a site near Radersburg, MT and the other from a site outside Boulder, MT on the Beaverhead-Deerlodge National Forest.

All plants used in the experiments were progeny of the plants extracted in the field or of the hybrid plants obtained from CSU. These progeny were simply made by cloning the original parent plant. Clones were made by taking 4-cm stem cuttings of the parent plant and dipping them in Clonex[®] Rooting Compound (Hydronamics International, Lansing, MI) before placing them in a soil medium located in UV stabilized Ray Leach “Cone-tainers” (Stuewe and Sons, Tangent, OR). Each clone was then covered by an inverted 50-ml BD Falcon™ Blue Max centrifuge tube (Fisher Scientific, Logan, UT) to maintain a more constant temperature and humidity range. The tubes were removed when the clones were well established and growing. After 3-4 weeks, when the clones were large enough, they were transplanted into 17.5 x 13.3 x 13.5 cm plastic pots for further development.

The soil medium used in both the cone-tainers and the pots was a 50-50 mix by volume of MSU PGC soil mix (MSU mix) and 47 Sunshine Mix #1 (Sun Gro Horticulture, Bellevue, WA). The MSU mix is a 1:1:1 ratio by volume of mineral soil, Canadian sphagnum peat moss, and washed concrete sand with 0.45 kg of Aqua-Gro

2000G wetting agent (Aquatrols, Paulsboro, NJ) added per cubic meter of mixed soil.

The soil was steam sterilized before use.

The plants were grown in a greenhouse with GE Multi-Vapor MVR1000/C/U lights (GE Lighting Global, Bucyrus, OH), also located in the PGC. The temperature was maintained at 22° C during the day (lights on) and 20° C during the night (lights off).

There is a 1.5° range above or below those temperatures before the heating or cooling systems begin running. During the fall and winter months (October – March) a 16-hour photoperiod was maintained with the lights running from 0600 to 2000 h.

During the spring and summer months the supplemental lighting was not used. Plants were watered daily and fertilized weekly with a 400-ml solution containing 100 ppm Jack's Professional 20-20-20 water soluble fertilizer (J. R Peters Horticulture, Allentown, PA). The solution was prepared using a Siphonex proportioner (Hozon, Earth City, MO).

Each plant was covered with a 7.5 x 30.5-cm cylindrical cage made from transparent polycarbonate plastic before weevils were introduced. Each cylinder was open on one end where it was placed over the plant and closed at the opposite end by an 8-cm diameter cap of mite-proof mesh attached to the cylinder with silicone. In addition to the mesh cap, each cylinder had four 4-cm diameter holes to allow ventilation. Each hole was covered with mite-proof mesh attached with silicone. Each cage was placed over the plant and pushed 1.5 cm into the soil. The base of each cage was then covered with white bunker sand (Lane Mountain, CO) to prevent the weevils from crawling out from the bottom of the cage. An additional 1.5 cm hole facilitated introduction of the

weevils in each cage. This hole was filled with cotton plug to prevent escape of the insects after introduction.

The experiment was conducted in a greenhouse at the Forestry Sciences Laboratory, USDA Forest Service located at 1648 South 7th Avenue in Bozeman, MT. The layout for the experiment was a complete randomized block design with a light intensity gradient that decreased, then increased again as one moved south (Fig.1). Light intensity was measured using a LI-COR data logger (model LI-1400) in every position in each block and then averaged to get the overall block light intensity.

Differences in host suitability were assessed using two different interspecific weevil pairs (a *M. janthinus* female with a *M. janthiniformis* male; a *M. janthiniformis* female with a *M. janthinus* male) which were placed as separate treatments on the six different toadflax genotypes described above. In addition, intraspecific weevil pairs (a *M. janthinus* female with a *M. janthinus* male, a *M. janthiniformis* female with a *M. janthiniformis* male) were placed on their respective natural host to serve as a positive control. A total of three different weevil crosses were placed on the pure Dalmatian toadflax and pure yellow toadflax test plants: the two interspecific pairs, and one intraspecific pair. The four hybrid toadflax genotypes (two field collected, two-hand crosses as described above) received only the two interspecific weevil pairs. The total number of weevil pair-toadflax genotype treatments assessed was 14; each treatment was replicated 4 times. A plant from each weevil pair-toadflax genotype treatment was placed in one of four total blocks. Plants were randomly positioned within each block at the beginning of the experiment, and thereafter repositioned randomly within the same block

every week. Three pairs of weevils were placed on each plant on 1 June 2012. A week later, on 8 June, three more pairs of weevils were placed on each plant to achieve a total treatment density of 12 weevils/6 male-female weevil pairs on each plant.

