Effects of nematicidal seed treatments on the biology of the soybean cyst nematode, Heterodera glycines

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Effects of nematicidal seed treatments on the biology of the soybean cyst nematode, *Heterodera glycines*.

by

Jared Paul Jensen

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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Major: Plant Pathology

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ABSTRACT

Nematicidal seed treatments are a welcomed new tool to manage the soybean cyst nematode, *Heterodera glycines*, in soybean production. Results of field-based research on the effects of nematicidal seed treatments have been inconsistent and do not indicate how or when a seed treatment affects the nematode. This dissertation describes 1) the development of new methods to study *H. glycines* biology and 2) the use of new and traditional methods to study the effects of Avicta® (abamectin) and Clariva® (*Pasteuria nishizawai*) seed treatments on the biology of *H. glycines*.

Two new methods were developed to study the behavior of *H. glycines* second-stage juveniles (J2s) exposed to abamectin. The first method utilized a high-resolution flatbed scanner that automatically recorded images of populations of *H. glycines* every hour for 24 hours. A custom computer program then compared the locations of the nematodes in the population from one image to the next to calculate the percentage of nematodes in the population that moved. The second analysis method recorded videos of individual *H. glycines* J2s after incubation in abamectin. Computer analysis of the videos tracked the motion of individual nematodes by measuring the speed and change in curvature of points along the nematode body. Both methods measured paralyzing effects of abamectin on J2 movement and motion at concentrations of 1, 10, and 100 μg/ml. Sequential digital imaging combined with computer analysis provided quantitative data unavailable in manual microscopic observation of nematodes.

Controlled-environment experiments combined new methods (including movement analysis) with traditional methods in the greenhouse and laboratory to discern the effects of Avicta and Clariva seed treatments on specific life stages and processes of *H. glycines*. The
stages and processes analyzed included hatching, movement, penetration, development, and reproduction. As a seed treatment, Avicta, with the active ingredient abamectin, inhibited nematode movement and root penetration at soil depths 2 and 5 cm below the soil line. Clariva seed treatment, comprised of spores of *Pasteuria nishizawae*, inhibited juvenile movement, penetration at specific soil depths (2, 5, and 7 cm), and development in younger plants. Overall, both treatments were most effective when *H. glycines* were found outside the root and inside young plants (up to 20 days). Effects of either treatment were minimal in older plants (30 to 60 days) with larger root systems. Protection of younger plants early in the season, though, may still be sufficient to increase stand establishment and increase yield in *H. glycines*-infested soils.
CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

Dissertation Organization

This dissertation is organized into four chapters. This first chapter is the introduction, which provides a literature review of the soybean cyst nematode, management options, and nematicidal seed treatments. The second and third chapters contain method development and research results, written as separate journal manuscripts. The fourth chapter is a general summary of the research presented.

Introduction

Soybean (\textit{Glycine max}) is one of the most commonly grown crops in the Midwestern United States, second only to corn. After processing, soybeans can be used for animal feed, human consumption, ink, biofuels, milk replacements, cooking oil, plastics, and lubricants. Up to 70 percent of protein meal consumed by humans can be attributed to soybeans (SoyStats 2016). In 2016, the United States produced over 117 million metric tons (4.3 billion bushels) of soybeans, planted on about 33.7 million hectares (83.4 million acres) of land (USDA 2016). Iowa is consistently one of the nation’s leading soybean producing states. In 2016 Iowa farmers planted soybeans on about 3.8 million hectares (9.5 million acres), which produced approximately 15.4 million metric tons (571 million bushels), and yielding over $5.3 billion in sales (USDA 2016). Given that soybean production is a major industry in Iowa, it is important that farmers maintain profitability through management of yield-reducing stresses, like plant pathogens.
The large-scale planting of cash crops, such as soybeans, year after year fosters a favorable environment for growth of plant pathogens. In 2014, it was estimated that pathogens of soybeans were responsible for over 13.8 million metric tons (511 million bushels) of lost yield, in the US and Ontario, Canada (Allen et al. 2017). The soybean cyst nematode, *Heterodera glycines* Ichinohe, is the most economically harmful of the soybean pathogens, causing an estimated yield loss of over 348,000 metric tons (129 million bushels) in 2014 (Allen et al. 2017). That estimated yield loss translates to over $1 billion in lost revenue (Koenning and Wrather 2010; Schmitt 2004; Allen et al. 2017). One of the reasons the soybean cyst nematode is so damaging is because it is found in almost every soybean-growing region in North America (Tylka and Marett 2017).

**History of *H. glycines***

The soybean cyst nematode is thought to have evolved with soybean cultivation in China and Japan. The nematode was first reported in literature as *Heterodera goettingiana* (pea cyst nematode) in 1951 by Goffart, but later reclassified as a new species, *Heterodera glycines*, by Ichinohe in 1952 (Ichinohe 1952). Shortly after its classification, *H. glycines* was discovered in the United States in North Carolina in 1954 (Winstead et al. 1955) and in other states (Missouri, Tennessee, Arkansas, Illinois) over the next few years (Riggs 2004). It is believed that *H. glycines* was introduced to the United States through the movement of soil from *H. glycines*-infested regions of Asia, either through infested seed lots or soil used to inoculate soybeans with *Bradyrhizobium*, which likely explains its relatively vast distribution in a short amount of time (Hymowitz 1990; Riggs 2004). Since the 1970s, *H. glycines* has been detected in all 99 counties of Iowa (Tylka and Lang 2017).
Life Cycle of *H. glycines*

The life cycle of *H. glycines* starts with the nematode developing inside of an egg. Within the egg, the first-stage juvenile (J1) matures from a growing embryo, forming the muscular, nervous and secretory/excretory systems (Lauritis et al. 1983; Koenning 2004). While still in the egg, the developing juvenile undergoes its first molt into the second-stage juvenile (J2). J2s will reach full development before hatching from the egg (Lauritis et al. 1983; Koenning 2004).

Juvenile development and hatching is in response to a combination of factors such as soil temperature, moisture, time (diapause), soil chemistry, and genetics (Alston and Schmitt 1988; Sikora and Noel 1996; Niblack et al. 2006). Not all juveniles will hatch from eggs at the same time. This diverse set of hatching behaviors helps maintain the population when unexpected and unfavorable conditions occur (Niblack et al. 2006). Upon emergence, J2s migrate through small (150-250 µm in diameter) openings between soil particles and move towards growing soybean roots (Koenning 2004).

Using its stylet, the J2 pierces an epidermal root cell and moves intracellularly until it establishes a feeding site (Koenning 2004). A secreted mixture of enzymes, including cellulases, aid in nematode intracellular migration (Koenning 2004; Niblack et al. 2006). Once the J2 chooses a cell to feed on, usually in the inner cortex or vascular cylinder of the root, it secretes additional enzymes and proteins (effectors) through its stylet into the host cell, causing that cell to become a specialized feeding site known as a syncytium (plural: syncytia) (Gheysen and Mitchum 2011). Inside the syncytium, the secreted effectors cause changes to the cytoplasm, increase the number of ribosomes, form smaller secondary vacuoles, and open cell walls between adjacent cells (Gheysen and Mitchum 2011). All these changes create a large, multinucleate cell
that acts as a metabolic sink within the plant and provides the nematode with food to continue to develop.

The feeding J2 will grow in width then molt into a third-stage juvenile (J3). During the J3 stage, gonads will start to develop while the nematode continues to enlarge. The nematode will molt for a third time into the forth-stage juvenile (J4). Male J4s will revert to a vermiform shape that is curled within the cuticle, whereas female J4s become so large their posterior end protrudes through to the root surface (Koenning 2004). After one last molt, the nematodes enter the adult stage. Adult males will emerge as vermiform worms from the J4 cuticle and leave the root in search of females for mating. Males will mate with multiple females, and females will be mated by multiple males (Koenning 2004). Adult males no longer feed and eventually die after mating. Adult females continue to feed while producing eggs. Eggs are stored in two locations: inside the body of the female and deposited in a gelatinous matrix outside the posterior end of the female (Thompson and Tylka 1997). Once the female’s body cavity is entirely full of eggs, the female dies and her body eventually falls off the root. Over time, the cuticle of the dead female darkens and hardens to form a cyst. Cysts of *H. glycines* protect the developing eggs, so that eggs can withstand extreme conditions such as cold temperatures and lack of moisture. Within a cyst, eggs may remain viable for up to 11 years (Niblack et al. 2006). A single *H. glycines* generation, from egg to egg, takes about 25 to 30 days, depending on soil conditions including temperature and moisture (Da Rocha 2008). In Iowa, four to six nematode generations can occur in a single growing season, allowing for a rapid increase in population densities.
Management of *H. glycines*

Active movement of *H. glycines* is limited to just a few centimeters that J2s can traverse when searching for roots to infect. Most new *H. glycines* infestations are the result of passive dispersal of cysts and eggs through movement of cyst-containing soil from wind, farm equipment, water runoff, and animals (Riggs 2004). Once a field becomes infested with *H. glycines*, there are no economically practical means of eradication. To remain profitable, a farmer must manage *H. glycines* populations by keeping soil population densities in check. In Iowa, there are two means to manage *H. glycines*: grow a nonhost crop and grow *H. glycines*-resistant soybean varieties.

