Abundance and Distribution of two Unevenly Managed Root-Mining Insects for the Biological Control of Spotted Knapweed in Idaho

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Abstract

Widespread invasive environmental weeds are managed successfully using classical biological control. Few studies are quantifying success at spatial scales, despite a standard agreement that post-release evaluation should be done consistently. Systematic post-release monitoring is equally crucial in a weed biocontrol project as pre-release host specificity testing. This thesis's second chapter examines Idaho's unequally managed root-feeding biological control agents of *Centaurea stoebe* L. subsp. *micranthos*, an invasive herbaceous perennial plant. This research indicated that *Agapeta zoegana* L. (Lepidoptera: Tortricidae), a root-boring moth of *C. stoebe* native to Eurasia, is as prevalent as *Cyphocleonus achates* (Fåhr.) (Coleoptera: Curculionidae), a root-feeding weevil that is yearly grown, collected, and redistributed at a high cost to federal and state agencies. This review is intended to inform the biological control practitioners that *A. zoegana* is no longer uncommon as projected throughout Idaho, and moth rearing and redistribution could be considered if the rearing is feasible under semi-standardized conditions and economically viable. Chapter 2 also indicated that *A. zoegana* does not compete with *C. achates* in the field, which might cause additive damage to individual *C. stoebe* plants. If this effect increases to have detrimental impacts on population densities, it could enhance biological control. The severity of multiple *A. zoegana* herbivory was found to decrease aboveground biomass relative to no herbivory. Chapter 3 of this thesis examines the feasibility of rearing *A. zoegana* in greenhouse rearing systems. Results serve as a starting point to develop more refined rearing systems based on large tubs. Our data question the continuation of *C. achates* rearing and redistribution initiatives.

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Dedication

To my beloved family!

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Chapter 1: Review of Classical Biological Control of *Centaurea stoebe* in the United States

Nonnative or alien invasive plants (hereafter AIP) are plant species that can quickly and drastically alter biotic communities and ecosystems in the regions outside their native range into which they were introduced (Pyšek and Richardson, 2010; Qian and Ricklefs, 2006). AIPs tend to naturalize easily in new ranges partly because of reduced herbivore pressure compared to their native range (Blumenthal et al., 2009; Keane and Crawley, 2002). It is estimated that only about 1% of AIPs become invasive in an introduced region, but this is sufficient to cause severe harm to agriculture, forestry, native biodiversity, infrastructure, and human health, resulting in profound economic and environmental damage (Pimentel et al., 2005; Vilà et al., 2011; Williams et al., 2010; Williamson and Griffiths, 1996). Estimating the economic, environmental, and human health costs of AIPs can be challenging (Pimentel et al., 2005). While some studies estimate costs to be substantial, with $34 billion annually (Pimentel et al., 2005), there are only a few detailed accounts of the costs of specific AIPs affecting specific commodities or ecosystems (Duncan et al., 2004). Because of the widespread distribution, locations of invasions, and the general magnitude of the problem, AIPs are difficult to manage (Pimentel, 2009; Pyšek et al., 2012; Simberloff, 2011; Van Kleunen et al., 2015). The most common management practice in the United States is using herbicides; however, some AIPs are no longer controlled with herbicides because management is considered cost prohibitive (Culliney, 2005; DiTomaso, 2000; Pimentel, 2009; Sheley et al., 2011). In addition, for any management practice to be successful, AIPs require long-term control strategies (Culliney, 2005; Sheley et al., 2011). Increasingly, chemical and mechanical control means are often unsustainable, expensive, or risky (Clewley et al., 2012; Culliney, 2005; Kelton and Price, 2011; Sheley et al., 2011). Classical biological control of weeds is an alternative self-perpetuating and environmentally safe approach to the management of AIPs, which can be a successful strategy for the long-term control of widespread environmental AIPs (Clewley et al., 2012; Hinz et al., 2020; McFadyen, 1998; Schwarzländer et al., 2018; Seastedt, 2015; Van Driesche et al., 2010). If successful, it can also reduce or entirely replace the use of herbicides and their negative environmental effects (Myers & Cory, 2017), protect biodiversity, and restore ecosystem services impacted by invasive plants (Van Driesche, 2012). Furthermore, it can provide long-term control if effective agents are released, and the programs often outweigh the costs of developing and implementing them by a wide margin (Culliney, 2005; Harris, 1979; Page and Lacey, 2006; Wainger et al., 2018). For instance, Australian weed biological control efforts have shown a return of $23 for every $1 invested (Page and Lacey, 2006), and similar positive results have been observed in South Africa with benefit-cost ratios ranging from 8:1 for *Lantana camara* L. to as high as 709:1 for *Opuntia aurantiaca* Lindl. (Van Wilgen et al., 2004).

Classical biological control of weeds

Classical biological control of weeds (hereafter BCW) is an alternative approach to managing invasive weeds, which involves reuniting the invasive plant with host-specific, co-evolved natural enemies from its native range, known as biological control agents (hereafter BCA) (McFadyen, 1998; Schwarzländer et al., 2018). The enemy release hypothesis is often cited as the ecological framework for BCW (Keane and Crawley, 2002). Enemy release assumes that an alien naturalized plant species may experience drastic population increases and range expansions in their introduced range because of escape from co-evolved specialist natural enemies in their native range (Elton, 1958; Keane and Crawley, 2002). Consequently, reuniting the AIP with its co-evolved natural enemies would reverse the natural enemy escape and reduce the AIPs' competitive advantage in the introduced range (Keane and Crawley, 2002; Mitchell and Power, 2003). One of the concepts to explain the evolution of increased competitive ability (EICA) of AIPs is based on the hypothesis that resources of AIPs are no longer required to defend against herbivory and could be reallocated to growth (Blossey and Notzold, 1995). BCW aims to reduce an AIPs' competitive advantage by introducing their co-evolved key-herbivore natural enemies with the expectation that this action would sufficiently damage AIPs and eliminate their invasiveness (Schwarzländer et al., 2018; Shea et al., 2010). BCW is self-perpetuating and can cover large, remote, and challenging areas, making it a highly cost-effective control strategy with long-lasting ecological and economic benefits (Clewley et al., 2012; Culliney, 2005; Harms et al., 2021; McFadyen, 1998; Van Driesche, 2012). A BCA unable to control the target weed alone can still have a significant impact if effectively integrated with other management techniques (Lym, 2005). However, BCW is considered largely unsuitable for AIPs in agricultural cropping systems because of the continued soil and vegetation cover disruption associated with cropping systems (Westwood et al., 2018).

BCW is not without any risks: In certain instances – though rarely through deliberate introductions – BCAs have fed on or developed on native plant species, typically closely related confamilials or congeners to the targeted AIP (Hinz et al., 2019; McCoy and Frank, 2010; Müller-Schärer et al., 2020; Suckling and Sforza, 2014). The most significant direct effects are caused by two agents, namely *Cactoblastis cactorum* Berg. (Lepidoptera: Pyralidae) on native *Opuntia* spp. and *Rhinocyllus conicus* Froel. (Coleoptera: Curculionidae) on native North American *Cirsium* spp. (Havens et al., 2012; Jezorek et al., 2010; Louda et al., 2005; Pemberton and Liu, 2007; Zimmermann et al., 2000). Multiple BCAs may also compete with each other, compromising and hindering their ability to control the weed (Swope and Satterthwaite, 2012). BCAs may become food sources for predators or browsing ungulates who feed on weed tissues inhabiting juvenile BCA life stages, reducing their effectiveness in both cases (Ortega et al., 2004; Templeton, 2011). Furthermore, moderate levels of herbivory can stimulate over-compensatory growth or plant tolerance and may benefit AIPs rather than harming them (Callaway et al., 1999; Müller-Schärer et al., 2004). Additional potential downsides of BCW are the initial high costs to develop BCAs, especially if these fail to effectively control an AIP (Gassmann and Louda, 2001; Havens et al., 2019, 2012). In theory, introductions of nonnative BCAs could produce invasive species if a BCA causes nontarget effects (Gassmann and Louda, 2001; Havens et al., 2012; Louda et al., 2005, 2003; Louda and O'brien, 2002), although that risk is now considered minimal (Hinz et al., 2020, 2019; Suckling and Sforza, 2014). Finally, successfully controlling one AIP does not necessarily result in native vegetation recovery (Symstad, 2004). More likely, other AIPs already present in the habitat may use the additional resources; for example, in Idaho, the successful BCW program against *Hypericum perforatum* L. led *Centaurea solstitialis* L. and *Centaurea stoebe* L. to become more invasive (Campbell and McCaffrey, 1991). Overall, the potential benefits largely outweigh the drawbacks or risks associated with BCW, and if appropriately implemented, BCW is by far the most environmentally benign management strategy for AIPs (Culliney, 2005; Hinz et al., 2019; McFadyen, 1998).

Assessments of biological weed control programs

Over the past few decades, significant advancements have been made in target weed identification procedures, biological control candidate evaluation, and risk assessment of nontarget impacts (Ollivier et al., 2020; Paynter et al., 2020; Schaffner et al., 2018). As a result, less than 1% of intentional releases worldwide may adversely impact nontarget species populations, which indicates that procedures for determining agent host range pre-release have improved (Hinz et al., 2020, 2019).

To further improve BCW as a discipline, quantitative evaluations of each introduced BCA's post-release contribution to the control of a weed and its potential adverse nontarget effects are essential (Catton et al., 2016; Myers and Cory, 2017). Maintaining a perspective on the positive and negative impacts of BCAs is crucial to improve pre-release assessments of future biological control candidates and avoid needless additional species introductions (Catton et al., 2016; Myers and Cory, 2017). Schaffner et al. (2020) emphasize the importance of quantifying the economic benefits of BCAs after release and stress the necessity of systematic post-release monitoring to understand factors that influence weed population growth at the landscape scale.

The Idaho State Department of Agriculture, University of Idaho, Nez Perce Biocontrol Center, and federal land management agencies collaborated to develop the Standard Impact Monitoring Protocol (SIMP), a citizen-science monitoring program in Idaho, to help land managers track the effectiveness of BCAs against AIP (SIMP, 2023). SIMP has been a valuable tool for evaluating the landscape-level impacts of BCAs on AIP (Weed et al., 2018; Weed and Schwarzländer, 2014). However, most of the post-release evaluation studies tend to center on subjective assessments of the agent establishment, with a focus on individual plant-level evaluations (Dhileepan and Briese, 2003; Hinz et al., 2020; McClay, 1995; Schaffner et al., 2020; Thomas and Reid, 2007). Few studies have investigated the population-level impact of released agents on invasive weeds. For instance, Catton et al.

(2016) conducted a quantitative evaluation of *Cynoglossum officinale* L. (Boraginaceae). Schaffner et al. (2020) examined the effects on *Tamarix* spp. (Tamaricaceae), *Azolla filiculoides* L. (Salviniaceae), and *Ambrosia artemisiifolia* L. (Asteraceae). Seastedt and Knochel (2021) focused on *Centaurea stoebe* L. spp. *micranthos* (Gugler) Hayek (Asteraceae), while Weed et al. (2018) and Weed and Schwarzländer (2014) explored the impacts on *Linaria dalmatica* L. spp. *dalmatica* Mill. (Plantaginaceae). In addition to these individual studies, some meta-analyses and global reviews have assessed the overall population-level effects of released agents on invasive weeds. Clewley et al. (2012); Hinz et al. (2020, 2019); Schwarzländer et al. (2018) and Suckling and Sforza (2014) are notable examples of such comprehensive analysis. The lack of population-level quantitative assessments of AIPs is likely because they take several years or need to be conducted on larger spatial scales, both requiring substantial resources (Hinz et al., 2020, 2019; Morin et al., 2009; Schaffner et al., 2020; Schwarzländer et al., 2018). Comprehensive post-release monitoring programs can be challenging, especially given funding constraints (McCulloch et al., 2022; Schaffner et al., 2020).

A worldwide review concluded that 65.7% of targeted weeds were controlled to some extent, indicating that biological control agents have effectively controlled at least some invasive plant species, which assumingly resulted in environmental and economic benefits (Schwarzländer et al., 2018). Furthermore, 53.5% of the BCAs caused some level of control on the target weed (Hinz et al., 2020). Most studies found that BCAs considerably decreased target weeds’ flower and seed production, with a relatively large effect size (e.g., a 35% reduction in flower output and a 42% reduction in seed production) (Clewley et al., 2012).

***Centaurea stoebe*, a major weed in western North American grasslands**

Spotted knapweed (*Centaurea stoebe* L. subsp. *micranthos* (Gugler) Hayek (Asteraceae), syn. *Centaurea maculosa*) is indigenous to Europe (Ochsmann, 2000; Sheley et al., 1998). *C. stoebe* comprises two subspecies: *C. stoebe* spp. *micranthos,* which is a polycarpic tetraploid perennial, and *C. stoebe* spp. *stoebe,* which is a monocarpic diploid biennial (Broz et al., 2009; Hahn and Müller-Schärer, 2013; Mráz et al., 2011; Treier et al., 2009). While the plant has both tetraploid (2n = 36) and diploid (2n = 18) cytotypes in its native European range, only the tetraploid cytotype has been identified in North America following its invasion (Mráz et al., 2011; Treier et al., 2009).

C. stoebe spp. *micranthos* is a short-lived herbaceous perennial that forms rosettes with deep taproots averaging over 30 cm long (Sheley et al., 1998; Story et al., 2000; Watson and Renney, 1974). It has upright, pubescent, branched stems that can reach up to 100 cm in height, producing 1-6 stems per plant (Watson and Renney, 1974). Flower heads (capitula) are about 1.2 cm in diameter and occur singly or in clusters of 2-3 on branch ends (Watson and Renney, 1974). Each capitulum contains 25-35 pink and purple flowers, yielding about 26 seeds per flower head, and each plant typically has around 16 capitula (Sheley et al., 1998; Watson and Renney, 1974). Seeds are dark brown and 2-3 mm long (Sheley et al., 1998). Depending on soil moisture, seeds germinate in fall or early spring, and rosette growth begins in April (Watson and Renney, 1974). A single plant has the potential to yield 900 seeds, and areas infested with *C. stoebe* can have a soil seed bank of up to 40,000 seeds per square meter, which can remain viable for over eight years (Davis et al., 1993; Jacobs and Sheley, 1998; Schirman, 1981; Seastedt et al., 2007; Sheley et al., 1998).

During the first year, most plants form rosettes, although some plants may already produce flowers during this time (Schirman, 1981). Most plants will flower during their second and consecutive years and can live up to nine years (Sheley et al., 1998). *C. stoebe* bolts in early May and flowers during the summer, with black margin bract tips and obvious longitudinal veins (Sheley et al., 1998; Zouhar, 2001). The polycarpic life cycle of tetraploids gives them an advantage over diploids, leading to higher reproduction potential and promoting their invasion success in North America (Hahn and Müller-Schärer, 2013; Henery et al., 2010; Mráz et al., 2011; Treier et al., 2009).

Introduction and spread of *Centaurea stoebe* in North America

Centaurea stoebe was first recorded in North America in Victoria, British Columbia, in 1893 (Groh, 1944). The plant was likely introduced as a contaminant of alfalfa seed shipments from Europe and has since spread throughout the United States and Canada (Lejeune and Seastedt, 2001; Sheley et al., 1998; Watson and Renney, 1974). From coastal introductions, *C. stoebe* spread throughout the northern North American continent (Broennimann et al., 2014). *C. stoebe* is a highly invasive and economically damaging nonnative plant species in North America (Alper, 2004). Over the past 40 years, it has been the focus of extensive study and control efforts due to its aggressive invasiveness, resulting in the domination of rangelands and grasslands (Carson et al., 2014). *C. stoebe* is known for thriving in disturbed areas like riparian channels, roadsides, or resource-rich locations, but it can also persist in intact rangelands with low nutrient availability (Knochel and Seastedt, 2010). It mostly prefers mesic communities in semi-arid regions of the western U.S., and soil moisture levels highly influence its reproductive output (Corn et al., 2007; Pearson et al., 2017; Watson and Renney, 1974).

Seeds typically fall off plants, with mature open capitula being knocked off by animals or wind, and the bracts of the seed heads can easily adhere to animals or vehicles, enabling long-distance dispersal (Zouhar, 2001). *C. stoebe* covers over three million acres of rangeland and woodland ecosystems (Story and Piper, 2001a). *C. stoebe* is found in eight Canadian provinces/territories and 46 U.S. states and is categorized as noxious in 16 U.S. states (EDDMapS, 2023).

Impacts of *Centaurea stoebe* invasions

The invasion by *C. stoebe* results in millions of dollars annually to cattle and sheep ranchers due to the invasion of vulnerable rangeland (Bucher, 1984; DiTomaso, 2000). Hirsch and Leitch (1996) reported that along with two other minor knapweed species in Montana, *C. stoebe* caused $14 million in direct negative impacts and $28 million in indirect effects to the state annually. Harris and Cranston (1979) found that infestation of *C. stoebe* in Canada reduced forage production by more than 88% in over 74,000 acres of land. The presence of *C. stoebe* in an area leads to a decrease in available forage plants for both livestock and wildlife by 60% to 90%, causes an increase in surface water runoff, soil erosion, and stream sediments, and reduces water infiltration (DiTomaso, 2000; Jacobs and Sheley, 1998; Lacey et al., 1989; Watson and Renney, 1974). This can occur through increased phosphorous levels in its rhizospheres and alterations in the soil carbon and nitrogen pools (Hook et al., 2004; Thorpe et al., 2006; Zabinski et al., 2002). In addition to modifying soil characteristics, *C. stoebe* can also lead to a reduction in the richness and diversity of native plant species (Ortega and Pearson, 2005; Tyser and Key, 1988), which results in reduced food resources available for native herbivores (Bateman and Ostoja, 2012; Bultman and DeWitt, 2008; Litt and Steidl, 2010).

The invasion of *C. stoebe* affects not only aboveground processes but also impacts belowground systems, such as disturbing soil microbial and nematode communities and altering the structure of invertebrate arthropod food webs (Foster et al., 2021, 2020; García-De la Cruz et al., 2019). The presence of *C. stoebe* has an adverse effect on arbuscular mycorrhizae and decreases soil glomalin (Lutgen and Rillig, 2004), which is a glycoprotein that plays a significant role in sequestering soil carbon and nitrogen (Treseder and Turner, 2007). These disturbances in the belowground systems have been identified as contributing to the increased spread of *C. stoebe* (Akin-Fajiye and Gurevitch, 2020). The invasion of *C. stoebe* impacts belowground nutrient levels by decreasing soil nitrogen and carbon (Singh et al., 2022). Near monospecific *C. stoebe* stands have increased bare ground due to reduced soil organic matter and carbon compared to grasslands, and its ability to increase soil phosphorus levels may facilitate its invasion, resulting in larger patches (Fraser and Carlyle, 2011). In addition, *C. stoebe* has been reported to release catechin, a phytotoxic chemical into the soil that suppresses the growth of surrounding plants (Bais et al., 2003; Pollock et al., 2011; Reinhart and Rinella, 2011; Ridenour and Callaway, 2001; Stermitz et al., 2009). However, the allelopathic effects of *C. stoebe* are still debated (Blair et al., 2006, 2005; Chobot et al., 2009; Duke et al., 2009).

Management of *Centaurea stoebe*

Centaurea stoebe populations can be managed using cultural control (enhancing plant competition or preventing weed introduction), mechanical control (tillage, mowing, hand-pulling), herbicides, prescribed fire, ungulate grazing, and biocontrol (Ainsworth, 2003; DiTomaso, 2000; Ditomaso et al., 2006; Knochel and Seastedt, 2009). The densities of *C. stoebe* are adversely affected by competition from native plant species (Pokorny et al., 2005;

Rinella et al., 2007). Mechanical methods like mowing and hand-pulling can effectively prevent seed germination and deplete plant carbohydrate reserves (DiTomaso, 2000; MacDonald et al., 2013; Watson and Renney, 1974). However, mowing can harm soil structure, leading to reduced stability of soil aggregates and water retention ability (Lutgen and Rillig, 2004), and hand pulling is laborious and time-consuming, requiring persistent efforts for several years before seed dispersal (MacDonald et al., 2013).

At present, herbicides are widely used to manage *C. stoebe*, and the active herbicide ingredients that are currently registered for this purpose are 2,4-D, dicamba, picloram, clopyralid, aminopyralid, and aminocyclopyrachlor, all of which are classified as synthetic auxin herbicides (Mangin and Hall, 2016; Senseman, 2007). Recently, a *C. stoebe* population was found to be resistant to the commonly used herbicide clopyralid (3,6-dichloro-2-pyridine carboxylic acid), posing the question whether herbicides can be a sustainable long-term management strategy for the control of this AIP (Mangin and Hall, 2016). Another herbicide, picloram (4-Amino-3,5,6-trichloro-2-pyridine carboxylic acid), targeting *C. stoebe* can reduce native forb richness and diversity while promoting exotic grasses like *Bromus tectorum* L., which provides poor forage compared to native species it displaces and also is difficult to control (Crone et al., 2009; Ortega and Pearson, 2011, 2010; Rinella et al., 2009; Sheley et al., 2006).

Another method to control *C. stoebe* is prescribed burning, but its applications depend on site characteristics and environmental conditions, which demand constant and repeated effort over an extended period making it expensive (Ditomaso et al., 2006; Knochel and Seastedt, 2009). In addition, prescribed burning may favor reinvasion of *C. stoebe* (MacDonald et al., 2013) or invasions by other AIPs on the highly disturbed and bare land following burns

(Brooks et al., 2004; Seastedt et al., 2007). Native mammals and invertebrates may also act as additional unidentified control agents for *C. stoebe* (Milbrath and Biazzo, 2020). Sheep were found to control young and mature *C. stoebe* plants, suggesting grazing could limit its spread (Sheley et al., 1998). Cattle, on the other hand, prefer grasses over *C. stoebe* due to the plant's coarse nature and potential for irritation from rough stems and spines on floral bracts (Lacey et al., 1995; Sheley et al., 1998).

Classical biological control of *Centaurea stoebe*

In the United States, it is required that the effectiveness of a BCA candidate is assessed experimentally pre-release to minimize the risk of introducing agents that do not impair the targeted weed (APHIS, 2021; Hinz et al., 2019). Generally, BCW programs aim to introduce only as many agents as necessary to control the target weed successfully (Denoth et al., 2002). There are two competing hypotheses on biological control agent impact: The silver bullet hypothesis assumes that one biological control agent will reach outbreak densities post-release and successfully control a weed through overwhelming herbivore densities (Myers, 2004, 1985). The hypothesis also assumes that it is challenging to predict pre-release which BCA candidate may reach outbreak densities (Denoth et al., 2002; Myers, 1985; Seastedt et al., 2007; Stephens et al., 2013). Alternatively, it has been proposed that a combination of BCAs, typically occupying different niches, leads to cumulative stress, which best controls a target weed (Harris, 1981, 1973; Knochel and Seastedt, 2009; McEvoy and Coombs, 1999; Müller-Schärer and Schroeder, 1993; Seastedt et al., 2007; Seastedt and Knochel, 2021; Story et al., 2008). Biological control can have population-level effects on *C. stoebe* by directly reducing seed production and plant survival and indirectly decreasing its ability to thrive under favorable climatic conditions (Maines et al., 2013). This species' most effective management strategy is believed to combine biological controls with plant competition (Seastedt and Pyšek, 2011; Seastedt and Knochel, 2021; Sheley et al., 1998). For *C. stoebe,* a total of 13 BCA species have been introduced into the United States between 1973 and 1993 (Table 1.1). The first insect introduced in the United States in 1973 was the European seed head fly, *Urophora affinis* Frfld. The last most recently introduced biocontrol agent was also a seed head weevil, *Larinus obtusus* Gyll. in 1993 (Müller-Schärer and Schroeder, 1993). In total, eight seed head-feeding insects and five root-mining insects were approved and released during this period (Müller-Schärer and Schroeder, 1993) (Table 1.1). The seed head weevil *Larinus minutus* Gyll. and *Larinus obtusus* Gyll. are two of the most widely released BCAs of *C. stoebe* and are able to reduce their host plant fitness and plant densities (Myers et al., 2009; Stephens and Myers, 2013; Story et al., 2008; Wooley et al., 2011). Seed head-attacking BCAs have successfully reduced seed production by 50% to 95% (Story et al., 2008). However, the impact of reduced seed output on weed density depends on whether seedling recruitment is constrained by seed availability (Müller-Schärer and Schroeder, 1993). Meanwhile, the populations of *C. stoebe* are regulated by density-dependent mortality of seedlings, which involves insect communities on both the seed heads and roots, making them crucial for effective management (Harris, 2011; Knochel and Seastedt, 2009, 2010; Müller-Schärer and Schroeder, 1993; Seastedt et al., 2007; Seastedt and Knochel, 2021). Studies suggest *Larinus* spp. may not be essential for *C. stoebe* decline if the root weevil *Cyphocleonus achates* Fåhr is abundant (Story et al., 2006). Additionally, *Urophora* spp. is ineffective at reducing *Centaurea* to acceptable levels (Myers and Bazely, 2003). Furthermore, competitive interactions between seed heads attacking BCAs *Larinus* and *Urophora* species reduce overall seed head herbivory efficacy (LeJeune et al., 2005;

Seastedt et al., 2007; Smith and Mayer, 2005). *L. minutus* presence can also reduce the survival of *Terellia virens* Loew (Groppe and Marquardt, 1989). In addition, only one larva of *Metzneria paucipunctella* Zell. can survive per seed head due to strong intraspecific competition, which also attacks and destroys the larva of *Urophora* spp. and other insects found in seed heads (Englert, 1971; Story et al., 1991b).

A total of five root-mining BCAs were introduced between 1980 and 1988. Among these are the root moth *Agapeta zoegana* L., root weevil *Cyphocleonus achates* Fahr., and the root beetle *Sphenoptera jugoslavica* Obenb. (Müller-Schärer and Schroeder, 1993). There are several factors why root mining BCAs were considered for controlling *C. stoebe.* The short-lived perennial plant can produce large roots (Sheley et al., 1998; Watson and Renney, 1974). Damaging those tissues was considered superior to only reducing the reproductive output via seed head feeders (Knochel and Seastedt, 2010; Seastedt and Knochel, 2021; Smith, 2004; Story et al., 2006; Sturdevant et al., 2006). The perennial life history can also benefit the establishment of dense monospecific populations despite seed head biological control reducing the seed production (Story et al., 2008). For *C. stoebe*, seed head herbivory, even if it is reduced to 90% of reproductive output, it would be insufficient to affect the population biology of the plant because it has been shown to be seed unlimited (Müller et al., 1989; Myers and Risley, 2000; Story et al., 2008). Therefore, to be effective, seed reduction needs to be combined with BCAs weakening the plant growth or causing plant mortality outright, such as root mining biocontrol agents (Blossey and Hunt-Joshi, 2003; Knochel et al., 2010; Knochel and Seastedt, 2010; Maines et al., 2013; Seastedt et al., 2007; Seastedt and Knochel, 2021; Story et al., 2006, 2008). Root mining BCAs also may be advantageous because they are better protected from predators and adverse environmental conditions aboveground (Egli and Olckers, 2017; Feeny, 1976; Müller et al., 1989; Simelane, 2010), providing them with greater resilience to environmental fluctuations (Johnson et al., 2015). Root mining BCA candidates are more difficult to develop and study because of their hidden life history and require greater effort during pre-release host specificity assessments (Blossey and Hunt-Joshi, 2003; Knochel and Seastedt, 2009). For those reasons, root-mining herbivores may in some instances be disregarded as BCA candidate species.

The root-mining moth, *Pelochrista medullana* Staudinger (Lepidoptera: Tortricidae), was released in 1984 in Montana, but the establishment was sluggish, and the abundance of the moth is considered limited in the United States (Winston et al., 2014). *P. medullana* was first released in Canada in 1986 and is moderately abundant (Winston et al., 2014). Another root-mining moth *Pterolonche inspersa* Stgr. (Lepidoptera: Pterolonchidae) was first released in the U.S. in 1986 but has not been detected since 2012 except in one region in Montana, perhaps because of the successful control of *Centaurea diffusa* Lam. by *Larinus* spp., or the seed head weevils prevented the establishment of the moth (Herron-Sweet et al., 2015; Winston et al., 2014). In Canada, *P. inspersa* was first released in 1986, and the moth is moderately abundant (Winston et al., 2014).

Three root-mining insects released for the biological control of *C. stoebe* are well established in the United States, i.e., *Sphenoptera jugoslavica* Obenberger (Coleoptera: Buprestidae), *Cyphocleonus achates* Fåhraeus (Coleoptera: Curculionidae) and *Agapeta zoegana* L. (Lepidoptera: Tortricidae) and are discussed in more detail below.

