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The Life History of Larinus Minutus, a Biological Control Agent of Invasive Knapweeds, and its Dispersal from Release Sites in Arkansas

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ABSTRACT

Larinus minutus Gyllenhal, a biological control agent of invasive knapweeds, has become established in several states and provinces since initial North American introduction in 1991. In order to reduce growing spotted knapweed populations in Northwest Arkansas, *Larinus minutus* (a biological control agent of spotted knapweed) was released annually from 2008-2011. Little is known about the larval development of this species, although the widespread use of this insect has provided research describing detailed host range and generalized life history. The speed and extent of the spread of this weevil from release sites following introduction have not been reported. This research described the larval development of *L. minutus* and its spread from release sites. Overwintered adult weevils were field collected and allowed to mate for two days for larval development studies. Females were placed individually into a mesh cage attached to a capitulum and allowed ~24 hours to oviposit. Randomly-collected caged capitula were dissected biweekly and head capsule measurements recorded. Once a majority of larva pupated, alternate day observations were conducted on remaining caged capitula to determine average emergence date. Two cohorts (occurring at full and late-flower) were used to observe season-related development differences. Two larval instars were observed from head capsule data analyzed with Hcap, a computer program that analyzes frequency distributions to determine instar number, mean head capsule width, instar range, and optimal separation points. Compared to previously published observations, all developmental stages were accelerated and one fewer stage was observed. Release sites were surveyed with transect sampling in winter of 2011 and 2012 to describe average *L. minutus* spread following introduction. Sampling included collection of 100 capitula per quadrat along each transect for later dissection and timed visual observation to record positive infestation. GPS coordinates were recorded at each sample location to determine

distance from a release site. Collected data were analyzed with a diffusion equation to describe the spread from a release site. This research shows two years post release, an annual increase of infested capitula, up to 21%, and spread from a release point, up to ~225 m can be expected.

This thesis is approved for recommendation
to the Graduate Council.

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CHAPTER 1

INTRODUCTION

Introduction

Spotted knapweed, *Centaurea stoebe* ssp. *micranthos* (Gugler) Hayek, is one of the most widespread and problematic weeds of the introduced *Centaurea* in North America. Spotted knapweed is found in 46 states and 7 Canadian provinces (USDA 2013). While a number of options including herbicides and cultural practices exist for managing knapweeds, biological control is typically used as it is the most cost efficient and sustainable. The classical biological control of invasive knapweeds is a longstanding program in North America that has culminated in the importation of 13 natural enemies of *Centaurea spp.* from 1970-93 (Müller-Schärer and Schroeder 1993) with redistribution of particular agents ongoing (Minteer et al. 2011).

Larinus minutus Gyllenhal was first released in North America in 1991 for the biological control of invasive knapweeds (Müller-Schärer and Schroeder 1993). The weevil is thought to be one of the most successful agents due to reductions of knapweed that followed its introduction in Colorado and British Columbia (Seastedt et al. 2003, Myers 2008). The weevil has been established in 8 states (Lang et al. 1996, Story 2002, Minteer et al. 2011) and despite its widespread distribution and use, relatively little is known of its larval development besides a generalized life history.

Adult forms of *L. minutus* overwinter near the base of the plant and become active in spring. Mating is observed approximately 4 weeks later with oviposition taking place in the florets of newly open capitulum soon thereafter (Groppe 1990, Kashefi and Sobhian 1998). Upon hatching, larvae move to the center of the capitulum and complete development over the course of 3-4 weeks and pupate within the capitulum (Groppe 1990). After emergence from pupation, adults consume nearby knapweed, preferring flowers to other parts of the plant, and overwinter until the next season (Groppe 1990).

Because the life history of *L. minutus* is so generalized, this provided an opportunity to better describe the larval development. The preliminary observations made in Groppe (1990) did not report a range, variance, or frequency of each instar (3 were reported), and thus I suspect the reported number of instars and corresponding head capsule widths may be inaccurate due to an insufficient sample size. Furthermore, an average development time for each instar was not determined. The first objective of this thesis research was to address these inadequacies with regular sampling of discretely established, field-based cohorts, of protected and contained immature *L. minutus* from oviposition until emergence as adults. Two cohorts were setup to determine any seasonal differences in *L. minutus* development.

As *L. minutus* is credited with providing partial control of knapweeds in British Columbia and Colorado (Seastedt et al. 2003, Myers 2008) it is important to document what happens to a site in the years following introduction. This information can be useful to future biological control efforts. Research conducted by Minteer (2012) introduced *L. minutus* to 37 different sites in northwest Arkansas from 2008-2011. The objective of chapter 3 was to describe changes in *L. minutus* infestation levels and spread from release points as a site ages. This was accomplished by grouping release sites by year of *L. minutus* introduction and using transect sampling to estimate infestation levels and spread, and analyzing these data with an exponential decay function when possible.

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CHAPTER 1

LITERATURE REVIEW

Taxonomy and Nomenclature

Due to the high morphological variation and use of inappropriate characters, spotted knapweed has undergone numerous name changes and is difficult to classify (Ochsmann 2001). A study utilizing molecular and morphological techniques found North American spotted knapweed belongs to *Centaurea stoebe* L. subsp. *micranthos* (Gugler) Hayek, and not *Centaurea stoebe* L. subsp. *stoebe*. Both subspecies are separated by ploidy level with tetraploids and diploids belonging to subsp. *micranthos* and subsp. *stoebe* respectively (Ochsmann 2001). A list of accepted synonyms, as well as previous names, for *C. stoebe* subsp. *micranthos* is presented in Ochsmann (2001).

Biology and habitat

Spotted knapweed is a biennial or short-lived perennial forb Eurasian in origin and can be diploid or tetraploid. A ploidy ratio study (Treier et al. 2009) of spotted knapweed populations in Europe and North America found that both tetraploid and diploid forms exist in mostly non-overlapping populations in Europe while the tetraploid form comprises 98% of the populations sampled in North America. Both ploidy forms possess different life histories with diploids and tetraploids exhibiting monocarpic and polycarpic life cycles respectively. In general the plant is a pioneer species that quickly invades and takes advantage of disturbances in soil (Watson and Renney 1974).

As this project deals with North American spotted knapweed, only the biology of the tetraploid form will be considered. Spotted knapweed starts off as an achene that after sprouting overwinters as a basal rosette. Upon the start of the next growing season the plant will bolt and can produce over 15 stems (Watson and Renney 1974). The stems can than grow >1 m with branching occurring in the upper half. Branches are terminated with capitula that are covered in

characteristic brown triangular tips that give spotted knapweed its name. During flowering, each capitulum produces 20 to 50 pink or purple tubular florets (Watson and Renney 1974, Winston et al. 2010) and after pollination, each capitulum produces an average of 30 achenes (Winston et al. 2010). Depending on growing conditions, the annual viable seed production of one spotted knapweed plant is estimated at 350-20,000 seeds (Watson and Renney 1974). After the growing season, individual stems will senesce and the plant will overwinter again as a rosette. While achenes are the primary form of reproduction in spotted knapweed, the plant can employ lateral shooting to produce new rosettes (Watson and Renney 1974).

Range and invasion success

Spotted knapweed was first introduced to North America as a contaminant of hay in the late 1800s (Winston et al. 2010) and is reported from all states except Oklahoma, Texas, Mississippi, and Alaska (USDA 2013). Treier et al. (2009) suggests that both ploidy forms were introduced to North America but that the tetraploid form has outcompeted the diploid form and is the dominant ploidy type. Overall, spotted knapweed is estimated to infest over 2.9 million ha in the United States (DiTomaso 2000) with the largest populations found in the Northwestern and central states were it is a rangeland pest. The plant is estimated to spread at a rate of 10-24% annually (Duncan et al. 2004). Dispersal of knapweed achenes occurs by wind, water, attachment to animals or vehicles, and as contaminants of hay (Winston et al. 2010). Once knapweed has been established at a site, only 0.1 percent of the total produced seeds are required to survive to maintain knapweed densities (Schirman 1981).

The polyploidy exhibited by North American spotted knapweed is thought to be a contributing factor to the plants establishment success. In comparison to a diploid, a tetraploid produces additional florets, smaller capitulum, a lower number of capitulum per plant, extra

accessory rosettes, and significantly less seeds per plant in one season (Henery et al. 2010, Mráz et al. 2011). Since the tetraploid spotted knapweed has the ability to flower numerous times, higher total seed fecundity can be attained in comparison to the monocarpic diploid form. This may have given tetraploids the competitive edge in North American establishment (Treier et al. 2009, Henery et al. 2010).

In addition to the success of the tetraploid form in North America, spotted knapweed may have undergone additional evolutionary changes after introduction. The evolution of increased competitive ability (EICA) hypothesis states that following introduction to an area lacking specialist co-evolved natural enemies, genotypes of the invasive species that invest in reproductive ability or biomass accumulation rather than herbivory defense, whichever is the most beneficial, will be favorably selected (Blossey and Nötzold 1995). In comparison to the European tetraploid counterpart, North American tetraploids exhibit a lower level of gene expression involved with plant defense (Broz et al. 2009), a significant increase in reproduction capacity (Henery et al. 2010), and an increased tolerance to drier continental climates (Treier et al. 2009). These observations seem to support the EICA hypothesis but all three publications assert the need for further testing in order to rule out other possibilities such as a founder effect (Broz et al. 2009, Treier et al. 2009, Henery et al. 2010) that could account for these differences.

