




2017

## INVASION DYNAMICS OF THE EXOTIC LIANA *EUONYMUS FORTUNEI* (TURCZ.) HAND.-MAZZ. (WINTERCREEPER)

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INVASION DYNAMICS OF THE EXOTIC LIANA *EUONYMUS FORTUNEI*  
(TURCZ.) HAND.-MAZZ. (WINTERCREEPER)

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DISSERTATION

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A dissertation submitted in partial fulfillment of the  
requirements for the degree of Doctor of Philosophy in the  
College of Arts and Sciences  
at the University of Kentucky

By

Todd Jeffrey Rounsaville  
Lexington, Kentucky

Director: Dr. Mary Arthur, Professor of Forest Ecology  
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2017

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## ABSTRACT OF DISSERTATION

### INVASION DYNAMICS OF THE EXOTIC LIANA *EUONYMUS FORTUNEI* (TURCZ.) HAND.-MAZZ. (WINTERCREEPER)

Elevated atmospheric CO<sub>2</sub> has been implicated as a driver of increased liana abundance worldwide. Known as disturbance creators and beneficiaries, lianas possess the potential to significantly influence forest ecosystems. I investigated the early-invasion dynamics of *Euonymus fortunei* (wintercreeper), an evergreen liana that is invading forests in eastern North America, disrupting native plant communities and ecosystem functions.

Wintercreeper is widely cultivated as an ornamental groundcover, frequently invading natural areas via asexual stem growth. Invasion of remote natural areas is dependent upon seed transport and may occur less frequently. I examined the mechanisms of seed dormancy by conducting a 'move-along' experiment using fresh and after-ripened seeds. Additionally, I sought to characterize the nature of seed dispersal by birds by deploying seed traps within an invaded forest in central Kentucky. Wintercreeper seeds displayed conditional nondeep physiological dormancy. Although germination occurs at high velocity following cold stratification, a cold period was only facultative to break dormancy. While fresh seeds had greater germination rates (98.6%) compared to after-ripened seeds (85.7%), after-ripened seeds experienced earlier germination (41 days across all treatments). My findings also indicate that bird-mediated seed dispersal occurs throughout the winter from seeds that after-ripen on maternal liana phanerophytes. Overall, these data suggest wintercreeper seeds are capable of dispersing and recruiting in areas with mild to non-existent winters.

The invasion of natural areas by exotic invaders is regulated by biotic and abiotic processes, which influence the invader's success or failure. I studied the vegetation and soil effects of paired invaded (INV), uninvaded (NAT), and 'restored' (RES) sites on the germination and survival of wintercreeper seeds and seedlings. The effect of aril (with vs. without) was also tested, both in the field and *in vitro*. In the field, total germination and first year survival were 55.6% and 24.2%, respectively, across treatments. Total germination was unaffected by treatments, yet vegetation ( $P = 0.0016$ ) and aril ( $P = 0.001$ )

treatments significantly influenced germination rates over time, including delayed germination of seeds with arils. The proportion of germinated seedlings that survived was significantly different based on vegetation ( $P = 0.054$ ) and aril ( $P = 0.071$ ) treatments after the first winter of growth, but not prior to the first winter. The proportion of seedling survival was significantly lower among seeds dispersed with an aril and seeds sown within INV treatments; there were no interactive effects.

Finally, I examined how seedling density and growth habit (horizontal vs. vertical) influenced plant survival, growth, and allometry. I also tested extractable soil C, N, P, K, Ca, and Mg prior to planting and after 17 months of soil conditioning. I found evidence that increased planting density negatively influenced growth among individual plants (lower survival, basal diameter, shoot mass, root mass). At the plot level, high-density plantings yielded greater stem length, and shoot, root, and combined biomass, indicating positive frequency dependence for this species. Soil analyses indicated C, N, P, Ca, and Mg significantly increased ( $P < 0.05$ ) over the course of the experiment. I conclude that in wintercreeper, prominent asexual propagation leads to aggregate populations whose total contributions to above- and belowground biomass are positively correlated with density, even though individual plant fitness is not.

Altogether, this work provides insight into how wintercreeper invasions occur at broad and fine scales. This information will provide a foundation for future wintercreeper studies and aid land managers in their prevention and control strategies.

KEYWORDS: Biological Invasion, *Euonymus fortunei*, Liana, Seed Germination, Seedling Growth

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\_\_\_\_\_  
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April 28, 2017  
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Date

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*To my family*

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## Chapter One

### Introduction and background

#### Overview

Since Elton's seminal "The Ecology of Invasion by Animals and Plants" was published (1958), the discipline of biological invasions has grown to encompass a diverse field of study. Increased global connectivity (globalization) has no doubt facilitated opportunities to study both the pathways and consequences of biological invaders. This field of research is important due to invasive species' potential to influence human health, agriculture, species diversity, and ecosystem function. In many cases, researchers are compelled to include nonindigenous and invasive species in their studies, simply because of these species' ubiquity (and thus influence) within natural systems. Alternatively, invaders may become the central focus of research that seeks to understand the nature of their success in novel environments, and the resulting fate of these invaded ecosystems.

Among those who study biological invasions, there is not a strict consensus of how to define the term 'invasive species'. Although there is agreement that invasive species can spread and self-sustain within a new environment, contention arises where *impact* (ecological or economic) is included in the definition (Lockwood et al. 2013). The primary concern when invoking an *impact* statement is the inherent human subjectivity, rather than the empirical evidence that comes from documenting the spread of populations. Still, there is agreement that biological invasions are themselves a *process* as opposed to binary classification in which an organism is either invasive or non-invasive.

Blackburn et al. (2011) have provided the scientific community with a ‘unified’ model of the invasion process which incorporates previous models from both plant and animal literature (Fig. 1.1). The utility of such a model is manifold; it provides consensus for invader terminology, outlines a series of stages and barriers to invasion, and provides a framework for management. For those studying a given nonindigenous species, this model may be advantageous for identifying what barrier(s) have precluded additional spread, potentially influencing management concerns and recommendations. The ‘unified’ model (Fig. 1.1) also provides utility for species that (by any definition) have become ‘invasive’ by passing through each barrier during the process of invasion. For example, we can intuitively ascertain that failure (at any stage) during the process, leads to a breakdown (failure) in invasion. Determining which barrier(s) are most limiting to spread can help guide management in the most efficient way possible. For example, the invasive biennial *Alliaria petiolata* (garlic mustard) is noted to have rather complex population dynamics, and studies show that not only is targeted management of adults more efficient than control of basal rosettes, but targeting rosettes with herbicide can actually increase population densities over time (Pardini et al. 2009). Additionally, studies that elucidate the complexities of the invasion process can aid the prevention of a given invader from reaching additional susceptible environments. For plants whose spread is due largely (or wholly) to heavy seed production (propagule pressure), empirical evidence of this has led to legislative action including outright bans of sale and propagation of certain species (Drew et al. 2010; Oregon Department of Agriculture 2017). Finally, by determining what traits have facilitated the colonization of known

invasive species, we can hope to gain predictive power for future invasions, particularly for closely related species (congeneric species and conspecific varieties).

Among terrestrial plant invaders, the ornamental plant industry is the leading contributor to the introduction of invasive species (Lockwood et al. 2013). This is especially true of woody invaders in North America, of which 99% were intentionally introduced, including 85% for landscaping alone (Martin et al. 2009). Some of these species are widely recognized as invaders, including *Acer platanoides* (Norway maple), *Celastrus orbiculatus* (Oriental bittersweet), *Euonymus alatus* (burning bush), and *Paulownia tomentosa* (Empress tree), and their status as such has led to numerous ecological studies (Dirr 1998). Still many other alien plants which span the continuum of invasion have not been studied at all, including some that are widespread in native ecosystems. One of these species is the evergreen liana *Euonymus fortunei* (wintercreeper), which is the central focus of this dissertation.

### **History of *Euonymus fortunei* introduction**

Exactly 20 years after Robert Fortune identified wintercreeper (named in his honor as *Elaeodendron fortunei* Turcz.) in China, the first specimens were introduced to cultivation in 1865 (Graves, 1940). Wintercreeper quickly became a desirable landscape plant because it was easy to establish in cultivation and provided year-round interest due to its evergreen foliage. Leaf mutations are noted to be common, with a variety of shapes and color sports that are readily propagated from vegetative cuttings (Dirr 1998). Dirr (1998) lists 53 varieties and named cultivars in commerce which include a plethora of variegated leaf patterns, growth habits (spreading, climbing, shrubby), and ultimate size

at maturity. This high diversity is unsurprising overall, given that *Euonymus fortunei* is recognized as the most widespread and polymorphic of ~130 *Euonymus* species worldwide (Ma et al. 2008). Additionally, wintercreeper has two morphologically diverse life phases. Juvenile plants are typically small-leaved groundcovers, which become sexually mature after ascending trees, during which leaf size and shape change significantly. The combined influence of natural phenotypic diversity, maturity-induced phase change, taxonomic confusion/hybridization, and propensity to mutate and revert under cultivation (Dirr 1998), present a significant challenge for tracking individual genotypes.

Due to early involvement in botanical exploration of China by C.S. Sargent and E.H. Wilson (Graves 1940), the Arnold Arboretum seems to possess the earliest records of *E. fortunei* introductions to North America (Table 1.1). The earliest of these records appear to be cultivars ‘Carrierei’ and ‘Reticulata’, introduced to the Arnold Arboretum in 1880 and 1885, respectively. Additional clones and cultivars steadily appeared in the early 20<sup>th</sup> century, some of which originated from cultivated plants in Europe (Graves 1940). Still, there is great confusion in regard to both wild origin/provenance (virtually unknown) and garden origin of plants. For example, the small-leaved cultivar ‘Minimus’ was allegedly introduced from France in 1912 (Graves 1940; Dirr 1998) but other records indicate it was accessioned (#6269\*A) in Boston, Massachusetts as early as November 15, 1910 (Arnold Arboretum 2017).

Of particular interest to the study of wintercreeper invasion is the ‘purple’ form, which is recorded as both *E. fortunei* var. *coloratus* and as a cultivar, ‘Coloratus’. This form remains available commercially, and is noteworthy because it is phenotypically

similar to the vast majority of invasive populations in Eastern North America (personal observation). According to Graves (1940), the origin of this plant dates back to 1914 when F.N. Meyer made a wild (seed) collection from Shensi, China (Fig. 1.2). As Graves (1940) notes, this form is quite similar to the type specimen save for the reddish-purple winter coloration, which is not known among wild populations. At this time in his career, Meyer was working for the USDA whom he had instructed to send one-quarter to one-half of all his seeds to Charles Sargent at the Arnold Arboretum (Cunningham 1984). The immediate fate of the purple clone remains unclear, but the ‘Coloratus’ cultivar came to the Arnold Arboretum as a plant on March 21, 1922, was accessioned as #15434\*A, and removed from the collection sometime after 2011 (Arnold Arboretum 2017). Records indicate that ‘Coloratus’ was first accessioned at the New York Botanical Garden (# R456/35) in 1935, and had come from Vassar College (Jon Peter, NYBG Plant Records Manager, personal correspondence). In all likelihood, it had entered the nursery industry in the 1920’s, if not before. In Kentucky, there is little to no phenotypic diversity in naturalized populations, which all resemble ‘Coloratus’. The earliest records of naturalized wintercreeper in Kentucky date to 1922, and based on herbarium records did not become widespread until the 1980’s (Liang 2010).

These records serve to provide support for a substantial ‘lag time’ that preceded invasion in Kentucky, where my research took place. Invaded areas in central Kentucky exist as dense groundcover monocultures (100% forest floor cover) that appear to reduce species diversity and prevent natural forest succession and regeneration. Similarly, neighboring states have experienced similar invasions, evident from the growing amount of ecological research being generated, particularly by Dr. Heather Reynolds’ research

group (Swedo et al. 2008; Smith and Reynolds 2012, 2015; Bauer and Reynolds 2016) at Indiana University, as well as others in Ohio and Kentucky (Hertzberg 2011; Conover et al. 2016; Mattingly et al. 2016; Bray et al. 2017). Indication from EDDMaps (2017) that *Euonymus fortunei* has naturalized across Eastern North America (Wisconsin to Maine, and south from Texas to Florida), but is rarely recognized as an invader, is cause for concern. Especially troubling is the ability of wintercreeper to invade undisturbed old-growth forests, as opposed to many invaders that are primarily associated with canopy gaps and disturbance (Zouhar 2009). Knowing that wintercreeper experiences a lag time of invasion, areas experiencing preliminary stages of colonization may greatly benefit from ecological studies to aid early detection and prevention.

The overall goal of this work was to (1.) Provide a historical narrative for the introduction and use of wintercreeper in North America (Chapter One); (2.) Investigate the ecological processes that promote (or impair) wintercreeper invasion (Chapters Two, Three, and Four); and (3.) Present the implications of this research and provide insight for future studies (Chapter Five). I chose to study this system (wintercreeper) based in part on personal experience with it as a prolific invader of natural areas in Kentucky. More importantly, I chose to study wintercreeper because its status as an invasive species remains equivocal in the horticultural domain, and there is little empirical evidence to suggest otherwise. Thus, the desirable outcome of this work is to provide evidence of invasibility in the hopes of future regulatory action; to elucidate what mechanisms (if any) facilitate this species invasion in order to aid prevention and management; and to propose potentially valuable follow-up studies that can help protect natural areas from additional invasion.

Figure 1.1. A proposed unified framework for biological invasions by Blackburn et al. (2011). Copyright © 2011 Elsevier Ltd. All rights reserved. doi:10.1016.

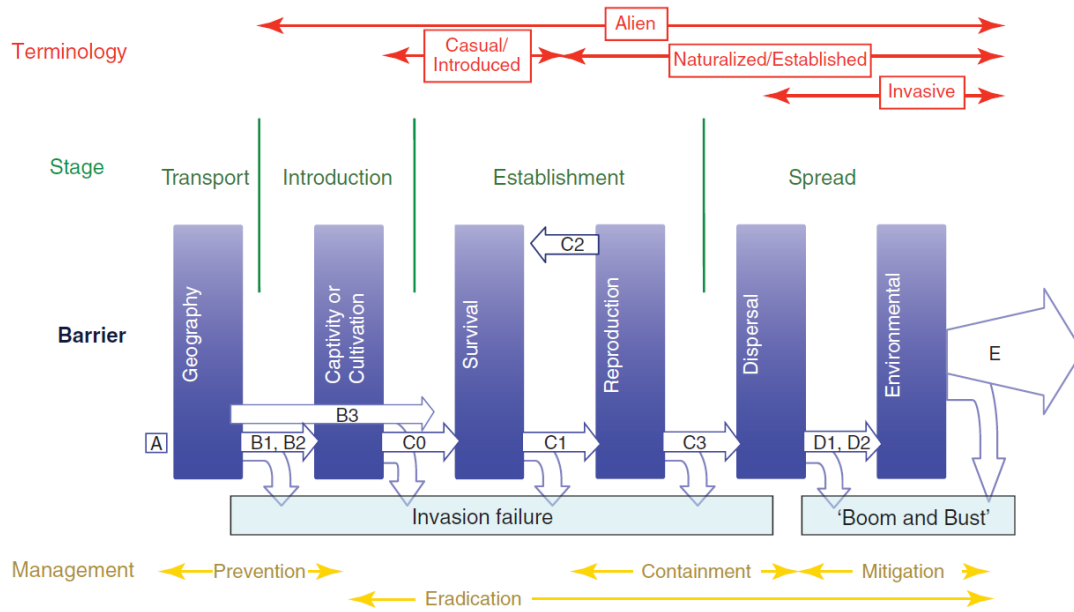




Table 1.1. Earliest accession records for *Euonymus fortunei* varieties and cultivars at the Arnold Arboretum prior to 1930. Data provided by the Arnold Arboretum (2017).

Name	Accession Number	Date accessioned	Source	Received as
<i>E. fortunei</i>	7219*A	March 15, 1908	E.H. Wilson, China	seed
<i>E. fortunei</i> var. <i>vegetus</i>	15407*A	November 16, 1921	unknown	cutting
<i>E. fortunei</i> var. <i>radicans</i>	15409*A	November 16, 1921	unknown	plant
<i>E. fortunei microphylla</i>	6268*A	November 15, 1910	unknown	plant
<i>E. fortunei multiflora</i>	6270*A	November 15, 1910	unknown	plant
‘ <i>Carrierei</i> ’	163*A	January 20, 1880	unknown	cutting
‘ <i>Coloratus</i> ’	15434*A	March 21, 1922	unknown	plant
‘ <i>Minimus</i> ’	6269*A	November 15, 1910	unknown	plant
‘ <i>Reticulata</i> ’	14571*A	April 15, 1885	unknown	plant
‘ <i>Silver Queen</i> ’	15408*A	July 17, 1918	unknown	plant

Figure 1.2. Photograph taken by F.N. Meyer (1914) of *Euonymus fortunei* growing in situ (Shensi, China). Copyright © 2004, President and Fellows of Harvard College, Arnold Arboretum Archives; all rights reserved.



## Chapter Two

### **Seed dynamics of the liana *Euonymus fortunei* and implications for invasibility**

#### **Introduction**

In recent decades, lianas have gained attention as significant drivers of temperate and tropical forest dynamics (Gerwing et al. 2006). The lianescent life form is that of a structural parasite, perpetually in competition with host trees for light, water, and nutrients (Castagneri et al. 2013). Lianas are formidable competitors, largely due to their ability to focus carbon (C) allocation to primary growth, with lower investments in tannins, lignin, cellulose, and leaf mass area in comparison to host trees (Asner and Martin 2015). Elevated CO<sub>2</sub> levels have been shown to promote liana establishment (Wang et al. 2011), and gains in liana abundance further influence C dynamics by reducing C storage and sequestration through a reduction in tree biomass (Van der Heijden et al. 2015). Considering the well-documented increase in atmospheric CO<sub>2</sub> levels worldwide (Lotfiomran et al. 2016), lianas have the potential to increase in abundance in both native and novel environments. Interestingly, many of the growth trends displayed by lianas are indicative of invasive plant species, e.g., higher CO<sub>2</sub> assimilation rate, standing biomass, net primary productivity, shoot-root ratio, and specific leaf area, in comparison to liana tree hosts or native species (Ehrenfeld 2003; Asner and Martin 2015). Given the invasive possibilities, a better understanding of liana dispersal and colonization within susceptible environments is an important consideration for forest management and plant conservation.

One such invader is *Euonymus fortunei* (Turcz.) Hand.-Mazz., a liana of rising ecological concern. Among roughly 130 species of *Euonymus* L. (Celastraceae), *E. fortunei* is regarded as both the most widespread and polymorphic species (Ma et al. 2008). *Euonymus fortunei* occurs naturally throughout China, as well as parts of India, Japan, Korea, Thailand, and Vietnam, and it is cultivated on every continent except Antarctica (Ma et al. 2008). High intraspecific diversity in phenotype, and (potentially) genotype, has facilitated the introduction of numerous clones within the horticultural trade of North America, where it is colloquially known as ‘wintercreeper’. Cultivation of wintercreeper began as early as 1865 (Graves 1940), and to this day its evergreen (and in some clones, variegated) foliage and adaptability make it a desirable groundcover for a broad range of garden environments. Only recently has the scientific literature regarding *E. fortunei* begun to shift from horticultural [i.e. pest management and nursery growth optimization (Cockfield and Potter 1986; Cole et al. 2005; Guo et al. 2007)] to ecological, the latter in response to the species’ invasiveness, both potential and realized.

Recent *E. fortunei* investigations have helped elucidate plant-soil feedback mechanisms (Smith and Reynolds 2012, 2015; Bray et al. 2017) as well as methods for eradication and native community restoration (Bauer and Reynolds 2016; Conover et al. 2016; Mattingly et al. 2016). These studies have begun to provide an urgently needed narrative for *E. fortunei* community ecology and control methods, which will be critical for forest restoration efforts. Nevertheless, little is currently known about the dynamics of *establishment*, specifically that of seed dormancy and dispersal. In viewing biological invasions as a process (*sensu* Blackburn et al. 2011), seed dynamics can serve as barriers between certain stages of invasion, thus influencing the degree of success for a given

species. Therefore, a better understanding of seed dynamics as invasion barriers can also guide management strategies in space and time, given that: 1. The frequency of long distance dispersal events (for terrestrial plants: seed) is considered the most important contributor to the rate of geographic spread (Lockwood et al. 2013; Merow et al. 2011), and 2. Seed dormancy influences the persistence of propagules in the soil seed bank (Chambers and MacMahon 1994). Additionally, propagule pressure is positively correlated with invasiveness and widely regarded as the most important factor for successful establishment (Colautti et al. 2006; Simberloff 2009), in part because as the number of seeds increases, so does the potential for dispersal events and seed bank introductions (among other factors). Although it has never been formally quantified, my observations suggest that propagule pressure is high in wintercreeper based on the volume of seeds that is consistently produced each growing season.

Wintercreeper is an evergreen liana with two distinct life forms. Chamaephytic juveniles with small leaves root adventitiously along the forest floor, often forming thick mats of vegetation that have been shown to alter soil chemistry, bacterial communities, and litter decomposition rates (Bray et al. 2017). When a vertical host (e.g. tree) is found, so begins the transition to a phanerophytic adult with larger leaves. Adult morphs ascend as structural parasites, and lateral branching precedes the capacity to flower and set seeds. Invasive populations of wintercreeper exhibit a flowering period that extends over several weeks in June/July, with fruit maturation occurring in October/November (personal observation), which is consistent with phenologic reports from natural populations in China and Japan (Masaki et al. 1994; Ma et al. 2008).

To my knowledge, there have been no formal investigations into the nature of *Euonymus fortunei* seed dispersal, nor the mechanisms of seed dormancy-break and germination. The fruits of *Euonymus* spp. are conspicuous and taxonomically indicative of the genus. Wintercreeper produces a pinkish-white capsule, from which ~4 diaspores are suspended and enclosed by an orange seed envelope. Though it is regularly referred to as an aril, the seed covering is more correctly characterized as a caruncle due to its micropylar origin (Zhang et al. 2011). No explicit accounts of seed dispersal have been given for wintercreeper, but authors frequently note the attractively colored diaspores of *Euonymus* spp. as being bird-dispersed (Brizicky 1964a; Thomas et al. 2011). Personal observations from the field suggest that bird-dispersal of wintercreeper may be minor to nonexistent, given that: 1. A significant number of diaspores are retained on fruiting specimens throughout the winter, and 2. A large quantity of intact diaspores collect on the forest floor beneath fruiting specimens. These observations suggest that dispersal may be largely (or wholly) due only to gravity, and thus without animal-mediated (long-distance) dispersal.

