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Investigating the Relationship Between Invasive Garlic Mustard Alliaria petiolata and Blacklegged Ticks Ixodes scapularis

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By

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ABSTRACT

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Garlic mustard, *Alliaria petiolata*, is an invasive herb that has been studied for its chemical allelopathy, ability to hinder plant growth of its competitors, and potential to inhibit entomopathogenic fungi. It has been suggested that these fungi, which infect and kill insects and arthropods like blacklegged ticks, *Ixodes scapularis*, may be negatively impacted by garlic mustard presence. This study investigated the potential relationship between garlic mustard and blacklegged tick populations through two experiments. One experiment measured the effect of garlic mustard soil and leaf extract on waxworm survival, where waxworms were used as a bioassay for the presence of fungi. The second experiment measured the abundance of blacklegged ticks found among plots of varying densities of garlic mustard and native herbaceous plant species. My results indicate that garlic mustard soil significantly increases waxworm survival ($P < 0.0001$), however, there was no significant relationship between garlic mustard prevalence and tick abundance. The discrepancy in results between my lab and field studies lead me to conclude that waxworms are more susceptible to entomopathogenic fungi than ticks are, and are therefore a poor model for blacklegged tick survival. These findings indicate that controlling garlic mustard populations will likely have no effect on tick populations and the spread of tick-borne diseases.

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Introduction

The blacklegged tick, *Ixodes scapularis*, is a growing public health concern as it is a vector for many diseases in the United States. Most notably, the blacklegged tick transmits *Borrelia burgdorferi*, the agent of Lyme disease- the most common vector-borne illness in North America (Centers for Disease Control and Prevention, 2015). To understand why blacklegged ticks are more prevalent in certain areas over others, and in an effort to prevent the spread of disease, it is important to understand the blacklegged tick's relationship with the environment. This study investigates the role of invasive versus native plant species as an environmental factor of blacklegged tick survival.

Understanding invasive plant species and their implications on the environment is vital to maintaining a healthy ecosystem. A native plant species is considered to be one that arrived to an area unassisted by humans, and successfully colonized there. Biological invasion, on the other hand, occurs when human establishment of a species that is not native to an area is successful, and that population persists and spreads. This establishment may be intentional or not. Either way, invasion has ecological implications that can be cause for concern. Among the reasons invasive species are problematic include their ability to harm native species by outcompeting for resources like water and light, or by introducing new pathogens that are detrimental to native species (Simberloff, 2013). Another negative characteristic is that some invasive plant species, like Honeysuckle and Japanese Barberry, have been seen to have a direct relationship with tick abundance, and therefore may be helping to spread tick-borne diseases (Allan et al. 2010; Thota, 2016; Williams and Ward, 2010).

Alliara petiolata, otherwise known as garlic mustard, is an invasive exotic that was introduced to North America in the 1800s from Europe (Davis et al., 2012). It is thought to have been introduced to America as a culinary herb and for medicinal purposes (Hayes and Holzmueller, 2012). As a vegetable, garlic mustard provides high levels of vitamin C and vitamin A. As a medicine, garlic mustard can be used as a sweat-inducing drug and to treat gangrene and ulcers (Cavers et al., 1979). Presently, garlic mustard can be found in North America from southern Ontario to around the Midwestern and Northeastern regions of the United States (Hayes and Holzmueller, 2012).

Garlic mustard is a biennial plant in which seedlings emerge and grow into young rosettes during the first year and flower stalks are formed in its second growing season. The species grows between 30 and 100 cm tall and commonly invades moist areas near rivers as well as forest edges and areas that have been disturbed (Hayes and Holzmueller, 2012). Garlic mustard can be distinguished from other flowering plants within its family by its distinct garlic-like scent (Cavers et al., 1979). Due to its invasive nature, garlic mustard has replaced many native spring ephemerals. This may be a result of its rapid growth in the early spring and late fall (Cavers et al., 1979), which allows it to heavily colonize land before native species emerge. The ability of garlic mustard to outcompete native herbs thereby decreases their abundance and diversity. Garlic mustard may also have the ability to inhibit growth of trees and herbs through chemical allelopathy (Stinson et al., 2006), by targeting mycorrhizae, fungi that exhibits a mutualistic relationship with plants by aiding in uptake of water and nutrients while gaining carbohydrates from the plant after it photosynthesizes (Pace, 2003). Chemical aspects of garlic mustard have been thoroughly investigated, and have been found to potentially have characteristics that are pathogenic to some organisms (Blazevic and Mastelic, 2008).