Plants were assessed daily, watered as needed, and visual observations were made of the total number of mating events taking place during 1 hour in the morning and 1 hour in the afternoon. On 22 June 2012 the adult weevils and cages were removed and the plants remained in the greenhouse for an additional 70 days to allow for F1 progeny development. The stems were then cut from each plant and placed in individual size 70 (0.211-mm) nylon mesh bags that were 30-cm wide and a height of 1-m. These were held in the MSU cold storage facility located in the PGC for the duration of the winter. During March 2013, stems were dissected to record the number of live and dead adults, pupae, larvae, and eggs, and number of emergence holes.

Data were initially analyzed using an ANOVA treating the light intensity as a factor and also as a co-variate in an ANCOVA. The results from using light intensity as a blocking factor or a co-variate in the four ANOVA's showed there was no significant light effect. Light was removed as a factor in the model. A separate ANOVA was conducted for mean mating events day⁻¹, for total number of eggs laid, for percentage survival (the number of weevils that survived to adulthood divided by the total number of eggs laid), and for adults cm⁻¹ of stem as the dependent factor in each analysis.

Genetic Confirmation of F1 Hybrid Weevils

To establish that these were F1 hybrid progeny an additional analysis was conducted using individuals dissected from stems of the naturally occurring host plants in

May 2013. The host stems were from the same Missoula, MT and Ovando, MT toadflax populations from which the parents for the F1 hybrids were collected in 2011. Emerging adults were stored in ethanol after carefully noting the identity of the host plant. Progeny from the interspecific and intraspecific laboratory pairings were also stored in alcohol and sent to Dr. John Gaskin, USDA- ARS in Sidney MT for DNA analysis as described in the next paragraph.

Insects were stored in alcohol and genomic DNA was extracted from the head of each insect using a Qiagen QIAamp Micro Kit (Qiagen, Valencia, CA). 2-uL of the final 50-uL extraction were used to perform AFLP analysis, which followed Vos et al. (1995) with modifications as in Gaskin and Kazmer (2009). All 15 selective primer combinations of *MseI* + CAA, CAC, CAT, CTA, or CTC and *EcoRI* + AAG, ACC, or ACT were pre-screened for PCR product quality and number of variable loci using eight samples, and the most polymorphic primer pair was chosen (*MseI* + CAA/ *EcoRI* + ACC). AFLPs were produced on an Applied Biosystems (ABI; Foster City, CA) 3130 Genetic Analyzer. Loci were initially scored by the fragment analyzer software GeneMapper v 4.0 (ABI). These 32 loci were then manually screened, making this a semi-automatic scoring method, as suggested by Papa *et al.* (2005).

NTSYS-pc ver.2.1 software (Rohlf 1994) was used to calculate the Dice (1945) similarity coefficient: $2a/(2a+b+c)$ where a = number of bands present in both samples, b and c = number of bands present in only one or the other sample, respectively. Principal Coordinates Analysis (PCoA) was performed on Dice similarity coefficients using the DCENTER and EIGEN modules of NTSYS (Fig. 10).

Assessing Reproductive Viability of F1 Hybrids

A subsequent experiment was conducted to determine if the F1 weevils were reproductively viable. Stems from the first experiment containing hybrid progeny were dissected and the number of F1 adults was recorded. Males and females from the same plant were collected and 3 pairs (3 males, 3 females) were placed on a fresh clone of the same toadflax genotype they had developed on. The pure Dalmatian toadflax plants from the first experiment produced minimal weevil progeny, so tests of F1 reproductive viability of progeny from Dalmatian toadflax could not be included in this experiment. The remaining five toadflax genotypes (i.e., YT-O6; YT-O6 x DT-M6; DT-M6 x YT-O6; Radersburg hybrid and Boulder River hybrid) produced enough F1 adult progeny for further experimentation. Each of these five suitable host toadflax genotypes received two different weevil treatments, one male and one female offspring from reproductive pairing of a *M. janthinus* female with a *M. janthiniformis* male, or one male and one female offspring from a reproductive pairing of a *M. janthiniformis* female with a *M. janthinus* male. This resulted in 10 treatments that were each replicated twice, for a total of twenty study plants. The second experiment was conducted in the PGC greenhouse at Montana State University under identical conditions to those described above.

The weevil pairs were placed on the plants on 16 April 2013 and removed 30 April 2013, when the cages were also removed. The plants were then grown under the environmental conditions as previously described, for 75 days until 15 July, when the stems were dissected to collect any adult F2 weevils.

Results

Number of Hybrid Eggs

An ANOVA was used to determine the effect of the toadflax genotype, weevil phenotype, toadflax genotype*weevil phenotype on the number of eggs per stem. The results indicate a strong toadflax genotype effect ($F = 8.04$, d.f. = 5, $p = 0.00002$) and a strong toadflax genotype*weevil pairing interaction ($F = 4.02$, d.f. = 10, $p = 0.0009$) (Figure 8). The effect of toadflax genotype is most evident with the greatest number of F1 eggs found in the pure yellow toadflax and the DT X YT genotype. These results also suggest that *M. janthinus* females were overall much more fecund than *M. janthiniformis* females, depositing significantly more eggs in all toadflax genotypes except pure Dalmatian toadflax. The greatest number of eggs recorded were generated by female *M. janthinus* paired with male *M. janthiniformis* on the DT x YT hybrid; oviposition was not significantly different from that observed for the intraspecific *M. janthinus* positive control on pure yellow toadflax.