Sphenoptera jugoslavica

The root-feeding beetle *Sphenoptera jugoslavica* Obenberger (Coleoptera: Buprestidae) is native to Romania, Bulgaria, northern Greece, and northeastern Turkey (Harris and Shorthouse, 1996). In 1980, the buprestid collected in Greece was first released in the United States, primarily to control *Centaurea diffusa* (Winston et al., 2014). After its introduction in 1987, *S. jugoslavica* has also been found in the roots of *C. stoebe* since the buprestid has spread naturally and with anthropogenic assistance through deliberate redistributions (Winston et al., 2014). Adult *S. jugoslavica* emerges from *Centaurea* roots between July and August, and females lay eggs in the root crowns of rosette plants (Myers et al., 2009). The oviposition period lasts from the end of July until mid-September (Harris and Shorthouse, 1996). Larvae develop in the roots in fall and spring, pupation occurs in the roots, and the new generation beetles emerge early the following summer (Harris, 2011; Myers et al., 2009). Although adults feed on the foliage of *Centaurea* spp., the larval mining in the roots causes more damage to plants (Powell and Myers, 1988; Zwölfer, 1976). Depleted root stores often result in surviving plants being stunted and producing fewer flowers (Powell and Myers, 1988). While *C. stoebe* is susceptible to attack, the biocontrol agent's abundance and effect are not as large compared to its preferred host plant *C. diffusa* (Julien and Griffiths, 1998). Callus, a vascular bypass, and swelling around the larva constitute compensatory or tolerating plant responses (Harris, 2011; Müller-Schärer et al., 2004). Even though multiple *S. jugoslavica* eggs are laid on plants, typically only one larva develops, or if two should develop, one is typically stunted (Harris and Shorthouse, 1996; Winston et al., 2016).

In British Columbia, where *C. stoebe* rosette growth was halted by drought in summer (July and August), plants were damaged by *S. jugoslavica* herbivory (Harris and Shorthouse, 1996). Attacked rosette plants turned into semelparous perennials that didn't produce seeds for up to five or more years, and the reproductive output was 50% compared to plants only attacked by seed head BCAs (Harris, 2011). There is no evidence of competitive interactions between *S. jugoslavica* and *A. zoegana* or *C. achates* (Bourchier et al., 2001; Stinson et al., 1994). It was hinted that the annual relative densities of *C. achates* and *S. jugoslavica* might be negatively correlated due to differential success in different climates and rosette selection in various habitats (Seastedt et al., 2007). Nevertheless, these two species mutually reinforce each other, resulting in a combined impact surpassing either species' effect in isolation (Seastedt et al., 2007).

Agapeta zoegana

Agapeta zoegana L. (Lepidoptera: Tortricidae) is a root-feeding moth that was imported from Austria and Hungary and released in Montana in 1984 against *C. stoebe* (Müller-Schärer & Schroeder, 1993; Story et al., 1991a). In North America, *A. zoegana* has one generation per year, while in its native European range, it can be bivoltine or even have three generations per year (Müller et al., 1988). Adults are about 1 cm long and bright yellow (Fitzpatrick, 1989). Adult moths emerge from June through August, mate, and lay their eggs singly or in clusters on the leaves and stems of *C. stoebe* (Corn et al., 2009; Story et al., 1991a). Females can lay between 150 and 400 eggs in their lifetime (Fitzpatrick, 1989). Within two weeks, larvae hatch and begin mining in the cortical tissues of the taproot of *C. stoebe* (Müller et al., 1988; Story et al., 2000). The larvae of *A. zoegana* feed behind a silken web on root cortex tissues, overwinter and pupate in roots, and emerge as adults during the following late spring and summer (Müller et al., 1989; Story et al., 1991a). Larvae develop through six instars and mostly overwinter as third or fourth instars in North America (Corn et al., 2009) while as final instars in Eurasia (Müller et al., 1988). Early instar larvae feed on the epidermal tissues of the root crown, while older larvae feed on the cortex and endodermis of the root (Story et al., 1991a). The moth does not avoid plants that have been attacked, as multiple larvae can be present in the same root (Story et al., 2000). Moths are short-lived, with a longevity of 10 to 14 days, and the sex ratio of adults is nearly equal throughout the season (Story et al., 1991a). Adults mate on the day of their emergence, and females typically begin to lay eggs during the second night (Müller et al., 1988; Story et al., 1991a). *A. zoegana* lays eggs on rosettes of *C. stoebe* of all ploidy levels and attacks all phenological developmental stages (Collins and Müller-Schärer, 2012). *A. zoegana* shows a strong preference for mature, larger *C. stoebe* with correspondingly large roots in North America, whereas it typically targets rosettes in Europe (Müller et al., 1988; Story et al., 2000). Different attack preferences between European diploid and North American tetraploid species may be due to differences in plant biology (Story et al., 2000). In addition, larger plants, especially those that have bolted, tend to experience greater damage from *A. zoegana* infestation (Smith and Story, 2003). *A. zoegana* larvae are capable of belowground migration from one plant to another and can locate a new host plant within a short distance of 10 cm if the root of their host plant is consumed (Müller et al., 1988). *A. zoegana* is primarily found in semi-natural, undisturbed areas such as dry grasslands, steppic biotopes, and south-facing slopes, as well as ruderal habitats such as gravel quarries in its native range. However, it is rare or not present in areas with highly variable populations of *C. stoebe* (Müller et al., 1988).

Root-mining by the *A. zoegana* larva causes considerable root damage, with heavily attacked roots often truncated and necrotic below the feeding area, resulting in abnormal proliferation of small lateral roots (Story et al., 2000). Several laboratory and greenhouse studies have shown that *A. zoegana* is capable of reducing the survival of *C. stoebe* rosettes (Müller, 1989b; Müller et al., 1988; Muller-Scharer, 1991). In dense populations with mostly small rosettes, a single larva can destroy multiple rosettes during development (Müller et al., 1988). *A. zoegana* was found to decrease the height, aboveground biomass, number of stems, and number of capitula of *C. stoebe* during the post-release assessment at the field release site of the moth (Story et al., 2000). *A. zoegana* might have caused stress to mature knapweed plants, allowing for greater survival of seedlings by preventing older plants from monopolizing moisture and soil nutrients (Story et al., 2000). The results were inconclusive, but there were some indications that *A. zoegana* may be decreasing the density of bolted plants (Story et al., 2000).

The first successful establishment of *A. zoegana* was recorded in Montana in 1990, with 16% of roots infested and an average of 1.2 larvae per infested root obtained through augmentative releases and field cages (Story et al., 1991a). This moth rapidly expands its range across the United States, although local populations are declining because of insect migration (Winston et al., 2016, 2014). *A. zoegana* establishment has been observed in several studies. During a survey conducted in its native range of eastern Austria and northwestern Hungary, the larval establishment of *A. zoegana* was calculated as 23.6%, whereas in central Hungary and France, the percentage of roots attacked was 8% (Müller et al., 1988). The post-release assessment of *A. zoegana* in the field found a mean of 0.37 larvae per root at the release site and 0.07 at the control site, where the percentage establishment was 22% and 31.8% at the release site and 5.9% and 22.3% at the control site for two census dates (Story et al., 2000). In manipulative experimental plots, where groups of *C. stoebe* plants were exposed to varying numbers of adult *C. achates*, the average number of *A.*

zoegana larvae per root ranged from 1.6 to 4.2 (Corn et al., 2006). Furthermore, during the sampling of roots from five *C. stoebe*-dominated sites in Montana in late fall and early spring, it was found that *A. zoegana* larvae had established in 31% of the roots (Corn et al., 2009). Additionally, field collection in Montana over two years at 11 sites revealed the presence of 91 and 400 larvae, respectively (Sturdevant and Dewey, 2002). The percentage of roots infested with *A. zoegana* larva was found to be 43% from a survey of 86 sites in Montana, Idaho, and Washington (Clark et al., 2001b). In Montana, Herron-Sweet et al. (2015) reared *C. stoebe* roots from 19 sites, observing a mean of 6.9 *A. zoegana* adults emerge per site when present. The available data for comparing laboratory and field studies are still scarce.

However, studies indicate that *A. zoegana* does not immediately reduce *C. stoebe* fecundity and may trigger compensatory plant growth, although root mining tends to reduce the survival of immature plants (Müller, 1989b; Muller-Scharer, 1991). In addition, the reduction of rosette density could not be confirmed by a study at the field release site of *A. zoegana* (Story et al., 2000). Furthermore, without grass competition, *A. zoegana* herbivory was not found to reduce biomass, fecundity, and plant height (Muller-Scharer, 1991). Another controlled greenhouse study found that the feeding by the moth larvae on the taproot's lower parts did not reduce root or stem biomass because new roots grew above the feeding location to compensate for the loss of water and nutrients (Steinger and Müller-Schärer, 1992). After two years in enclosed garden plots where Idaho fescue (*Festuca idahoensis* Elmer) was grown as a competitor grass, *A. zoegana* infestation had no impact on the aboveground biomass of *C. stoebe* (Callaway et al., 1999). Additional studies suggest that the presence of *A. zoegana* may enhance *C. stoebe*'s allelopathic effects due to the increased release of catechin from the roots when it is attacked by the moth (Thelen et al., 2005). It was documented that *A. zoegana* herbivory on *C. stoebe* causes compensatory growth and has adverse effects on native grasses (Callaway et al., 1999; Ridenour and Callaway, 2003).

Additionally, Newingham et al. (2007) suggested that *A. zoegana* has competitive domination over several native grass species. Furthermore, Sturdevant et al. (2006) conducted a multi-site study and found no evidence linking *A. zoegana* to a decrease in *C. stoebe* populations. It appears that damage caused by *A. zoegana* to the root cortex is less severe than that caused by the development of *C. achates* in the central vascular tissue (Steinger and Müller-Schärer, 1992). Nevertheless, Story et al. (2000) argue that *A. zoegana*'s preference for larger, mature knapweed plants is likely due to their oviposition and larval attack behavior rather than a response from the plant to the moth infestation. Studies suggest that *A. zoegana* could significantly decrease knapweed vigor with higher herbivory densities and following multiple years of consistent attack (Corn et al., 2007; Ridenour and Callaway, 2003; Story et al., 2000).

Cyphocleonus achates

Cyphocleonus achates Fåhraeus (Coleoptera: Curculionidae) is a root-mining weevil of Eurasian origin that was first released in the United States in 1988 in Montana for the biological control of *C. stoebe* (Müller-Schärer and Schroeder, 1993; Stinson et al., 1994). *C. achates* exhibits a univoltine life cycle in North America, while in Europe, it displays a multivoltine nature annually (Stinson et al., 1994; Wikeem et al., 1999). The adult *C. achates* weevils are brown-gray mottled in color and are relatively large, measuring 13-15 mm in length (Stinson et al., 1994; Winston et al., 2016). Adults appear in summer between July and October (Corn et al., 2009), typically three to four weeks following *A. zoegana* (Mosley et al., 2016). During their adult life cycle of 30-60 days, the weevils feed on *C. stoebe* leaves and lay one to three eggs per day on the root or root crown (Corn et al., 2009; Stinson et al., 1994). The eggs hatch after ten days, and the larvae develop through four instars while feeding on the central vascular tissue of roots during the subsequent fall and spring (Stinson et al., 1994). *C. achates* larvae induce a gall-like structure in the taproot of *C. stoebe*, where they feed, overwinter, and pupate semi-sessile (Stinson et al., 1994). Most larvae overwinter as first instars (Corn et al., 2009; Stinson et al., 1994). Weevils grow particularly fast in *C. stoebe* roots in sandy soil (Story et al., 1996). Warm soil temperatures are required for *C. achates* larval development; therefore, the weevil thrives on sunny, south-facing slopes with light soils (Lang, 1997).

Cyphocleonus achates reduces plant biomass and kills plants outright by damaging the xylemic tissues above the feeding site, preventing water transportation (Corn et al., 2007; Steinger and Müller-Schärer, 1992). *C. achates* can also affect the vertical distribution of roots, impacting water relations and competition, as is evident from the increase in fine root production above damaged areas (Seastedt and Knochel, 2021). Earlier studies have indicated that *C. achates* can impede the growth of *C. stoebe* plants (Stinson et al., 1994) or diminish seed production (Müller and Schroeder, 1989; Müller and Steinger, 1990; Stinson et al., 1994).Various post-release assessments focusing on specific field release sites or common garden experiments have shown that *C. achates* reduces the biomass of *C. stoebe* (Corn et al., 2007, 2006; Jacobs et al., 2006; Knochel and Seastedt, 2010). *C. achates* also resulted in a decline in flower and/or seed production in assessments at specific field release sites, several individual study sites, or greenhouse experiments (Jacobs et al., 2006; Knochel et al., 2010; Knochel and Seastedt, 2010; Seastedt et al., 2007). Additionally, rosettes have been found to die prematurely because of *C. achates* exposure (Knochel et al., 2010). Multiple long-term studies conducted over periods of 10 to 20 years at field release sites, including short-term common garden experiments, have reported a decline in the population of *C. stoebe* following the release of *C. achates* (Corn et al., 2007, 2006; Gayton and Miller, 2012; Jacobs et al., 2006; Seastedt and Knochel, 2021; Story et al., 2006). The adverse impact of *C. achates* was also observed for related species *Centaurea diffusa* L. (Asteraceae) on its density, size, and seed-head diameter (Van Hezewijk and Bourchier, 2012).

The first report for the establishment of *C. achates* was recorded in Montana in 1993, obtained through augmentative releases and field cages (Story et al., 1997). It was established quickly and has increased to outbreak populations in some areas in Montana (Story et al., 2006). Efforts to rear *C. achates* through mass rearing and artificial diets have yielded successful results (Goodman et al., 2006; Story et al., 1996; Tomic-Carruthers, 2009). The weevil is collected, reared, and intensively redistributed throughout areas where *C. stoebe* is invasive (Seastedt et al., 2007; Story and White, 2010; Sturdevant et al., 2006). The species' limited dispersal ability due to its inability to fly enables it to flourish in clusters of interconnected *C. stoebe* plants; however, its distribution may be restricted in areas lacking this plant, requiring substantial efforts to enhance its population and spread it across regions infested with *C. stoebe* (Rondeau, 2007; Seastedt and Knochel, 2021; Story et al., 2006).

Cyphocleonus achates establishment has been observed in various studies. Stinson et al. (1994) found 36% to 86% of *C. stoebe* plants attacked by *C. achates* with an average of 2.46 late instar larvae per root in the field in its native range of Austria and Romania. On specific field release site, Knochel and Seastedt (2010) observed 78% to 82% of plants attacked by *C. achates* in the plots with 2 to 2.24 weevils per root, which is comparable to the weighted average of 1.8 weevils per plant in long term study of 20 years by Seastedt and Knochel (2021) conducted in several individual field release sites. Similarly, Seastedt et al. (2007) found 34% of plants attacked by *C. achates*, with a range of 0.02 to 0.40 weevils per root, and Story et al. (2006) reported adult numbers of 0.05 to 0.69 *C. achates* per square meter in post-release assessments focusing on several individual field release sites. Meanwhile, Wooley et al. (2011) recovered an average of 2.6 *C. achates* weevils per root, with the number of weevils per root ranging from zero to nine in a study at a specific *C. achates* field release site. In greenhouse experiments, Knochel et al. (2010) observed an average of 2.36 weevils per root, while caged common garden plots had 41% of plants infested with 0.74 weevils per root. In manipulative experimental plots, Corn et al. (2006) reported 9 to 11 larvae per root, while Corn et al. (2007) reported a range of 11.9 to 13.5 larvae per root; however, the weevil densities in the enclosures ranged from 0.3 to 0.9 larvae per root, which was significantly lower than treatment plots.

When 48 release sites for *C. achates* were surveyed, of which 46 had received *C. achates* releases, Sturdevant et al. (2006) reported 0.08 to 0.3 larvae per root and 6.9 to 21.4 adult weevils per site, with 75% of the sites showing establishment. While sampling roots from five *C. stoebe*-dominated sites in Montana in late fall and early spring, it was found that *C. achates* larvae had established in 16% of the roots (Corn et al., 2009). Herron-Sweet et al. (2015) found an average emergence of 2.9 *C. achates* adults per site in a study of 19 sites in Montana. In 16 sites Sturdevant and Dewey (2002) sampled, *C. achates* were established in 69% of the sites based on larval sampling and in 56% of the sites based on adult sampling. Meanwhile, Clark et al. (2001b) found *C. achates* larval infestation in 35.6% of the roots sampled from 45 sites in Montana, Idaho, and Washington. Additionally, in Michigan, Carson et al. (2014) found low numbers of *C. achates* in only five of the six sites where it was released.

However, some studies suggest no quantifiable impact of root feeding by *C. achates* on *C. stoebe* populations (Carson et al., 2014; Ortega et al., 2012; Steinger and Müller-Schärer, 1992; Sturdevant et al., 2006). This is because the insects may not have been present at the release sites for a long enough period to reach the required densities that would have an impact on *C. stoebe* (Carson et al., 2014; Sturdevant et al., 2006). Studies suggest *C. stoebe* can show compensatory growth because of *C. achates* feeding (Ortega et al., 2012; Steinger and Müller-Schärer, 1992; Wooley et al., 2011), and some studies also suggest that drought, potentially compounded by the impacts of *C. achates*, might cause the decline of *C. stoebe* populations (Pearson et al., 2017; Sturdevant et al., 2006). No significant impact of *C. achates* was observed on the physiology, including water use efficiency of *C. stoebe*, which is advantageous for *C. stoebe*, particularly in arid conditions, due to which *C. achates* reduces *C. stoebe* densities during dry years, but the plant compensates for damage in wet years (Wooley et al., 2011). Additionally, Thelen et al. (2005) reported that *C. stoebe* exudes higher amounts of catechin from its roots when attacked by *C. achates*. Another important constraint of the performance of *C. achates* is a lack of sufficiently large plants to support the full development of a single larva (Bourchier et al., 2001).

Objectives

This research project has two objectives. Firstly, I aimed to assess the distribution and abundance of the two main root-mining biocontrol agents of *C. stoebe*, *A. zoegana*, and *C. achates* across a larger spatial scale (State of Idaho) to find out whether the BCAs populations vary according to their release history in the state, whether densities are sufficient to inflict damage of *C. stoebe* and whether the species coexist or negatively interact in their shared invasive host plant. Secondly, I tried to assess the practicality of mass-rearing *A. zoegana* for redistribution purposes. Additionally, I aimed to calculate the overall cost per moth of three simple rearing methods to evaluate whether a rearing could be cost-effective.

Table 1.1. Classical biological control agents introduced for the *Centaurea stoebe* in the United States. Data extracted from Müller-Schärer and Schroeder (1993) & Winston et al. (2016).

Niche	Order/Family	Species	Common name	Year introduced	Feeding damage	Impact	Main host[1]	Availability
Seed-head	Diptera: Tephritidae	*Urophora affinis* Fraunfeld	Banded knapweed gall fly	1973	Seeds	Moderate to high	S/D	Readily available
Seed-head	Diptera: Tephritidae	*Urophora quadrifasciata* Meigen	UV knapweed seed-head fly	1980	Seeds	Moderate to high	S/D	Readily available
Seed-head	Lepidoptera: Gelechiidae	*Metzneria paucipunctella* Zeller	Knapweed seed-head moth	1980	Seeds	Low	S	Widespread in ID, MT, OR, WA
Root	Coleoptera: Buprestidae	*Sphenoptera jugoslavica* Obenberger	Bronze knapweed root borer	1980	Roots	Moderate to high	D	Readily collected from OR and WA
Root	Lepidoptera: Tortricidae	*Agapeta zoegana* L.	Sulfur knapweed moth	1984	Roots	Moderate	S	Becoming widespread; low numbers
Root	Lepidoptera: Tortricidae	*Pelochrista medullana* Staudinger	Brown-winged knapweed root moth	1984	Roots	Low to Moderate	S/D	No longer established
Root	Lepidoptera: Pterolonchidae	*Pterolonche inspersa* Staudinger	Grey-winged knapweed root moth	1986	Roots	Low to Moderate	S/D	No longer established
Root	Coleoptera: Curculionidae	*Cyphocleonus achates* Fåhraeus	Knapweed root weevil	1988	Roots	Moderate to high	S	Widely established and increasing

Table 1.1. continued

Seed-head	Coleoptera: Curculionidae	*Larinus minutus* Gyllenhal	Lesser knapweed weevil	1991	Seeds, foliage	Very high	D	Widely established and increasing
Seed-head	Diptera: Tephritidae	*Terellia virens* Loew	Green clearwing knapweed fly	1992	Seeds	Low	S	Established but not in high densities
Seed-head	Diptera: Tephritidae	*Chaetorellia acrolophi* White	Knapweed peacock fly	1992	Seeds	Low	S	Established but not in high densities
Seed-head	Coleoptera: Curculionidae	*Bangasternus fausti* Reitter	Broad-nosed knapweed seed-head weevil	1992	Seeds	Low to Moderate	D	Readily collected from OR and WA
Seed-head	Coleoptera: Curculionidae	*Larinus obtusus* Gyllenhal	Blunt knapweed flower weevil	1993	Seeds, foliage	Moderate	S/D	Widely established but not dense

[1] S: Spotted knapweed (*Centaurea stoebe* L.), D: Diffuse knapweed (*Centaurea diffusa* L.)

Chapter 2: Abundance and Distribution of two Unevenly Managed Root-Mining Insects for the Biological Control of Spotted Knapweed in Idaho

Abstract

Assessing post-release outcomes is an essential but neglected component of classical biological weed control programs to measure the effectiveness of biocontrol agents and evaluate any potential unintended effects on other species. Thirteen biological control agents were introduced into the USA between 1973 and 1993 for the biological control of spotted knapweed *Centaurea stoebe* L. subsp. *micranthos* (Gugler) Hayek (Asteraceae). Among them are the root-mining weevil *Cyphocleonus achates* (Fåhraeus) (Coleoptera: Curculionidae) and the root-mining moth *Agapeta zoegana* L. (Lepidoptera: Tortricidae), first released in 1988 and 1984, respectively. In this study, we aimed to assess the distribution, abundance, and interaction between the two root miners and estimate their potential impact on a spatial scale (State of Idaho). We focused on these biocontrol agents because extensive efforts have been made to rear and redistribute *C. achates*, while similar efforts for *A. zoegana* have been nonexistent. We randomly collected plants on five census dates between 2019 - 2022 (n = 2,460) at 37 field sites representing all areas in the state of Idaho with *C. stoebe* invasions. Collection times coincided with the presence of larval feeding of the biocontrol agents. Roots were dissected for larval presence, and below and aboveground plant matter was dried. We found that attack rates of *A. zoegana* and *C. achates* are similar, with an overall 9.23 ± 0.91% (mean ± SE) of *C. stoebe* roots attacked by *A. zoegana* and 9.67 ± 0.79% attacked by *C. achates*. Based on all *C. stoebe* plants analyzed, the number of larvae per root was 0.30 ± 0.05 for *A. zoegana* and 0.21 ± 0.04 for *C. achates,* respectively, at field sites. Our data indicated no competitive adverse interaction between *A.*

zoegana and *C. achates* in roots attacked by both herbivores. We also found that attacks by more than one larva of *A. zoegana* or heterospecific attack reduced the aboveground dry weight of *C. stoebe* plants. Both root-mining biocontrol agents are widely distributed throughout areas in Idaho invaded by *C. stoebe.* Still, observed attack rates and the resulting injury to *C. stoebe* roots are most likely insufficient to control the rangeland weed. Our study calls into question costly efforts to distribute *C. achates* while completely neglecting such efforts for *A. zoegana*.

Introduction

Classical biological control of weed (BCW hereafter) programs for alien invasive plants (AIP hereafter) involves selection of the target weed, exploration and host-specificity testing of biological control agent (BCA hereafter) candidates, and their rearing, release, and post-release monitoring and evaluation (Hinz et al., 2019; Müller-Schärer and Schaffner, 2008; Schaffner et al., 2018). Recent advances in molecular target weed population structure, BCA candidate identification, and improved pre-release risk assessments have improved the discipline (Gaskin et al., 2011; Hinz et al., 2020, 2019; Paynter et al., 2020; Schaffner et al., 2018; Schwarzländer et al., 2018). However, in some countries heavily invested in BCW, such as the USA (Harms et al., 2020), there is still a lack of quantitative post-release assessments of BCW programs, albeit mandated by the responsible federal regulatory agency, the United States Department of Agriculture's Animal Plant Health Inspection Service (USDA APHIS) (APHIS, 2021). Post-release assessments are necessary to identify potential nontarget effects of released agents, provide the basis to assess the effectiveness of BCAs and the overall success of BCW programs, and in justifying resources spent in the past or future for this IAP management practice (Clewley et al., 2012; Havens et al., 2019; Hinz et al., 2020, 2019; Schaffner et al., 2020; Schwarzländer et al., 2018).

Monitoring initiatives mostly focus on biocontrol agent presence and abundance, statistically assessed at the plant level (Hinz et al., 2020; Schaffner et al., 2020), but few studies quantitatively evaluate the population-level impact of released agents on invasive weeds (e.g., Catton et al., 2016; Clewley et al., 2012; Hinz et al., 2020, 2019; Schaffner et al., 2020; Schwarzländer et al., 2018; Seastedt and Knochel, 2021; Weed et al., 2018; Weed and Schwarzländer, 2014). In Idaho, a citizen-science monitoring program called Standard Impact Monitoring Protocol (SIMP) has been developed to help land managers track the effectiveness of BCAs against AIP (SIMP, 2023). However, the lack of quantitative post-release monitoring data is widely acknowledged (Clewley et al., 2012; Hinz et al., 2020, 2019). Current post-release monitoring of community and ecosystem-level BCW is inadequate due to financial, geopolitical, and logistical limitations and overreliance on early results without careful quantification (McCulloch et al., 2022; Schaffner et al., 2020).

Centaurea stoebe L. subsp. *micranthos* (Gugler) Hayak (Asteraceae) has been the focus of extensive study and control efforts due to its aggressive invasiveness, resulting in the domination of rangelands and grasslands (Carson et al., 2014). In North America, *C. stoebe* covers over three million acres of rangeland and woodland ecosystems (Story & Piper, 2001). Hirsch and Leitch (1996) reported that in Montana alone, *C. stoebe*, along with two other minor knapweed species, contribute to an annual economic impact exceeding $42 million. *Centaurea stoebe* is found in eight Canadian provinces/territories and 46 US states and is categorized as noxious in 16 US states (EDDMapS, 2023). A BCW program for *C. stoebe* was in place from 1973 to 1993, during which thirteen BCAs, including eight seed-head and five root BCAs, were introduced (Müller-Schärer and Schroeder, 1993). Since 2008, SIMP has been a valuable tool for land managers to monitor the progress of *C. achates*, *Larinus* spp., and *Bangasternus fausti* Reitt. in the management of *C. stoebe*, which is achieved by tracking vegetation cover and densities of AIPs, and population sizes of BCAs (SIMP, 2023).

Several long-term studies conducted over 15 to 30 years show that introducing BCA to decrease seed production and plant survival of *C. stoebe* has reduced its population and even eliminated it in some cases (Gayton and Miller, 2012; Seastedt and Knochel, 2021; Story et al., 2008). Seed-head insects have significantly reduced *C. stoebe* seed production for many years (Story et al., 1991b; Story and Piper, 2001a; Story et al., 1989, 2008). However, certain seed-head insects may not coexist harmoniously due to interspecific and intraspecific competition (Englert, 1971; Groppe and Marquardt, 1989; Seastedt et al., 2007; Smith and Mayer, 2005; Story et al., 1991b). Additionally, in the absence of root BCAs, seed-head BCAs have a restricted ability to influence plant densities because of the competitive superiority and perennial habit of *C. stoebe* (Story et al., 2008). Therefore, to be effective, seed reduction needs to be combined with BCAs such as root mining biocontrol agents that weaken plants or cause outright plant mortality (Blossey and Hunt-Joshi, 2003; Knochel et al., 2010; Knochel and Seastedt, 2010; Maines et al., 2013; Myers and Risley, 2000; Seastedt et al., 2007; Seastedt and Knochel, 2021; Story et al., 2006, 2008). However, root BCAs are occasionally overlooked in BCW due to operational challenges or difficulty working with them (Blossey and Hunt-Joshi, 2003; Erb and Lu, 2013; Willsey et al., 2017). Additionally, root-mining BCA candidates are more difficult to assess because of their hidden life history and require more significant effort during pre-release host specificity assessments (Blossey and Hunt-Joshi, 2003; Knochel and Seastedt, 2009). The root moth *Agapeta zoegana* L., the root weevil *Cyphocleonus achates* Fåhr. and the root beetle *Sphenoptera jugoslavica* Obenb., are currently established in the US, whereas the root moth *Pelochrista medullana* Stgr., and the root moth Pterolonche *inspersa* Stgr. are considered rare or no longer established in the US (Winston et al., 2014).