There are no reports related to seed dormancy in wintercreeper, but as Nikolaeva (1969) noted concerning the genus as a whole, seeds of most species are in a state of deep dormancy and incapable of germinating without a period of cold stratification. Baskin and Baskin (2014) reported that the number of days of cold stratification necessary to break dormancy in several *Euonymus* species was 139 (*E. americana*), 60 (*E. atropurpureus*), 75 (*E. maackii*), 180 (*E. sacrosacta*), and 105 (*E. verrucosa*). In *Euonymus maackii* and *E. verrucosa*, loss of dormancy is promoted when a warm stratification period precedes cold stratification (Baskin and Baskin 2014). *Euonymus*

*alatus*, another significant invader of North American forests, was reported to break dormancy following 90 days of cold stratification (Dirr and Heuser 1987). However, when *E. alatus* seeds were sown in a variety of natural environments, Brand et al. (2012) reported no germination until the second or third spring, suggesting that warm followed by cold stratification was necessary to break dormancy. One exception to deep dormancy in *Euonymus* may be *E. nanus*, for which Nikolaeva (1969) reported that no seed dormancy existed.

I can conclude from prior seed investigations that dormancy in *Euonymus* spp. is endogenous, rather than exogenous (Baskin and Baskin 2014; Brizicky 1964a; Kollman et al. 1998), thus the embryo is dormant. Embryological studies reveal that polyembryos occur in some (but not all) species of *Euonymus* (Brizicky 1964b). Polyembryony, the presence of two or more embryos within a single seed, can influence plant fitness through modification of seed mass, seedling survival, and genetic (sporophytic vs. gametophytic) inheritance (Oka et al. 2016). Brizicky (1964b) found polyembryos in eight out of fifteen species of *Euonymus*. Polyembryony has never been reported for *Euonymus fortunei*, but given the frequency of its occurrence in congeners, I suspected it could be a factor influencing establishment dynamics.

Severity of wintercreeper invasions (density and % cover) appears to be positively correlated with proximity to human development, specifically, in areas where wintercreeper was cultivated residentially or commercially (personal observations). In some cases, it appears that ‘escape’ from cultivation was the result of vegetative (asexual) stem growth. Diminishing degrees of colonization as a function of distance from cultivated plants despite their abundant and regular seed production is not entirely

surprising given that frugivorous birds often deposit most consumed seed <5 m from the host canopy (Masaki et al. 1994; Viswanathan et al. 2015). My field observations of *E. fortunei* colonization indicate a lack of recruitment in uninvaded natural areas near (<5 m) to fruiting lianas, suggesting that one or more seed-related barriers could exist. Given the deep-dormancy seemingly is inherent in *Euonymus* spp., it is possible that unmet endogenous or exogenous stimuli prevent germination. The abundance of fruit persisting on maternal lianas and fallen beneath them could be evidence for lack of dispersal, or overproduction. In the case of the latter, I would expect dispersal is occurring but recruitment is prevented by other means.

Lacking any published data related to *E. fortunei* seed dormancy and ecology, I chose to test two hypotheses that may explain a lack of seed recruitment. H<sub>1</sub>: a dormancy-breaking requirement (e.g. extreme cold temperature, or temperature sequence) is not being met, thus seed recruitment cannot occur. H<sub>2</sub>: seeds are not being transported (animal dispersed) away from the maternal parent. Testing these hypotheses will expand the knowledge base of wintercreeper ecology within its introduced range by examining for the first time several facets of *Euonymus fortunei* seed dynamics. It will also inform the scientific community's collective understanding of plant invasions in eastern North America, particularly concerning lag effects and predictive power for alien plant species currently characterized as non-invasive. More immediately, this research will aid land managers seeking to prioritize management strategies in the prevention and eradication of wintercreeper.



## Materials and Methods

### *Study site*

In central Kentucky where the study took place, natural areas tend to exhibit a strong dichotomy in regard to wintercreeper establishment: those that are largely devoid of wintercreeper and those that approach 100% cover throughout. One notable exception to this personal observation is a site commonly referred to as ‘Scott’s Grove’, a 20 ha deciduous forest in southern Jessamine County (Bloom et al. 2002). Cross sections taken from mature wintercreeper stems at Scott’s Grove indicate that the species has been present for at least 30 years. Despite this, there exists a patchwork of invasion across the forest in which heavily invaded (100% cover) regions with fruiting colonies of wintercreeper exist as islands within an otherwise native and largely homogeneous forest (Fig 2.1).

### *Seed germination*

With so few published studies on *E. fortunei* invasion dynamics, it is worthwhile to comment on the genetics of my study population. The plants growing in Scott’s Grove are phenotypically similar to the clone more commonly referred to as ‘purple wintercreeper’ (a synonym for *E. fortunei* ‘Coloratus’). The ‘Coloratus’ cultivar is distinguished by the reddish-purple coloration of winter foliage, a trait that is not known in wild (i.e. old world) populations. Instead, a single origin of this phenotype originated from a 1914 seed collection by Frank N. Meyer in Shensi, China (Graves 1940). Although it is plausible the plants used in this study are ‘Coloratus’ clones resulting from

apomixis (potentially combined with abortion of recombinant embryos), I cannot discount outcrossing with other horticultural clones.

*Euonymus fortunei* seeds were collected twice for the purpose of testing both ‘fresh’ (ripe) and after-ripened seeds. The first collection (Exp. 1, ‘fresh seed’) was made on November 3, 2012, when the fruit capsules were naturally dehisced to expose the orange diaspores. The second collection (Exp. 2, ‘after-ripened seed’) was made on February 2, 2013, at which time the mature diaspores had been attached to the maternal lianas for approximately 3 months. Both seed collections were taken from the same ~30 mature lianas, which had naturally invaded Scott’s Grove in Jessamine County, Kentucky.

For each collection, seeds from all lianas were bulked and a random subset was used for the germination study. The caruncle of each seed was removed by gently rubbing with a paper towel. No further cleaning or sterilization was performed, and the cleaned seeds were immediately placed on the germination substrate. Experimental units consisted of 50 cleaned seeds evenly distributed in a 90 mm Petri dish on 5 mm of white quartz sand moistened with distilled water, and three dishes were used for each treatment and control. For the duration of the germination studies, a sheet of clear plastic wrap was used to enclose the Petri dish to prevent excessive drying.

Identical ‘move-along’ trials were conducted for both seed collections, each consisting of eight sets of three dishes of seeds (Table 2.1). Move along experiments are germination phenology studies used to determine what temperatures or temperature sequences are required for dormancy break (Baskin and Baskin 2003). Move along treatments (1-3) were cycled among incubators in a progression designed to mimic

seasonal temperatures of central Kentucky, starting in the winter (treatments 1 and 2) and summer (treatment 3). The remaining five sets of seeds functioned as controls (4-8), and they were held at the same day/night temperature regime for the duration of the experiment.

The incubator environment was set to cycle between a day/night (respectively) temperature for winter (5/1° C), early spring/late autumn (15/6° C), late spring/early autumn (20/10° C), and summer (25/15° C). The day vs. night temperature cycles each lasted for 12 h per day. The incubators provided 14 h of cool white fluorescent light (20  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , 400-700 nm) each day. The lights came on 1 h prior to the start of the high temperature and remained on for 1 h after the start of the low temperature. Unlike all other incubators, the 1° C incubator used for the winter treatment and control did not provide light, nor did it cycle temperature. For practical purposes, the incubators themselves could not be replicated, thus each treatment was comprised of three pseudo-replications. Each experimental unit was checked on a weekly basis to record number of germinated seeds and provide supplemental distilled water as necessary. A seed was scored as 'germinated' when the radicle exceeded 1 mm in length. All Petri dishes remained in their respective move-along cycles or control temperatures until all seeds had either germinated or rotted. Germinated seeds were discarded to prevent overcrowding within Petri dishes. When seeds with two or more radicles were observed, I recorded the number of polyembryos. A sample of 50 polyembryonic seeds were transferred to pots containing sterile potting media in a greenhouse environment. These seeds were allowed to develop into seedlings in order to assess polyembryo survival.

### *Seed dispersal*

Gravity-traps were used to characterize seed dispersal. Each seed-trap consisted of a shallow athletic training cone whose top and bottom diameters were 8 and 28 cm, respectively. The cones were inverted, and affixed with plastic zip-ties to a 15.2 cm long section of PVC pipe with an internal diameter of 14.6 cm. A plastic cup with small drainage holes was inserted inside the PVC housing that allowed seed to be safely retained and later counted. A hole was drilled and tapped at the base of the PVC housing to accept a setscrew, which held the cup in position firmly against the inverted cone above. Finally, two more plastic zip-ties threaded through holes in the PVC housing attached the entire seed-trap to a piece of metal rebar, 122 cm above the forest floor.

A total of 90 seed-traps were constructed and deployed in a 9 x 10 grid format within the study forest (Fig 2.2). The forest had a *Quercus* spp./*Fraxinus* spp./*Acer saccharum* dominated canopy and minimal understory due to the absence of shrubs and winter-dormancy of herbaceous vegetation. A 10 m spacing between traps was chosen to balance proximity to heavy-fruited wintercreeper phanerophytes with areas that were completely devoid of wintercreeper. This yielded a study site with an area of 7200 m<sup>2</sup> (80 x 90 m) and a total seed-trap area of 5.54 m<sup>2</sup> [ $(\pi 14 \text{ cm}^2) 90$ ] or 0.077% of total area. The duration of this dispersal study was from November 1, 2014 through May 2, 2015. Data were collected every 2 weeks at which time seeds were removed from the traps and counted. To test viability, seeds collected in the traps were sown in a greenhouse environment using sterile potting media.

### *Statistical analysis*

Statistically speaking, my primary interest was comparing the germination rates (speed) across the eight treatments/controls. That only some of the experimental units reached complete germination presented a challenge for modeling these data. All traces of linearity in the germination rates vanished near 34 weeks. Thus, fitting a linear model where each treatment received its own slope would miss much of the information contained in these data.

To better model these data, I considered both the fact that only a few experimental units reached full germination and that there were repeated measurements taken on each experimental unit over time. I thus focused on the time taken to exceed pre-specified levels of percentage germination. Because all controls and treatments reached at least 75% germination (>38/50 seeds), I deemed 75% to be the greatest percentage of interest. Below 75%, I considered increments of 15% down to 15% itself. Increments of 15% were chosen to provide a tradeoff between significant changes in percentage germination (not too small) and jumps in percentage germination so large they may inappropriately group together treatments (not too large). These two concerns led to a multivariate analysis of variance (MANOVA) with the response variables as the number of weeks to exceed  $p$ th% germination, where  $p$  is a vector of the five percentages of interest: 15, 30, 45, 60, and 75. This MANOVA perfectly adapts to effects of the repeated measures, correlations across time for each of the experimental units. The MANOVA was fit using the statistical software R (R. Core Team 2014).

A small sample size of seeds collected as part of the dispersal experiment rendered statistical analysis inappropriate. Instead, data were summarized qualitatively.

## Results

### *Seed germination*

Overall seed germination was high among both experiments, and fresh (exp. 1) and after-ripened (exp. 2) seeds had mean total germination values of 98.6 and 85.7%, respectively. Among fresh seeds, the mild winter treatment, winter control, and early spring control all achieved complete (100%) germination after 17, 33, and 36 weeks, respectively. No after-ripened treatments reached 100% germination, although the mild winter treatment and mild winter control had the highest germination (90% each). While germination was lower for after-ripened seeds than for fresh seeds across all treatments/controls, I also found that after-ripened seeds began germinating earlier than fresh seeds (Figs 2.3, 2.4). Germination rates (time between first and last germination observation) ranged from three to 40 weeks for fresh seed given winter treatment and after-ripened seeds given winter control, respectively (Figs 2.3, 2.4). Nevertheless, germination rates did not always show uniformity between experiments or treatments. For example, temperatures used for winter (1°) and mild-winter (5/1°) treatments, as well as winter (1°) and mild-winter (5/1°) controls consistently yielded faster germination in fresh seeds (vs. after-ripened), taking 3 (vs. 12), 4 (vs. 9), 11 (vs. 40), and 15 (vs. 24) weeks, respectively. Conversely, the summer treatment (25/15°), and early spring (15/6°), late spring (20/10°), and summer (25/15°) controls consistently yielded faster germination in after-ripened seeds (vs. fresh), taking 9 (vs. 36), 13 (vs. 19), 6 (vs. 34), and 8 (vs. 25) weeks, respectively.

Fresh seeds held at 25° C (summer control) began germinating within 2 weeks, indicating that cold stratification is not required for dormancy breaking. Even so, fresh

seeds held at 1° C (winter control) began germinating after 23 weeks, and when fresh seeds at 1° C were transferred to 15/6° C (winter move-along treatment), germination rates were extremely rapid. I conclude from these findings that *Euonymus fortunei* seeds display conditional nondeep physiological dormancy, in which cold stratification is not required *per se*, yet it can delay germination, and alter (depending on fresh vs. after ripened) germination rates.

The MANOVA yielded an overall effect of treatment that was statistically significant [ $F(35,160) = 4.35, p < 0.0001$ ]. Statistically, this indicates that at least one treatment's mean vector (a vector of average weeks to exceed pth% germination for each value of p) was different from the other treatments mean vectors. For example, at constant early spring temperatures fresh seeds responded differently than after-ripened seeds (Fig 2.5); i.e. the control at 15/6° C consistently germinated faster in experiment two than in experiment one. Because at least one control, e.g. early spring, is grouped differently across experiments, there is intuitive evidence that there exists a statistical difference between experiments. Indeed, the overall effect of experiment was found to be statistically significant [ $F(5,28) = 327.61, p < 0.0001$ ]. Since both main effects were highly significant, I also checked the interaction between experiment and treatment. The experiment by treatment interaction was statistically significant [ $F(35,160) = 3.63, p < 0.0001$ ].

I observed the presence of polyembryos in 38 out of 156 wintercreeper seeds (24.4% polyembryony) that had germinated over a two-week period towards the beginning of experiment 1. In actuality, this may be a conservative value since seeds were discarded when any one radicle emerged and grew >1 mm. Although a few

polyembryonic seeds (n=4 out of 156 tested) had three embryos, the majority (97.4%) yielded two. Upon moving approximately 50 polyembryonate seeds to a greenhouse environment, I observed 100% survival of all embryos. Seedlings that originated within the same seed all appeared to develop and grow normally.

### *Seed dispersal*

In total, the 90 seed traps deployed within the study forest collected 29 diaspores and 5 cleaned seeds over the course of the winter (Fig 2.2). All diaspores were collected from traps directly beneath or in close proximity to heavy-fruited adult lianas. Conversely, 'clean seed' was found both under and away from fruiting lianas, suggesting that for these seeds, the diaspores had been consumed and expelled by birds. Although only five cleaned seeds were captured during the study, they were collected between early December and late March with no obvious temporal trend. Intact diaspores showed a strong pulse of dispersal at the end of winter, during which 24 of 29 seeds were recorded between February 21<sup>st</sup> and April 4th (Fig 2.6). The 5 cleaned seeds captured in the traps germinated and grew normally in a greenhouse environment.

### **Discussion**

Results from the move-along germination experiment indicate that *Euonymus fortunei* seeds are highly viable and exhibit conditional nondeep physiological dormancy. I therefore reject H<sub>1</sub> (that failure to recruit may be due to an unmet dormancy-breaking requirement) based on my findings that all treatments/controls germinated between 78.6 (winter control) and 100% (mild winter move-along treatment, winter and early spring



controls). The ability of wintercreeper seeds to germinate with or without cold stratification or temperature cycling can be regarded as facultative dormancy, which ultimately serves to protect seeds from premature germination (Dillon and Reichard 2014). Based on the system put forth by Baskin and Baskin (2004), *E. fortunei* seed exhibits Type 2 nondeep physiological dormancy in which the minimum temperature for germination decreases from high to low during the progression from dormancy to non-dormancy.

After-ripening is a process in which dry storage of seeds can release physiological dormancy and thus promote germination (Baskin and Baskin 2014). After-ripened seeds for exp. 2 were collected after approximately 3 months of dry, cold storage while attached to the maternal lianas. When comparing fresh seeds (exp. 1) and after-ripened seeds (exp. 2), I found that the 3-month-old after-ripened seeds germinated faster (greater slope) than fresh ones at all five control temperatures (Fig 2.4). Thus, dormancy-break (after-ripening) had occurred while seeds were attached to the mother plant during winter. Similarly, when cold stratified seeds (winter and mild winter move-along treatments) were transferred to 15/6° C (early spring), I observed that after-ripened seed germinated 2 weeks earlier than fresh ones (Fig 2.3). These results are unsurprising overall, given that after-ripening can lead to more uniform and higher velocity germination due to widening the range of temperatures necessary to germinate, altering sensitivity to regulatory plant-hormones, and modifying light/darkness requirements (Finch-Savage and Leubner-Metzger 2006).

While after-ripened seeds appear to germinate earlier and faster than fresh ones [by 41.1 days (5.9 weeks) across treatments/controls], I also found that total mean

germination percentage was lower for after-ripened seed across all treatments/controls (85.7% for after-ripened vs. 98.6% for fresh) (Figs 2.3, 2.4). This may be the result of excessive drying of the seeds during after-ripening, during which the aril darkens and withers over the course of the winter (personal observation). One might conclude from these observations that a trade-off exists between seeds that are dispersed early vs. allowed to after-ripen. Seeds that reach the forest floor in early winter may ultimately have greater viability via cold/moist stratification, yet they are simultaneously at greater risk of seed predation by rodents or microorganisms (Chambers and McMahon 1994). Alternatively, seeds left to after-ripen above ground may compensate for an abiotic reduction in viability by avoidance of biotic predation. Of course, one can only speculate about the level of post-dispersal seed predation for wintercreeper, though Kollmann et al. (1998) found that *Euonymus europaeus* seeds had an extremely thin testa and experienced low levels of rodent predation in comparison to other fleshy fruits.

One anomalous result of this experiment was the fresh vs. after-ripened germination of the early spring control (15/6° C) in comparison to the other control temperatures. Based on my modeling (Fig 2.5), fresh seeds maintained at 15/6° C exhibited a similar germination pattern to the colder controls [winter (1° C) and mild winter (5/1° C)]. This pattern is highly disparate compared to after-ripened seed maintained at 15/6° C, which resembled that of warmer controls [late spring (20/10° C) and summer (25/15° C)]. This is insightful considering 15/6° C was the median temperature among the five controls, and experienced both a comparatively warm ‘day’ temperature (15° C) as well as a comparatively cold ‘night’ temperature (6° C). Thus, it is possible the ‘night’ temperature (6° C) of the early spring control was sufficient to hold

the seeds in a state of dormancy for 17 weeks, similar to the winter and mild winter controls. By contrast, the late spring control began germinating within 3 weeks, suggesting a 'night' temperature of 10° C was insufficient to maintain dormancy. Although after-ripening appears to have made germination conditions more favorable (i.e., earlier germination vs. fresh seeds for all corresponding temperatures), Fig 2.4 reveals that germination uniformity (velocity) was inconsistent among control temperatures. For example, uniformity of germination was greater for late spring and summer controls when after ripened, yet greater for winter and mild winter controls when fresh. These results provide further evidence that cold stratification of at least 6° C, while facultative, ultimately acts to prolong seed dormancy, and in turn increases germination velocity.

Hormonal cues also may play a role in wintercreeper's seed dormancy. Abscisic acid (ABA) is a plant growth hormone that is known to induce dormancy and inhibit germination of seeds (Baskin and Baskin 2004). Thammina et al. (2012) found that in *Euonymus alatus*, a prolonged period of dormancy is likely maintained by a continual biosynthesis of ABA. In many plants, cold stratification has been correlated to a loss in ABA, although ABA loss alone may not confer non-dormancy (Baskin and Baskin 2014). The level of, and sensitivity to, endogenously produced ABA differs among plants, and it is further affected by other hormones known to break dormancy and promote germination, notably the gibberellins (GA) and ethylene (Baskin and Baskin 2004). Exogenously applied GA<sub>3</sub> overcame dormancy in *Euonymus europaeus* seeds, yet the embryonic pathways to overcome dormancy were different in comparison to that of cold stratification (Béranger-Novat and Dawidowicz-Grzegorzewska 1992). Taken altogether,

the ability of fresh seeds to germinate within 3 weeks at 20/10° (late spring) and 25/15° C (summer), but 17+ weeks at 1° (winter), 5/1° (mild winter), and 15/6° C (early spring) may be the result of a complex interaction between temperature, hormones, or other factors.

Upon capturing clean (bird-dispersed), viable seed within the seed-traps, I must reject my second hypothesis (H<sub>2</sub>) that the failure of seedlings to recruit away from wintercreeper populations may due to an absence of avian dispersal. Despite small sample numbers, the seed-trap experiment provided qualitatively enlightening insights. First and foremost, my observations indicate that bird-mediated (potentially long-distance) dispersal occurred. In the context of this study, I have no way of knowing how far the bird dispersed seed traveled before reaching the seed traps. Still, in modeling the European starling-mediated dispersal of the closely related liana *Celastrus orbiculatus* (Oriental bittersweet) in the northeastern United States, Merow et al. (2011) estimated a local bird dispersal distance of 2.14 km annually. Furthermore, personal observations over the course of the study revealed the presence of both wintercreeper diaspores and cleaned seed in mammalian scat on the forest floor. While only five ‘cleaned’ seeds were caught during the study, seed traps accounted for only 0.077% of the 7,200 m<sup>2</sup> study site, suggesting that the entire area would have been subjected to 6,494 bird-dispersed seed alone. By the same logic, some 37,662 diaspores would have fallen independently. It is possible that I would have caught more seeds if a different seed-trap design had been employed, as post-data collection simulated seed drop tests conducted indoors illustrated that a significant number of seeds bounced out of the inverted plastic cones. By

extension, this suggests that seed dispersal is actually higher than the speculative numbers reported here.

Ultimately, practical interest in these data is centered upon the invasive dynamics of *Euonymus fortunei*, particularly in how this species increases in local abundance and its potential for geographical spread. In central Kentucky, there are two likely fates for wintercreeper seeds, best exemplified herein as the winter move-along treatment (bird dispersal of fresh seed in early winter) and early spring control (after ripening followed by spring abscission). The winter move-along treatment suggests that after 12 weeks at 1° C, exposure to 15/6° C (early spring temperature) initiates rapid wintercreeper germination after two weeks (Fig 2.3). Historically, central Kentucky reaches 15° C on March 26<sup>th</sup>, and experiences a low of 6° C on April 9<sup>th</sup> (U.S. Climate Data, 2016). Alternatively, if held indefinitely at 1° C, fresh seed will still germinate after 23 weeks (Fig 2.4, winter control). These results indicate that naturally cold/wet stratified wintercreeper seeds will remain dormant but germinate with great velocity during the onset of spring temperatures. Even so, a large proportion of wintercreeper seeds will also remain aboveground to after-ripen until early spring. These diaspores began germinating within 4 weeks at early spring temperatures (Fig 2.4, after-ripened seeds at 15/6° C), experiencing minor reductions to total germination compared to fresh seeds of the aforementioned winter control. These two natural scenarios for recruitment confer wintercreeper with alternative seed fates. This divergence in seed conditioning methods allows a means for seed persistence within variable environments (Cochrane et al. 2014), which can be particularly advantageous for exotic species.