Hybrid Adult Percentage Survival

Percentage survival was calculated as the percentage of live adults obtained from total number of eggs deposited. The ANVOA determined the effect of toadflax genotype, weevil phenotype, toadflax genotype*weevil phenotype on percent survival to adulthood. There is a strong effect of toadflax genotype ($F = 2.69$, d.f. = 5, $p < 0.0001$) (Figure 9). This is primarily due to the lack of survivors on the pure Dalmatian genotype, with only the intraspecific pairing yielding any live offspring. The survival of hybrid F1 weevils was greatest on hybrid plants. A trend was detected for offspring from female *M.*

janthiniformis to have a higher percent survival compared to offspring of female *M. janthinus*. However, these values were not found to be significantly different indicating that further study is needed for clarification.

Hybrid Adults cm⁻¹ of Stem

This was a discrete measure of the total number of weevil progeny found for the entire length of host stem and determines the overall suitability of toadflax genotypes for the development of hybrid F1 progeny from each weevil pairing. An ANOVA again determined the effect of toadflax genotype, weevil pairing, and toadflax genotype*weevil pairing on average surviving adults cm⁻¹ stem. The results indicate a toadflax genotype effect ($F = 6.7$, d.f. = 5, $p < 0.0001$) (Figure 10), but no effect of weevil pairing or interaction between toadflax genotype and weevil pairing. Similar to the results reported for percent survival (Figure 9), this assessment shows that hybrid plants were best suited for the production of hybrid weevils, with the DT x YT cross being an optimal toadflax genotype for the production of F1 hybrid weevils. Even though a significant weevil pairing influence was not detected, hybrid weevil pairings with *M. janthinus* as the female parent produced a greater number of offspring than hybrid pairings with *M. janthiniformis* as the female parent (Figure 10).

Genetic Confirmation of F1 Hybrids

The results of the AFLP analyses are shown in Figure 11. Data corresponding to the three sources of weevils separate well into three distinct clusters. A cluster for individuals sourced from Dalmatian toadflax appears at one end of the array while a

cluster for individuals sourced from yellow toadflax appears at the other end (Figure 11). The scores for the laboratory produced hybrid weevils form a cluster that is intermediate between the clusters for Dalmatian toadflax and yellow toadflax sourced individuals, with little overlap. The identities of weevils collected from the same source locations had been previously confirmed to the species level by Dr. Ivo Toševski, Department of Plant Pests, Institute for Plant Protection and Environment, Zemun, Serbia using the molecular methods described in Toševski et al. (2011).

Viability of F1 Hybrid Progeny

Results reported to this point confirmed that mating between *M. janthinus* and *M. janthiniformis* could produce hybrid F1 offspring. However, it was still necessary to determine if these hybrid *Mecinus* were reproductively viable. A follow-up experiment conducted in spring 2013 addressed this uncertainty (Table 9). The results of that study lead us to believe that F1 hybrid weevils are reproductively viable. However, this result was not obtained without some complications. When the plants were dissected after the same duration of development as the previous experiments and the adult F2 progeny of the F1 hybrid parents were counted, we also found emergence holes, meaning that some F2 adults had emerged and were foraging. The plants were no longer enclosed in cages so the origin of the emerged F2 individuals could not be addressed with certainty. This compromised our ability to make accurate comparisons of the viability of host genotypes for production of the F2 generation. The experiment was therefore terminated.

Discussion

For weevil pairings with *M. janthiniformis* as the female parent, the production of F1 eggs is numerically consistent but lower than pairings with *M. janthinus* as the female parent, which produced a variable, but greater, number of F1 eggs (Figure 8). This result may indicate that *M. janthinus* females are more fecund than *M. janthiniformis* females in pairings with congeneric males. Congenerically paired *M. janthinus* females did especially well on the DT x YT genotype, depositing nearly 50 eggs. It is unclear if there is any significant relationship between the parental origins of the toadflax hybrid genotypes compared to the F1 weevil hybrids. The toadflax genotype effect detected in the ANOVA is likely driven by the refractory nature of pure Dalmatian toadflax and the Boulder River hybrids, which had the fewest F1 hybrid eggs. These two genotypes were also refractory to conspecific pairs in the earlier experiments. Clones from the pure Dalmatian toadflax plant field collected in Missoula proved to be the least suitable hosts among all toadflax genotypes tested, although the naturally occurring hybrid from Boulder River was similarly low in suitability. Conversely, synthetic reciprocal hybrid crosses using the Dalmatian toadflax parent plant were generally quite suitable when compared to other toadflax genotypes and thus, it is unclear why this parent genotype was so limiting.

