INVESTIGATIONS INTO THE ONTOGENESIS AND HOSTS OF THE MEADOW CYST NEMATODE, *HETERODERA PRATENSIS,* IN MICHIGAN

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ABSTRACT

In 2018, *Heterodera pratensis* was detected in Michigan in a serendipitous sequencing event; *Heterodera* cysts were identified in a soil sample from a golf course in St. Clair County with morphological data matching that of published measurements and confirmed by the USDA laboratory in Beltsville, MD. This was believed to be the first detection of this nematode in North America.

To better understand the life cycle of *H. pratensis, Poa annua* and *Agrostis stolonifera* were inoculated with second stage juveniles, and plants were destructively sampled 1-155 days post inoculation. Field data were also collected from two greens on an infected golf course in Cheboygan, Michigan. For two years, the population of *H. pratensis* and temperature information were monitored. The total life cycle, from inoculation to cyst formation, in the growth chamber was 70 days on creeping bentgrass and 71 days on annual bluegrass, with no secondary egg hatch, suggesting only a single generation annually. In the field study, J2s had a major hatch in the spring and adults were observed between 60-90 days.

To understand the host range and possible threats to small grain producers and other turfgrasses, 17 grass species were screened. Nine of 17 were considered good hosts, 4 were poor hosts, and 4 were non-hosts, based on the finial population-initial population reproduction ratio (P_f/P_i). *H. pratensis* developed on wheat and barley, which were not previously noted as hosts.

This thesis is dedicated to my daughters and husband.
Katelyn, who is old enough to remember,
Zoe, who is young enough to be blissfully unaware,
and Austin, the most supportive partner in the world.

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TABLE OF CONTENTS

LITERATURE REVIEW

Nematodes: An Introduction

Nematodes are the most numerous animals on earth and may be found in almost any ecological niche. They may be separated into two groups: parasitic and free living. Within the parasitic group, there are plant and animal parasites (Chitwood and Chitwood, 1974). Plant parasites may be further categorized into ectoparasites, which only feed from the outside of the plant, migratory endoparasites, which feed and move into and out of foliar or root tissues, and sedentary endoparasites, which enter root tissue and form specialized feeding cells, the final feeding sites for these nematodes (Chitwood and Chitwood, 1974; Evans et al., 1993). The most economically important of the sedentary endoparasitic plant-parasitic nematodes are the root knot (*Meloidogyne* spp.) and cyst nematodes; the latter are the focus of this thesis.

Cyst nematodes are named for the female carcass, or cyst, that encases the eggs. The cyst protects eggs from desiccation, predators, and other threats to the offspring (Perry et al., 2018). Cyst nematodes, obligatory endoparasites, form specialized cells called syncytia. These multinucleated cells, formed from many cells, act as the feeding sites for the cyst nematodes. These nematodes drain nutrients from the host plant until the females complete their life cycle and fall from the roots (Escobar and Fenoll, 2015). These nutrient sink forming parasites are responsible for significant yield losses worldwide.

There are eight genera of cyst nematodes, the most diverse and destructive genus being *Heterodera. (*Perry et al., 2018). This genus can be further broken into seven species groups, all with slightly different morphological, biological, and host characteristics. Nematodes in the *H. schachtii* species group have been known to cause, on average, 30-40% yield loss in soybean and sugar beet, but can cause an upward of 60-100% loss depending on the environmental

conditions. (Lima et al., 2017; Wright et al., 2018; Clark and Hillocks, 2014; University of Minnesota Extension, 2020.) Species in the *H. avenae* species group are similarly damaging, but they feed almost exclusively on monocots, including turfgrasses and small grains (Smiley et al., 2017).

The *H. avenae* species are typically classified as cereal cyst nematodes; 12 known species comprise this species group, 10 of which are in the *H. avenae* species complex, the main nematode being *H. avenae* (Subbotin et al., 2010; Smiley et al., 2017). Originally, several of what are now considered different species, were thought to be different pathotypes of *H. avenae.* Many of these were distinguished using lines of resistance in wheat, oats, and barley (Anderson 1959, 1961; Cotton 1962, 1963; Fiddian and Kimber, 1964). As more populations of *H. avenae* were investigated, and pathotype screening became more complex; there was suspicion of different unidentified species rather than simply different pathotypes.

Cook and Williams (1972) discussed pathotype and morphometric differences across and within geographic regions (Cook, 1975) several of which have now been resolved into new species within the *H. avenae* species complex (Subbotin, 2018) or rediscovered as populations of *H. filipjevi* (Smiley et al., 2017). Five species have been added to the species complex since the early 1990s, some significant pathogens of grains, and others only found on grasses, and not reported to parasitize grains. The Australian and Chinese cereal cyst nematode (CCN), *H. australis* and *H. sturhani,* are two species found to infect grains (Smiley et al., 2017). There is controversy around their new species status; some authors do not believe they are actually distinct from *H. avenae* due to a lack of morphological evidence (Peng et al., 2016; Vanstone et al., 2008). In depth studies are lacking for two other species, *H. aucklandica* and *H. riparia,* since they do not seem to parasitize any significant agricultural grasses (Subbotin, 2010B). The

final species, and the focus of this thesis, *H. pratensis* has not been found to parasitize grains, but is known to attack important cool season turfgrass species.

Nematodes on Cool Season Turfgrasses in North America

Golf courses, lawns, athletic fields, and other turfgrass hold some of the most diverse populations of plant parasitic nematodes. A New York survey from 1978 found 10 genera across 16 golf courses, with most courses having four or more genera (Murdoch et al., 1978), cysts being present in 13% of the samples. Another New York survey in 1991, based on samples submitted to the Cornell Nematode Diagnostic laboratory, found nine genera between 18 samples, 33% of which contained cysts (Hummel and Gruttadaurio (Eds), 1991). A survey from Ontario, by Yu et al. (1998), had very similar results, finding nine different genera but with a lower detection of cysts; they only detected *Heterodera* spp. on one golf course. A 1993 survey from Michigan reported 12 different types of plant parasitic nematodes across 106 samples; cyst nematodes were reported at 10.1% (Warner et al.). An extensive survey done by Zeng et al. in 2012 showed higher diversity across the Carolinas. They detected 22 genera and 29 different species, one of which was an unidentified *Heterodera* species. It appears, turfgrasses support a large diversity of plant-parasitic nematodes.

Few other ‘crops’ provide such a uniquely ideal environment for nematodes as turfgrass. It is a perennial system, with a host under constant stress due to high levels of maintenance, often grown on sand that is frequently watered. For most, if not all golf greens, these conditions allow nematodes to move easily and provide ample roots to parasitize (Crow, 2017; LaMondia et al., 2018). Golf courses are highly maintained to produce a consistent playing surface and to keep the turf appearance acceptable; however, many of the management practices that keep golfers

happy may exacerbate nematode damage, especially during times of high stress (Jordan, 2005; Nelson, 1995; Crow, 2017, 2005a).

Most nematodes that feed on cool season turf are root parasitic that cause turf decline and symptoms such as leaf chlorosis, thinning, or wilting (Smiley et al., 1992). As is generally accepted, on many plant species, the feeding by plant-parasitic nematodes on roots does not result in characteristic secondary (above ground) symptoms. These symptoms are not specific to nematodes let alone any specific species of nematode, so even though the general symptoms of nematode feeding are known, the specific impact of most species of nematodes are not well known. This is especially true for cyst nematodes found in northern climates on cool season turfgrasses (LaMondia et al., 2018; Smiley et al., 1992; Crow, 2005a).

There are two species of cyst nematode commonly found on cool season turfgrasses in the USA, *H. ustinovi (*syn. *H. iri)* and *Punctodera punctata.* These nematodes are thought to cause chlorosis, thinning of the turf, and root stunting (Joseph et al., 2018; Horne, 1965; Radice et al., 1985). Several other species have the potential to cause damage to turfgrasses within the *H. avenae* group of cyst nematodes. *H. mani* and *H. hordecalis* are known to parasitize some species within the genera *Lolium* and *Festuca,* but only *H. mani* exists in the USA, found only in California and the Pacific Northwest (Subbotin et al., 2010; Smiley et al., 2017). Three other species are known to feed on various turf species including *Agrostis* spp. and *Poa* spp. These being *H.filipjevi, H. avenae,* and *H. pratensis.* The former two are known to cause significant damage to grains in the US Pacific Northwest, but little is known regarding their virulence on turfgrass (Subbotin et al., 2010). The final species, *H. pratensis,* was previously only found in the northeastern hemisphere, but like *H. ustinovi*, it was known to parasitize some major turfgrass species.

In 2018, *H. pratensis* was detected in Michigan in a serendipitous sequencing event; *Heterodera* cysts were identified in a soil sample from a golf course in St. Clair County. They were sequenced to confirm species identity of what was thought, at the time, to be *H. ustinovi*. *H. pratensis* was described by Gabler et al. in 2000. What was originally thought to be a population of *H. avenae* was re-examined morphometrically and separated from other closely related species using protein isoelectric focusing (IEF) and rDNA restriction fragment length polymorphism (RFLP). Since *Heterodera pratensis* is a recently described species and was not previously known in North America, there is very little information available. Most of the information contained in this literature review will discuss the impacts of the closely related *Heterodera avenae*.

Pathogenicity and Economics

Michigan has about 10 million acres of farmland with approximately 35,000 acres of oats, 490,000 acres of wheat, 215,000 acres of barley, (Michigan department of agriculture and rural development, 2020), and is reported to have around 1.89 million acres of turfgrass (Michigan Turfgrass Foundation, 2020), with about 10,000 acres of overlap between these, as sod is counted as an agricultural product (United States Department of Agriculture, National Agriculture Statistics Service Michigan Field Office, 2018-2019). Small grains in Michigan are worth 191 million dollars (MDARD, 2020) and the golf industry is said to have a 4.2 billion dollar impact on Michigan's economy annually (Michigan.gov, 2019). These data suggest there is approximately 2.63 million acres of food for cereal cyst nematodes (CCN), and the economic impact is unknown. There are little data on CCN in Michigan, most pathogenicity studies are from Australia and the Pacific Northwest (PNW).

In 1983, *H. avenae* was detected in Michigan, and in 1986 Burnett reported that there was a reduction in the dry weight and yield on spring wheat and oats. Barley appeared less impacted but did support development. In China, *H. sturhani*, closer to *H. pratensis* biologically than *H. avenae,* is reported to reduce wheat yields between 10-40% (Peng et al., 2007, 2016). Similar yield loss, caused by *H. avenae*, has been reported in the PNW as Smiley et al. (1994) reported up to a 50% yield reduction in annual winter wheat. They observed fasciculation (witches brooms) of roots. In 2005, they showed yield reductions in spring wheat, again annually planted, showing more symptoms than wheat in a rotation. It was observed that the younger the wheat plants were when the nematodes infected during the spring, the greater the negative impact of the nematodes. Reductions in root weight and shoot weight have also been reported to be caused by *H. filipjevi* and *H. avenae* on various varieties of susceptible and tolerant spring wheat (Smiley and Yan, 2015). Spring barely is susceptible to root damage and yield loss but seems to tolerate nematode feeding better (Marshall and Smiley, 2016).

Life Cycle of Cereal Cyst Nematodes

Cereal cyst nematodes in the *H. avenae* complex are reported to have similar life cycles (Nicol and Rivoal, 2008; Smiley et al., 2017). They produce a single generation a year from egg hatch to cyst formation. The eggs overwinter in cysts. Second stage juveniles (J2s) emerge from eggs in two distinct patterns, depending on ecotype (Rivoal and Cook, 1993). In temperate regions, when soil temperatures begin to rise in spring, the majority of J2s hatch from their eggs, but in Mediterranean climates, they hatch in late fall to early spring (Rivoal and Cook, 1993). The Mediterranean ecotype appears to enter dormancy in the summer and requires a period of hot, dry weather followed by cool, wet weather (Jing et al., 2014; Rivoal, 1986) to break it. The temperate ecotype may have a more optional diapause, with some egg hatch in the cool falls and

most of the egg hatch in the wet springs, as the temperatures start rising after the winter. The highest percentage of hatch is usually obtained after a cold period (Smiley et al., 2005; Williams and Beane, 1979). The northern/temperate pathotype of *H. avenae* is present in the PNW and Michigan, and *H. pratensis* appears to follow the same hatching pattern (Albrecht et al., 2007; Burnett, 1986).

Like other cyst species, the first molt takes place within the eggshell. Once soil temperatures are between 2 and 20°C, the J2 hatches and begins searching for roots to parasitize (Albrecht et al., 2007; Jing et al., 2014; Williams and Beane, 1979). While in the soil, the J2 swims through the thin film of water surrounding soil particles, using chemotaxis to locate potential host plant roots to feed on. Once located, the J2 uses its stylet to penetrate the root cells and enters the cell to search for a suitable feeding site, where it signals the plant to initiate syncytial formation. Once the site is established, the nematode begins to grow and molt once again, entering the third stage juvenile (J3) swollen stage. In the next stage, fourth stage juvenile (J4), the females and males are distinguishable within the root system. Syncytia of the males begin to atrophy, but the female's syncytia continue to sustain them (Toumi, 2018; Bohlmann, 2015).

Adult males will shed their J4 cuticle, then exit from the root, where they will begin their search for females to mate. Females, on the other hand, will continue to feed and swell, until they rupture the plant's epidermal cells. Once they erupt through the root exterior, the males are able to mate them, and fertilized egg production may begin. Females use their feeding sites until the plant dies or their bodies are completely full of eggs. When this happens, the white females will die, their bodies turning brown and falling from the root. The eggs inside her cyst lie dormant until the next growing season when soil temperature and/or moisture will break the dormancy

and the cycle repeats (Rivoal, 1986; Smiley et al., 2005).

The time it takes a female to complete its life cycle is largely determined by environmental factors, mostly temperature. Lower temperatures increase the duration of the life cycle and higher temperatures shorten it (Banyer and Fisher, 1971; Jing et al., 2014). Wu et al. (2014) reported that it took females between 83-99 days to fully develop in the roots of winter wheat in China. Reports from the Slovak republic have some *H. avenae* life cycles as short as 56 days on spring oats and 63- 84 days in winter wheat (Smiley et al., 2017; Sabová et al., 1985).

Known Host Range of *H. pratensis* and *H. avenae*

H. avenae has a long list of reported hosts; most of these overlap with those of *H. pratensis*. The main noted distinction is that European and Asian populations of *H. pratensis* are only known to infect non-agricultural/non-grain grasses, and this is used to separate it from *H. sturhani,* which mainly infects cereals. An attempt was made to infect barley and wheat with *H. pratensis*, since it has been found in a barley field and is noted to be associated with small grains (Smiley and Nicol, 2009; Gäbler et al., 2000). That attempt failed, and it seems to be considered insignificant on those crops. For comparison, *H. avenae* is a major pathogen of cereals, mainly wheat, oats, and barley, and is known to parasitize and sustain itself between grain crops on non-grain, typically weedy grasses (Smiley et al., 1994, 2005; Gill and Swarup, 1971). The known hosts of both species are listed below (**Table 1)**.