Although it has been suggested that garlic mustard allelochemical compounds negatively impact other plants (Stinson et al., 2006; Vaughn and Berhow, 1999; Prati and Bossdorf, 2003), there has been little evidence to further support that this is actually the case. A study by McCarthy and Hanson (1998), which tested the allelopathic effects of garlic mustard extract on radish, lettuce, winter rye, and hairy vetch seeds and seedlings, saw no appreciable link between ability to germinate and extract application. Another study, conducted by Rodgers et al. (2008), revealed that impact of garlic mustard is species-dependent, as some native species, like boxelder maple, had increased seedling growth when exposed to garlic mustard, while other native species, like Jack-in-the-pulpit, saw slowed growth when exposed to garlic mustard.

Research has shown that these allelochemical compounds that garlic mustard possess also inhibit the growth of entomopathogenic fungi, a fungi known to infect arthropods (Keesing et al., 2011). The potential of garlic mustard to hinder entomopathogenic fungi may be advantageous or detrimental. Eliminating arthropods that serve important ecological purposes like pollination and decomposition would not be beneficial, however, eliminating arthropods like ticks, which serve as vectors for a multitude of diseases, would be valuable to humans

(Keesing et al., 2011). Two species of entomopathogenic fungi, *Beuveria bassiana* and *Metarhizium anisoplae* are known to negatively impact blacklegged tick survival. Vaicekonyte and Keesing (2012) explored the relationship between garlic mustard and entomopathogenic fungi, showing that removal of garlic mustard from soil allowed entomopathogenic fungi to establish at higher levels than prior to the removal. Although Keesing et al. (2012) found an association between garlic mustard presence and inhibition of fungi through the use of waxworms as a bioassay, this effect of garlic mustard may not be reciprocated with tick infection, and garlic mustard may actually cause a reduction in population of ticks (see Rollins et al., 2006 and Malcomb et al., 2009).

Because garlic mustard may impact ticks, it is important to understand not only the properties and development of garlic mustard, but to understand these characteristics of ticks as well. The blacklegged tick is a three-host tick, in which a blood meal is required for the tick to molt into its next life stage. All ticks have an 'inactive' egg stage followed by three active life stages. These life stages are larvae, nymph, and adult. Appearance of male and female ticks is the same in all life stages except for the adult stage (Sonenshine, 1993). With *Ixodes scapularis*, the female is larger and has red coloration around its dorsal scutum (shield; TickEncounter Resource Center).

After hatching from the initial embryonated egg stage, larvae spend days acquiring a sufficient blood meal from a host before dropping and molting in a protected environment, like leaf litter, into the nymphal stage. This process of acquiring a meal is initiated by questing behavior after larvae climb the low vegetation they have dispersed into after hatching. During questing, the tick will stretch out its forelegs while holding onto the vegetation with its lower legs (Centers for Disease Control and Prevention, 2011). This behavior allows the tick to detect a potential host, like a mammal or bird, and latch on as it brushes past. Once a suitable host is found, the tick will insert its mouthpart and feeding tube into the animal to obtain a meal. The nymphs replicate these behaviors, and emerge as adults after molting (Sonenshine, 1993). Before or during female feeding, the male and female adults will mate and the female will drop and seek shelter before laying thousands of eggs and then dying. The male remains on the host, seeking further meals and mating opportunities before dying (Sonenshine, 1993). Pathogens can be transmitted between the three active life stages of ticks in a single blood meal, via transmission through a host (Levi et al., 2015). It is common to see larvae and nymphs low to the ground, as they quest at the height of their most common hosts, small mammals. Adults are found at the tips of vegetation, where they are likely to encounter larger hosts, like deer. A full life cycle may be completed in under a year, but the average life cycle of *Ixodes scapularis* is around two years (Centers for Disease Control and Prevention, 2011). Nymphs are the life stage of major concern in the transmission of pathogens, and their small size and seasons of activity, which are concurrent with the seasons of outdoor activity of many humans- spring and summer, enhance this sentiment (Levi et al., 2015).