Figure 9 shows that the interspecific weevil pairs had the best percent survival to F1 hybrid adults on clones of hybrid toadflax genotypes. Every treatment, with the exception of those on clones of the pure Dalmatian toadflax plant, had at least 50% survival. Survival on clones of the Radersburg field-collected hybrids, and on clones of

both of the synthetic hybrids was above 70 percent. This percent survival was greater than anything we saw in the experiments conducted in 2012 (Chapter 3). The two genotypes that were least viable for F1 hybrid weevil survival were clones of the pure yellow and pure Dalmatian parent plants. This could indicate that viable F1 hybrid weevils need viable clones of hybrid host toadflax genotypes to have the greatest success.

Figure 10 depicting live adult progeny cm^{-1} of stem provides an overall picture of how effective the each toadflax genotype was as a host for developing hybrid F1 weevil populations. Most congeneric weevil pairings were fairly productive on hybrid toadflax genotypes. Interspecific pairings were universally highly productive on the DT x YT hybrid, yielding a similar number of F1 adults cm^{-1} of stem. Hybrid offspring of *M. janthiniformis* females outnumbered hybrid offspring of *M. janthinus* females only on clones of the field-collected Radersburg hybrid ($p < 0.05$). On clones of pure yellow toadflax, the number of adult offspring cm^{-1} of stem generated by *M. janthinus* females paired with *M. janthiniformis* males was nearly double the number of progeny produced by conspecific *M. janthinus* weevil pairs and quadruple the number of progeny cm^{-1} of stem produced by *M. janthiniformis* females paired with *M. janthinus* males ($p < 0.05$).

The AFLP analysis of putative F1 hybrid progeny is robust and confirms that these laboratory offspring are indeed hybrid offspring. By resampling the weevil populations that were the source of the initial parents for confirmation we have made the separation into 3 distinct clusters compelling.

Finally, the experiment on the reproductive viability of F1 hybrid weevil conducted in spring 2013 did not end as expected. The F2 weevils may have emerged

earlier due to the season they were placed on the plants, which occurred concurrent to when the insects are active in the wild. Even without comparative statistics, we did find live F2 adults inside stems of all but 4 plants. Unfortunately, it is not possible to ascertain which F1 weevil pairs performed best on the host toadflax genotypes, yet the F1 generation was quite reproductively viable and could potentially live independently on hybrid populations of toadflax.

Conclusions

Survival of F1 offspring from crosses of *Mecinus janthinus* and *Mecinus janthiniformis* was higher on hybrid toadflax plants than on either of the parental toadflax species. This result may have significant implications for biological control targeting both toadflax species. The obvious implication from these results is that hybrid weevil populations will perform better on hybrid populations of toadflax. These experiments did not assess the damage hybrid weevils can cause to the host plant in comparison to the parent weevil species. However, increased survival leads to the conclusion that more damage would likely be inflicted by hybrid weevils based purely on the higher available numbers of stem mining larvae. These data were obtained in no-choice trials, so this does not imply that a *Mecinus* species will prefer to copulate with the congener. Further, field trials need to be conducted to determine whether if hybrid populations can be reared naturally.

The F1 hybrid adults did reproduce successfully when paired on the same plant genotypic substrate that they emerged from. This is an exciting result, but requires further investigation. In the wild conditions would not be as controlled, so the outcome of

backcrosses and introgression on the effectiveness of hybrid biocontrol agents is unknown. Further research is needed, because these findings have important implications for the potential use of hybrid *Mecinus* in hybrid toadflax management.

Tables and Figures

Plant Number	Toadflax		Dead Adults	Live Adults
	Genotype ¹	F1 <i>Mecinus</i> Pair ²		
1	YT	YT/DT	0	0
2	YT	YT/DT	0	0
3	YT	DT/YT	0	2
4	YT	DT/YT	0	2
5	RD*	YT/DT	0	9
6	RD*	YT/DT	2	8
7	RD*	DT/YT	1	5
8	RD*	DT/YT	0	4
9	BR*	YT/DT	0	8
10	BR*	YT/DT	0	0
11	BR*	DT/YT	0	24
12	BR*	DT/YT	0	4
13	DT x YT	YT/DT	3	9
14	DT x YT	YT/DT	2	27
15	DT x YT	DT/YT	0	10
16	DT x YT	DT/YT	0	4
17	YT x DT	YT/DT	0	0
18	YT x DT	YT/DT	2	0
19	YT x DT	DT/YT	2	18
20	YT x DT	DT/YT	1	9

Table 7. Reproductive success of F1 generation *Mecinus* species hybrids. Toadflax genotype, *Mecinus* F1 cross, number of live and dead F2 adult progeny recovered.

*Denotes field collected, molecularly confirmed hybrid. ¹Maternal parent in toadflax hybrid listed first.

²Maternal parent in F1 *Mecinus* cross listed first.