Table 1: Hosts of *Heterodera pratensis* and *H. avenae* (Gäbler et al., 2000; Gill and Swarup, 1971; Smiley et al., 2017; Subbotin et al., 2010B). Genera containing multiple host species listed without species name.
* Indicates shared host species with *H. pratensis*

Hosts of *Heterodera pratensis*	**Hosts of *Heterodera avenae***			
Agrostis stolonifera	*Agropyron*	*Bromus*	*Phalaris*	*Sorghum*
Cynosurus cristatus	*Agrostis**	*Dactylis**	*Phleum*	*Trisetum*
Dactylis glomerate	*Alopecurus*	*Echinochloa*	*Poa**	*Triticum*
Poa annua	*Anisantha*	*Festuca**	*Polypogon*	*Vulpia*
Elyumus repens	*Arrhenatherum*	*Hordeum*	*Secale*	*Zerna*
Festuca pratensis	*Avena*	*Koeleria*	*Senebiera pinnatifida*	*Zea*
Lolium perenne	*Brachypodium*	*Lolium**	*Setaria*	

Nematode Control in Turfgrass

Since European populations of *H. pratensis* are not known to infect important grain crops, no work has been done to evaluate current resistance genes against it. Furthermore, little has been done on turfgrass resistance to nematode feeding, especially for cyst nematodes. In 2011, different bermudagrass cultivars were evaluated for sting nematode resistance (Pang et al.), and they found that some were more tolerant to root feeding than others. Likewise, some research has been done on Kentucky bluegrass tolerance (Nelson, 1995) against the lesion nematode, *Pratylenchus neglectus*. *Poa annua* seems to either be more attractive than some *Agrostis* sp. or more susceptible (Jordan, 2005). Typically, nematode control is recommended before the turf is established, either by planting less susceptible turf species, using non-infected sod (Kirckpatrick; Nelson, 1995), or by fumigating (Bird, 1988; Crow, 2014). On most push up-greens, fumigation is not possible or is ineffective long term, and complete renovation is expensive (LaMondia, 2018; Crow, 2014). Tolerance and resistance may be a future option for nematode and cyst management, but little has been published at this point.

There are several nematicides labeled for turfgrass use in the United States that, if applied at the right time, may prove effective in the control of *H. pratensis.* Curfew (Corteva Agriscience, Indianapolis, IN) is a soil fumigate with an active ingredient of 1,3-Dichloropropene(1,3-D). It kills nematodes free in the soil and is a contact nematicide (Crow, 2014) that is not labeled in Michigan but is labeled for use on cyst nematodes. Indemnify (Bayer Crop Science US, Harrisburg, SD) is another broad spectrum nematicide labeled for turfgrass with an active ingredient of Fluopyram; it is not labeled specifically for cyst nematodes. Although it has some systemic properties, it has the most impact on nematodes in the soil, and cyst nematodes in plant roots will not be impacted by this nematicide (Waldo, 2019). However, J2s moving in the soil may be killed, which may reduce the initial levels of nematodes invading root tissue in the beginning of the year. Nimitz Pro G (Adama Agricultural Solutions, Raleigh, NC), active ingredient of Fluensulfone, is labeled as a systemic and contact nematicide. It seems to show some efficacy in Florida but is not labeled specifically for cyst nematodes (Crow, 2017). Divanem (Syngenta Crop Protection AG, Basel**,** Switzerland), active ingredient Abamectin, is a contact nematicide. It seems to remain in the thatch layer, not impacting soil nematodes much. During the hatch of root knot J2s, Divanem seems to be partly effective, which means it may be effective against cyst nematode J2s as well, even though it is not specifically labeled for them (Crow, 2017).

Cereal Cyst Nematode Control in Grains

For *H. avenae* infection in grains, resistance and crop rotation are the main tools for management. In 1959, Andersen tested four *Hordeum vulgare,* barley, varieties and two *Avena sativa,* oat, varieties on 99 populations of *H. avenae.* They found no single variety resistant to all populations. They found that when oat was resistant, it appeared to have more complete

resistance across populations than that of barley. Now, 19 genes have been identified and shown to provide resistance to *H. avenae* and other CCN. Eleven cereal cyst nematode resistance (Cre) genes are in wheat, Cre 1 being the most effective in Australia and the PNW (Smiley et al., 2011; Karelov et al., 2019). It should be noted that most of the Cre genes come from wild relatives of wheat and not specifically cultivated wheat. Five *H. avenae* resistance (Rha) genes are found in barley, and Rha 2 and 3 seem to be effective against PNW populations (Smiley et al., 2011). Finally, three resistance genes are in oats, but they are not named.

Variations in effectiveness of resistance is important to consider when breeding local cultivars. Even though a variety is resistant to Australian CCNs, the resistance may not be effective in the PNW. Smiley et al. (2005) found plants with the Cre1 and Cre 8 genes to be susceptible to the Oregon CCN populations tested, although they were resistant in Australia. Conversely, in 2011 they found cultivars containing Cre1 genes to be the most effective form of resistance in wheat. This suggests that not all cultivars will have the same level of resistance even though the same genes may be present. Two species were later identified in the PNW which may help to explain the differences in resistance (Pariyar, 2016; Smiley and Yan, 2015).

In infested fields, crop rotations to a non-host or a poor host are effective in boosting wheat yields in subsequent years (Smiley et al., 1994, 2005). Significant population declines in *H. avenae* have been reported under non-host and resistant crops, 42 to 80 percent decrease recorded in CCN numbers in a single year (Rivoal and Cook, 1993). Although this can help boost yields with minimal rotation in annual cropping systems, this strategy is not as useful for most golf courses since turfgrasses are perennial crops, and rotation is cost prohibitive. Overseeding with less susceptible varieties could be a management option, if varieties are identified and become available. In the southern US overseeding has shown to increase the

number of sting nematodes on greens (Crow et al., 2005.) and grains in the earlier growth stages have been found to be more susceptible to feeding (Smiley et al., 2005). Any attempts to include rotations and overseeding as a nematode management strategy must be carefully considered in the overall management plan for each golf course, lawn, and sports field, so as not to cause more damage than good.

Although there are some potential control techniques for CCN in grains and non-grain grasses, the lack of biological data in the literature for specific nematode species makes it difficult to make targeted strategies for control. Investigating the life cycle and host of range of *H. pratensis* can lay the groundwork for future research into controlling this nematode. These studies can also reveal more of the differences and similarities to other populations of *H. pratensis* and other species in the *H. avenae* species complex.

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RESERARCH OBJECTIVES

1) To monitor the life cycle and developmental rate of *Heterodera pratensis* on creeping bentgrass (*Agrostis stolonifera*) and annual bluegrass (*Poa annua* var. *annua*) in a controlled environment and compare it to the field life cycle over two years.
2) To identify a potential time frame for *Heterodera pratensis* control on cool season turf.
3) To investigate the host range of the Michigan population of *Heterodera pratensis*.

CHAPTER 1:
LIFE CYCLE OF *HETERODERA PRATENSIS*, THE MEADOW CYST NEMATODE, ON CREEPING BENTGRASS (*AGROSTIS STOLONIFERA)* AND ANNUAL BLUEGRASS (*POA ANNUA* VAR. *ANNUA)* UNDER GROWTH CHAMBER AND FIELD CONDITIONS

Abstract

Little is known about the specific life cycles of the different nematodes within the *Heterodera avenae* species complex, especially the species that only infect non-grain grasses like *Heterodera pratensis*. In turf, nematicides may be utilized throughout the growing season, but knowing the life cycle of specific nematodes can allow for targeted pesticide applications during the most vulnerable points in their life cycles, decreasing the amount of nematicides used and saving money.

To better understand the life cycle of *H. pratensis,* month old seedlings of creeping bentgrass (CBG), *Agrostis stolonifera* 'Declaration' and field-collected annual bluegrass (ABG), *Poa annua* var. *annua* were inoculated with 148-339 newly hatched second stage juveniles (J2s). One seedling was used per container and plants were destructively sampled daily starting one day post inoculation (DPI) until 96 DPI. Samples were collected once every 5 days from 96 to 121 DPI. A final sampling point was taken between 147-155 DPI. Field data were also collected from two putting greens on a *H. pratensis* infected golf course in northern Michigan. For two years, the population of *H. pratensis* was tracked from early May until late July.

The life cycle, from inoculation to cyst formation, in the growth chamber, was 70 days in CBG and 71 days in ABG, with no secondary egg hatch, suggesting only a single yearly generation. In the field study, J2s had a major hatch in the spring, then followed a similar life cycle to the growth chamber experiment.

Introduction

The *Heterodera avenae* species complex is a closely related group of 10 cyst nematode species, all with slight morphological differences. *H. avenae* and *H. filipjevi* are the most economically important species found in the United States. In the Pacific Northwest (PNW) yield loss caused by *H. avenae* in heavily infested fields can be as high as 50% in continuous wheat rotations (Smiley, et al. 1994, 2005). The average loss due to *Heterodera* spp. in the PNW is estimated around 10% (Smiley, et al., 2009). Three other species in the *H. avenae* species complex, *H. pratensis, H. ustinovi,* and *H. mani*, are found in the United States on turfgrass (Subbotin, et al., 2010; Smiley et al., 2017). Less is known about these species' biology, hosts, and damage potential. This is especially true for *H. pratensis* which, in 2018, was detected for the first time in North America on a golf course in Michigan (Stouffer-Hopkins et al., 2018).

The life cycle of *H. pratensis* is currently extrapolated from its close relative *H. avenae*, with a single generation per year (Subbotin, et al., 2010; Smiley et al., 2017). For species in the *H. avenae* group, eggs are in the soil of infected fields. Second stage juveniles (J2s) hatch from eggs and search out roots to infect. The J2s enter the roots, find suitable cells to establish feeding sites, and begin to feed. J2s are vermiform, in the *H. pratensis* type population the documented average length of the J2s body is 535.0 (490-575) µm. J2s from the Michigan population are 496.0 (460-530) µm (Gabler et al., 2000; Handoo, 2002; Stouffer-Hopkins et al., 2018).

The third and fourth stage juveniles appear as swollen worms within the root tissue. It is at this point females and males diverge in appearance. Males will shed their swollen juvenile skin and emerge as long vermiform worms, exiting the root tissue altogether. *H. pratensis* males from the type population were 1170.0 (1050-1330) µm, on average. Females will continue to swell, eventually partly erupting from the root tissue. Their white bodies are visible with the

naked eye on the root exterior. They fill their bodies with eggs, eventually hardening their cuticle and dying, turning into a cyst. Cysts of *H. pratensis* are lemon shaped, no longer resembling the worm shape of the J2s. From the type-population the average length of the cysts was 675.0 (530-800) µm with a width of 570.0 (400-685) µm. From the Michigan population the average length of a cyst was 643.6 (570-710) µm, with a width of 553.0 (500-630) µm (Gabler et al., 2000; Handoo, 2002; Stouffer-Hopkins et al., 2018). After egg production, the life cycle is complete and the brown cyst will help protect the eggs until they can hatch. Although the life cycle is similar, different cyst nematodes in the *Heterodera avenae* species complex have different host ranges, temperature requirements, and life cycle durations.

Other than what is known about the genus *Heterodera*, little has been reported about the specific life cycle of *H. pratensis*. Different species, and even different populations within the same species, may have varied life cycles depending on host and environmental conditions. The life cycle of *H. avenae* in China has been reported from 83-99 days, but in Slovakia from 56-84 days due to variations in temperature and moisture during early infection and development (Wu et al., 2014; Sabová et al., 1985). The benefit of knowing specifics about a species life cycle is the ability to target different life stages with pesticides. This can help when forming control strategies and mitigating damage to host plants.

The first objective of this study was to investigate the length of the life cycle and the various life stages of *H. pratensis* on creeping bentgrass and annual bluegrass under spring simulated spring temperatures in a controlled environment. The second objective was to observe *H. pratensis* in a field setting, over a two-year period, and monitor the life stages across seasonal and temperature changes.

Materials and Methods

Seed Preparation

Creeping bentgrass, *Agrostis stolonifera* 'Declaration' and annual bluegrass, *Poa annua var. annua,* were the chosen hosts for this study. The annual bluegrass seed was collected from an annual bluegrass green at the Michigan State University Hancock Turfgrass Research Center, East Lansing MI. Clippings were collected in a mower bag in the spring during seed fill, dried for two months, and then seeds were separated from grass blades using a South Dakota Seed Blower (Seedburo Equipment Company, Des Plaines, IL). Both grasses were seeded into a sand and vermiculite mixture and grown in trays for a month. The seedlings were then transplanted into SC(10) Ray Leach "Super Cell" UV cone-tainers (Stuewe and Sons., Inc., Tangent, OR) filled with autoclaved topdressing sand. One plant per cone-tainer was allowed to establish for two weeks before inoculation. Seedlings that died were replaced before inoculation.

Inoculum Preparation

Soil samples were collected from two infected greens at a golf course near Cheboygan, Michigan. They were kept at 2 ºC for 2-3 months to help break dormancy (Williams and Beane, 1979). Cysts were extracted from the soil using a modified centrifugation-flotation technique (Jenkins, 1964). Approximately 2.5 gallons of soil were emptied into a 10L bucket filled with 7L of water. The soil was stirred by hand in the water until no soil clumps were felt. Once the soil had been mixed it was poured over a VWR 8" Stainless Steel Test Sieve USA series # 16 nested over a USA series # 100 sieve (VWR International. Radnor, PA). The top sieve was removed, and the contents of the bottom sieve were transferred into eight 100 mL centrifuge tubes. These were placed and spun in a Block Scientific centrifuge at 1750 rpm for four minutes (Block Scientific, Bellport, NY). After four minutes the tubes were removed and the water decanted, so

only the soil pellets at the bottom remained. Then, approximately 80 mL of a 1.25 g/mL sucrose solution was poured into each tube. The soil pellets were resuspended into the solution and the samples were placed back into the centrifuge and spun for two additional minutes at 1750 rpm. The tubes were removed from the centrifuge and the solution was decanted over a VWR 8" Stainless Steel Test Sieve USA series # 400 (VWR International. Radnor, PA). The samples were rinsed in the sieve with running water for 10 seconds then poured into sterile specimen collection cup and stored at 2 ºC until used for inoculum.