It is interesting to note that unlike most previously studied *Euonymus* taxa, *E. fortunei* exhibits extremely weak dormancy. Using freshly matured seed, I observed germination within two and three weeks for summer and late spring controls, respectively (Fig 2.4). The ecological significance of this relates to the facultative requirement for cold stratification in wintercreeper, quite unlike all previously reported species of *Euonymus* [other than *E. nanus* (Nikolaeva 1969)]. In central Kentucky where this research took place, germination was prevented by low seasonal temperatures during the winter. On the other hand, the ability to germinate without cold stratification, combined with the confirmation of bird-mediated dispersal, suggests that range expansion southward and/or to areas with milder winters is highly probable. Recent reports certainly validate this in Texas and other areas in the southeast (EDDMaps 2016; Nesom 2010). Ultimately, the rate of spread is further compounded by the popularity of wintercreeper for landscaping, as the plant is unregulated and readily available commercially. The broad landscape-adaptability exhibited by wintercreeper, coupled with the seed dormancy and animal dispersal data, and the well supported finding that liana establishment is promoted with elevated CO<sub>2</sub> levels (Wang et al. 2011; Van der Heijden et al. 2015), further position it as a species capable of additional range expansion in response to climate change.

To the narrative of wintercreeper colonization and adaptability there is another interesting finding that deserves further study: polyembryony. Polyembryony is the production of two or more embryos within a seed, and has been documented in some (but not all) species of *Euonymus* (Brizicky 1964b). Polyembryos may originate from a variety of tissue types, with or without fertilization, but are generally the same among

species (Brizicky 1964b). The particular embryonic origin of polyembryos can lead to alternative outcomes of ecological consequence, including changes to ploidy (if endosperm derived) or asexual (non-recombinant) reproduction via apomixis (Thurlby et al. 2012). Among *Euonymus* species exhibiting polyembryony, the vast majority of seeds were polyembryonate (vs. monoembryonate), and while two, three, or four embryos were most common, *E. americanus* had up to ten embryos per seed (Brizicky 1964b). There are no previous reports of polyembryony in *E. fortunei*, but I estimated at least 24.4% of the seeds were polyembryonic. Brizicky (1964b) reported that adventitious embryony from the inner integument was found for all documented polyembryonic *Euonymus* species as well as *Celastrus scandens* (Celastraceae), thus I presume it is also the case for *E. fortunei*. Since fertilization is required for adventitious embryo formation (Brizicky 1964a), it is likely that polyembryonic wintercreeper seeds contain both a sexually-recombinant novel genotype as well as one (or more) maternally cloned genotypes. In theory, this strategy may be another means of increased fitness: recombinant embryos see increased genetic variation to aid colonization of novel environments, while amegaspermy simultaneously confers local adaptation (Thurlby et al. 2012; Verhoeven et al. 2010). More directly, in their study of the invasive vine *Vincetoxicum rossicum*, Ladd and Cappucino (2005) found that mortality was higher among individual embryos of polyembryonic seeds, yet the probability of at least one polyembryo surviving was greater than for monoembryonic seeds (100% vs 71%), overall. Thus polyembryony, as a vehicle for increased propagule pressure, may aid invasibility by compounding the amount of establishment opportunities per dispersal unit.

## Conclusions

I found that invasive populations of *Euonymus fortunei* possess several divergent strategies that may aid fitness at local scales while also contributing to range expansion. Wintercreeper seeds were highly viable and exhibited conditional nondeep physiological dormancy. In Kentucky, dormancy break may occur via natural cold-stratification at the soil level or after-ripening of seeds on maternal plants. Each strategy may be advantageous in varying environmental conditions, yet dormancy break by either method was shown to be facultative. Movement by humans (cultivated plants) and animals (seeds) is likely to contribute to range expansion, upon which a lack of obligate dormancy barriers coupled with polyembryonic seeds may further aid the movement of wintercreeper into natural areas. These findings suggest that seeds are an important driver for wintercreeper invasion dynamics, and land managers may benefit from the targeted removal of phanerophytic individuals. The patchwork invasion of the study forest used for this experiment is not attributable to physiological seed barriers or a lack of seed rain; thus future studies should consider the influence of soils, native vegetation, and seed-herbivory. Though the body of data implicating *Euonymus fortunei* as a significant invasive species is still small, I hope these findings will help reduce or regulate the intentional cultivation of the species and aid future research.



Table 2.1. Outline of treatments and controls used for wintercreeper seed germination experiments<sup>z</sup>

Move-along treatments	Progression of temperatures (C°)							
	1°	15/6° <sup>y</sup>	20/10°	25/15°	20/10°	15/6°	20/10°	1°
1. winter								
2. mild winter	5/1°	15/6°	20/10°	25/15°	20/10°	15/6°	20/10°	5/1°
3. summer	25/15°	20/10°	15/6°	5/1°	15/6°	20/10°	25/15°	
weeks at temperature	12	4	4	12	4	4	4	12

Controls<sup>x</sup>

4. winter (1°)
5. mild winter (5/1°)
6. early spring (15/6°)
7. late spring (20/10°)
8. summer (25/15°)

<sup>z</sup>Modified from Baskin and Baskin (2003).

<sup>y</sup>Simulated day/night cycled temperatures. 'Day' temperatures receive 12h of the 14h daily photoperiod.

<sup>x</sup>Held at constant temperatures

Figure 2.1. Site characteristics of ‘Scott’s Grove’, the experimental forest used during the study. (A: left) photograph of one invaded ‘island’ (arrows indicate invasion boundary), (B: top right) map of site location (red star) in Kentucky (Kentucky Geological Survey, 1979), (C: bottom right) historical average temperatures for central Kentucky (°C) (U.S. Climate Data, 2016)

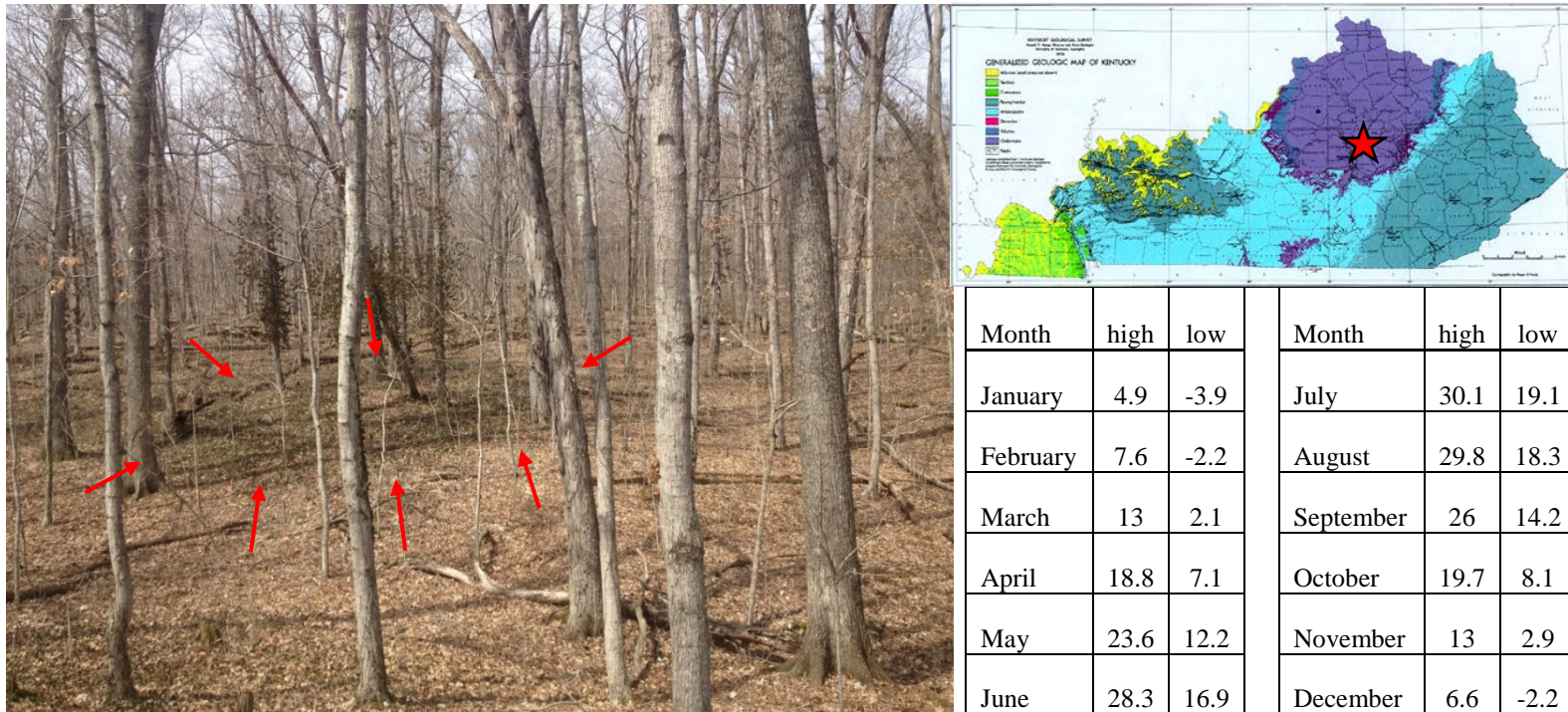


Figure 2.2. Experimental layout of seed rain study in relation to fruiting lianas and amount of seeds captured in seed traps. All line intersections represent seed trap locations (n=90) using a 10 m spacing between traps. Gray circles represent approximate area of mature, fruiting wintercreeper phanerophytes (using trees as structural hosts), which was measured in the field during seed-trap installation. Arrows and numbers indicate total number of seeds collected during the study; intact diaspores shown underlined (n=29 total), cleaned (i.e. bird dispersed) seed is not underlined (n=5 total)

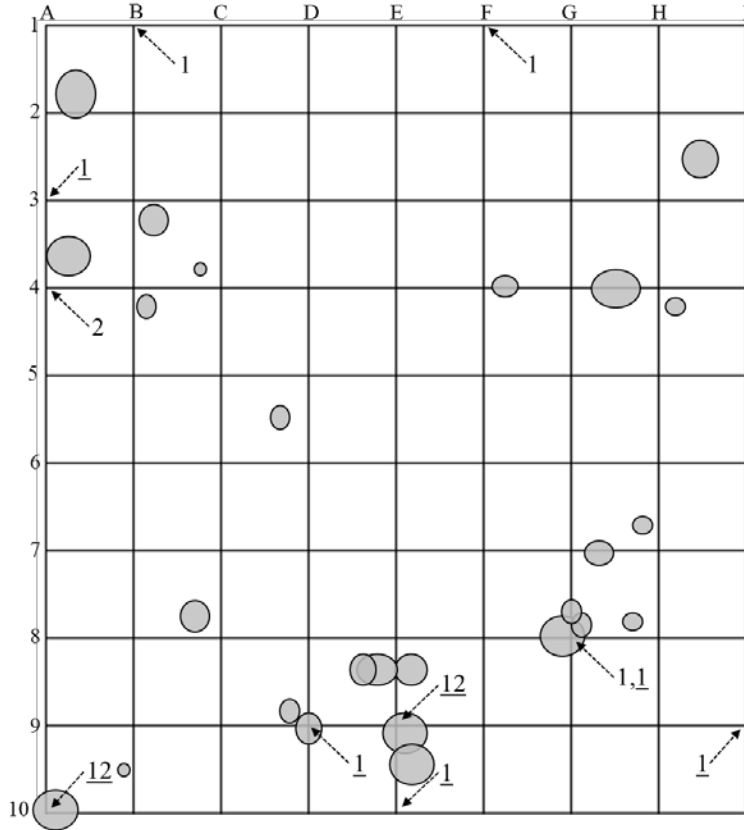


Figure 2.3. Move-along treatment germination rates for exp. 1: fresh seeds (solid shapes) vs. exp. 2: after-ripened seeds (hollow shapes). Transition between incubators occurred at weeks 12, 16, 20, and 32, and 36, as noted in Table 1. Error bars represent SEM

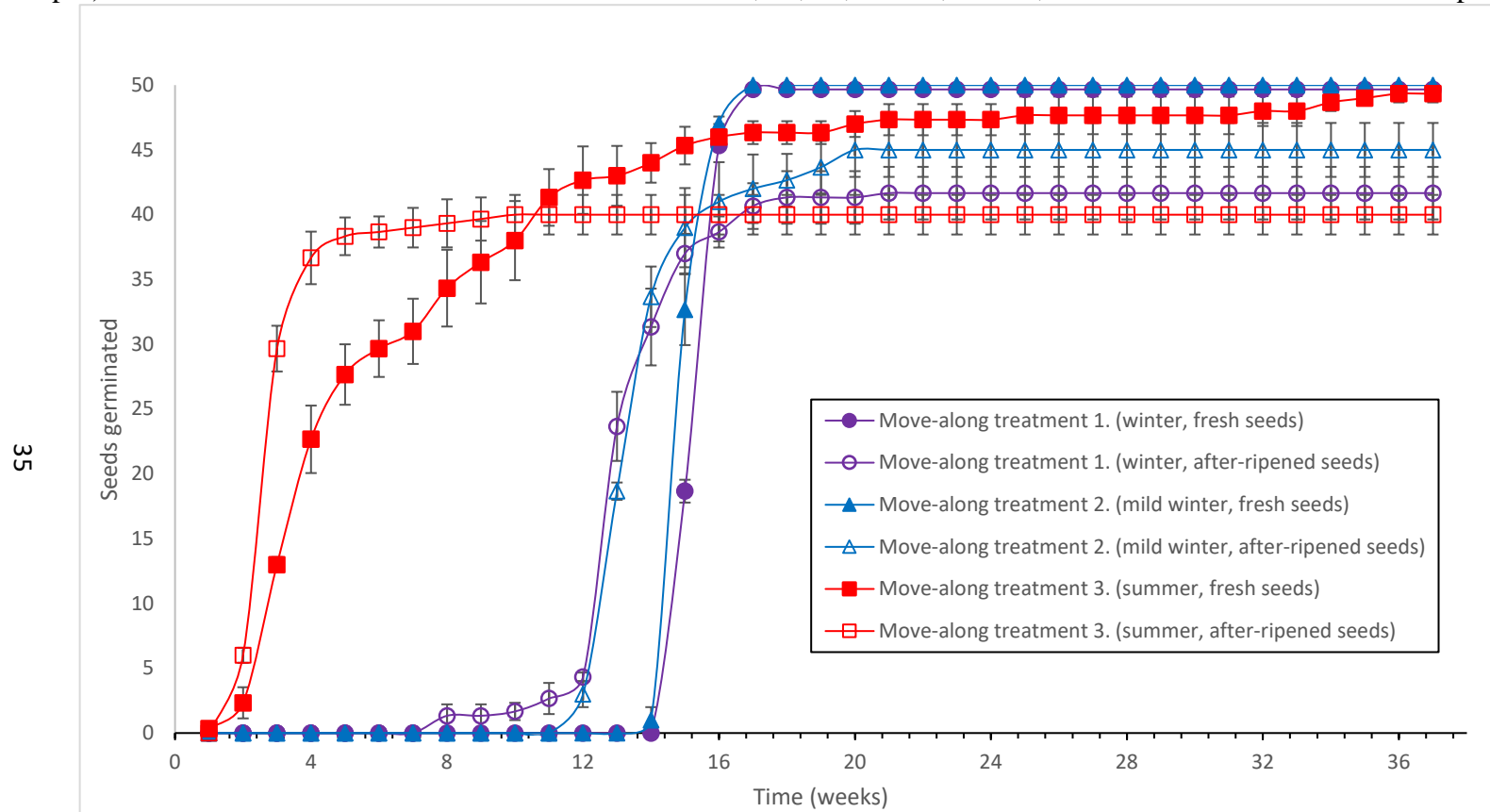


Figure 2.4. Control temperature germination rates for exp. 1: fresh seeds (solid shapes/lines) vs. exp. 2: after-ripened seeds (hollow shapes/dashes). Error bars represent SEM

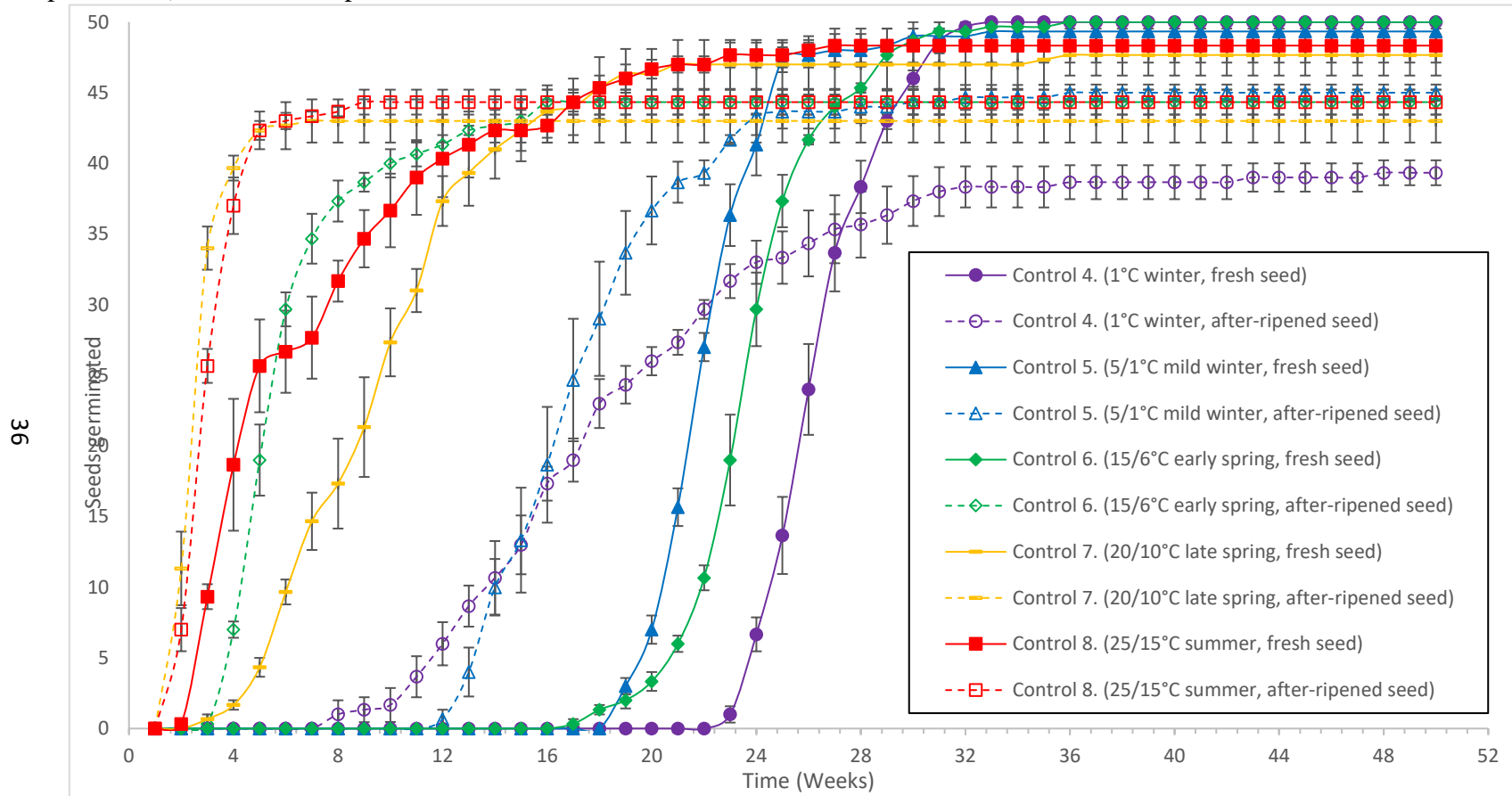
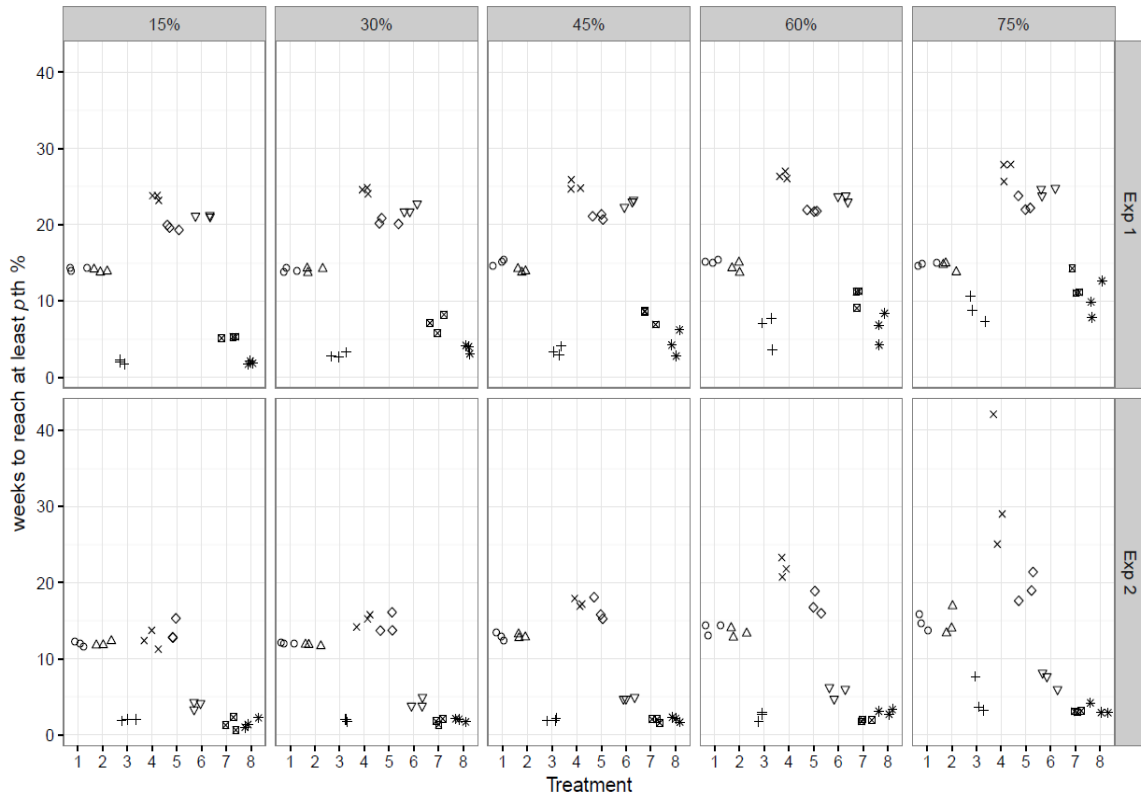
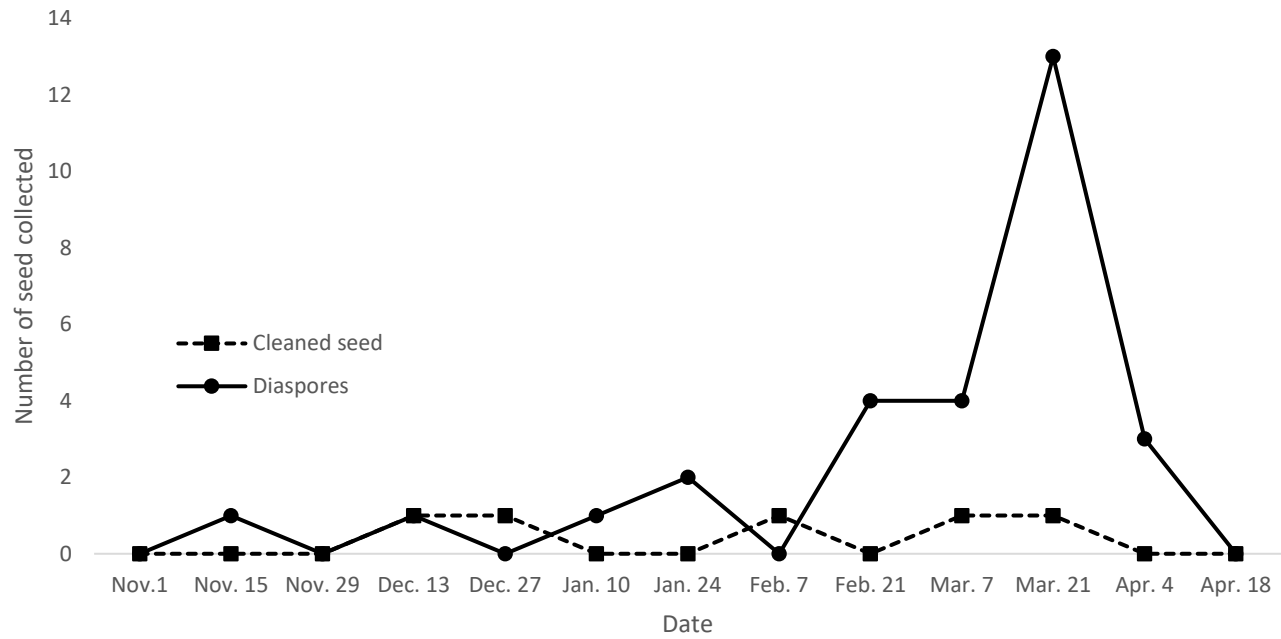