My project is to investigate the relationship between garlic mustard and blacklegged tick presence. Two experiments were conducted to explore this relationship. The first experiment measured survival of waxworms (used as a bioassay for fungi) to determine the effects of garlic mustard soil, control soil, garlic mustard extract, and control extract on the survival of the waxworms. The second experiment compared the abundance of *Ixodes scapularis*, (blacklegged ticks) found among invasive *Alliaria petiolata* (garlic mustard) to the abundance of *Ixodes scapularis* found among native, herbaceous plants. Data were collected from the Albany Pine Bush Preserve, Reist Sanctuary, DiCaprio Park, and Woodlawn Sportsmen's Club, all in the Schenectady, NY area. My hypothesis is that there will be a greater abundance of ticks among plots where garlic mustard is present, and that waxworm survival will be higher in treatment jars containing garlic mustard soil and/or watered with garlic mustard extract, due to its ability to inhibit entomopathogenic fungi. Confirming the existence of a relationship between the two species would give insight on ways to manipulate the spread of Lyme disease through the removal or maintenance of garlic mustard populations.

Methods

Waxworm Experiment

Because garlic mustard is thought to kill entomopathogenic fungi, which adversely impacts tick survival, waxworms were used as a standard bioassay for presence of fungi and allelopathic chemicals in order to determine the effects of soil and plant leaf and root extract on the survival of blacklegged ticks (Vaicekonyte and Keesing, 2012). Plant extract was created from garlic mustard and Virginia creeper plant leaves and roots collected from Reist Sanctuary. Leaves and roots were air dried for 24 hours and then placed in a drying oven at 60 °C for 24 hours. Once dry, the leaves were weighed so that extracts of equal concentration could be created. Each plant extract had a concentration of 0.025 g/mL (in deionized water). After 24 hours, the plant leaves and roots were strained out of the extract, and the extract was stored in the refrigerator for the remainder of the experiment.

Waxworm larvae were kept refrigerated at 8 °C for approximately 5 days before the experiment, and at room temperature (21°C) throughout the experiment. I filled 100 clear Wheaton straight sided glass jars (7 cm H x 6 cm W) halfway with soil collected from Reist Sanctuary. The soil was sieved through 2mm openings to remove any sticks, rocks, or other debris. There were five sets of conditions; 20 jars filled with garlic mustard soil and 'watered' with garlic mustard extract, 20 jars filled with garlic mustard soil and 'watered' with Virginia creeper extract, 20 jars filled with control soil and 'watered' with garlic mustard extract, 20 jars filled with control soil and 'watered' with Virginia creeper extract, and 20 jars filled with control soil and 'watered' with deionized water. Soil was collected from the same sites the leaves were collected from, however the control soil was not necessarily collected from Virginia creeper areas, so the experiment was not completely balanced. Following the protocol of Keesing et al. (2012), and adjusting calculations according to the volume of the jars I used, I placed 5 healthy looking and active waxworms in each jar and added 1 mL of deionized water to each jar on the first day, day 0, to initiate moisture and humidity in the jars. The jars were covered with cheesecloth secured by a rubber band and the lids of the jars were gently placed on top to discourage waxworm escape. Every day, the jars were rotated so that the waxworms were exposed to all areas of the soil in the jar. Every two days, starting on day 0, 1 mL of plant extract was added to the jars, and every two days, starting on day 1, the number of waxworms alive was counted and recorded. Dead waxworms were immediately disposed of. The experiment ran for 15 days, where on day 15 any waxworm that remained alive could be considered to be a 'survivor'.