Allelopathy has also been suggested as another factor considered having an impact on invasion success and establishment. The allelopathic advantage against resident species hypothesis put forth by Callaway and Ridenour (2004) suggests an invasive plant in possession of an allelochemical will compete better with plants in an invaded range in comparison to coevolved plants in its native range. The assumption is that the native plants will have evolved allelochemical defenses that the plants in the introduced range lack.

After soil infested with spotted knapweed was found to inhibit the growth and development of crops (Fletcher and Renney 1963), it was suggested that the plant releases a growth chemical, which would later be defined as an allelochemical, that inhibited the growth of competing plants. Later research extracted cnicin, a sesquiterpene lactone, from spotted knapweed and found that when applied at varying concentrations, the germination of tested plants was inhibited (Kelsey and Locken 1987). While little research has been done on the allelopathic potential of cnicin since then, one study found that while cnicin did not prevent germination, it can inhibit seeding growth (Schabes and Sigstad 2007). Cnicin may also aid knapweed establishment via different means. Large herbivores find cnicin to be bitter tasting and will avoid its consumption if possible (Watson and Renney 1974, Kelsey and Locken 1987). As a result, other species of plant are overgrazed and any competitive effects to spotted knapweed are reduced.

Perhaps the most researched compound implicated in the allelopathic potential of spotted knapweed is (-)-catechin. After developing a hexane extraction technique, Bais et al. (2002) claimed to have to isolated racemic catechin from root exudates of spotted knapweed and that the (-)-catechin form was phytotoxic. Additional research (Bais et al. 2003) reported high levels of catechin in the soil surrounding spotted knapweed and that (-)-catechin damages surrounding root systems by creating reactive oxygen species. When (-)-catechin was applied to the roots of diffuse knapweed (*Centaurea diffusa* Lam.), *Arabidopsis thaliana* (L) Heynh, and spotted knapweed at soil representative levels, reactive oxygen species were created which resulted in large-scale cell death in diffuse knapweed and *A. thaliana*. Spotted knapweed in comparison only produced low levels of reactive oxygen species and failed to exhibit any necrosis.

In an attempt to assess the evolution of increased competitive ability hypothesis, a study (Blair et al. 2005) comparing catechin production rates between European and North American spotted knapweed found the hexane extraction technique developed by Bais et al. (2002) to be non-reproducible and catechin to be insoluble in hexane. After developing a new technique to extract catechin, Blair et al. (2005) was unable to find any catechin in soil samples from spotted knapweed field sites. Catechin has been reported as highly unstable in soils (Inderjit et al. 2008) and only present at extremely low concentrations (Perry et al. 2007). Since then, Bais et al. (2002) has been retracted and an erratum has been posted for Bais et al. (2003). Both publications acknowledge the non-reproducible nature of the hexane extraction.

The reactive oxygen species mechanism of (-)-catechin has also been contested. Research (Duke et al. 2009) attempting to recreate the formation of reactive oxygen species reported by Bais et al. (2003) found root death did not occur following catechin application, even after being left in media containing (-)-catechin for four days. After being placed in (-)-catechin free media, tested plants resumed healthy root growth within two days. Additionally, Duke et al. (2009) found that catechin actually inhibits the formation of reactive oxygen species. No explanation has been presented to account for the differences in results between the two studies (Bais et al. 2003, Duke et al. 2009) since then.

Finally, the lack of specialist natural enemies attacking knapweeds, prior to biological control efforts in North America, is thought to have contributed to the invasion success and spread of knapweeds by allowing them to escape the herbivory pressure that native plant life presumably experiences (Müller-Schärer and Schroeder 1993). This idea, in combination with the assumption that an invasive will be highly unlikely to experience a host switch from a native specialist natural enemy comprises assumptions made by the enemy release hypothesis (ERH)

(Keane and Crawley 2002). By escaping herbivory pressure, an invasive plant may be better able to compete with native plants in the acquiring of nutrient and space resources. While a native plant should be evolved to its corresponding native environment, and thus exert a relatively high pressure on competing invasive plants, anthropologic manipulation of local conditions can decrease the competitive ability of a native plant and allow an opportunity for an invasive plant to establish (Keane and Crawley 2002). Knapweeds are pioneer species that quickly colonize disturbed habitats such as along roads, railways, places of refuse, and overgrazed rangeland (Watson and Renney 1974), however they have a difficult time establishing in areas under cultivation (Harris and Cranston 1979). A combination of reduced competition from native plants as a result of disturbance and escape from herbivory pressure seem to be two factors conducive to knapweed establishment and spread.

Ecological and Economic impacts

The direct and indirect effects of spotted knapweed and two other knapweeds, *C. diffusa* and *Rhaponticum repens (L.)* Hidalgo, are estimated to cost Montana $42 million dollars annually based on an infestation of over 2 million acres (Hirsch and Leitch 1996). An infestation of diffuse and spotted knapweed of 30,000 ha in British Columbia reduced available forage by up to 88% (Harris and Cranston 1979). Ingestion of large amounts of diffuse and spotted knapweed can lead to toxic symptoms in horses (Maddox 1979). Spotted knapweed-dominated sites experience increased surface water runoff, soil sedimentation yields, and interrill erosion (Lacey et al. 1989). Invasion of spotted knapweed is related to reductions in plant community composition (Tyser and Key 1988).

Chemical Control

The use of herbicides is an established and effective method of controlling knapweed populations. Dicamba, clopyralid and picloram, and 2,4-D will effectively control knapweed if properly applied (Müller-Schärer and Schroeder 1993). However since most formulas are broad spectrum or specific to a particular group of plants, detrimental effects to native plants can occur (Synder and Shephard 2007) and surface water runoff can lead to the contamination of nearby water bodies (Hirsch and Leitch 1996). The use of herbicides as a primary means of knapweed control is economically unfeasible due to the wide area in which knapweed has invaded and the relative low monetary value of the land on which the weed is a pest (Maddox 1979). Herbicides are best used in spot treatment of knapweed in recently invaded areas in order to prevent the establishment of a knapweed seed bank (Harris and Cranston 1979).

Cultural control

One cultural management option is the use of herbivore grazing. While cattle and horses avoid spotted knapweed (Cheeseman 2006), sheep and other wildlife have been observed grazing on knapweeds (Wright and Kelsey 1997, Olson and Wallander 2001). As a consequence, prescription grazing using sheep has been proposed as a control method of spotted knapweed (Launchbaugh and Hendrickson 2001). Additional research investigating the potential of grazing as a component of knapweed management must be conducted as 22 percent of consumed achenes remain viable after passing through a sheep's digestion system which may contribute to spread (Wallander et al. 1995) especially given only 0.1 percent of seed is needed for contamination (Schirman 1981).

Controlled burning can provide another management technique in certain situations. While fire has been shown to successfully reduce knapweed populations in Midwestern grass

prairies (Wintson et al. 2010), in Montana a controlled fire of a forested site created a disturbance conducive to knapweed colonization with an approximate sixfold increase of spotted knapweed occurring within 2 years of burning (Sheley et al. 1998). Additionally, knapweeds have a deep taproot that allows the plant to survive burning (Wintson et al. 2010).

Cultivation, hand pulling, and mowing can also be used to impede the spread of spotted knapweed, however these options are time and cost prohibited due to the widespread distribution of knapweeds. These techniques are best used to control spotted knapweed in limited areas (Winston et al. 2010).

Biological control

Due to difficulties with other control measures, biological control is the most economic and long lasting solution to knapweed infestation (Harris and Cranston 1979). The knapweed biological control program was first started in 1961 with field surveys of spotted knapweed in Western Europe and by 1971, 12 natural enemies had been discovered and the host ranges of 10 studied (Schroeder 1985). The first agents investigated and introduced were those that attack the seeds and flowering parts of knapweeds in an attempt to establish control via seed reduction (Schroeder 1985). Releases were made from 1970-1976 of three seed feeders and one root borer (Müller-Schärer and Schroeder 1993). On the assumption that seed reduction alone would provide insufficient control, an investigation of natural enemies that attack the roots and rosettes of knapweeds was conducted by CIBC from 1979 to 1983 at 37 European locations (Schroeder 1985) and resulted in the release of four root-feeding species from 1982-1987 (Müller-Schärer and Schroeder 1993). With introduced species totaling eight, Harris (1991) suggested attack of the soft achene stage could result in further seed reduction. As a consequence, five additional achene feeders were released from 1991-1993 (Müller-Schärer and Schroeder 1993). Overall,

from 1970-1993, 13 insect species were imported and established in North America (Müller-Schärer and Schroeder 1993). Since then, no additional introductions have taken place, however foreign exploration for additional natural enemies to exploit the foliage and root crowns of rosettes, niches of spotted knapweed phenology currently unattacked, has been suggested (Smith 2001, Story 2002).

Reductions of spotted knapweed densities in Montana were observed after the introduction of *Cyphocleonus achates* Fahraeus (Story et al. 2006). Prior to *C. achates* introduction, six other agents were already established but failed to reduce densities (Story et al. 2006). As infestation of spotted knapweed by *C. achates* can stress the plant to mortality or reduced vigor (Corn et al. 2006) it has been implicated in being the most effective natural enemy of spotted knapweed (Myers 2008). As multiple natural enemies were present prior to *C. achates* establishment, reductions of spotted knapweed density could also be the result of a cumulative stress threshold being reached (Story et al. 2006). Likewise, introduction of *C. achates* with three other natural enemies in a spotted knapweed dominated site in Colorado resulted in a 93 percent reduction in spotted knapweed over a 8 year period (Carney and Michels 2010). Research investigating the combined effects of multiple natural enemies including *C. achates* found an overall decrease in knapweed performance and vitality and projects knapweed biological control can be attained by utilizing multiple species of biological control agents (Knochel and Seastedt 2010).