Figure 2.5. Boxplot of germination data for exp. 1 fresh seeds and exp. 2 after-ripened seeds



Treatment	Symbol	Starting temp (°C)
1. move-along (winter)	O	1°
2. move-along (mild winter)	△	5/1°
3. move-along (summer)	+	25/15°
4. control (winter)	×	1°
5. control (mild winter)	◇	5/1°
6. control (early spring)	▽	15/6°
7. control (late spring)	⊠	20/10°
8. control (summer)	*	25/15°

Figure 2.6. Temporal pattern of seed-rain for cleaned seed (bird-dispersed) and diaspores (gravity-dispersed) collected in seed traps for the whole study area



## Chapter Three

### **Seed dispersal and site characteristics influence germination and seedling survival of the invasive liana *Euonymus fortunei* (wintercreeper) in a rural woodland**

#### **Introduction**

The field of plant invasion ecology continues to grow exponentially, in part because the negative effects of many exotic plants have simply grown too large to ignore (Lockwood et al. 2013). In the United States alone, estimated costs (damages and control) of ~5,000 exotic plant species exceed \$35 billion annually (Pimentel et al. 2005). Although most of the damage-associated costs come directly from agricultural losses, they do not overshadow the threat to native species and ecosystems. Reduction of biodiversity and disruption of ecosystem processes are among the negative effects of exotic plant invaders that remain difficult to quantify monetarily (Theoharides and Dukes 2007; Xu et al. 2014). Nevertheless, the inherent value of indigenous species and ecosystems continues to drive invasion ecology research due to implications for invader prevention and remediation of natural areas (Reichard and Hamilton 1997; Byers et al. 2002; Leicht-Young and Pavlovic 2015).

With an estimated 25,000 nonindigenous plant species currently in the United States (Pimentel et al. 2005), it remains a serious challenge to determine which of these possess the capacity to invade natural ecosystems. Simultaneously, government agencies and private organizations are faced with developing policies and regulations concerning the import of new exotic species, which themselves have the potential to cause economic and environmental damage (Lockwood et al. 2013; Xu et al. 2014). Given the number of species to consider, it is advantageous (and often compulsory) to seek trends among



successful invaders and susceptible environments in order to manage and regulate invasion. Recent meta-analyses continue to shed light on trends that promote invasion, including propagule pressure (Simberloff 2009), interspecific hybridization (Hovick and Whitney 2014), soil chemistry and nutrient cycling (Weidenhamer and Callaway 2010; Vilà et al. 2011; Castro-Díez et al. 2014), plant chemical and physical defenses (Felker-Quinn et al. 2013), and community richness (Levine et al. 2004; Fridley et al. 2007). Still, the utility of these analyses for predicting invasive species or susceptible communities falls short of panacea in many regards.

While meta-analyses can help us elucidate various generalities among invaders, we must also recognize that the invasion of any given species will have its own degree of specificity (Byers et al. 2002). Additionally, because meta-analyses are inherently comprised of retrospective analysis among species already deemed invasive, the invasion ecology of slow-establishing species may be underreported (Reichard and Hamilton 1997). Lag times, the period between introduction and recognized invasion, are driven by a variety of biotic and abiotic processes, and can span for decades (Crooks and Soulé 1999). For example, among 184 woody invaders, Kowarik (1995) determined average lag times for shrubs and trees were 131 and 170 years, respectively. By unknowingly excluding data of nonindigenous species still on the trajectory to becoming ‘invasive’, one may fail to capture certain life-history demographics. In one such instance, closed canopy (i.e., undisturbed) forests are regularly implicated as being resistant to invasion (Gorchov et al. 2014), yet these results may be confounded by the fact that the majority of invaders are intentionally introduced, shade intolerant species (Martin et al. 2009). Thus, species-level studies can be particularly important to identify the specific set of

traits that beget invasibility, and consequently, use them to guide prevention and management strategies (Leicht-Young and Pavlovic 2015).

*Euonymus fortunei* (Turcz.) Hand.-Mazz. (hereafter, wintercreeper) is an Old-World liana whose invasion of North America merits closer investigation due to the species trajectory of invasion and (in some regards) uncharacteristic ecology. Although various wintercreeper clones were introduced into the European and North American nursery trades beginning in 1865 (Graves 1940), only in the past decade have the ecological impacts become great enough to warrant attention. Currently, wintercreeper has been reported in 31 states between Maine and Florida, and west to Wisconsin and Texas (EDDMaps 2017). Nevertheless, documented occurrences are sparse and extremely patchy at the county level, and only six states have added wintercreeper to informal regulatory lists (EDDMaps 2017). Kentucky, where my investigations took place, lies at the center of wintercreeper's invaded range, where (along with bordering states) its invasion is most severe. The first report of wintercreeper having escaped from cultivation into a natural area of Kentucky occurred in 1922 (Liang 2010). Liang (2010) found that by 1980, the species had only been observed in three, non-contiguous counties, but the number of counties rose to 10, 32, and 47 by 1990, 2000, and 2008, respectively. Considering this species' decadal lag time in Kentucky, its widespread (albeit patchy) distribution in eastern North America, and recent first occurrence in states such as Texas (Nesom 2010), it is reasonable to expect further invasion in years to come.

Ecological investigations of wintercreeper have begun only in the past decade, and focus largely on post-invasion, community-level processes and interactions (*see*: Smith and Reynolds 2015; Bauer and Reynolds 2016; Mattingly et al. 2016; Bray et al.

2017). To my knowledge, there are no published studies that directly consider the processes that lead to, or promote, invasion of wintercreeper. Understanding the factors that facilitate wintercreeper invasion into new environments would be of great utility for land managers seeking to prioritize resources for early detection and management. Given that monetary resources will always be limited, and the costs of eradication are likely to increase substantially based on area of infestation (Lockwood et al. 2013; Xu et al. 2014), the need for these data may be especially urgent. This is particularly true for wintercreeper, which unlike the overwhelming majority of introduced woody ornamentals, is able to colonize mature, undisturbed forests that may receive less monitoring than forests closer to urban areas (Martin et al. 2009; Zouhar 2009). Among Byers et al. (2002) list of 12 key research questions intended to prioritize and manage nonindigenous species, two directly address pre-invasion barriers: “what limits a species spread?” and “what makes a particular habitat vulnerable to invasion?” To examine the factors that ultimately lead to establishment success or failure, consideration must be given to the characteristics of the invader itself as well as that of the host community, and their interaction (Calviño-Cancela and Rubido-Bará 2013).

Analyses of soil differences in paired invaded vs. uninvaded soils are routinely employed in invasion studies (Ehrenfeld 2003). In some cases, significant differences between sites implicate the invader as a ‘driver’ of change to soil nutrient pools and nutrient cycling (Leicht-Young et al. 2009; Weidenhamer and Callaway 2010).

Alternatively, Iannone III et al. (2015) determined that significant differences in pH, total carbon (C), total nitrogen (N), and calcium (Ca) pre-dated colonization of European buckthorn, and therefore may have promoted invasion. When an invader *is* driving

belowground changes, the direction and magnitude of change can differ greatly based on community composition, the invader's abundance, soil type, and environmental factors (Ehrenfeld 2003; Brewer and Bailey 2014). For example, decomposition rates in Oriental bittersweet plots tended to be greater than in paired uninvaded plots (overall), yet among individual sites the opposite was sometimes true, or there was no difference at all (Leicht-Young et al. 2009). Plant-soil feedback, which occurs when a plant influences the rhizosphere to alter the growth of itself or other species, has recently been implicated as a driver of wintercreeper invasion (Smith and Reynolds 2012, 2015). While negative plant-soil feedback favors species coexistence, wintercreeper exhibited neutral to positive feedback when grown in soils conditioned by itself and five functionally different native species (Smith and Reynolds 2015). Positive plant-soil feedbacks facilitate invasion and monodominance via effects on allelochemicals, soil nutrients, and soil microorganisms, yet the specific drivers for wintercreeper invasion are not fully understood (Schradin and Cipollini 2012; Smith and Reynolds 2015).

Here, I investigate the factors that may limit (or promote) wintercreeper establishment (i.e., seedling recruitment), by examining the effects and interactions of seed dispersal pathway, plant community, and soil properties. These variables are admittedly only a few of many that may be implicated during establishment, and they were chosen based on my observations in the field along with preliminary data from other studies. I chose a study forest that had been colonized by wintercreeper more than 30 years prior (Rounsaville et al. 2017a), yet remained strongly heterogeneous with regard to invaded vs. uninvaded patches. Using the same study site, Rounsaville et al. (2017a) concluded that fruits (diaspores) were primarily gravity dispersed beneath maternal

lianas, yet seeds were also being distributed by birds to uninvaded regions within the forest. Thus, accounting for these uninvaded regions in light of annual (consistent) propagule introduction was my primary objective. Seed dispersal method ultimately represents seeds that fell to the ground beneath maternal lianas (with an intact aril) vs. seeds that were cleaned (aril removed) via avian digestion (potential for long-distance dispersal). Plant community and soil properties each corresponded to paired native (uninvaded), invaded, and restored (wintercreeper removed) sites. I hypothesized that the heterogeneous colonization of wintercreeper at the study site was due to failure of native (uninvaded) sites to support seedlings. Therefore, I expected at least one of the factors associated with uninvaded sites (native vegetation, animal dispersed cleaned seeds, or soil chemistry/texture) to negatively influence germination or survival.

## **Materials and Methods**

### *Study site*

Field studies were conducted within a 20 ha forest site known as Scott's Grove in Jessamine County, Kentucky (37.7741, -84.6103). Soils at Scott's Grove consist of McAfee silt loam (6-12% slopes) and McAfee-Rock Outcrop Complex (6-20% slopes) (Bloom et al. 2002). The forest can be characterized as mature, deciduous, and dominated by sugar maple (*Acer saccharum*), hickory (*Carya glabra*, *C. ovata*), ash (*Fraxinus americana*, *F. quadrangulata*), and oak (*Quercus alba*, *Q. muhlenbergii*, *Q. shumardii*). The understory is predominately open, with numerous sinkholes and limestone outcroppings. A shrub layer is largely absent at the site, restricted to *Lonicera maackii* and *L. fragrantissima*, two invasive shrub species that have begun to colonize the

forest edge and canopy gaps. There is no evidence of significant forest disturbance in at least 60 years prior to this study (Bloom et al. 2002). Mean monthly temperatures and total precipitation during the study period were obtained from the Lexington Bluegrass Airport Weather station, along with historical averages (Fig 3.1) provided by the National Oceanic and Atmospheric Administration (NOAA, 2016). Average annual precipitation for the region is 115 cm; over the course of this study (October 2014 to May 2016) precipitation was 28 cm above average (NOAA, 2016).

Scott's Grove was selected due to the nature of wintercreeper invasion at the site: a mosaic of patches containing 100% wintercreeper cover amidst an otherwise uninvaded landscape. This is atypical of wintercreeper invasions of central Kentucky, which tend to approach complete forest cover or be absent altogether (Bray et al. 2017). Wintercreeper invasion within Scott's Grove occurred at least 30 years prior to this study (Rounsaville et al. 2017), and isolated colonies of fruiting maternal individuals could be found throughout the forest.

#### *Field germination and survival*

Germination studies were conducted in the field using protective enclosures ('seed frames'). The seed frames were deployed at three vegetation conditions (sites): 'native' (NAT), 'invaded' (INV), and 'restored' (RES). NAT vegetation sites were those that completely lacked wintercreeper, and were at least 5m away from the edge of wintercreeper invasion. Native vegetation within NAT frames was sparse during the course of this study, with *Toxicodendron radicans* being most prevalent, and infrequent occurrences of *Agrimonia* sp, *Carex* spp, *Elymus* spp, and *Polymnia canadensis*. INV

vegetation sites were those consisting of 100% wintercreeper cover on the forest floor. Prostrate wintercreeper stems root adventitiously on the forest floor, forming a thick mat of evergreen shoots. There were no additional plant species present within INV vegetation sites, with the exception of small *Toxicodendron* root-sprouts. RES vegetation sites were identical to INV sites, but the wintercreeper stems and roots within each frame were carefully extracted to minimize soil disturbance. The RES sites are therefore representative of a 'restored' condition. Seed frames for the three site categories were placed within 15m of each other at each of four blocks (replications). The replications occurred along a transect that ran lengthwise through the center of Scott's Grove. Each replication was placed at the first well-defined invaded/uninvaded location that occurred along the transect >100m from the previous block.

Seed frames were constructed and installed at Scott's Grove on October 5, 2014. The frames were assembled using untreated, dimensional (3.8 x 14cm) pine lumber. Each frame consisted of a 65 x 65cm square, whose perimeter was carefully trenched into the soil to a depth of 12cm. Trenching was performed to protect seeds from burrowing mammalian seed-predators during winter stratification. Tree roots were severed when necessary during trenching, and the soil and litter inside of each frame was left as undisturbed as possible. Due to the nature of adventitious stem-rooting of wintercreeper, the ground vines within the INV treatment remained perfectly healthy and active during the study, despite being severed to accommodate frame installation. An additional, identically sized square frame was mounted on top of each entrenched frame and connected on one side with two small hinges. The frame assemblies remained in place

for four weeks, during which time any disturbed soil was allowed to settle, and naturally abscised tree leaves collected within the frames.

Wintercreeper diaspores were collected from approximately 15 mature individuals at Scott's Grove on October 26, 2014. The diaspores were bulked and a random subset of 1200 were removed for the study. The subset of wintercreeper diaspores was randomly divided in half; 600 were left intact while another 600 were manually stripped of their aril (i.e., cleaned seeds). On November 2, the seeds were distributed within the seed frames. The inside of each frame was partitioned down the middle by attaching a 2 x 10cm board (buried 8cm deep) to the entrenched half of the frame. Aril treatments (N=aril removed vs. Y=aril remained) were randomly assigned to one side of each frame in a split-plot design. Each treatment consisted of 50 seeds, which were distributed evenly within their respective split-plot. Seeds were lightly incorporated amongst the leaf litter and vegetation to help put them in contact with soil. A single layer of 3mm mesh window screen was stapled onto the top of the frame assembly to exclude seed predators during the winter, while also allowing light and precipitation to enter the frames. The mesh screen also prevented additional wintercreeper seeds from being naturally dispersed within the frames. When germination began during spring of 2015, I concluded that the risk of seed deletion (predation) and addition (seed-rain) had passed, and the mesh screens were removed for the remainder of the study.

On the first date germination was recorded (May 2, 2015), I observed noticeably lower numbers of seeds in the uncleaned (aril = Y) seed treatments had germinated. At this time, I began tracking individual seedlings based on the time-interval when germination occurred. Plastic-coated metal wires (1mm diameter) were cut into 6 cm



segments and twisted into rings at the base of newly germinated seedlings. Three colors were used to differentiate between germination 'phase': orange represented 'early' germination (before May 3), blue for 'mid' germination (May 3 through June 12), and green for 'late' germination (June 13 and onward). Germination data were collected every two weeks in 2015, until September 5, when I felt confident no more germination would occur. Thus on September 5 a final tally of total germination was made, and survival was recorded in all plots by counting the total number of seedlings. Seedlings were allowed to overwinter in their uncovered frames and total survival (post-winter) was assessed a second time on May 1, 2016.

### *Soil sampling*

Whole-plot soil samples were collected on May 1, 2016. Ten cores of upper mineral soil (0-5 cm) were extracted from each frame, and bulked together for each sample. Samples were screened through a 2 mm sieve and stored at 5° C prior to analysis. Approximately 2 g (dry weight) of soil were sent to University of Kentucky Regulatory Services for analysis on June 8, 2016. Soils were analyzed for extractable phosphorus (P), potassium (K), calcium (Ca), and magnesium (Mg), using Mehlich-3 protocols (Mehlich 1984). Soil pH was determined using a 1:1 ratio of soil and water. Total soil carbon (C) and nitrogen (N) were determined using a vario MAX CNS Macro Elemental Analyzer (Elementar Analysensysteme GmbH, Germany). Cation exchange capacity (CEC) was determined using ammonium saturation of exchange sites and analysis of saturated ammonium using an ammonium ion-selective electrode. Inductively Coupled Plasma Spectrophotometry (ICP) was used to quantify bases on soil exchange

sites. Base saturation (%) was determined as [(total bases/CEC) x 100]. Soil texture was determined using the micro-pipette method (Miller and Miller 1987).

### *Aril Effects in Vitro*

Based on preliminary field observations that aril presence delayed germination, I conducted a germination study *in vitro* to compare the effect of aril tissue in a more controlled setting. Wintercreeper diaspores were collected on December 20, 2015 from the same 15 Scott's Grove lianas that provided seed for the field study. The bulked diaspores were randomly divided into groups of 300 and prepared for three treatments. Two of the three treatments were identical to those used in the field study: 'cleaned' (aril removed) and 'uncleaned' (intact diaspores). The third treatment ('cleaned +aril') consisted of seeds that were cleaned, but whose removed aril tissue remained in contact with seeds as part of the experimental unit. In this way, the 'cleaned +aril' and the 'uncleaned' seeds each contain aril tissue, but the arils in the former treatment do not act as a direct barrier to oxygen, light, or water. Each treatment was replicated six times for a total of  $n = 18$  experimental units. Experimental units consisted of 50 cleaned seeds evenly distributed in a 90 mm diameter Petri dish on 5 mm depth of white quartz sand moistened with distilled water. Supplemental water was supplied as necessary for the duration of the experiment. All experimental units were immediately placed within an incubator that cycled between  $5/1^{\circ}$  C every 12 hours, to replicate the average winter day/night (respectively) temperatures in central Kentucky. On March 15, after 12 weeks of cold-stratification, the Petri dishes began cycling through additional incubators set to average seasonal temperatures as a 'Move-Along' experiment (Baskin and Baskin, 2003).

The temperature (day/night) progression among incubators consisted of 5/1° C (winter: 12 weeks), 15/6° C (early spring: 4 weeks), 20/10° C (late spring: 4 weeks), 25/15° C (summer: 12 weeks). All incubators provided a 14 h photoperiod (20  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , 400-700 nm, cool white fluorescent light); the lights came on 1 h prior to the start of the high temperature and remained on for 1 h after the start of the low temperature. Seeds were scored as 'germinated' when the radicle had reached or exceeded 3 mm in length. Germination data were collected daily until all seeds had either germinated or perished.

### *Statistical analysis*

For the field study, total germination by site and aril treatment, as well as their interaction, was compared using Analysis of Variance (ANOVA) following square root transformation of the data to achieve normality. A cumulative logit model was used to analyze the multinomial dataset of germination for each phase (early, middle, and late). Estimates were used to determine if treatments or their interaction yielded a different germination response for each phase. Since the model assumed the effect of each treatment is the same for each category of response, a Chi-squared test for proportional odds assumption was used to determine if a more complex model was warranted. I used a two-way ANOVA to test for differences in total seedling survival between sites and aril treatments, for both pre-winter (September '15) and post-winter (May '16) data. I also wanted to determine if seedling survival proportions were significantly different between treatments. Survival proportions were calculated as the number of surviving seedlings / number of germinates (for each split-plot), and a logistic regression was used to test the effects of site and aril on pre- and post-winter survival probabilities. The differences of

treatment LS Means were tested with a Tukey-Kramer adjustment for multiple comparisons. Soil nutrient and cation exchange data for whole-plots were compared using ANOVA, and means within categories were compared using Tukey's honestly significant difference (HSD) tests. Soil texture data were arcsine transformed because each response was a percentage that sums to one. A Multivariate Analysis of Variance (MANOVA) was performed on these correlated data to test whether the mean proportion of sand, silt, and clay differed between blocks and sites. Due to low site replications ( $n = 4$ ), I set  $\alpha = 0.10$  for analysis of field collected data. Data from the *in vitro* germination study were modeled using local polynomial regression (LOESS), with confidence bands for the germination curves, and  $\alpha = 0.05$ . All statistical analyses were performed using SAS software (Version 9.4).

## Results

### *Field germination*

Germination within the seeds frames was observed between May 2 and June 27, 2015, during which time 667 seeds (55.6%) germinated across treatments. My analysis revealed that neither site, aril, nor their interaction yielded significantly different amounts of total germination. However, further analysis of seed germination as a function of time (phase) revealed that the main effects of site [ $F(2,662) = 6.53, p = 0.0016$ ] and aril [ $F(2,662) = 59.97, p < 0.0001$ ] each significantly influenced germination. Early, middle, and late phases contributed 69, 25, and 6% to total germination, respectively (Table 3.1). Seeds with an aril (Y) consistently germinated more slowly than seeds lacking an aril (N). For example, early phase germination percentages for Y vs. N aril were 59% (vs.