Growing a nonhost crop is an easy and effective way to manage *H. glycines* populations and is a practice that most farmers perform through their regular cropping rotations. Iowa farmers commonly follow a two-year crop rotation sequence, using corn (*Zea mays*) as the nonhost crop alternating with soybeans. As an obligate biotroph, *H. glycines* have a highly specialized relationship with soybeans that allows them to form syncytia via secreted effectors. In nonhosts, *H. glycines* are unable to form a syncytium. Hatched *H. glycines* J2s that are unable to find a suitable host, form and maintain a syncytium eventually will die of starvation. After one year of growing a nonhost crop, there can be a 5 to 50 percent decrease in *H. glycines* egg density in the soil (Tylka, personal communication). With reduced nematode population densities, soybean yields can increase by 10 to 40 percent following one year of nonhost crop (Koenning et al. 1993). However, since *H. glycines* hatching is affected by a variety of factors (host presence, genetics, time, environment, etc.), only a proportion of the *H. glycines* egg population will hatch when a nonhost crop is grown (Koenning et al. 1993; Riggs 2004). If a nonhost crop is grown for more than one consecutive growing season, there will be less of a
decline in the *H. glycines* egg density in the soil in second or third year of corn compared to the first season (Tylka, personal communication). The unhatched eggs remain viable and only will hatch when timing and environmental conditions are favorable. Although growing nonhost crops reduces nematode population densities, it is not feasible to use as the only management strategy to eliminate *H. glycines*.

Growing *H. glycines*-resistant soybeans currently is the most effective way to maintain favorable soybean yields in nematode-infested fields. When *H. glycines* J2s feed on a resistant soybean variety, the plant mounts a response that kills the developing syncytium, somewhat comparable to a hypersensitive response to other plant pathogens. Soybean resistance to *H. glycines* is conferred through a combination of various resistance genes (Rhg). The underlying mechanism of resistance is still unknown (Mitchum 2016), but histological studies have observed dilation of the rough endoplasmic reticulum, apoptosis, nuclear degeneration, and necrosis (Endo 1965; Kim et al. 1987; Kim et al. 2012). The timing of histological responses varies between resistance sources (i.e. Peking vs PI 88788), yet the end result still reduces the number of nematodes that develop to maturity (Kim et al. 2010). Similar to feeding on a nonhost, *H. glycines* J2s that are unable to develop and maintain a syncytium eventually will die of starvation. In the field, resistant varieties produce more yield and result in lower end-of-season *H. glycines* soil egg population densities when compared to susceptible varieties (Tylka et al. 2016). On average, *H. glycines*-resistant varieties yield 0.3 to 0.9 tons per hectare (5 to 15 bushels per acre) more than susceptible varieties (Tylka and Mullaney 2017).

Due to effectiveness in diverse locations and affordability (no price difference compared to seeds of susceptible varieties), planting *H. glycines*-resistant varieties has become a common practice in soybean production. Of the seven major sources of *H. glycines* resistance (Niblack et
al. 2002), PI 88788 is arguably the most popular among soybean breeders most likely due to the ease with which *H. glycines* resistance genes are incorporated into progeny breeding lines (Tylka, personal communication). This may explain why resistance sourced from PI 88788 parents is incorporated in over 97 percent of commercially available soybean varieties in Iowa (Tylka and Mullaney 2017). Repeated use of the same source of resistance, though, has brought selection pressure against *H. glycines* populations in favor of populations that have adapted to grow on varieties with PI 88788 source of resistance. Instances of this shift in *H. glycines* populations have been documented in several soybean-producing states, such as Missouri, Illinois, and Iowa (Mitchum et al. 2007; Niblack et al. 2008; McCarville et al. 2017). Resistance-management schemes exist, like the six-year rotation described by Tylka and Mullaney (2017), but can only be accomplished when there is diversity in the *H. glycines*-resistant varieties available. Newer management options are needed to help maintain yield and reduce *H. glycines* field populations.

**Seed Treatments**

The notion of treating a seed with a biological, chemical, or physical process or additive is one of earliest known practices in disease management (Munkvold 2009; Munkvold et al. 2014). Seed treatments offer many advantages to the farmer that help save time, input costs, and yield. By using a treatment to the seed, farmers are able to apply the product directly to the spermosphere (Nelson 2004) to protect the vulnerable seed and seedling, ensure uniform application of product per plant, reduce the overall amount of product applied per field, and the reduced risk of exposure when the product is applied to the seed in commercial facilities (Munkvold et al. 2014). There has been a noticeable increase in the popularity of seed treatments
over the past 10 to 15 years due to the increased value of seeds and the desire to protect the investment. Combinations of pesticides, fertilizers, and polymers help ensure healthy germination and growth to maximize yield. Farmers have embraced the promise of seed treatments so much, it is estimated that the seed treatment market will grow from $1 billion in sales in 2002 to over $4 billion in 2017 (Munkvold 2014). Seed treatments, with their streamlined delivery mode and decreased risk, have created opportunities for active ingredients that would have never been viable if applied on a field-wide scale as soil or foliar treatments.

**Nematicides as Seed Treatments**

The increase in seed treatment usage has yielded a new means of nematode management: nematicides applied as seed treatments. Alone, field-scale application of nematicides, either granular or fumigants, has been found to have adverse environmental impacts (Monfort et al. 2006; Cabrera et al. 2009a; Zasada et al. 2010) and is cost-prohibitive for soybean production in the Midwest. When formulated as seed treatments, though, nematicides become an easy-to-use and cost-effective tool in managing soil nematode populations. Syngenta Crop Protection® (Greensboro, NC) has developed two nematicidal seed treatments marketed towards soybean production in the Midwest, Avicta® and Clariva®.

Avicta is a general nematicidal seed treatment that has been marketed for use in corn, cotton, and soybean production. Abamectin, the active ingredient of Avicta, is a blend of avermectins B1A and B1B, belonging to the broader chemical family of avermectins (Wislocki et al. 1989, Faske and Star 2006). Avermectins are 16-membered macrocyclic lactones produced by the soil-dwelling bacterium *Streptomyces avermitilis*, with insecticidal, nematicidal, and
acaricidal properties (Burg et al. 1979, Bull et al. 1984, Wislocki et al. 1989). Avermectins are neurotoxins that disrupt gamma-aminobutyric acid (GABA) neurotransmitters and open up glutamate-gated ion channels, leading to paralysis (Bull et al. 1984; Faske and Starr 2006). Avermectins are noted for low water solubility, short half-life (21 to 56 days in soil), minimal movement within the plant, and high affinity for organic matter (Putter et al. 1983; Bull et al. 1984).

Since discovery in 1979 (Burg et al. 1979), avermectins have been evaluated for nematicidal activity in a variety of applications including injections, dips, sprays, and soil applications, and sold under a variety of trade names such as Avid®, Vertimec®, Agri-mek®, and Affirm® (Garabedian and Van Gundy 1983; Roberts and Matthews 1995; Jansson and Rabatin 1998; Cabrera et al. 2012). It wasn’t until the early 2000s that abamectin was explored for use as a seed treatment (Abawi et al. 2003; Becker et al. 2003). Combining the active ingredient with adhesives to stick to the seed and plant tissues would, in theory, make abamectin favorable as a seed treatment by limiting dispersal to only the rhizosphere.

As a seed treatment, abamectin is attractive because it has limited mobility in the rhizosphere, degrades after a short amount of time, and has limited uptake in above-ground plant tissues. Abamectin seed treatments have shown some success at controlling Meloidogyne species, Pratylenchus zea, and Heterodera schachtii (Abawi et al. 2003; Becker et al. 2003; Cabrera et al. 2009a; Cabrera et al. 2009b). In 2011, Syngenta introduced Avicta seed treatment for use on soybeans throughout the US, with marketed protection against H. glycines and many other nematodes (McClement 2011). Small-plot studies in Iowa from 2013 to 2015 found inconsistent yield increases using Avicta seed treatment; a significant yield increase was only observed at one site-year (Clifton et al. 2017).
Clariva is a biologically based nematicidal seed treatment comprised of spores of *Pasteuria nishizawai*, an obligate, endospore-forming parasite of *H. glycines*. *Pasteuria nishizawai* was first observed in Japan by Nishizawa who noticed a decline in *H. elachista* populations and attributed the loss to *P. penetrans* (Nishizawa 1987). Further studies of this bacterium revealed that it was not *P. penetrans*, but a newly discovered *Pasteuria* species that is an obligate parasite of *H. glycines*, namely *P. nishizawai* (Sayre et al. 1991a; Sayre et al. 1991b). Similar to other *Pasteuria* species, *P. nishizawai* endospores attach to the cuticle of J2s in the soil. A germ tube from the attached spore penetrates the body of the nematode once the J2 enters into the root. Microcolonies form inside the body of the nematode in late J2 or early J3 stages. The microcolonies will fragment and multiply throughout the body of the J4. Sporulation occurs in J4 and young adult females. Spores then are released into the environment once the nematode dies and disintegrates and the process starts again. (Sayre et al 1991b; Atibalentja et al. 2004; Noel et al. 2005).

Most published studies of *Pasteuria* species had only relied on morphological characterization due to the inability to obtain a pure in vitro culture for molecular analysis (Atibalentja et al. 2004). A single abstract (Hewlett et al. 2002) has been published on the ability to mass-produce pure culture of *Pasteuria penetrans* growing the bacterium in filtered broth of liquid culture with an associated soil bacterium. Since then, Pasteuria Biosciences filed a patent in 2004 claiming a large-scale fermentation process for *Pasteuria* species (Wilson and Jackson 2013). Syngenta Crop Protection later purchased Pasteuria Biosciences® and subsequently released Clariva seed treatment for soybeans in 2014.