The root-feeding moth *Agapeta zoegana* L. (Lepidoptera: Tortricidae) was imported from Austria and Hungary to Montana in 1984 as a biological control agent against *C. stoebe* (Müller-Schärer & Schroeder, 1993; Story et al., 1991a). The larvae of *A. zoegana* feed behind a silken web on root cortex tissues, overwinter and pupate in roots, and emerge as adults during the following late spring and summer (Müller et al., 1989; Story et al., 1991a). Larvae develop through six instars and mostly overwinter as third or fourth instars in North America (Corn et al., 2009). *Agapeta zoegana* reduces *C. stoebe* survival, biomass, or reproductive potential (Müller et al., 1988; Müller et al., 1989; Muller-Scharer, 1991; Story et al., 2000). However, the moth can enhance *C. stoebe*’s allelopathic effects (Thelen et al., 2005), has competitive domination over natural grass species (Newingham et al., 2007), or has no significant impact on *C. stoebe* vigor (Corn et al., 2007). *A. zoegana* herbivory causes the compensatory growth of *C. stoebe* (Steinger and Müller-Schärer, 1992) and also has adverse effects on native grasses (Callaway et al., 1999; Ridenour and Callaway, 2003). The moth has extended its geographical distribution throughout the United States. However, despite its expansion, the local populations of this moth are diminishing due to insect migration (Winston et al., 2016, 2014).

The root-mining weevil *Cyphocleonus achates* Fåhr. (Coleoptera: Curculionidae) of Eurasian origin was initially imported to North America in 1988 for biological control of *C. stoebe*

(Müller-Schärer and Schroeder, 1993). Adults appear in summer between July and October (Corn et al., 2009), typically three to four weeks following *A. zoegana* (Mosley et al., 2016). The larvae develop through four instars while feeding on the central vascular tissue of roots during the subsequent fall and spring (Stinson et al., 1994). It was established quickly and has increased to outbreak populations in some areas in Montana (Story et al., 2006). The weevil is collected, reared, and intensively redistributed throughout areas where *C. stoebe* is invasive (Corn et al., 2006; Seastedt and Knochel, 2021; Sturdevant et al., 2006). Several common garden and manipulative experiments have shown that *C. achates* reduces the biomass of *C. stoebe* (Corn et al., 2007, 2006; Jacobs et al., 2006; Knochel and Seastedt, 2010). *Cyphocleonus achates* also resulted in declining flower and/or seed production in common garden and greenhouse experiments (Jacobs et al., 2006; Knochel et al., 2010; Knochel and Seastedt, 2010; Seastedt et al., 2007). Several studies have also reported population-level impacts of *C. achates* on *C. stoebe* after the release of *C. achates* (Corn et al., 2007, 2006; Gayton and Miller, 2012; Jacobs et al., 2006; Seastedt and Knochel, 2021; Story et al., 2006). However, some studies suggest *C. stoebe* can tolerate and overcompensate for *C. achates* root herbivory (Ortega et al., 2012; Steinger and Müller-Schärer, 1992; Wooley et al., 2011), and others suggest that drought, potentially compounded by the impacts of *C. achates*, might cause the decline of *C. stoebe* populations (Pearson et al., 2017; Sturdevant et al., 2006). Because of intensive rearing programs, this weevil can now be found across much of the Northwest of North America (Winston et al., 2016). The weevil is also successfully reared using artificial meridic diets (Goodman et al., 2006; Tomic-Carruthers, 2009). The release of these agents differs considerably in that *C. achates* is reared and redistributed more frequently than *A. zoegana* (Winston et al., 2016, 2014).

We aimed to investigate the distribution of *C. achates* and *A. zoegana* on a spatial scale of Idaho along environmental gradients, the intensity and proportion of attack, and the variability of belowground herbivory by these species. Based on the greatly differing rearing and redistribution programs, we hypothesized that *C. achates* is more abundant than *A. zoegana* across our study region. We also sought evidence for competitive or facilitating interactions between the two species based on their occurrence in the host. And finally, we tried to infer from the abundance of belowground herbivory whether these BCAs impact *C. stoebe* plant populations.

Materials and Methods

Study sites

Release history information of *A. zoegana* and *C. achates* in Idaho was obtained from the Nez Perce Bio-Control Center in Lapwai, Idaho, which maintains an up-to-date database of all documented weed biocontrol releases in the State of Idaho. The release information included release coordinates of sites for each species, date of release, the number of insects released, insect life stage released, and environmental parameters of the release sites. The database included 49 releases of *A. zoegana* and 3,760 releases of *C. achates* in Idaho between 1993 and 2021 (Fig.2.1).

For this study, we selected 37 different sites in the State of Idaho to collect *C. stoebe* plants with the assistance of land managers in local, state, and federal governments. Sites were randomly chosen without knowledge of BCA release history or presence. Care was taken to locate sites in all areas of the state where *C. stoebe* is known to be invasive. Most field sites were located in highly disturbed roadside right of ways, rangeland, fallow land, or open forests (see Table 2.1 for site information). The number of study sites from which plants were collected ranged from four to 32, depending on the year and census date. Sampling in 2019 was preliminary and mainly aimed to confirm that *A. zoegana* is established in Idaho. Generally, entire plants, including roots, were randomly chosen at sites by collaborators and dug to ensure roots were collected entirely and undamaged. Plants were bagged and cooled until shipped via bonded carrier or transported by car to our laboratory at the University of Idaho. If possible, 30 *C. stoebe* were collected at each site with minimal disturbance to other individuals and surrounding vegetation (see Table 2.1 for the number of plants collected). Upon arrival at the University of Idaho, plants were stored in black plastic contractor bags (114 cm height, 55 cm width, 0.07 mm of thickness) in a cold room at 4° C until dissection. The timing of plant collections varied depending on the phenology of the plant in different locales in Idaho and the availability of collaborators to collect *C. stoebe*. Generally, plants were collected at the time of greatest overlap of mature *A. zoegana* and *C. achates* larvae in roots, respectively (late May to the beginning of July). However, we also sampled plants between October and early November 2021 to ensure that herbivory by younger larvae did not drastically differ from that of late instar herbivory.

Plant dissections

All roots were carefully washed and airdried for two to three hours before analysis. For all plants that arrived at the laboratory with all aboveground plant material intact, this was cut at the root crown, bagged, and dried in paper bags at the University of Idaho Parker Research Farm at 44° C for 72 to 144 hrs., depending on plant size. The weight was measured with a precision of 0.1 g.

To obtain measurements representing orthogonal diameters, each root's diameter was measured twice, one centimeter below the cut root crown, using an electronic digital caliper (Model 01407A, Neiko, China) with a precision of 0.01 mm. The two measurements were then averaged. The roots were meticulously dissected by creating multiple longitudinal sections that extended from the cortex to the endodermis. Additionally, horizontal sections were made, all of which were carried out under a stereomicroscope to ensure precision and accuracy in the examination process. This dissection aimed to identify the presence of larval instars of *A. zoegana*, *C. achates*, or *S. jugoslavica*. A no. 23 blade surgical scalpel was used for the dissection process. All herbivore larvae found were extracted and preserved in 5 ml transparent glass vials filled with 70% ethanol for subsequent instar determination. Roots that showed signs of mining but where no larvae were found during dissections were considered unattacked, as *C. stoebe* is a perennial plant, and mining could have occurred in previous years. After the complete dissection of each root, all the root material was carefully bagged in brown disposable paper lunch bags (Great value™, 3.63 kg volume, 31 cm height, 15 cm width, 10 cm girth) and dried in a drying cabinet at the University of Idaho Parker Research Farm at 44º C for 72 to 144 hrs., depending on root size, and weighed to the precision of 0.1 g.

Intensity of herbivory and proportion of plants attacked at field sites

Herbivory intensity was defined for this study as the mean number of larvae per *C. stoebe* root at a study site. The number of entirely unattacked *C. stoebe* roots was higher throughout the study than expected. Therefore, the intensity of herbivory was assessed twice: once by considering all the attacked roots and then by excluding those roots that were not attacked by herbivore species. For the proportion of *C. stoebe* roots attacked at a study site, we differentiated attacks by one or more larvae of *A. zoegana*, *C. achates*, *S. jugoslavica*, and roots attacked by more than one herbivore species.

A generalized linear mixed model (hereafter GLMM) with a lognormal distribution was used to assess the herbivore intensity for each species across years, with year as a fixed effect and sites within the year as a random effect.

A GLMM with a binary distribution was used to assess the herbivory proportion at each site and year, with site as a fixed effect and plants as a random effect. Finally, to assess the proportion of plants attacked by each larval species with respect to no herbivory, and compare between the species, a GLMM assuming a binary distribution was used, considering the year, species, and their interaction as fixed effects and sites within the year as a random effect.

Plant size and belowground biocontrol attack

To assess whether the intensity of root herbivory differed intra-specifically and inter-specifically with respect to root size, we used the root crown diameter (hereafter RCD) measured in mm as a predictor for root size. We previously established the correlation between RCD and root dry weight for *C. stoebe* and found a significant positive correlation (range: $r^2 = 0.599 - 0729$, $P < 0.0001$). A Poisson distribution GLMM with a dummy variable regression was used to estimate whether the intensity of root herbivory differed with root size for the three species. The model accounted for the fixed effect of species and the continuous effect of average root size and considered sites within years as random effects. Single degree of freedom contrasts was used to investigate potential differences in intercept and slope terms among species. To assess if RCD is different between the three species among infested roots, a lognormal distribution GLMM ANOVA was used, with species as a fixed effect and site by species interaction as a random effect.

Additionally, the proportion of attacks was classified into nine root size classes to assess the difference in the proportion of plants attacked by each of the three species. Root size classes were determined based on RCD and included: class 1 (0 - 4.99 mm), class 2 (5 - 9.99 mm), class 3 (10 - 14.99 mm), class 4 (15 - 19.99 mm), class 5 (20 -24.99 mm), class 6 (25 - 29.99 mm), class 7 (30 - 34.99 mm), class 8 (35 - 39.99 mm), and class 9 (roots exceeding 40 mm). For each species, a beta distribution GLMM ANOVA was fit separately to assess overall differences in the proportion of plants attacked by different species relative to nine root size classes. The model used root size classes as a fixed effect and the number of attacks in each class as a random effect.

Belowground biocontrol intensity in relation to geographic factors

To investigate how the belowground biocontrol herbivory intensity by *A. zoegana* or *C. achates* relates to the elevation and latitude of the study site, we utilized GPS Visualizer (https://www.gpsvisualizer.com) to extract elevation data from the coordinates of the sites (See Table 2.1. for the coordinates of sites). Data were pooled across years with sites unique to their coordinates. To conduct this analysis, the proportion of plants attacked by each BCA was relative to the proportion of plants that particular BCA did not attack. A binary distribution GLMM was used to calculate the proportion of herbivory by each species, with sites as fixed effects and plants as random effects. A simple linear regression test was performed using GraphPad Software. (2023). GraphPad Prism (Version 9.5.1 for Windows) [Computer software]. San Diego, CA, USA, which estimated the relationship between the proportion of plants attacked and the latitude and elevation of the collection sites.

Belowground biocontrol herbivory and BCA release history near study sites

We used the QGIS Development Team. (2022). QGIS (Version 3.26.3) [Computer software]. Open-Source Geospatial Foundation to determine all *A. zoegana* and *C. achates* releases made within a 10 km radius of our study sites. We obtained data from the Idaho biocontrol release database at the Nez Perce Bio-Control Center in Lapwai, ID. This was done to evaluate the proportion of plants attacked by *A. zoegana* and *C. achates* in relation to the number and timing of previous biological control releases. We analyzed the total number of BCA individuals released per species, the year of the most recent releases, the number of releases made, and the number of BCA individuals in the most recent release within a 10 km radius (see Appendix K for release details). Using GraphPad Software. (2023). GraphPad Prism (Version 9.5.1 for Windows) [Computer software]. San Diego, CA, USA, we conducted a simple linear regression test to determine the correlation between the proportion of plants attacked by *A. zoegana* or *C. achates* and the aforementioned factors. The proportion of plants attacked by each BCA was relative to the proportion of plants that particular BCA did not attack.

Belowground biological herbivory intensity and aboveground *C. stoebe* plant weight

We pooled all 2020, 2021, and 2022 plant data to assess whether belowground herbivory intensity affected aboveground biomass production. We only included plants (n = 1,435) that arrived with intact aboveground biomass, excluding those where collaborators had removed the aboveground biomass. Similarly, we categorized belowground herbivory intensities as follows: one or more larvae of *A. zoegana*, one or more larvae of *C. achates*, one or more larvae of *S. jugolsavica*, and more than one larva total of *A. zoegana* and *C. achates.* A GLMM dummy variable regression model was used to estimate the relationship between belowground biomass (with the aforementioned herbivory intensities) and aboveground biomass. The model assumed a normal distribution with species intercepts and belowground weight slopes as fixed effects. The belowground biomass was divided into five weight classes: BW1 (< 10) g, BW2 (10 to < 20) g, BW3 (20 to < 30) g, BW4 (30 to < 40) g, and BW5 (40 to < 60) g. The model was fitted to aboveground biomass, with five belowground weight classes defined. Separate residual variance estimates were used across five belowground weight classes to account for heterogeneity. Single degree of freedom contrasts was used to investigate differences in slope estimates between different herbivory intensities.

Interactions between *A. zoegana* and *C. achates* larvae in *C. stoebe* roots

To study whether *A. zoegana* and *C. achates* larvae attacks resulted in competitive or facilitative interactions, we compared observed and expected numbers of co-occurrence for the conspecific and heterospecific attacks. The expected number of attacks was based on the proportion of single attacks of each species and the total number of attacks. A chi-square analysis, summed across years, was used to compare observed and expected numbers for each of three cases, where years acted as replicates for analysis: *AA* (represents conspecific *A. zoegana*), *CC* (Conspecific *C. achates*), and *AC* (Heterospecific *A. zoegana* and *C. achates*).

Statistical analyses were conducted with the statistical software package SAS Institute Inc. (2021). SAS Version 9.4 [Computer software]. Cary, NC, SAS Institute Inc.

Results

Intensity of herbivory and proportion of plants attacked at field sites

We found larvae of all three root-feeding biological control agents during root dissections of *C. stoebe* plants. The presence of *A. zoegana* and *C. achates* varied independently among sites and years, and that of *S. jugoslavica* was comparatively low in all the years except late 2021 (Fig.2.2; Appendix A).

The intensity of root-herbivory by *A. zoegana* and *C. achates* was consistent among census dates regardless of whether data were analyzed for all plants (Fig. 2.3a) or excluding plants that were not attacked at all (Fig 2.4b); *A. zoegana* ($F_{4,54} = 0.98$, $P = 0.43$ and $F_{4,28} = 1.54$, $P = 0.22$; Fig. 2.3a, b, respectively, inner brackets) and *C. achates* ($F_{4,55} = 2.48$, $P = 0.055$ and $F_{4,30} = 2.46$, $P = 0.067$; Fig. 2.3a, b, respectively, inner brackets), but differed for *S. jugoslavica* ($F_{4,40} = 3.64$, $P = 0.0128$ and $F_{4,19} = 5.52$, $P = 0.004$; Fig. 2.3a, b, respectively, inner brackets). Including unattacked *C. stoebe* plants, there were 0.30 ± 0.05 SE *A. zoegana* larvae, 0.21 ± 0.04 SE *C. achates,* and 0.18 ± 0.04 SE *S. jugoslavica* larvae per root at field sites throughout the study period (Fig. 2.3a, Appendix B). When only considering *C. stoebe* plants attacked by one or more species, the intensity of the attack was 1.65 ± 0.09 SE for *A. zoegana,* 1.52 ± 0.07 SE for *C. achates*, and 1.19 ± 0.06 SE *S. jugoslavica* larvae per root (Fig. 2.3b; Appendix D). There was no difference in the herbivory intensity when considering all plants sampled between *A. zoegana* and *C. achates* ($t_{67} = 1.87$, $P = 0.066$) and between *C. achates* and *S. jugoslavica* ($t_{67} = 0.69$, $P = 0.494$), but there was less herbivory by *S. jugoslavica* larvae per root when compared to *A. zoegana* ($t_{67} = 2.34$, $P = 0.022$) (Fig. 2.3a; Appendix C). Similarly, when only attacked plants were analyzed, herbivory intensity did not differ between *A. zoegana* and *C. achates* ($t_{67} = 1.32$, $P = 0.191$).

Herbivory intensity was lower for *S. jugoslavica* when compared to *A. zoegana* ($t_{67} = 4.85$, $P < 0.0001$) or *C. achates* ($t_{67} = 3.84$, $P = 0.0003$) (Fig.2.3b; Appendix E).

There was a significant interaction between herbivore species and census date on herbivory intensity for all plants ($F_{8,67} = 6.51$, $P < 0.0001$), and plants attacked by the root-mining BCA ($F_{8,67} = 3.82$, $P = 0.0010$), suggesting that environmental factors affected abundance.

When only considering plants attacked by one of the root herbivore species at a field site versus plants not attacked by any herbivore, *A. zoegana* larvae were found in 9.23 ± 0.91% (mean ± SE) of plants, *C. achates* larvae were found in 9.67 ± 0.79%, *S. jugoslavica* larvae were found in 6.61 ± 0.66%, and multiple species of herbivore were found in 5.48 ± 0.60% of plants (Fig. 2.4). Across all sites and years, 65.08 ± 1.32% of all plants analyzed were unattacked by any species (n = 2,460) (Fig 2.4; Appendix F).

The percentage of plants attacked by *A. zoegana* and multiple species was consistent among census dates ($F_{4,92} = 1.87$, $P = 0.123$ and $F_{4,92} = 0.42$, $P = 0.79$; Fig. 2.4a, d, respectively, upper brackets), but differed for *C. achates* and *S. jugoslavica* ($F_{4,92} = 4.13$, $P = 0.004$ and $F_{4,92} = 8.71$, $P < 0.0001$; Fig. 2.4b, c, respectively, upper brackets).

Insect species ($F_{4, 12183} = 399.24$, $P < 0.0001$), census date ($F_{4, 92} = 3.13$, $P = 0.018$), and their interaction ($F_{16, 12183} = 14.15$, $P < 0.0001$) had a significant effect on the percentage of plants attacked suggesting that environmental factors affected the rate of plants attacked.

There was no difference in the percentage of plants attacked by *A. zoegana* and *C. achates* ($t_{12183 =}$ -0.36, $P = 0.716$), as well as multiple species and *S. jugoslavica* ($t_{12183 =}$ -1.27, $P = 0.204$), but percentages of plants attacked did differ for *A. zoegana* compared to multiple species, *S. jugoslavica*, and unattacked plants, for *C. achates* compared to multiple species, *S.*

jugoslavica and unattacked plants, for multiple species compared to *S. jugoslavica* and unattacked plants and *S. jugoslavica* compared to unattacked plants (Appendix G).

Plant size and belowground biocontrol attack

There was a positive relationship between the root size measured as root crown diameter and the number of larvae found in roots for each of the three species, *A. zoegana*, *C. achates*, and *S. jugoslavica* ($F_{3,840} = 43.34$; $P < 0.0001$; Fig. 2.5a, Fig. 2.5b, Fig. 2.5c, respectively; Appendix H). However, there was no significant difference between the three species for the number of larvae found in roots with increasing root size ($F_{3,840} = 1.03$; $P = 0.377$, Appendix H).

Within attacked plants, the results indicated significant variations in RCD among different species occurrences ($F_{2,75} = 5.38$; $P = 0.007$). The mean root size attacked by *A. zoegana* was 17.78 ± 0.72 mm (mean ± SE), by *C. achates* was 17.42 ± 0.73 mm, and by *S. jugoslavica* was 15.51 ± 0.73 mm (Appendix I).

When attacked roots were placed in classes of root size (Fig. 2.6), the percentage of plants attacked across the root classes only differed for *A. zoegana* ($F_{8, 9} = 3.6$, $P = 0.037$; Fig. 2.6a), but not for *C. achates* ($F_{8, 8} = 2.37$, $P = 0.122$; Fig. 2.6b), *S. jugoslavica* ($F_{7, 12} = 0.72$, $P = 0.66$; Fig. 2.6c), or at least one herbivore species, ($F_{8, 2} = 3.64$, $P = 0.233$; Fig. 2.6d).

Belowground biocontrol intensity in relation to geographic factors

The proportion of plants attacked by *A. zoegana* ($P = 0.0052$; Fig. 2.7a) and *S. jugoslavica* ($P = 0.026$; Fig. 2.7c) increased with the latitude of field sites. In contrast, the proportion of plants attacked by *C. achates* larvae did not differ with latitude ($P = 0.836$; Fig. 2.7b). Increasing elevation of *C. stoebe* study sites was marginally negatively correlated with the proportion of *C. stoebe* plants attacked by *A. zoegana* (*P* = 0.0504; Fig. 2.7a) and negatively correlated with the proportion of *C. stoebe* plants attacked by S. *jugoslavica* (*P* = 0.0022; Fig. 2.7c). Conversely, increasing elevation of *C. stoebe* sites was positively correlated with the proportion of plants attacked by *C. achates* (*P* = 0.0187; Fig. 2.7b).

Belowground biocontrol herbivory and BCA release history near study sites

There was no relationship between the proportion of *C. stoebe* plants attacked at field sites with the total number of *A. zoegana* released at respective sites (*P* = 0.254; Fig.2.8a), the year of most recent *A. zoegana* release (*P* = 0.199; Fig.2.8b), the number of *A. zoegana* releases made at the site (*P* = 0.187; Fig.2.8c), or the number of *A. zoegana* most recently released (*P* = 0.216; Fig.2.8d). Similarly, there was no relationship between the proportion of plants attacked by *C. achates* at field sites with the total number of *C. achates* weevils released at sites (*P* = 0.988; Fig.2.9a), the number of *C. achates* releases (*P* = 0.635; Fig.2.9c), or the number of C. *achates* most recently released (*P* = 0.165; Fig.2.9d). There was, however, a marginal negative relationship between the proportion of plants attacked by *C. achates* and the year of the most recent release (*P* = 0.057; Fig.2.9b).

Belowground biological herbivory intensity and aboveground *C. stoebe* plant weight

Regardless of the presence of the biological control agent species or herbivory level, the *C. stoebe* root dry weight was a good predictor for the aboveground dry weight (range r^2 = 0.767–0.928) (Fig. 2.10; Appendix M). The result also showed that the herbivory levels ($F_{8, 1363}$ = 2.25, *P* = 0.022) and the interaction of herbivory levels and belowground weight ($F_{8, 1363}$ = 139.69, *P* < 0.0001) were significant predictors of aboveground weight (Fig. 2.10, Appendix M).

There was no difference between the slope of the regression for unattacked *C. stoebe* plants with those of plants attacked by one larva of *A. zoegana* or *C. achates* ($F_{1, 1363}$ = 1.13, P = 0.288; $F_{1, 1363}$ = 0.77, P = 0.77, respectively, Fig. 2.10). But slopes of the regression for unattacked plants differed from that for plants attacked by at least two larvae of *A. zoegana* ($F_{1, 1363}$ = 4.95, P = 0.0262) (Fig 2.10). Plants attacked by at least 2 *C. achates* did not affect the relationship between root dry weight and aboveground dry weight of plants differently than unattacked plants ($F_{1, 1363}$ = 3.36, P = 0.067, Fig. 2.10). The slopes of the regression for plants attacked by at least two larvae of *A. zoegana* did not differ significantly from plants attacked by at least two larvae of *C. achates* ($F_{1, 1363}$ = 0.19, P = 0.665, Appendix N). *Centaurea stoebe* plants attacked by two or more *A. zoegana* larvae had 28.36% ± 14.85 SE less biomass than unattacked plants. Plants attacked by two or more *C. achates* larvae had 21.21% ± 14.27 SE less biomass than unattacked plants.

Interactions between *A. zoegana* and *C. achates* larvae in *C. stoebe* roots

We rejected our hypothesis of independence within and between the species *A. zoegana* and *C. achates* due to significantly higher proportions of plants attacked by conspecific *A. zoegana* larvae (*AA*) (χ^2 = 169.217, df = 4, P = 0), conspecific *C. achates* larvae (*CC*) (χ^2 = 324.46, df = 4, P = 0), and also plants with heterospecific *A. zoegana* and *C. achates* larvae (*AC*) (χ^2 =18.386, df = 4, P = 0.001), than the expected proportion of either combination (Fig. 2.11, Appendix O). The occurrence of the hetero species, more than predicted, suggests no competitive interaction and possibility of facilitation between these two species.

Discussion

As expected, we found *A. zoegana* and *C. achates* widespread at field sites throughout the State of Idaho in our surveys. We also found the root-mining buprestid *S. jugoslavica,* initially released for controlling *Centaurea diffusa* and *C. stoebe*, to be widespread in our surveys. Our analysis showed that the interactions between belowground BCA species and year had a significant effect on the intensity of the attack. This indicates that environmental factors such as climate and habitat can influence the performance of the BCA species and their interactions with *C. stoebe*. Therefore, to gain a comprehensive understanding of species occupancy and abundance, both biotic and abiotic factors should be considered (Harms et al., 2020; Wilson and Fox, 2021).

In our comprehensive area-based investigation, we observed consistent levels of herbivory inflicted by *A. zoegana* and *C. achates* on *C. stoebe* roots across the years 2019 to 2022. We observed an average of 0.21 ± 0.04 *C. achates* larva per root, comparable to previous reports of 0.08 to 0.3 larva per root at multiple *C. achates* release sites (Sturdevant et al., 2006). Our observations were also similar to the manipulative experimental plots inside the enclosures, where the larval intensity ranged from 0.3 to 0.9 larvae per root (Corn et al., 2007), and caged common garden plots, where the number of weevils per root was 0.74 (Knochel et al., 2010). However, our larval intensity was lower than 1.8 to 2.24 weevils per root reported in other post-release assessments that focused on specific field release sites or several individual study sites (Knochel and Seastedt, 2010; Seastedt and Knochel, 2021). It was also lower than the larval intensity of 9 to 13.5 larvae per root found in manipulative common garden experiments (Corn et al., 2007, 2006). The larger number of larvae per root in those manipulative plots may be an artifact of initial weevil densities higher than those typically encountered in open populations (Corn et al., 2007). Additionally, the percentage of roots attacked by *C. achates* was consistently low throughout our study when compared to previous post-release studies conducted at multiple sites or specific field sites (Clark et al., 2001b; Corn et al., 2009; Knochel and Seastedt, 2010; Seastedt et al., 2007; Sturdevant et al., 2006; Sturdevant and Dewey, 2002). Overall, our findings suggest that the release of *C. achates* has not led to a significant increase in herbivory in *C. stoebe* roots in our study area throughout five census dates.

For *A. zoegana*, the intensity of herbivory was constant between the years, in contrast to previous findings (Story et al., 2000; Sturdevant and Dewey, 2002). The average number of larvae per root in our study (0.30 ± 0.05) was like that reported by Story et al. (2000) following the release of *A. zoegana* in a natural field setting (0.37 larvae per root). However, the percentage of plants attacked in their study (22% to 31%) was much higher than in our study (9.23 ± 0.91%). Notably, even the control site in their study showed the establishment of *A. zoegana* increasing from 5.9% to 22.3% during two census dates. Their finding suggests that *A. zoegana* has the capability to persist and expand in natural environments without any human intervention, as evidenced by their study's use of a natural field setting with realistic moth populations. Additionally, we found that the mean number of larvae in infested roots was 1.65 ± 0.09, comparable to the average of 1.2 larvae per infested root reported in Montana four years after the initial release of *A. zoegana* (Story et al., 1991a). However, the percentage of plants attacked in our study was consistently lower than in other studies conducted at multiple sites (Clark et al., 2001b; Corn et al., 2009; Sturdevant and Dewey, 2002) or in the manipulative experimental plots (Corn et al., 2006). These differences could be due to ecological factors like climatic adaptation and soil moisture conditions, which influence the establishment of *A. zoegana* in various locations (Fitzpatrick, 1989).