86%), 58% (vs. 85%), and 42% (vs. 75%) for NAT, RES, and INV sites, respectively (Table 3.1). Pairwise comparisons made among sites showed that NAT and RES did not differ in germination response [ $t(662) = -0.25, p = 1.00$ ], yet NAT vs. INV [ $t(662) = 3.32, p = 0.0028$ ] and RES vs. INV [ $t(662) = 2.98, p = 0.0091$ ] significantly differed for all three phases (Fig 3.2). I found that germination among the six combinations of treatments resulted in four distinct groupings that remained consistent between phases despite proportional shifts in germination (Fig 3.2). In other words, NAT and RES sites grouped together by aril (N and Y; groups 1 and 2, respectively), and the INV site produced different responses at each aril category (groups 3 and 4). The logistic procedure used to test the germination modeling (score test for the proportional odds assumption) returned a  $p$ -value of 0.423, indicating the tests were appropriate (Peterson and Harrell 1990).

### *Seedling survival*

The total number of surviving seedlings did not differ by site, aril, or their interaction for pre-winter (September 2015) or post-winter (May 2016) datasets (Table 3.2). Similarly, the proportion of germinated seeds that survived near the end of the 2015 growing season did not differ significantly between sites [ $F(2,15.71) = 1.51, p = 0.251$ ] or aril treatments [ $F(1,15.42) = 2.91, p = 0.108$ ]. Post-winter survival rates recorded in May 2016 differed significantly based on site [ $F(2,15.38) = 3.55, p = 0.054$ ] and aril [ $F(1,14.68) = 3.79, p = 0.071$ ]; there was no interaction between the two treatments (Table 3.2). I found that for seedlings tallied during the post-winter measurements, seeds initially sown with an aril (Y) yielded a significantly higher survival percentage (47.7%)

than did seeds without an aril (33%). Multiple comparison tests for survival indicated no differences between sites in September '15. In May '16, I found site relationships were similar to those in the germination study: NAT and RES sites did not differ from each other [ $t(14.25) = 0.15, p = 0.987$ ], but the proportion of surviving seedlings among the INV site were significantly lower compared to NAT [ $t(15.99) = 2.3, p = 0.086$ ] and RES [ $t(16.21) = 2.42, p = 0.069$ ] sites.

### *Soil analysis*

Among all soil chemistry variables, only extractable Mg differed among sites (Table 3.3). NAT and INV sites were significantly different, but not different from RES [ $F(2,6) = 4.32, p = 0.069$ ]. Magnesium concentrations (mg/g) were higher in INV soils (0.37) than NAT soils (0.15), while RES soils (0.26) were intermediate. I conducted the analysis without RES site data to test for differences in the two naturally occurring sites (NAT and INV), and found no additional significant differences between sites. Despite a lack of significant differences in other soil parameters, all of the responses I tested for (CEC, base saturation, pH, C, N, P, K, and Ca) were numerically higher in INV vs. NAT soils (Table 3.3). Analysis of soil texture revealed no significant differences between blocks or sites using the four common MANOVA tests (Wilks' Lambda, Pillai's Trace, Hotelling-Lawley Trace, and Roy's Greatest Root). The  $p$ -values returned for all four test statistics yielded a greater effect by block (vs. site), suggesting soil texture different across the study forest.

### *Aril effects in vitro*

Treatments that included aril tissue *in vitro* significantly delayed germination (cleaned +aril) and led to high mortality (aril intact) in comparison to cleaned seeds (Fig 3.3). Seeds with an intact aril reached only 6.4% total germination, and following 79 days of cold stratification the remaining seeds had all rotted and were discarded. Germination of cleaned seeds and clean +aril each reached 100% germination after 43 and 59 days, respectively. For these two treatments, confidence bands indicate that germination curves differed between days 18 and 38, during which time ‘cleaned +aril’ seeds experienced delayed germination compared to cleaned seeds.

### **Discussion**

Over approximately a two-month period of field germination, the *total* number of germinated seeds did not differ significantly based on site or aril treatment, but there was a significant response for *timing* of germination. The significant effect of site and aril on germination proportions over time (phase) provide insights into the role vegetation (site) and dispersal method (aril treatment) may play in the invasion process. Under natural conditions wintercreeper seeds are produced in great quantities, the majority of which are gravity-dispersed (with aril) directly beneath maternal lianas, and to a lesser extent, seeds are bird-dispersed (no aril) to invaded and uninvaded areas (Rounsaville et al. 2017a). I found that a greater proportion of seeds with no aril germinated during the early phase, but the opposite was true for middle and late phases, across all vegetation treatments (Fig 3.2). These results are consistent with findings for *Euonymus europaeus*, in which seeds with intact arils exhibited a delay in field germination compared to seeds without arils

(eight vs. five weeks, respectively) following a stratification period (Bezdeckova et al. 2009).

In comparison to the *in vitro* germination study of aril effects, I found a similar delay in germination for both treatments containing aril material (Fig 3.3). There, seeds with an intact aril also experienced low germination and high mortality that may be attributable to the aril's impediment of oxygen, light, and/or water. It is possible that poor germination caused by intact arils is important to prevent vivipary, which would be advantageous given the propensity of wintercreeper seeds to after-ripen on maternal vines, and their facultative dormancy requirement (Rounsaville et al. 2017a). In contrast, when removed aril tissue was included with cleaned seed (cleaned +aril), germination lagged behind that of cleaned seeds without arils, despite total germination for both treatments eventually reaching 100% (Fig 3.3). Still, the former treatment (cleaned +aril) I believe is more indicative of aril-enclosed seeds used for the field experiment as a result of microbial and environmental aril degradation. The processes that led to the observed aril-mediated delay of germination are difficult to account for in the context of this work, and may not be the same between the lab and field experiments. For example, allelochemicals produced by invasive species can be autotoxic, and their direct effects can be stronger within the largely sterile environment of a Petri dish (Lankau 2010; Bauer et al. 2012).

My experimental design included a restored (RES) site treatment that was selected based on two considerations. First, it represents a condition relevant to land managers who have eradicated wintercreeper growth, and thus understanding future biotic resistance potential would be beneficial (Mattingly et al. 2016). Second, it



provides additional insight into the specific influence of conspecific chamaephytes (by comparison with INV site response) upon seed and seedling response. Germination proportions by phase were always the same for NAT and RES sites, yet consistently different for INV for both aril treatments (Fig 3.2). These results are interesting, given that total numbers of germinates did not differ significantly among sites or aril treatment. A potential influence upon germination time is leaf litter, which may delay or prevent germination by blocking light and acting as a physical barrier to seedling growth (Molofsky and Augspurger 1992; Chambers and MacMahon 1994). For example, Oriental bittersweet, an invasive, Celastraceous liana like wintercreeper, had significant reductions in seedling emergence through intact leaf litter compared to an equal mass of fragmented litter (Ellsworth et al. 2004). Sites with wintercreeper invasion have been shown to have higher soil moisture and faster rate of leaf litter decomposition compared to paired uninvaded sites (Bray et al. 2017). Because of this, I expected greater amounts of leaf litter among NAT and RES sites would contribute to a delay in germination compared to INV sites. Instead, I found that within aril treatments, INV sites had smaller proportions of early phase germination and greater proportions in middle and late phases (Fig 3.2).

Differences in soil biotic communities have also been documented between wintercreeper invaded and uninvaded sites, and may persist even years after wintercreeper removal (Bray et al. 2017). Soil biotic communities are known to influence seed mortality (Chambers and MacMahon 1994; Baskin and Baskin 2014), and if they were to ultimately affect germination of wintercreeper seeds, I would anticipate RES and INV sites to respond similarly, which they did not. One similarity between

NAT and RES sites is their exposure to sun, which is blocked from seeds and soil of INV sites due to wintercreeper's dense mat of evergreen foliage. Although light alone is not facultative for wintercreeper germination (Rounsaville et al. 2017a), early phase germination occurred at a time when deciduous trees remained dormant, and as a result NAT and RES sites would be subjected earlier to increased soil and seed temperatures due to increased sun exposure. Because a transition to early spring temperatures (15/6 °C) led to high velocity germination of wintercreeper seeds in the lab experiment (Fig 3.3), it is reasonable to expect that exposure to sun (i.e., earlier warmer temperatures) produced similar phase patterns for NAT and RES sites. Light intensity at Scott's Grove changes dramatically throughout the year. Photosynthetic photon irradiance (PPI) at Scott's Grove rarely exceeded  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$  during the summer, yet PPI increased by 1-2 orders of magnitude during the winter when deciduous trees were dormant (Bloom et al. 2002). During the study, the middle phase of germination was marked by a significant reduction in rainfall, which was 8.1 cm (61%) below average during May (Fig 3.1). During this time, respective proportions of germination for each aril treatment were highest in the INV sites (vs. NAT and RES), which Bray et al. (2017) found had significantly higher soil moisture in spring. It is possible that excessive drying (increased sunlight and decreased precipitation) of NAT and RES sites in May irreversibly arrested germination during middle and late phases, since the radicles of most seeds emerged at the soil surface.

Ultimately, seedling survival (as opposed to germination) serves as a stronger metric for invasibility, and therefore provides greater utility for predicting conditions that promote invasion. Having experimentally tested how/where seeds disperse (with or

without arils in invaded vs. uninvaded sites), I acknowledge that these processes are likely to occur in uneven ratios. For example, as a function of distance from fruiting lianas, NAT sites are likely to experience less seed rain overall, most of which will be cleaned (animal dispersed) seeds, as compared to INV sites (Rounsaville et al. 2017a). Thus, survival proportions (rather than totals) are of particular interest. The survival data (Table 3.2) are of interest due to the implications of survival as a precursor to population growth and spread.

I first recorded survival rates in September 2015, prior to leaf senescence of canopy trees and exposure to cold temperatures. At this time, the overwhelming majority of seedlings had not produced any true leaves, and consisted only of cotyledons atop a 2-5 cm hypocotyl. No significant treatment differences were detected in September, although survival was numerically the lowest within INV sites (48.7%), and lower still among seeds that were sown without arils (51.4%) (Table 3.2). By May 2016 when I recorded post-winter survival, % survival had decreased from 63.6% (pre-winter) to 43.5% across treatments. I was surprised to find that most seedlings had overwintered as cotyledons; only after nearly a year were their first true leaves starting to emerge. I observed a significant effect of both site and aril treatment on May survival proportions (there was no interaction between site and aril). The significant effect of aril in May 2016 again yielded higher survival among seeds sown with arils. This is interesting given that aril was non-significant in September, and arils themselves are ephemeral artifacts, long since degraded. I would have anticipated an aril effect during the first season, especially since a significant aril effect on germination phase might have led to mortality based on abiotic influence. For example, significantly higher early-phase

germination of seeds without arils may lead to greater susceptibility to an early-spring killing frost. Alternatively, as canopy trees leaf out and light intensity declines, early germinates may benefit from greater C gains (Graves, 1990).

Post-winter aril effects on survival are made further curious due to their independence from vegetation treatment. There are no published accounts of wintercreeper aril chemistry, though Thomas et al. (2011) note the arils of *Euonymus europaeus* are both toxic (protein synthesis inhibition) and high in nitrogen (1.23 mg total N per aril) compared to other fleshy-fruited shrubs. Implicating N as a driver of survival is unlikely given the modest contribution of aril N over the course of nearly one year. On the other hand, soil microorganisms play a significant role in seed and seedling mortality, and arils frequently contain anti-microbial compounds (Gallery et al. 2010). Seed coats of some plants harbor bacteria that antagonistically inhibit the growth of various soil fungi (Baskin and Baskin 2014). Although beyond the scope of this study, it is plausible that arils may aid survival via the suppression of soil microorganisms that may otherwise lead to seedling mortality.

Site significantly influenced survival measured in May 2016, at which point INV survival (26.5%) was lower than that of NAT (48.4%) and RES (47%) (Table 3.2). Similar to germination phase response, I found no difference between NAT and RES sites, yet both differed significantly from INV. Thus, lacking an interaction with aril, one may conclude that in RES (wintercreeper removed) sites, INV soil legacy effects such as microbial community composition (Bray et al. 2017) do not influence seedling survival. Similarly, the soil parameters that were investigated did not differ between RES and INV sites (Table 3.3). Thus, reduced survival (and increased mortality) among INV sites is

most likely the result of physical and/or chemical influence by the established wintercreeper chamaephytes. Chemical action (allelopathy) is a common component of exotic plant invasions that acts to the detriment of native species and sometimes to conspecifics of the invader itself (Pisula and Meiners 2010; Bauer et al. 2012). Considering wintercreeper's propensity to grow in monoculture, in addition to non-significant differences in germination among site within this study, it is unlikely that wintercreeper, if allelopathic, is autotoxic. Further, Smith and Reynolds (2012, 2015) found wintercreeper performed better when grown in self-conditioned soil, and concluded that neutral to positive plant-soil feedback is likely to promote its invasion. Positive feedback notwithstanding, wintercreeper seedlings within INV sites competed for resources (particularly sunlight) with preexisting wintercreeper vines, unlike seedlings in NAT and RES sites. Seedlings lacked true leaves during the first growing season and were typically the same height or shorter than ground stems, thereby reducing photosynthetic capabilities and C gains relative to NAT and RES sites. Though I did not measure seedling growth, it is highly plausible that competition for sunlight, more than other factors, increased seedling mortality in INV sites.

The analysis of Scott's Grove soils revealed that the differences between soil parameters (0-5 cm depth) were non-significant with the exception of extractable magnesium (Table 3.3). It is highly likely that the lack of replication ( $n = 4$ ) contributed to the lack of significant differences, as Bray et al. (2017) found significant differences in % C and % N when examining paired NAT/INV sites ( $n = 10$ ) within the same study forest. Nevertheless, INV soils had consistently higher soil nutrient concentrations compared to NAT soils. Elevated soil nutrients (most commonly C and N) are frequently

associated with exotic plant invasions (Ehrenfeld 2003; Weidenhamer and Callaway 2010). This is unsurprising overall, considering invaders often display greater aboveground net primary productivity (ANPP) compared to native competitors (Vilà et al. 2011). Greater C gains from increased ANPP may facilitate root growth, thereby further aiding nutrient gains including vertical transport from deeper regions of the soil profile. This process frequently acts as a feedback loop for invaders, as increased N and other nutrients yield higher quality litter and increase decomposition rates (Ehrenfeld 2003). Among paired invaded/uninvaded plots, Oriental bittersweet was found to have higher rates of N mineralization and decomposition, and while P levels were similar among paired plots, invaded soils had significantly higher pH, K, Ca, and Mg levels (Leicht-Young et al. 2009).

I found highly significant differences between NAT and INV soils in extractable Mg. Elevated soil Mg concentrations have been noted among other plant invasions, though frequently in conjunction with other base cations (Blank and Young 2002, 2004; Rodgers et al. 2008; Leicht-Young et al. 2009). Rodgers et al. (2008) attributed higher base cation (Ca and Mg) availability in garlic mustard invasions to plant-mediated increases in pH, which occurred via root exudation. Blank and Young (2004) hypothesized enzyme activity used by invaders to stimulate low resource soils to induce mineralization of N, Ca, and Mg. Process notwithstanding, Mg plays a critical role in photosynthesis as the central atom of the chlorophyll molecule (Shaul 2002). In general, lianas possess higher photosynthetic potential (vs. host trees) due to consistently lower leaf mass per unit area (LMA) (Kazda 2015), thus higher Mg reserves could further contribute to photosynthetic efficiency in a light-limited environment such as a forest

understory. High levels of foliar Mg can also aid plants under drought stress by inhibiting photosynthesis (Shaul 2002). Magnesium has not been well studied as a driver of invasion, and although increased Mg levels may facilitate metabolic processes among wintercreeper and Oriental bittersweet invasions (Leicht-Young et al. 2009), its effect can be neutral to negative for other invaders (Dassonville et al. 2008; McGrath and Binkley 2009). Although I found evidence that extractable Mg decreases following wintercreeper removal, I cannot conclusively say if wintercreeper directly increases this cation or preferentially invades soils where it is high. Future studies to determine if Mg or other soil nutrients promote invasion would be advantageous to help monitoring and prevention of wintercreeper.

I hypothesized that the heterogeneous invasion of wintercreeper at the study site was due to a negative influence on seeds or seedlings by at least one treatment associated with uninvaded sites. Based on my data, I may fail to reject this hypothesis based on seed dispersal method alone, and suggest that the overall processes of landscape spread are likely to be more nuanced and deserving of further attention in the future. Seed dispersal method is implicated as a limiting factor at uninvaded sites because cleaned seeds (no aril) ultimately yielded significantly lower rates of survival. Bird dispersal of viable, cleaned seeds is documented to occur within my study forest, yet it is highly unlikely that gravity dispersed seeds (with aril) would be dispersed to uninvaded sites (Rounsaville et al. 2017a). Although seeds with arils exhibited higher rates of survival (vs. without arils), I expect them to be preferentially dispersed among INV sites, which yielded significantly lower survival than other vegetation treatments. Given equal numbers of seeds, it is logical to assume natural patterns of seed dispersal and vegetation

(site) type may equalize survival, and indeed, I found no significant interaction among those treatments. Because seed dispersal density is negatively correlated with distance from origin (Nathan and Muller-Landau 2000), NAT sites should be expected to incur less propagule pressure in general, and thus reduced opportunity for germination and survival. That aril-mediated survival became significant only post-winter is suggestive of this tissue producing a microbial effect, which may benefit seedlings via suppression (antagonism) of detrimental microbes or beneficial symbioses with seedlings.

## **Conclusions**

For land managers seeking to prioritize wintercreeper prevention or control, my findings provide new insight for management techniques. First, germination occurs in both invaded (INV) and uninvaded (NAT) sites in equal ratios, but survival (proportion) is greater in the latter. The amount of seed input may ultimately regulate number of seedlings surviving in the field, thus areas closer to fruiting lianas have a greater likelihood of recruitment. Second, sites of wintercreeper eradication may temporarily become more invasible, based on soil legacy effects and increased survival in RES sites in comparison to INV. Therefore, eradication strategies should include elimination of all nearby seed sources as well as follow-up monitoring. Third, additional evidence was found that wintercreeper invaded soils are associated with higher nutrient concentrations, particularly magnesium. If high resource soils ultimately give wintercreeper an advantage, they may prove more susceptible to invasion, even if wintercreeper is a post-invasion driver of soil change (*sensu* Iannone III et al. 2015). Finally, the inability to definitely answer Byers et al. (2002): “what makes a particular habitat vulnerable to



invasion?" is itself, enlightening. In general, as spatial-scales increase so does the richness (i.e. invasibility) of exotic species (Fridley et al. 2007). A growing body of evidence suggests that multiple life history traits may aid wintercreeper success [e.g., asexual spread, modified nutrient cycling (Bray et al. 2017), plant-soil feedback (Smith and Reynolds 2012), facultative seed dormancy and polyembryony (Rounsaville et al. 2017a)], thus precluding a simple characterization of invasion for this species.

Accordingly, if vulnerable habitats cannot be definitively categorized, there should instead be efforts made to improve techniques for more widespread monitoring, and thus achieve success via early detection and eradication. For a species with decadal lag times and slow establishment rates (Liang 2010), there may be cause for optimism. Future studies will be imperative to better understand and control this invader.

Figure 3.1. Monthly temperature and precipitation data recorded during the experiment along with historical (1948-2016) averages. Error bars represent monthly high and low temperatures. Data were recorded at the Lexington Bluegrass Airport (Kentucky, USA, 38.0408°, -84.6058°) and provided by the National Oceanic and Atmospheric Administration.

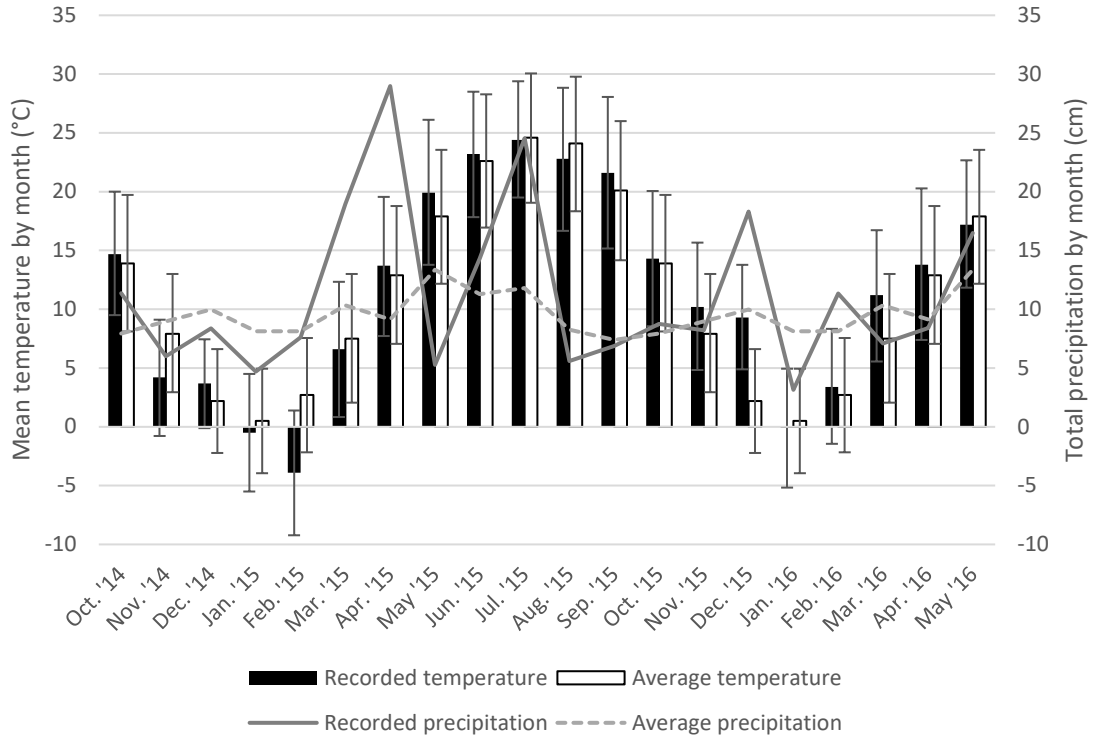


Table 3.1. Seed germination response for site (NAT, RES, and INV), aril (presence or absence), and phase (early, middle, or late). Values for ‘n’ represent number of germinated seeds for each category, ‘prop’ represents the proportion of germinated seeds and sum to one within each category [aril (by site), site, phase]. Totals by aril (by site) appear as plain text, totals by site appear **bolded**, totals by phase appear *italicized*. A total of 1200 seeds were sown, and total germination across treatments was 667 seeds. A total of 400 seeds were sown within each site (n = 3), and 200 seeds were sown for each aril\*site treatment (n = 6).