Dragging Experiment

To measure the abundance of blacklegged ticks found among garlic mustard and native herbaceous plant species, field studies were conducted between April and June, 2016. Field sites included Reist Sanctuary, located on the Schenectady-Niskayuna border in New York, the Albany Pine Bush Preserve in Albany, New York, and DiCaprio Park in Schenectady, New York. One hundred square meter plot 'sub-sites' were constructed at each location. Sub-sites were selected based on density of garlic mustard and native herbivorous plants, so that sites with high density of garlic mustard, medium density, and low density could be paired with plots high, medium, and low in density of native herbaceous plants. Once sub-sites were determined and plots were measured and marked off with flags, I estimated percent ground coverage of garlic mustard, native herbaceous plants, *Lonicera*, and native shrubs. I additionally recorded the location coordinates, date, time, weather conditions, canopy coverage, and amount of woody debris on the ground.

Two methods of tick collection were used in the study, dragging/flagging (Morlando et al., 2012) and tick traps (Solberg, 1992). The cloths were dragged in one-minute intervals for ten minutes, and in between each interval the cloths were inspected for ticks. All ticks found were recorded and categorized by life stage. For the first tick found in each site, the minute interval it was found in was recorded. The second tick collection method involved the use of tick traps. Dry ice-baited tick traps were modeled after the basic set-up of the tick traps constructed by Solberg (1992). Once the traps were set in the field, they were left for 24 hours. After retrieval of the traps, the area they were placed in was further scanned for ticks using the dragging/flagging method.

Results

Tick Collection

One-way regression analyses were conducted to evaluate which plot-type yielded the most ticks (Figure 1). It was more difficult than expected to find large patches of *A. petiolata* that were in areas of appropriate tick habitat, e.g. not on edges and rather in closed canopy sites. In plots that used the dragging method for tick collection (Figure 1 A-C), there was no significant correlation between the number of ticks collected and the % abundance of *A. petiolata*, % abundance of other herbs, or % abundance of total herbs ($P>0.05$). Similarly, in the plots where CO_2 traps were placed and subsequent dragging occurred (Figure 1 D), there was no significant correlation between the number of ticks found and the % abundance of *A. petiolata* ($P>0.05$).