Cysts were removed from storage and placed in 15 mL of tap water in a Falcon 60x15mm sterile petri dish (Corning Inc., Corning, NY) and left on a lab bench, at 70 ºF for 3-7 days, to allow second stage juveniles (J2s) to hatch. The J2s were moved to a sterile specimen collection cup using a syringe. Using a dissecting microscope at 40x magnification, a 2 mL aliquot from each cup was examined and specimens enumerated, to estimate the initial density of nematodes per cup. At the base of the transplanted seedlings, 1 mL of water, containing 148-339 J2s, were syringed into the soil of each cone-tainer. Plants were lightly watered with approximately 30 ml before and after inoculation to prevent nematode desiccation.

Experimental Set Up and Soil and Root Analysis

The growth chamber utilized was set at 21 ºC for 14 hours and 18 ºC for 10 hours daily, with a 14-hour photoperiod. Starting one day post inoculation (DPI), four plants per grass species were destructively sampled for 96 consecutive days, then once every five days until 121 DPI, with a final sampling point between 147-155 DPI. During the final sampling point, the average number of cysts per plant, offspring, eggs and J2s per cyst, and the overall reproductive value were calculated. For each sampling point, root and top lengths and weights were measured. For maintenance, the grasses were trimmed every 35 days.

Plants were destructively sampled at each time point and using a modified centrifugation-flotation method (Jenkins, 1964) the nematodes were extracted from the soil. Cone-tainers containing approximately 100cc of soil were emptied into a 10L bucket filled with 7L of water. The soil was stirred by hand in the water until no soil clumps were felt. For samples processed 1-35 DPI the roots, from the cone-tainers, were rinsed and removed from the water with minor agitation and set aside for plant measurements and root staining. For samples processed 35-155 DPI the roots were rinsed in the bucket, then the outer root surfaces were rubbed by hand (root scrubbing) to encourage females for come off in the water. The plants were set aside for additional measurements. Once the soil had been mixed and the roots scrubbed the soil was poured over a VWR 8" Stainless Steel Test Sieve USA series # 16 nested over a USA series # 400 sieve (VWR International. Radnor, PA). The top sieve was removed, and the contents of the bottom sieve were transferred into a 100 mL centrifuge tube. This was spun in a Block Scientific centrifuge at 1750 rpm for four minutes (Block Scientific, Bellport, NY). After four minutes the tube was removed and the water decanted, so only the soil pellet at the bottom remained. Then, approximately 80 mL of a 1.25 g/mL sucrose solution was poured into the test tube. The soil pellet was resuspended into the solution and the sample was placed back into the centrifuge and spun for two minutes at 1750 rpm. The tubes were removed from the centrifuge after two minutes and the solution was decanted over a VWR 8" Stainless Steel Test Sieve USA series # 400 (VWR International. Radnor, PA). The sample was rinsed in the sieve with running water for 10 seconds then poured into a 10 mL Pyrex test tube (Corning Inc. Corning, NY). Samples were stored at 2 ^{o} C until counted.

Roots were scrubbed at 35 DPI to remove female nematodes during soil washing. Nematode life stages were identified and counted under a dissecting microscope at 40x

magnification. Second stage juveniles were extracted from the soil and counted until 10 DPI. After 10 days in the soil, most of the remaining J2s from the initial inoculum were dead, likely due to starvation, and were too decayed to identify clearly, thus were omitted from enumeration. Swollen juveniles[1], males, and females were counted in the soil between 1 DPI and 50 DPI where after only females and cysts were enumerated. For the final destructive sampling, the remaining samples were processed, the cysts and females were counted then crushed and the fecundity measured by counting the eggs and J2s within them.

Roots were processed using a Sodium-Hypochlorite-Acid-Fuchsin Method (Byrd et al., 1983). Roots were rinsed during the soil washing process then cleared in a diluted bleach solution, so nematodes could be visualized through the cell walls. Plants 1-15 DPI were placed in a 4:1 water to bleach solution for four minutes, and plants 16 DPI and older were placed in a 2:1 water to bleach solution for four minutes. Plants were removed from the bleach after four minutes and rinsed in water for 45 seconds, to remove any remaining bleach. They were then placed into a beaker with 40 mL of tap water, and 1 ml of stock acid-fuchsin-stain solution was added, to stain the nematodes in the roots. The beaker was placed onto a hot plate and the roots and solution were boiled for 30 seconds. After 30 seconds of boiling, the roots were removed and rinsed in tap water for 30 seconds to remove excess stain. These were patted dry and placed into 30 ml of acidified glycerin, to de-stain the plant cells while leaving the nematodes stained. These were brought to a boil on the hot plate. Once boiled the roots were placed into a Falcon 60x15mm sterile petri dish (Corning Inc., Corning, NY) with enough glycerin to cover them and stored at 70 ^{o} F until analyzed.

[1] Normally, swollen juveniles would not be found outside the root. However, due to how fine and thin the roots of these grass species were swollen juveniles were found in the soil during destructive sampling and counted.

Once processed the roots were sandwiched into one layer between the top and bottom of an inverted 150 x 20mm VWR glass petri dish (VWR International, Radnor, PA.), and the stained nematodes were counted. All life stages were counted in the roots. After 50 DPI, the soil procedure was sufficient to collect most of the nematode data, as very few nematodes were detectable in the root tissue, and root analysis was stopped.

Seasonal Tracking

For two years, a population of *H. pratensis* was tracked from early May until late July on two putting greens from a single golf course in northern Michigan. Soil temperature data was taken in 2017- 2019 from two Michigan State University (MSU) weather stations, one in Gaylord, and one in Petoskey, which were then averaged to estimate soil temperatures at the sampling site (Enviroweather, 2017-2019, Soilconditions). Air temperature was taken from two MSU weather, one in Gaylord and one in Hawkes, which were then averaged to estimate air temperature at the sampling site stations (Enviroweather, 2017-2019, Weathersummary). Soil samples were collected and shipped overnight by Fed Ex to the Michigan State Plant and Pest Diagnostics Laboratory in East Lansing, MI.

Samples were processed using a modified centrifugation flotation method (Jenkins, 1964). Using a scoop, 100cc of soil was taken from the sample and emptied into a 10L bucket filled with 7L of water. The soil was stirred by hand in the water until no soil clumps were felt. Once the soil had been mixed it was poured over a VWR 8” Stainless Steel Test Sieve USA series # 16 nested over a USA series # 400 sieve (VWR International. Radnor, PA). The top sieve was removed, and the contents of the bottom sieve were transferred into a 100 mL centrifuge tube. This was spun in a Block Scientific centrifuge at 1750 rpm for four minutes (Block Scientific, Bellport, NY). After four minutes the tube was removed and the water

decanted, so only the soil pellet at the bottom remained. Then, approximately 80 mL of a 1.25 g/mL sucrose solution was poured into the test tube. The soil pellet was resuspended into the solution and the sample was placed back into the centrifuge and spun for two minutes at 1750 rpm. The tube was removed from the centrifuge after two minutes and the solution was decanted over a VWR 8" Stainless Steel Test Sieve USA series # 400 (VWR International. Radnor, PA). The sample was rinsed in the sieve with running water for 10 seconds then poured into a 10 mL Pyrex test tube (Corning Inc. Corning, NY) then stored at 2 ºC.

Nematodes were then counted using light microscopy under a dissecting microscope at 40x magnification. All life stages were counted, including J2s free in the soil. Empty cysts were not counted. Females and cysts were crushed using a tissue homogenizer, and the released eggs and J2s were enumerated.

Statistical Analysis and Experimental Design

Growth Chamber

Four replicates each of two grass species were set up in a completely randomized factorial design in a single growth chamber. The data analysis for this paper was generated using SAS software, Version 9.4M2 of the SAS System for Windows. Data were analyzed using the glimmix (GLM) procedure (SAS Institute Inc., 2018, Cary, NC, USA.), followed by LS means to separate significant differences among days and grass species during the life cycle. P values below 0.05 were significant in all analyses. The best fit model of log(x+1) was used to help normalize a Poisson distribution, and each life stage was analyzed. Day and plant species were evaluated for significant differences and interactions. The Corr procedure (SAS Institute Inc., 2018, Cary, NC, USA.) was used to look for correlations between the different nematode life stages and top and root growth parameters. For the final sampling point, only plant species significance was evaluated, a log transformation was used to normalize the data then it was analyzed using the GLM procedure (SAS Institute Inc., 2018, Cary, NC, USA.).

Field Data Analysis

Seven dates were used from 2018 and 2019, with two replicates from each date. Date representation and grouping was used for comparison across years since there was a slight difference in sampling dates. Dates between 1-10th of the month were considered E-Early, 10-20th were M-Mid, and 20-29th were L-Late. The Mixed procedure (SAS Institute Inc., 2018, Cary, NC, USA.) was used to analyze interactions of date and year on five life stages: cysts, egg and J2s (from the crushed cysts and females), free J2s in the soil, males, and white females. The Corr and Reg procedures (SAS Institute Inc., 2018, Cary, NC, USA.) were used to look at correlation and regression fit, respectively, for the life stages and average soil and air temperatures.

Results

Nematode Life Stages and Plant Growth in a Growth Chamber

Although root and top growth weight (above ground biomass; leaf and stem tissue) and length were recorded, no significant correlations were found among any growth parameter or any nematode life stage, inside or outside of the root system.

Significant differences were observed by day for all nematode developmental stages. Juvenile penetration of the roots was significantly different ($P<.05$) between plant species. J2s were observed in annual bluegrass (ABG) roots 1-9 days post inoculation (DPI) and in creeping bentgrass (CBG) 1-14 DPI, and day 29 DPI (**Figure 1)**. Some Syncytia were observed as early as 3 DPI. Swollen juveniles were observed in the roots 8–26 DPI and in the soil 11-28 DPI, with no significant differences between grass species.

Male development overlapped with swollen development, and there were significant differences between plant species. Males were observed at significantly different levels in the roots of CBG 14-28, 31, 35, 36, and 37 DPI. Males were detected in the root tissue of ABG at statistically significant levels from 15-27 DPI (**Figure 2)**. Males were observed in the soil from 21-50 DPI when decay of males was significant, and they were no longer counted. For counts of males in the soil, no significant differences were observed between grass species (data not shown).

White female development was significantly different between ABG and CBG ($P<.05$). Eggs were detected as early as 26 DPI in white females. In CBG roots, white females were observed at 15-41 DPI, except for 36 and 37 DPI **(Figure 3A)**. In ABG Statistically significant development was observed mostly between 22-36 DPI, although it was sporadic in that range; development on 17, 22, 24, 26, 29-31, 33, 35-36, and 42 DPI was statistically different (**Figure**

3B). Females in the soil were observed at much higher levels after root scrubbing was used, 35-121 DPI in CBG and 35-95 DPI in ABG (**Figures 3 A and B)**.

Cyst development was observed in CBG at statistically significant levels at 36,43, 50, 52, 63, 64, 66, 71-73, 77, and 84-121 DPI. Cysts development in ABG had a general range of 71-121 DPI with 51, 53, 57, 71, 73-79, 81-84, 86, 87 89 90-92, 94, 95, and 96-121 DPI all having significant differences in development (**Figure 3B)**. In ABG white female development overlapped with cyst formation 71-96 DPI, then tapered off after 95 DPI, suggesting a transition period where both were present. In CBG white female development diminished at 93 days but was still significant until day 121. These data suggest the total life cycle of *H. pratensis* from inoculation to cyst formation could be as early as 51 DPI in ABG with the majority of development after 71 DPI, and 70 DPI in CBG with the earliest development at 36 DPI **(Appendix: Figure A1 and A2)**.

Nematode Reproduction and Fecundity

The average nematode offspring per plant and final reproductive values[2] were not significant between plant species **(Table 2A and 2B)**. The means were similar suggesting the nematode developed equally well on both species. Fecundity of *H. pratensis* on ABG was statistically higher than on CBG (**Table 2C**).

[2] Similar to the population replacement rate in higher order mammals.

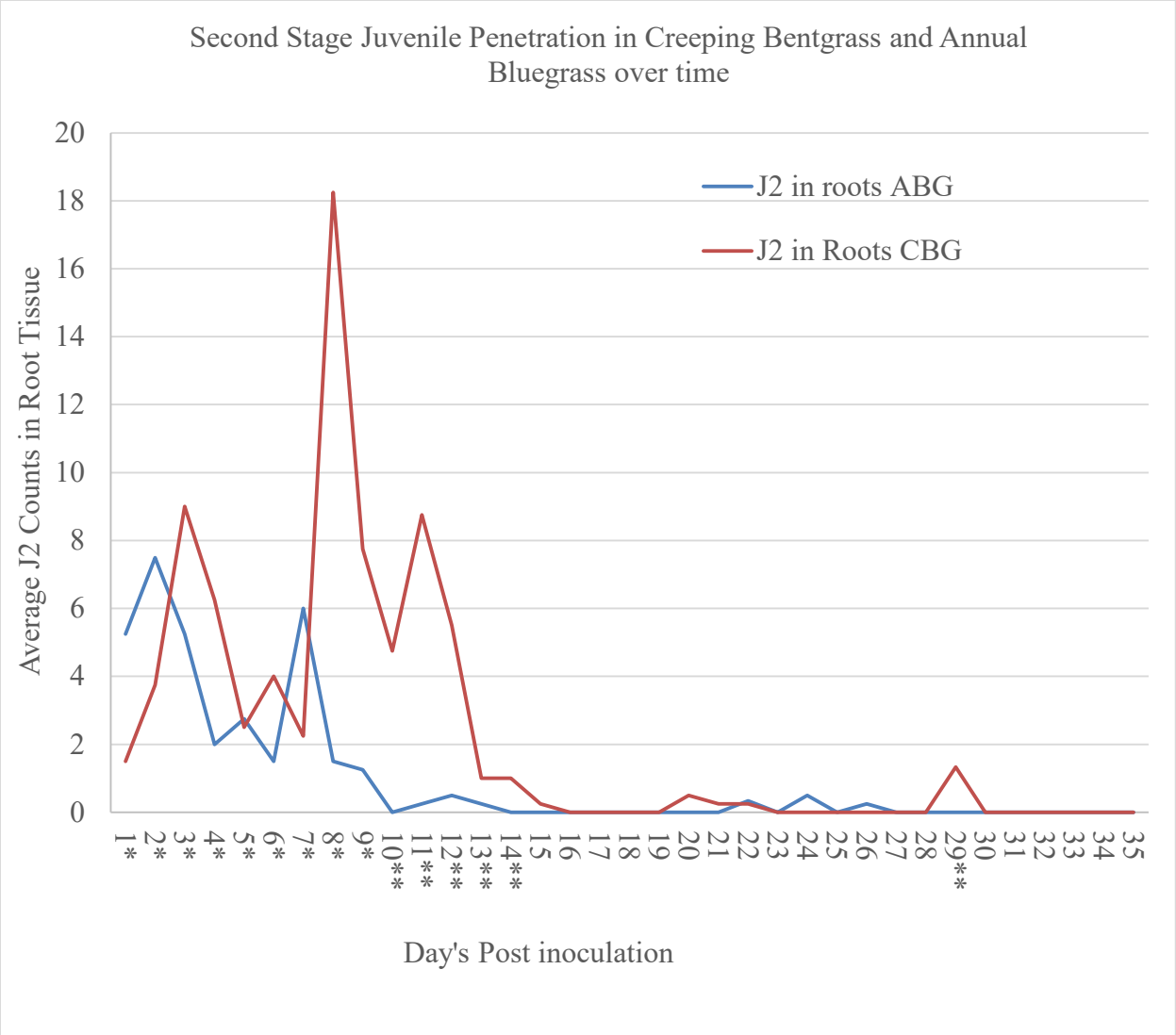

Figure 1: Average *Heterodera pratensis* second stage juveniles (J2) in roots of *Poa annua* var. *annua* (ABG) and *Agrostis stolonifera* (CBG). The average J2 counts from four replicates, for each grass species are shown. The days post inoculation (DPI) and grass species interactions were separated using Least Significant Means with P< .05. ABG at 1-9 DPI were significantly different and CBG at 1-14, and day 29 DPI were significantly different.
* Indicates DPI with Significant differences for both grass species
** Indicates DPI with Significant differences for CBG only