Larinus minutus

A number of *Larinus* spp. have been investigated and utilized for the biological control of invasive weeds (Groppe et al. 1990, Jordan 1995, Lang et al. 1996, Gültekin et al. 2008, Briese 2000). After undergoing host specificity testing from 1985-1989 (Groppe 1990), *Larinus*

minutus Gyllenhal was first released in North America in 1991 for the biological control of diffuse and spotted knapweeds (Müller-Schärer and Schroeder 1993) from initial collections of adult weevils in Greece and Romania (Lang et al. 1996). Since then, the weevil has been redistributed and established in Arkansas, Colorado, Indiana, Minnesota, Montana, Oregon, Wyoming, and Washington (Lang et al. 1996, Story 2002, Minteer et al. 2011).

Myers (2008) suggested that *L. minutus* alone provides sufficient control of diffuse knapweed as decreases of diffuse knapweed in British Columbia and Colorado, both areas with longstanding knapweed biological control programs, did not occur until after *L. minutus* establishment (Seastedt et al. 2003, Myers 2008). This reduction can likely be attributed to large-scale seed destruction exhibited by larval feeding, which typically destroys 100% of achenes in infested capitula (Kashefi and Sobhian 1998), and adult feeding of rosette leaves, seedlings, and the parenchyma of bolting stems which can kill plants (Myers et al. 2009).

***Larinus minutus* biology**

Larinus minutus overwinters as an adult in the debris surrounding knapweed sites and leaves these sites in spring (Kashefi and Sobhian 1998). Overwintered adults consume nearby knapweed, preferring flowers relative to other parts of the plant, and are observed mating approximately 4 weeks later (Groppe 1990, Kashefi and Sobhian 1998). Once mated, females oviposit in newly opened flowers that under laboratory conditions hatch in 3-4 days (Kashefi and Sobhian 1998). For the next 3-4 weeks in the capitulum, the larva reportedly passes through 3 instars and consumes all surrounding achenes (Groppe 1990). The mature larva then constructs a pupation chamber from remaining material in the capitulum and pupates (Kashefi and Sobhian 1998). After emergence from pupation, adults consume nearby knapweed, again preferring

flowers to other parts of the plant, and overwinter in the surrounding soil until the next season (Groppe 1990).

Spread from release sites

As release of a biological control agent is akin to that of an invading organism, quantification of invasion dynamics can provide useful information (Fagan et al. 2002). Indeed, numerous diffusion and integrodifference equations can be used to model and describe the spread of an invading organism, or alternatively a biological control agent (Kot et al. 1996). Determination of the factors affecting the spread of a newly introduced agent greatly improves the application of these models.

In general, spread is typically described by monitoring of sites at different distances from the release location. For example, *Galerucella calmariensis* L. and *G. pusilla* Duftschmid, both chrysomelid biological control agents of purple loosestrife, were estimated to disperse 15, 46 and 69 m from initial release sites (~40 m^2) for each successive year after (McAvoy et al. 1997). This study however didn't describe infestation rates relative to distance from the release sites. Another study monitored and described the dispersal of biological control agent, *Rhinoncomimus latipes* Korotyaev (Coleoptera: Curculionidae), from release sites on mile-a-minute weed over the course of four years by setting up monitoring points along concentric circles around the release point (Lake et al. 2011). In doing this, researchers were able to determine average spread for the weevil to be 1.5 to 2.9 m per wk and at 4 months following release, weevils had spread beyond 25 m, the largest concentric circle that was monitored.

L. minutus has been reported as spreading up to ~2 km 2 years from a release made in California (Woods and Popescu 2001). There have been no published, detailed studies describing *Larinus* spp. dispersion from release sites in North America. Given the potential of *L.*

minutus in providing successful control of knapweed, formulation of a model describing spread and impact can provide valuable information to the implementation of future biological control programs.

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CHAPTER 3

THE LARVAL DEVELOPMENT OF *L. MINUTUS*

Introduction

Spotted knapweed, *Centaurea stoebe* ssp. *micranthos* (Gugler) Hayek, is an invasive plant native to Eurasia. Initial introduction occurred in northwestern North America (Watson and Renney 1974) and it can now be found in 46 states and 7 Canadian provinces (USDA 2013). Spotted knapweed reduces available forage for livestock and wildlife (Watson and Renney 1974) and, increases soil surface water runoff and stream sediment yield (Lacey et al. 1989). Spotted knapweed, along with other invasive knapweeds, has been the target of classical biological control attempts because control through the widespread and continual use of herbicides is economically impractical (Maddox 1979). From 1970-93, 13 natural enemies of spotted knapweed were imported and established in North America (Müller-Schärer and Schroeder 1993).

Larinus minutus Gyllenhal (Col: Curculionidae) was first introduced to North America in 1991 for biological control of *Centaurea spp.* (Lang et al. 1996). Since then, the weevil has become established in Arkansas, Colorado, Indiana, Minnesota, Montana, Oregon, Wyoming, and Washington (Lang et al. 1996, Story 2002, Minteer et al. 2011). *Larinus minutus* is credited with providing partial control of knapweeds in British Columbia and Colorado, which are areas with longstanding knapweed biological control programs (Seastedt et al. 2003, Myers 2008). Knapweed densities in these areas decreased only after introduction of *L. minutus*. This reduction was attributed to large-scale seed destruction and adult feeding (Myers et al. 2009). Larval *L. minutus* feeding destroys 100% of achenes in infested capitula (Kashefi and Sobhian 1998). Despite the widespread use of *L. minutus*, relatively little is known about its larval development.

L. minutus overwinters as an adult in debris near knapweed sites and leaves these sites in spring (Kashefi and Sobhian 1998). Once active, adults feed on, seedlings and new shoots of knapweed. *Larinus minutus* prefers feeding on flowers, once they are present, relative to other parts of the plant. After feeding, adults mate approximately four weeks later (Groppe 1990). A mated female will chew a small hole in the floret of a newly opened capitulum to prepare a site for oviposition. An egg is then directly oviposited into the empty space and under laboratory conditions eggs hatch in 3-4 days (Kashefi and Sobhian 1998). During the next 4 weeks, the larva reportedly passes through three instars, moving deeper into the capitulum and consuming all surrounding achenes. Prior to pupation, the mature larva constructs a cocoon from remaining material in the capitulum (Kashefi and Sobhian 1998). After pupation, the newly emerged adult feeds on nearby knapweed and overwinters in surrounding soil until the following spring (Groppe 1990).

The number of larval stages in insects is recognized by creating a frequency distribution of recorded measurements, usually taken from the width of the head capsule, with each peak corresponding to an instar (Daly 1985). Additional support for instar number can be obtained by plotting the logarithmic mean of each peak against that of the presumed instar number (Dyar 1890). A straight line should be produced, provided full representation of data for each instar and size increases of each subsequent instar progress geometrically with a corresponding constant growth ratio (Daly 1985). Deviation from a straight line suggests a missed instar. In the event a frequency distribution produces overlapping, non-discreet peaks, various alternative techniques have been developed to determine instar numbers (Caltagirone et al. 1983, Schmidt 1996, Logan et al. 1998).

The objective of this study was to better define larval development of *L. minutus* with regular sampling of discretely established, field-based cohorts, of protected and contained immature *L. minutus*.

Materials and Methods

This experiment was conducted at a spotted knapweed plot in full-flower at the University of Arkansas Agriculture Experiment Station located in Washington County Arkansas. Full-flower was defined as a narrow timeframe (1-2 wk) in which the vast majority (~80%<) of knapweed capitula were in a state of bloom (late May to early June in northwest Arkansas).

At the onset of full-flower, approximately 600 active adult weevils were collected from the spotted knapweed plot and divided into mating sub-colonies, each containing 100 adults. Sub-colonies were maintained in an environmental chamber held at 25° C with a 16:8 (L:D) h photoperiod and provisioned with flowering spotted knapweed capitula for food. After two days, weevils were removed from sub-colonies and sexed using a method described by Kashefi (1993) until 250 females were identified. Females were then transferred individually to lidded 1 oz clear plastic cups for handling and transport.

Newly-opened capitula (250) were selected throughout the plot. Each selected capitulum was hand pollinated with a phenologically similar capitulum of a different plant, by rubbing the florets of each respective capitulum together, to ensure development of the achenes. Mesh cages (ca. 13 cm x 8 cm) were attached to the stem below the capitulum with a fishing line drawstring. Mesh cages were only attached to newly-opened capitula, as females have been noted to oviposit in capitula of that phenological stage (Kashefi and Sobhian 1998). Once the cage was in place on the pollinated capitulum, a female was transferred from the plastic cup into a mesh cage. All 250 sexed females were transferred individually to a mesh-caged capitulum.

Caged females were allowed 24 h for oviposition after which they were destroyed to prevent excessive oviposition and capitulum damage via feeding. Female destruction was accomplished mechanically without cage removal to prevent damage to the capitulum or stem. Three days after the caged female was killed, 20 caged capitula were collected in an unbiased manner twice-weekly. After collection, caged capitula were dissected and developing *L. minutus* removed. For each capitulum, the number of larvae present and corresponding head capsule widths were measured using an ocular micrometer within a stereomicroscope. Once dissections suggested a majority of *L. minutus* had pupated, remaining caged capitula were observed every other day for adult emergence. Upon emergence, adults were sexed and collection date was recorded.