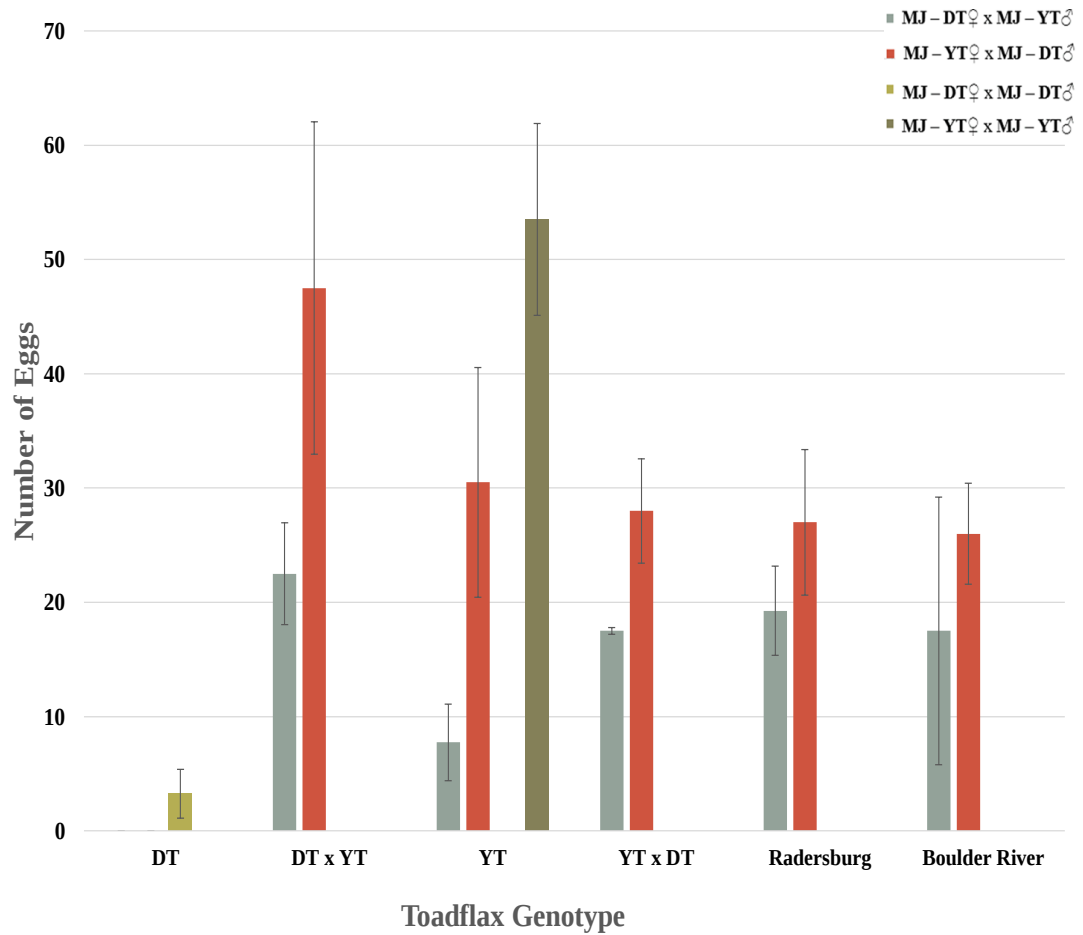


Figure 8. Mean number of eggs laid in 2012 experiment ($\pm$ standard error) for each set of weevil pairings. Pairs include interspecific reciprocal crosses of *M. janthinformis* with *M. janthinus* on six toadflax genotypes. Intraspecific weevil pairs were tested only on pure clones of their respective natural host to generate positive controls. There is a significant effect of toadflax genotype and a significant interaction between insect and toadflax genotype ($p < 0.05$).