One of the principles of biological control argues that BCW is successful because BCA densities are greater in the introduction area than in the native range because of the natural enemy release of the agents (Keane and Crawley, 2002). The only parasitoid species associated with *C. stoebe* biocontrol agents in the introduced region are recorded for two species of *Urophora* gall fly (Gillespie, 1983; Kovach, 2004; Lang and Richard, 1998; Lang et al., 2003; Marshall, 2007; Marshall et al., 2004; Turner et al., 1990), and only once for *A. zoegana*, where it is not suffering high mortality compared to its native range (Herron-Sweet et al., 2015). This is consistent with observations that natural enemies are rare for *A. zoegana* and *C. achates* in their released ranges. On the other hand, the percentage of plants attacked by *A. zoegana* in our study was considerably lower than the survey conducted in its native range of Austria and Hungary, which was 23.6%. However, it was comparable to the percentage of roots attacked of 8% in central Hungary and France, as observed by Müller et al. (1988). Furthermore, the average number of larvae per root for *C. achates* in our study was substantially lower than the average of 2.46 larvae per root in its native range of Austria and Romania (Stinson et al., 1994). Also, the percentage of plants attacked by *C. achates* in our study was considerably lower than its native range of Austria and Romania, which ranged from 36% to 86% (Stinson et al., 1994), thus, calling the notion of enemy release into question for both *A. zoegana* and *C. achates.*

The mean number of *A. zoegana* and *C. achates* larvae in roots increased with root size, consistent with previous research (Collins and Müller-Schärer, 2012; Knochel and Seastedt, 2010; Maines et al., 2013; Smith and Story, 2003; Story et al., 2000). However, the relationship was not decisive for *C. achates*, as there was no relationship between the root size and the proportion of plants attacked, contradicting previous findings that suggested *C. achates* primarily infests larger roots (Knochel and Seastedt, 2010; Müller et al., 1988; Stinson et al., 1994; Volovnik, 2010; Wooley et al., 2011). These inconsistencies may be because most previous assessments did not assess the belowground biocontrol herbivory of *C. stoebe* on a larger spatial scale or because the overall proportion of plants attacked in this study was not large. It could also be attributed to methodological differences like sampling techniques and data analysis methods, or the context-dependent nature of herbivory where other factors such as plant health and nutrient availability might have interacted with root size to influence herbivory patterns.

Rearing and redistribution programs for *A. zoegana* and *C. achates* differed considerably in the State of Idaho, as is likely the case for most other western states of the USA where *C. stoebe* is problematic. While *C. achates* has been a focus of educational programs and has been heavily reared and redistributed, no such programs exist for *A. zoegana*. Our hypothesis that, as a result, *C. achates* is more abundant than *A. zoegana* must be rejected. There was no difference in the herbivory intensity, or the percentage of plants attacked by these two root herbivores at study sites across Idaho. We specifically used root dissections to assess the realized herbivory (number of larvae in a root), as has been recommended for these herbivores (Clark et al., 2001b), as preferable to indirect measurements such as timed adult insect counts or insect sweep net samples (Oehmichen et al., 2017). It has been argued that redistribution programs of the flightless *C. achates* are necessary because of the slow growth and low dispersal of this BCA (Carson et al., 2014; Paynter and Bellgard, 2011; Rondeau, 2007). However, our study showed that despite repeated releases and large release numbers at sites, there was no correlation between release numbers and herbivory rates, calling into question the effectiveness of redistribution programs.

Environmental gradients affected the distribution of BCAs in our study. We found *A. zoegana* more prevalent at study sites in northern regions, which may be attributed to a general southward movement of *A. zoegana* following large redistribution efforts in British Columbia (Bourchier et al., 2001). Furthermore, the northern prevalence of the moth is apparently not the result of climatic drivers based on the elevational distribution of this agent as the moth was found less commonly at higher-elevation study sites. Hence, climate cannot explain the more common northern distribution of *A. zoegana.* Like other insects, cooler temperatures associated with higher elevations may approach an agent's lower physiological limit (Fitzpatrick, 1989; Gutiérrez et al., 2016; Harms and Cronin, 2020; Román-Palacios and Wiens, 2020; Salcido et al., 2020; Wilson et al., 2007). In contrast, the proportion of plants attacked by *C. achates* was less at lower elevation study sites, which is surprising because the weevil is found in its native range in summer dry, hot grasslands habitats at low elevations in Romania and Austria (Stinson et al., 1994).

Generally, larger releases of BCAs are associated with better odds of the establishment of agent populations (Corn et al., 2006; Grevstad, 1999; Memmott et al., 2005, 1998; Paynter et al., 2016; Powell et al., 2000; Story et al., 2000), but our study did not observe such association between release size and herbivory rates. Theoretically, newly released BCAs are expected to experience high population growth rates owing to the availability of ample resources (Carson and Landis, 2014). In addition, it is expected that the populations of introduced BCAs would decline over time due to increased predation pressure (Herron-Sweet et al., 2015). However, our results indicate a slight inverse correlation between the recency of

C. achates releases and attack proportion, which aligns with other studies that suggest *C. achates* initially has a slow population growth rate that may accelerate subsequently (Carson et al., 2014; Story et al., 1997; Story and Stougaard, 2006). The spatial scale in this study included sites along environmental gradients, and despite the limited number of environmental factors assessed, our results indicate that these gradients have an impact on the abundance and distribution of *A. zoegana* and *C. achates,* although not necessarily in a uniform manner. Thus, achieving consistent belowground biocontrol herbivory can be challenging due to the impact of environmental gradients on the effectiveness of BCAs, as pointed out by Harms et al., 2020. This challenge can be especially pronounced in the case of *C. stoebe,* which can adapt to different environments and habitats (Závada et al., 2022).

We found no indication of competitive interactions between *A. zoegana* and *C. achates* coinhabiting roots of *C. stoebe* in this study, contrary to predictions of negative interactions between the two BCA species sharing an invasive weed host (Lazarus and Germino, 2021). The observed frequency of heterospecific occurrence of species in *C. stoebe* roots was much higher than predicted, indicating that there may be facilitation between *A. zoegana* and *C. achates* sharing the same host plant. Facilitation could be attributed, at least in part, to niche separation within the root tissue and differences in the phenology of their attack reducing competition (Müller et al., 1989; Müller, 1988; Steinger and Müller-Schärer, 1992; Stinson et al., 1994), coupled with unknown effects of one agent that increase host suitability for the other. Our study does not contradict predictions that *A. zoegana* and *C. achates* have cumulative negative feedback on *C. stoebe* plant fitness (Collins and Müller-Schärer, 2012; Stinson et al., 1994). The increase in *A. zoegana* abundance and distribution in Idaho should, therefore, not negatively interfere with any impact *C. achates* may have on *C. stoebe.*

We did not find any indication that the observed levels of *C. achates* herbivory across study sites in Idaho impaired aboveground biomass production, consistent with some previous studies (Carson et al., 2014; Ortega et al., 2012; Pearson et al., 2017; Sturdevant et al., 2006) but in stark contrast to other studies (Corn et al., 2007, 2006; Jacobs et al., 2006; Knochel and Seastedt, 2010). The impact of *C. achates* was likely influenced by factors such as nutrient and water availability, as observed in other studies (Pearson et al., 2017; Sturdevant et al., 2006; Wooley et al., 2011) or mitigated by plant tolerance (Müller-Schärer et al., 2004; Ortega et al., 2012; Steinger and Müller-Schärer, 1992), which were not examined in our research. In contrast to *C. achates*, more than one *A. zoegana* larvae reduced *C. stoebe* aboveground biomass, in line with a study by Story et al. (2000) conducted at field sites. This finding is significant as it contributes to the ongoing debate surrounding the impact of *A. zoegana* herbivory on the survival and growth of *C. stoebe*. While some studies have reported the reduced survival of rosettes (Müller, 1989b; Muller-Scharer, 1991), others have found no significant impact (Callaway et al., 1999; Sturdevant et al., 2006), compensatory growth of *C. stoebe* or even negative effects on native grasses due to *A. zoegana* herbivory (Callaway et al., 1999; Newingham et al., 2007; Ridenour and Callaway, 2003). The reduction in *C. stoebe* biomass due to heterospecific herbivory by *A. zoegana* and *C. achates* aligns with prior predictions, indicating that *C. achates* complements *A. zoegana*, leading to an additive negative impact on the fitness of *C. stoebe* (Collins and Müller-Schärer, 2012; Stinson et al., 1994). Assessing the impact of either belowground BCA was not the main aim of this study, and aboveground biomass production is only an indirect albeit reliable indicator for the impact of root herbivores (Müller, 1988). Regardless, we found, as expected, that aboveground biomass reduction is root herbivory density dependent. The findings reported in this study align with previous research that has demonstrated the negative impacts of belowground herbivory on knapweeds (Bourchier and Van Hezewijk, 2013; Collins and Müller-Schärer, 2012; Jacobs et al., 2006; Knochel and Seastedt, 2009, 2010; Knochel et al., 2010; Müller et al., 1989; Müller et al., 1988; Seastedt and Knochel, 2021; Van Hezewijk and Bourchier, 2012). However, it is surprising that *A. zoegana* appears to have a more significant per-capita impact than *C. achates* in our study, a detail thus far not reported to our knowledge. This observation contradicts the previously established conclusions that *C. achates* inflicts more damage than *A. zoegana* (Corn et al., 2007; Steinger and Müller-Schärer, 1992; Story et al., 2006).

Regardless of the effects of environmental gradients on the BCAs, the absence of competitive interactions between the BCAs and their overall similar abundance and distribution, the result of this areawide assessment suggests that the observed herbivory intensity (larvae per plant), particularly the percentage of plants attacked at study sites are likely insufficient to inflict population-level control in *C. stoebe. C. achates* disperses slowly since the insect is flightless, which may have implications for its ability to effectively control *C. stoebe* (Carson et al., 2014; Paynter and Bellgard, 2011), although outbreak densities of that biocontrol agent and related successful control of *C. stoebe* have occurred (Gayton and Miller, 2012; Jacobs et al., 2006; Seastedt et al., 2007; Story et al., 2006). Likewise, the distribution of *A. zoegana* throughout the State of Idaho is impressive, given that release records include only approximately 8,500 moths over the last 30 years. However, given that BCW is expected to be self-perpetuating and that both agents have been released for 40 years, it seems unlikely that the observed abundances of both BCAs will experience a significant increase, which is likely necessary for effective control of *C. stoebe*.

Table 2.1. Study sites for *Centaurea stoebe* root herbivory survey 2019 to 2022.

Site Number	Coordinates		Site name	County	Number of Plants Sampled by Year					Habitat type
	Latitude (ºN)	Longitude (ºW)			2019	2020	2021	Late 2021	2022	
1	48.5637	-116.8256	Priest Lake	Bonner	-	30	18	-	30	Open forest
2	48.5637	-116.8256	Priest Lake (From the fire pit)	Bonner	-	-	30	-	-	Open forest
3	48.4711	-116.8550	Coolin	Bonner	-	-	30	-	30	Fallow land
4	47.9522	-116.6057	Farragut	Kootenai	30	30	30	30	30	Fallow land
5	47.9033	-116.7684	Brunner’s Road	Kootenai	30	30	30	30	30	Open forest
6	47.5536	-116.3672	Cataldo	Kootenai	30	30	30	30	30	Right of way
7	46.5194	-116.0504	Band Mill	Clearwater	30	30	30	30	-	Open field, dry conifer on the perimeter
8	46.4664	-116.2380	Orofino	Clearwater	-	30	30	30	-	Open field
9	46.4663	-116.2374	Orofino White Eagle	Clearwater	-	30	30	30	-	Open field, dry conifer on the perimeter
10	46.4439	-116.8362	Spalding	Nez Perce	-	30	30	30	-	Railroad Right of way/open field
11	46.2328	-116.0257	Feed Store	Lewis	-	4	15	-	-	Railroad Right of way
12	46.2181	-116.0290	Hill Street Bridge	Lewis	-	17	30	30	-	Right of way
13	46.1518	-115.9155	Penny Cliffs	Idaho	-	12	21	24	-	Right of way
14	46.1416	-115.9415	Maggie Creek	Idaho	-	-	9	-	-	Right of way
15	46.1370	-115.5916	Selway	Idaho	-	12	-	-	-	Right of way
16	46.1307	-115.7887	Dumpster Beach	Idaho	-	11	19	9	-	River edge/ Sand
17	45.8918	-116.0429	HWY 14 Mt ID Grade	Idaho	-	20	20	19	-	Right of way

Table 2.1 continued

18	45.8291	-115.9668	Mt Idaho Grade	Idaho	-	15	-	-	-	Right of way
19	45.8291	-115.9668	Castle Creek	Idaho	-	30	20	20	-	Open forest/ Bio Site
20	45.8239	-115.9167	Road 484	Idaho	-	14	21	21	-	Right of way
21	45.8121	-115.6816	Golden Road-N	Idaho	-	-	20	30	-	Right of way/ Riverbank
22	45.8121	-115.6816	Golden Road-S	Idaho	-	-	-	16	-	Right of way
23	45.8034	-115.6958	IDT Pullout	Idaho	-	30	19	-	-	Right of way/ Riverbank
24	45.3755	-114.0903	Salmon-Challis	Lemhi	-	15	-	30	-	*
25	44.8986	-116.0905	Mc Call IDL	Valley	-	-	30	30	-	*
26	44.3285	-112.2041	Spencer- Private land	Clark	-	-	5	30	-	Rangeland
27	44.3283	-112.1890	Spencer Ranch	Clark	-	-	30	30	-	Rangeland
28	44.1341	-111.3060	Mesa Falls Rd	Fremont	-	30	30	30	-	Open forest
29	44.0730	-115.5680	Lowman	Boise	-	30	30	30	-	Open forest
30	43.8250	-111.9180	Cartier Slough	Madison	-	30	30	30	-	Open forest
31	43.8170	-115.8250	Idaho City	Boise	-	30	30	30	-	Open forest
32	43.7812	-114.5050	Mountain View	Blaine	-	30	20	30	-	*
33	43.7341	-114.3702	Lake Creek	Blaine	-	26	20	30	-	Open forest
34	43.7198	-114.3781	HWY 75	Blaine	-	30	20	30	-	*
35	43.5969	-111.6400	South Fork	Bonneville	-	30	30	30	-	Open forest
36	42.6950	-114.2908	BLM Gate	Jerome	-	5	30	-	-	*
37	42.1493	-114.9929	Jarbige	Twin Falls	-	3	-	-	-	*

- indicates no plants collected from the site in a given year, * indicates no information available for habitat type

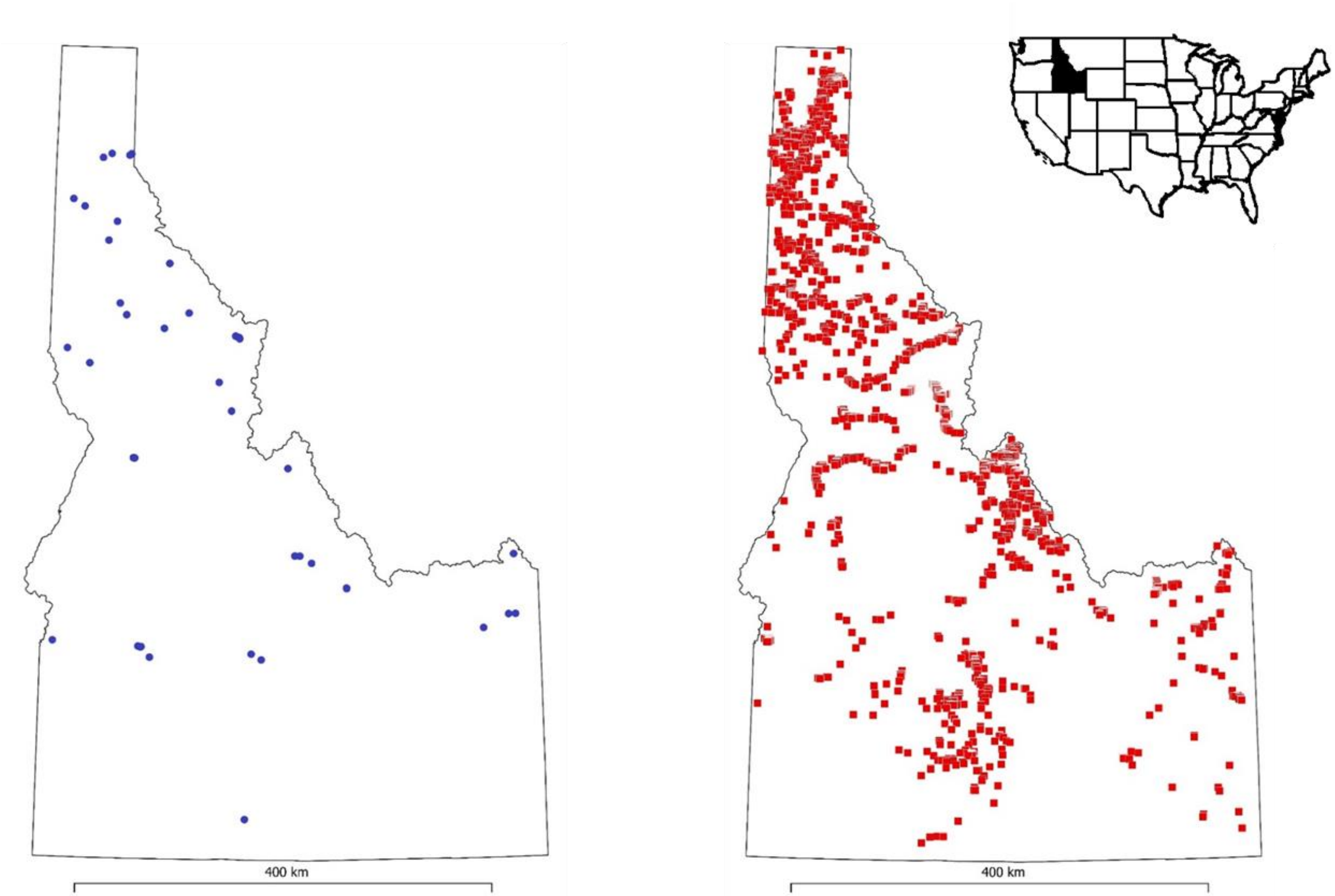
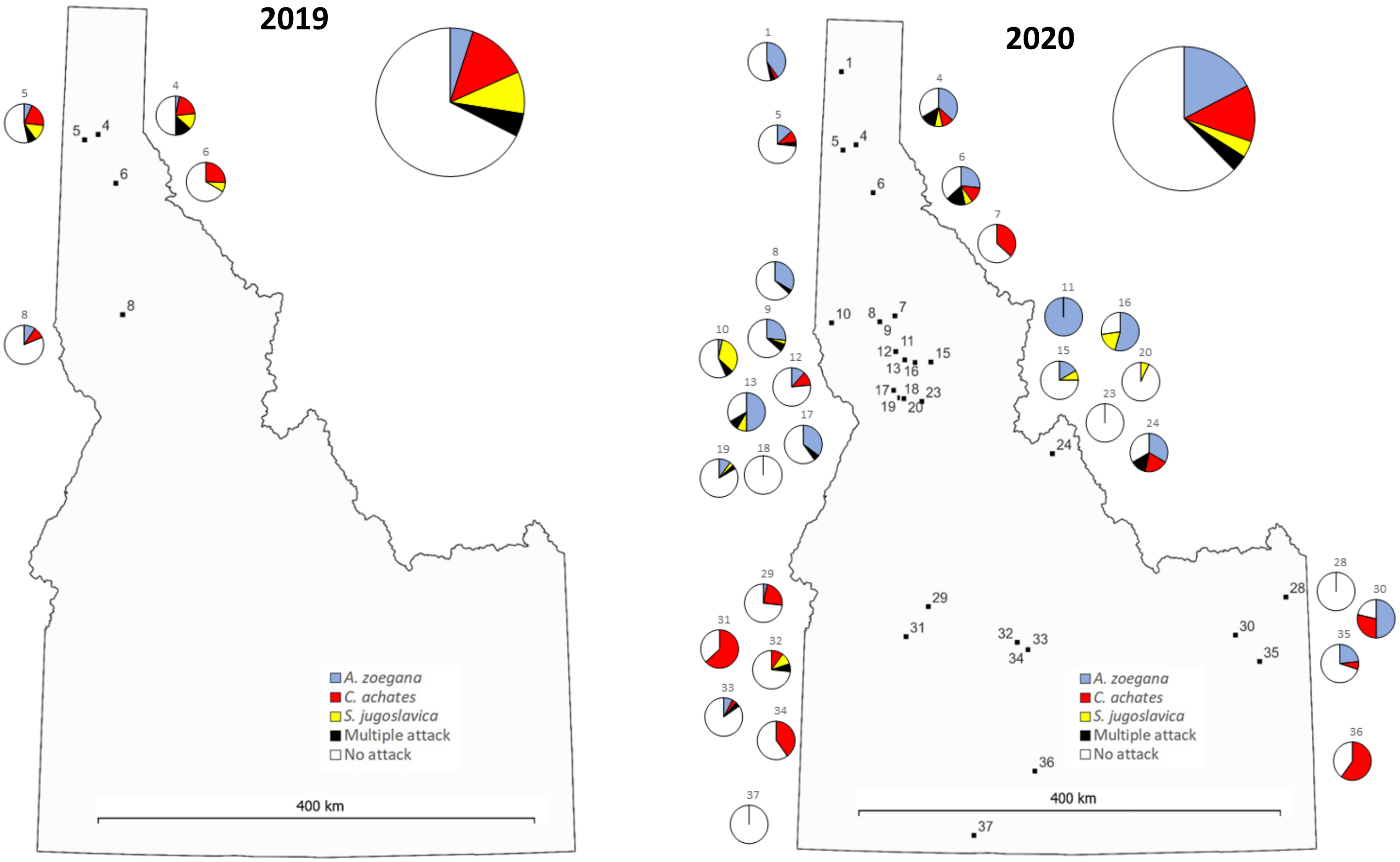


Fig. 2.1: Documented releases of *Agapeta zoegana* and *Cyphocleonus achates* in Idaho between 1993-2021. *A. zoegana* (blue symbols): n = 49 releases, ∑8,501 moths. *C. achates* (red symbols): n = 3,760 releases, ∑428,910 weevils. Data source: Nez Perce Tribe Bio-Control Center, Lapwai, Idaho, USA.


2019
2020
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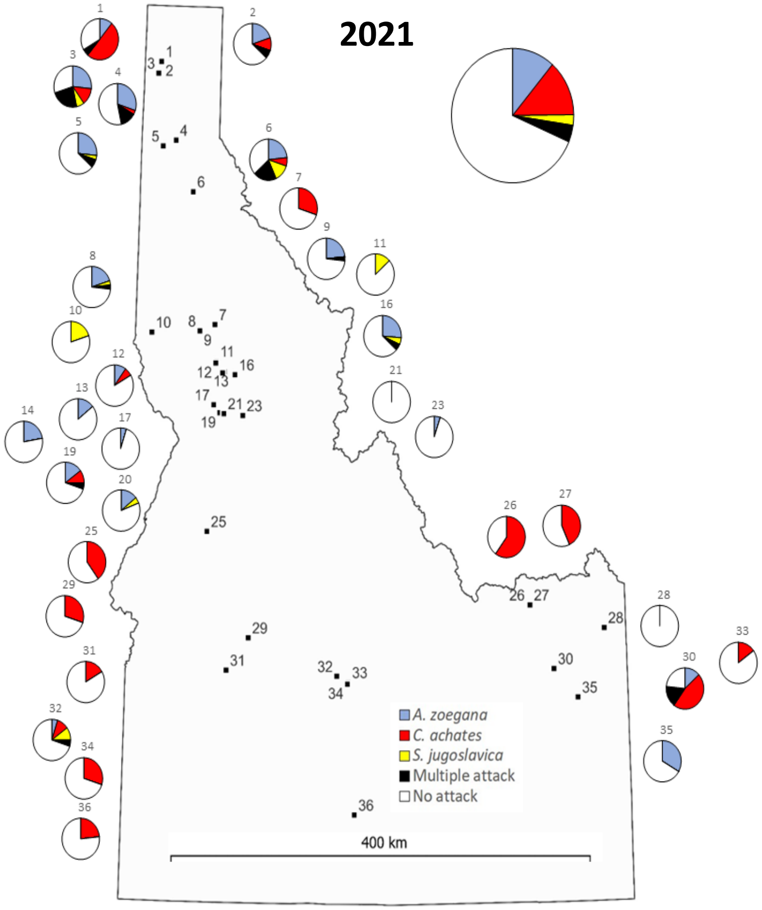
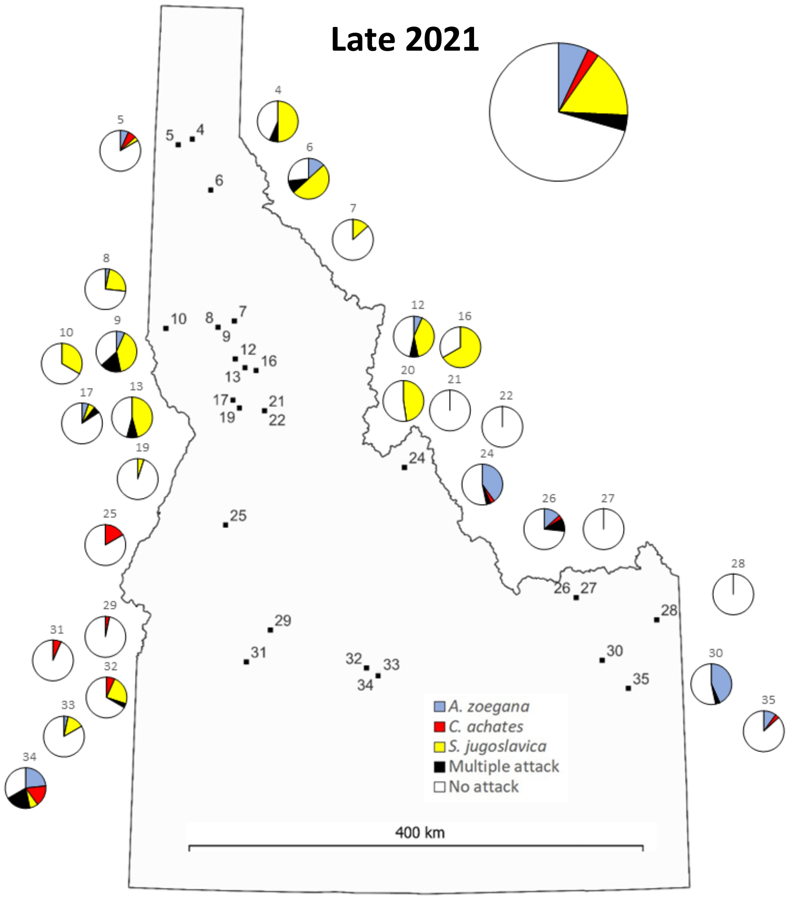
36
37
A. zoegana
C. achates
S. jugoslavica
Multiple attack
No attack
400 km


2021
A. zoegana
C. achates
S. jugoslavica
Multiple attack
No attack
400 km


Late 2021
A. zoegana
C. achates
S. jugoslavica
Multiple attack
No attack
400 km

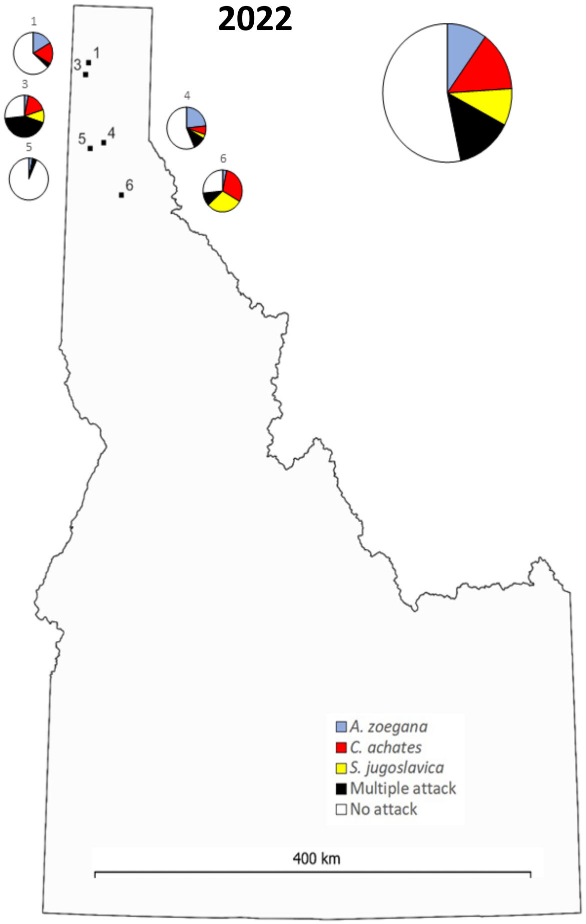


Fig. 2.2: Visualized summary of proportions of *Centaurea stoebe* plants at 37 study sites in Idaho with belowground herbivory by *Agapeta zoegana* (blue), *Cyphocleonus achates* (red), *Sphenoptera jugoslavica* (yellow), multiple species (black), or without root herbivores (white), between 2019 and 2022. Larger pie charts represent the mean attack for the respective census dates. (See text and Appendix A for additional information).