A second, late-flower, cohort was set up in mid-June to examine seasonal effects on *L. minutus* development. Late-flower occurs after a vast majority of knapweed capitula are no longer in bloom and individual stalks begin to senesce. At this point, a much smaller number of capitula can still be found in various stages of bloom that can support *L. minutus* development. Although the late-flower cohort followed the same experimental procedures as the full-flower cohort, biweekly collection of 20 randomly selected caged capitula was increased to 30 after preliminary data suggested accelerated larval development and poor representation of later instars.

Head capsule measurements from both cohorts were combined for Hcap analysis (Logan et al. 1998). The Hcap program determines instar number, mean head capsule width and SD within an instar, number of larvae in an instar, optimum instar separation points, and probabilities of instar misclassification from head capsule data. The mean development time and standard error for each life stage were determined from caged capitula dissection data for both

cohorts. Using calculated mean development times as a reference, a mean daily temperature was determined for each life stage from weather data taken from a meteorological station at the Arkansas Agriculture Experiment Station.

Results

The frequency distribution produced by Hcap indicates *L. minutus* in northwest Arkansas undergoes two larval instars (Fig. 1). Overall, head capsule widths ranged from 0.38 mm to 1.02 mm. The mean, standard deviation, number of larvae in an instar, size ranges, and instar misclassification probability calculated by the Hcap program are presented in Table 1.

Three days following oviposition, only eggs were present in the first full-flower collection, but by the second collection date, 7 d after oviposition, only L_1 larvae were found. This suggests eggs hatched 3-7 d following oviposition for the full-flower cohort. For the late-flower cohort, L_1 larvae were the most common life stage 3 d from oviposition suggesting a majority of egg hatch occurred in 1-3 d.

Instar development times and corresponding mean daily temperatures for both cohorts are presented in Table 2. Pupae were present in dissections 17-24 d from oviposition in the full-flower cohort and 6-17 d for the late-flower cohort. Development from oviposition to pupation occurred in 21.6 ± 1.0 d (n=9) for the peak-flower cohort and 16.8 ± 0.5 (n=28) d for the late-flower cohort. Twice-weekly collections of caged capitula were terminated at 24 and 17 d for full and late-flower, respectively, after dissections suggested pupae were the most prominent life stage. Adult emergence occurred 26.9 ± 0.8 d (n=9) and 25.6 ± 0.4 d (n=25) from oviposition for full and late-flower respectively.

Discussion

In this study, *L. minutus* was found to have two larval instars with head capsule widths at 0.51 ± 0.07 mm and 0.88 ± 0.07 mm. My results disagree with reports from a previous publication specifying three instars with corresponding head capsule widths at 0.5, 0.7, and 1.0 mm (Groppe 1990). Groppe's three head capsule widths fall within the range of measurements recorded in the present study. These observations (Groppe 1990) did not report a range, variance, or frequency for each instar, thus we suspect the reported number of instars and corresponding head capsule widths are inaccurate due to an insufficient sample size.

Alternatively, our study may have recorded a reduction in instar number for *L. minutus*. Although the number of larval instars is typically thought to be immutable within a species, intraspecific variability of instar number, otherwise known as developmental polymorphism (Schmidt and Lauer 1977), has been documented in >100 species and in most major orders (Esperk et al. 2007). A summary of publications reporting developmental polymorphism found variation of instar number commonly attributed to temperature, photoperiod, humidity, food quality and quantity, and sex (Esperk et al. 2007). This study was not designed to specifically address these factors, as the occurrence of two instars was unexpected. However, the possibility of their influence on larval *L. minutus* instar number could not be assessed as Groppe (1990), did not report developmental conditions.

Based on occurrence of pupae from dissections, an accelerated larval development rate, in comparison to that reported in Groppe (1990), was observed in both cohorts. Likewise, differences in the average daily temperatures experienced by the full and late-flower cohort are suspected to be the reason for differences in larval developmental rates. An increase in temperature can also have other effects in insect development. Head capsule widths of instars of

Listronotus bonariensis Kuschel (Coleoptera: Curculionidae) were shown to vary widely between seasons and even between generations (Goldson et al. 2001). In the chironimid *Glyptotendipes tokunagai* Sasa, higher temperatures induced decreases in both development times and head capsule widths (Baek et al. 2012). If higher temperatures can reduce the overall body size in *L. minutus*, laboratory larval development studies will elucidate this relationship and may provide an explanation for the discrepancy in mean head capsule width of this study and that of Groppe's (1990).

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Figure 1. Head capsule frequency distribution of *L. minutus* produced by Hcap from collections in northwest Arkansas. The solid line represents the distribution of each instar.

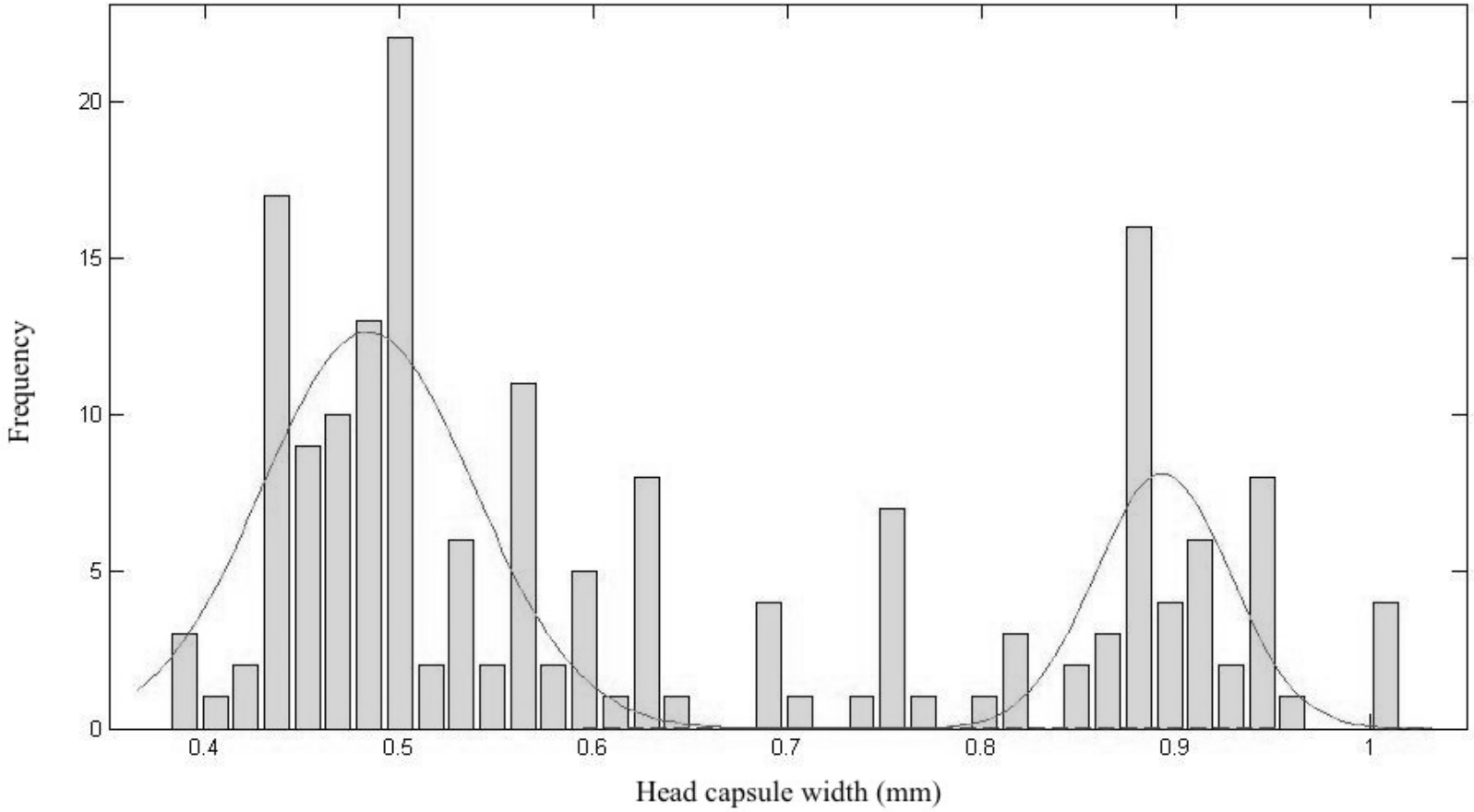

Table 1. Descriptive statistics for *Larinus minutus* head capsule width data calculated by Hcap.

Larval instar	Sample size	Mean ± SD (mm)	Size range (mm)	Probability of Misclassification		
				i as i-1	i as i+1	Total
1	120	0.51 ± 0.07	0.36-0.73	0.000000	0.000017	0. 000017
2	59	0.88 ± 0.07	0.73-1.03	0.032077	0.000000	0.032077

Table 2. Average larval development times (days) and daily temperatures for two cohorts of *Larinus minutus*. All times are measured from oviposition. Mean daily temperature is calculated from the daily maximum and minimum temperature between oviposition and mean development time for each stage. Full-flower was defined as a narrow 1-2 wk time period in which >80% of the capitula were in a state of bloom within the knapweed plot. Late-flower was occurred after full-flower and is characterized by <20% of the capitula in a state of bloom.

Cohort	Instar	n	Mean Development Mean ± SE (days)	Mean Daily Temp. (C°)	Range (days)
Full-flower	L1	33	9.55 ± 0.67	23.8	6-20
	L2	8	13.12 ± 0.66	23.52	10-17
Late-flower	L1	56	4.11 ± 0.23	29.95	3-10
	L2	42	11.52 ± 0.32	29.84	6-17

CHAPTER 4

SPREAD OF *L. MINUTUS* AT RELEASE SITES IN NORTHWEST ARKANSAS

Introduction

Spotted knapweed, *Centaurea stoebe* ssp. *micranthos* (Gugler) Hayek, is an invasive plant native to Eurasia. The plant was initially introduced to the North American Pacific Northwest (Watson and Renney 1974) and is now found in all but four states (USDA 2013). Ecological and economic damage from spotted knapweed infestations include increased stream sediment yield, soil surface water runoff, and reduction in available forage for livestock and wildlife (Watson and Renney 1974, Lacey et al. 1989).