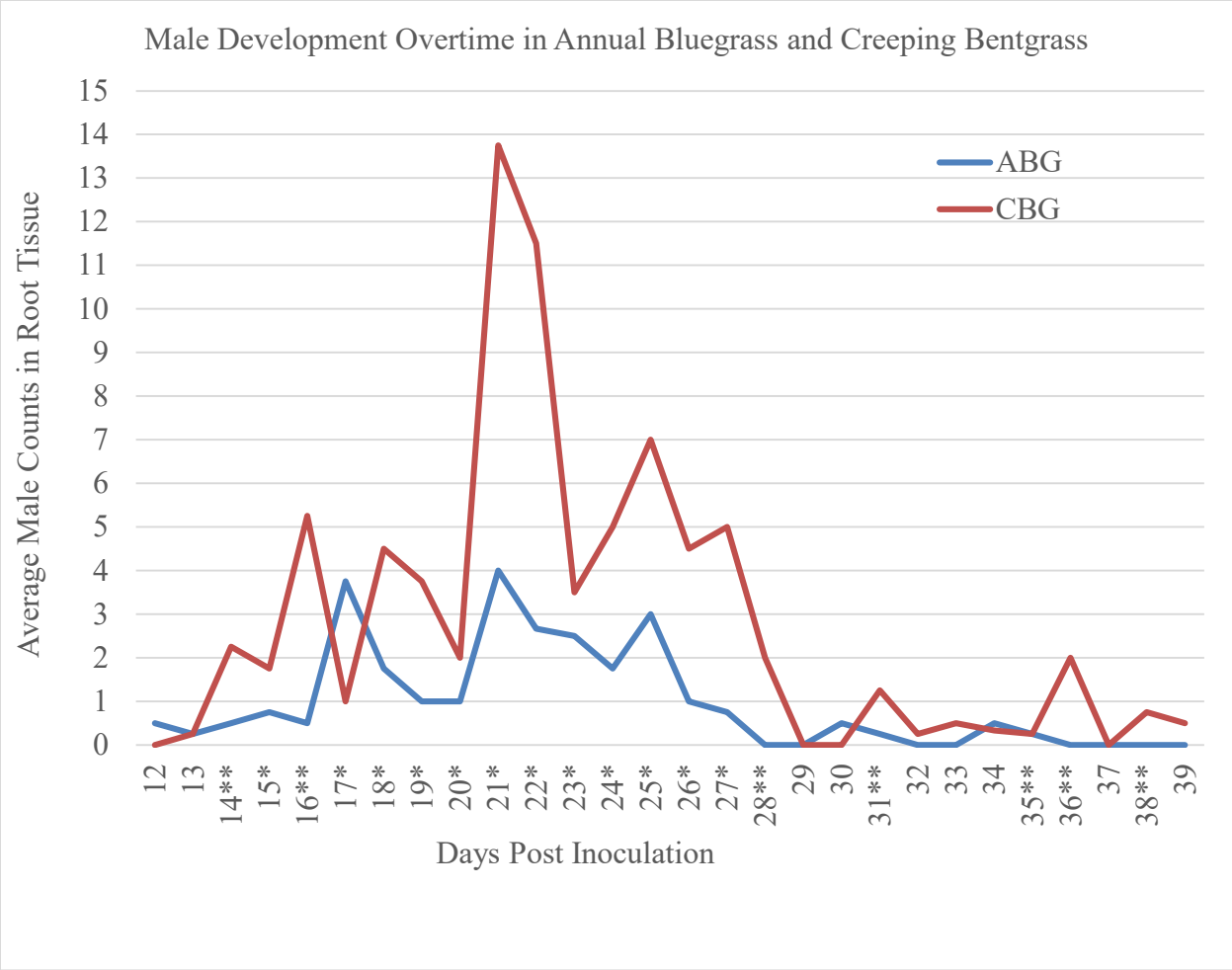

Figure 2: Average *Heterodera pratensis* males in roots of *Poa annua* var. *annua* (ABG) and *Agrostis stolonifera* (CBG). The average male counts from four replicates for each grass species are shown. The day post inoculation (DPI) and grass species interactions were separated using Least significant means with a (P< .05). Males were detected at significantly different levels in ABG roots 15-27 DPI and in CBG 14-28, 31, 35-37 DPI.
* Indicates DPI with Significant differences for both grass species
** Indicates DPI with Significant differences for CBG only

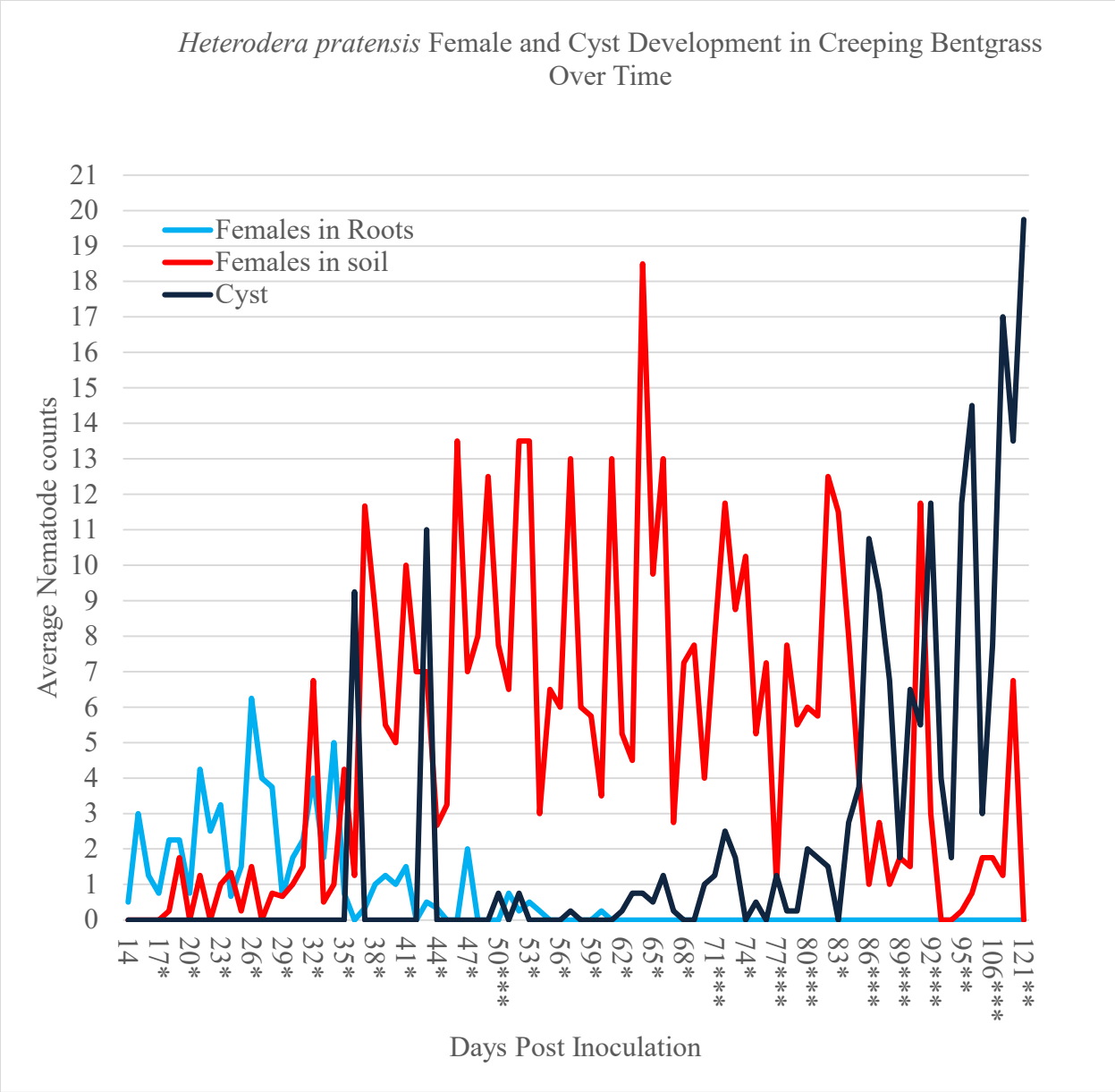

Figure 3A: Average counts of *Heterodera pratensis* white females in roots and soil, and cysts on *Agrostis stolonifera* (CBG). The average counts from four replicates, for each development stage, are shown. The days post inoculation (DPI) and grass species interactions were separated using Least Significant Means with a (P< .05). White females were detected in roots at significantly different levels in CBG at 15-35, and 38-41 DPI and cyst were detected 36,43, 50, 52, 63, 64, 66, 71-73, 77, and 84-121 DPI.
* Indicates significant white female development (in both roots and soil)
** Indicates significant cyst development
*** Indicates significant white female and cyst development

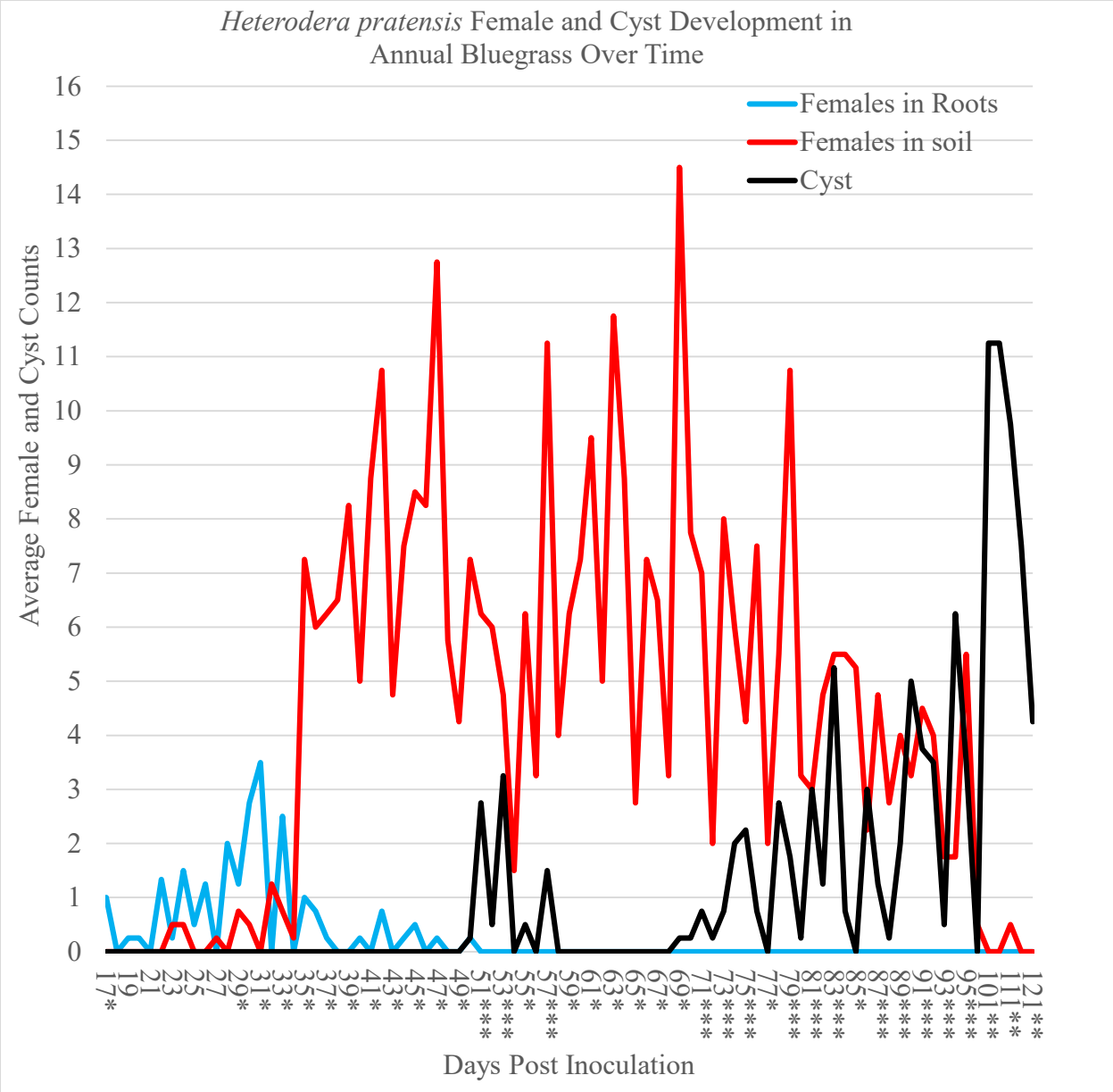

Figure 3B: Average counts of *Heterodera pratensis* white females in root tissue and soil and cysts on *Poa annua* var. *annua* (ABG). The average nematode counts from four replicates, for each developmental stage, are shown. The day post inoculation (DPI) and grass species interactions were separated using Least significant means with a (P< .05). Females were detected at significantly different levels in ABG roots on 17, 22, 24, 26, 29-31, 33, 35-36, and 42 DPI. Cysts development in ABG had a general range of 71-121 DPI with significant differences at 51, 53, 57, 71, 73-79, 81-84, 86, 87 89 90-92, 94, 95, and 96-121 DPI.
* Indicates significant white female development (in both roots and soil)
** Indicates significant cyst development
*** Indicates significant white female and cyst development

Table 2A: Summary statistics for offspring (eggs and J2) per plant at the final sampling date on Creeping Bentgrass and Annual Bluegrass. Different letters indicate statistical differences between CBG and ABG.
N is the number of cysts crushed for each measurement.