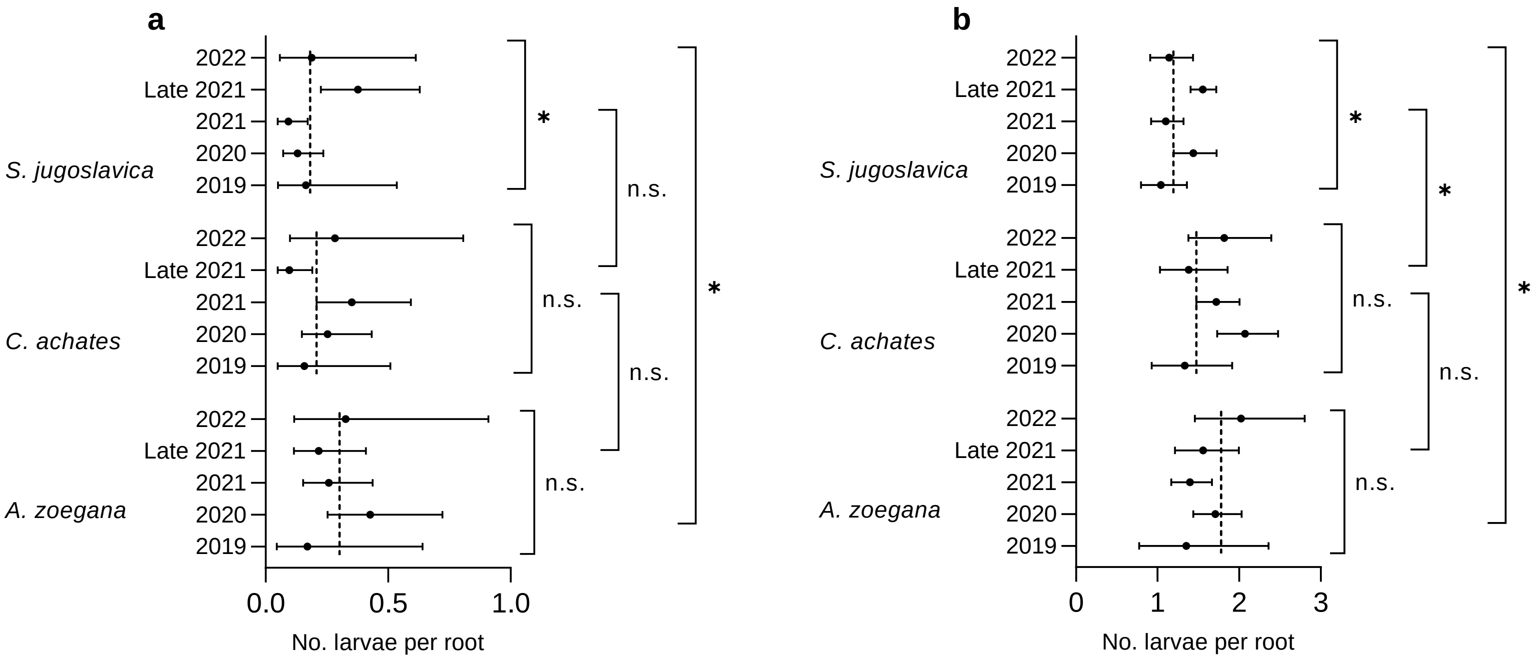


Fig. 2.3: Intensity of root herbivory by *S. jugoslavica*, *C. achates*, and *A. zoegana* in *C. stoebe* roots at five census dates between 2019 - 2022. Mean ± CI number of larvae per root including unattacked roots (a) or excluding unattacked roots (b) at 37 field sites in Idaho. Vertical dotted lines represent the average across the five census dates. Inner brackets test for differences within species among census dates, and brackets test for differences between species. *, $P < 0.05$; n.s., not significant (See text, Appendix B, C, D, and E for further details).

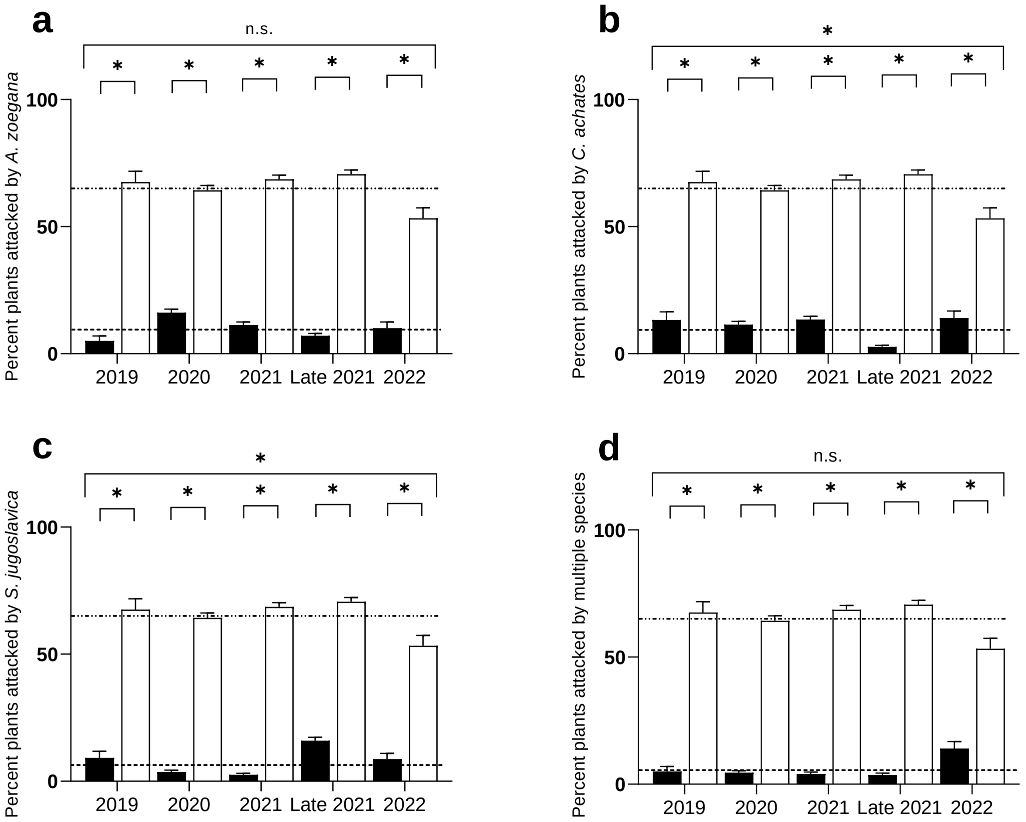


Fig. 2.4: Percentage plants (± SE) attacked (black bars) by *A. zoegana* (a), *C. achates* (b), *S. jugoslavica* (c), and plants attacked by multiple species of herbivore (d) versus plants not attacked by any of the biological control agents (white bars). Horizontal dotted lines represent the overall average for respective percentages of attacked and unattacked plants. Lower brackets indicate whether percentages between attacked and unattacked plants differed in a given year, and upper brackets indicate whether herbivory percentages differed across the study period. *, $P \leq 0.05$; n.s.: not significant (See text and Appendix F for details).

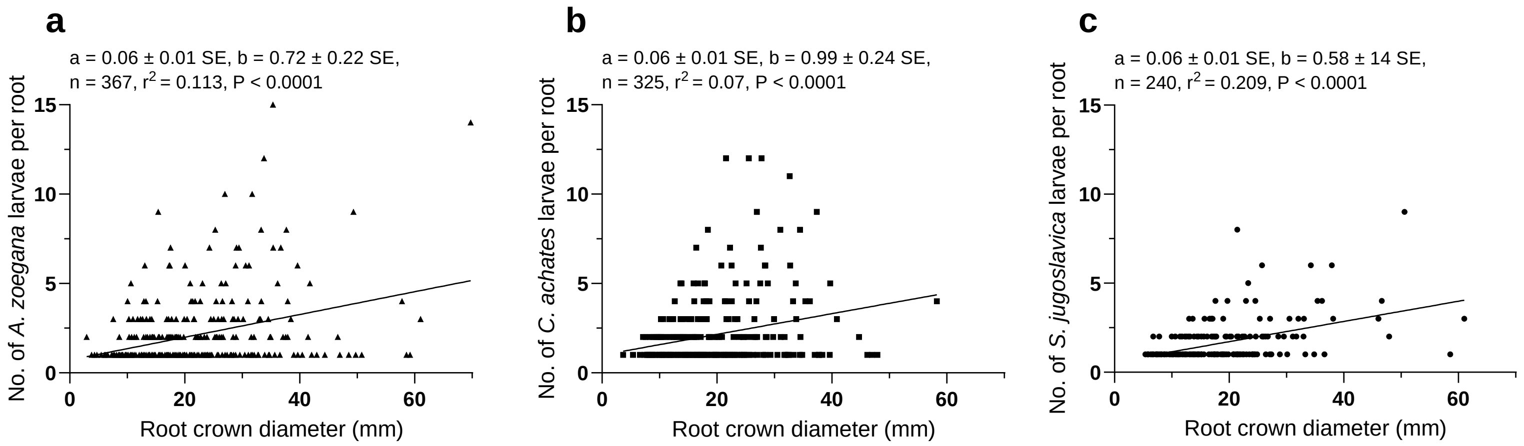


Fig. 2.5: Relationship between root size measured as root crown diameter and the number of larvae of *A. zoegana* (a), *C. achates* (b), and *S. jugoslavica* (c) found in roots.

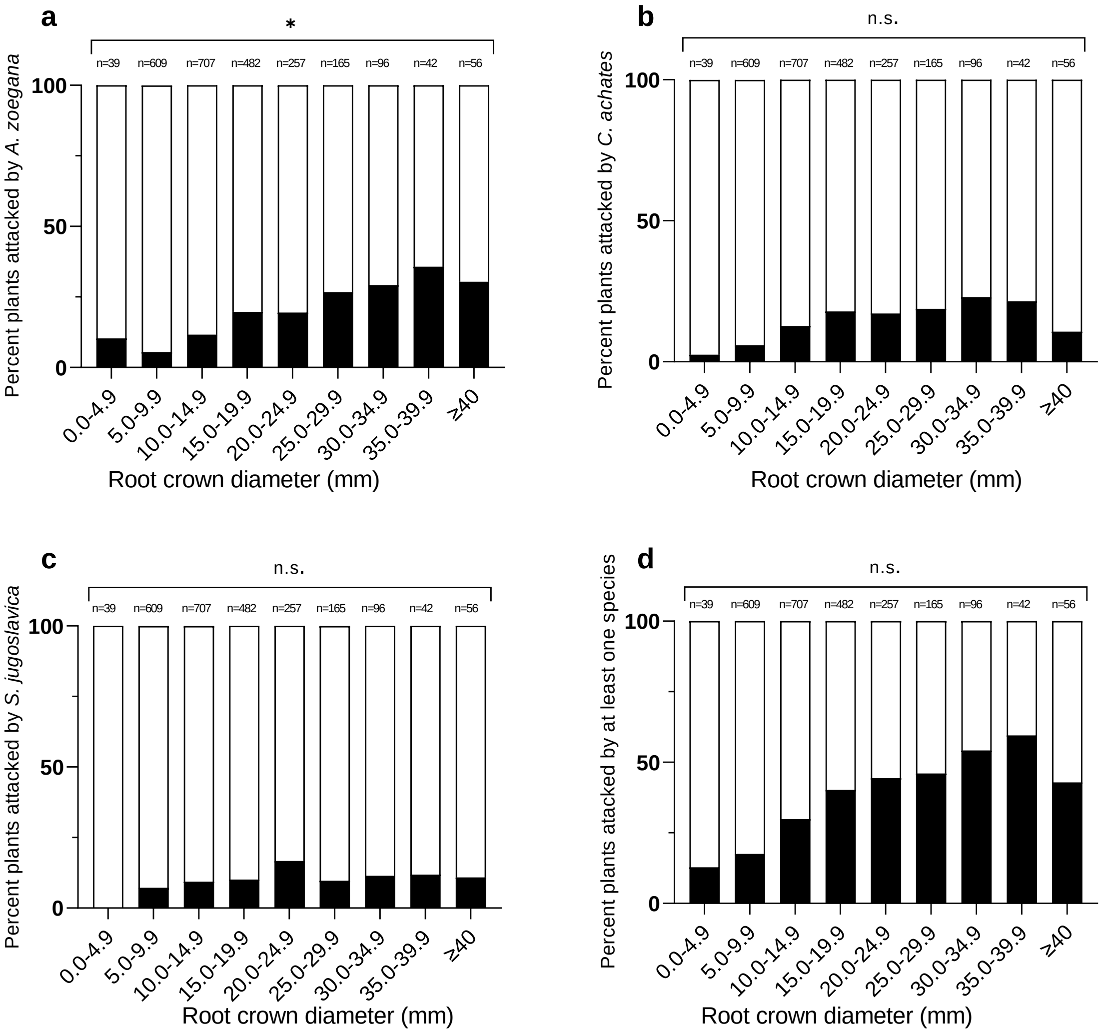


Fig. 2.6: Percent *C. stoebe* roots attacked by *A. zoegana* (a), *C. achates* (b), *S. jugoslavica* (c), and cumulative attack by at least one herbivore (d) in nine root crown diameters classes. Brackets indicate a test for differences of attack between root size classes*, $P \leq 0.05$; n.s., not significant.

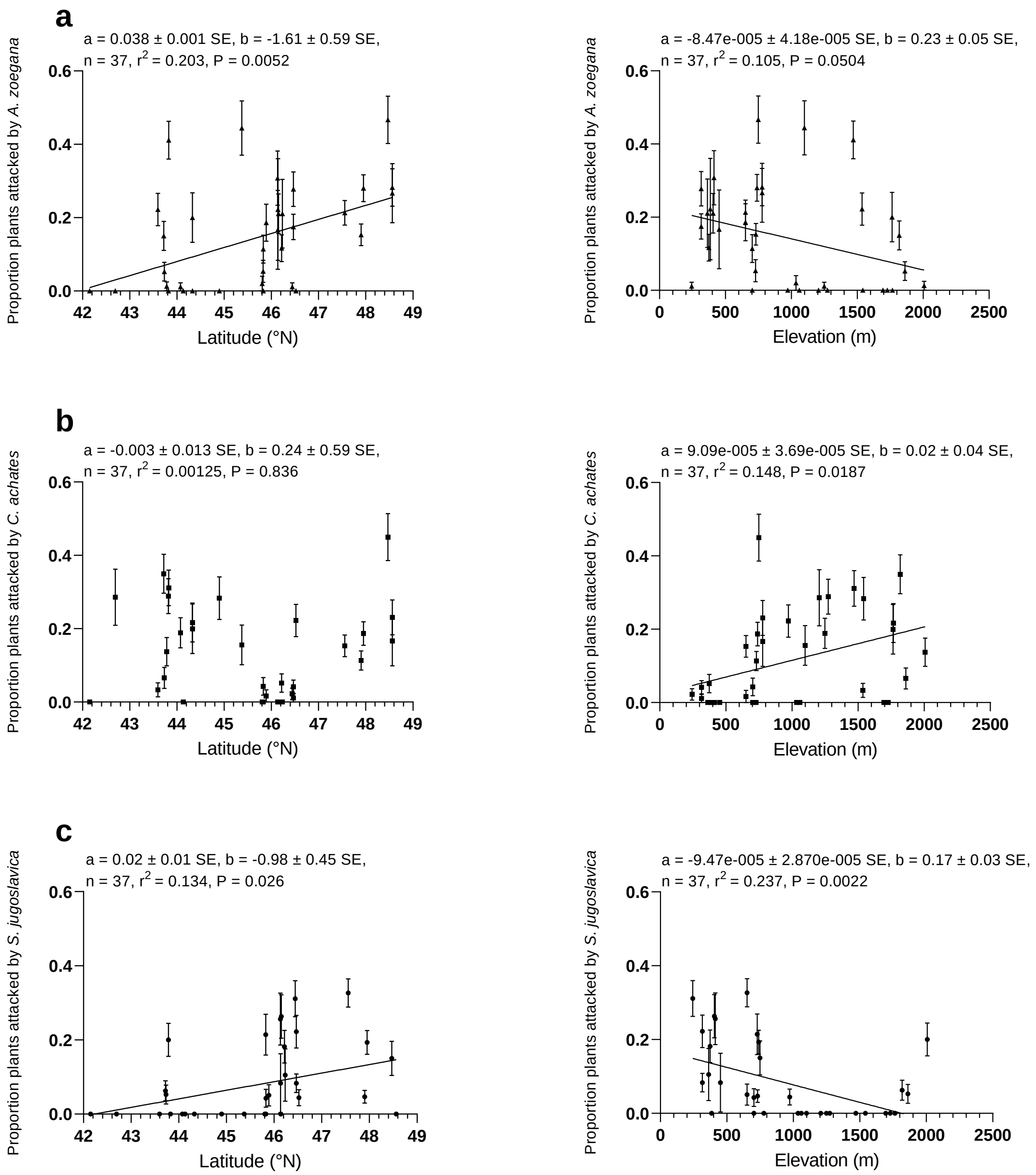


Fig. 2.7: Relationship between latitude (left graphs) and elevation (right graphs) of *C. stoebe* study sites and the proportion of *C. stoebe* plants attacked at respective sites by *A. zoegana* (a), *C. achates* (b), *S. jugoslavica* (c).

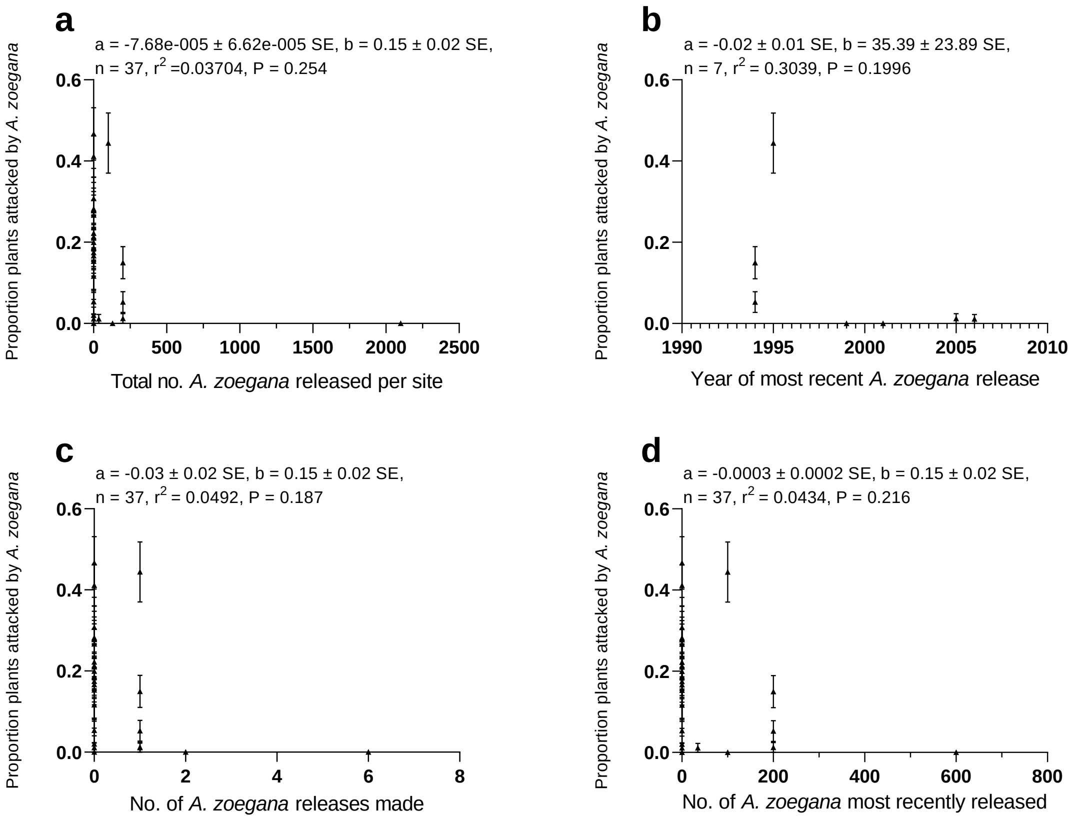


Fig. 2.8: Relationship between the proportion of *C. stoebe* plants attacked by *A. zoegana* at field sites with the total number of insects released at or nearby respective sites (a), the year of the most recent release (b), the total number of releases made (c), and the number of individual insects most recently released (d).

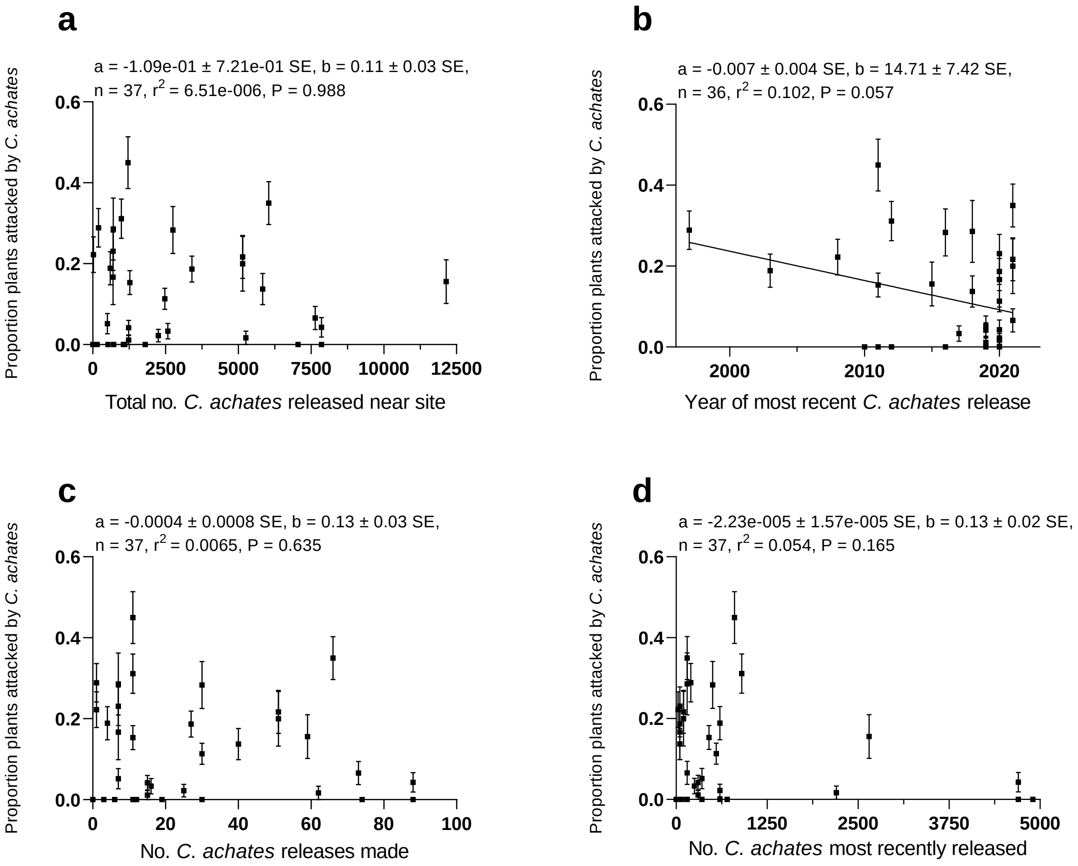


Fig. 2.9: Relationship between the proportion of *C. stoebe* plants attacked by *C. achates* at field sites with the total number of insects released at or nearby respective sites (a), the year of the most recent release (b), the total number of releases made (c), and the number of individual insects most recently released (d).

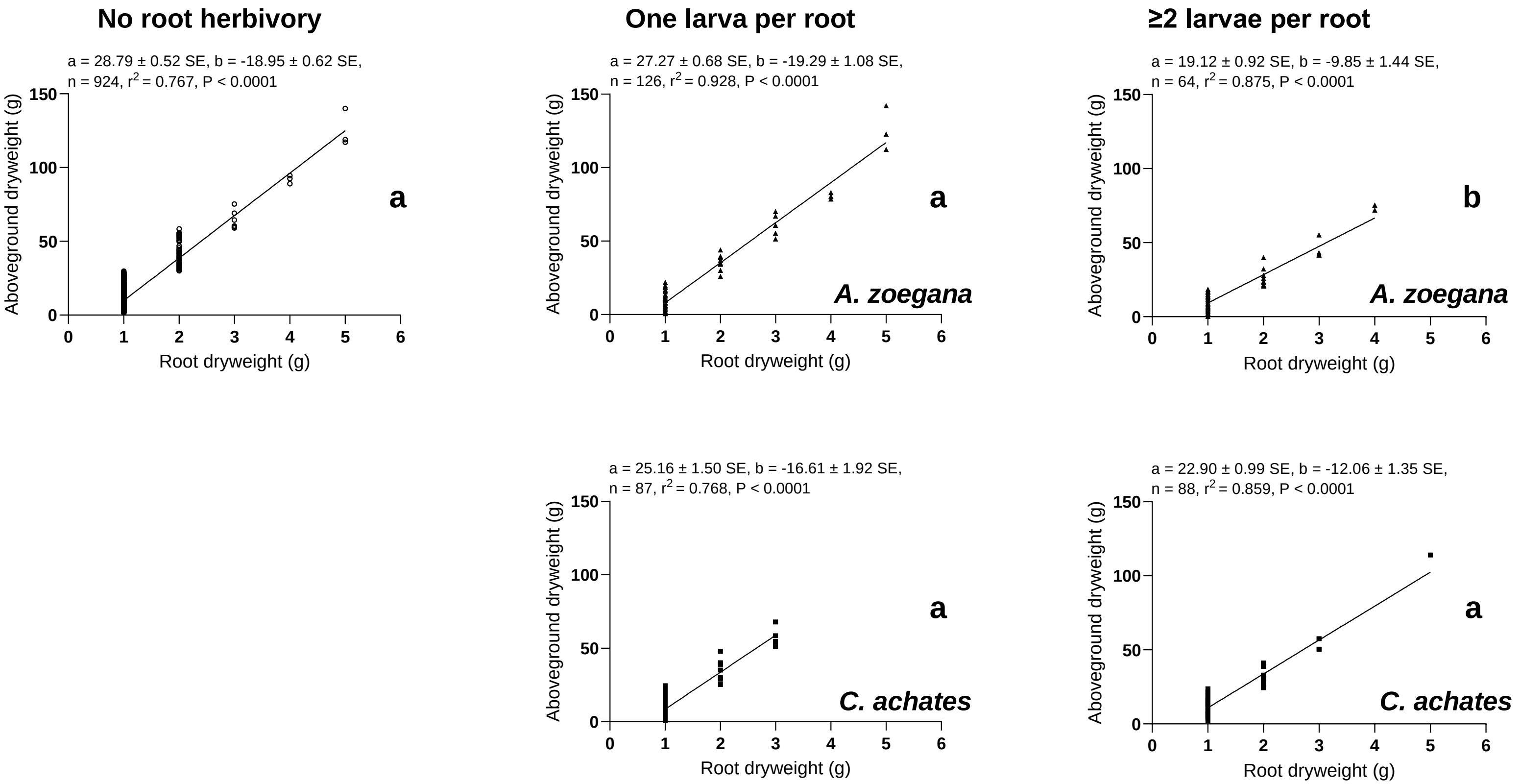


Fig. 2.10: Relationship between *C. stoebe* root dry weight and aboveground dry weight for plants not attacked by root-mining biological control agents (left graph), attacked by one larva of *A. zoegana* and *C. achates* respectively (graphs center column), and attacked by two or more *A. zoegana* and *C. achates*, respectively (graphs right column). Differing letters to the right of regression models denote significant differences between slopes of respective graphs, $P < 0.05$ Generalized linear mixed model. (See text and Appendix N for further details).