In small-plot and strip-trial experiments in Iowa, Clariva-treated soybeans resulted in inconsistent yield effects across locations. In strip-trial experiments, Clariva-treated beans
yielded higher than beans without Clariva in five out of 18 strip-trials, yet no yield differences were measured in small-plot experiments (Bissonnette et al. 2018). Only two locations (one each in 2014 and 2015) had significant reduction in end-of-season soil egg population density in plots with Clariva seed treatment relative to untreated controls. In general, with Clariva seed treatment, there was no correlation between reductions in end-of-season egg population densities and increases in yield (Bissonnette et al. 2018).

**Overview and Objectives**

The biology of *H. glycines* and its intimate relationship with soybeans makes it a challenging pathogen to manage. Crop rotation with a nonhost and growing *H. glycines*-resistant soybean varieties are proven methods that maintain yield while managing nematode population densities. However, repeated use of the same source of resistance has led to a shift in *H. glycines* populations, selecting for populations that have adapted to PI 88788 resistance. To maintain profitability, farmers must look for additional means to manage damaging nematode populations.

Nematicidal seed treatments are an emerging tool farmers can use to add another layer of protection to early season soybean crops. Syngenta Crop Protection has developed two such seed treatments, Avicta and Clariva, marketed at controlling *H. glycines* in Midwest soybean production. Small-plot field research of these treatments has generated inconsistent results for effects on nematode population densities and yield. Little is known on how these seed treatments may affect the biology of *H. glycines*.

The objectives of this research were twofold: 1) to develop new greenhouse and laboratory methods to study the biology of *H. glycines*, and 2) to use these methods and
traditional methods to study the effects of Avicta and Clariva seed treatments on the biology of *H. glycines*. Further study of how these seed treatments function may help Iowa farmers decide how best to manage their *H. glycines* field populations.


CHAPTER 2. MOVEMENT AND MOTION OF SOYBEAN CYST NEMATODE, HETERODERA GLYCINES, POPULATIONS AND INDIVIDUALS IN RESPONSE TO ABAMECTIN

A paper submitted to the journal Phytopathology

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Abstract

Two new in vitro methods were developed to analyze plant-parasitic nematode behavior, at the population and the individual organism levels, through time-lapse image analysis. The first method employed a high-resolution flatbed scanner to monitor the movement of a population of nematodes over a 24-hour period at 25°C. The second method tracked multiple motion parameters of individual nematodes on a microscopic scale, using a high-speed camera. Changes in movement and motion of second-stage juveniles (J2s) of the soybean cyst nematode, Heterodera glycines Ichinohe, were measured after exposure to a serial dilution of abamectin (0.1 μg/ml to 100 μg/ml). Movement and motion of H. glycines were significantly reduced as the concentration of abamectin increased. The effective range of abamectin to inhibit movement and motion of H. glycines J2s was between 1.0 μg/ml and 10 μg/ml. Proof-of-concept experiments for both methods produced one of the first in vitro sensitivity studies of H. glycines to abamectin. The two methods developed allow for high-throughput analysis of nematode movement and
motion, and provide objective and data-rich measurements that are difficult to achieve from conventional microscopic laboratory methods.

**Introduction**

In the United States, the soybean cyst nematode, *Heterodera glycines* Ichinohe, is arguably the principal yield-reducing pathogen of soybeans year after year (Koenning and Wrather 2010; Allen et al. 2017). Nematode-protectant seed treatments are an emerging tool for managing *H. glycines*. Seed treatments are an attractive management option because they deliver a concentrated active ingredient (i.e., nematicide) directly around the developing seedling, while minimizing the overall amount of chemical input used in the field (Munkvold et al. 2014). Additionally, combining the use of nematode-protectant seed treatments with *H. glycines*-resistant soybean cultivars may reduce selection pressure for increased nematode virulence and prolong the efficacy of *H. glycines* resistance. The emergence of, and interest in, nematode-protectant seed treatments has renewed the need to develop newer nematicides, and methods to screen and study the effects of such compounds.

Juvenile mortality, as indicated by lack of movement, is a major defining factor of nematicide activity. A common laboratory method used to assess nematode mortality is observations of nematode movement using a light microscope. But some nematodes, such as *H. glycines*, may be immobile when observed for a short period of time but still alive (Schroeder and MacGuidwin 2010). To confirm nematode death, the observer must visually score the nematode response after prodding with a fine needle probe (Cayrol 1986), stain the nematode with a vital stain (Carins et al. 1960; Ogiga and Estey 1974; Hooper 1986; Schroeder and MacGuidwin 2010), or stimulate the nematode with a basic compound, such as sodium...
hydroxide (Chen and Dickenson 2010; Xiang and Lawrence 2016). These methods, are effective, but they subjectively measure only the presence or absence of movement. Analysis of movement and motion with time-lapse imaging can serve as an alternative in vitro method to quantitatively study nematode behavioral response to nematicides and may allow detection of subtle changes in nematode behavior.

Sequential, digital imaging has been used to investigate nematode movement since the 1980s. Several movement-analysis methods have been developed to study Caenorhabditis elegans and range from high-throughput analysis observing multiple nematodes to low-throughput, data-intensive motion analysis on individuals (Dusenbery 1985; Peet et al. 1990; Husson et al. 2012; Njus et al. 2015). These techniques, however, have not been used with plant-parasitic nematodes, which are much smaller and often exhibit sedentary or slow-moving behavior (Husson et al. 2012). Recently, image analysis and nematode-tracking techniques have been adapted to be used for studies with plant-parasitic nematodes. For example, Beeman et al. (2016) used images obtained from a high-resolution flatbed scanner to monitor nematode chemotaxis in response to live soybean seedling root exudates in a microfluidic chip.

The adaptation of time-lapse image analysis tools to study plant-parasitic nematodes would provide researchers unbiased, high-throughput methods to aid in the development of new nematicides. Data-rich analyses of nematode behavior will enable the detection of both lethal and sub-lethal effects of nematicides, potentially resulting in identification of new nematicidal compounds with a wide range of modes of action. In this study, the tracking of the location of populations of nematodes is referred to as movement whereas the tracking of points on an individual nematode is referred to as motion. The objective of this study was to develop new
image-analysis techniques to study the movement and motion of *H. glycines* second-stage juveniles (J2s) at the population and individual nematode levels, respectively.

**Materials and Methods**

**Nematode preparation.** The *H. glycines* populations used in these experiments were maintained in greenhouse culture pots on *H. glycines*-susceptible soybeans, *Glycine max* cv Williams 82, grown in Fruitfield coarse sand collected from Muscatine, IA was naturally infested with *H. glycines* (HG type 2.5.7). *H. glycines* females were removed from 30-day-old soybean roots by spraying the roots with a water stream, with the roots placed on a 850-µm-pore sieve nested over a 250-µm-pore sieve. The females collected on the lower, 250-µm-pore sieve were separated from soil particles and other debris via sucrose centrifugation using a 1,362 g/L sucrose/water solution (Jenkins 1964). Next, the females were crushed using a rotating rubber stopper against a 250-µm-pore sieve to release the eggs (Faghihi and Ferris 2000). The eggs were collected on a 25-µm-pore sieve nested under a 75-µm-pore sieve and were further isolated from debris through a second round of sucrose centrifugation with a 454 g/L sucrose/water solution (Jenkins 1964). The eggs were rinsed with water then incubated on a 30-µm-pore sieve over a thin film of tap water at 25°C for 72 hours to collect hatched J2s (Wong et al. 1993). To obtain active nematodes, collected J2s were once more placed on a 30-µm-pore sieve in one centimeter of tap water. After one hour, J2s that moved through the sieve were collected in the water below and used for movement and motion analysis experiments.

**Abamectin incubation.** Reagent-grade abamectin (Alfa Aesar, Haverhill, MA, >97 percent purity), a known nematicide (Kass et al. 1984), was dissolved in 100 percent acetone, then serially diluted to final concentrations of 100 µg/ml, 10 µg/ml, 1 µg/ml, and 0.1 µg/ml abamectin.
in 1 percent acetone in sterile distilled water. The 1 percent acetone solvent acted as a control treatment and it is henceforth referred to as the 0 μg/ml abamectin concentration. Active populations of *H. glycines* J2s, collected as described above, were incubated in one of the abamectin concentrations or in sterile distilled water in the dark at 25°C for two hours. Next, the incubated J2s were rinsed three times with sterile distilled water to remove excess abamectin. Heat-killed J2s, boiled in water for 5 minutes, were included as a negative control treatment. In summary, *H. glycines* J2s were incubated in one of five different concentrations of abamectin, or in sterile water or heat-killed. The movement and motion of J2s from each treatment were analyzed with the two methods described below.

**Movement analysis of nematode populations.** Fifteen to sixty *H. glycines* J2s from each treatment were added to individual wells of a 24-well plate containing 11.5 percent Pluronic F127 gel (Sigma Aldrich, St. Louis, MO) (Sasaki-Crawley 2012). The plates were placed onto an EPSON Perfection V750 Pro scanner, positioned inside a temperature-controlled Plexiglass® housing at 25°C, where scanned images at 2,400 dpi were captured every hour for 24 hours. Using a custom program written in MATLAB (Mathworks Inc., Natick, MA), the location of every nematode at each time point on the images was first manually identified. Thereafter, the program determined X and Y coordinated for each nematode. A nematode was considered moving if it traversed at least 300 μm between successive images, as this distance was found to be an effective minimum threshold for movement in preliminary experiments (Figure 1 A). Using a minimum movement threshold of 300 μm removed small artifacts in the data caused by displacement of heat killed nematodes due to scanner vibrations, nematode settling, or other external factors. The percentage of movement of nematodes in each treatment was calculated at hours 0, 8, 16, and 24 and also averaged over the 24-hour period (Table 1). For each treatment,
populations of 15 to 60 nematodes were analyzed for six replications over 24 hours with two experimental runs.