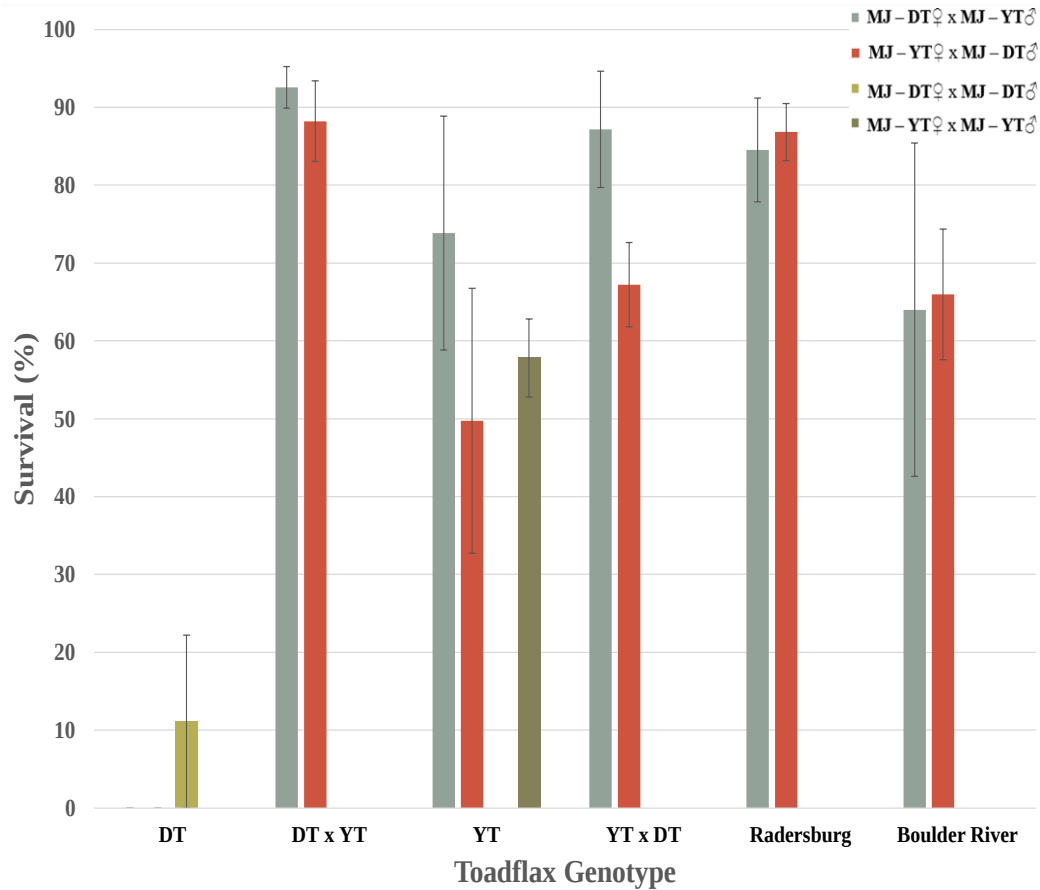


Figure 9. Mean percent survival in 2012 experiment ($\pm$ standard error) for each set of weevil pairings. Pairs include interspecific reciprocal crosses of *M. janthinformis* with *M. janthinus* on six toadflax genotypes. Intraspecific weevil pairs were tested only on pure clones of their respective natural host to generate positive controls. There is a significant effect of toadflax genotype only ($p < 0.05$).