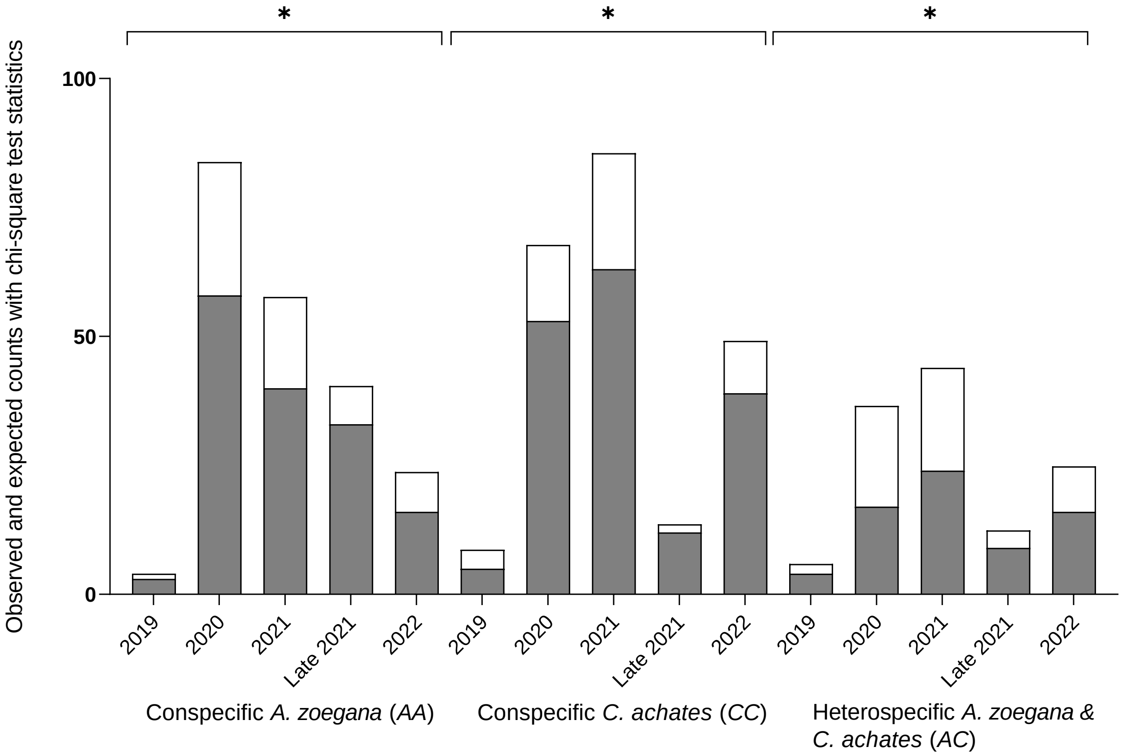


Fig. 2.11: Test of independence within conspecific *A. zoegana* (*AA*), conspecific *C. achates* (*CC*), and between heterospecific *A. zoegana* and *C. achates* (*AC*) across the five census dates. Gray bars represent the observed numbers of each case (*AA*, *CC*, or *AC*), and white bars indicate the expected numbers for each case across years. Brackets indicate chi-square test statistics between observed and expected numbers for each case where years acted as replicates for analysis. df = 4; *, $P < 0.05$. (See Appendix O and text for further information)**.**

Chapter 3: Costs of Greenhouse Propagation for Root Mining Moth *Agapeta zoegana* in Spotted Knapweed Biological Control: A Comparative Feasibility Study

Abstract

Centaurea stoebe L. subsp. *micranthos* is an invasive Eurasian perennial which is a problematic weed across North America. Despite the use of chemical, physical, and mechanical methods, long-term control of *C. stoebe* has been challenging. Biological control using host-specific natural enemies, such as the root-boring moth, *Agapeta zoegana* L. (Lepidoptera: Tortricidae) is a potential solution. This will require large scale releases of the agents. This study aimed to develop a cost-effective greenhouse-rearing method for mass production of adult *A. zoegana*. Three methods: two greenhouses, and one lab rearing from the field collected roots were compared. The rearing costs per moth differed among these methods. The costs for greenhouse-rearing methods were $17.75 per moth using single-plant pots and $13.40 per moth using six-plant tubs. The cost for laboratory rearing from field-collected plants was $14.50 per moth. These costs included expenses associated with labor, consumables, supplies, and the operational costs for rearing. The costs were found to be high due to the short-term production approach, which required larger initial investments. They also included costs for pots and tubs in the greenhouse from which no moths emerged. If only pots and tubs where emergence occurred are considered, the costs per moth would be $3.61 per moth for those reared in single-plant pots and $10.42 per moth for those reared in tubs. The emergence of the moth per infected root was highest for the pots and lowest for those reared in lab. Using a method developed as part of this study to sex the moths based on cloacal apertures, this study also investigated the external morphology of moths to distinguish between sexes to determine if the rearing methods affected the sex ratio of

emerging adults. No bias was found. This study indicates that rearing *A. zoegana* as a biological control agent using the methods tested here would be expensive. Based on these results, however, an improved, less labor intensive and more cost-effective method using large tubs could be pursued. Fine-tuning the rearing methods based on these findings will be crucial in mitigating mortality rates.

Introduction

Mass rearing of beneficial insects involves the cost-effective production of a large number of disease-free individuals on a production line to maximize fertile females' production efficiency and minimize labor and space requirements (Finney and Fisher, 1964; Moran et al., 2014). Mass rearing of weed biological control agents (BCA hereafter) can be an essential element of classical biological weed control (BCW hereafter) since the success of programs depends on the areawide establishment and quick population increase of BCA populations to maximize their impact on the target weed (Halbritter and Wheeler, 2019; Harms et al., 2020). Consistent and frequent releases of the largest possible number of weed BCA enhances the likelihood of establishment and reduces the time required for adequate control (Hill et al., 2021). Weed BCAs have high benefit-to-cost ratios, and mass rearing can provide benefits for BCA population expansion especially for those agents that are univoltine or have a long development time, offering additional economic and environmental benefits (Gilkeson, 2019; Moran et al., 2014; Page and Lacey, 2006; Van Wilgen and De Lange, 2011).

Despite the potential for mass-rearing insects for weed biological control, it is uncommon to do so, as the primary method involves dispersing agents through human intervention or natural means across different field sites (Van Driesche, 2002; Van Driesche et al., 2010).

Also, the mass production of weed BCAs in non-crop areas lacks significant commercial motivation (Moran et al. 2014). During the 1920s and 1930s, Australia executed an immensely successful program against prickly pear cacti, marking the pioneering utilization of mass-rearing techniques against weeds (Dodd, 1940; Goeden and Andres, 1999). Several successful examples of mass-rearing weed BCAs using field cages or greenhouse using potted or planted weed specimens exist, such as *Galerucella calmariensis* L. and *G. pusilla* Duft. (Coleoptera: Chrysomelidae) (Blossey and Hunt, 1999), *Mogulones crucifer* Pallas (Coleoptera: Curculionidae) (Smith et al., 2009), *Omphalapion hookeri* Kirby (Coleoptera: Apionidae) (McClay and De Clerck-Floate, 1999), *Carvalhotingis visenda* Drake and Hambleton (Hemiptera: Tingidae) (Dhileepan et al., 2010), *Evippe* spp. (Lepidoptera: Gelechidae), *Prosopidopsylla flava* Burkhardt (Hemiptera: Psyllidae) (van Klinken et al., 2003), *Cyphocleonus achates* Fahr. (Coleoptera: Curculionidae) (Story et al., 1996; Story and White, 2010), *Agapeta zoegana* (Story et al., 1994), *Pseudophilothrips ichini* Hood (Thysanoptera: Phlaeothripidae) (Cuda et al., 2008; Halbritter et al., 2021), and *Tetramesa romana* Walker (Hymenoptera: Eurytomidae) (Moran et al., 2014). These mass-reared BCAs have successfully established field populations and demonstrated significant impact, indicating the effectiveness of mass-rearing approaches. Successful rearing of weed BCAs on an artificial meridic diet includes root-feeding weevils *Hylobius transversovittatus* Goeze (Coleoptera: Curculionidae) and *C. achates* (Blossey et al., 2000; Goodman et al., 2006; Tomic-Carruthers, 2009). Although some weed BCAs can be reared effectively using alternate hosts, or prey species, there is limited data on the propagation of weed BCAs (Blossey et al., 2000; Bolckmans et al., 2015; Hill et al., 2021; Moran et al., 2014). The studies exploring the costs associated with mass- rearing BCAs are relatively scarce.

Regarding univoltine species like *A. zoegana*, *C. achates*, and *M. crucifer*, the previous research has indicated rearing costs ranging from US $0.08 to $2.00 per individual (Smith et al., 2009; Story et al., 1996, 1994). Rearing *H. transversovittatus* on artificial diet incurred a cost of $0.50 (Blossey et al., 2000). Conversely, multivoltine BCAs suitable for outdoor rearing, like *Hydrellia pakistanae* Deonier (Diptera: Ephydridae) and *Cyrtobagous salviniae* Calder (Coleoptera: Curculionidae) were reared at remarkably low costs of $0.002 to $0.008 per insect, respectively (Harms et al., 2009a, 2009b). In case studies where distribution costs were included alongside rearing expenses, the cost per individual of BCAs ranged from $0.06 to $8.40 for *Megamelus scutellaris* Berg (Hemiptera: Delphacidae) in South Africa and *Neolema ogloblini* Monros (Coleoptera: Chrysomelidae) in New Zealand (Moran et al., 2023). Furthermore, rearing *T. romana* in a greenhouse incurred a cost of approximately $0.25 per insect (Moran et al., 2014).

Spotted knapweed (*Centaurea stoebe* L. subsp. *micranthos* (Gugler) Hayek (Asteraceae), syn. *Centaurea maculosa*) is indigenous to Europe (Ochsmann, 2000; Sheley et al., 1998). It is a short-lived herbaceous perennial that forms rosettes with deep taproots averaging over 30 cm long (Sheley et al., 1998; Story et al., 2000; Watson and Renney, 1974). It was first introduced in North America in Victoria, British Columbia, in 1893 (Groh, 1944), from Europe and has since spread throughout the United States and Canada (Lejeune and Seastedt, 2001; Sheley et al., 1998; Watson and Renney, 1974). Over the past 40 years, it has been the focus of extensive study and control efforts due to its aggressive invasiveness, resulting in the domination of rangelands and grasslands (Carson et al., 2014). It poses a challenge for management and restoration due to the deep taproot, high seed production rate, long seed viability in the soil, and competitive nutrient uptake (Carey et al., 2004; Davis et al., 1993;

DiTomaso, 2000; Herron et al., 2001; Schirman, 1981). *Centaurea stoebe* populations can be managed using cultural control (enhancing plant competition or preventing weed introduction), mechanical control (tillage, mowing, hand-pulling), herbicides, prescribed fire, ungulate grazing, and biocontrol (Ainsworth, 2003; DiTomaso, 2000; Ditomaso et al., 2006; Knochel and Seastedt, 2009). However, these methods have proven laborious, time-consuming, unsustainable, or posing an environmental threat (Ditomaso et al., 2006; Lutgen and Rillig, 2004; MacDonald et al., 2013; Mangin and Hall, 2016). An alternative to these methods is classical biological control, which involves reuniting host-specific, co-evolved natural enemies of the weed from their native range (McFadyen, 1998; Schwarzländer et al., 2018). It has been proposed that a combination of biocontrol agents, typically occupying different niches, leads to cumulative stress, which best controls a target weed (Knochel and Seastedt, 2009; Müller-Schärer and Schroeder, 1993; Seastedt et al., 2007; Seastedt and Knochel, 2021; Story et al., 2008).

For *C. stoebe*, 13 BCA species have been introduced into the United States between 1973 and 1993, including eight seed-head-feeding insects and five root-mining insects (Müller-Schärer and Schroeder, 1993). The seed head weevil *Larinus minutus* Gyll. and *Larinus obtusus* Gyll. are two of the most widely released BCAs of *C. stoebe* (Myers et al., 2009; Stephens and Myers, 2013; Story et al., 2008). Seed-head feeding BCAs have significantly reduced *C. stoebe* seed production in field populations (Story et al., 1991b, 2008, 1989; Story and Piper, 2001b). However, for the effective management of *C. stoebe*, seed reduction needs to be combined with BCAs causing plant mortality outright, such as root mining biocontrol agents (Blossey and Hunt-Joshi, 2003; Knochel and Seastedt, 2010; Maines et al., 2013; Seastedt et al., 2007; Seastedt and Knochel, 2021; Story et al., 2008). Even though the establishment rates and impact of belowground herbivores for BCW are the same as or higher than those for aboveground herbivores, root mining BCA are understudied and are more difficult to develop due to their hidden life history (Blossey and Hunt-Joshi, 2003; Knochel and Seastedt, 2009). Three root-mining insects released for the biological control of *C. stoebe* are well established in the United States, i.e., *Sphenoptera jugoslavica* Obenberger (Coleoptera: Buprestidae), *Cyphocleonus achates* Fåhraeus (Coleoptera: Curculionidae) and *Agapeta zoegana* L. (Lepidoptera: Tortricidae) (Winston et al., 2014). The root-mining moth, *Pelochrista medullana* Staudinger (Lepidoptera: Tortricidae), and the root-mining moth *Pterolonche inspersa* Stgr. (Lepidoptera: Pterolonchidae) are either considered not established or the abundance is limited in the U.S (Winston et al., 2014).

Agapeta zoegana L. (Lepidoptera: Tortricidae) is a root-feeding BCA of *C. stoebe* introduced from Austria and Hungary to the U.S. for its management (Müller-Schärer and Schroeder, 1993; Story et al., 2000). In North America, *A. zoegana* has one generation per year, while in its native European range, it can be bivoltine or even have three generations per year (Müller et al., 1988). Adult moths emerge from June through August, mate, and lay their eggs singly or in clusters on the leaves and stems of *C. stoebe* (Corn et al., 2009; Story et al., 1991a). Females can lay between 150 and 400 eggs in their lifetime (Fitzpatrick, 1989). The larvae of *A. zoegana* feed behind a silken web on root cortex tissues, overwinter and pupate in roots, and emerge as adults during the following late spring and summer (Müller et al., 1989; Story et al., 1991a). Female moths typically mate on the same day they emerge and start laying eggs on the second night (Fitzpatrick, 1989; Story et al., 1991a). Several laboratory and greenhouse studies have shown that *A. zoegana* can reduce the survival of *C. stoebe* rosettes (Müller, 1989b; Müller et al., 1988; Muller-Scharer, 1991). *A. zoegana* was found to decrease the height, aboveground biomass, number of stems, and number of capitula of *C. stoebe* during the post-release assessment at the field release site of the moth (Story et al., 2000). There were some indications that *A. zoegana* may be decreasing the density of bolted plants (Story et al., 2000). Studies also suggest that *A. zoegana* could decrease *C. stoebe* plant vigor at higher herbivory densities and following multiple years of attack (Corn et al., 2007; Ridenour and Callaway, 2003; Story et al., 2000).

The successful colonization of *A. zoegana* in the U.S. cost $1.3 per insect using field cages (Story et al., 1994). Artificial diets designed for *Choristoneura fumiferana* Clem. (Lepidoptera: Tortricidae) were effective for rearing *A. zoegana*, providing valuable data on larval survival for future mass-rearing strategies (Raina et al., 2006). However, field cage rearing was a preferred propagation method for maintaining a synchronized and cold-hardy population of *A. zoegana* (Story et al., 1994). To achieve higher herbivory levels, releasing more than one pair of moths per spotted knapweed plant has been suggested for field cage-rearing purposes (Powell et al., 2000). Prior to implementing a rearing program for a BCA, it is crucial to acquire a comprehensive understanding of the biology of the both the agent and the targeted invasive species, to develop effective methods for rearing both organisms on a larger scale (Sørensen et al., 2012).

The moth is not readily available for collection and redistribution, in part because of its nocturnal life history (Fitzpatrick, 1989). Additionally, *A. zoegana* is a univoltine species with a limited collection window due to its annual production of only one generation, appearing for about three months (Müller et al., 1988; Story et al., 1991a). Moreover, adults cannot be recollected in the field as the moths are too delicate to be collected by sweep netting and are unlikely to be attracted to sugary baits (Fitzpatrick, 1989). Consequently *A.*

zoegana redistribution has been limited. The database Nez Perce Bio-Control Center in Lapwai, Idaho documents only 49 releases of *A. zoegana* with a total of 8,501 moths between 1993 and 2021 in Idaho. On the other hand, another root-mining BCA of spotted knapweed, the weevil *Cyphocleonus achates* Fahr. (Coleoptera: Curculionidae) has been released 3,760 times with 428,910 weevils during that period. Therefore, *A. zoegana* rearing would be helpful for the propagation of the moth for redistribution.

In this study, we describe and compare laboratory and two greenhouse rearing efforts for *A. zoegana* and their associated costs to boost BCA numbers available for *A. zoegana* redistribution. Specifically, we conducted an evaluation of the resources needed to rear moths from field-collected plants in a laboratory setting, and the resource requirements for moths reared from plant transplants placed in individual pots, as well as those placed in tubs containing multiple plants in the greenhouse.

Materials and Methods

To investigate the feasibility of providing larger numbers of *A. zoegana* for field releases, costs for three rearing and propagation methods were compared. These were 1) the collection and subsequent rearing of moths from infested plants in the field, 2) rearing of *A. zoegana* on individually potted plants in a greenhouse, and 3) rearing of *A. zoegana* in larger tubs including six plants in the greenhouse. All experiments were conducted in entomological laboratories at the University of Idaho or in greenhouses at The University of Idaho Parker Research Farm, both in Moscow, ID.

Rearing of *A. zoegana* from infected plants collected in the field

To rear *A. zoegana*, *C. stoebe* plants were collected at field sites at the time mature larvae were expected in the roots. In 2019 and 2020, respectively, roots were collected from four *C. stoebe* infestations in northern Idaho. These sites were Farragut (47.95219° N, -116.606° W), Brunner's Road (47.90331° N, -116.768° W), Cataldo (47.55361° N, -116.367° W) and Priest Lake (48.56372° N, -116.826° W). Collection of plants took place in 2019 and in 2020. In 2019, a total of 349 *C. stoebe* plants were collected (Farragut, n=254, Brunner's Road, n=15, Cataldo, n=80) and in 2020, a total of 359 plants were collected (Farragut, n=139, Brunner's Road, n=3, Cataldo, n=188, Priest Lake, n=29). Plant collections focused on large plants as *A. zoegana* has been reported to preferably attack large plants (Smith and Story, 2003). Plants were bagged in black plastic contractor bags (114 cm height, 55 cm width, 0.07 mm thick), and transferred in the cooler to the University of Idaho within the same day of collection.

At the University of Idaho, a plant rearing substrate was prepared consisting of a 1:2-part mix of washed playground sand and vermiculite (Thermo-rock West, Inc, Chandler, AZ). Approximately 2 l of purified water were added to approximately every 16 l of substrate and mixed to moisten the substrate and to prevent *C. stoebe* roots from drying out prematurely. 1.89 l plastic containers (Glad® Deep Dish Food Storage Container with interlocking lids; 21.9 cm length, 16.3 cm width, 10 cm height) were used to store *C. stoebe* roots collected in the field. The aboveground biomass of field-collected plants was trimmed, and the root crown diameter of each root was measured twice to obtain orthogonal diameters. Measurements were taken one centimeter below the root crown using an electronic digital caliper with a precision of 0.01 mm (Model 01407A, Neiko, China). All roots were placed individually in labeled containers and buried in the moistened sand-vermiculite mixture to preserve root tissues. Lids were punctured with a pinning needle to promote aeration and prevent mold growth. All containers were arranged on six-tiered shelving units and maintained in the laboratory at 24 ºC, with a light dark cycle of 16:8 hours. Moth emergence was monitored daily for all containers. Morphological differences, including variations in abdomen shape and cloacal apertures, have proven to be useful in distinguishing between male and female *A. zoegana* (Fitzpatrick, 1989; Powell et al., 2000). To determine the gender of each moth, observations were made immediately after emergence under a stereomicroscope. Given the lack of existing visual references for sexing *A. zoegana*, sketches were included depicting the ventral surface of the abdomen of both male and female moths (Fig 3.1).

Greenhouse rearing

The experiments were carried out in 2019 and 2020. In general, plants sown in 2018 were used for greenhouse rearing experiments in 2019. For cost estimates, 2020 plant germination was used as the benchmark data because of the similarity in costs associated with the supplies and labor, as well as the utilization of same seed during the germination process.

To rear *A. zoegana* in greenhouses, a combination of newly emerged adults obtained from the previous year's greenhouse rearing and *A. zoegana* adults obtained from the laboratory rearing of field collected infested roots were used. In the first year of the study, experimental setups established by a previous graduate student were used solely for collecting moths from the greenhouse, not for recording purposes. One pair of moths irrespective of being greenhouse or laboratory-reared moths were set up in a mating cage for 72 to 96h immediately following their emergence. The mating cage was constructed from a 7.5 l translucent polypropylene food storage container with a round bottom (27.5 cm height, 18.6 cm diameter), lined with paper towels and fitted with a mesh cloth circular lid for ventilation. The mating cages were kept in laboratory at ambient temperature of 24 ºC and an L:D of 16:8 hours. While it is not common for the moths to feed on nectar (Fitzpatrick, 1989), a 10% sucrose solution in 3 ml petri dishes and fresh *C. stoebe* flowers were provided to the moths in mating cages. The flowers were inserted into 125 ml Erlenmeyer flasks that were half-filled with water, creating an environment conducive to mating behavior. It was observed that the moths utilized the provided resources. To ensure their availability, the sucrose solution and flowers were replenished every 48 hours. Each mating cage was appropriately labeled with relevant information about the moths, enabling the tracking of the breeding population.

For greenhouse propagation of *A. zoegana*, all *C. stoebe* plants were grown from seeds collected in 2015 from a population in Moscow, Idaho (46.7317° N, -116.9983° W). Seeds were germinated in January 2020 in black plastic pots with a volume of 0.6 l, 12 cm height, 10 cm width, and 8 cm diameter (Grower's solution, Cookeville, TN) using a soil mix comprising one part washed playground sand and three parts of Pro-Mix BX Mycorrhizae (Premier Tech, Quakertown, PA). Trace elements (2.5 g), chelated iron (1.25 g), limestone (47.5 g), triple super phosphate (47.5 g), and Osmocote® (187.5 g) were added to every 12 kgs of the sand-soil mixture. Pots were placed on a tray and maintained at 16 ºC during the day and 11 ºC at night, with a 12:12 h L:D cycle in a greenhouse at Parker Research Farm. Trays were watered as needed, considering the observed seed germination rate of 80% to 85%.

All seedlings were transplanted after 10 weeks during the last week of March 2020 into individual pots or tubs (see below) using the same aforementioned soil mix. Following transplant, plants were maintained at 18:11 ºC and 12:12 h L: D.

Individual pot propagation

Black plastic tree seedling pots (T-pot Three, Stuewe and Sons, Inc, Tangent, OR, USA) with a volume of 7.6 l, 19.7 cm diameter, and 31.8 cm height were used. Pots were filled with the soil mix, to 5 cm below the pot rim. Metal wires (approximately 70 cm long) were bent into a U-shape and pushed into pots to support a mesh cover. A regular mesh paint strainer with a volume of 18.92 l (28.9 cm height, 25.4 cm width, Trimaco Supertuff, Morrisville, NC) was used to cover the pot from the top. A garden strap (Velcro® Brand One Wrap® Garden ties, Manchester, NH) was used to tie the mesh cage to the pot.

In 2019, 42 individually potted *C. stoebe* propagated since 2018 were used for the rearing. Between July 18 and August 16, 2019, one mated pair of *A. zoegana* were released onto each of the 42 mesh covered plants. In 2020, 76 individually potted *C. stoebe* received one pair of *A. zoegana* moths between June 29 and August 6, 2020.

All plants were pruned twice throughout the following 12 months, once in the summer and once in the winter to prevent mesh cages from being overgrown with vegetation and to facilitate collection and retrieval of F1 generation moths. Additionally, if any potential predators, such as spiders and ants were spotted within the cage, they were manually crushed by hand.

To collect F1 generation moths, transparent plastic vials (50 ml volume, 8 cm height) were used. The garden strap Velcro bands, which were originally used to secure the mesh cage to the pot, were untied, providing a convenient means for collecting the moths. After the collection, the bands were properly secured to prevent the escape of the moths. At the expected time of moth emergence between late May and mid-August, rearing plants were visually inspected daily for adult moth sightings. Any adult moths discovered were recorded and sexed before being placed in a mating cage.

Plant tub propagation

In addition to the individually potted *C. stoebe*, oval plastic tubs (Tuff Stuff Products KMT 102 Oval Tank, Terra Bella, CA) with a volume of 113.5 l (30.5 cm height, 49 cm width, 104 cm length) were used for the rearing of *A. zoegana*. The setup of tubs was like that of individually potted plants with the following exceptions: When filling tubs with soil mixture, pots were filled to 10 cm from the tub rim. Six *C. stoebe* plants were transplanted into each tub. In 2019, seven tubs were set up for rearing. To cover tubs, PVC pipe and fittings (1.27 cm diameter) was used to create a rectangular frame (65 cm length, 41 cm width, 121 cm height). A ‘Bug Enclosure Mesh’ (D & M Tarps & More LLC, Lewiston, ID) measuring 7 cm length, 42 cm width, 101.6 cm height with a 10 cm elastic bungee incorporated at the bottom seam for proper fit was used to cage plants in the tubs. A cutout of 25 cm was made in the middle of the cage, and stick-on Tape (Velcro® Brand, Manchester, NH), which was used to allow easy release of parental and collection of F1 generation *A. zoegana*. Four pairs of *A. zoegana* were released in each tub. Tubs were set up in 2019 between July 18 and August 16. In 2020, eleven tubs were set up for the rearing of *A. zoegana* between June 29 and July 25.

Comparisons of the cost efficiency of the *A. zoegana* rearing methods

The costs of rearing out *A. zoegana* from field collected plants were compared, rearing them in individual potted plants in a greenhouse, and rearing them in larger tubs that hold six *C. stoebe* plants in a greenhouse on a per moth basis. The costs for all inputs and labor were estimated as precisely and comprehensively as possible to arrive at realistic costs for collecting or rearing the biological control agent. All costs for the 2019 and 2020 rearing efforts were added. Cost estimates were based on consumables, labor, and operational costs excluding expenses for reusable hardware like pots and tubs. Returns and cost efficiency (US dollars/moth) for each rearing method were based on the included costs and the yield in adult moths per pot or tub.

Results

Costs of rearing *A. zoegana* in the laboratory from field collected *C. stoebe* roots

The total cost of rearing moths in the laboratory from field collected *C. stoebe* roots in 2019 and 2020 was $5,117.52. The rearing resulted in 353 emerging moths. This resulted in an average cost of $14.50 per moth reared (Table 3.1, Appendix P). Moths emerged from 32.06% of the rearing containers (n = 227), for an average of 1.56 ± 0.20 SE moths per infected *C. stoebe* root. The overall sex ratio was 1.15: 1 female: male. 67.94% (n = 481) containers had no *A. zoegana* emerging. Labor represented the largest portion of the costs, accounting for 43.29%, followed by supplies and consumables for 27.77%, and operational costs for 22% of the total costs.

Cost of rearing *A. zoegana* in individually potted *C. stoebe* plants and in tubs containing six plants

A total of 207 moths emerged from individually potted *C. stoebe* plants, for a cost of $3,673.80 (Table 3.2, Appendix P). A total of 374 moths emerged from tubs, each containing six *C. stoebe* plants, for a cost of $5,012.47 (Table 3.3, Appendix P). The cost per moth for these two methods was $17.75 for individually potted plants and $13.40 for six-plant tubs. For individually potted plants, moths emerged from 24 (20.34%) of 118 pots used in the experiment. 94 potted plants (79.66%) had no moth emergence. For potted plants with successful emergence, a mean of 8.63 ± 2.11 SE moths emerged from each plant. Moths emerged from 14 (77.78%) of the 18 tubs used in the experiment. For those tubs with successful emergence, a mean of 26.71 ± 4.34 SE moths emerged per tub, or 4.45 ± 0.73 SE moths per plant.

Labor costs were predominant across both rearing methods, comprising 72.35% and 51.45% of the total costs, followed by cost of supplies and consumables accounting for 29.6% and 39.6%, and the remaining portion, 10.85% and 8.60%, was attributed to operational expenses for pots and tubs, respectively.

Sex ratio of *Agapeta zoegana* reared from *C. stoebe* plants propagated in pots or tubs

Moths emerged from potted plants from 7 June to 28 June 2020 (n = 11 pots producing moths). Of the total 123 moths that emerged, the sex ratio was approximately 1:1; 60 (48.78%) males and 63 (51.22%) females (Fig. 3.2). Moths from tubs emerged from 31 May 31 to 3 August 3, 2020 (n = 5 tubs producing moths). Of the total 117 moths that emerged, the sex ratio was approximately 1:1; 60 (51.28%) males and 57 (48.72%) females (Fig. 3.2).

Moths emerged from potted plants from May 20 to August 3, 2021 (n = 13 pots producing moths). Of the total 84 moths that emerged, the sex ratio was 1.1: 1; 44 (52.38%) males and 40 (47.62%) females (Fig. 3.3). Moths from tubs emerged from June 6 to August 19, 2021 (n = 11 tubs producing moths). Of the total 257 moths that emerged, the sex ratio was 1:1.29; 112 (43.58%) males and 145 (56.42%) females.

In 2021, moths emerged from nine tubs from the 2020 cohort, and two tubs from 2019 cohort that had previously shown moth emergence in 2020.

Moth emergence among field collected individually pot and tub propagated plants

A chi-square test was used to compare moth's emergence among different propagation methods, i.e., lab rearing from field collected plants, rearing individually in potted *C. stoebe* plants, or reared in tubs containing six plants. The expected number of moths for each rearing method was estimated by dividing the number of moths proportionally across the total number of propagated plants in each method. Tubs were counted as six plants. Emergence rates differed across the three rearing methods ($\chi^2 = 53.15$, df = 2, $P < 0.0001$).