Aril	Site												Phase totals		
	Native (NAT)				Restored (RES)				Invaded (INV)						
	no	yes		no	yes		no	yes		no	yes		<i>Phase</i>	<i>n</i>	<i>prop.</i>
<i>Phase</i>	<i>n</i>	<i>prop.</i>	<i>n</i>	<i>prop.</i>	<i>n</i>	<i>prop.</i>	<i>n</i>	<i>prop.</i>	<i>n</i>	<i>prop.</i>	<i>n</i>	<i>prop.</i>	<i>Phase</i>	<i>n</i>	<i>prop.</i>
<i>Early</i>	141	0.855	57	0.593	88	0.848	62	0.58	74	0.746	39	0.42	<i>Early</i>	<b>461</b>	<b>0.691</b>
<i>Mid.</i>	19	0.124	34	0.327	12	0.13	37	0.335	19	0.213	44	0.431	<i>Mid.</i>	<b>165</b>	<b>0.247</b>
<i>Late</i>	4	0.021	7	0.08	3	0.02	9	0.084	7	0.041	11	0.148	<i>Late</i>	<b>41</b>	<b>0.061</b>
Aril totals by site	164	1.00	98	1.00	103	1.00	108	1.00	100	1.00	94	1.00			
		<i>n</i>		<i>prop.</i>		<i>n</i>		<i>prop.</i>		<i>n</i>		<i>prop.</i>			
<b>Site totals</b>	<b>262</b>		<b>0.393</b>		<b>211</b>		<b>0.316</b>		<b>194</b>		<b>0.291</b>		<b>667</b>	<b>1.00</b>	

Figure 3.2. Germination proportions among all site (NAT, RES, INV) and aril (N, Y) combinations for early, middle, and late phases. Combinations that share the same group (symbol) within each phase are not significantly different ( $\alpha = 0.10$ ).

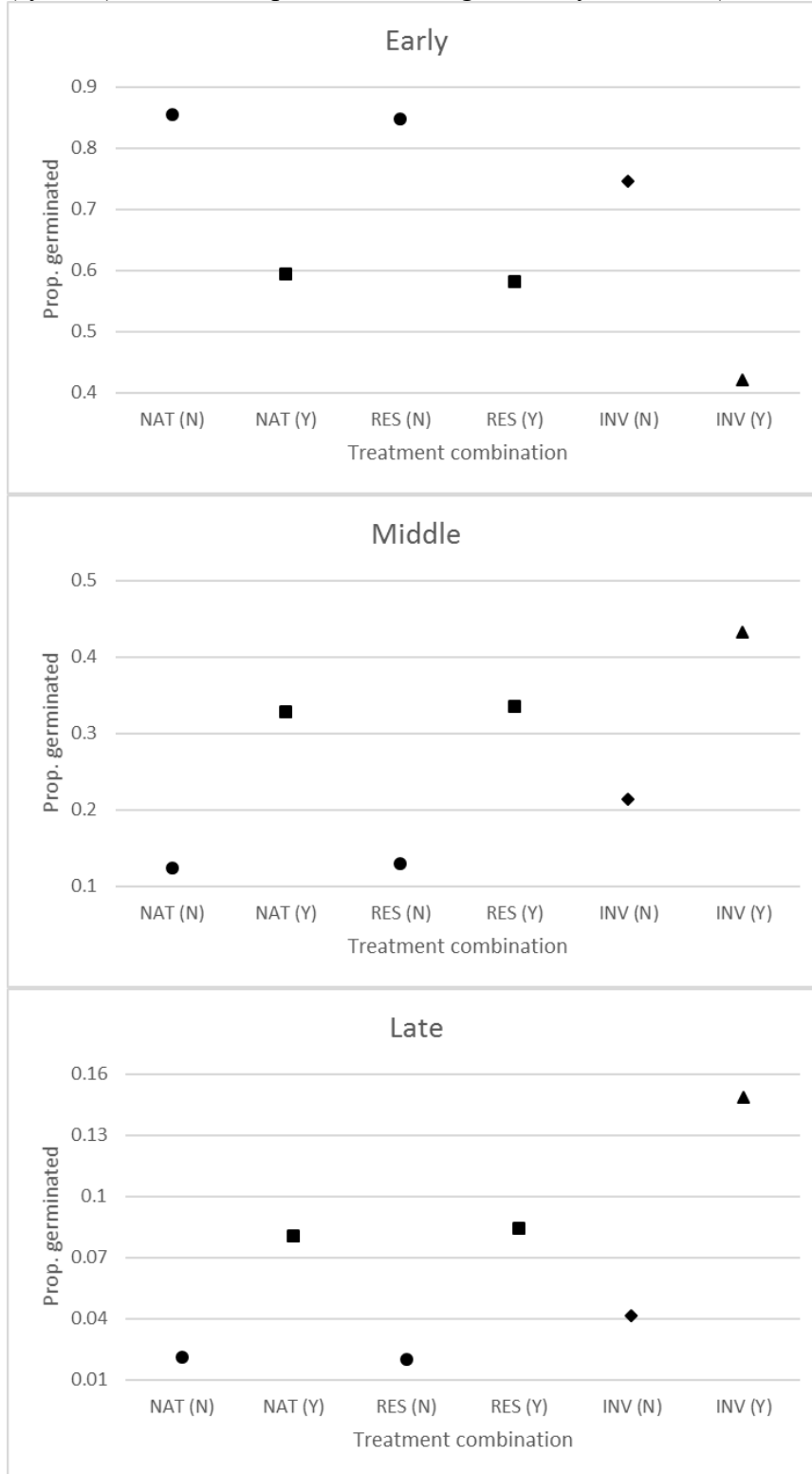


Table 3.2. Seedling survival in September 2015 and May 2016 for each site and aril treatment. The number (n) of surviving seedlings is out of a possible 400 for each of three sites, or 600 for each aril treatment. Proportion (prop.) of surviving seedlings = [seedlings observed (by date) / total germination]. *p*-values represent Type I tests of fixed effects, \* indicates significant differences between treatments ( $\alpha = 0.10$ ).

Site	Sep. 2015		May 2016	
	n	prop.	n	prop.
NAT	173	0.607	114	0.484
RES	131	0.692	108	0.47
INV	120	0.487	68	0.265
<i>p</i>	0.508	0.251	0.327	0.054*

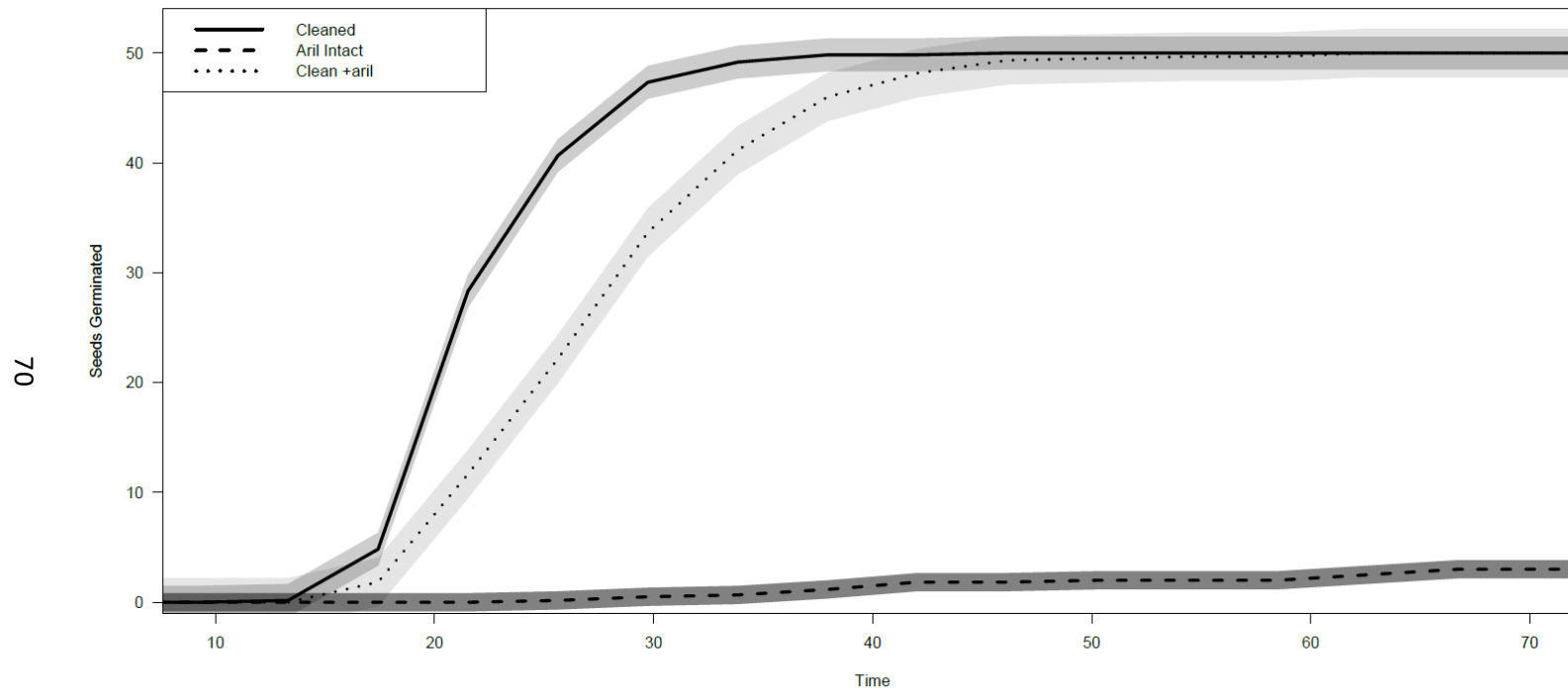
  

Aril	Sep. 2015		May 2016	
	n	prop.	n	prop.
N	212	0.514	139	0.33
Y	212	0.676	151	0.477
<i>p</i>	1	0.108	0.773	0.071*

Table 3.3. Mean values  $\pm$  SE for soil parameters among sites. Values followed by a different letter, within a column, are significantly different ( $p \leq 0.1$ ).

Site	CEC (meq/100g)	% Base Sat.	P (mg/g)	K (mg/g)	Ca (mg/g)	Mg (mg/g)	pH	% C	% N
NAT	19.453 $\pm$ 4.419 A	78.32 $\pm$ 11.22 A	0.007 $\pm$ 0.001 A	0.128 $\pm$ 0.03 A	2.112 $\pm$ 0.63 A	0.152 $\pm$ 0.029 A	4.96 $\pm$ 0.34 A	3.46 $\pm$ 0.86 A	0.24 $\pm$ 0.07 A
RES	30.295 $\pm$ 4.755 A	102.6 $\pm$ 12.31 A	0.009 $\pm$ 0.001 A	0.163 $\pm$ 0.025 A	3.838 $\pm$ 0.929 A	0.261 $\pm$ 0.039 AB	5.72 $\pm$ 0.49 A	5.78 $\pm$ 1.43 A	0.45 $\pm$ 0.12 A
INV	32.28 $\pm$ 4.718 A	99.43 $\pm$ 7.65 A	0.008 $\pm$ 0.002 A	0.174 $\pm$ 0.007 A	3.549 $\pm$ 0.567 A	0.366 $\pm$ 0.059 B	5.76 $\pm$ 0.39 A	5.86 $\pm$ 1.33 A	0.48 $\pm$ 0.12 A
<i>p</i>	0.132	0.255	0.337	0.373	0.191	0.069	0.362	0.361	0.28

Figure 3.3. Germination response of cleaned seeds and aril treatments in vitro. Day 1 is the time seeds were moved from 12 weeks of cold stratification (5°C) to simulated spring temperatures (15°C). Shaded regions represent confidence intervals ( $\alpha = 0.05$ ) for each treatment.



## Chapter Four

### **Juvenile plants of the temperate evergreen liana *Euonymus fortunei* (wintercreeper) exhibit density dependence and alter biomass allocation and soil nutrient concentrations**

#### **Introduction**

Scientific interest in lianas (woody vines) has grown exponentially in the past few decades (Schnitzer et al. 2015). Well over a century since Charles Darwin's (1865) foundational studies on climbing plants, there are a number of reasons why liana research has now taken off so dramatically. Foremost, liana influence upon forest ecosystems is disproportionately greater than their modest contributions to aboveground biomass (Burnham 2015). Lianas drive forest dynamics disproportionately because of their behavior as structural parasites, perpetually in competition with trees. As lianas compete for above- and belowground resources, they negatively influence tree growth, diversity, fecundity, and survival (Matthews et al. 2016). Through their direct effect on trees, forest-level processes can be drastically altered by lianas; including reductions in carbon (C) sequestration and transpiration, increased nutrient mineralization, and delayed forest regeneration and succession (Ladwig and Meiners 2010; Kazda 2015; van der Heijden et al. 2015; Campanello et al. 2016).

The influence of liana growth on ecosystems becomes particularly noteworthy considering a well-documented increase in liana abundance worldwide. Elevated atmospheric CO<sub>2</sub> concentrations and temperatures have been implicated as drivers of increased liana abundance (Condon et al. 1992; Ladwig and Meiners 2010; Wang et al. 2011) in temperate and tropical forests. Lianas are unique compared to other woody



plants due to their ability to direct C allocation to primary growth, with comparatively lower investments in secondary growth and defense compounds (Asner and Martin 2015). As a result, increases in liana abundance can release more C from terrestrial stocks by competitively reducing tree biomass (van der Heijden et al. 2015).

Additionally, as natural agents and beneficiaries of disturbance, lianas are highly effective at capitalizing on increased nutrient availability following tree fall, due to their comparatively fast growth rates (Kazda 2015). Because lianas are more abundant along forest edges and gaps (Ladwig and Meiners 2010), increased gap and edge formation from disturbance events and forest fragmentation are expected to further promote liana recruitment (Matthews et al. 2016). Given the ongoing trends of increased CO<sub>2</sub> emissions and forest fragmentation (Berry 2008), lianas are poised to play a greater role in ecosystem processes for the foreseeable future.

Convergent evolution of the lianescent growth habit has conferred a series of unique ecological attributes that are indicative of the group, but often quite unlike those of trees and shrubs. As young plants (seedlings), both light-demanding and shade tolerant species of *Bauhinia* lianas allocated more biomass to leaves than did trees species of the same genus (Cai et al. 2007). Liana seedlings also have consistently higher photosynthetic capacity and lower leaf construction costs than trees, further enabling rapid colonization following disturbance (Santiago et al. 2015; Campanello et al. 2016). As climbing plants establish, aboveground growth may occur both horizontally and vertically in space, and development of adventitious roots and shoots can lead to dense population monocultures (Gerwig et al. 2006; Leicht-Young et al. 2011). Density is an important regulator of population growth and survival, due to plant competition for

resources. The so-called self-thinning law (density-dependent mortality) predicts that over time, intraspecific competition can lead to mortality and reduced yields as a consequence of overcrowding (Yoda et al. 1963; Watkinson 1980). While very few studies have investigated the effect of density among climbing plants, there is preliminary evidence that they may be less prone to self-thinning than self-supported plants. For example, in two species of *Celastrus* lianas, mortality was significantly lower among high-density planting treatments (Leicht-Young et al. 2011). Similarly, higher planting densities of the perennial vine *Vincetoxicum rossicum* responded with higher biomass and greater seed-set than did low and medium density plantings (Cappuccino 2004). The potential for climbers to exhibit positive density dependence may be attributable to their ability to escape competition by growing outward and upward away from neighboring plants (Klimeš and Klimešová 1994; Letcher and Chazdon 2009). Positive frequency dependence is typically predicted to reduce species diversity (Molofsky and Bever 2002), raising further concern in light of increased global liana abundance.

The combination of traits that promote abundance within indigenous forests also aids the invasion of exotic liana species within novel environments. These traits, including rapid growth, higher net primary productivity, greater standing biomass, and positive association with disturbance tend to be indicative of plant invaders in general (Ehrenfeld 2003; Asner and Martin 2015). Temperate forests have substantially lower liana diversity than tropical forests (Ladwig and Meiners 2015), and it is believed that low native liana diversity and density in North America may provide empty niches for exotic species to exploit (Gentry 1991; Leicht-Young and Pavlovic 2015).

Approximately 9% of plant invaders in eastern North America are lianas, some of which

(e.g. Oriental bittersweet, Japanese honeysuckle, and kudzu) are particularly widespread and problematic (Ladwig and Meiners 2010; Leicht-Young and Pavlovic 2015). Still, there has been limited research documenting liana invasion ecology in temperate forests. Considering a documented increase of temperate liana abundance, and their potential to disrupt native ecosystems, additional studies will be important to understand and potentially mitigate their invasion processes (Leicht-Young and Pavlovic 2015).

In this study, I investigate the establishment dynamics of *Euonymus fortunei* (hereafter: wintercreeper), an evergreen liana invader of North America. Introduced from temperate Asia as an ornamental groundcover in 1907, wintercreeper is widely cultivated and still sold as a landscape plant (Zouhar 2009). Only in the past decade has wintercreeper gained recognition as an invasive species, and accordingly, investigations of its ecology are now beginning to emerge. Wintercreeper has been reported to colonize diverse environments from high-light urban areas to old-growth forests, where it tends to form dense monocultures that competitively exclude native species (Swedo et al. 2008; Zouhar 2009; Song and Li 2016). Smith and Reynolds (2012, 2015) have implicated plant-soil feedback as a driver of invasion, though it remains unclear if feedback is mediated by abiotic vs. biotic factors. Studying paired invaded vs. uninvaded plots, both Swedo et al. (2008) and Bray et al. (2017) noted significant differences between soil biotic communities, as well as abiotic factors including soil nutrients, soil moisture, and pH. Still, it is unknown if the observed differences in invaded sites were pre-existing or wintercreeper mediated.

To my knowledge, there are no published reports of wintercreeper establishment at the seedling stage. This early phase of development and growth may be important to

better understand wintercreeper invasion dynamics for several reasons. First, seedling establishment is subject to occur amidst intraspecific competition. Strong intraspecific competition (i.e., high seedling density) may influence seedling growth and survival positively via Allee effects (Cappuccino 2004), or negatively due to self-thinning (Yoda et al. 1963). Second, wintercreeper seedlings are incapable of self-supported vertical growth (personal observations), thus depending on where seeds germinate, seedlings will either climb a tree for support or grow horizontally until a suitable support is encountered. Vertical growth may allow seedlings to escape ground-level competition and provide greater access to light, potentially accelerating above- and belowground growth. Third, soil nutrient concentrations are routinely found to differ between paired invaded and uninvaded sites in a variety of plant invaders (Weidenhamer and Callaway 2010). There is evidence that total soil carbon, nitrogen, and extractable magnesium are higher in wintercreeper invaded sites (Bray et al. 2017; Rounsaville et al. 2017b), but it remains to be determined if wintercreeper is a beneficiary (i.e., preferential invader) or driver of these differences. I designed an experiment to examine the influence of growth orientation (horizontal or vertical) and planting density on wintercreeper seedling survival and development. I also tested soil carbon and nutrient concentrations pre- and post-planting to determine if wintercreeper was influencing their availability. In central Kentucky where the study took place, invaded sites are typically dense (layered stems) monoculture patches of 100% forest floor cover. Based on available liana data and my observed all-or-nothing invasion trend for wintercreeper, I specifically tested the following hypotheses: (H<sub>1</sub>) high planting density will positively influence survival, growth, and biomass allocation; (H<sub>2</sub>) access to vertical support will positively influence

plant growth and biomass allocation; and (H<sub>3</sub>) soil carbon and nutrient concentrations will increase after conditioning by wintercreeper.

## **Materials and Methods**

### *Experimental design*

*Euonymus fortunei* diaspores were collected and mixed from six naturally invaded populations in Lexington, Kentucky on January 4, 2015. A random subset of 300 seeds were prepared for germination by gently removing and discarding the aril tissue. Seeds were sown in 50-cell plug trays containing PRO-MIX potting media (Premier Tech, Quakertown PA), and transferred to a heated greenhouse. All seeds germinated within 4 weeks, and the resulting seedlings were maintained in the greenhouse (maximum low: 15°C, maximum high: 25°C) prior to being moved into the field.

My experiment took place at the State Botanical Garden of Kentucky on the University of Kentucky campus. I chose a level, open field site to minimize any influence of tree competition and provide uniform light to all treatments. The field consisted predominately of turfgrass (*Festuca spp.*, *Lolium spp.*, *Poa spp.*) with minor amounts of common weeds (*Taraxacum officinale*, *Viola sororia*). Prior to this experiment, the field was mowed to between 5-20cm height for at least 25 years, and before that it was cultivated in row crops. A 7 x 7m site was chosen for the experiment and sprayed with glyphosate to kill pre-existing vegetation on May 17, 2015. To more accurately represent the light conditions of a forest understory, I constructed a stand-alone shade structure on May 31. The shade structure was prepared on the margin of the plot, using eight 9 x 9cm pieces of treated lumber as load-bearing vertical supports on the

corners and sides of the plot. The shade structure stood 3m tall after burying the 4.3m long vertical supports in the ground. The vertical supports were reinforced with 2 x 15cm horizontal braces across the top and sides, and then black, knitted polyethelene fabric providing 50% shade (A.M. Leonard, Piqua OH) was used to enclose the entire structure. The greenhouse-grown seedlings were moved into the shade structure on June 14, 2015, where they remained in the plug-trays for 2 weeks to adjust to light and temperature conditions of the plot. On June 28, the entire plot was hand-weeded to remove reemerging weeds and then the seedlings were planted into their respective treatments. At the time of planting, the seedlings were uniform in root development and stem length (30-35cm). Immediately after planting, the entire plot was watered using a sprinkler to aid seedling establishment. No additional irrigation was provided for the remainder of the experiment, and whole plot hand-weeding occurred as necessary until the treatments were harvested on November 27, 2016 (17 months of field growth). Between December 6, 2015 and March 27, 2016, the shade fabric covering the ceiling of the structure was removed to allow snowfall (approximately 20 cm total) to both cover the horizontal liana stems as it would naturally, and to prevent excessive load on the shade structure.

My experimental design was a factorial combination of plant density and growth orientation. There were three levels of plant density (low, medium, and high), in which 2, 6, or 14 seedlings were planted in a 1 x 1m plot, respectively. Two levels of growth orientation (horizontal and vertical) were achieved with the absence or presence of a vertical support, respectively. Vertical support was provided by driving a 1.5m metal post into the center of each appropriate treatment. Hackberry (*Celtis occidentalis*) trees with a basal diameter between 8-10cm were harvested from a nearby forest, cut into 3m

lengths, and affixed to the metal posts using zip ties. The factorial experiment yielded six treatments (3 densities x 2 growth habits) that were replicated six times in a randomized complete block design (RCBD) for a total of  $n=36$  experimental units. Plots within each block were adjoining, and blocks were separated by a narrow (20 cm) walking lane. Seedlings used in all density treatments were planted toward the center of each plot, and were spaced on 10cm centers. For those treatments in which a vertical support was provided, all seedling stems were directed vertically toward the central support, and gently held in place with a piece of yarn. This was done to encourage vertical growth and adhesion to the provided support. A total of 268 seedlings were planted across treatments.

#### *Plant survival and growth*

After 17 months of growth, all experimental units were harvested for data analysis. Harvesting occurred at a time when I felt further growth of roots and shoots would confound experimental data by influencing neighboring treatments. I assessed the following plant-response parameters: 1. survival (%), 2. plant morphological development (basal diameter, internode distance, and stem length), 3. plant dry mass (shoots, roots, and total), and 4. growth allometry [specific leaf area (SLA), shoot to root ratio (S/R), specific stem length (SSL), leaf mass fraction (LMF), stem mass fraction (SMF), and root mass fraction (RMF)] (Table 4.1). To accurately assess these variables, individual plants were carefully removed from treatment plots to keep all parts intact. Adventitious stem-roots were present on horizontal and vertical vines, and were gently pried away from the soil and hackberry supports, respectively. Primary root systems

were relatively shallow and fibrous, and were carefully lifted from the soil using a spading fork.