Spotted and other invasive knapweeds, have been the target of classical biological control with a total of 13 natural enemies imported and established in North America (Müller-Schärer and Schroeder 1993). Of these, *Larinus minutus* Gyllenhal was first introduced to North America in 1991 (Lang et al. 1996). The weevil has since become established in Arkansas, Colorado, Indiana, Minnesota, Montana, Oregon, Wyoming, and Washington (Lang et al. 1996, Story 2002, Minteer et al. 2011). Introduction of *L. minutus* to sites in British Columbia and Colorado, areas in which other natural enemies have already been released, resulted in a decrease of diffuse and spotted knapweed density (Seastedt et al. 2003, Myers 2008). Larval *L. minutus* feeding destroys 100% of achenes in infested capitula (Kashefi and Sobhian 1998) and adult feeding, may kill bolting stems (Myers et al. 2009).

Larinus minutus was reported as spreading up to 140 m in one year from 2 release sites in Washington state (Whaley 2002) to ~2 km 2 years from a single release location in California (Woods and Popescu 2001). Formulation of a model describing spread and impact of *L. minutus* can provide valuable information in the implementation of future biological control programs. This study was designed to evaluate the local spread of *L. minutus* at multiple release sites in Arkansas.

Methods

Larinus minutus spread and infestation rates were documented by sampling along transects from late fall to late winter in 2011-2012 and 2012-2013 at *L. minutus* release sites. Sampling that occurred in 2011-2012 and 2012-2013 will hereafter be referred to as 2011 sampling and 2012 sampling, respectively. The year of initial release of *L. minutus* varied among release sites (2009-2011). Data were collected from 20 release sites in 2011 and 23 sites in 2012 (Table 1). While *L. minutus* was introduced at 37 sites in northwest Arkansas (Minteer 2012), mowing, urban development and yearly variation in knapweed patch size eliminated some of the release sites for sampling.

When establishing sampling transects, initial transects began at the release point so as to pass through the area with the most knapweed. A second transect, approximately perpendicular to the initial transect, was then similarly created. Four transects were established in this manner, with each new transect approximately perpendicular to the last created transect. Additional transects were created in-between the initial four transects in large knapweed patches in order to ensure a more complete description of density of *L. minutus* across the knapweed-infested site.

The total number and direction of transects was generally limited by spotted knapweed abundance as the weed is patchily distributed. Circular sampling quadrats with a ~7 m radius were established every ~15 m along each transect. Plastic flagging was used to mark the center and the perimeter of each quadrat to ensure that only the knapweed within a quadrat was assessed and that adjacent quadrats did not overlap. A quadrat was established at the next available knapweed patch along that transect in the event a quadrat contained no knapweed.

The coordinate of the center of each quadrat were recorded with a GPS device (Garmin Nüvi 500, Garmin Ltd., Kansas City, MO). Observations during this experiment placed

coordinate accuracy within ~2 m. These coordinates were later used to determine the distance of a quadrat from the point of initial release of *L. minutus* at a given site visual searches were conducted for 3 minutes or until an emergence hole was observed, whichever came first. A transect was terminated when no emergence holes were observed during the visual searches of two successive quadrats during 2011 sampling. In 2012, sampling transects terminated when no more knapweed could be found along the transect, or when further sampling was not possible because of the extension of the transect into posted private property. Transects never went beyond ~1 km, and most terminated within the first 300 m, as sites were bounded by anthropogenic features, natural boundaries, and the patchy distribution of knapweed. This change was made after observations from 2011 suggested weevil distribution at a given site could occur in a non-continuous manner and that the weevil could spread further than what was expected.

Greater than 100 capitula were collected in an unbiased manner throughout a quadrat and saved for subsequent dissection to determine percent infestation. Capitula were approached and collected from an angle that prevented observation of any potential emergence holes in order to prevent sampling bias. An infestation percentage was determined from a maximum of 100 dissected capitula, even if >100 were collected for each sampling circle. In the event <100 capitula were present within a quadrat, all capitula were collected and an infestation percentage was still determined. Capitula were classified as infested if a *L. minutus* emergence hole was observed, or if dissection revealed evidence of complete *L. minutus* development.

Release sites were grouped by years from release, and an average maximum distance of quadrats with *L. minutus* infested capitula was calculated in order to calculate yearly changes in maximum distance of *L. minutus* infestation from the release point at each site. An average *L.*

minutus infestation was calculated from all sampling quadrats within the first 50 m from the release point, hereafter referred to as the area of release, for release sites that recovered *L. minutus* from capitula collections. After these initial values were calculated, release sites were then grouped by year and an overall average *L. minutus* infestation at the area of release was determined.

Data were analyzed with a one-way ANOVA with maximum distance at which *L. minutus* were detected or percent infestation at the release area as the response variables and years since release as the fixed factor. Data on maximum distance were log-transformed and those on percent infestation were arcsine-square root transformed to ensure that they conformed to the assumptions of the ANOVA. Posthoc pairwise comparisons of means across the fixed factor were made using Tukey's HSD tests. Evidence of strong ($p<0.05$) effects are used to make inferences.

Knapweed is patchily distributed and *L. minutus* is univoltine. Both of these factors place limits on the distance *L. minutus* can move within a season. Consequentially, high levels of infestation were expected at the point of release and would likely decrease with distance from the release point. An exponential decay function would adequately describe this expected local population increase (i.e. at the release point) and spread of *L. minutus* following introduction. Diffusion equations are an established method in describing the spread of an invading or introduced organism and provide a first step in evaluating ecological factors involved with resultant spread (Rudd and Gandour 1985, Andow et al. 1990).

The exponential decay function $y = Ae^{-Bx}$ was fitted to transect sampling data with a Levenberg-Marquardt nonlinear least-squares algorithm in R to quantitatively describe spread at each of the release sites (R Development Core Team 2012). In the above equation, y is the

predicted %-infested capitula, and x is the distance from the release point. A is the estimate of %-infested capitula at the initial release location (i.e., x=0). B represents the rate at which density of *L. minutus* declines relative to distance from initial release point ('decay in weevil abundance' hereafter). The smaller the absolute value of B, the more gradual the decline in abundance from the initial release point. An approximate value of A and B based on the data were used as starting values for the iterative fitting of the exponential decay function using the algorithm mentioned earlier; the analysis provides the best fit parameter estimates for A and B given the data.

The exponential decay function was fit only to release sites that fulfilled the following criteria: 1) more than 10 quadrats were sampled per year, and 2) Sampling occurred in both collection years. The rationale for these criteria were that criterion 1 ensured that only sites with adequate data were used to understand the spread patterns, and criterion 2 enabled an examination of difference in spread patterns across sampling years. Five sites (sites 3, 4, 15, 16 and 31) met both of these requirements. Data from sites that did not fit the above criteria are presented as scatter plots displaying %-infested capitula in relation to distances from the release point.

Results

Although *L. minutus* infestation was recorded at most sites in both sampling years, there were some release sites in which the weevil was not recovered by the sampling method used (Table 1). Of the 20 release sites sampled in 2011, *L. minutus* infestation was not recorded at four sites (1, 20, 27 and 30). Likewise, weevil infestations were not documented from four of the 23 sites sampled in 2012 (9, 11, 20 and 27).

Release sites sampled in 2012 generally established quadrats at further distances from the release point than 2011, as the criteria used in making a decision to end a sampling transect were modified in the second year (Figures 1, 2, & 3). Sampling transects in 2012 would rarely go beyond 500 m due to the bounded nature of the release sites. Infestation was documented between 0–309 m and 0–622 m from all release sites from 2011 and 2012 sampling periods respectively. The average maximum distance of *L. minutus* infestation increased by ~60 m and ~100 m between 0–1 yr and 1–2 yr from release respectively but decreased ~25 m between 2–3 yr from release (Table 2). There was a difference in average maximum distance in relation to years since release ($F=2.946$; d.f.=3,30; $P=0.049$), although there were no significant differences observed between any 2 years ($P<0.05$). There was no difference in the average percent infestation in relation to years since release ($F=2.607$; d.f.=3,25; $P=0.074$), or between any 2 years ($P<0.05$) (Table 2).

The r^2 values of the exponential fit for sites 3, 4, 15, 16 and 31 were 0.58, 0.06, 0.68, 0.26 and 0.72 respectively in 2011 and 0.03, 0.37, 0.47, 0.51, and 0.20 in 2012 respectively. A significant increase in %-infested capitula was observed at sites 3, 4, and 15 while an increase was not observed at sites 31 and 16 ($P<0.05$, Table 3). The decay in weevil abundance (*B*) at sites 3 and 15 were lower between years suggesting that *L. minutus* spread from the release location ($P<0.05$, Table 4). *Larinus minutus* releases were made in 2009 for site 31, whereas releases in sites 3, 4, 15, and 16 were made in 2010. Infestations at the initial release point were ~5–15% based on the exponential decay model (Table 3) for 2010 release sites sampled in 2011. Infestation levels were higher for these sites from 2012 sampling 2 years after release (~38–48% at initial release point, Table 3).