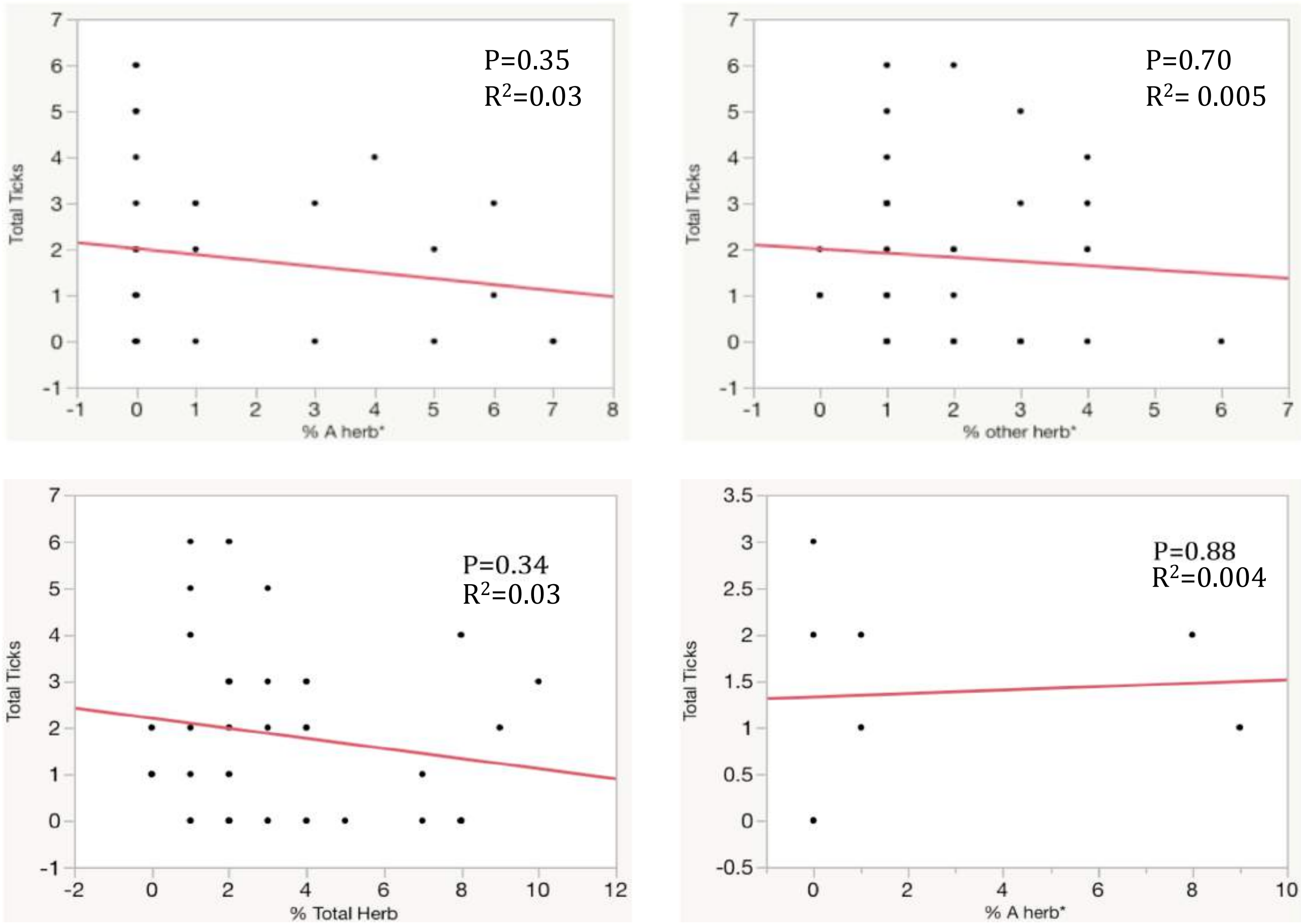

Figure 1. Total number of ticks found among drag plots containing varying densities of *Alliaria petiolata* (A.), other herbaceous plants (B), and *Alliaria petiolata* and other herbaceous plants (C). Data were collected in Reist Sanctuary, the Albany Pine Bush Preserve, and DiCaprio Park in New York. (D) Shows total number of ticks collected via both CO_2 traps and dragging at sites containing various densities of *Alliaria petiolata*. These data were collected in Reist Sanctuary, the Albany Pine Bush Preserve, and Woodlawn Sportsman Club in New York.

Waxworm Survival

We *a priori* chose day 11 for analysis of data via a two-way ANOVA with soil type (*Alliaria* or control) and extract type (*Alliaria* or Virginia creeper) as the main effects on waxworm survival because very few waxworms survived to the end of the experiment. The 2-way ANOVA (Table 1) reveals that soil type is the only significant variable on the mean number of waxworms alive on day 11 of the study (Figures 2 and 3; P value for soil <0.0001). The number of waxworms alive on day 11 of the study in control conditions was not significantly different from the number of waxworms alive in Virginia creeper water and control soil, but was significantly different than every other treatment.