Host	N	Mean	Std Dev	Minimum	Maximum
CBG	29	1910.90a	2353.04	0	10656
ABG	41	1658.56a	1763.74	0	6013

Table 2B: Summery statistics for *Reproduction (R). Final population/ initial population (P_f/P_i) on CBG and ABG at the final sampling date. Different letters indicate statistical differences between creeping bentgrass (CBG) and annual bluegrass (ABG).
N is the number of cysts crushed for each measurement.
* Indicates zero values, plants where no nematodes were detected, were removed for this table's calculations.

Host	N	Mean	Std Dev	Minimum	Maximum
CBG	24	9.5a	9.9	0.2	43.7
ABG	33	8.4a	7.14	1.3	24.6

Table 2C: Summery statistics for the *Fecundity (Offspring / Cyst) on Annual Bluegrass and Creeping Bentgrass on the final sampling date. Different letters indicate statistical differences between CBG and ABG.
N is the number of cysts crushed for each measurement.
* Indicates zero values, plants where no nematodes were detected, were removed for this table's calculations.

Host	N	Mean	Std Dev	Minimum	Maximum
CBG	24	236.38a	117.4	50	495
ABG	33	289.2b	67.5	117	405

Field Monitoring

Significant differences were detected across days for cysts, eggs and J2s (offspring), J2s free in the soil[3], males, and females. The date by year interaction was significant for males, cysts, and eggs and J2s all having higher counts in 2019, than 2018 (**Figures 4, 5, and 6 respectively)**.

J2s free in the soil were observed in both 2018 and 2019 for 30 days in May, especially early and mid-May, then the numbers declined and had a slight peak at the end of July. Males were observed in both years for 30 days in June starting in mid- June through late June. White females were observed for 60 days in both years in mid-June until late July. Cysts, excluding empty ones, were at the highest counts in late July and early May and lowest throughout June. Cysts could be observed throughout the year, which may be accounted for by cysts remaining in the soil from previous years. The offspring from the crushed females and cysts were highest in mid -July and lowest in mid-June. The overall life cycle was 80-90 days from early May to the end of July.

J2s free in the soil had a regression coefficient (R^2) of .4176, and a correlation coefficient of -.474 with soil temperature showing a moderate liner relationship, although if sampling had taken place sooner this relationship may have been different. Females had a R^2 of .5285 and a correlation coefficient of .56 with soil temperature, also having a moderate linear relationship at the end of the growing season.

[3] These are second stage Juveniles that hatched from eggs and are mobile in the soil but have not located a host yet.

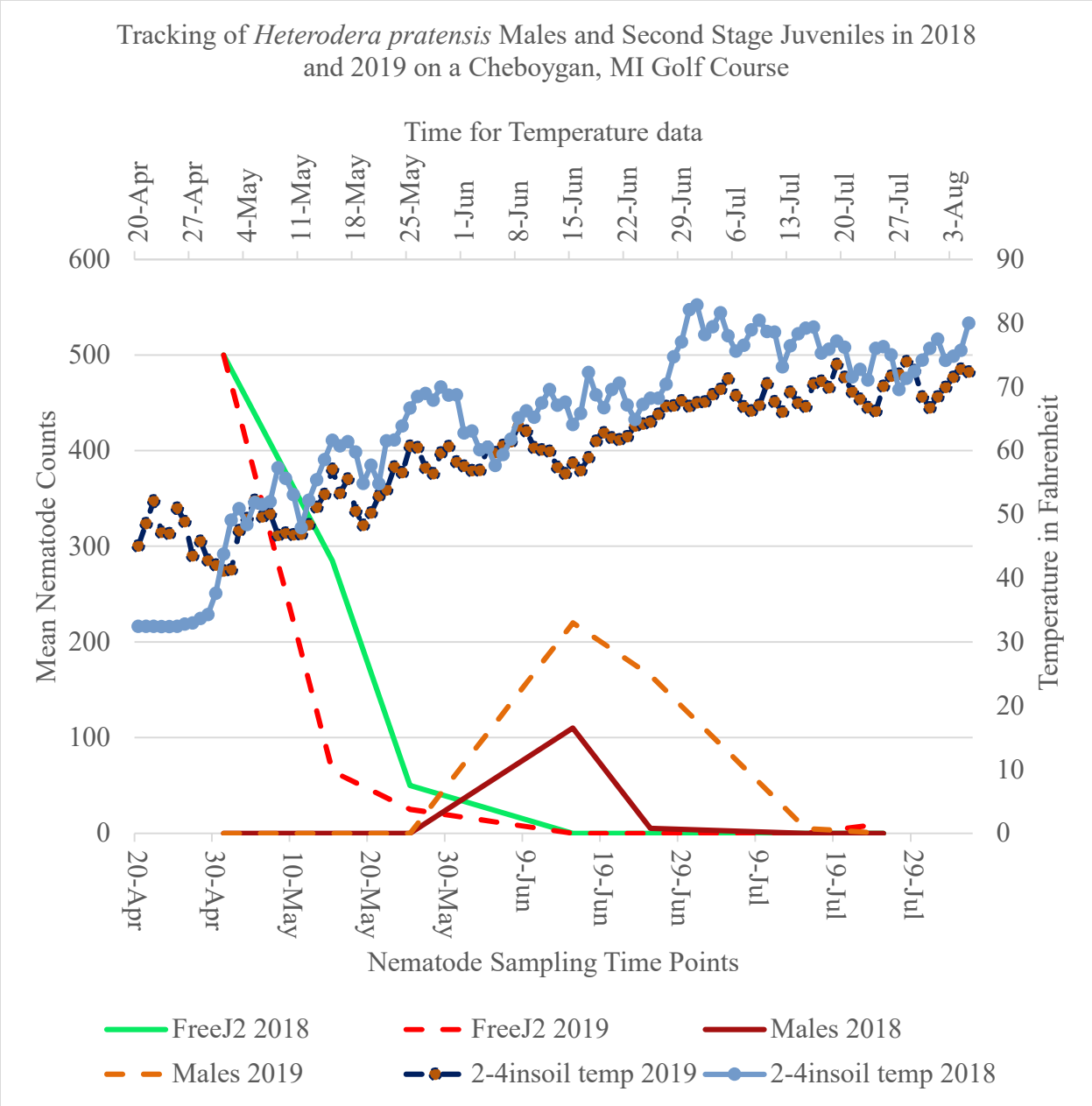

Figure 4: Field monitoring of males and J2s free in the soil in Cheboygan, Michigan. Counts from two greens were averaged at each time point. Years and sampling dates were separated using LS-means to look for overall model interactions (P<.05), and the date by year interaction was significant for males counts.

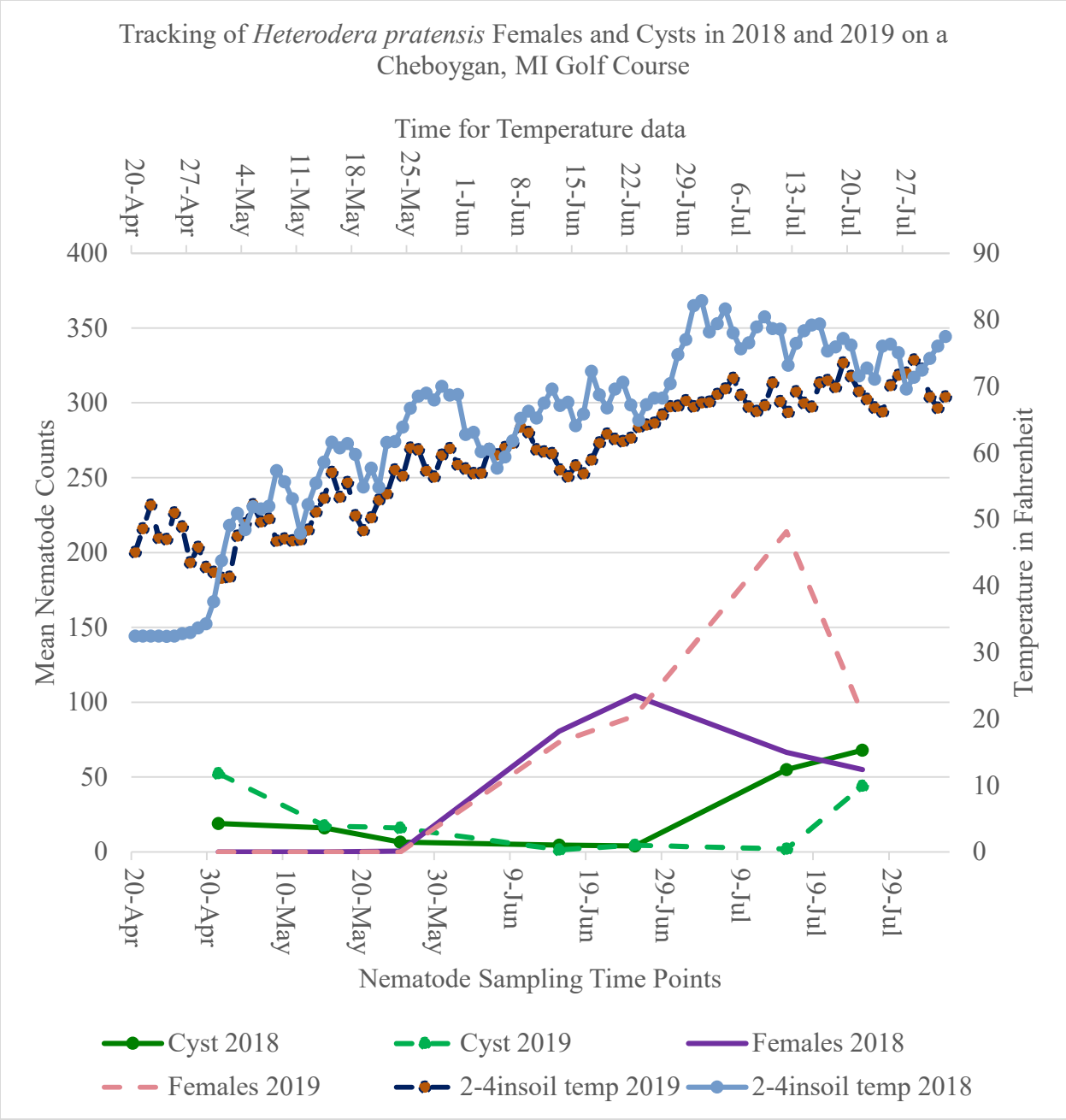

Figure 5: Field monitoring of cysts and females in Cheboygan, Michigan. Counts from two greens were averaged at each time point. Years and sampling dates were separated using LS-means to look for overall model interactions (P<.05), and the date by year interaction was significant for cysts. Significant differences were detected across days for cysts and females.

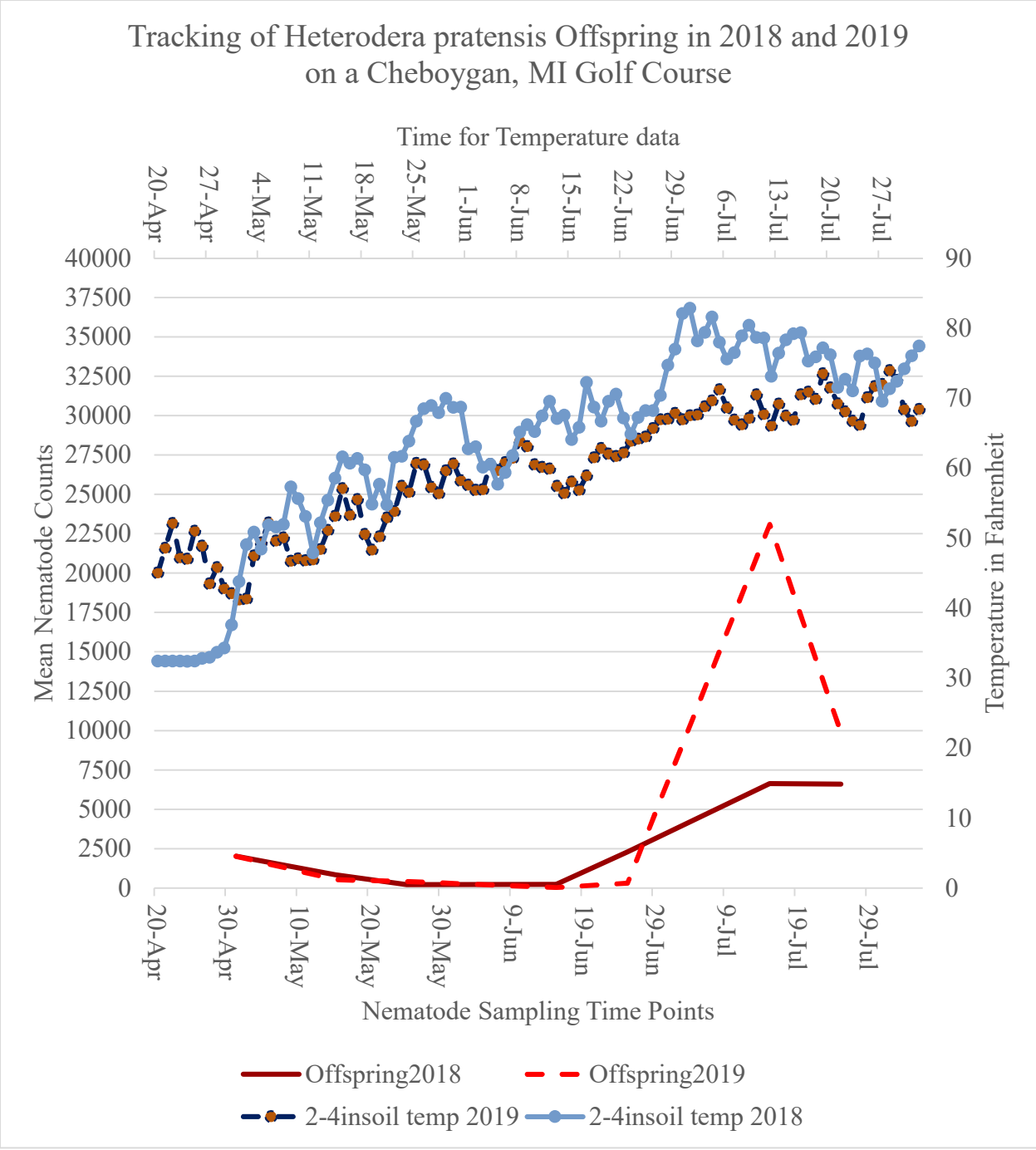

Figure 6: Field monitoring of offspring in Cheboygan, Michigan. Counts of second stage juveniles (J2) plus eggs (offspring) from two greens were averaged at each time point. Years and sampling dates were separated using LS-means to look for overall model interactions (P<.05). The counts for offspring were significantly different between years and across dates.