**Motion analysis of individual nematodes.** Nematode suspensions from each treatment were placed on a microscope slide with coverslip. 10-15 individual J2s of each treatment were observed using bright-field microscopy with overhead lighting (Leica LED 5000 light attached to Leica M205 C stereoscope) at 63x magnification. The motion of each nematode was recorded for one minute with a high-speed camera (QICAM 12-bit color fast 1394), at a frame rate of one image every 0.1 seconds. Videos were captured at 0 and 24 hours after incubation. The videos were analyzed with another custom program written in MATLAB that tracked 13 evenly spaced points along the nematode body (Figure 1 B). In the preliminary experiments, thirteen tracking points were determined to be a sufficient number for *H. glycines* (data not shown); more tracking points are needed with longer nematodes such as *Brugia malayi* (Njus and Pandey 2017). An X and Y coordinate was assigned for each tracking point at successive time points within the software, and these coordinates were used by the program to calculate speed and change in curvature of the nematodes. Speed, in µm/sec, was calculated as the change in distance per unit time between successive images. Curvature, was calculated using the Menger curvature method (Korta et al. 2007) in which curvature is represented as the radius of a circle that passes through three points, centered on the point of interest (Figure 1 B). The change in curvature, in µm⁻¹, was calculated as the change in radius length between successive time points. Lastly, the MATLAB program combined all the tracking points of an individual nematode to produce a contour plot, which illustrated the changes in nematode curvature over time. The motion of at least six individual J2s was analyzed per treatment, and the experiment was conducted twice.
Data analysis. All statistical analyses were completed using JMP Pro 13 (SAS Institute, Cary, NC). Analysis of variance (ANOVA, $P=0.05$) was used to determine significance of effects of factors for each assay. A repeated measures analysis ($P=0.05$) was used to determine significance of effects for the percent movement of the population averaged over 24 hours. When significant treatment effects were detected, means were separated using Tukey’s honestly significance difference (HSD, $\alpha=0.05$) test. The means tested were abamectin concentration, time, body location (individual motion analysis only), and experimental run. For the motion analysis of individual nematodes, these effects were calculated for both the movement parameters, speed and change in curvature.

Results

Movement analysis of nematode populations. When averaged over the 24 hours, the percentage of movement of heat-killed J2s was 1.6 percent compared to the 42.1 percent movement in water and 43.0 percent in 0 $\mu$g/ml abamectin. The highest concentration of abamectin (100 $\mu$g/ml) significantly reduced nematode movement to 1.9 percent of the population, about a 95 percent reduction compared to the observed movement of the population incubated in water. The lowest concentrations of abamectin (0 $\mu$g/ml and 0.1 $\mu$g/ml) did not reduce population movement relative to the water treatment (Table 1). J2s in all treatments displayed varied movement at the beginning of the experiment then movement became more stable over the final 10 hours (Figure 2).

Motion analysis of individual nematodes. The motion of individual $H. \text{glycines}$ J2s was illustrated in contour plots with data from representative J2s, selected arbitrarily, from each
treatment (Figure 3 A-G). In these plots, the change in curvature was plotted for all 11 possible sets of tracking points over the 60-second observation period. An active worm had shorter wavelengths and higher frequencies of motion, as depicted by recurring vertical bands of shorter widths (e.g. in control). In contrast, an inactive worm had longer wavelengths and lower frequencies or motion represented by wider or non-changing bands (as shown in higher abamectin concentrations). Higher numbers of vertical lines throughout a contour plot indicated more changes in curvature. In the lowest abamectin concentrations (0 μg/ml and 0.1 μg/ml), the nematodes exhibited multiple changes in curvature, similar to that in the water control. The J2s incubated in the highest abamectin concentration (100 μg/ml) exhibited no change in curvature, similar to the heat-killed negative control.

The pattern of the contour plots revealed that most of the nematode motion occurred at the head and tail tracking points, therefore these two points were further analyzed quantitatively. Average speed of motion and change in curvature were both affected by abamectin concentration and varied by body location, time of image capture, and experimental run (data not shown). The average speed of the head and tail tracking points for the heat-killed treatment was 0.3 μm/sec and 0.2 μm/sec, respectively. The average speed of the water treatment was 4.8 μm/sec and 3.2 μm/sec for the head and tail, respectively. As the concentrations of abamectin increased to 10 and 100 μg/ml, speed of nematode motion was reduced across both body locations at 0- and 24-hour observation times (Figure 4 A and B, respectively) to levels similar to that of the heat-killed control nematodes. Similar results were obtained with change in curvature data collected at both 0 and 24 hours (Figure 5 A and B, respectively). The average change in curvature for the heat-killed treatment was $1.6 \times 10^{-4}$ and $1.5 \times 10^{-4}$ μm$^{-1}$ for the head and tail, respectively, whereas change in curvature at these points on the nematode body in the water treatment was $1.98 \times 10^{-3}$
and 7.6 x 10^{-4} \mu m^{-1} for the head and tail, respectively. The lowest concentrations of abamectin (0 and 0.1 \mu g/ml) did not affect nematode speed and curvature; motion of the head and tail was similar to the water control. Motion of the head tracking point was significantly different from the tail when measured for both speed or change in curvature across all treatments.

**Discussion**

Determining the response of plant-parasitic nematodes to management compounds often is a time-intensive, low-throughput task. Assessing movement and motion based on observing nematodes through a microscope yields a single, binary “moving or not moving” observation. Also, it is difficult to distinguish subtle differences in nematode behavior through visual observation. Manual observation methods are labor intensive and can be prone to inter-operator variability and error from operator fatigue, resulting in false positive and false negative assessments. Therefore, there is a need to develop computer-based nematode observation techniques to accurately assess and quantify the behavior of plant-parasitic nematodes. Two new methods were developed that objectively quantify plant-parasitic nematode behavior through movement analysis of nematode populations and motion analysis of individual nematodes.

Movement analysis of nematode populations measured the percentage of individual J2s in a population of nematodes that have moved between each observation. The sensitivity of this technique was validated by comparing percentage of movement of nematode populations treated with log-scale dilutions of abamectin. As the concentration of abamectin increased, the percentage of movement of the nematode populations from each treatment decreased (Figure 2). The two highest concentrations of abamectin (10 \mu g/ml and 100 \mu g/ml) inhibited percentage of
movement of *H. glycines* J2s to levels similar to that of the heat-killed, negative control. Moderate decreases in movement also were observed at the intermediate 1 μg/ml abamectin concentration. These results indicate that high concentrations of abamectin completely inhibit nematode movement, which indicates nematode death. However, intermediate concentrations of abamectin may have sub-lethal effects, by slowing down movement without killing the nematode. Additional sub-lethal effects can be difficult to quantify by manual techniques but can be computed by computer-based tracking techniques.

During the first few hours of the experiment, increased movement was observed from nematode populations in all treatments. After a few hours, the movement of the nematodes gradually decreased and remained relatively consistent for the remainder of the observation period. The increase in movement can be attributed to two factors, the delay in effects of abamectin and settling of the nematodes in the semi-solid medium, 11.5 percent pluronic gel, used in the experiment. Using a concentration of pluronic gel lower than the commonly used 23 percent (Sasaki-Crawley et al. 2012) allowed for increased visibility of nematodes in the scanned images, however, the semi-solid media allowed the nematodes to sink down from the surface until they settled on the bottom of the wells.

If a similar experiment were conducted with a range of abamectin concentrations using manual observation methods, the task would be immensely time consuming and only a fraction of the number of experimental observations and replications could be conducted within the same timeframe. Automating this process with the high-resolution scanner and custom software increased the productivity potential of such experiments while also adding a level of quantitative data that could not be previously estimated. Observations of nematode movement over a 24-hour period, rather than a single observation, also allows the researcher to detect the duration of
effects of a nematicide, to determine if there are any quenching effects of the treatment or time-delayed responses. In the experiments described herein, abamectin had an immediate and lasting effect at higher concentrations (10 μg/ml and 100 μg/ml) on *H. glycines* J2s without recovery of nematode mobility.

Motion analysis of individual nematodes measured microscopic changes in speed and in curvature of *H. glycines* J2s. The sensitivity of this technique was determined by measuring differences in nematode motion at 13 different body locations every 0.1 seconds following exposure to various concentrations of abamectin. Plotting whole-body changes in curvature through contour plots (Figure 3 A-G) allowed for visualization of changes in nematode motion over time. Motion, when measured by change in curvature, cascades across the nematode body starting in the anterior or head moving towards the posterior or tail (Lebois et al. 2012). Treatments with lower abamectin concentrations (0 μg/ml and 0.1 μg/ml) and the water control showed waves of changes in curvature moving across the body over time, as represented by thin vertical bands in the contour plots. In contrast, nematodes exposed to higher concentrations of abamectin (10 μg/ml and 100 μg/ml), and the heat-killed negative control, showed no changes in curvature over time, as shown by the lack of vertical bands.