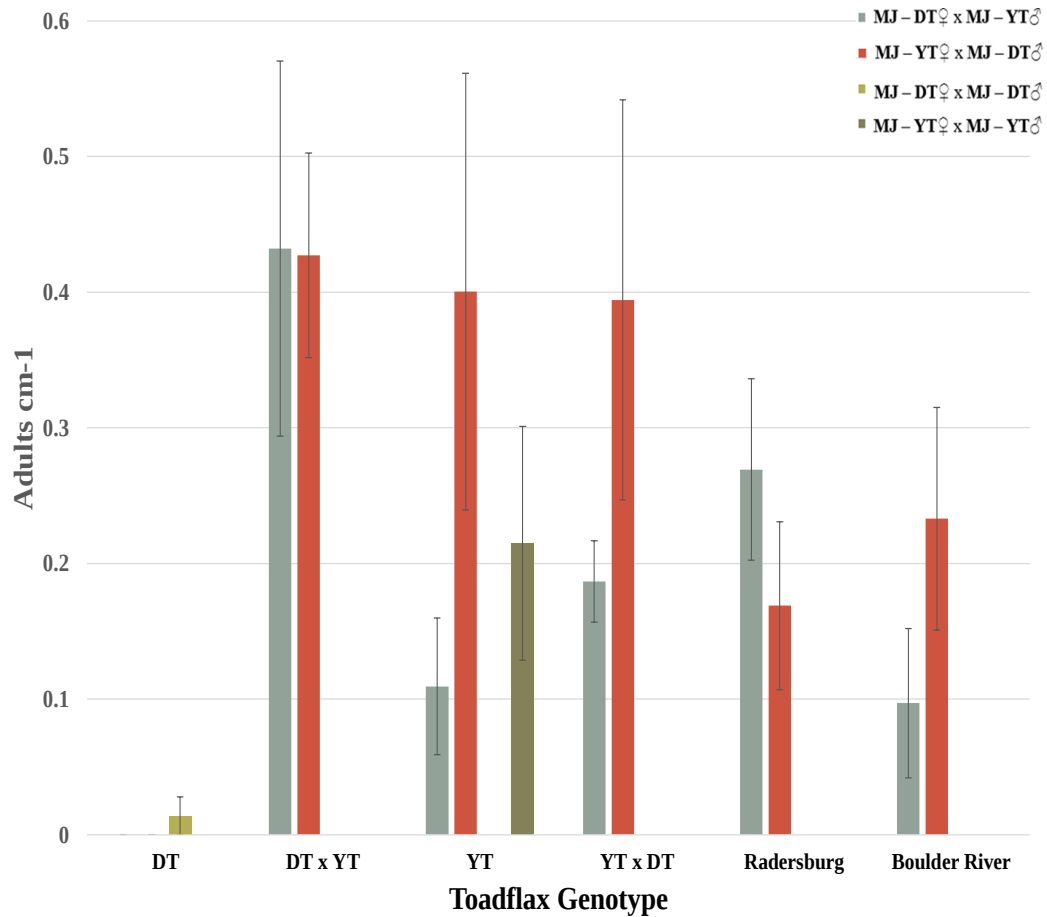


Figure 10. Mean number of adults cm^{-1} of stem ($\pm$ standard error) for each set of weevil pairings. Pairs include interspecific reciprocal crosses of *M. janthinformis* with *M. janthinus* on six toadflax genotypes. Intraspecific weevil pairs were tested only on pure clones of their respective natural host to generate positive controls. There is a significant effect of toadflax genotype and a significant interaction between insect and toadflax genotype ($p < 0.05$).

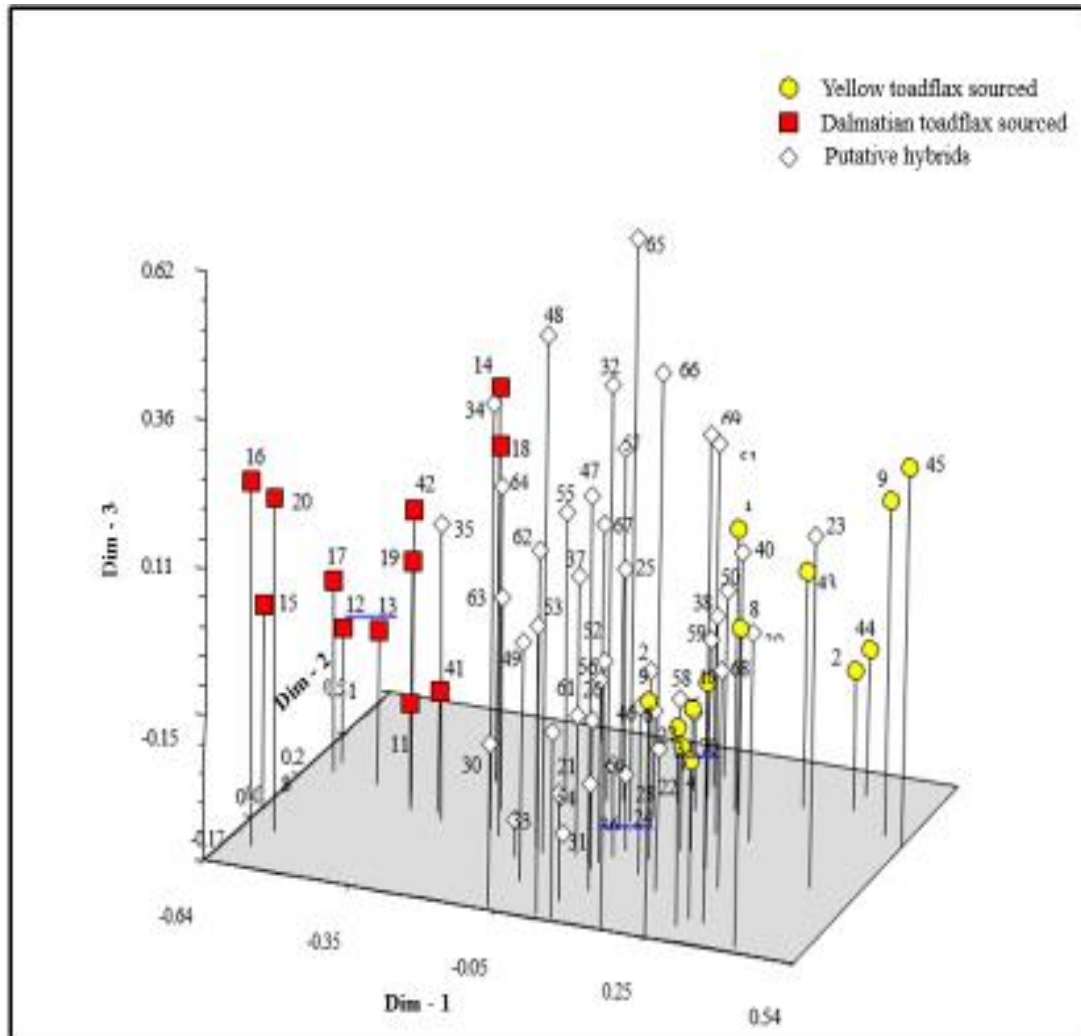


Figure 11. Principal Coordinates Analysis (PCoA) of AFLP data for F1 *Mecinus* reared on different *Linaria vulgaris* (yellow toadflax), *L. dalmatica* (Dalmatian toadflax), and their putative laboratory hybrids. The coordinate dimensions cluster the experimental hybrids between the adult weevils emerged from yellow toadflax and those emerged from Dalmatian toadflax.