Discussion

During this study, *A. zoegana* was strictly univoltine, completing only one generation per year, as has been reported for the species (Müller et al., 1988; Powell et al., 2000; Story et al., 1994). In this study, the costs associated with rearing *A. zoegana* were calculated using plants collected from moth infested field sites. Moths were reared on individually potted *C. stoebe* plants and in tubs containing six plants in a greenhouse. The moths were reared twice in 2019 and 2020. Depending on the rearing method, the estimated cost per moth ranged between $14.50 (lab reared), $17.75 (greenhouse propagated on individually potted plants), and $13.40 (greenhouse propagated on tubs), respectively. Under ideal conditions, considering the costs associated with only the emerged containers, the estimated cost per moth ranged between $4.64 (lab propagated), $3.61 (greenhouse propagated on single potted plants), and $10.42 (greenhouse propagated on tubs). Of the three methods, rearing from plants in the tubs was the most cost effective per moth emerged, rearing from individually potted plants was the least cost effective and laboratory rearing was intermediate.

In these experiments, most pots (94/118) or several tubs (4 of 18) produced no moths. The high proportion of pots yielding no moths could have been caused by mortality of the female moths before oviposition, or death of the rearing plant before the larvae completed development. At least 70% of plants with no emergence were found dead in the greenhouse in early spring suggesting winter plant mortality because of suboptimal overwintering conditions. Plants were not dissected but the remainder of failures must be attributed to oviposition failures or larvae death. The low success rate of pot rearing makes the method cost ineffective. If rearing failures could be eliminated the cost efficiency would increase substantially to as low as an estimated $3.61 per moth, based on the costs and emergence just from successful pots in the greenhouse in this study. In comparison to pots, tubs exhibited a lower incidence of complete failure, with only four instances of no emergence. This can be attributed to the low probability of simultaneous failure for all six plants in a tub. However, considering the infected roots, the average moth emergence rate was relatively lower for tubs. Consequently, the proportion of tubs that did not produce any moths is not significantly different from pots if the plants are considered independent. Given the higher input costs to set up tubs, it is still concerning that four tubs did not produce any insects. We observed most of the plant mortality following the winter for those four tubs, which may have been caused by suboptimal overwintering conditions combined with an attack by secondary greenhouse pests such as *Myzus persicae* Sulzer (Hemiptera: Aphididae). If accounting only for tubs where emergence occurred, the cost per moth would have decreased to $10.42 per moth.

When comparing our findings to other studies on mass production of weed BCA, we found that labor costs for mass-producing *A. zoegana* were comparable. Similarly, insect collection accounted for a significant portion of labor costs in other mass rearing efforts of weed BCA, for example 32% in a study on the rearing of *C. achates* in field mass production (Story et al., 1996). Labor costs for insectary rearing of *Mogulones crucifer* Pallas (Coleoptera: Curculionidae) accounted for 78.3% of total expenses (Smith et al., 2009). About 72.8% of the total costs for field propagation of *A. zoegana* were from labor costs, with moth collection accounting for nearly half of the labor in a study by Story et al. (1994). In that study the cost of mass-rearing *A. zoegana* was estimated at $1.32 per moth using field cages.

Our results indicate that rearing *A. zoegana* in a single-plant pot is not cost effective, at least with the failure rate that occurred in our study. Fewer offspring emerged than moths were released on pots because of the large number of pots setup that did not produce any offspring. It may have been possible that the pots did not allow the *C. stoebe* plants to develop an adequately large taproot in comparison to the larger volume tubs. However, this seems unlikely because we used tree-training pots that were taller than most *C. stoebe* roots collected in the field. Additionally, a single *A. zoegana* larva can destroy multiple rosettes during its development (Müller et al., 1988).

Our studies show that the production method can significantly influence the cost of rearing *A. zoegana* and highlight the importance of carefully considering the costs of different production methods when developing BCA rearing systems. It is also important to note that while labor costs are a significant component of overall costs, they could be defrayed if, once developed, a rearing method could be undertaken by volunteers or as part of educational program or supported externally as an extension service activity. In this study, we opted for rearing methods using plants instead of developing an artificial diet in the laboratory. First, this would likely have been much more labor intensive and thus expensive (Raina et al., 2006). Second, our aim was to ensure the moth's phenology was synchronized with the plant's phenology, a requirement for a system to be used by land managers or the public.

In our study, the evaluation of rearing methods reveals a complex picture of success rates and cost effectiveness. When considering the percentage of emerged containers, lab rearing showed an intermediate success rate of 32%, while pots and tubs displayed success rates of 15.25% and 77.78%, respectively. However, focusing on moth emergence based on infected roots reveals a different story, with pots exhibiting a significantly higher emergence rate per plant (8.63 ± 2.11 moths), followed by tubs (4.45 ± 0.73 moths), and lab containers (1.56 ± 0.20 moths). Analyzing the cost per moth, tubs emerged as the most cost-effective method, with a remarkably low cost of $13.45 per moth, closely followed by lab rearing at $14.50 per moth. In contrast, pots proved to be the most expensive approach, with a cost of $17.75 per moth. Highlighting the case of ideal conditions, considering the cost of rearing only for pots and tubs where emergence occurred, the production cost per moth would be highest for tubs ($10.42), followed by lab rearing ($4.64), while the lowest cost would be attributed to pots ($3.61).

To further enhance moth production for propagating *A. zoegana*, employing larger tubs could yield greater success rates since simultaneous mortality of all plants is infrequent. It is worth noting that a single surviving plant has demonstrated the potential to generate a substantial number of moths, as evidenced by a single pot producing as many as 45 moths. However, it is important to acknowledge that the rate of plant survival in pots is low. Moreover, our study focused on the cost aspect, and under observed circumstances, tub rearing proved to be the most cost-effective option. As an additional cost-saving measure, substituting purchased horticultural soil with locally sourced soil could further reduce the expenses associated with tubs.

Irrespective of the rearing method employed, the production of *A. zoegana* faces inherent constraints due to its univoltine life history (Müller et al., 1989). However, given the potential of *A. zoegana* in effectively controlling *C. stoebe* infestations (Müller, 1989; Müller et al., 1988; Muller-Scharer, 1991; Story et al., 2000), we strongly recommend overwintering of the containers rather than relying on low temperature adjustment in the greenhouse. Refining the rearing methods based on these findings will be crucial in mitigating mortality rates. This study serves as a crucial starting point for the development of refined rearing systems, specifically emphasizing the use of larger tubs, which holds the potential for substantial reductions in labor costs associated with rearing *A. zoegana*.

Table 3.1. Activities and resources used for mass rearing *Agapeta zoegana* from field collected *Centaurea stoebe* roots in the laboratory in 2019 and 2020, respectively.

Activity	Resources and inputs 2019	Cost in USD 2019	Resources and inputs 2020	Cost in USD 2020	Average costs in USD
Field collection of plants at 3 sites	Labor: 21h	315.00	Labor: 28h	420.00	367.50
	Mileage for 280 miles	162.40	Mileage for 280 miles	159.60	161.00
Rearing mixture preparation	Labor: 22.8h (container filling for 349 containers, root cutting, measurements, setup of containers, labeling)	342.00	Labor: 23.5h (container filling for 359 containers, root cutting, measurements, setup of containers, labeling)	352.50	347.25
	Supplies (vermiculite, sand, containers, etc.)	649.58	Supplies (vermiculite, sand, containers, etc.)	661.78	655.68
Larval rearing	Labor: 26.2h (mating cage preparation, replacing flowers, checking emergence and gender identification)	393.00	Labor: 26.2h (mating cage preparation, replacing flowers, checking emergence and gender identification)	393.00	393.00
	Supplies and consumables	55.00	Supplies and consumables	55.00	55.00

Table 3.1. continued

Storage space	Laboratory space lease (10m^2 at \$53.8m^{-2} year^{-1})	179.33	Laboratory space lease (10m^2 at \$53.8m^{-2} year^{-1})	179.33	179.33
	Laboratory operating costs (4 months at \$100 month^{-1}	400.00	Laboratory operating costs (4 months at \$100 month^{-1})	400.00	400.00
Total		**2,496.31**		**2,621.21**	**2,558.76**

Table 3.2. Activities and resources used for mass rearing *Agapeta zoegana* in the individual potted *Centaurea stoebe* plants in the greenhouse in 2019 and 2020, respectively.

Activity	**Resources and inputs 2019**	**Cost in USD 2019**	**Resources and inputs 2020**	**Cost in USD 2020**	**Average costs in USD**
Growing plants in a greenhouse	Greenhouse space lease (6.5m^2 at 6.73m^{-2}year^{-1})	43.75	Greenhouse space lease (9.75m^2 at \$6.73m^{-2}year^{-1})	65.62	54.69
	Greenhouse Operating Costs (12 months at \$12.5 month^{-1})	150.00	Greenhouse Operating Costs (12 months at \$12.5 month^{-1})	150.00	150.00
Germination	Labor: 8.23h (watering, soil mixing, soil filling, seeding and labeling)	123.45	Labor: 13.96h (watering, soil mixing, soil filling, seeding and labeling)	209.40	166.43
	Supplies (soil mix: 0.058m^3 and nutrients)	29.91	Supplies (soil mix: 0.116m^3 and nutrients)	85.83	57.87
Growing knapweed	Labor: 28.75h (watering, soil mixing, soil filling, transplanting, and labelling)	431.25	Labor: 49.46h (watering, soil mixing, soil filling, transplanting, and labelling)	741.90	586.58
	Supplies (soil mix: 0.242m^3 and nutrients)	191.45	Supplies (soil mix: 0.438m^3 and nutrients)	340.77	266.11
Cage setup	Labor: 20.5h	157.50	Labor: 19h	285.00	221.25

Table 3.2. continued

	Supplies (fence wire, cage, and Velcro)	106.68	Supplies (fence wire, cage, and Velcro)	193.04	149.86
Collecting and releasing moths	Labor: 8.75h	131.25	Labor: 15.8h	237.00	184.13
Total		**1,365.24**		**2,308.56**	**1,836.92**

Table 3.3. Activities and resources used for mass rearing *Agapeta zoegana* in the tubs containing six *Centaurea stoebe* plants in the greenhouse in 2019 and 2020, respectively.

Activity	Resources and inputs 2019	Cost in USD 2019	Resources and inputs 2020	Cost in USD 2020	Average costs in USD
Growing plants in a greenhouse	Greenhouse space lease (8.85m^2 at 6.73m^{-2}year^{-1})	59.56	Greenhouse space lease (13.28m^2 at \$6.73m^{-2} year^{-1})	89.37	74.47
	Greenhouse Operating Costs (12 months at \$12.5 month^{-1})	150.00	Greenhouse Operating Costs (12 months at \$12.5 month^{-1})	150.00	150.00
Germination	Labor: 8.23h (watering, soil mixing, soil filling, seeding and labeling)	123.45	Labor: 13.96h (watering, soil mixing, soil filling, seeding and labeling)	209.40	166.43
	Supplies (soil mix: 0.058m^3 and nutrients)	23.11	Supplies (soil mix: 0.116m^3 and nutrients)	59.83	41.47
Growing knapweed	Labor: 39.83h (watering, soil mixing, soil filling, transplanting, and labelling)	597.45	Labor: 58h (watering, soil mixing, soil filling, transplanting, and labelling)	870.00	733.73
	Supplies (soil mix: 0.058m^3 and nutrients)	331.71	Supplies (soil mix: 0.116m^3 and nutrients)	424.03	377.87

Table 3.3. continued

Cage setup	Labor: 5.6h	84.00	Labor: 8.8h	132.00	108.00
	Supplies (PVC pipes, cage, and Velcro)	445.69	Supplies (PVC pipes, cage, and Velcro)	700.37	573.03
Collecting and releasing moths	Labor: 14.6h	219.00	Labor: 22.9h	343.50	281.25
Total		**2,033.97**		**2,978.5**	**2,506.25**

Table 3.4. Summary table for propagation of *Agapeta zoegana* in laboratory and greenhouse (2019- 2020).

***Agapeta zoegana* propagation summary**	**Laboratory**	**Single potted plant**	**Six-plant tubs**
Number of containers used	708	118	18
Number of containers with emergence	227	24	14
Total number of moths emerged	353	207	374
Moth emergence rate per infected root	1.56 ± 0.20	8.63 ± 2.11	4.45 ± 0.73
Cost of propagation per moth	$14.50	$17.75	$13.40
Costs associated with containers with emergence	$4.64	$3.61	$10.42

Fig. 3.1: *Agapeta zoegana* adult moth sketch; female (left) with rounded abdomen and tapered cloacal aperture; male (right) with flat abdomen and ventrally directed cloacal aperture. Black arrows indicate the location of cloacal aperture.

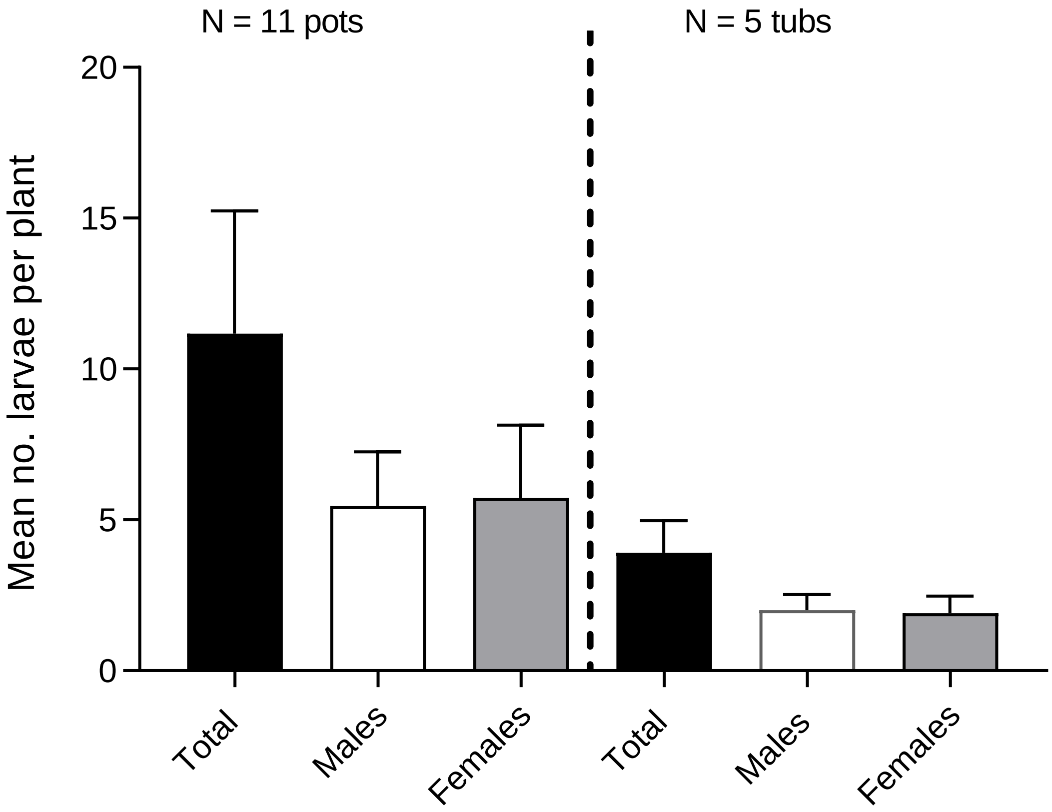


Fig. 3.2: Emergence of *Agapeta zoegana* moths from individual potted *C. stoebe* plants (left) and tubs containing six plants (right) in 2020. Values are means ± SE. N, number of replicates of pots and tubs, respectively with moth emergence (see text for details).

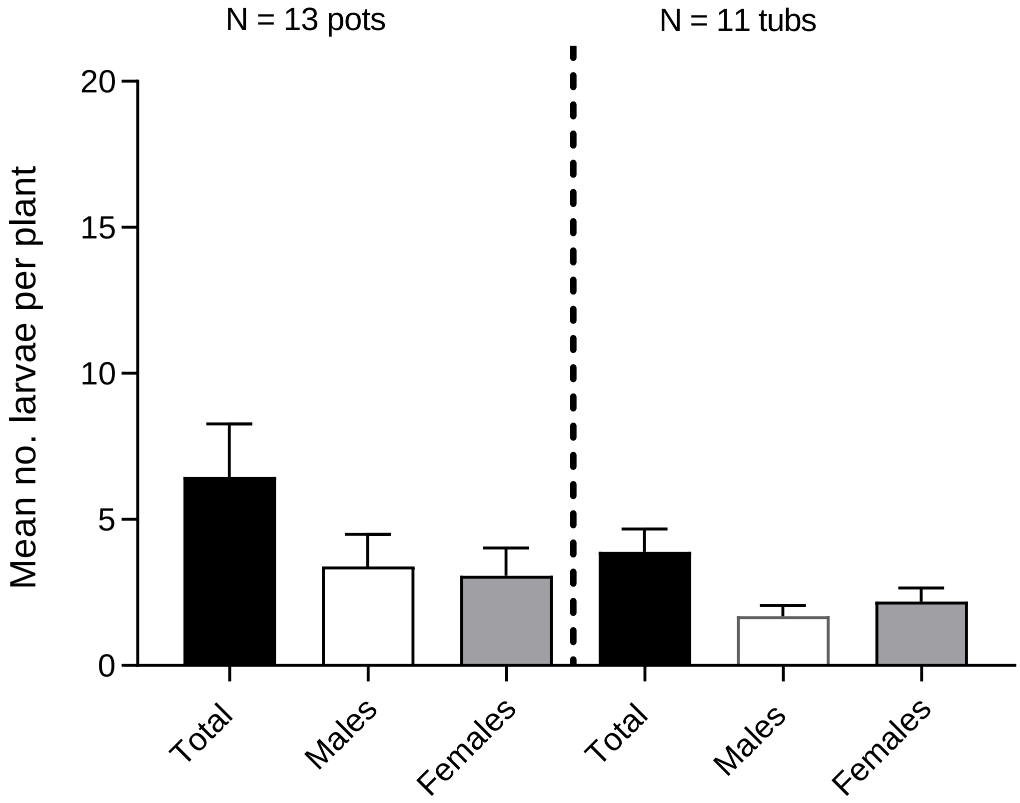


Fig. 3.3: Emergence of *Agapeta zoegana* moths from individual potted *C. stoebe* plants (left) and tubs containing six plants (right) in 2021. Values are means ± SE. N, number of replicates of pots and tubs, respectively with moth emergence (see text for details).

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Appendix A. Proportion (Mean ± SE) of *Centaurea stoebe* plants at 37 study sites in Idaho without root herbivores, with root herbivory by *Agapeta zoegana*, *Cyphocleonus achates*, *Sphenoptera jugoslavica*, more than one species of the root herbivore (multiple species), between 2019 and 2022.

Year	**Study site[1]**	**Number of plants (n)**	**Proportion of plant herbivory**				
			No herbivory (Mean ± SE)	***A. zoegana*** (Mean ± SE)	***C. achates*** (Mean ± SE)	***S. jugoslavica*** (Mean ± SE)	**Multiple species[2]** (Mean ± SE)
2019	4	30	0.50 ± 0.09	0.03 ± 0.03	0.2 ± 0.07	0.13 ± 0.06	0.13 ± 0.06
	5	30	0.53 ± 0.09	0.07 ± 0.05	0.2 ± 0.07	0.13 ± 0.06	0.07 ± 0.05
	6	30	0.87 ± 0.06	0	0.03 ± 0.03	0.10 ± 0.05	0
	8	30	0.80 ± 0.07	0.10 ± 0.05	0.10 ± 0.05	0	0
Average			0.68 ± 0.04	0.05 ± 0.02	0.13 ± 0.03	0.09 ± 0.03	0.05 ± 0.02
2020	1	30	0.53 ± 0.09	0.40 ± 0.09	0.03 ± 0.03	0	0.03 ± 0.03
	4	30	0.33 ± 0.09	0.37 ± 0.09	0.10 ± 0.05	0.07 ± 0.05	0.13 ± 0.06
	5	30	0.73 ± 0.08	0.13 ± 0.06	0.10 ± 0.05	0	0.03 ± 0.03
	6	30	0.37 ± 0.09	0.27 ± 0.08	0.13 ± 0.06	0.07 ± 0.05	0.17 ± 0.07
	7	30	0.63 ± 0.09	0	0.37 ± 0.09	0	0
	8	30	0.63 ± 0.09	0.33 ± 0.09	0	0	0.03 ± 0.03
	9	30	0.63 ± 0.09	0.27 ± 0.08	0	0.03 ± 0.03	0.07 ± 0.05
	10	30	0.57 ± 0.09	0.03 ± 0.03	0	0.33 ± 0.09	0.07 ± 0.05
	11	4	0	1.00 ± 0.00	0	0	0
	12	17	0.76 ± 0.1	0.12 ± 0.08	0.12 ± 0.08	0	0
	13	12	0.33 ± 0.14	0.50 ± 0.14	0	0.08 ± 0.08	0.08 ± 0.08
	15	12	0.75 ± 0.13	0.17 ± 0.11	0	0.08 ± 0.08	0
	16	11	0.27 ± 0.13	0.55 ± 0.15	0	0.18 ± 0.12	0
	17	20	0.60 ± 0.11	0.35 ± 0.11	0	0	0.05 ± 0.05

Appendix A continued

	18	15	0	0	0	0	0
2020	19	30	0.83 ± 0.07	0.10 ± 0.05	0	0.03 ± 0.03	0.03 ± 0.03
	20	14	0.93 ± 0.07	0	0	0.07 ± 0.07	0
	23	30	1.00 ± 0.00	0	0	0	0
	24	15	0.33 ± 0.12	0.33 ± 0.12	0.2 ± 0.1	0	0.13 ± 0.09
	28	30	1.00 ± 0.00	0	0	0	0
	29	30	0.73 ± 0.08	0.03 ± 0.03	0.23 ± 0.08	0	0
	30	30	0.20 ± 0.07	0.47 ± 0.09	0.27 ± 0.08	0	0
	31	30	0.37 ± 0.09	0	0.63 ± 0.09	0	0
	32	30	0.73 ± 0.08	0	0.10 ± 0.05	0.10 ± 0.05	0.07 ± 0.05
	33	26	0.85 ± 0.07	0.08 ± 0.05	0.04 ± 0.04	0	0.04 ± 0.04
	34	30	0.60 ± 0.09	0	0.40 ± 0.09	0	0
	35	30	0.70 ± 0.08	0.23 ± 0.08	0.07 ± 0.05	0	0
	36	5	0.40 ± 0.22	0	0.60 ± 0.22	0	0
	37	3	1.00 ± 0.00	0	0	0	0
Average			0.61 ± 0.02	0.17 ± 0.01	0.12 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
2021	1	18	0.33 ± 0.11	0.11 ± 0.07	0.50 ± 0.12	0	0.06 ± 0.05
	2	30	0.63 ± 0.09	0.20 ± 0.07	0.10 ± 0.05	0	0.07 ± 0.05
	3	30	0.30 ± 0.08	0.27 ± 0.08	0.13 ± 0.06	0.07 ± 0.05	0.23 ± 0.08
	4	30	0.53 ± 0.09	0.30 ± 0.08	0.03 ± 0.03	0	0.13 ± 0.06
	5	30	0.63 ± 0.09	0.27 ± 0.08	0	0.03 ± 0.03	0.07 ± 0.05
	6	30	0.37 ± 0.09	0.23 ± 0.08	0.07 ± 0.05	0.13 ± 0.06	0.20 ± 0.07
	7	30	0.70 ± 0.08	0	0.30 ± 0.08	0	0
	8	30	0.73 ± 0.08	0.20 ± 0.07	0	0.03 ± 0.03	0.03 ± 0.03
	9	30	0.73 ± 0.08	0.23 ± 0.08	0	0	0.03 ± 0.03
	10	30	0.80 ± 0.07	0	0	0.20 ± 0.07	0

Appendix A continued

2021	11	15	0.87 ± 0.09	0	0	0.13 ± 0.09	0
	12	30	0.83 ± 0.07	0.10 ± 0.05	0.07 ± 0.05	0	0
	13	21	0.86 ± 0.08	0.14 ± 0.08	0	0	0
	14	9	0.78 ± 0.14	0.22 ± 0.14	0	0	0
	16	19	0.63 ± 0.11	0.26 ± 0.10	0	0.05 ± 0.05	0.05 ± 0.05
	17	20	0.95 ± 0.05	0.05 ± 0.05	0	0	0
	19	20	0.70 ± 0.10	0.15 ± 0.08	0.10 ± 0.07	0	0.05 ± 0.05
	20	21	0.81 ± 0.09	0.14 ± 0.08	0	0.05 ± 0.05	0
	21	20	1.00 ± 0.00	0	0	0	0
	23	19	0.95 ± 0.05	0.05 ± 0.05	0	0	0
	25	30	0.60 ± 0.09	0	0.40 ± 0.09	0	0
	26	5	0.40 ± 0.22	0	0.60 ± 0.22	0	0
	27	30	0.57 ± 0.09	0	0.43 ± 0.09	0	0
	28	30	1.00 ± 0.00	0	0	0	0
	29	30	0.70 ± 0.08	0	0.30 ± 0.08	0	0
	30	30	0.23 ± 0.08	0.13 ± 0.06	0.47 ± 0.09	0	0.17 ± 0.07
	31	30	0.83 ± 0.07	0	0.17 ± 0.07	0	0
	32	20	0.70 ± 0.10	0.05 ± 0.05	0.10 ± 0.07	0.10 ± 0.07	0.05 ± 0.05
	33	20	0.85 ± 0.08	0	0.15 ± 0.08	0	0
	34	20	0.70 ± 0.10	0	0.30 ± 0.10	0	0
	35	30	0.67 ± 0.09	0.33 ± 0.09	0	0	0
	36	30	0.77 ± 0.08	0	0.23 ± 0.08	0	0
Average			0.69 ± 0.02	0.11 ± 0.01	0.13 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
Late 2021	4	30	0.43 ± 0.09	0	0	0.50 ± 0.09	0.07 ± 0.05
	5	30	0.83 ± 0.07	0.07 ± 0.05	0.07 ± 0.05	0.03 ± 0.03	0
	6	30	0.27 ± 0.08	0.13 ± 0.06	0	0.50 ± 0.09	0.1 ± 0.05

Appendix A continued

	7	30	0.87 ± 0.06	0	0	0.13 ± 0.06	0
	8	30	0.73 ± 0.08	0.03 ± 0.03	0	0.23 ± 0.08	0
	9	30	0.37 ± 0.09	0.07 ± 0.05	0	0.40 ± 0.09	0.17 ± 0.07
Late 2021	10	30	0.67 ± 0.09	0	0	0.33 ± 0.09	0
	12	30	0.47 ± 0.09	0.07 ± 0.05	0	0.40 ± 0.09	0.07 ± 0.05
	13	24	0.46 ± 0.10	0	0	0.46 ± 0.1	0.08 ± 0.06
	16	9	0.33 ± 0.16	0	0	0.67 ± 0.16	0
	17	19	0.84 ± 0.08	0.05 ± 0.05	0	0.05 ± 0.05	0.05 ± 0.05
	19	20	0.95 ± 0.05	0	0	0.05 ± 0.05	0
	20	21	0.52 ± 0.11	0	0	0.48 ± 0.11	0
	21	30	1.00 ± 0.00	0	0	0	0
	22	16	1.00 ± 0.00	0	0	0	0
	24	30	0.53 ± 0.09	0.40 ± 0.09	0.03 ± 0.03	0	0.03 ± 0.03
	25	30	0.83 ± 0.07	0	0.17 ± 0.07	0	0
	26	30	0.73 ± 0.08	0.13 ± 0.06	0.03 ± 0.03	0	0.1 ± 0.05
	27	30	1.00 ± 0.00	0	0	0	0
	28	30	1.00 ± 0.00	0	0	0	0
	29	30	0.97 ± 0.03	0	0.03 ± 0.03	0	0
	30	30	0.53 ± 0.09	0.43 ± 0.09	0	0	0.03 ± 0.03
	31	30	0.93 ± 0.05	0	0.07 ± 0.05	0	0
	32	30	0.67 ± 0.09	0	0.07 ± 0.05	0.23 ± 0.08	0.03 ± 0.03
	33	30	0.83 ± 0.07	0.03 ± 0.03	0	0.13 ± 0.06	0
	34	30	0.33 ± 0.09	0.23 ± 0.08	0.17 ± 0.07	0.07 ± 0.05	0.20 ± 0.07
	35	30	0.87 ± 0.06	0.10 ± 0.05	0.03 ± 0.03	0	0
Average			0.71 ± 0.02	0.07 ± 0.01	0.03 ± 0.01	0.16 ± 0.01	0.04 ± 0.01
2022	1	30	0.63 ± 0.09	0.17 ± 0.07	0.17 ± 0.07	0	0.03 ± 0.03

Appendix A continued

	3	30	0.27 ± 0.08	0.03 ± 0.03	0.17 ± 0.07	0.10 ± 0.05	0.43 ± 0.09
	4	30	0.57 ± 0.09	0.23 ± 0.08	0.07 ± 0.05	0.03 ± 0.03	0.10 ± 0.05
	5	30	0.93 ± 0.05	0.03 ± 0.03	0	0	0.03 ± 0.03
	6	30	0.27 ± 0.08	0.03 ± 0.03	0.30 ± 0.08	0.30 ± 0.08	0.10 ± 0.05
Average			0.53 ± 0.04	0.10 ± 0.02	0.14 ± 0.03	0.09 ± 0.02	0.14 ± 0.03

[1] Site names for respective study site numbers are listed in Table 2.1; [2] Proportion of plants attacked by more than one species of root herbivore; Results are generalized linear mixed models, assuming a binary distribution.