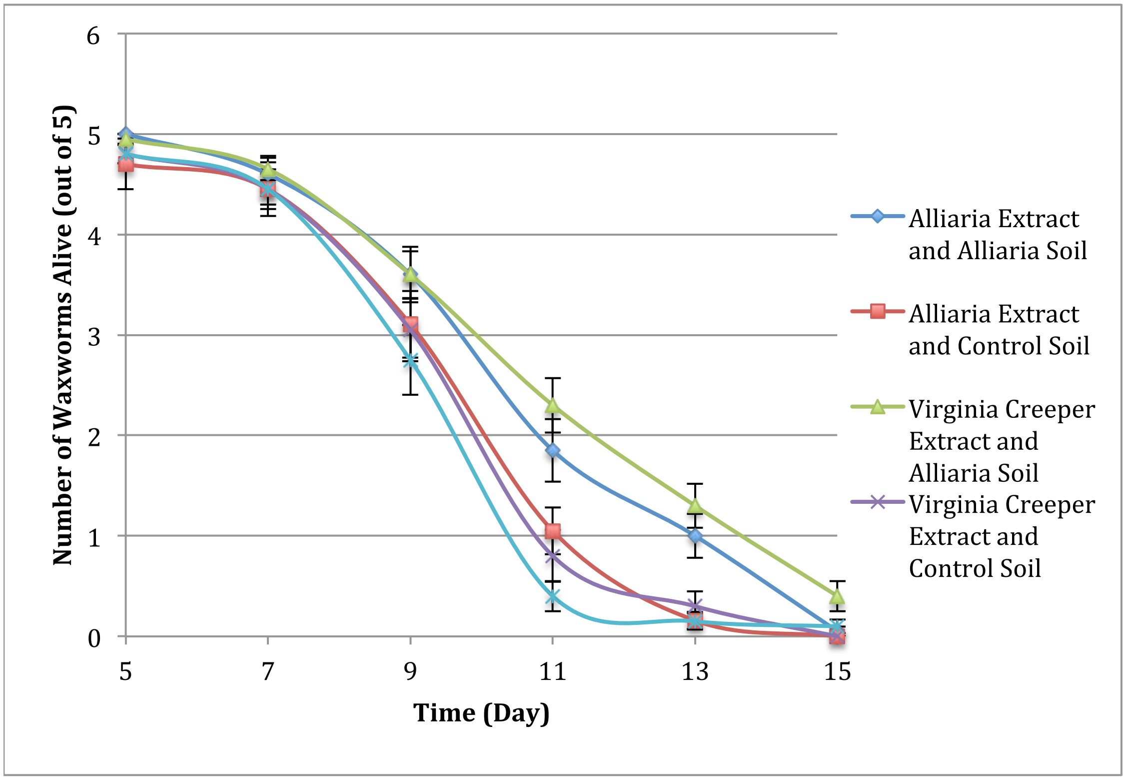

Figure 2. Mean number of waxworms alive on day 11 in response to four treatments of varying soil and water conditions. Each treatment contained 20 jars. Vertical bars are +/- 1 SE.

Table 1. Two-way ANOVA on factors affecting the survival of waxworms on day 11

Source of Variation	df	MS	*F*
Intercept	3	9.70	6.65***
Soil	1	26.45	18.13***
Extract	1	0.20	0.14^{ns}
Extract*Soil	1	2.45	1.68^{ns}
Error	76	1.46	