As the concentration of abamectin increased, there was a significant decrease in both the average speed (Figure 4) and average change in curvature (Figure 5) of the nematodes in the experiments at both 0 and 24 hours. The changes in nematode behavior, when analyzed for average speed, corresponded to similar changes in curvature. Observations of motion at different tracking locations along the nematode body enabled comparison of the response of different body areas to a given treatment. Greater changes in motion (both speed and change in curvature) were observed at the head compared to tail for all treatments. This difference in motion of the
head versus the tail likely is due to the presence of chemosensory organs at the anterior portion of the nematode being first affected by the abamectin (Lebois et al. 2012). Measurements were taken at 0 and 24 hours to determine if the effects of abamectin diminish over time. On average, there was a 6 percent decrease in both speed and change in curvature after 24 hours across all treatments. This overall decrease in motion over time can be possible attributed to a variety of factors, such as a time delay in the effects of abamectin within the nematode or a decrease in nematode viability after experimental preparation and handling.

Speed measurements are strong indications of nematode movement, but can be influenced by outside disruptions to the experimental setup, such as table vibrations affecting the microscope/camera stability or changes in the volume of water in the microscope slide. Curvature measurements were additional and complementary to speed measurements in the motion analysis assay. Calculating the change in curvature in conjunction with speed helped determine if any outside influences caused changes in nematode movement. Because nematode movement is the result of contracting and relaxing of dorsal and ventral muscles (Perry and Moens 2013), changes in curvature must be present for movement to occur. If changes in speed occur without corresponding changes in curvature, then movement was most likely due to outside influences, whereas if changes in speed are accompanied by changes in curvature, then the observations of motion were likely true movement of the nematode.

Comparable studies of nematode movement through manual user observation could not yield the same quality and quantity of consistent, unbiased data as studies conducted with these methods. With the aid of computer software, motion analysis of individual nematodes measured and quantified microscopic changes in nematode positioning that likely would otherwise go unnoticed to average human observation. Individual motion analysis has the sensitivity to
measure minute changes in nematode movement, like subtle twitches, enabling the researcher to measure the effects of the target nematicide and determine if any sub-lethal effects exist. This is especially important when studying nematodes such as *H. glycines*, which may enter into a semi-immobile state (Schroeder and MacGuidwin 2010), where they may exhibit minimal movement.

At first, the data observed across abamectin concentrations using both methods were analyzed as continuous variables and were fitted to logistic curves. However, such analyses had low $R^2$ values for their fitted logistic curves (data not shown). Alternatively, the treatments were treated as discrete, categorical variables. And because the treatments were treated as categorical, an effective concentration ($EC_{50}$) or inhibitory concentration ($IC_{50}$) of abamectin could not be calculated. Consequently, this study refers to the optimal concentration of abamectin for nematicidal activity as an effective range.

Both methods described herein provided quantitative data of nematode behavior, but on different scales. Each analysis can be performed independent of the other, but results from both methods were complementary. Movement analysis of nematode populations provided a high-throughput technique to screen multiple treatments and test multiple replications simultaneously. Measuring nematode movement on the population scale allowed for the observation of large numbers of nematodes in a small amount of time and space. This method would be ideal for screening the efficacy of potential nematicides in a research and development setting. Once a candidate nematicide has been identified, motion analysis of individual nematodes would provide information on how the nematicide affected the behavior of the nematode on a single organism level. Results from motion analysis of individual nematodes can then be used to determine the minimum concentration required of the candidate nematicide for lethal or sub-lethal effects by comparing measurements of speed and change in curvature to controls.
There have been several studies on the effectiveness of abamectin on various plant-parasitic nematodes such as *Heterodera schachtii*, *Meloidogyne incognita*, *Pratylenchus zeae*, and *Rotylenchulus reniformis* (Faske and Starr 2006; Cabrera et al. 2009), but no reports about *in vitro* effects of abamectin on *H. glycines*. Using abamectin in both assays described herein not only demonstrated the specificity of each analysis, but also provided the first *in vitro* sensitivity data of *H. glycines* to abamectin. This study demonstrated the effective range of abamectin, with irreversible effects, between 1 and 10 μg/ml, and sub-lethal effects at concentrations as low as 0.1 μg/ml. The *in vitro* lethal range of abamectin on *H. glycines* obtained here was similar to what was reported for *M. incognita* at 1.56 μg/ml (Faske and Starr 2006), but far different from the 32.9 μg/ml reported for *R. reniformis* (Faske and Starr 2006).

These methods may be viable tools for nematicide research and assist in the development of new products for the emerging nematode-protectant seed treatment market. Future work with these methods need not be limited to nematicides, but can incorporate other microorganisms including biological control candidates, root leachates, other nematodes, and soil extracts to develop a better understanding of how the soil environment plays a role in nematode movement.

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Table 1. Movement of *Heterodera glycines* second-stage juveniles after exposure to abamectin concentration during several observation intervals. The numbers presented are mean percentages of movement values. Means were calculated from two experimental runs with six replications per run. Means within the same observation period column followed by the same letter were not significantly different according to Tukey’s honest significance difference test ($\alpha=0.05$).

Denotes a single observation interval during the entire 24-hour experiment. Average indicates the percentage of movement of *H. glycines* J2s, for a given abamectin concentration, averaged over the whole 24-hour observation period.

<table>
<thead>
<tr>
<th>Treatment (μg/ml)</th>
<th>Hour 0&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Hour 8&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Hour 16&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Hour 24&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Averaged&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
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<tr>
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<td>43.04 A</td>
<td>35.3 A</td>
<td>36.96 A</td>
<td>43.02 A</td>
</tr>
<tr>
<td>0.1</td>
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<td>35.38 A</td>
<td>32.7 A</td>
<td>31.18 A</td>
<td>39.01 A</td>
</tr>
<tr>
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<td>5.73 B</td>
<td>10.41 B</td>
<td>9.78 B</td>
<td>9.81 B</td>
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<tr>
<td>10</td>
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<td>2.29 B</td>
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<td>4.99 B</td>
<td>2.81 B</td>
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<tr>
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<td>0.48 B</td>
<td>1.09 B</td>
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</tr>
<tr>
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<td>2.86 B</td>
<td>0.19 B</td>
<td>1.68 B</td>
</tr>
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<td>38.3 A</td>
<td>31.71 A</td>
<td>31.91 A</td>
<td>42.19 A</td>
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</table>
Figure 1. Methods to measure nematode behavior in movement analysis of nematode populations and motion analysis of individual nematodes. A) Preliminary experiments tracked movement of two nematode populations over 24 hours. Circles represent individual nematodes. Blue circles indicate that nematode had moved since the previous observation. Red circles indicate that nematode did not move from since the previous observation. B) Diagram of 13 evenly spaced tracking points (yellow circles) used for motion analysis of individual nematodes. Curvature was measured by calculating the reciprocal of the radius, R, of a circle that passed through three points, the tracking point of interest and its two adjacent points.
Figure 2. Movement of *Heterodera glycines* second-stage juveniles over time after treatment with abamectin. The percent movement of each treatment population was calculated every hour for 24 hours through sequential digital imaging on a high-resolution flatbed scanner. Data includes two experimental runs and 6 replications per run.
Figure 3. Contour plots of whole body change in curvature of *Heterodera glycines* second-stage juveniles after treatment with abamectin. Contour plots track changes in curvature at 11 body locations along the nematode body, over 60 seconds of observation. The frequency of the alternating color in the plot indicates its curvature movements initiated at the head and cascaded down to the tail. An active nematode is represented by shorter vertical bands (shorter wavelength) while an inactive nematode is depicted by broader bands (longer wavelengths). Each plot displays the movement of a single representative *H. glycines* J2 after exposure to an abamectin treatment of 0 μg/ml (A), 0.1 μg/ml (B), 1 μg/ml (C), 10 μg/ml (D), 100 μg/ml (E), heat-killed negative control (F) and water (G).
Figure 4. Speed of motion of *Heterodera glycines* second-stage juveniles after treatment with varying abamectin concentrations. Bars represent average speeds (µm/sec) of the head (black bar) and tail (gray bar) over 60 seconds of observation at 0 (Figure 4A) and 24 (Figure 4B) hours after incubation. The speed of motion was analyzed separately for the head (capital letters) and tail (lowercase letters). Capital letters above the black bars and lowercase letters above the grey bars indicate differences in speed according to Tukey’s honestly significant difference (α=0.05).
Figure 5. Change in curvature of motion of *H. glycines* second-stage juveniles after treatment with abamectin. Bars represent average changes in curvature (1/µm) over 60 seconds of observation at 0 (Figure 4A) and 24 (Figure 4B) hours after incubation. Black bars represent the anterior tracking point (head), gray bars represent the posterior tracking point (tail). The change in curvature was analyzed separately for the head (capital letters) and tail (lowercase letters). Capital letters above the black bars and lowercase letters above the grey bars indicate differences in change in curvature according to Tukey’s honestly significant difference (α=0.05).

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Abstract

Nematicidal seed treatments are a relatively new strategy for managing plant-parasitic nematodes in row crops. Two such seed treatments, Avicta® (abamectin) and Clariva® (*Pasteuria nishizawai*) are marketed by Syngenta® for use against *Heterodera glycines* in soybean production in the upper Midwest. The specific effects of these seed treatments on the biology of the nematode have not been previously studied. The effects of Avicta and Clariva on *H. glycines* hatching, movement, attraction, penetration, development, and reproduction were determined in controlled-environment experiments. Avicta inhibited juvenile movement and penetration at the seed depth and 3 cm below the seed. Clariva inhibited juvenile movement and penetration at 3 and 5 cm below the seed and development within the roots of young plants. Both seed treatments affected nematodes in 10- and 20-day-old plants, but effects developing nematodes in older plants (30 and 60 days) with larger root systems were not detected. These results provide details
of the specific mechanisms of early season protection provided by Avicta and Clariva seed treatments.