Discussion

Overall, the large number of sites at which *L. minutus* infestation was recorded indicates successful establishment of populations of this species at the majority of the release sites. Monitoring of release sites following initial introduction found *L. minutus* present at all but two release sites (Minteer 2012). The consistent annual increase in both the average %-infested capitula within the area of release, and the maximum distance of infestation implies yearly population growth and spread following introduction to a site. Despite this, it is obvious that establishment of *L. minutus* populations at some sites remain less successful and have lower levels of infestation. Sites sampled 3 yr post introduction failed to follow the general pattern of yearly increase in terms of both maximum distance and average %-infestation at the area of release. This was likely due to the smaller number of sites that fell within this category. Equally, this may be the result smaller knapweed patches at these sites.

Emergence holes were observed in 2011 at site 30 and in 2012 at sites 20 and 27 during visual inspection from the six sites in which sampling failed to record *L. minutus* infestation. This indicates that the weevil is present at these release sites despite infested capitula not being collected. This result may possibly be due to either a clumped distribution of *L. minutus* such that 100 capitula per quadrat represents an inadequate sample size to capture establishment, and/or a reduced knapweed density. Given that the sampling regime in this study failed to record infestation at sites in which *L. minutus* was present, future sampling programs should retain visual searches in order to increase chances of observing *L. minutus* infestation.

Release sites analyzed with the exponential decay function revealed that localized increase and spread was evident at all sites except site 31. At site 31 similar infestation levels were reported from both sampling years at the area of release. Weevils in this area had an

additional year for population growth in comparison to the other 2010 introduction sites as *L. minutus* was introduced in 2009 at this site. As a consequence, site 31 had high infestation levels (~30%) at the release site during 2011 sampling relative to sites 3, 4, 15, and 16 (~5–15%). This also likely explains why the 2011 infestation levels at site 31 (~30%) were similar to 2012 infestation levels at sites 3, 4, 15 and 16 (~39–48%).

There was also no significant difference in percent infestation between sampling years at site 16 (Table 3). A severe reduction of knapweed near the release site in 2012 was possibly a result of a drought in combination with mowing and grass competition, which reduced the competitive ability of knapweed. However, of the release-site-knapweed that could be sampled at this site in 2012, plants were typically stunted and were not as robust in comparison to the previous year. This may have reduced survival of larval *L. minutus* by reducing achene production. A high larval mortality would result in lower *L. minutus* infestation levels from quadrats as only capitula in which an emergence hole was present were counted as infested.

An increase in *L. minutus* population was evident from significant increases of release-site-knapweed infestation levels between sampling years 2011 and 2012 at the remaining sites (3, 4, and 15) (Table 3). Of these, site 3's knapweed was the most like a monoculture for both sampling years, with a high level of knapweed coverage. Mowing from nearby businesses contained knapweed in this area to an absolute distance of ~180 m from the release point in 2012. The absolute value of B at site 3 was significantly smaller in 2012 than in 2011, indicating that local spread has occurred at this bounded site (Table 4).

Both sites 4 and 15 had healthy, robust knapweed in 2011 with reduced competition from other plants. These sites also had large knapweed populations in 2012. Both of these factors likely led to the significant *L. minutus* population increase observed at the release point at both

sites. At site 4, although sampling in 2011 only extended to ~200 m, there was a marked increase in infestation levels at the same distance in 2012 suggesting successful spread of the weevil from the release site. There was a similar pattern at site 15 in which a clear increase in infestation was recorded at ~300 m, the edge of that site's sampling in 2011. These data suggests an outward expansion of *L. minutus* from the release point after population growth occurs.

These data suggests that in the years following introduction of *L. minutus* to a release site, increases in infestation and spread can be expected. The high number of sampled release sites in which *L. minutus* was recovered suggested that *L. minutus* was likely present at all release sites, although sometimes at non-detectible levels with the sampling regime used in this study. Capitula infestations up to ~21% may be expected within the area of release two years post introduction given an average release of ~750 *L. minutus*. Furthermore, *L. minutus* could be expected to spread at least ~225 m from the release point two years post release based on these studies. These findings have implications for future spotted knapweed biological control programs that utilize *L. minutus*. Increases in both the number of release sites and their proximity to each other would be an appropriate approach for the rapid and sustained suppression of spotted knapweed within a confined area. If rapid suppression is not a priority distances between release points could be increased. These conclusions were supported by analysis of release sites using the exponential decay equation, which showed a yearly increase of capitula infestation and spread from the release point. Diffusion models, like the exponential decay function used in this study, are valuable tools in investigating the spread of an introduced organism (Andow et al. 1990); my findings support the utility of such approaches. Future studies should continue transect sampling of release sites to observe how colonization progresses

across these release sites. Novel patches of knapweed, in which no releases of *L. minutus* were made, should also be monitored and sampled in order to capture instances of long-distance dispersal of individual gravid females.

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Table 1. *Larinus minutus* release sites sampled over course of study.

Site	Lat.	Long.	Weevil Release Date	# Weevil Released	Sampling Year			
					2011		2012	
					Sampled Yes/No	Recovery Yes/No	Sampled Yes/No	Recovery Yes/No
1	36.31833	-94.18419	6/29/10	600	Yes	No	Yes	Yes
2	36.30461	-94.18047	6/29/10	600	Yes	Yes	Yes	Yes
3	36.322325	-94.18543	6/29/10	600	Yes	Yes	Yes	Yes
4	36.2985	-94.16965	6/25/10 & 7/2/10	1000 & 800	Yes	Yes	Yes	Yes
5	36.354611	-94.175259	6/25/10	700	No	N/A	Yes	Yes
8	36.334011	-94.162925	6/24/11	1500	Yes	Yes	Yes	Yes
9	36.20656	-92.99887	7/6/10	400	No	N/A	Yes	No
11	36.17681	-93.53625	7/1/10	300	No	N/A	Yes	No
12	36.23018	-93.5303	7/1/10	600	Yes	Yes	No	N/A
13	35.90324	-93.93130	6/25/11	600	Yes	Yes	Yes	Yes

14	35.91032	-93.93620	6/25/11	600	Yes	Yes	Yes	Yes
15	36.25772	-93.63195	7/1/10	1200	Yes	Yes	Yes	Yes
16	36.25634	-93.63939	7/1/10	1200	Yes	Yes	Yes	Yes
20	36.17105	-93.91383	6/25/10	700	Yes	No	Yes	No
21	36.05995	-94.12745	6/29/10	900	Yes	Yes	Yes	Yes
22	36.10334	-94.0061	7/1/10	800	No	N/A	Yes	Yes
23	36.10069	-94.05145	7/1/10	400	No	N/A	Yes	Yes
25	36.120473	-94.153211	7/2/10	400	Yes	Yes	No	N/A
27	36.03448	-94.18479	6/13/09	700	Yes	No	Yes	No
29	35.92496	-94.19784	6/14/09	700	Yes	Yes	Yes	Yes
30	35.96508	-93.99424	6/25/11	800	Yes	No	Yes	Yes
31	36.10053	-94.18552	6/16/09	700	Yes	Yes	Yes	Yes
33	35.9845	-94.19894	6/15/09	700	Yes	Yes	Yes	Yes
34	36.07678	-94.19767	6/13/09	600	Yes	Yes	Yes	Yes
35	36.111396	-94.162396	6/26/11	700	Yes	Yes	Yes	Yes

Table 2. The average maximum distance and average infestation for sites of a certain release year.

Years post release	Average maximum distance *L. minutus* recorded (m) Mean ± SE	Average infestation in first 50 m (%) Mean ± SE
0	64.38 ± 37.49 (n=4)	2.83 ± 0.83 (n=4)
1	126.1 ± 25.53 (n=13)	5.27 ± 1.28 (n=11)[1]
2	225.46 ± 53.41 (n=13)	20.76 ± 5.80 (n=12)
3	205.7 ± 47.72 (n=4)	19.75 ± 1.79 (n=2)

[1]Reductions in sample size between average maximum distance and average infestation are a result of some sites not having knapweed within the first 50 m.

Table 3. *A* values estimated by the exponential decay function $y = Ae^{-Bx}$.

Site number	2011-2012 Mean ± SE	2012-2013 Mean ± SE
3	15.33 ± 2.48 a[1]	38.58 ± 4.69 b
4	5.57 ± 2.08 a	44.4 ± 9.85 b
15	11.81 ± 1.4 a	39.74 ± 6.20 b
16	14.41 ± 3.78 a	47.83 ± 20.83 a
31	30.09 ± 4.68 a	24.34 ± 5.52 a

[1]Means in a row followed by the same letter are not significantly different (P<0.05). 95% CI were used.

Table 4. *B* values estimated by the exponential decay function $y = Ae^{-Bx}$.

Site number	2011-2012 Mean ± SE	2012-2013 Mean ± SE
3	$1.6 \times 10^{-2} \pm 3.9 \times 10^{-3}$ a[1]	$9.6 \times 10^{-4} \pm 1.4 \times 10^{-3}$ b
4	$4.8 \times 10^{-3} \pm 4.9 \times 10^{-3}$ a	$5.6 \times 10^{-3} \pm 2.0 \times 10^{-3}$ a
15	$3.1 \times 10^{-2} \pm 4.4 \times 10^{-3}$ a	$1.2 \times 10^{-2} \pm 3.0 \times 10^{-3}$ b
16	$1.6 \times 10^{-2} \pm 6.9 \times 10^{-3}$ a	$3.0 \times 10^{-2} \pm 1.4 \times 10^{-2}$ a
31	$2.3 \times 10^{-2} \pm 7.1 \times 10^{-3}$ a	$4.0 \times 10^{-3} \pm 3.0 \times 10^{-3}$ a

[1]Means in a row followed by the same letter are not significantly different ($P<0.05$). 95% CI were used.