***= P<0.001; ns= nonsignificant

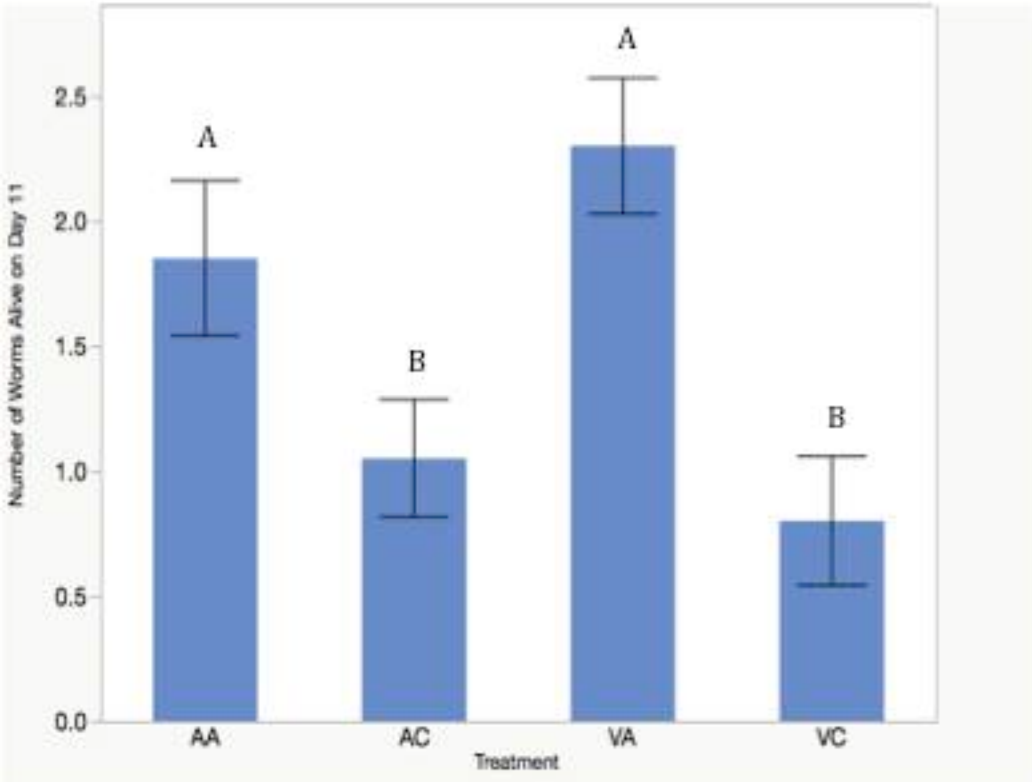

Figure 3. Mean number of waxworms alive on day 11 in varying treatment conditions. The treatment conditions are *Alliaria* extract and *Alliaria* soil (AA), *Alliaria* extract and control soil (AC), Virginia creeper extract and *Alliaria* soil (VA), and Virginia creeper extract and Control soil (VC). Bars not connected by the same letter are significantly different. Error bars are +/- 1 SE.

Discussion

The tick collection and waxworm survival experiments yielded inconsistent results regarding the effects of garlic mustard on tick abundance. Since entomopathogenic fungi are deleterious to tick survival, it was expected that any adverse effect of garlic mustard on fungi would result in increased tick survival, and therefore a greater abundance of ticks found among garlic mustard. The lab study using waxworms as a bioassay for entomopathogenic fungi was consistent with this hypothesis, as it showed garlic mustard soil to have a significant positive effect on waxworm survival. However, these results were not consistent with the field data. In the field there was no correlation between the number of ticks found and the abundance of garlic mustard. Therefore, the data does not support my original hypothesis, but rather supports the null hypothesis that there is no significant difference between the abundance of ticks found among garlic mustard and the abundance of ticks found among other herbaceous plant species.

The results of the waxworm experiment suggest that garlic mustard may still have the potential to reduce tick abundance in the field, even though the field results did not reflect this. The lack of a significant effect of garlic mustard extract in the lab is corroborated by the absence of relationship between garlic mustard and tick abundance in the field. Field studies have shown that the effect of garlic mustard on plants is also inconsistent**.** Garlic mustard leaf extract has been seen to affect germination of certain plant species while root extract had a greater impact on others (Cipollini, 2016). Even the impact of garlic mustard on germination of the same plant species has varied from one study to another (Cipollini and Greenawalt, 2016). In a lab study, garlic mustard extract hindered pale touch-me-not, *Impatiens pallida,* seed survival and growth. When extracts were applied at lower concentrations that are more comparable to those found in the field, however, there was no apparent impact of the extract on its growth (Barto and Cipollini, 2009). Due to the results of these and other studies, the impact of garlic mustard allelopathy in nature is uncertain.