Survival was calculated as the number of seedlings planted / number of plants harvested within each experimental unit. Basal diameter was recorded for all surviving seedlings by taking the average of two, 90° offset measurements at the point just above where the root transitioned to stem, and are reported as the mean of all stem diameters per experimental unit. Internode distance was calculated using the longest stem per experimental unit. I started 20cm from the distal end of the longest stem (avoiding under-developed primary growth), and calculated the mean distance of the next ten successive internodes (measured from the base of each bud). Stem length was calculated as the mean of the three longest stems per experimental unit at medium and high planting densities. The low-density treatment, having only a potential maximum of two stems, was the mean value of the two longest stems.

I separated all roots (including adventitious stem roots) from shoots (leaves + stems) on freshly harvested plants. Roots required additional rinsing to carefully remove soil and organic debris from the fine root systems. Separated shoot and root networks for each experimental unit were transferred to paper bags and moved to a walk-in oven at 60°C for one week before taking dry-mass measurements. Total biomass values represent the sum of shoot and root dry mass. I also calculated *average* shoot and root biomass as the quotient of total biomass values / number of surviving plants per experimental unit.

Leaves for SLA analysis were harvested from the stems used for stem length measurements. Beginning 20cm from the distal end and working toward the proximal



end, 10 (medium and high density) or 15 (low density) successive leaves were collected from each stem, for a total of 30 leaves per experimental unit. The fresh leaves were analyzed using a LI-3100C area meter (LI-COR, Lincoln, Nebraska) to determine total area, and oven dried at 60°C for one-week prior to taking dry-weight measurements. The oven dried mass of leaves separated for SLA measurements was included in shoot mass and leaf mass fraction calculations for each experimental unit. Shoot to root ratios were calculated using dry mass values (shoots/roots). Specific stem length (SSL) was determined by dividing stem length by average stem mass, for each experimental unit. Finally, dried shoots were separated and weighed as individual leaf and stem fractions. I determined leaf, stem, and root mass fractions by dividing the dry mass of each component by the total dry mass per experimental unit.

### *Soil sampling*

Soil samples were collected on July 1, 2015 (pre-conditioning) and November 15, 2016 (post-conditioning) from the 18 experimental units that lacked vertical supports. Three cores of upper mineral soil (0-10 cm) were extracted at random from each experimental unit, and bulked for each treatment unit. Samples were sieved through a 2 mm sieve and stored at -62° C prior to analysis. Approximately 2 g (dry weight) of soil were sent to University of Kentucky Regulatory Services for analysis on December 16, 2016. Soils were analyzed for extractable phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and zinc (Zn), using Mehlich-3 protocols (Mehlich 1984). Total soil carbon (C) and nitrogen (N) were determined using a vario MAX CNS Macro Elemental Analyzer (Elementar Analysensysteme GmbH, Germany).

### *Statistical analysis*

I used a two-way Analysis of Variance (ANOVA) to test for differences in plant growth among planting densities (low, medium, and high) and orientation (horizontal vs. vertical). The same model was used to compare soil nutrient concentrations based on planting density (low, medium, and high) and time of collection (pre- and post-conditioning). Preliminary soil analysis indicated significant increases in exchangeable nutrients and total C occurred over the course of study. Because increased organic matter (C) is likely to influence total N and extractable nutrients, I decided to analyze soil data a second time using C as a covariate in the ANOVA model. This analysis was intended to determine if increases in organic matter would also predict changes in other soil nutrients. Data were checked for normality and homogeneity of residuals, and transformed when necessary to satisfy ANOVA assumptions. Least Squares Means were used to test for differences of planting density among treatments. A Multivariate Analysis of Variance (MANOVA) was performed on leaf, stem, and root mass fraction data to test whether the mean proportion of each component differed between planting density or growth orientation. All statistical analyses were performed using SAS software (Version 9.4).

## **Results**

### *Plant survival and growth*

Wintercreeper survival was significantly lower [ $F(2,25) = 14.2, p < 0.0001$ ] among high density plantings (89.9%) than at low (100%) and medium (98.6%) densities (Table 4.2). Nevertheless, survival was high overall, and no fewer than 11 out of 14

plants (78.6%) survived among all high density experimental units. Neither growth orientation nor the interaction of growth orientation and density had a significant effect on plant survival.

Plant morphological responses of diameter and stem length were significantly different in response to planting density (Table 4.2). Basal diameters of low and medium density plantings were significantly larger [ $F(2,25) = 5.62, p = 0.0096$ ] than that of high density. Stem length (average of longest stems) was significantly greater [ $F(2,25) = 8.64, p = 0.0014$ ] at medium and high densities compared to low density. Although stem length did not differ significantly between medium and high density treatments, they were on average 22% longer than low density stems. Internode length did not differ among planting densities. There were no significantly different responses of plant morphological characters resulting from growth orientation treatment.

Total (plot-level) shoot, root, and combined biomass were positively correlated with wintercreeper density and were significantly different [ $F(2,25) = 59.95, p < 0.0001$ ;  $F(2,25) = 80.29, p < 0.0001$ ; and  $F(2,25) = 64.88, p < 0.0001$ , respectively] between the three density treatments (Table 4.1). Significant differences were also found for average (individual plant) shoot, root, and combined biomass, but the trends were unlike that of the plot-level findings. Instead, I found high planting density yielded significantly lower average shoot [ $F(2,25) = 6.07, p = 0.0071$ ], root [ $F(2,25) = 6.42, p = 0.0056$ ], and combined [ $F(2,25) = 6.31, p = 0.006$ ] biomass values compared to low and medium densities, which did not differ from one another.

Although total shoot, root, and combined biomass tended to be greater on vertically oriented treatments (vs. horizontally oriented), only for roots was the difference

significant [ $F(1,25) = 11.46, p = 0.0023$ ]. There was a nearly significant [ $F(1,25) = 4.15, p = 0.0523$ ] effect of growth orientation on average root biomass, which tended to be greater for vertically oriented treatments. Despite significant main effects of density, and in some cases growth orientation, on plot-level and plant-level (average) biomass responses, there were no interactive effects found.

Among all allometric growth responses, only SSL was significantly influenced by planting density [ $F(2,25) = 11.59, p = 0.0003$ ]. I found SSL was greatest among high density plantings (0.129) compared to low (0.083) and medium (0.083) (Table 4.3). The MANOVA for leaf, stem, and root mass fractions indicated that neither planting density nor growth orientation significantly influenced plant allocation for these components (data not presented). The univariate ANOVA nevertheless indicated that growth orientation led to a significant difference in SMF [ $F(1,25) = 4.65, p = < 0.0409$ ], in which horizontally grown treatments yielded a greater proportion of dry stem mass (0.582) than vertical treatments (0.567). Despite the dependent relationship that exists between leaf, stem, and root mass fractions, LMF and RMF were not significantly influenced by growth orientation. However, I found some evidence that lower SMF among vertical treatments was compensated by an increase in RMF, though nonsignificant at  $\alpha = 0.05$  [ $F(1,25) = 3.38, p = < 0.0777$ ].

### *Soil conditioning*

Following 17 months of soil conditioning, most of the nutrient concentrations I measured had changed significantly, and appeared to be independent of plant density (Table 4.4). Concentrations of total carbon [ $F(1,15) = 71.80, p < 0.0001$ ], total nitrogen

[ $F(1,15) = 44.89, p < 0.0001$ ], and extractable phosphorus [ $F(1,15) = 14.54, p = 0.0017$ ], calcium [ $F(1,15) = 65.95, p < 0.0001$ ], and magnesium [ $F(1,15) = 23.51, p = 0.0002$ ] all significantly increased over the course of the study. Soil concentrations of K were nearly significant [ $F(1,15) = 4.17, p < 0.0591$ ], and unlike other nutrients tended to decrease in response to conditioning.

Further analysis of soil nutrients using C as a covariate eliminated the significant effect of time as a predictor of total N and extractable Mg (Table 4.5). This analysis suggested that soil C significantly predicted increases in N [ $F(1,14) = 96.72, p < 0.0001$ ], P [ $F(1,14) = 1.4, p < 0.0001$ ], Ca [ $F(1,14) = 30.71, p < 0.0001$ ], and Mg [ $F(1,14) = 26.65, p = 0.0001$ ]. Unlike N and Mg, the influence of time remained significant for P [ $F(1,14) = 7.4, p = 0.0166$ ] and Ca [ $F(1,14) = 12.33, p = 0.0035$ ]. Although soil C had no influence upon K ( $p = 0.2557$ ), using C as a covariate did lead to a significant effect of time [ $F(1,14) = 5.01, p = 0.042$ ], in which extractable K concentrations decreased (0.278 to 0.248 mg/g) in response to soil conditioning (Tables 3.4, 3.5).

## **Discussion**

My hypothesis ( $H_1$ ) that wintercreeper survival and growth would be positively correlated with density was not supported by survival data, while plant growth responses suggest both positive and negative influence at high densities (Table 4.6). That both positive and negative frequency dependence appear to exist for wintercreeper presents an interesting interpretation of density effects for this liana.

By the end of the experiment, survival was lower among high density wintercreeper plantings, as compared to low and medium densities. These findings run

counter to that of *Celastrus orbiculatus* and *C. scandens* (Leicht-Young et al. 2011), close relatives of wintercreeper (Celastraceae), which to my knowledge are the only other liana species to have been assessed for intraspecific density-based survival. For each *Celastrus* species, low and medium density plantings displayed similar survival rates, yet they were significantly lower than that of high density plantings. Thus the observed decline in survival for wintercreeper is more characteristic to that of trees, in which proximity to intraspecific neighbors can lead to negative density dependence (Wang et al. 2012). Among all plant types, populations are predicted to experience self-thinning (mortality) due to competition for available resources when density exceeds a certain threshold (Norberg 1988). Given the survival results, I conclude that the chosen densities of 2, 6, and 14 seedlings m<sup>-2</sup> provided an appropriate appraisal of density based self-thinning.

Further support for the supposition that high-density mortality was influenced by resource competition comes from data at the individual plant level. Average diameter was measured for all surviving seedlings, and was significantly smaller for seedlings within high density plantings (Table 4.2). Similarly, average root biomass and average shoot biomass were significantly lower at high density than for low and medium densities (Table 4.2). These findings are consistent with nearly all density studies, in which average plant mass is reduced at higher densities (Poorter et al. 2012). In birch tree seedlings, Aphalo and Rikala (2006) found that dry mass was unaffected by planting density, likely due to compensatory responses such as increased plant height and thinner, larger leaves among high density plants. However, these birch seedlings were grown in individual pots supplied with equal amounts of water and nutrients. Given that soil

nutrients were unaffected by density in the study (Table 4.4), it is possible that competition for water could have reduced average seedling root mass at high densities. Water is an especially important resource for lianas because of their substantially larger xylem vessels (compared to trees) which carry greater risk of drought-induced cavitation (Santiago et al. 2015).

I found evidence that wintercreeper may ultimately be more successful at high densities, despite declines in survival and individual plant growth. This is best exemplified by total (plot-level) biomass, which was positively correlated with density for shoots and roots (Table 4.2). My findings that plot-level biomass increases with density, even if average plant biomass does not, is consistent with other intraspecific density experiments of climbing plants (Cappuccino 2004; Leicht-Young et al. 2011). By contrast, the opposite trend is typically observed for trees, i.e., total standing biomass tends to decrease with plant density (Puri et al. 1994; Johansson 2007; Forster et al. 2007). Unlike trees which are comparatively sessile, it is hypothesized that lianas can be successful at high densities due to their ability to ‘escape’ from competition and seek out resources (Letcher and Chazdon 2009; Wang et al. 2012). I found that stem length was longer in medium and high density plantings compared to low density, which lends support to the ‘escape’ hypothesis. It is also interesting to note that while high density seedlings yielded significantly longer stems compared to low density seedlings, average (per-plant) shoot biomass at high density was significantly lower. This suggests that seedlings at low density were more inclined to ‘fill out’ available space with a greater proliferation of secondary branching, while higher density seedlings placed greater

investment in a main stem. These findings were independent of growth orientation, thus both vertically and horizontally oriented plantings showed the same trend in allocation.

The somewhat paradoxical findings that wintercreeper seedlings display both positive (total biomass) and negative (survival, average individual biomass) frequency dependence are attributable to the growth habit exhibited by wintercreeper.

Wintercreeper is most commonly encountered as an understory species forming a thick groundcover monoculture. This is in contrast to the majority of liana species worldwide that are adapted to the high light environments encountered following gap creation and ultimately secured after overtopping host tree canopies (Cai et al. 2007). Although adult wintercreeper phanerophytes use trees for support, they do not reach or overtop the forest canopy (Rounsaville et al. 2017a). Thus the high propensity for adventitious rooting and forest-floor layering among wintercreeper chamaephytes quickly obscures the individuality of seedlings. In other words, the survival and growth of individual wintercreeper seedlings may become trivial in the context of density dependence within an aggregate, clonally propagating population. Certainly, genotype can play a role in other invasion processes (e.g., plant physical and chemical defenses, intraspecific admixture) outside the context of this experiment. Nevertheless, I believe the overall influence of density upon *total* biomass to be the strongest indicator of positive density dependence in wintercreeper.

My hypothesis (H<sub>2</sub>) that vertically oriented treatments would positively influence plant growth and biomass was only supported by total root biomass (Table 4.1). Nevertheless, the effect of vertically supported growth on root mass is particularly interesting for several reasons. First, if vertical growth was to confer escape from ground



level competition (particularly for light, which is further limited by overlapping ground-stems), I would expect an increase in shoot mass, stem length, or leaf mass per area (there were no differences for these parameters). Second, horizontally oriented seedlings tended to have greater occurrence of adventitious stem-roots, and they were carefully extracted from the soil and included within the measurements of root (belowground) biomass. I therefore would have expected greater root mass in horizontally oriented seedlings, yet the opposite was true and total root mass of vertically grown experimental units was  $48.5 \pm 6.3$  g, compared to  $37.6 \pm 5.2$  g.

Greater belowground biomass of vertically oriented seedlings is difficult to account for in the context of this experiment. It is possible that ascending stems will be subject to increased wind loading, even if using a tree for support. Wind loading studies conducted on tree seedlings suggest that root architecture may be modified by wind, but total root mass remains unchanged (Stokes et al. 1995; Tamasi et al. 2005). In wintercreeper, vertically oriented vines undergo a marked transition of leaf morphs during phase change from juvenile to adult, in which leaf area increases substantially. This life history is identical to the evergreen liana English ivy (*Hedera helix*), whose ascending adult leaves are larger, thicker, have greater stomatal frequency, more chloroplasts, and higher net photosynthesis than juvenile leaves (Bauer and Bauer 1980). It would be reasonable to assume adult wintercreeper leaves could accelerate root growth via increased C gains, yet at the time of harvest (November 2016), all vertically oriented vines remained in a juvenile phase. Based on ascending stem height (between 1.5 -2.2 m), the study plants may have been preparing for (or in the early stages of) phase change. Roots are known to influence phase change via hormonal cues, and increased root mass

would be important for lianas to support higher transpiration rates of adult leaves (Frydman and Wareing 1973; Poethig 1990). Vertically oriented vines tended to have larger basal diameter than those grown horizontally. Although the difference in diameter was nonsignificant ( $p = 0.1002$ ), wintercreeper stems provide no structural support, and increased diameter would be important to provide increased amounts of water and nutrients to adult foliage and reproductive organs, and may be further indication of phase change preparation. It is likely that given more time, ascending (adult phase) wintercreeper stems would yield more significantly different trends in morphological parameters such as diameter, as well as biomass allocation.

Analysis of wintercreeper seedling allometry suggests that stems have significant plasticity during early establishment (Table 4.3). The majority of dry mass in wintercreeper plants was allocated to SMF across all treatments, which is a common trend for climbing plants seeking to access the forest canopy for sunlight, or to capitalize on resources following disturbance (Poorter et al. 2012). Accordingly, simulations based on allometric biomass equations predict that over time (having colonized a suitable environment, or parasitized trees for support) lianas will shift a greater fraction of biomass to leaves (Wyka et al. 2013). This trend is markedly different from that of trees, whose contribution to SMF is lowest as seedlings yet forms the majority of total biomass as adults (Poorter and Nagel 2000). Previous studies tend to demonstrate that as crowding (density) increases, SMF increases as well in an effort to capture light (Poorter et al. 2012). Interestingly, I found that orientation, *not* density, significantly influenced SMF. This may be due to wintercreeper's profound tolerance of deep shade, thus light may not be a limiting resource. That SMF was greater on horizontally oriented plants

suggests ‘seeking’ behavior of young vines. Evidence that soil nutrients were consistent among experimental units may indicate that plants were not seeking additional resources *per se*, but instead, a vertical support. Not only is vertical growth obligatory for sexual reproduction in wintercreeper, but in Kentucky forests where deer pressure is heavy on wintercreeper (personal observations), vertical growth is the only means to escape significant herbivory. I found SSL (stem length/stem dry mass) to be significantly greater at high density compared to low and medium density, and similar between growth orientation treatments. The trend for SSL to increase under crowding conditions is well known, and occurs in response to plants seeking to intercept light (Poorter et al. 2012). Nevertheless, I recognize that the SSL calculations use the average stem length (of two or three longest stems) per plot along with the average stem mass, thus the values I present may be slightly greater than expected.

I found support for the hypothesis (H<sub>3</sub>) that soil nutrient concentrations would increase in response to wintercreeper conditioning. However, although C, N, P, Ca, and Mg significantly increased, I also found evidence of a significant decrease in the concentration of K (Table 4.5). Overall, these data validate the few previous reports of wintercreeper being associated with higher total soil C, N, and extractable Mg (Bray et al. 2017; Rounsaville et al. 2017a).

Data from this study suggest there are multiple drivers of soil nutrient changes in response to wintercreeper. First, there is a clear correlation between increased soil organic matter (C) and total N, and extractable P, Ca, and Mg. The common trend among invaders to increase soil nutrient pools (particularly C and N), is typically attributed directly to their growth. Compared to co-occurring natives, invasive plants tend to have

greater biomass and net primary productivity (Ehrenfeld 2003). Increased net primary productivity is caused by traits such as high SLA or net photosynthetic rate, thereby increasing plant C gains and ultimately increasing C pools in litter, soil, and soil microbes (Liao et al. 2008). This can lead to a positive feedback cycle for plants, in which increased root mass can provide access to N and other nutrients that were previously unexploited, and increased soil C can aid soil microbes with N fixation (Liao et al. 2008). The feedback cycle is further accelerated by high quality litter and faster decomposition rates that are common among invasive species (Weidenhamer and Callaway 2010), including wintercreeper (Bray et al. 2017). If high quality litter ultimately suggests nutrient losses for a plant, and rapid mineralization makes those nutrients available to other species, wintercreeper's tendency for monodominance is likely to reduce the loss of those nutrients within a population.

I observed modest contributions of naturally abscised wintercreeper leaves, which may contribute to the increase in soil C detected prior to plant harvesting (post-conditioning). Increased C input to soils may also result from the necromass of pre-existing grass roots within the study plot. Personeni and Loiseau (2004) found that in *Lolium* and *Dactylis* plots sprayed with glyphosate (similar to our study), 15-25% of C remained within incubated root necromass after 18 months. At the same time, the authors found that < 14% of initial *Lolium* C had stabilized in the soil, and < 2% for *Dactylis*. Thus, over roughly the same timeframe (17 months), I expect a rather small influence of grass necromass C would have influenced the post-conditioning samples, as most of it would be lost via C mineralization or removed when roots were sieved from soil samples. Furthermore, when including C as a covariate, the effect of planting density approached

significance for N ( $p = 0.0645$ ), compared to  $p = 0.6275$  without C in the model. This suggests N (which was influenced by C, but not time) showed a trend of increasing where plant density (and therefore shoot biomass and litter input) was greater.

In addition to soil nutrient concentration increases via organic matter deposition, the independent effect of time (conditioning) also increased extractable P and Ca, and decreased K. Therefore, it may be plausible that other direct, active processes mediated by wintercreeper contributed to the significant changes in extractable soil nutrients. Schneider et al. (2001) found that soil P can increase via the production of phosphatase enzymes from roots and microorganisms (Schneider et al. 2001). Alternatively, garlic mustard increased soil pH through root exudates, which Rodgers et al. (2008) hypothesized led to an increase in extractable P and base cations in the soil. I did not measure soil pH or soil enzymes thus it is possible one or both of these factors may have influenced increased P or Ca concentrations.

Nevertheless, Rounsaville et al. (2017b) noted wintercreeper invaded sites tended to have higher pH than uninvaded sites (5.76 vs. 4.96), while Swedo et al. (2008) noted the opposite trend, where invaded sites were significantly lower (6.34 vs. 7.12). Despite the opposing trends, the pH of invaded sites were more similar to one another. Thus because pH influences nutrient availability in soil, it may be advantageous for wintercreeper to modify soil pH to an optimal level (Dassonville et al. 2008). The decrease in soil K only became significant ( $p = 0.042$ ) when C was included in the model as a covariate, likely due the loss of one error degree of freedom (Table 4.5). It is plausible that decreased soil K was influenced by greater demand of this nutrient by wintercreeper. Plants require large amounts of K, and when aboveground plant parts are

harvested the soil supply of K can be quickly drained (Brady and Weil 2002).

Additionally, K has the highest resorption rate of all essential nutrients (Vergutz et al. 2012), which may account for why this nutrient was not predicted by organic matter as were N, P, Ca, and Mg.

While soil C increased twofold during the experiment, N and Mg were the only soil nutrients whose increase was fully predicted by C (as opposed to time alone) (Table 4.5). Previous studies have found significant increases in C, N, and Mg (but not other nutrients) in wintercreeper invaded soils, which may suggest that deposition of wintercreeper leaf litter is the major (or only) driver of soil change in natural populations over time. In paired uninvaded/invaded sites Bray et al. (2017) found C and N (averaged 0-10 cm depth) to be 3.2/6.2% and 0.22/0.5% (respectively), and were significantly different between sites. Rounsaville et al. (2017b) reported significant differences for soil Mg (0-5cm), which was 0.15/0.37 mg/g among uninvaded/invaded wintercreeper sites. These values are similar to the findings of this study for pre- and post-conditioning total C (2.49 and 4.5%), total N (0.24 and 0.34%), and extractable Mg (0.15 and 0.18 mg/g), particularly if comparing the pre-conditioning values to that of uninvaded soils (Table 4.4).