Figure 1. Sites sampled in 2011 and 2012 following releases of *Larinus minutus* adults in the summer of 2009.

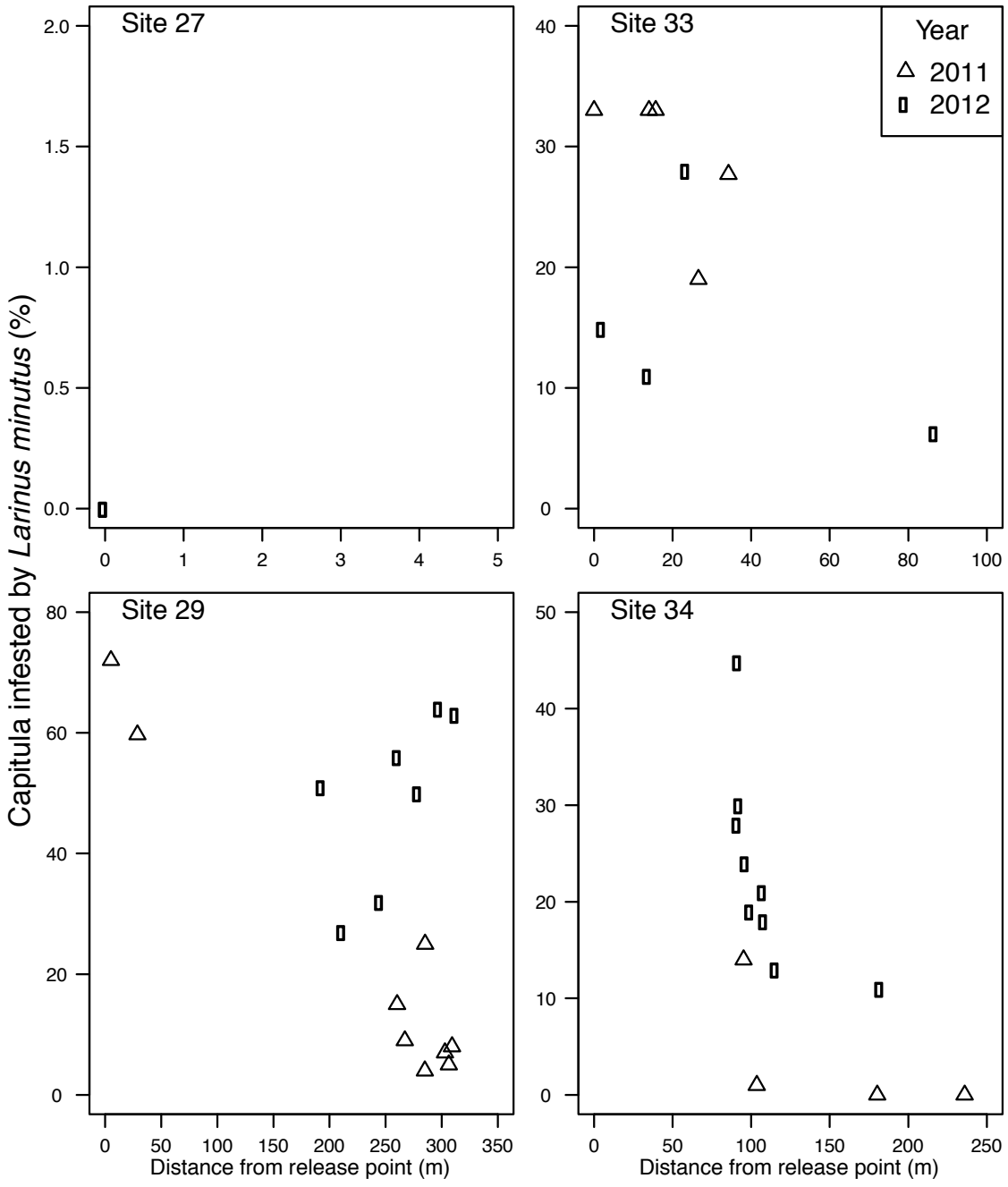


Figure 2. Sites sampled in 2011 and 2012 following releases of *Larinus minutus* adults in the summer of 2010.

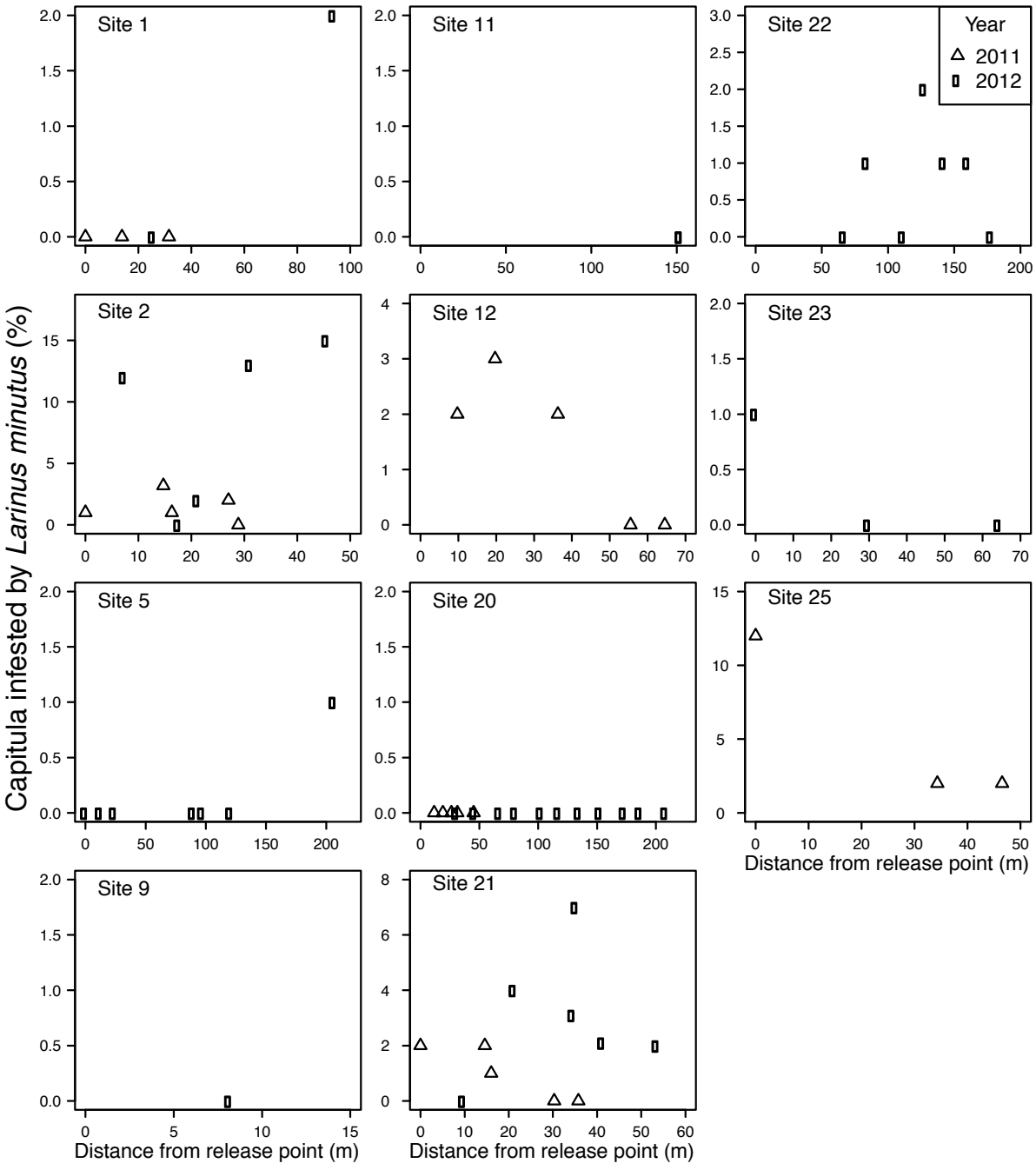


Figure 3. Sites sampled in 2011 and 2012 following releases of *Larinus minutus* adults in the summer of 2011.