Garlic mustard allelopathy is dependent on the substances the plant releases. Garlic mustard has been found to possess 44 volatile sulfur-containing compounds, which include glucosinolate and cyanide derivatives (Blazevic and Mastelic, 2008). The effectiveness of these compounds as allelopathic agents may be dependent on when and where garlic mustard is being studied. These compounds, specifically the glucosinolate compounds, were found to decline as garlic mustard populations grew older and became better established (Lankau et al., 2009). Because plant enzymes degrade glucosinolates quickly, glucosinolates must be able to work well in small doses in order to be effective allelopathic agents (Cipollini, 2016). Other microbes that inhibit the ability of garlic mustard to flourish may also interfere with garlic mustard allelopathy**.** Therefore, the location and age of the garlic mustard populations might have played a role in the discontinuity of results (Cipollini, 2016).

In addition to this variation of garlic mustard impact seen in the field, there is also variation in the impact of garlic mustard seen between the field and in the lab. The mechanism by which garlic mustard extract is leached into the soil in the environment is intrinsically different from how extract is collected and administered in the soil in lab conditions. Previous published literature regarding the allelopathy of garlic mustard and its relationship with entomopathogenic fungi has been primarily in lab settings, and therefore the findings may not fully carry over into the field setting. Vaicekonyte and Keesing (2012) saw that removing garlic mustard from soil resulted in increased levels of entomopathogenic fungi. My study revealed a relationship that is consistent with their finding, as waxworm survival was increased due to some component of garlic mustard soil (the soil helped to suppress the entomopathogenic fungi likely to kill the wax worms).

The results of field studies reported by Rollins et al. (2006) in their unpublished undergraduate paper revealed that there was no significant difference between the survival of engorged ticks that were placed in the field in plots containing garlic mustard versus those placed in control soil plots. Even when *B. bassiana* was added to both control and garlic mustard soils tick infection rates were not significantly different between the two soil types. Much like my study, their study yielded significant results in regards to waxworm survival. The results revealed that waxworm fungal infection was greater in control soil than in garlic mustard soil. Malcomb et al. (2009) again saw similar results in their unpublished undergraduate research paper, where field studies revealed that garlic mustard plots protected waxworms from fungal infection, but no significant results were found in regards to engorged tick survival. These two papers, as well as the research I conducted, show that it is likely that waxworm survival is not a good model for tick survival, due to the high susceptibility of waxworms to fungal infection compared to ticks. One study saw low fungal spore germination and slow growth of fungi, *B. bassiana* in particular, on nymph blacklegged tick cuticles, which could be due to the many protective layers that a cuticle is comprised of (Flor, 2006). In addition to impeding fungal growth, the cuticle acts as a barrier, making it difficult for the fungi to infiltrate and cause infection and subsequent mortality of the ticks (Flor, 2006). Compared to the thick cuticles of ticks, waxworms are softer and therefore presumably more susceptible to fungal infection.

My results reveal there is a discrepancy between the impact of garlic mustard on waxworm survival in the lab and tick survival in the field. Because there are so many factors that influence garlic mustard allelopathic toxicity, like soil microbes, plant age, density, location, and competition, follow-up studies are necessary (Cipollini, 2016). What is preventing garlic mustard from exhibiting a direct relationship with tick abundance? Keesing et al. (2011) and Vaicekonyte and Keesing (2012) have created the impression that garlic mustard removal would lead to a decline in tick abundance, and therefore would help prevent the spread of tick-borne diseases. Their results were conducted in lab settings and their conclusion, which was made without ever looking in the field, implies that garlic mustard could be a human health risk. I am not aware of any published literature that indicates that there is a higher density of ticks found among garlic mustard plants. My work corroborates the unpublished work of Rollins et al. (2006) and Malcomb et al. (2009), indicating that there is no reason to believe that garlic mustard has an effect on tick survival or on tick-borne disease risk.

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