**Introduction**

Continued improvement of yield potential through optimization of traits and technology has made seeds the most valuable agricultural input for the soybean farmer. With such a large investment in soybean seed, the agriculture industry has turned its focus to protecting the seed and young seedling against early season diseases and insect damage (Munkvold 2009). Fungicidal, insecticidal, and nematicidal seed treatments are an efficient and cost-effective means to deliver protection against pests and pathogens at the time of planting. Advantages of this approach include a reduced amount of chemical applied per field, continuous early season delivery of chemical within the soil, and fewer non-target effects, among others. The global seed treatment market has grown from $1 billion in 2002 to an estimated $4 billion in 2017 (Munkvold 2014).

Traditional soil-applied granular and fumigant nematicides are environmentally hazardous and cost prohibitive for use in large-scale row cropping (Monfort et al. 2006, Zasada et al. 2010). Nematicidal seed treatments are a welcomed new tool for nematode management in soybean production. In the upper Midwest, the soybean cyst nematode, *Heterodera glycines* Ichinohe, is the principal yield-reducing pathogen of soybeans (Allen et al. 2017). Until a decade ago, the main means of managing *H. glycines* were rotation of non-host crops and *H. glycines*-resistant soybean varieties (Niblack and Tylka 2012). Syngenta Crop Protection (Greensboro,
NC) has developed two nematicidal soybean seed treatments, Avicta® and Clariva®, for managing *H. glycines*.

Abamectin, the active ingredient of Avicta, is a small molecule that was known to have nematicidal effectivity as early as the 1980s (Sasser and Kirkpatrick 1982, Van Gundy 1983, Nordmeyer and Dickson 1985). Due to the low water solubility and short half-life of abamectin (Putter et al. 1981, Bull et al 1984), the compound received little attention in agriculture until it was formulated as a seed treatment. As a broad-spectrum chemical nematicide, abamectin has been shown to have activity against *Pratylenchus zeae, Meloidogyne incognita, Heterodera schachtii*, and *Rotylenchulus reniformis* (Faske 2006, Cabrera 2009, da Silva et al. 2016).

Clariva is a biologically based seed treatment containing spores of *Pasteuria nishizawai*, an obligate, endospore-forming bacterial parasite of *H. glycines* (Sayre 1991). For many years it was not possible to produce large quantities of *Pasteuria* for use in fields for nematode biocontrol. But in 2004, Pasteuria Biosciences succeeded in developing a large-scale fermentation process for *Pasteuria* species (Wilson and Jackson 2013), and subsequently, Syngenta Crop Protection developed and launched the seed treatment Clariva in 2014.

Field-based studies of nematicidal seed treatments are useful to assess if the products affect yields and nematode population densities and to what extent (Barham et al. 2005, Vitti et al. 2014, Tylka et al. 2015, Bissonnette et al. 2018). But such field studies do not reveal specifically how or when a treatment works. To assess the specific benefits of a seed treatment, controlled-environment experiments are necessary. In this paper, a combination of greenhouse and laboratory assays were used to determine details of the effects of Avicta and Clariva on specific activities and life stages of *H. glycines*. 
Materials and Methods

We conducted experiments to assess the effects of Avicta and Clariva seed treatments on nematode hatching, movement, attraction, root penetration, development within roots, and reproduction. The first two methods described immediately below explain how *H. glycines* eggs, second-stage juveniles (J2s), and leachates from soil planted with treated seeds were collected. Each subsequent section describes the experiments conducted to test the effects of Avicta and Clariva seed treatments on an individual life stage or process of *H. glycines*. All seed used in the described experiments was harvested in 2015. Untreated seed were sent to Syngenta personnel in Stanton, MN, where each seed was treated with approximately 0.1 mg of Avicta or 1 x 10⁷ spores of Clariva.

Nematode egg and juvenile preparation. Populations of *H. glycines* used in laboratory and greenhouse experiments were maintained in greenhouse culture pots on *H. glycines*-susceptible soybeans, *Glycine max* cv Williams 82, grown in Fruitfield coarse sand collected from Muscatine, IA, naturally infested with *H. glycines* (HG type 2.5.7). Eggs and second-stage juveniles (J2s) were collected from adult *H. glycines* females exposed on the surface of 30-day-old soybean roots. The adult females were dislodged from soybean roots placed on a 850-µm-pore sieve nested over a 250-µm-pore sieve by spraying the roots with a stream of tap water. The adult females collected on the 250-µm-pore sieve were separated from soil and root debris through sucrose centrifugation using a 1,362 g/L sucrose solution (Jenkins 1964). Eggs were released by crushing the females using a rotating rubber stopper on the surface of a 250-µm-pore sieve (Faghihi and Ferris 2000). The eggs then were collected on a 25-μm-pore sieve nested under a 75-µm-pore sieve. A second round of sucrose centrifugation with 454 g/L sucrose/water solution further separated the eggs from debris (Jenkins 1964). The eggs were rinsed with water
on a 25-µm-pore sieve to remove the sucrose solution and then were used in experiments or were set up for collection of hatched J2s.

To collect J2s, eggs were placed on a 30-µm-pore sieve and submerged in a layer of tap water at 25°C for 72 hours (Wong et al. 1993). Hatched J2s moved through the pore openings in the sieve into the water below. To obtain active J2s for in vitro experiments, hatched J2s that were collected after 72 hours were placed on a 30-µm-pore sieve again and set in a layer of tap water. After one to two hours, the J2s that moved through the sieves were collected in the water below and used for experiments.

**Collection of soil leachate.** In a polystyrene 6-well microplate (Thermo Fisher Scientific Inc., Waltham, MA), a 30-µm-pore sieve containing 6.5 g of sterile 1:1 sand/soil mixture was placed in each well. Next, one seed of *H. glycines*-susceptible soybean (cv Williams 82) treated with either Avicta or Clariva was planted in the soil in each sieve in the 6-well plate. Untreated seed and unplanted (fallow) soil were included as controls. Sterile distilled water (2 ml) was added to each sieve. The plates were covered with lids and placed in an incubator for 24 hours at 25°C in the dark. Next, 2.5 ml of sterile distilled water was added to each sieve, and the leachate that flowed through the sieve was collected in the well below. Soil leachates from five wells with the same treatment were collected and combined into a sterile 50-ml conical tube and stored at 4°C until used. New leachate was collected for each run of the experiments described below.

**Hatching experiments.** In a sterile, polystyrene 6-well microplate, 3 ml of a single treatment solution were added to each well. Treatment solutions consisted of 500 µl of a soil leachate (described above) combined with 2.5 ml of sterile distilled water. Control treatments were 0.1 mg of Avicta solution, 1x10^7 spores of Clariva solution, sterile distilled water, and 5.5mM ZnSO₄, all made to a final volume of 3 ml with sterile distilled water. Next, a 30-µm-pore sieve
was added to the two left-most wells in each well plate. Each row of the 6-well plate served as an experimental unit. Between 300 and 400 *H. glycines* eggs were added to each sieve. The plates were covered with a lid, sealed with Parafilm®, and incubated in the dark at 25°C. The sieves (containing the unhatched eggs) were transferred to the middle wells of the plate after three days and to the right-most wells after seven days. After each transfer, the plates were re-covered, sealed with Parafilm, and incubated in the dark at 25°C. On the 14th day, the remaining contents of each sieve were washed out with water into a clean, empty well of a new plate. The number of J2s that hatched at days 3, 7, and 14 were counted. The number of unhatched eggs from the remnants washed from the sieve on day 14 also was counted. Cumulative percentage hatch was calculated by dividing the total number of hatched juveniles (on days 3, 7, and 14 combined) by the total number of nematodes in the experimental unit (hatched juveniles and unhatched eggs). The experiment was conducted twice with four replications per treatment per run.

**Movement analysis experiments.** Active J2s in 100 μl of water (collected as described above) were incubated in a solution of 500 ml of soil leachate (collected as described above) diluted with 500 ml of sterile distilled water for two hours in 1.5 ml microcentrifuge tubes in the dark at 25°C. Control treatments consisted of 1 ml solutions of 0.1 mg of Avicta solution, 1x10⁷ spores of Clariva solution, sterile distilled water, and heat-killed J2s in sterile distilled water.

To isolate the J2s from the incubation solutions, each treatment was spun at 2,000xg for three minutes in a mini centrifuge (Thermo Fisher Scientific Inc., Waltham, MA) to concentrate the nematodes in a pellet. The top 500 ml of treatment solution supernatant were removed and 500 ml of 545 g/L sucrose solution were added. Each tube was vortexed to resuspend the nematodes with the sucrose solution, then spun again at 2,000xg for three minutes. Clean nematodes were contained in the sucrose supernatant, and debris from the soil leachates were
contained in the pellet. The top 500 ml supernatant was removed, added to a new 1.5 ml microcentrifuge tube, and mixed with 500 ml of sterile distilled water.