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SUMMARY

This thesis sought to address the factors complicating effective and consistent biological control of Dalmatian and yellow toadflax ; namely the naturally occurring hybrid toadflax populations resulting from the cross pollination of yellow and Dalmatian toadflax located at multiple sites in Montana, Colorado, Washington and Idaho and the recent discovery of new data showing that European field populations of this biological control agent actual consist of two cryptic species: *M. janthinus* which feeds on yellow toadflax and *M. janthiniformis* which feeds primarily on Dalmatian toadflax. Both species are present in North America and feeding on their respective host plants. All early releases were considered to be *M. janthinus*, but it is actually the recently described *M. janthiniformis* that has been most successful, with considerable impact on populations of Dalmatian toadflax.

Most releases made in North America on yellow toadflax were actually sourced from populations nearby that had established on Dalmatian toadflax, and were thus *M. janthiniformis*. Given the historical complications in management of the two toadflax species by what are now considered two species of *Mecinus*, we can see how urgent the problems posed by hybrid toadflax are.

The questions addressed here included: Which *Mecinus* species should be selected for deployment? How effective is either weevil species against hybrid toadflax? Are the two *Mecinus* species behaviorally *and* reproductively isolated? How can we improve the overall exotic toadflax biological control program?

Which species should be selected for deployment? The results from Chapter 2 illustrate that the answer to this question depends on each toadflax population independently. Our bioassays support the idea that each *Mecinus* species is host specific because of its respective host plant's volatile profile. Therefore, populations of hybrid toadflax that appear to have a larger genetic contribution from yellow toadflax would be more suitable for *M. janthinus*; conversely a population that appears to favor genetic contributions from Dalmatian toadflax would be more likely to be suitable for *M. janthiniformis*. Future study in this area should seek to address what specific compounds attract each *Mecinus* species. Perhaps synthetic attractants could then be developed to streamline establishing individuals from already existing *Mecinus* populations to new locations of pure or hybrid toadflax.

How effective is either weevil species against hybrid toadflax? The results from Chapter 3 were complicated, but encouraging. The problem that hybridization in toadflax poses to the development of *M. janthinus* and *M. janthiniformis* from yellow and Dalmatian toadflax, respectively, can potentially be overcome. *Mecinus janthiniformis* appears to be a better overall agent in terms of actual development in hybrid populations, but both species showed promise. The most encouraging aspect is that no hybrid that we tested appears to inhibit either species from development and thus, should not limit field establishment. This encourages land managers to make larger releases of both species onto hybrid populations of toadflax. I believe this would allow for more genetic variability to overcome any subtle changes in the hybrid toadflax populations. Another implication is that *M. janthiniformis* would be the preferred agent to use when long term

establishment and impact is the goal on most populations of hybrid toadflax. Further research in this area should include field studies to see if similar results can be obtained on established populations of hybrid toadflax.

*Are the two *Mecinus* species behaviorally and reproductively isolated?*

Establishing interspecific mating pairs on hybrid and pure toadflax genotypes, we saw variable numbers of putative F1 hybrid weevils. Thanks to the efforts in molecular genetics by Dr. John Gaskin, USDA, ARS in Sidney, Montana, it is clear that the F1 hybrid weevil progeny were a genetic combination of the *M. janthinus* population in Ovando and the *M. janthiniformis* population found in Missoula. This illustrates that the speciation between these two is in early stages in terms of genetic separation.

Biologically, they could be considered the same species under forced, no-choice conditions. Unfortunately, we could not readily determine which individual F2 hybrid weevil came from which F1 hybrid weevil parent and thus cannot comment on which toadflax genotype will best support the development of successful populations of hybrid *Mecinus*.

These results suggest that there are no barriers to successfully achieving this, especially when using a hybrid toadflax genotype as the target population. Further laboratory research needs to be conducted to establish larger population of hybrid *Mecinus* and furthermore, field studies would then need to be conducted to assess their potential and establish their host preferences. Using F2 and later generations of hybrid *Mecinus* might also be effective against pure Dalmatian and yellow toadflax. For the overall experiment in Chapter 4, we need to consider the poor suitability of the Dalmatian

toadflax parent that was used. This experiment should be expanded, using a larger variety of plants should be to better support the current results.

How can the overall exotic toadflax biological control program be improved?

The data I have collected and the conclusions drawn from these will greatly improve the overall biological program to control Dalmatian, yellow and hybrid toadflax populations. We now have a better understanding of the mechanism that drives host specificity of both *Mecinus* species. We see that hybrid toadflax can potentially be controlled by these same species and under the right conditions, these stem mining weevil species can produce fertile F1 hybrid offspring to perhaps better counter the threat posed by hybrid toadflax populations. There are now more and better resources available for use by land managers and researchers in the ongoing struggle against invasive toadflax populations.

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