Discussion

This study reaffirmed that the life cycle of *H. pratensis* is similar to other species in the *H. avenae* complex. Development, from J2 to Cysts, of *H. pratensis* in both the growth chamber study and the field study were in the time frame of 60-90 days, with some very early development and slight differences between hosts. In the field study, J2s free in the soil were likely detectable before our initial May sampling dates. As soil temperature rises between 2-10 ºC (40-50 ºF), *H. avenae* egg hatch in the soil is significant (Albrecht et al., 2007; Jing et al., 2014) and root penetration begins. Based on the soil temperatures, peak egg hatch probably took place between April 1st and May 31st. Earlier sampling dates may have revealed the beginning of J2 hatch and extended the life cycle length in the field slightly.

The slight peak in egg hatch at the end of the year suggests a smaller invasion before winter (Sabová et al., 1985). Otherwise, there seems to be only one major J2 hatch per year, following a similar pattern to the northern eco-type reported by Rivoal in 1986. This is encouraging from a management point of view. The tentative window for spraying nematicide would be when the J2s are in the soil, early in the spring as soil temperatures start to approach 50 º F (10 ºC), which is their most vulnerable time. This is similar to root knot nematode treatment window (Crow et al., 2017). There seems to be a moderate correlation between soil temperature and the J2s free in the soil so monitoring soil temperatures should lead to knowing the ideal window to spray a particular turfgrass area, but this needs to be investigated further. At the very least, soil below 50^0F (10 ºC) should be sampled for free J2s and sprayed accordingly, assuming snow is not an obstacle.

In the field and growth chamber studies, most of the nematode life stages had similar duration when they were present in the soil, except for J2s free in the soil; in the growth chamber

experiment they were only counted for 15 days but in the field they were present for 30. In the field studies, there was a 20-day period when no adult life stages were detectable in the soil, likely due to juvenile development inside the roots. Even though there were differences in environmental temperatures and moisture, males were detectable in the soil for 30 days in the field and growth chamber. Females were detectable for 60 days in the field, but detection differed between CBG and ABG in the growth chamber. In ABG white females were detectable in the soil for 66 days, but in CBG white females were detectable for almost 100 days from 19 DPI until 116 DPI. CBG diverging from the field data by more than a month could be indicative of a longer transition period of females to cysts for *H. pratensis* on some hosts.

In the field data cysts were present at every sampling point, left over from previous years. Eggs and J2s, that were present around 60 days after J2s were detected free in the soil were indicative of new cyst formation. In the growth chamber cyst formation was more constant CBG and ABG around 70 DPI. The early cyst development was likely due to slight changes in the host plant and environmental conditions that forced some females to senesce early. Although female and cyst development was not as clear cut as the other life stages in the growth chambers the general time frames still line up with reported life cycles of *H. avenae* (Wu et al., 2014; Smiley et al., 2017; Sabová et al., 1985). Both studies give support to the similarity of *H. pratensis* and *H. avenae* development.

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APPENDIX

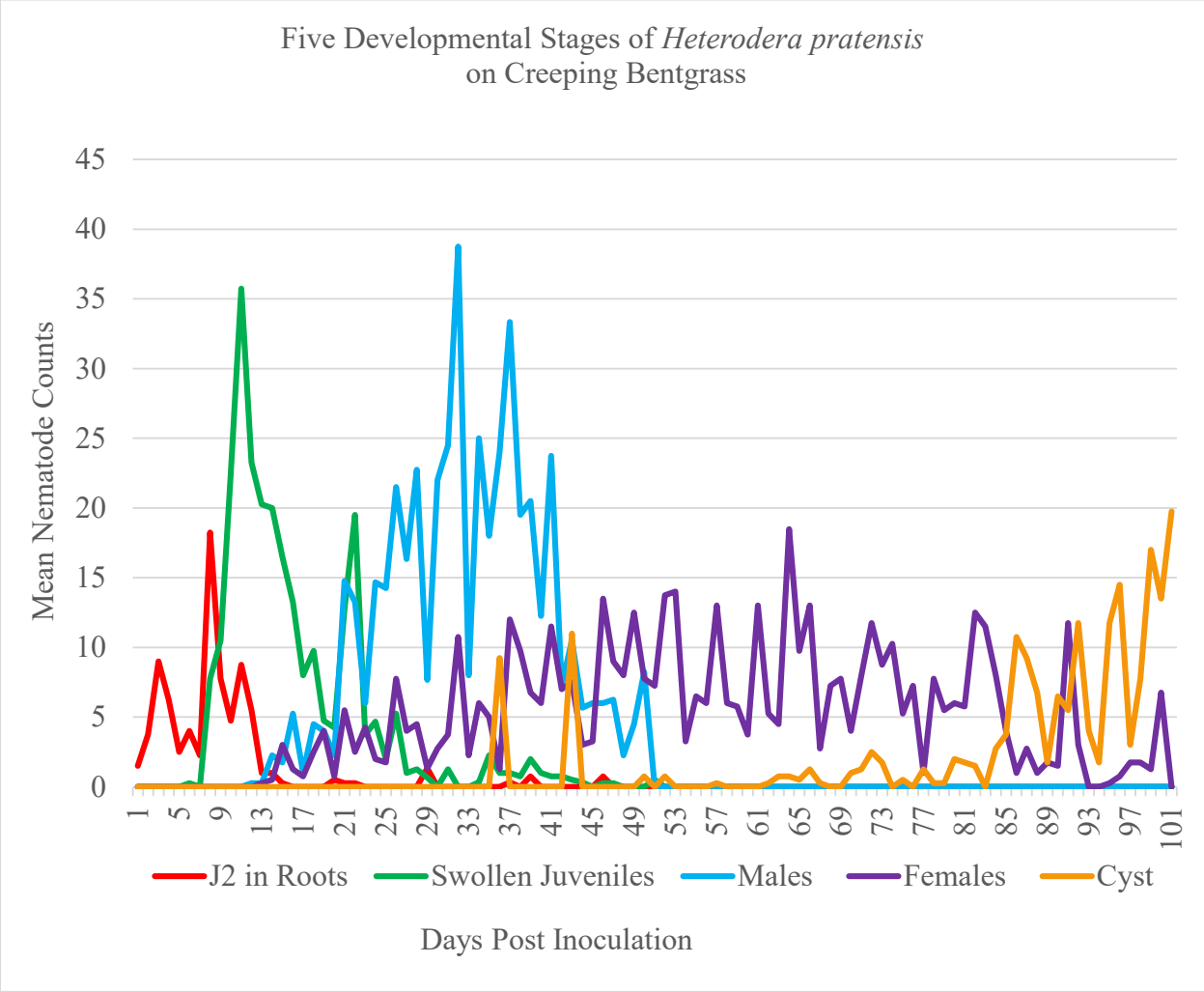

Figure A1: Life cycle of *Heterodera pratensis* on *Agrostis stolonifera* under growth chamber conditions. The average nematode counts of four replicates are shown. The life stages found in the roots and soil were added together except for the second stage juveniles (J2). Only J2s were used as initial inoculum so only those counted in the roots are graphed. The total life cycle was as early as 36 days from inoculation to cyst formation, with the bulk of development happening after 84 DPI.

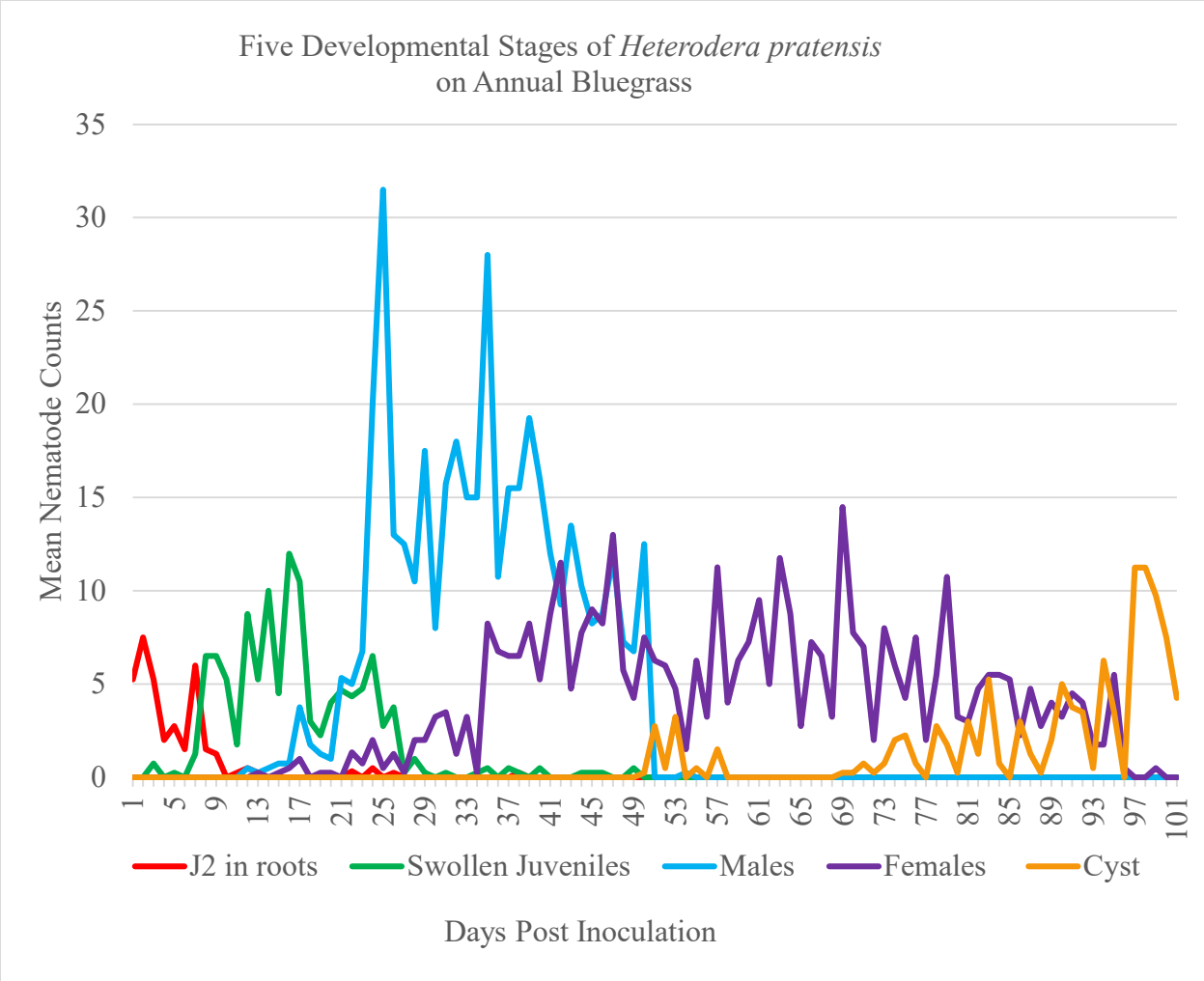

Figure A2: Life cycle of *Heterodera pratensis* on *Poa annua* var. *annua* in growth chamber. The average nematode counts of four replicates are shown. The life stages found in the roots and soil were added together except for the second stage juveniles (J2). Only J2s counted in the roots are graphed. The total life cycle was 71 days from inoculation to cyst formation. With some early cyst development around 50 days.

CHAPTER 2:
EXPLORATION INTO THE HOST RANGE OF *HETERODERA PRATENSIS* ON COOL SEASON TURFGRASS AND SMALL GRAINS OF MICHIGAN

Abstract

Heterodera pratensis in Europe and Asia is known to infect several different grass hosts including creeping bentgrass, *Agrostis stolonifera,* and annual bluegrass, *Poa annua*, but has not been successfully reared on barley or wheat. Since the closely related species, *H. avenae,* is known to have many different pathotypes, and Michigan grows 740,000 acres of small grains annually, it is important to better understand the host range of Michigan's population of *H. pratensis.* Seventeen plant species were screened, including three that are known hosts of Euro-Asian populations. Ten non-grain grasses: creeping bentgrass (CBG); annual bluegrass (ABG) one field collected and one cultivated variety; Kentucky bluegrass; perennial and annual ryegrass; rough bluegrass; colonial bentgrass; velvet bentgrass, and wood bluegrass were also evaluated. Seven small grains: white millet; sorghum; corn; soft red wheat; barley; oats, and rye were also included.

Seeds were germinated and infected with 1132-1206 nematode eggs and second stage juveniles then placed in a growth chamber set at 25 ºC for 14 hours and 18 ºC for 10 hours daily. Four replications from each plant species were destructively sampled at days 14, 35, and 145. Of the ten non-grain grasses tested, seven were considered good hosts, meaning their reproductive value (R), final population (Pf)/ initial population (Pi), was greater than 1, which included rough bluegrass *(P. trivialis)*, cultivated ABG (*P. annua L)*, wood bluegrass (*P. nemoralis)*, CBG (*A. stolonifera)*, annual rye grass *(L. multiflorum)*, field collected ABG (*P. annua var annua)* and Kentucky bluegrass (*P. pratensis)*. Three of the non-grain grasses were poor hosts with a R below 1 but more than 0, including perennial ryegrass (*Lolium perenne)*, colonial bentgrass

(*Agrostis capillaris),* and velvet bentgrass (*A. canina)*. Of the grains one, rye (*Secale cereal)*, was a poor host. Four were non-hosts, with an R of 0, white millet (*Panicum miliaceum),* sorghum *(Sorghum bicolor),* corn (*Zea mays),* and oats *(Avena sativa)*. The final two, soft red wheat *(Triticum aestivum)* and barley (*Hordeum vulgare)*, were good hosts, with barley having a higher R than CBG.

Introduction

The full extent of *Heterodera pratensis* host range is largely unknown; it is a relatively new species and was originally found to only infest grass (Gäbler et al., 2000). It was detected in a grassland and reared in a greenhouse on several different grass hosts, with failed attempts to rear it on barley 'City' and wheat 'Tinos' (Gäbler et al., 2000), with no other attempt reported in the literature. A population of *H. pratensis* was found in a barley field, but it was undetermined if its host was barley or some other weedy grass (Gäbler et al., 2000). According to the International Maize and Wheat improvement center database (CIMMYT), 'Tinos' wheat does not contain any resistance genes to cereal cyst nematodes (CCN), thus *H. pratensis* was and is still largely considered to only parasitize nonagricultural grasses (Smiley et al., 2017).

Though, if *H. avenae* and the other nematodes in its species complex are reflected on, alternate pathotypes and virulence levels in different populations are common and should be considered possible with *H. pratensis* as well. CCN resistance genes are sometimes effective and other times not (Smiley et al., 2005, 2011), and the host range of the North American population of *H. pratensis* may differ from Euro-Asian populations. The objectives of this study were to further elucidate the host range of *H. pratensis*, testing its ability to infect novel small grains and non-grain grasses, as well as known grass hosts of the type population. Michigan grows 740,000 acres of small grains annually (USDA, 2018-2019) and has over 1.8 million acres of turfgrass

(Michigan Turfgrass Foundation, 2020), so it is important to know the potential these nematodes hold to infect them.