Nematodes incubated in leachates of soil planted with treated seeds and in control solutions were analyzed using motion analysis of individual nematodes described by Jensen et al. 2017. J2s in 30 ml of suspension from the treatments described above were placed on a microscope slide with coverslip and observed with overhead light microscopy (Leica LED 5000 light attached to Leica M205 C stereoscope) at 63x magnification (6.3x magnification, 10x eyepiece, and 1x camera). Images were taken of an individual J2 every 0.1 seconds for 60 seconds with a high-speed camera (QICAM 12-bit color fast 1394). Then the images were converted into a video, and individual J2s were subjected to movement analysis using a second, custom MATLAB program that calculated the speed and change in curvature of tracking points along the nematode body. For each treatment, 8-15 J2s were analyzed at the anterior-most tracking point in each of two experimental runs.

**Juvenile attraction experiments.** Active J2s (15 to 60) were added to the center nematode entry port of a microfluidic chemotaxis chip filled with sterile distilled water, as described by Beeman et al. (2017). Soil leachate (30 μl), collected as described above, was added to a treatment port of a randomly assigned chip (1-14), lane (1-4), and side (left or right) combination. Sterile distilled water was used as the control in the opposite treatment port for each lane. Control solutions tested included 0.1 mg of Avicta, 1x10^7 spores of Clariva, 500 mM CaCl2, and 500 mM KNO3. Also sterile distilled water was added to both ports of one lane in each chip to make sure there was no bias for movement of nematodes to one side of the chip or the other. Once the soil leachate treatments and control solutions were added, the chips were placed in an inverted, square, 10 cm petri dish, sealed with Parafilm, then incubated at 25°C in the dark.
After 24 hours, the chips were examined under a stereoscope, and the number of J2s in each side resting chamber (treatment or control) and in the center nematode entry port was counted. The number of J2s in each side of the lane was divided by the total number of J2s in all three areas of the lane to determine percentage. Juveniles found in the treatment resting chamber were considered attracted to the treatment whereas juveniles in the control resting chamber were considered repelled by the treatment. The percentage of juveniles in the treatment resting chamber was compared to the percentage of juveniles in the control resting chamber to determine if there was a significant treatment effect. There were five to six replications per treatment per run, and the experiment was conducted twice.

**Experiments to measure penetration of juveniles at discrete soil depths.** Falcon 50 ml conical centrifuge tubes (Fisher Scientific, Waltham, MA) were inverted to stand on the cap, the conical tips of the tubes were removed, and three 3.175-mm diameter holes were drilled into the sides at 2, 5, and 7 cm from the top. Once fabricated, the tubes were filled with approximately 50 cm$^3$ of sterile 1:1 sand/soil. One seed of *H. glycines*-susceptible soybean cv Williams 82 treated with Avicta or Clariva seed treatment was planted per tube, about 2 cm deep. Untreated Williams 82 and untreated *H. glycines*-resistant soybean cv Jack were planted in other tubes as controls.

Each tube was watered initially with 10ml of tap water. The tubes were arbitrarily arranged in a plastic box then placed in a growth chamber with 16-hour day length, at 50 percent humidity, and at 25°C. Each tube received 5 ml of tap water each day for days two through seven. On the sixth day, approximately 200 active J2s suspended in 150 μl water were pipetted through the pre-drilled holes to the center of the soil column at one of three soil depths (2, 5, or 7 cm), and the tubes were incubated for an additional 24 hours. On the seventh day, the plants were removed from the tubes, and the roots washed free of soil and patted dry. The roots were
separated from the stems at the soil line. The mass of each root system was recorded, roots were frozen for 12 hours at -20°C, and then thawed.

To isolate the penetrated J2s, each thawed root system was cut into 1.5-cm-long pieces and macerated with 75 ml of tap water using a household blender on high for 30 seconds, similar to what was described by Ruan et al. 2012. The macerated mixture was poured through a 250-µm-pore sieve nested over a 75-µm-pore sieve nested over a 25-µm-pore sieve. The root debris in the 250-µm-pore sieve were macerated further by pushing the material through the sieve with a rotating rubber stopper (Faghihi and Ferris 2000). The macerated root mixture was flushed with water through the middle 75-µm-pore sieve, and the released J2s were collected on the bottom 25-µm-pore sieve.

The number of penetrated J2s was counted via microscopic observation and compared among treatments on a per gram of root per basis. A proportion was calculated as the number of penetrated nematodes of a treatment at a set location, for a single replication, per gram of root, divided by the average number of penetrated nematodes at that same location of the untreated Williams 82 control per gram of root, over all replications. After this, the proportion of all untreated Williams 82 treatments was set to 1.0, as previously explained by Halbrendt et al. 1992. Three to four replications were planted for each treated seed and control seed for each run of the experiment, and the experiment was conducted twice.

**Experiments to measure total juvenile penetration and development.** Cone-tainers with a volume of 150 cm³ were filled sandy soil naturally infested with *H. glycines* with an initial egg density of 1,500 eggs per 100 cm³. One seed of Williams 82 treated with Avicta or Clariva seed treatments was planted in each cone-tainer, about 2 cm deep. Seeds of untreated Williams 82 or untreated Jack were planted as controls. Plants were grown in a growth chamber in 16-hour day
length, at 50 percent humidity, at 25°C, and watered with 20 ml of tap water every 2 days for 10, 20, or 30 days.

On the designated sample dates, plants were removed from the cone-tainers and their root systems were carefully dipped in water to remove soil. The stems were removed from the roots at the soil line and discarded, the roots were patted dry, and root mass was recorded. The 30-day-old roots were placed on a 850-μm-pore sieve nested over a 250-μm-pore sieve and were sprayed with water to dislodge adult females that were exposed on the root surface. The females were stored in labeled 50 ml conical tubes. Individual root systems were frozen for 12 hours at -20°C then thawed and cut into 1.5-cm-long segments, added to 75 ml of tap water in a household blender, and pulsed on high 10 times then blended on high for 30 seconds. The macerated mixture was poured through a stack of sieves with pore sizes 850, 250, 150, 75, 45, and 25 μm. Each sieve separated developmental stages of *H. glycines* due to differences in nematode size among the life stages (Raski 1949). Adult females were captured on 250-μm-pore sieves, fourth-stage juveniles on 150-μm-pore sieves, third-stage juveniles on 75- and 45-μm-pore sieves, and second-stage juveniles 25-μm-pore sieves.

The top 850-μm-pore sieve was flushed with water 10 times, removed from the stack, and its contents discarded. The second sieve, with 250 μm pores, was flushed with water 10 times and the contents were transferred to a 50-ml conical tube. Each subsequent sieve was flushed with water 10 times, then removed from the stack, and its contents transferred to a labeled conical tube. Overall, the nematodes contained within a root were collected in five different tubes based on the sieves on which the macerated mixture was captured. The nematodes obtained from each sieve were then counted under a stereoscope with overhead lighting. Adult females
previously collected from 30-day-old roots were combined with the contents recovered from the 250-μm-pore sieve of the same root system, and these adult female nematodes were counted.

Some of the adult vermiform males may have left the roots and moved into the soil for mating, therefore it was not possible to get a complete count of the number of adult males, and consequently, males were excluded from the tallies. The presence of root debris similar in size made it necessary to collect J3s on sieves of two different size. Therefore, the number of nematodes on the 75- and 45-μm-pore sieves were combined. The number of nematodes in a specific life stage was divided by the total number of nematodes found in the entire root system to give a percent distribution of each developmental stage. These percentages then were converted to a proportion, given as the percentage of nematodes in a given life stage, for a single replication (root system), divided by the average percentage of nematodes in the same life stage for all replications of the untreated Williams 82 control. The proportion for all untreated Williams 82 treatments was set to 1.0, as previously explained by Halbrendt et al. 1992. Five replications were planted for each treatment and control in each run of the experiment, and the experiment was conducted twice.

**Female maturity and reproduction experiments.** Small and large cone-tainers (150 cm³ and 600 cm³, respectively) were filled with Fruitfield coarse sand collected from Muscatine, IA, that was naturally infested with *H. glycines* with an initial egg population density of 1,500 eggs per 100 cm³ of soil. One seed of either Jack or Williams 82, treated with either Avicta or Clariva, was planted about 2 cm deep. Untreated Jack and Williams 82 were planted as controls. One cone-tainer of each treatment was arbitrarily placed in an 11-L bucket. Small and large cone-tainers were planted in separate buckets. Construction sand was added to the fill the spaces
around the cone-tainers. The buckets were placed in a water bath kept at 25°C inside a
greenhouse.

Each cone-tainer was watered with tap water until the soil was kept saturated every day
for the first five days, then every two days until the end of the experiment. Small cone-tainers
were grown for 30 days and large cone-tainers were grown for 60 days. After 30 days, plants
were removed from the small cone-tainers and the roots were carefully dipped in water to
remove soil adhered to the roots. Each root system was placed onto a 850-μm-pore sieve nested
over a 250-μm-pore sieve. With a stream of water, adult females exposed on the root surface
were removed from the root and collected on the 250-μm-pore sieve. These females were stored
at 4°C until counted. Root systems were separated from stems at the soil line and patted dry, and
the mass of each root system was recorded.