Overall, these findings are consistent with other plant invaders in North America that tend to influence (or be associated with) increased soil nutrients, for example European buckthorn (> C, N, Ca), garlic mustard (>N, P, Ca, Mg), Japanese stiltgrass (>P, Ca, Mg), and Oriental bittersweet (>Ca, Mg, K) (Rodgers et al. 2008; Leicht-Young et al. 2009; McGrath and Binkley 2009; Iannone III et al, 2015). Future studies will be

necessary to more accurately assess the mechanism(s) that drive soil changes among wintercreeper sites, especially within more natural (forest) conditions.

## **Conclusions**

This study found that juvenile wintercreeper growth and biomass allocation were strongly influenced by planting density. Although orientation had a comparatively smaller influence upon growth, I found vertically oriented stems to have significantly higher root mass. This finding is interesting given that vines had not undergone a phase change to adult, thus increased root biomass may occur in preparation. This finding suggests that host (support) trees will naturally face increased belowground competition from wintercreeper. Concentrations of total carbon and nitrogen, and extractable phosphorus, calcium and magnesium were significantly increased over the course of the study. The processes that mediate changes in soil nutrient concentrations among wintercreeper seedlings may be advantageous for this invader's establishment in disturbed or nutrient-poor soils. The observations that wintercreeper drives nutrient changes may help explain how increases in total biomass are sustained as planting density increases. Most interestingly, these results provide evidence that for wintercreeper, the influence and importance of 'individual plants' is lost over time, as aboveground stems adventitiously root, and coalesce into an aggregate monoculture. In this way, total above- and belowground biomass only increased with density, even if individual plant fitness did not. These findings may be particularly relevant for future studies of the ecological impacts of wintercreeper.

Table 4.1. Plant response variables with transformation method and p-values for planting density and growth orientation. \* significant at  $\alpha = 0.1$ ; \*\* significant at  $\alpha = 0.05$ .

	Transformation	Density	Orientation	Dens*Orien
Survival				
Percent survival	arcsine sqrt	< 0.0001**	0.3529	0.7874
Morphology				
Diameter	none	0.0096**	0.1002	0.9603
Internode distance	none	0.3172	0.9312	0.4025
Stem length	none	0.0014**	0.363	0.9456
Dry mass				
Total shoot	none	< 0.0001**	0.1712	0.8209
Total root	none	< 0.0001**	0.0023**	0.1964
Total combined	none	< 0.0001**	0.0902*	0.7538
Average shoot	none	0.0071**	0.2884	0.8516
Average root	none	0.0138**	0.0842*	0.8171
Average combined	none	0.006**	0.2157	0.8725
Allometry				
SLA (Specific leaf area)	log n	0.3242	0.6556	0.5207
S/R (Shoot to root ratio)	log n	0.7456	0.0719*	0.0919*
SSL (Specific stem length)	arcsine sqrt	0.0003**	0.2418	0.9785
LMF (Leaf mass fraction)	arcsine sqrt	0.6135	0.8577	0.6039
SMF (Stem mass fraction)	arcsine sqrt	0.3286	0.0409**	0.2167
RMF (Root mass fraction)	arcsine sqrt	0.7125	0.0777*	0.0956*



Table 4.2. Plant survival, morphological response, and biomass means  $\pm$  SE for planting density and growth orientation. Low, medium and high density plantings contained 2, 6, and 14 seedlings per  $1\text{m}^2$  plot. Values represent actual means (back transformed data). Letters (within rows) indicate values that are significantly different ( $\alpha = 0.05$ ).

	Planting density			Growth orientation	
	Low	Medium	High	Horizontal	Vertical
Survival (%)	$1 \pm 0.0^A$	$0.9861 \pm 0.0139^A$	$0.8988 \pm 0.024^B$	$0.9511 \pm 0.0196$	$0.9722 \pm 0.0131$
Diameter (mm)	$9.6083 \pm 0.6466^A$	$10.04833 \pm 0.2619^A$	$8.38 \pm 0.2943^B$	$8.9861 \pm 0.3529$	$9.705 \pm 0.4076$
Internode (cm)	$4.0041 \pm 0.1268$	$3.7567 \pm 0.1032$	$3.7958 \pm 0.1294$	$3.8583 \pm 0.0976$	$3.8461 \pm 0.1029$
Length (m)	$1.6092 \pm 0.083^A$	$1.95 \pm 0.057^B$	$1.9658 \pm 0.0591^B$	$1.8783 \pm 0.076$	$1.805 \pm 0.056$
Total shoot mass (g)	$72.76 \pm 10.83^A$	$205.96 \pm 15.05^B$	$286.41 \pm 21.75^C$	$177.04 \pm 24.35$	$199.71 \pm 25.64$
Total root mass	$16.5 \pm 2.3^A$	$46.32 \pm 3.89^B$	$66.18 \pm 5.28^C$	$37.55 \pm 5.21^A$	$48.46 \pm 6.26^B$
Total dry mass	$89.26 \pm 12.97^A$	$252.28 \pm 18.79^B$	$352.59 \pm 26.36^C$	$214.58 \pm 29.51$	$248.17 \pm 31.71$
Avg. shoot mass	$36.38 \pm 5.41^A$	$34.89 \pm 2.57^A$	$22.89 \pm 1.83^B$	$29.51 \pm 2.95$	$33.27 \pm 3.48$
Avg. root mass	$8.25 \pm 1.15^A$	$7.83 \pm 0.64^A$	$5.27 \pm 0.41^B$	$6.37 \pm 0.6$	$7.87 \pm 0.78$
Avg. dry mass	$44.63 \pm 6.48^A$	$42.73 \pm 3.18^A$	$28.16 \pm 2.2^B$	$35.87 \pm 3.52$	$41.14 \pm 4.21$

Table 4.3. Mean values for specific leaf area (SLA), shoot-root ratio (S/R), specific stem length (SSL), leaf mass fraction (LMF), stem mass fraction (SMF), and root mass fraction (RMF) among planting density and growth orientation treatments. Letters (within rows) indicate values that are significantly different ( $\alpha = 0.05$ ).

Parameter	Definition	Units	Planting density			Growth orientation	
			Low	Medium	High	Horizontal	Vertical
SLA	Leaf area/leaf dry mass	cm <sup>2</sup> g <sup>-1</sup>	130.16	123.64	125.09	127.04	125.55
S/R	(leaf + stem dry mass)/root dry mass	g g <sup>-1</sup>	4.38	4.52	4.41	4.64	4.24
SSL	Longest stem length/avg. stem dry mass	m g <sup>-1</sup>	0.083 <sup>A</sup>	0.083 <sup>A</sup>	0.129 <sup>B</sup>	0.103	0.093
LMF	leaf dry mass/total plant dry mass	g g <sup>-1</sup>	0.236	0.236	0.243	0.238	0.239
SMF	stem dry mass/total plant dry mass	g g <sup>-1</sup>	0.573	0.582	0.569	0.582 <sup>A</sup>	0.567 <sup>B</sup>
RMF	root dry mass/total plant dry mass	g g <sup>-1</sup>	0.191	0.183	0.224	0.18	0.194

Table 4.4. Soil nutrient concentration means  $\pm$  SE for total C, total N, and Mehlich-3 extractable P, K, Ca, and Mg in response to planting density and soil conditioning. Values represent actual means (back transformed data). Letters (within rows) indicate values that are significantly different ( $\alpha = 0.05$ ).

trans.	Density			Time		
	Low	Medium	High	Pre-conditioning	Post-conditioning	
C (%)	log n	3.5368 $\pm$ 0.5322	3.4981 $\pm$ 0.346	3.4423 $\pm$ 0.3286	2.4871 $\pm$ 0.0435 <sup>A</sup>	4.4977 $\pm$ 0.3159 <sup>B</sup>
N (%)	log n	0.2869 $\pm$ 0.0251	0.28 $\pm$ 0.014	0.3019 $\pm$ 0.0198	0.2432 $\pm$ 0.0042 <sup>A</sup>	0.3361 $\pm$ 0.0162 <sup>B</sup>
P (mg/g)	none	0.044 $\pm$ 0.0051	0.0413 $\pm$ 0.0032	0.0432 $\pm$ 0.003	0.0374 $\pm$ 0.0019 <sup>A</sup>	0.0482 $\pm$ 0.0035 <sup>B</sup>
K (mg/g)	none	0.2613 $\pm$ 0.0161	0.2749 $\pm$ 0.0153	0.2523 $\pm$ 0.0179	0.2779 $\pm$ 0.0119	0.2478 $\pm$ 0.0139
Ca (mg/g)	log n	1.3968 $\pm$ 0.1958	1.4503 $\pm$ 0.2144	1.367 $\pm$ 0.1418	1.0903 $\pm$ 0.0871 <sup>A</sup>	1.7191 $\pm$ 0.1611 <sup>B</sup>
Mg (mg/g)	log n	0.1653 $\pm$ 0.0099	0.1687 $\pm$ 0.0089	0.1614 $\pm$ 0.0068	0.1481 $\pm$ 0.004 <sup>A</sup>	0.1821 $\pm$ 0.0069 <sup>B</sup>

Table 4.5. (A) Influence of planting density, time, and density x time on soil nutrient concentrations, and (B) Influence of planting density, time, and density x time on soil nutrient concentrations, using C as a covariate. *p*-values in **bold** are significant ( $\alpha = 0.05$ ).

(A)								
	df trt.	df err.	C	N	P	K	Ca	Mg
Density	2	10	0.9535	0.6275	0.8613	0.4784	0.8923	0.746
Time	1	15	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>0.0017</b>	0.0591	<b>&lt;0.0001</b>	<b>0.0002</b>
Density*time	2	15	0.8746	0.543	0.9238	0.3369	0.3844	0.7484

(B)								
	df trt.	df err.		N	P	K	Ca	Mg
carbon	1	14		<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	0.2557	<b>&lt;0.0001</b>	<b>0.0001</b>
Density	2	10		0.0645	0.4485	0.4895	0.7422	0.7454
Time	1	14		0.5187	<b>0.0166</b>	<b>0.042</b>	<b>0.0035</b>	0.7401
Density*time	2	14		0.0616	0.5191	0.3701	0.1626	0.6465

Table 4.6. Plant response trends among planting density and growth orientation. Symbols that differ (within the same row) among density and orientation treatments indicate significant differences ( $\alpha = 0.05$ ), where (-) equals lowest value, (+) equals higher value, (++) equals highest value. Treatment rows denoted with (0) did not significantly differ.

	Planting Density			Growth Orientation	
	Low	Medium	High	Horizontal	Vertical
Survival					
Percent survival	+	+	-	0	0
Morphology					
Diameter	+	+	-	0	0
Internode distance	0	0	0	0	0
Stem length	-	+	+	0	0
Dry mass					
Total shoot	-	+	++	0	0
Total root	-	+	++	-	+
Total combined	-	+	++	0	0
Average shoot	+	+	-	0	0
Average root	+	+	-	0	0
Average combined	+	+	-	0	0
Allometry					
SLA (Specific leaf area)	0	0	0	0	0
S/R (Shoot to root ratio)	0	0	0	0	0
SSL (Specific stem length)	-	-	+	0	0
LMF (Leaf mass fraction)	0	0	0	0	0
SMF (Stem mass fraction)	0	0	0	+	-
RMF (Root mass fraction)	0	0	0	0	0

## Chapter Five

### **Conclusions and future directions**

#### **Synthesis**

The research conducted for this dissertation represents some of the first studies performed in the context of *Euonymus fortunei* establishment. I have done my best to include and cite the modest amount of additional wintercreeper literature throughout. Although these studies (collectively) are rather few in number, they have been published in the past decade and suggest that ecological interest in wintercreeper is on the rise. My impression is that wintercreeper invasions reached a critical mass in the Ohio River Valley 10-20 years ago, and thus stimulated research among several researchers within the region. A glance at wintercreeper's contemporary range (EDDMaps 2017) places Indiana, Kentucky, and Ohio at the center of the invader's broad distribution in eastern North America, indicating favorable conditions of the region.

Taken together, preliminary wintercreeper data provide a narrative that is both informative and alarming. Informatively, there is evidence that wintercreeper invaded sites influence ecosystems on multiple scales: achieving monodominance via neutral to positive plant-soil interactions (Smith and Reynolds 2012, 2015), limiting forest recruitment and succession (Mattingly et al. 2016), and to some extent, influencing the biotic and abiotic soil environment (Swedo et al. 2008; Bray et al. 2017). Alarmingly, there is little (if any) evidence to indicate resistance within ecosystems. Personal observations in Kentucky over the past six years include wintercreeper's ability to grow in densely shaded forests as well as full-sun urban environments. Although

wintercreeper does not seem to tolerate chronically saturated soils, it routinely persists in floodplain environments, and also grows well within dry and/or rocky sites where soil depths are < 2 cm. Still, light saturation and soil moisture are likely to regulate the speed at which wintercreeper grows, and future studies could provide context for rate of growth and establishment under diverse conditions. I also observed significant herbivory within sites subject to deer pressure. Strong deer pressure effectively reduces forest-floor cover of juvenile vines, but in turn directs growth vertically to reproductively capable vines. Thus, herbivory may slow or prevent establishment within new sites, but vertically established vines will escape herbivory and create strong propagule pressure in the immediate vicinity.

One of the objectives of my research was to characterize what (if any) processes could limit the spread and establishment of cultivated and naturalized wintercreeper populations. Here again, I repeatedly failed to identify any process as such. Seed dormancy (unlike most *Euonymus* spp.) is facultative, and both avian and mammalian dispersers transport seeds to uninvaded sites where germination and survival were equal to and greater than invaded sites, respectively. Nevertheless, these data indicate that failure to recruit and establish at some sites is regulated by other factors. I found that cleaned (bird dispersed) seeds did yield lower survival, the reason for which is unclear and may be related to microorganisms associated with aril tissue, a subject deserving of further study. Still, cleaned seeds are likely to be the only type reaching uncolonized habitats, and their dispersal may be rather limited (especially in comparison to after-ripened, gravity dispersed diaspores).

It seems highly plausible that propagule pressure is a major regulator of wintercreeper invasions. In addition to being consistently correlated with invasiveness (Simberloff 2009), propagule pressure has the potential to be quite high for wintercreeper, an r-selected species. This is made further evident based on my findings of polyembryony in wintercreeper, effectively increasing the number of survival/establishment opportunities per dispersal unit. Future studies to investigate aril tissue chemistry would be helpful to determine how nutritious diaspores are, as these results may influence dispersal in space and time. For example, what species consume wintercreeper seeds, and what are their migratory patterns?

A study of great value to wintercreeper invasion dynamics would be one of population genetics. As previously noted, the ‘Coloratus’ cultivar appears to be the main invasive phenotype. However, in cultivation, I have never observed the multitude of variegated forms to actually reproduce, nor have I seen a variegated plant in the wild. This begs the question: is ‘Coloratus’ a particularly successful genotype, or simply widespread because of its seemingly unique ability to mature physiologically? Wintercreeper presents a fascinating opportunity to study population genetics because all plants in cultivation represent genetic clones, and intraspecific hybridization between clones may be rare. Thus it would be valuable to know if naturalized populations are genetically identical to ‘Coloratus’ vs. hybrid progeny. The former scenario is certainly within the realm of possibilities. Fertilization is required to yield polyembryos in *Euonymus* (Brizicky 1964b), yet the apomictic (maternal clone) polyembryos can still develop if the recombinant embryo aborts. For this reason, genetic sampling of



polyembryos and maternal plants would help explain the role of genotype in the invasion process.

I conducted preliminary sampling of 2C DNA genome sizes in 2014 as a rough survey for potential polyploidization in wintercreeper (Table 5.1). Polyploidy is known to influence speciation via reproductive isolation as well as interspecific hybridization, and can also play a role in adaptability due to novel genome rearrangement (Soltis and Burleigh 2009). Flow cytometric analysis was conducted using the protocols of Rounsaville et al. (2011), and *Lolium perenne* was used as an internal standard with a genome size = 5.6 pg (Wang et al. 2009). Genome sizes were calculated as:  $2C = \text{genome size of standard} \times (\text{mean fluorescence value of sample} / \text{mean fluorescence value of standard})$ . I found that genome sizes of naturalized populations were similar in comparison to wild provenance *E. fortunei*, indicating no change in ploidy (Table 5.1). Thus, intraspecific hybridization between introduced clones would not have been prevented by differences in ploidy, and the role of genetic admixture should be considered as a contributor toward invasion success in wintercreeper.

The research conducted as part of this dissertation had one broad goal: to investigate the processes that contributed to wintercreeper invasion in an effort to guide land managers with early detection and management. I found evidence to suggest that wintercreeper behavior in Eastern North America is similar to that of Eastern Asia: an opportunistic generalist. Wintercreeper's broad geographic range and tolerance of abiotic conditions has allowed it to persist in cultivation for over a century. What appears to be a relatively recent proliferation in certain natural areas cannot be directly explained by my

work. However, I speculate that uninvaded natural areas (fine and broad scales) have not persisted as such due to natural (biotic) resistance. Rather, their status as uninvaded ecosystems is regulated by lower propagule pressure more than any other factor. Thus, proactive efforts toward eliminating seed sources (i.e. mature, vertical vines) and especially regulating this species in commerce may make a profound difference for the future spread of this invader. Increased atmospheric CO<sub>2</sub> and anthropogenic disturbances are expected to further promote recruitment by lianas such as wintercreeper. Perhaps the most beneficial direction for additional wintercreeper research is that of applied approaches for removal and restoration. What are the most effective strategies for eliminating wintercreeper in urban and forested environments? What are the ecological legacies (e.g. soil, seed bank, microorganisms) left by this invader following removal? What additional actions may be necessary to restore natural ecosystems to a desirable state? These questions are relevant for *all* plant invaders, and addressing them in a timely manner is of great benefit to native species and ecosystems.

Table 5.1. 2C DNA genome sizes among various *Euonymus fortunei* clones and North American native *Euonymus* species.

Taxon	Source	Accession number	2C genome size (pg)
<i>Euonymus fortunei</i>			
juvenile form	UK Arboretum Woods	NA	1.64
adult form	UK Arboretum Woods	NA	1.76
seedling	Scott's Grove	NA	1.79
small-leaved form	UK Arboretum Woods	NA	1.84
wild provenance (China) 1	Morton Arboretum	570-2001	1.62
wild provenance (China) 2	Morton Arboretum	394-2004	1.63
<i>Euonymus americanus</i>			
<i>Euonymus americanus</i>	UK Arboretum	2014-025-A	2.26
<i>Euonymus americanus</i>	UK Arboretum	2013-029-B	2.17
<i>Euonymus atropurpureus</i>	UK Arboretum	2013-030-B	1.08
<i>Euonymus obovatus</i>	UK Arboretum	2000-3517-A	1.14

Appendix 2.1. Germinating polyembryonate wintercreeper seeds with emergence of two radicles.



Appendix 2.2. Implementation of seed traps at the seed rain study site within Scott's Grove. Ascending wintercreeper phanerophytes visible in background.



Appendix 2.3. Evidence of animal (mammalian) dispersed seed within the Scott's Grove study forest.



Appendix 3.1. Cleaned seeds germinate on bare mineral soil late April 2015.



Appendix 3.2. Seed frame split plot design within an uninvaded (NAT) site condition on May 2, 2015. Cleaned seeds (left side) germinated earlier than did those sown with intact arils (right).

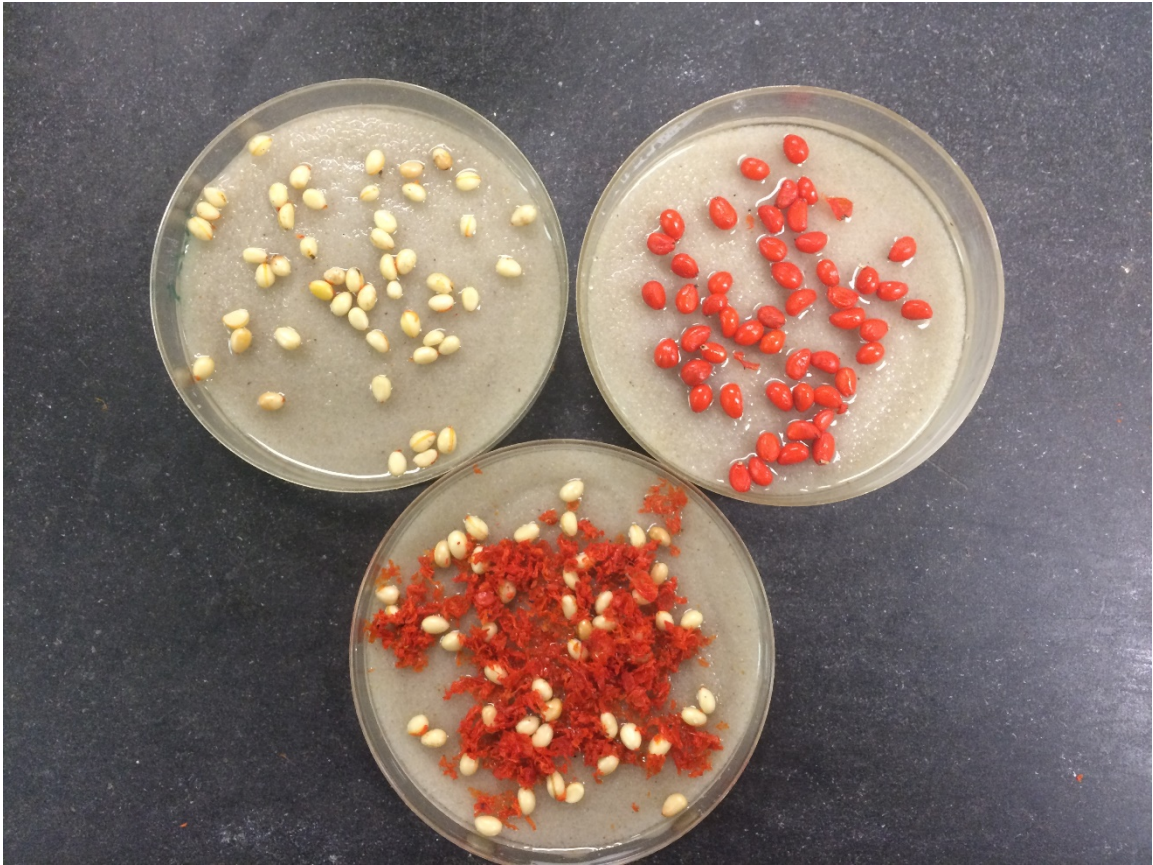




Appendix 3.3. Seedlings marked by germination phase using colored wires July 10, 2015. Most seedlings overwintered without production of true leaves.



Appendix 3.4. Seed treatments used for in vitro aril studies. Clockwise from top left: cleaned, aril intact, cleaned + aril.



Appendix 3.5. Mean  $\pm$  SE soil texture components for uninvaded (NAT), restored (RES), and invaded (INV) sites.

Site	Soil texture		
	%Sand	%Silt	%Clay
NAT	18.2 $\pm$ 3.4	64.5 $\pm$ 4.1	17.3 $\pm$ 2
RES	20.2 $\pm$ 4	57.9 $\pm$ 5.1	21.9 $\pm$ 4.3
INV	21 $\pm$ 4.8	57.6 $\pm$ 4.3	21.4 $\pm$ 1.3

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