Materials and Methods

Seeds

The seeds of nine turfgrasses, seven grains, and one wild grass were screened. Germination rates were calculated by germinating seeds using a moisture chamber in a Falcon 100x15mm sterile petri dish (Corning Inc., Corning, NY) and counting the ratio of sprouted seeds to non-sprouted seeds after a week, except for Kentucky bluegrass (*P. pratensis*) and rough bluegrass (*P. trivialis*), which took two weeks to germinate. 10 seeds were used for the grain species and 20 seeds for the non-grain grass species.

Creeping bentgrass (CBG) (*Agrostis stolonifera*) seed with a 63% germination rate was used during the first replication, and a 50% germination rate in the second replication, so a slightly higher seeding rate was used during the second replication (**Table 3)**. *Poa annua var annua* was collected from an annual bluegrass (ABG) green, with a 99% germination rate. Clippings were collected in the spring during seed fill, dried for two months and the seeds were separated from grass clippings using a South Dakota Seed Blower (Seedburo equipment company, Des Plaines, IL). Kentucky bluegrass (*P. pratensis*) was used with a 90% germination rate. Perennial ryegrass and annual ryegrass both had an 85% germination rate.

Rough bluegrass (*P. trivialis*), annual bluegrass (*P. annua L*,) colonial bentgrass (*Agrostis capillaris*), velvet bentgrass (*A. canina*) and wood bluegrass (*P. nemoralis*) were all obtained from the Germplasm Resource Information Network with an 80% germination rate. All of the grains, White millet (*Panicum miliaceum,*) sorghum (*Sorghum bicolor*), corn (*Zea mays*),

soft red wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), oats (*Avena sativa*), and rye (*Secale cereal*), had a 98% germination rate.

Table 3: Grass species and seeding rate used to screen *Heterodera pratensis* and test the host status. * Indicates a species not known to host *H. avenae.*
** indicates a known host of *H. pratensis*.
+ indicates the wild/non-turf grass.

Common name/Cultivar	**Scientific name**	**Seeding rate Seeds/Cone.**
Corn 'DK-52-84'	*Zea mays*	2
Soft Red Wheat 'Starburst'	*Triticum aestivum*	3
Barley	*Hordeum vulgare*	3
Sorghum	*Sorghum bicolor*	3
Rye 'Wheeler'	*Secale cereal*	3
White Millet *	*Panicum miliaceum*	3
Oats 'IDA'	*Avena sativa*	3
Annual Bluegrass ** Accession number: PI 236900	*Poa annua L.*	5
Rough Bluegrass 'Laser' Accession number: PI537439	*P. trivialis*	5
Kentucky Bluegrass 'Barenbrug'	*P. pratensis*	5
Creeping Bentgrass 'Declaration' **	*Agrostis stolonifera*	*5/8*
Velvet Bentgrass 'Kingstown' Accession number: PI578526	*A. canina*	*5*
Colonial Bentgrass 'Duchess', * Accession number: PI600936	*A. capillaris*	*5*
Annual Ryegrass 'Lone star'	*Festuca perennis* *(syn Lolium multiflorum)*	*3*
Perennial Ryegrass 'Silver Dollar' **	*Lolium perenne*	*3*
Annual Bluegrass (field collected) **	*P. annua var annua*	*5*
Wood Bluegrass 'S-92' + Accession number: PI 325464	*P. nemoralis*	*5*

Inoculum Preparation

Soil samples were collected from two infected greens at a golf course near Cheboygan, Michigan. They were kept at 2 ºC for 2-3 months to help break dormancy (Williams and Beane, 1979). Cysts were extracted using a modified centrifugation-flotation technique (Jenkins, 1964). Approximately 2.5 gallons of soil were emptied into a 10 L bucket filled with 7 L of water. The soil was stirred by hand in the water until no soil clumps were felt. Once the soil had been mixed the soil was poured over a VWR 8" Stainless Steel Test Sieve USA series # 16 nested over a USA series # 100 sieve (VWR International. Radnor, PA). The top sieve was removed, and the contents of the bottom sieve were transferred into eight 100 mL centrifuge tubes. This was spun in a Block Scientific centrifuge at 1750 rpm for four minutes (Block Scientific, Bellport, NY). After four minutes the tubes were removed and the water decanted, so only the soil pellets at the bottom remained. Then, approximately 80 mL of a 1.25 g/mL sucrose solution was poured into each tube. The soil pellets were resuspended into the solution and the samples were placed back into the centrifuge and spun for two additional minutes at 1750 rpm. The tubes were removed from the centrifuge and the solution was decanted over a VWR 8" Stainless Steel Test Sieve USA series # 400 (VWR International. Radnor, PA). The samples were rinsed in the sieve with running water for 10 seconds then poured into sterile specimen collection cup and stored at 2 º C until used for inoculum.

Once cysts were extracted, they were poured into a VWR 8" Stainless Steel Test Sieve USA series # 100 nested over a USA series # 650 sieve (VWR International. Radnor, PA) and manually crushed against it using a rubber widget under gently running water to release the eggs and second stage juveniles. These were then poured into a sterile specimen cup and 2 mL were

taken and enumerated, under a dissecting microscope at 40x magnification, to estimate the initial amount of nematodes per cup.

Experimental Set Up and Soil and Root Analysis

Seeds were planted into SC(10)U cell cone-tainers (Stuewe and Sons., Inc., Tangent, Oregon) containing autoclaved coarse sand and allowed to germinate at 70 ºF. Planting the seeds prior to inoculation was to help ensure the presence of roots for the nematodes. Once 50% of the cones, of a species, had at least one plant they were inoculated with 1132-1206 nematode eggs and second-stage juveniles. These were syringed into the soil of each cone-tainer at the base of the seedlings. They were then placed in a growth chamber set to 25 ºC for 14 hours and 18 ºC for 10 hours daily, with a 14 hour photoperiod. Plants were lightly watered before and after inoculation to prevent nematode desiccation.

Soil was processed at three time points and analyzed. Nematodes were extracted from the soil using a modified centrifugation flotation method (Jenkins, 1964). Cone-tainers containing approximately 100cc of soil were emptied into a 10L bucket filled with 7L of water. The soil was stirred by hand in the water until no soil clumps were felt. Roots were not scrubbed on day 14 to preserve the root quality for staining, but were scrubbed on day 35 and 140, using fingers to brush the root exterior, to dislodge females. Once the soil had been mixed and the roots scrubbed, or removed, the soil was poured over a VWR 8” Stainless Steel Test Sieve USA series # 16 nested over a USA series # 400 sieve (VWR International. Radnor, PA). The top sieve was removed, and the contents of the bottom sieve were transferred into a 100 mL centrifuge tube. This was spun in a Block Scientific centrifuge at 1750 rpm for four minutes (Block Scientific, Bellport, NY). After four minutes the tube was removed and the water decanted, so only the soil pellet at the bottom remained. Then, approximately 80 mL of a 1.25 g/mL sucrose solution was

poured into the test tube. The soil pellet was resuspended into the solution and the sample was placed back into the centrifuge and spun for two minutes at 1750 rpm. The tube were removed from the centrifuge after two minutes and the solution was decanted over a VWR 8" Stainless Steel Test Sieve USA series # 400 (VWR International. Radnor, PA). The sample was rinsed in the sieve with running water for 10 seconds then poured into a 10 mL Pyrex test tube (Corning Inc. Corning, NY) then stored at 2 ºC. Nematodes were counted under a dissecting microscope at 40x magnification. On day 140, cysts and females were crushed using a tissue homogenizer; eggs and second stage juveniles were counted.

Roots were stained on days 14 and 35 and processed using Sodium-Hypochlorite-Acid-Fuchsin Method (Byrd et al., 1983). Roots used for staining were rinsed during the soil washing process and cleared in a diluted bleach solution. Plants on day 14 were placed in a 4:1 water to bleach solution for four minutes, and plants on day 35 were placed in a 2:1 water to bleach solution for four minutes. Plants were removed from the bleach after four minutes and rinsed in water for 45 seconds, to remove any bleach that remained. They were then placed into a beaker with 40 mL of tap water, and 1 ml of stock acid-fuchsin-stain solution was added. The beaker was placed onto a hot plate and the roots in the solution were boiled for 30 seconds, to stain the nematodes inside of the root tissue. After 30 seconds of boiling, the roots were removed and rinsed in tap water for 30 seconds to remove excess stain. These were patted dry and placed into 30 ml of acidified glycerin, then brought to a boil on the hot plate, to de-stain the root tissue so the nematodes could be visualized. Once boiled the roots were placed into a Falcon 60x15mm sterile petri dish (Corning Inc., Corning, NY) with enough glycerin to cover them and stored at 70 ºF until analyzed. Roots were then sandwiched into one layer between the top and bottom of a

150 x 20mm VWR glass petri dish (VWR International, Radnor, PA.), and the stained nematodes were counted.

Experimental Design and Statistical Analysis

Seventeen plant species were evaluated in a completely randomized design. Four replications from each plant species were destructively sampled at day 14, day 35, and day 145. The experiment was repeated twice. *Agrostis stolonifera* was used as a control. The data analysis for this paper was generated using SAS software, Version 9.4M2 of the SAS System for Windows. Data were analyzed using the glimmix procedure (SAS Institute Inc., 2018, Cary, NC, USA), followed by LS means to separate significant differences between treatments. P values below 0.05 were significant in all analyses. The best fit model of log(x+1) was used to help normalize a Poisson distribution, and each life stage was analyzed. All life stages were analyzed at each time point. Additionally at the final time point, the average offspring (eggs + J2s) per plant, offspring per cyst and the overall reproductive value was calculated.

To evaluate the host status of the different grass species a reproductive value (R) was used. This was calculated by dividing the final population (Pf) of nematodes by the initial inoculum level (Pi). Non-hosts (R = 0) supported no nematode egg production. Poor hosts (R< 1) supported some egg production, but less than the initial inoculum level, so less than what it takes to replace the initial population. Good hosts (R> 1) were able to support enough egg development to replace the initial population.

Results

Host Status and Reproductive Values

Of the ten non-grain grasses tested, seven had R>1 and were considered good hosts including rough bluegrass *(P. trivialis)*, ABG (*P. annua L)*, wood bluegrass (*P. nemoralis)* CBG (*A. stolonifera)*, annual rye grass *(L. multiflorum)*, field collected ABG (*P. annua var annua)* and Kentucky bluegrass (*P. pratensis)* (**Table 4).** The latter two were statistically better hosts when compared to the control CBG. Perennial ryegrass (*Lolium perenne)*, colonial bentgrass (*Agrostis capillaris),* and velvet bentgrass (*A. canina)* were all poor hosts with a R<1.

Of the grains, only rye (*Secale cereal)* was a poor host. Four were non-hosts, with an R=0, including white millet (*Panicum miliaceum),* sorghum *(Sorghum bicolor),* corn (*Zea mays),* and oats *(Avena sativa)*. The final two grains, soft red wheat *(Triticum aestivum)* and barley (*Hordeum vulgare)*, were good hosts. Barley had better reproduction than CBG (**Table 4**).

Male Development

A few grasses had interesting male development compared to their host status (**Table 4**). On Kentucky bluegrass, both male development and R was statistically significant compared to the control, but male development was lower, and R was higher. Perennial ryegrass and rye were poor hosts, but male development was statistically similar to the control. Finally, corn was not considered a host, but it supported male development and was statistically similar to the control, making it an insufficient host (**Figure 7**)

Table 4: Reproductive summary statistics and host status for each host species.
Reproductive value (R) = Population final/ Population initial. This indicates host status R = 0 is a non-host, R<1 is a poor host, R>1 is a good host.
*Indicates development was statistically better than CBG (control)
+ indicates development was statistically worse than CBG (control)
'No' values are all statistically lower

Common name 'cultivar'	**Reproductive Value (R)**	**Offspring/cyst**	**Host**	**Supported Male Development**
Creeping Bentgrass 'Declaration'	1.4	102	Good	Yes
Kentucky Bluegrass 'Barenbrug'	4.47	174	Good*	Yes+
Annual Bluegrass (cultivated)	1.95	111	Good	Yes
Rough Bluegrass 'Laser'	2.3	113	Good	Yes
Wood Bluegrass 'S-92'	2.67	139	Good	Yes
Velvet Bentgrass 'Kingstown'	0.41	85	Poor+	Yes+
Colonial Bentgrass 'Duchess'	0.12	28	Poor+	Yes+
Perennial Ryegrass 'Silver Dollar'	0.65	94	Poor	Yes
Annual Bluegrass (Field collected)	3.78	102	Good*	Yes*
Annual Ryegrass 'Lone Star'	1.24	101	Good	Yes*
White Millet	0	0	No	No
Barley	4.24	173	Good*	Yes
Sorghum	0	0	No	No
Soft Red Wheat	2.98	169	Good*	Yes
Corn 'DK-52-84'	0	0	No	Yes
Rye 'Wheeler'	0.27	36	Poor+	Yes
Oats 'IDA'	0	0	No	No

Discussion

Grasses in the genus *Poa* were better hosts overall than the *Agrostis* spp. On golf greens, ABG is common either as a notorious weed or a welcome species. The presence of *H. pratensis,* could cause a decrease in the overall quality of the turf, especially if *P. annua* is the dominant species. Stands of Kentucky bluegrass may be damaged where *H. pratensis* is present, based on the high R of the nematode on that species. More sampling is required in Michigan to determine if this nematode species poses a threat to the average lawn owner or sports field manager.

Most of the hosts, good or poor, followed similar developmental patterns (**Figures 8** and **9)**, with the biggest differences in the final reproductive measurements showing that different hosts allow for varying multiplication rates, like *H. avenae* (Rivoal and Cook, 1993). Since *H. avenae* does not seem to be a large issue in Michigan and cereal cyst nematode (CCN) resistant genes are not common in grains grown in Michigan, the lack of development in 'IDA' oats, but development in both barley and wheat is notable. *H. avenae* is the oat cyst nematode and most other grain infecting CCNs can infect oats, as long as the oat cultivar contains no resistance genes and, 'IDA' oat is a cultivar bred to do well in Michigan (Michigan crop improvement association), based on the lack of development of *H. pratensis,* either it contains CCN resistance genes, or this population of *H. pratensis* cannot infect oat.

A small screening of 11 winter wheat lines was conducted (**Appendix: Table A1**) to see if *H. pratensis* was infectious on multiple Michigan cultivars. Eight of the eleven cultivars supported some female development, but not at statistically significant levels. None of the screened cultivars are known to have cereal cyst nematode resistance (CRE) genes, but a low initial inoculum level, 200 J2s may explain the low infection rate. Gabler et al. (2000) found no infection on 'Tinos' spring wheat, which also has no known CRE genes. Differences in *H.*

pratensis infection ability between different cultivars of spring and winter wheats should be investigated further.