Appendix B. Summary statistics for intensity of root herbivory by *A. zoegana*, *C. achates*, and *S. jugoslavica* in *C. stoebe* roots at five census dates between 2019 – 2022, including unattacked roots.

Year	**Herbivore species**	**Test statistics for Mean**			**95% confidence intervals**		
		t value	**df**	**p-value**	**Mean**	**Lower CI**	**Upper CI**
2019	*A. zoegana*	-2.68	54	0.0097	0.171	0.045	0.640
	C. achates	-3.16	55	0.0026	0.158	0.048	0.509
	S. jugoslavica	-3.09	40	0.0036	0.164	0.050	0.535
2020	*A. zoegana*	-3.25	54	0.002	0.427	0.252	0.721
	C. achates	-5.12	55	<0.0001	0.253	0.147	0.433
	S. jugoslavica	-6.97	40	<0.0001	0.130	0.072	0.235
2021	*A. zoegana*	-5.17	54	<0.0001	0.258	0.152	0.436
	C. achates	-4	55	0.0002	0.351	0.208	0.593
	S. jugoslavica	-7.8	40	<0.0001	0.093	0.049	0.171
Late 2021	*A. zoegana*	-4.82	54	<0.0001	0.217	0.115	0.409
	C. achates	-6.92	55	<0.0001	0.097	0.049	0.190
	S. jugoslavica	-3.86	40	0.0004	0.377	0.226	0.628
2022	*A. zoegana*	-2.19	54	0.033	0.327	0.117	0.909
	C. achates	-2.42	55	0.0191	0.283	0.099	0.806
	S. jugoslavica	-2.86	40	0.0067	0.188	0.057	0.612
Average total[1]	*A. zoegana*	-8	67	<0.0001	0.300	0.222	0.405
	C. achates	-10.71	67	<0.0001	0.208	0.155	0.278
	S. jugoslavica	-10.26	67	<0.0001	0.180	0.129	0.251

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[1] Average throughout the study period for each species of root herbivore. Test statistics are generalized linear mixed models, assuming a lognormal distribution. *P*- values < 0.05 significant.

Appendix C. Summary statistics for comparison of intensity of root herbivory by *A. zoegana, C. achates,* and *S. jugoslavica* throughout the study period, including unattacked roots.

Herbivore species comparison		**Test statistics for species comparison**			
Herbivore species 1	**Herbivore species 2**	**t value**	**df**	**p-value**	**Mean ± SE**
A. zoegana	*C. achates*	1.87	67	0.0663	0.37 ± 0.20
A. zoegana	*S. jugoslavica*	2.34	67	0.022	0.51 ± 0.22
C. achates	*S. jugoslavica*	0.69	67	0.4937	0.15 ± 0.21

Test statistics are generalized linear mixed models, assuming a lognormal distribution. *P*-values < 0.05 significant.

Appendix D. Summary statistics for intensity of root herbivory by *A. zoegana*, *C. achates*, and *S. jugoslavica* in *C. stoebe* roots at five census dates between 2019 – 2022, excluding unattacked roots.

Year	**Herbivore species**	**Test statistics for mean**			**95% confidence intervals**		
		t value	**df**	**p-value**	**Mean**	**Lower CI**	**Upper CI**
2019	*A. zoegana*	1.11	28	0.276	1.352	0.774	2.361
	C. achates	1.63	30	0.1141	1.333	0.929	1.914
	S. jugoslavica	0.32	19	0.7517	1.041	0.798	1.358
2020	*A. zoegana*	6.34	28	<0.0001	1.708	1.436	2.030
	C. achates	8.3	30	<0.0001	2.071	1.731	2.478
	S. jugoslavica	4.17	19	0.0005	1.436	1.197	1.723
2021	*A. zoegana*	3.82	28	0.0007	1.395	1.167	1.667
	C. achates	7.23	30	<0.0001	1.719	1.475	2.003
	S. jugoslavica	1.13	19	0.2707	1.102	0.921	1.318
Late 2021	*A. zoegana*	3.64	28	0.0011	1.557	1.213	1.998
	C. achates	2.24	30	0.0326	1.382	1.029	1.857
	S. jugoslavica	9.13	19	<0.0001	1.553	1.404	1.718
2022	*A. zoegana*	4.41	28	0.0001	2.021	1.457	2.802
	C. achates	4.41	30	0.0001	1.816	1.378	2.393
	S. jugoslavica	1.21	19	0.2411	1.141	0.907	1.435
Average total[1]	*A. zoegana*	10.1	67	<0.0001	1.646	1.491	1.816
	C. achates	9.32	67	<0.0001	1.522	1.391	1.666
	S. jugoslavica	3.62	67	0.0006	1.192	1.082	1.314

[1] Average throughout the study period for each species of root herbivore, excluding unattacked roots. Test statistics are generalized linear mixed models, assuming a lognormal distribution. *P*- values < 0.05 significant.

Appendix E. Summary statistics for comparison of intensity of root herbivory by *A. zoegana, C. achates,* and *S. jugoslavica* throughout the study period, excluding unattacked roots.

Herbivore species comparison		**Test statistics for species comparison**			
Herbivore species 1	**Herbivore species 2**	**df**	**t value**	**p-value**	**Mean ± SE**
A. zoegana	*C. achates*	67	1.32	0.191	0.08 ± 0.06
A. zoegana	*S. jugoslavica*	67	4.85	<0.0001	0.32 ± 0.07
C. achates	*S. jugoslavica*	67	3.84	0.0003	0.24 ± 0.06

Test statistics are generalized linear mixed models, assuming a lognormal distribution.

P- values < 0.05 significant.

Appendix F. Summary statistics for the proportion of plants attacked by *A. zoegana*, *C. achates*, *S. jugoslavica* and more than one species of root herbivore (Multiple species) versus un-attacked roots (No herbivory) in all the years.

Year	**Herbivore species**	**Test statistics for proportion difference**			**Estimate ± SE^2**	**Proportion of plant herbivory**
		t value	**df**	**p-value**		
2019	*A. zoegana*	-7.96	12183	<0.0001	-3.68 ± 0.46	0.05 ± 0.02
2020		-16.82	12183	<0.0001	-2.24 ± 0.13	0.16 ± 0.01
2021		-20.85	12183	<0.0001	-2.84 ± 0.14	0.11 ± 0.01
Late 2021		-20.97	12183	<0.0001	-3.46 ± 0.17	0.07 ± 0.01
2022		-7.34	12183	<0.0001	-2.33 ± 0.32	0.10 ± 0.02
2019	*C. achates*	-7.84	12183	<0.0001	-2.60 ± 0.33	0.13 ± 0.03
2020		-18.00	12183	<0.0001	-2.63 ± 0.15	0.11 ± 0.01
2021		-20.38	12183	<0.0001	-2.64 ± 0.13	0.13 ± 0.01
Late 2021		-18.53	12183	<0.0001	-4.46 ± 0.24	0.03 ± 0.01
2022		-6.80	12183	<0.0001	-1.95 ± 0.29	0.14 ± 0.03
2019	*S. jugoslavica*	-8.14	12183	<0.0001	-3.02 ± 0.37	0.09 ± 0.03
2020		-17.35	12183	<0.0001	-3.87 ± 0.22	0.04 ± 0.01
2021		-18.52	12183	<0.0001	-4.43 ± 0.24	0.03 ± 0.01
Late 2021		-19.70	12183	<0.0001	-2.54 ± 0.13	0.16 ± 0.01
2022		-7.47	12183	<0.0001	-2.49 ± 0.33	0.09 ± 0.02
2019	Multiple species	-7.96	12183	<0.0001	-3.68 ± 0.46	0.05 ± 0.02
2020		-17.87	12183	<0.0001	-3.64 ± 0.20	0.05 ± 0.01
2021		-20.10	12183	<0.0001	-3.94 ± 0.20	0.04 ± 0.01
Late 2021		-19.57	12183	<0.0001	-4.15 ± 0.21	0.04 ± 0.01
2022		-6.80	12183	<0.0001	-1.95 ± 0.29	0.14 ± 0.03
2019	No herbivory	_	_	_	_	0.68 ± 0.04
2020		_	_	_	_	0.64 ± 0.02
2021		_	_	_	_	0.69 ± 0.02

Appendix F continued

Late 2021		_	_	_	_	0.71 ± 0.02
2022		_	_	_	_	0.53 ± 0.04
Average total[2]	*A. zoegana*	-21.08	12183	<0.0001	-2.29 ± 0.11	0.09 ± 0.01
	C. achates	-24.70	12183	<0.0001	-2.24 ± 0.09	0.10 ± 0.01
	S. jugoslavica	-24.63	12183	<0.0001	-2.65 ± 0.11	0.07 ± 0.01
	Multiple species	-24.55	12183	<0.0001	-2.85 ± 0.12	0.05 ± 0.01
	No herbivory	_	_	_	_	0.65 ± 0.01

[1] Estimated difference of interaction of species and year Least Square Means; [2] Average throughout the study period for each root herbivore species, by more than one species of the root herbivore (Multiple species), and un-attacked roots (No herbivory). Test statistics are generalized linear mixed models, assuming a binary distribution. *P*- values < 0.05 significant.

Appendix G. Summary statistics for the comparison of proportion of plants attacked by *A. zoegana*, *C. achates*, *S. jugoslavica*, more than one species of root herbivore (Multiple species), and un-attacked roots (No herbivory).

Year	**Comparison of species**		**Estimate ± SE**[1]	**Test statistics for comparison**		
	Herbivore species 1	**Herbivore species 2**		**t value**	**df**	**p-value**
2019-2022	*A. zoegana*	*C. achates*	-0.05 ± 0.14	-0.36	12183	**0.7162**
2019-2022	*A. zoegana*	Multiple species	0.56 ± 0.16	3.54	12183	0.0004
2019-2022	*A. zoegana*	*S. jugoslavica*	0.36 ± 0.15	2.37	12183	0.0179
2019-2022	*A. zoegana*	No herbivory	-2.91 ± 0.12	-23.67	12183	<.0001
2019-2022	*C. achates*	Multiple species	0.61 ± 0.15	4.17	12183	<.0001
2019-2022	*C. achates*	*S. jugoslavica*	0.41 ± 0.14	2.94	12183	0.0033
2019-2022	*C. achates*	No herbivory	-2.86 ± 0.10	-26.61	12183	<.0001
2019-2022	Multiple species	*S. jugoslavica*	-0.20 ± 0.16	-1.27	12183	**0.2041**
2019-2022	Multiple species	No herbivory	-3.47 ± 0.13	-26.77	12183	<.0001
2019-2022	*S. jugoslavica*	No herbivory	-3.27 ± 0.12	-26.79	12183	<.0001

[1] Estimated differences of Species Least Square Means. Test statistics are generalized linear mixed models, assuming a binary distribution. *P*- values < 0.05 significant. Non-significant comparisons highlighted in bold.

Appendix H. Summary statistics for the relationship between root size measured as root crown diameter and the number of larvae of *A. zoegana*, *C. achates,* and *S. jugoslavica* found in roots.

Herbivore species		**Estimate ± SE**[1]	**Test statistics for increasing RCD and abundance**		
			t value	**df**	**p-value**
A. zoegana	Slope	0.026 ± 0.003	7.84	840	<0.0001
	Intercept	0.069 ± 0.095	0.73	840	0.4658
C. achates	Slope	0.029 ± 0.004	6.51	840	<0.0001
	Intercept	0.020 ± 0.108	0.18	840	0.8538
S. jugoslavica	Slope	0.032 ± 0.005	6.41	840	<0.0001
	Intercept	-0.183 ± 0.108	-1.5	840	0.135

[1] Estimated value for the interaction between average root crown diameter and abundance of each root herbivore species. Test statistics are generalized linear mixed model dummy variable regression model assuming a Poisson distribution. *P*-values < 0.05 significant.

Appendix I. Summary statistics for the test of difference in average RCD for plants attacked by *A. zoegana*, *C. achates*, and *S. jugoslavica* among attacked plants.

Herbivore species	**Estimate ± SE**[1]	**Test statistics**			**Exponentiated Estimate**[2]	**Exponentiated lower**	**Exponentiated Upper**
		t value	**df**	**p-value**			
A. zoegana	2.878 ± 0.039	74.5	75	<0.0001	17.78	16.46	19.2
C. achates	2.858 ± 0.04	74.5	75	<0.0001	17.42	16.08	18.86
S. jugoslavica	2.741 ± 0.045	74.5	75	<0.0001	15.51	14.19	16.95

[1] Estimated slope response (least square means) of average RCD for each species in infested roots; [2] Estimated average value of root size. Test statistics are generalized linear mixed model ANOVA assuming a lognormal distribution. *P*-values < 0.05 significant.

Appendix J. Proportion of plants attacked by each root herbivore species in relation to un-attacked plants by that root herbivore species pooled throughout the study period with respective site latitude and elevation.

Study site[1]	**Latitude (°N)**	**Elevation (m)**	**Proportion of plant herbivory**		
			A. zoegana	***C. achates***	***S. jugoslavica***
			Mean ± SE	**Mean ± SE**	**Mean ± SE**
1	48.56372	778.9	0.28 ± 0.05	0.23 ± 0.05	0
2	48.56372	778.9	0.27 ± 0.08	0.17 ± 0.07	0
3	48.47111	749.4	0.47 ± 0.06	0.45 ± 0.06	0.15 ± 0.05
4	47.952194	739.7	0.28 ± 0.04	0.19 ± 0.03	0.19 ± 0.03
5	47.903306	731.7	0.15 ± 0.03	0.11 ± 0.03	0.05 ± 0.02
6	47.553611	652.4	0.21 ± 0.03	0.15 ± 0.03	0.33 ± 0.04
7	46.51942	973.6	0	0.22 ± 0.04	0.04 ± 0.02
8	46.466389	316.1	0.18 ± 0.03	0.04 ± 0.02	0.08 ± 0.03
9	46.46632	316.1	0.28 ± 0.05	0.01 ± 0.01	0.22 ± 0.04
10	46.44392	244.4	0.01 ± 0.01	0.02 ± 0.02	0.31 ± 0.05
11	46.232806	363	0.21 ± 0.09	0	0.11 ± 0.07
12	46.218111	373.9	0.12 ± 0.04	0.05 ± 0.03	0.18 ± 0.04
13	46.151752	406.8	0.21 ± 0.05	0	0.26 ± 0.06
14	46.141556	385.5	0.22 ± 0.14	0	0
15	46.137	452.6	0.17 ± 0.11	0	0.08 ± 0.08
16	46.130661	413.4	0.31 ± 0.07	0	0.26 ± 0.07
17	45.891794	652.6	0.19 ± 0.05	0.02 ± 0.02	0.05 ± 0.03
18	45.829092	703.8	0	0	0
19	45.829062	703.4	0.11 ± 0.04	0.04 ± 0.02	0.04 ± 0.02
20	45.823895	727.8	0.05 ± 0.03	0	0.21 ± 0.05
21	45.812052	1061	0	0	0
22	45.812052	1061	0	0	0
23	45.803418	1036.1	0.02 ± 0.02	0	0

Appendix J continued

24	45.375446	1099.2	0.44 ± 0.07	0.16 ± 0.05	0
25	44.898613	1542.4	0	0.28 ± 0.06	0
26	44.32852	1763.9	0.20 ± 0.07	0.20 ± 0.07	0
27	44.32835	1767.2	0	0.22 ± 0.05	0
28	44.13411	1695.8	0	0	0
29	44.073	1249.5	0.01 ± 0.01	0.19 ± 0.04	0
30	43.825	1470.8	0.41± 0.05	0.31 ± 0.05	0
31	43.817	1274.1	0	0.29 ± 0.05	0
32	43.78117	2007.3	0.01 ± 0.01	0.14 ± 0.04	0.20 ± 0.04
33	43.73406	1861.3	0.05 ± 0.03	0.07 ± 0.03	0.05 ± 0.03
34	43.71983	1819.4	0.15 ± 0.04	0.35 ± 0.05	0.06 ± 0.03
35	43.59689	1537.4	0.22 ± 0.04	0.03 ± 0.02	0
36	42.6950254	1206.3	0	0.29 ± 0.08	0
37	42.149306	1728	0	0	0

[1] Site names for respective study site numbers are listed in Table 2.1. The results for proportion of root herbivory by each species are a generalized linear mixed model assuming a binary distribution.

Appendix K. The total number of *C. achates* released at or nearby respective sites, the number of releases made, the year of the most recent release, and the number of *C. achates* individuals in the most recent release within a 10 km radius.

Study site[1]	**Total numbers Released (sum)**	**Releases number (n)**	**Year of most recent release**	**Number in the most recent release**
1	700	7	8/6/2020	50
2	700	7	8/6/2020	50
3	1215	11	8/24/2011	800
4	3405	27	8/5/2020	50
5	2475	30	8/5/2020	550
6	1275	11	9/1/2011	450
7	25	1	8/18/2008	25
8	1236	15	7/19/2019	300
9	1236	15	7/19/2019	300
10	2253	25	8/6/2020	600
11	150	3	8/11/2010	150
12	504	7	7/25/2019	354
13	719	11	7/25/2019	354
14	719	11	7/25/2019	354
15	1800	30	8/9/2012	150
16	1050	19	8/17/2011	700
17	5257	62	8/14/2020	2200
18	7857	88	8/7/2020	4700
19	7857	88	8/14/2020	4700
20	7050	74	8/14/2020	4900
21	1100	12	8/13/2020	600
22	1100	12	8/13/2020	600
23	1050	12	8/13/2020	600
24	12141	59	8/13/2015	2650
25	2750	30	8/10/2016	500

Appendix K continued

26	5150	51	8/18/2021	100
27	5150	51	8/18/2021	100
28	0	0	-	0
29	600	4	8/5/2003	600
30	980	11	8/30/2012	900
31	200	1	7/24/1997	200
32	5835	40	8/11/2018	50
33	7645	73	8/19/2021	150
34	6045	66	8/19/2021	150
35	2580	16	8/4/2017	250
36	705	7	8/14/2018	150
37	525	6	8/5/2016	75

[1] Site names for respective study site numbers are listed in Table 2.1.

Appendix L. The total number of *A. zoegana* released at or nearby respective sites, the number of releases made, the year of the most recent release, and the number of *A. zoegana* individuals in the most recent release within a 10 km radius.

Study site[1]	**Total numbers Released (sum)**	**Releases number (n)**	**Year of most recent release**	**Number in the most recent release**
10	35	1	8/24/2006	35
24	100	1	7/15/1995	100
28	130	2	7/17/2001	100
31	2100	6	7/30/1999	600
32	200	1	7/22/2005	200
33	200	1	8/10/1994	200
34	200	1	8/10/1994	200

[1] Missing collection sites from 1-9, 11-23, 25-27, 29, 30 & 35-37 have no releases made within 10 km radius. Site names for respective collection site numbers are listed in Table 2.

Appendix M. Summary statistics for the regression of belowground herbivory intensity and aboveground *C. stoebe* plant weight.

***C. stoebe* herbivory intensity**		**Estimate ± SE[1]**	**Test statistics for herbivory intensity**			
			t value	**df**	**p-value**	**N[2]**
No herbivory	Slope	2.86 ± 0.10	27.97	1363	< 0.0001	924
	Intercept	1.43 ± 0.40	3.61	1363	0.0003	
A. zoegana	Slope	2.58 ± 0.25	10.4	1363	< 0.0001	126
	Intercept	-0.94 ± 1.17	-0.81	1363	0.4161	
More than one *A. zoegana*	Slope	2.05 ± 0.36	5.86	1363	< 0.0001	64
	Intercept	-0.69 ± 2.13	-0.33	1363	0.7446	
One *C. achates*	Slope	2.60 ± 0.29	9.22	1363	< 0.0001	87
	Intercept	-0.73 ± 1.34	-0.54	1363	0.5867	
More than one *C. achates*	Slope	2.26 ± 0.32	7.16	1363	< 0.0001	88
	Intercept	1.84 ± 1.60	1.15	1363	0.2508	
One *S. jugoslavica*	Slope	2.92 ± 0.43	6.87	1363	< 0.0001	42
	Intercept	0.05 ± 1.94	0.02	1363	0.9812	
More than one *S. jugoslavica*	Slope	1.24 ± 1.23	1.01	1363	0.3143	9
	Intercept	13.15 ± 8.93	1.47	1363	0.1413	
A. zoegana and *C. achates*	Slope	1.37 ± 0.48	2.85	1363	< 0.0001	39
	Intercept	1.82 ± 2.97	0.61	1363	0.5395	

[1] Estimated value of slope and intercepts for the regression of belowground herbivory intensity and aboveground *C. stoebe* plant weight; [2] Number of observations. Test statistics are generalized linear mixed model dummy variable regression model assuming a normal distribution. *P*-values < 0.05 significant.

Appendix N. Summary statistics for the comparison of slopes for the regression of belowground herbivory intensity and aboveground *C. stoebe* plant weight.

Comparison of slopes		**Test statistics for comparison of slopes**			
Herbivory intensity 1	**Herbivory intensity 2**	**F value**	**Ndf**[1]	**Ddf**[2]	**p-value**
No herbivory	One *A. zoegana*	1.13	1	1363	0.2881
No herbivory	More than one *A. zoegana*	4.95	1	1363	**0.0262**
No herbivory	One *C. achates*	0.77	1	1363	0.3819
No herbivory	More than one *C. achates*	3.36	1	1363	0.067
No herbivory	*A. zoegana* and *C. achates*	9.35	1	1363	**0.0023**
No herbivory	One *S. jugoslavica*	0.02	1	1363	0.9003
No herbivory	More than one *S. jugoslavica*	1.75	1	1363	0.1863
One *A. zoegana*	More than one *A. zoegana*	1.51	1	1363	0.2198
One *C. achates*	More than one *C. achates*	0.66	1	1363	0.4149
More than one *A. zoegana*	More than one *C. achates*	0.19	1	1363	0.6645

[1]Ndf: numerator degree of freedom; [2]Ddf: denominator degree of freedom. Test statistics are generalized linear mixed model dummy variable regression model assuming a normal distribution. *P*-values < 0.05 significant (highlighted in bold).

Appendix O. Chi-square (χ^2) test results for the test of independence between observed numbers and expected numbers of root herbivore species in each category of conspecific *A. zoegana* (*AA*), conspecific *C. achates* (*CC*) and heterospecific *A. zoegana* and *C. achates* (*AC*) between 2019 and 2022.

Observation	Year	Category[1]	Observed[2]	Expected[3]	Total roots	(χ^2)[4]
1	2019	*AA*	3.0000	1.0083	120	3.9340
2	2020	*AA*	57.9672	25.8449	664	39.9245
3	2021	*AA*	39.9796	17.6925	787	28.0749
4	Late 2021	*AA*	32.9964	7.4100	739	88.3481
5	2022	*AA*	16.0050	7.7067	150	8.9354
6	2019	*AC*	4.0000	1.9250	120	2.2367
7	2020	*AC*	17.0000	19.5316	664	0.3281
8	2021	*AC*	24.0000	19.9416	787	0.8260
9	Late 2021	*AC*	9.0000	3.4046	739	9.1959
10	2022	*AC*	16.0000	8.8400	150	5.7993
11	2019	*CC*	5.0040	3.6750	120	0.4806
12	2020	*CC*	52.9872	14.7605	664	98.9989
13	2021	*CC*	63.0387	22.4765	787	73.2006
14	Late 2021	*CC*	12.0014	1.5643	739	69.6378
15	2022	*CC*	39.0000	10.1400	150	82.1400

[1] Abbreviation used in category: *AA* = conspecific *A. zoegana*, *CC* = conspecific *C. achates* and *AC* = heterospecific *A. zoegana* and *C. achates*; [2] Observed number of root herbivore species; [3] Expected number of root herbivore species; [4] Chi-square tests of independence within or between root herbivore species (4 df).

Appendix P. Material details and cost for Laboratory and Greenhouse mass-rearing of *Agapeta zoegana*.

Materials	**Company**	**Capacity /Dimension**	**Type**	**Cost in USD**
Vermiculite	Therm-O-Rock West, INC, Chandler, Arizona 85226	0.1 cubic meter	#2 coarse	40.99
Rearing container	Glad Food Storage Container	Volume: 1.89 liters; 21.94 cm length, 16.35 cm width, 10 cm height	Plastic container with interlocking lids	1.22
Mating cage	Cambro Manufacturing Co., Huntington Beach Co.	Volume: 7.5 liters; 27.5 cm length, 18.56 cm diameter	Polypropylene food storage container	11
Contractor bags	Hefty Load & Carry Heavy Duty Contractor Large Trash Bags	Volume: 170 liters; Thickness: 0.07 mm; 114 cm height, 55 cm width; 22 counts	Black plastic contractor bags	21
Cotton rounds	WinCo regular cotton rounds	80 counts		3
Cotton balls	Walmart Stores, Inc.	200 counts		1.88
Paper towel	Bounty	One count	Soft Paper towel	2.49
Sugar	Western Family pure granulated sugar	1.8 kg	Granulated sugar	4.89
Erlenmeyer Flask	PYREX®	Volume: 125 ml	Stopper no. 5 -No. 4980	5
Sand	Moscow Building Supplies	0.38 m^3	Fine	48.98

Appendix P continued

Parafilm	Parafilm "M" Laboratory Film	One count, 3.87 m^2	All Purpose laboratory film	29.66
Metal shelf	Seville Classics	1.22 m length, 0.46 m width, 1.91 m height	6-Tier Ultra Durable Mobile Wire Shelving-Commercial Grade NSF Steel	160
Soil mix	Premier Tech Pro-Mix BX Mycorrhizae, Quakertown, PA	1 Bale = 0.107 m^3	Soil mix	42.8
Germination pots	Grower's Solution, Cookeville, TN	Volume: 0.6 liters (12 cm height, 10 cm width, 8 cm diameter)	Plastic seedling pots, #P107D	0.26
Tree pot Three	Stuewe and Sons, Inc, Tangent, OR, USA	Volume: 7.57 liters (31.8 cm height, 19.7 cm diameter)	Plastic pots	1.85
Pot cage	Trimaco super tuff, Morrisville, NC	Volume: 18.92 liters (28.91 cm height, 25.4 cm width)	Elastic top paint strainer bags	1.6
Velcro for pot	Velcro® Brand One Wrap® Garden Ties, Manchester, NH	5.49 m length, 1.2 cm wide	Cut-to-length Garden Strap	6.3
Fence wire	Spence Hardware Moscow	2.03 mm thickness; 1219.2 m	Hi-tensile fence wire	146

Appendix P continued

Tub cage	D & M Tarps & More LLC, Lewiston, ID	7 cm length, 42 cm width, 101.6 cm height with elastic bungee	Bug Enclosure Mesh	50
Velcro for tub	Velcro® Brand-Industrial strength, Model 90593, Manchester, NH	4.57 m length, 0.5 m width	Stick on Tape	14.39
Tub cage frames	Charlotte pipe, Spence Hardware & Supply, Inc.	90degree elbows (not threaded) - with inlets (4counts), 90degree elbow (threaded) - with inlets (4 counts), Adaptor (4 counts) PVC (1.27 cm * 6.1 m)	PVC	5.99
Tub	Tuff Stuff Products KMT 102 Oval Tank, Terra Bella, California, USA	Volume: 113.5 liters; 30.5 cm height, 49 cm width, 104 cm length)	Heavy-Duty Oval Tanks	23.31
Triple Super Phosphate (TSP)	Bonide Products Inc., Oriskany, New York	1.81 kg	TSP	10
Flower and Vegetable Osmocote	The Scotts Company LLC., Marysville, Ohio	3.63 kg	Osmocote	28

Appendix P continued

Dolomite limestone	Grow More Inc., Gardena, California	7.26 kg	Dolomite	33.5
Chelated Iron 10%	Grow More Inc., Gardena, California	680 g	Iron Chelate	16
Fertilizer	Miracle-Gro, Marysville, OH	3.4 kg	Water-soluble All-Purpose Plant Food	14.26
Sand	Moscow Building Supplies	0.38 m^3	Fine	48.98
Clear plastic 5 ml-snap-cap plastic vials	Thornton Plastics Company, Salt Lake City, UT	Volume: 50 ml; 8 cm height; 450 counts	No. 55-15 Snap-cap Plastic Vials	99.99
Plastic labels	Crafijie	360 counts	Plant Nursery Pot labels	8
Clear plastic pot saucer	Gardener's Blue Ribbon, MAT Inc., Long Grove, IL	25.4 cm diameter	Tree pot saucer	1.48
Nitrile gloves	Valiant Nitrile Exam Gloves, Powder-free	100 counts	Nitrile gloves	13.63