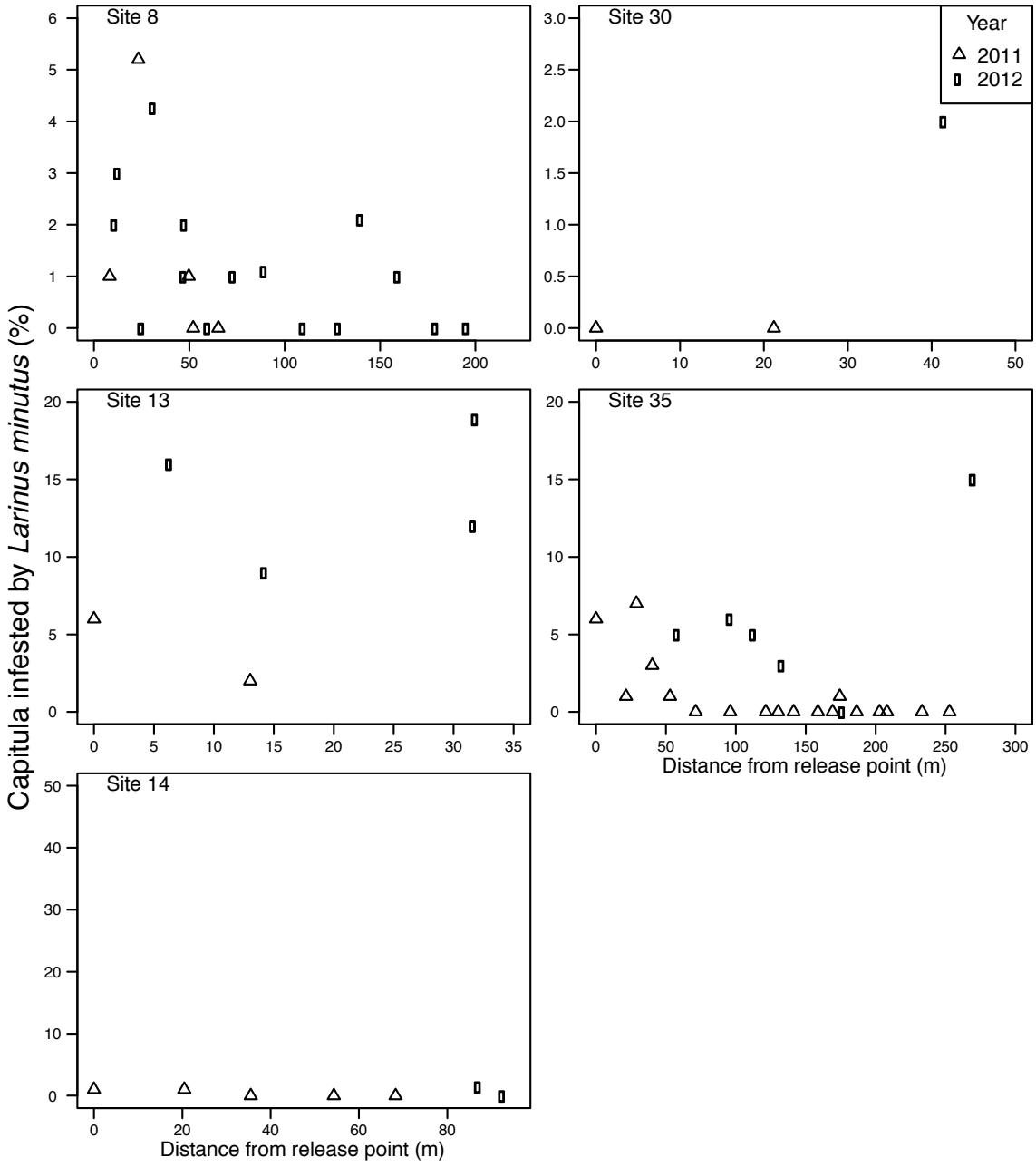
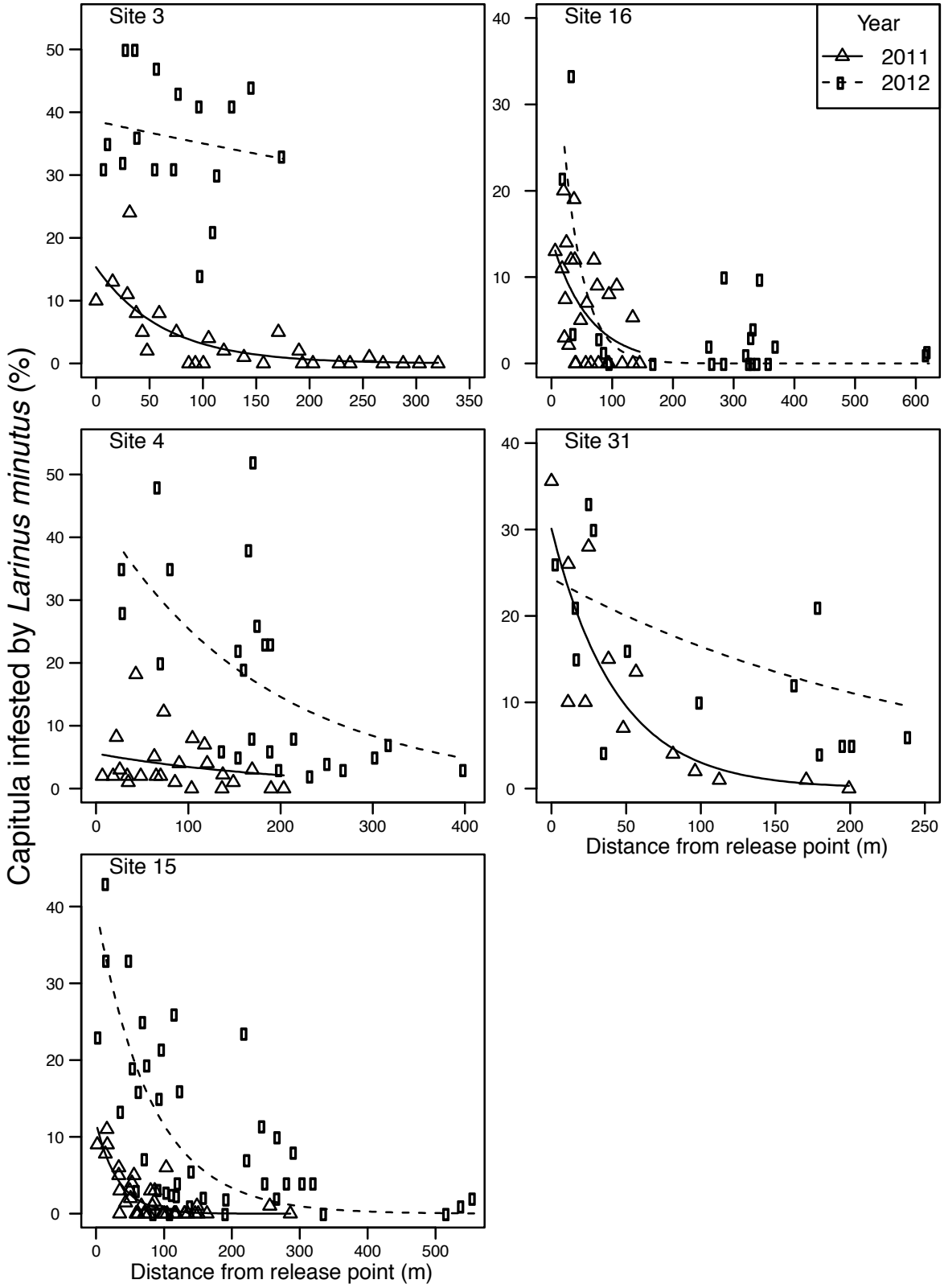


Figure 4. Sites for which the exponential decay function was fit.



CHAPTER 5

CONCLUSION

Conclusion

The objectives of this thesis were to better define the larval development of *L. minutus* and to describe spread from release sites in the years following introduction. This study calculated average development times of each larval instar and reported accelerated rates of development with fewer instars in comparison to previous publications. This research determined that annual increases in both capitula infestation rates and spread from a release site can be expected in the years following introduction.

Larinus minutus undergoes only two larval instars. This conflicts with a previous publication specifying three instars (Groppe 1990). My findings showed a large range in size within each instar. Since no rearing conditions, head capsule range, variance, or frequency were reported in Groppe (1990), I suspect the discrepancy in number of instars to be the result of an insufficient sample size in Groppe (1990). Developmental polymorphism (i.e., a shift in # of instars) can result from differences in temperature, photoperiod, and humidity (Esperk et al. 2007). Although it is possible that the differences in environmental conditions between the studies may have influenced instar number, I feel this is unlikely due to the clear findings of this study and the lack of methodology reported by Groppe (1990). Future multiple temperature laboratory development studies could fully assess the hypothesis that *L. minutus* undergoes temperature-induced developmental polymorphism. However, a more expansive collection of European *L. minutus* headcapsule data would be a more cost effective first step. Larvae developed in 24 and 17 d for full and late-flower cohorts respectively. A previous publication placed larval development as complete at 28 days (Groppe 1990). Multiple temperature laboratory development studies could also assess if these differences are a result of differences in temperatures from the weevils native range.

Increases in infestation and spread can be expected in the years following successful introduction of *L. minutus* to a site. There was fairly large variation in the level of *L. minutus* infestation increase among sites. This variation was likely due to factors such as constancy of knapweed between years, overall patch size, and a patchy spatial distribution that may reduce the amount of samplable knapweed. For all sites sampled, consistent increases in infestation at the area of release and in maximum infestation distance were observed, except for sites sampled three years from release. This was likely the result of the small number of sampled release sites fitting this category. An additional year of sampling when at least 10 more of these sites are at 3 years after release would provide a more robust analysis. This could be accomplished though sampling in late 2013. The sampling regimen used with this study (collection and dissection of 100 capitula) was inadequate at detecting low-level infestations with collection data only. Sample size could be increased significantly (e.g., to 1000 capitula), but this may not be cost or time effective. I believe it is imperative that future studies include a longer, standardized visual search component in order to record sites with low infestations. Although visual search is less precise as infested capitula are much more likely to be missed, the technique vastly increases the number of capitula sampled in the same period of time.

This study provides valuable reference information to future biological control programs utilizing *L. minutus*. In this study, an average release of ~750 *L. minutus* resulted in capitula infestations up to ~21% within the area of release and a spread of at least ~225 m from the release point two years post introduction. This spread distance is extremely conservative, as the study sites were largely bounded (geographically restricted). It is also evident that while the exponential decay function used in this study does not completely explain the observed patterns of spread, it does provide an essential first step in recognizing that additional variables influence

weevil spread. The percentage of knapweed coverage should be incorporated as a covariate of the exponential decay function to investigate knapweed amount in relation to capitula infestation levels. Future studies of *L. minutus* dispersal should attempt to sample and monitor novel patches of knapweed, of which no releases of *L. minutus* were made, to capture occurrences of long-distance dispersal. Findings from these studies, in addition to this study, can provide information in determining an optimal distance in which to make *L. minutus* releases.

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