The differences in male development can suggest the reasons the nematode's final population is high or low. A host may be easy to colonize for females and encourage larger female population, or the host may prevent female development by stopping large syncytia formation forcing more males to develop. The host may also just prevent initial penetration of the J2s, not allowing any development to happen. The male development on corn, but lack of female and egg development, is a potential tool if *H. pratensis* becomes a problem in row crops (Smiley et al., 2015). Since corn seems to attract the nematodes and allow for some penetration and development, but does not support reproduction, it could be used as a management tool in rotation, similar to trap crops or resistant varieties (Krall et al., 2000). Although the nematodes in the *H. avenae* species complex seem to hatch readily in water, additional reduction of a field population may be gained when a host can also entice hatching but maintain reproduction rate below one (Hauer et al., 2016) .

This is the first note of *H. pratensis* infecting grains. Most of the wheat and barley grown in Michigan does not come with CCN resistant genes, and growers may be vulnerable if their field were to be infected with a CCN. Since the impact of *H. pratensis* on grains is unknown, it is important to note it's potential to infect them. More research is needed to determine if *H. pratensis* can pose a real threat, especially if milder winters in Michigan may cause a cultural shift to growing spring wheat (Olson and Pennington, 2020).

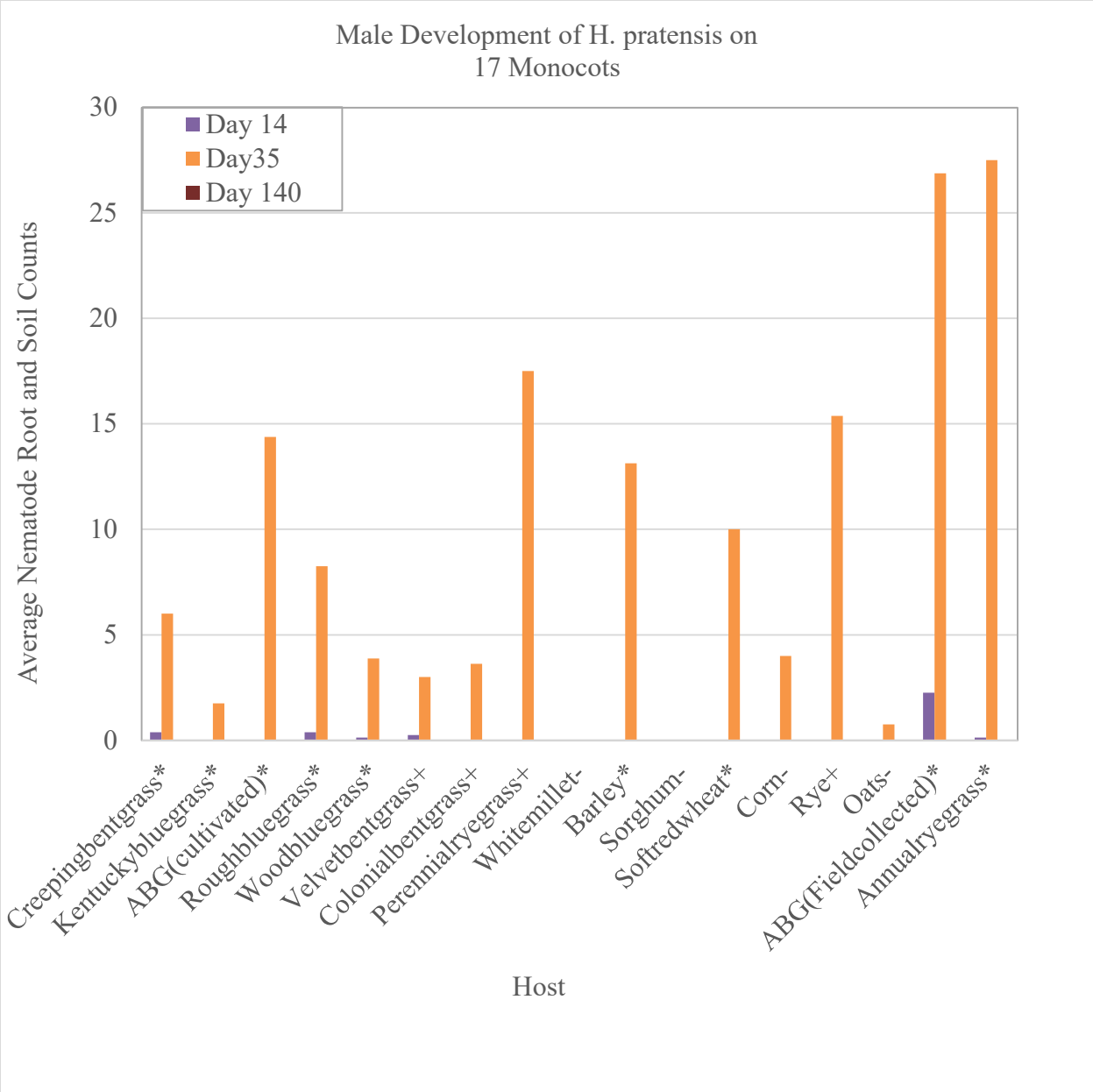

Figure 7: Average male development of *H. pratensis*, from 8 replicates, on 17 monocots at 14, 35, and 140 days.
***** Indicates grass species was a host for *H. pratensis.*
+ Indicates grass species was a poor host.
– indicates a non-host.

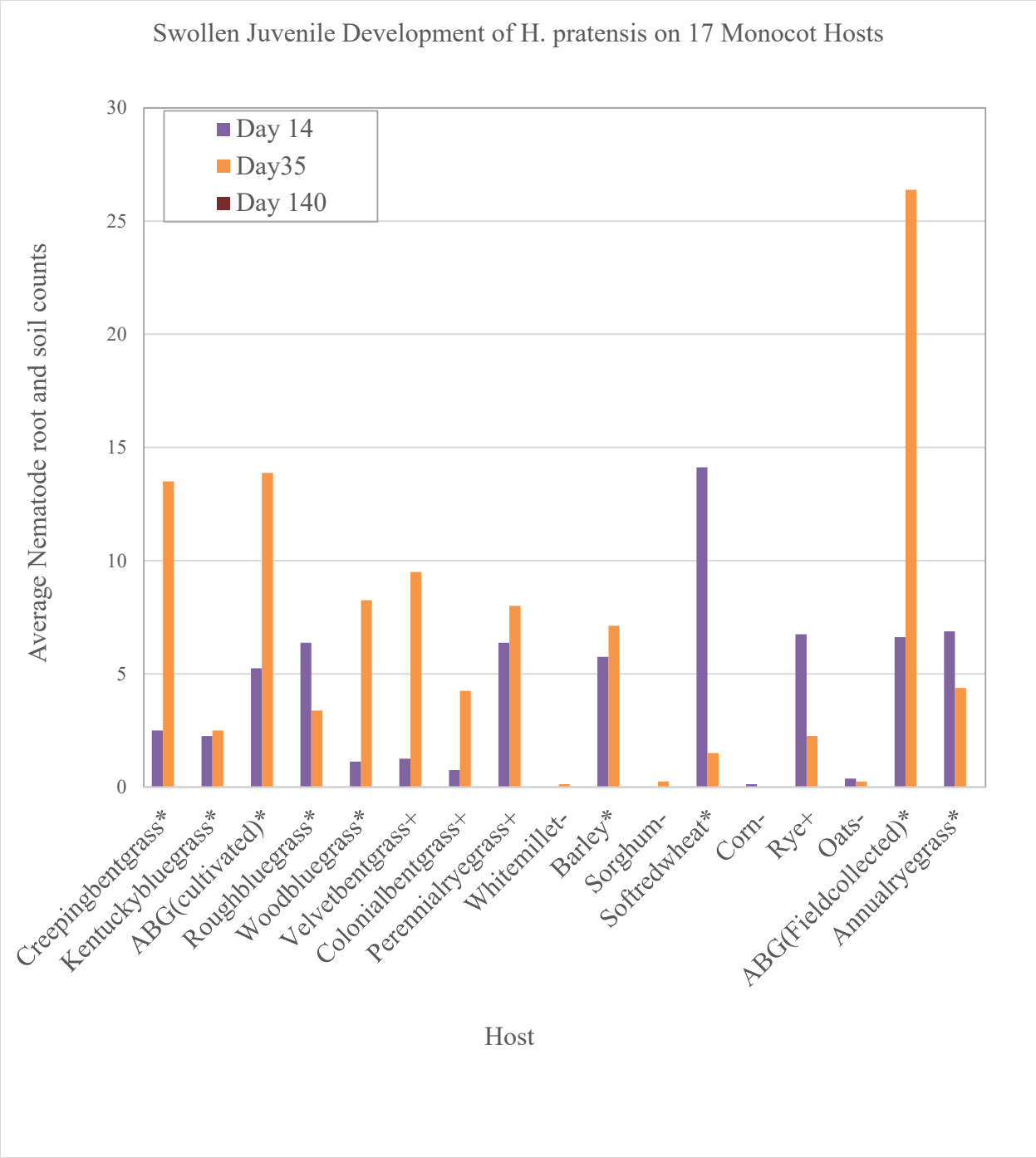

Figure 8: Average swollen juvenile development of *H. pratensis*, from 8 replicates, on 17 monocots at 14, 35, and 140 days.
* Indicates grass species was a host for *H. pratensis*
+ Indicates grass species was a poor host
– indicates a non-host

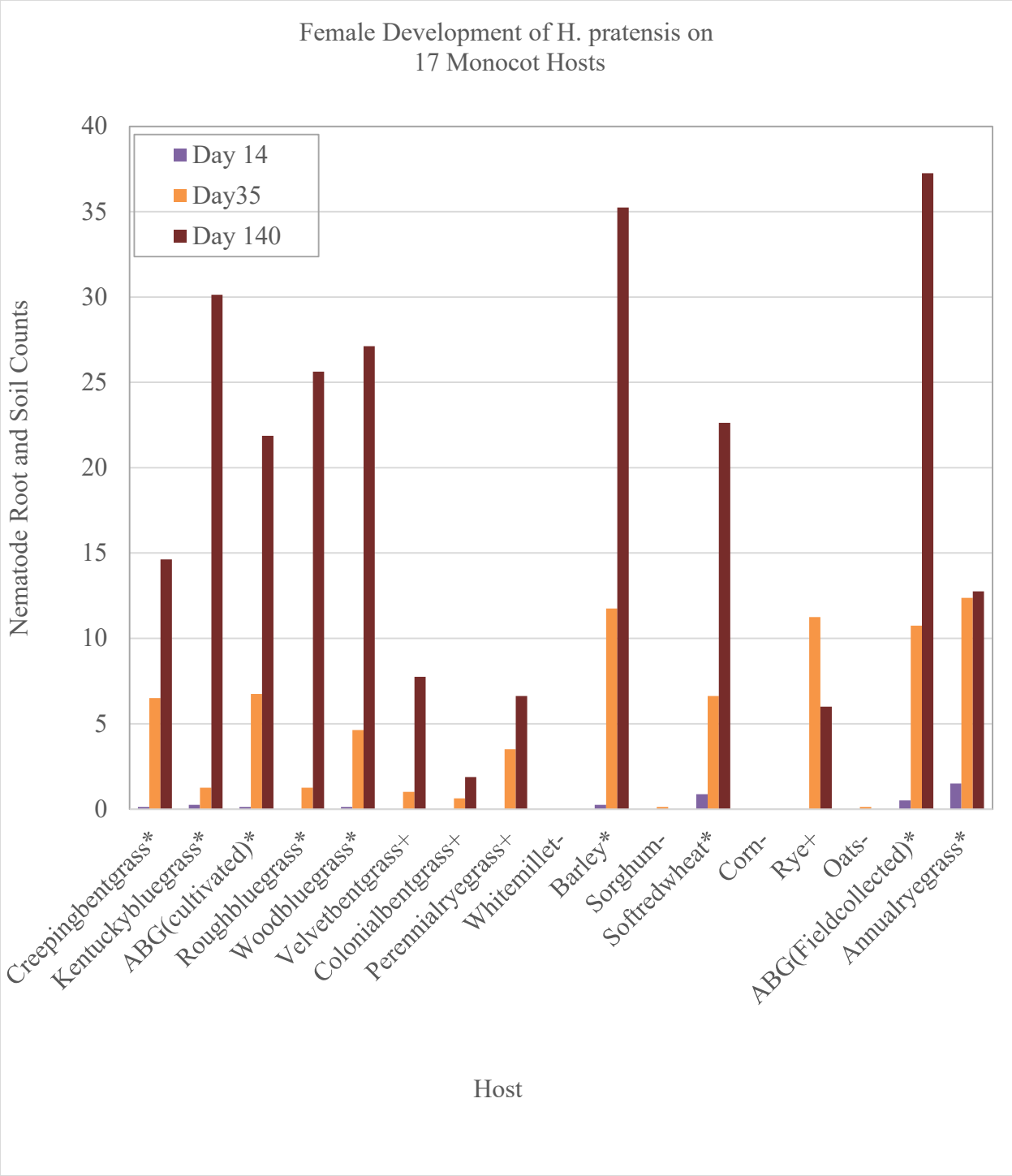

Figure 9: Average female development of *H. pratensis*, from 8 replicates, on 17 monocots at 14, 35, and 140 days.
* Indicates grass species was a host for *H. pratensis*
+ Indicates grass species was a poor host
– indicates a non-host

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APPENDIX

Table A1: Wheat Screening Summary Data. Eight out of 11 winter wheat verities had some development. The two hard wheat varieties had some development. Only ~224 J2s were used for inoculum, so lack of infection could be due to low inoculum.

Variety	Wheat Type	N Obs	Minimum Cysts	Maximum Cysts	Mean Cyst	Mean R	Mean Offspring/cysts
DF112R	soft red winter	4	0	2	1	0.369658	58.25
Hilliard	soft red winter	4	0	1	0.5	0.293803	68.75
KS05HW14	hard white winter	4	1	4	1.75	1.5	189.9375
MI14R1140	soft red winter	4	0	0	0	0	0
MI14W1039	soft white winter	4	0	0	0	0	0
Red Dragon	soft red winter	4	0	2	0.5	0.323718	37.875
SY100	soft red winter	4	0	1	0.75	0.592949	85.75
Shirley	soft red winter	4	0	2	1	0.934829	109.375
Starburst	soft red winter	4	0	1	0.5	0.540598	126.5
Whitetail	soft white winter	4	0	0	0	0	0
Zenda	hard red winter	4	0	1	0.5	0.193376	45.25