After 60 days, the contents of the large cone-tainers were emptied one by one into clean
11-L buckets. Each bucket was filled with 1L of water and the root systems were carefully
dipped repeatedly to remove soil adhered to the roots. Females were removed from roots as
described above, stored in labeled containers, and the mass of each root system recorded. Cysts,
the dead *H. glycines* females, were extracted from the soil in the bucket. The soil-water mixture
in the bucket was stirred clockwise at a constant speed for 20 seconds then let sit for 20 seconds.
Next, the suspension was poured through a 850-μm-pore size nested over a 250-μm-pore sieve
(Gerdemann 1955). Another 1L of tap water was added to the bucket and the process was
repeated once more. Cysts from the soil were collected from the 250-μm-pore sieve and stored at
4°C until counted.
Using a stereoscope with overhead lighting, the number of females and cysts (for the 60-day experiment) were counted for each plant. Next, the females and cysts were crushed using a rotating rubber stopper (Faghihi and Ferris 2000) to release the eggs. Eggs collected on a 25-μm-pore sieve were stained acid fuschin (Niblack et al. 1993) and counted.

The number of females and eggs from a single replication was divided by the mass of its root system to standardize nematode numbers for differences in root growth across replications. The number of females per gram of root, number of eggs per gram of root, and number of eggs per female per gram of root were compared for all treatments and controls. For both the 30- and 60-day experiments, there were six replications of each treatment per run with two experimental runs.

Data analysis. All statistical analyses were completed using JMP Pro 13 (SAS Institute, Cary, NC). Analysis of variance (ANOVA, P=0.05) with a fit model analysis, where treatment and experimental run were included in the effect tests, was used to determine significance of effects of treatment solutions for hatching, movement analysis, total juvenile penetration, and reproduction experiments. Significant treatment means were separated using Tukey’s honestly significant difference test (HSD, α=0.05). The means tested included percent hatch for the hatching experiment; speed and change in curvature for movement analysis experiments; nematodes per gram of root for total juvenile penetration experiments; and variety and treatment for reproduction experiments. A paired T-test was used to determine significant differences (P<0.05) between percent attraction or repulsion to a given treatment in attraction experiments. Proportions for both the spatial penetration and juvenile development experiments were compared against the null hypothesis (proportion is equal to 1.0) through a T-test with α=0.05.
Results

**Hatching experiments.** An average of two percent of the hatched juveniles in all treatments by day 3. Hatching increased in many, but not all, treatments by day 7. Similar hatch was observed on day 14 for all treatments except ZnSO₄ (Figure 1A). Cumulative percentage hatch was greatest in ZnSO₄, at 40 percent, and was lowest in Avicta solution, at 5 percent (Figure 1B). There were no significant differences in cumulative percent hatch of juveniles from eggs in leachates of soil planted with treated or untreated seeds compared to the fallow and water controls.

**Movement analysis experiments.** The movement of the head of individual *H. glycines* J2s was analyzed for two parameters, speed and change in curvature (Figure 2). The average speed of J2s incubated in water was 1.67 μm/sec and 1.39 μm/sec in leachate of fallow soil. There were no significant differences in J2 speed of movement in leachates of soil planted with untreated Williams 82, fallow soil, and the water control. Speed of movement of the J2s was significantly less in leachates of soil planted with seeds treated with either Avicta or Clariva than the water control. Similar treatment effects occurred with change in curvature. The average change in curvature for J2s incubated in water was 0.0011 μm⁻¹ and in leachate of fallow soil was 0.00081 μm⁻¹. There were no significant differences in change in curvature of J2s between those in the water control and in leachates of soil planted with untreated Williams 82 seed and fallow soil, but change in curvature was significantly less in leachates of soil planted with seeds treated with Avicta and Clariva compared to the water control.

**Juvenile attraction experiments.** Overall, 8 to 13 percent of the juveniles in the experiment moved either towards or away from the treatments. There was significant attraction of J2s
towards the KNO₃ positive control, at 15 percent. Also, there was significant repulsion of J2s from the CaCl₂ negative control, at 7 percent. There were similar numbers of nematodes in the left and right sides of chips when water was used in both ports of the same lane. And there were no significant differences in J2 attraction or repulsion for any of the soil leachate treatments or control solutions, with eight percent or less movement of J2s towards or away from any specific treatment (Figure 3).

**Experiments to measure penetration of juveniles at discrete soil depths.** Of the approximately 200 J2s introduced into the soil at each depth, 7 to 13.5 percent penetrated the untreated Williams 82 control. The average number of nematodes in the roots of the untreated control, by depth, was 14, 18, and 27 for 2, 5, and 7 cm, respectively. The highest number of J2s that penetrated a single plant was 59, and some plants had no nematodes penetrated. Significantly fewer J2s penetrated roots of seedlings grown from Avicta- or Clariva-treated seeds than roots of untreated Williams 82 when inoculated at the seed depth, which was 2 cm below the soil surface (Figure 4). Similar significant reductions in penetration were observed when J2s were inoculated at 5 cm deep, which was 3 cm below the seed. At 7 cm deep, or 5 cm below the seed, there were significantly fewer J2s in roots of seedlings grown from Clariva-treated seeds relative to those in the untreated Williams 82 roots, but not so for the Avicta treatment.

**Experiments to measure total juvenile penetration and development.** On average there were 245, 397, and 334 nematodes per gram of root in plants harvested at 10, 20, and 30 days, respectively. There were no differences in the total number of nematodes or the number of nematodes per gram of root among treatments (data not shown). That is, the total number of nematodes per root were not significantly different among Williams 82 treated with Avicta, Clariva, or left untreated, or untreated Jack.
The proportion of *H. glycines* developmental stages in roots grown from treated seed was compared to that in untreated Williams 82 at days 10, 20 and 30. After 10 days, the average number of nematodes on the untreated control at the J2 and J3 life stages was 87 and 156, respectively. There were significantly more (greater proportion) J2s present and significantly fewer J3s on Clariva-treated plants compared to the untreated control (Figure 5A). There were no differences in nematode development on Avicta-treated plants compared to the untreated control 10 days after planting. At 20 days, the average number of nematodes in the J2, J3, J4, and adult female stages for the untreated control was 20, 457, 147, and 63, respectively, and there were significantly more J3s and significantly fewer adult females on Avicta- and Clariva-treated plants compared to the untreated control (Figure 5B). After 30 days, the average number of nematodes at the J2, J3, J4, and adult female stages was 54, 122, 84, and 242, respectively, in the untreated control. There were significantly fewer J2s on both Avicta- and Clariva-treated plants than in untreated Williams 82, but similar proportions of nematodes at later life stages (Figure 5C). Overall, slower development was observed in Avicta-treated roots at 20 days and in Clariva-treated roots 10 and 20 days after planting. Differences in proportions of nematodes in different developmental stages were not detected 30 days after planting.

**Female maturity and reproduction experiments.** There were significant differences in numbers of *H. glycines* adult females and eggs between Jack (*H. glycines*-resistant) and Williams 82 (susceptible) varieties; therefore, each variety was analyzed separately. The average number of adult females on the untreated Williams 82 control at 30 and 60 days after planting was 136 and 397 females per gram of root, respectively. There were no differences in number of females per gram of root in either Avicta or Clariva treatments for either variety at 30 or 60 days (data not shown). The average number of adult females per gram of root on 30-day-old plants was 116
and 119 for Avicta and Clariva treatments on Williams 82, respectively. After 60 days, the average number of adult females was 472 and 427 for Avicta and Clariva treatments on Williams 82, respectively.

When standardized to plant root mass, the average number of eggs per gram of root on the untreated Williams 82 control was 16,232 and 79,648 for 30 and 60 days, respectively. There were no differences in the number of eggs per gram of root between treatments for either variety at 30 and 60 days (data not shown). The average number of eggs after 30 days on Williams 82 was 17,730 and 18,323 for Avicta and Clariva treatments, respectively. After 60 days, the average number of eggs per gram of root was 82,139 and 68,595 on Avicta and Clariva-treated Williams 82, respectively. The number of eggs per female per gram of root mass for the untreated Williams 82 control was 62 and 53 at 30 and 60 days, respectively. There were no differences among treatments for either variety at 30 and 60 days.

**Discussion**

The seed treatment Avicta significantly affected the most vulnerable life stage of *H. glycines*, the J2, which mostly exists outside the protection of the soybean root. Significant effects were observed on J2 movement and penetration at several soil depths. In previous experiments, we found that *H. glycines* was sensitive to abamectin at concentrations of 1 to 10 μg/ml (Jensen et al. 2017). A similar concentration of 1.56 μg/ml was reported to paralyze *M. incognita* (Faske and Starr 2006). When J2s were incubated in leachate from soil planted with Avicta-treated seed, movement was significantly inhibited. This inhibition likely also occurred when J2s were inoculated on seedlings grown from Avicta-treated seed. Significantly fewer J2s
penetrated roots when the nematodes were added near or 3 cm below the planted seed compared to roots untreated Williams 82. However, at 5 cm below the planted seed, there was no difference in J2 penetration on Avicta-treated plants compared to roots grown from untreated seed. These results are consistent with results of penetration of *M. incognita* and *R. reniformis* on roots of abamectin-treated cotton (Faske and Starr 2007). Because abamectin has low solubility in water and high affinity for organic matter (Wislocki et al. 1989), it is probable that as a seed treatment, most of the active ingredient will remain with the seed coat. It is likely that only small amounts of abamectin move with roots deep in the soil, providing minimal protection from *H. glycines*.

If the effects of Avicta are limited to the upper 3 cm or so of root systems, this may explain why minimal or no effects were observed in experiments where Avicta-treated plants were grown for 30 days or more. Although the penetrated nematodes developed slower in treated versus non-treated plants 20 days after planting, delayed juvenile development was not detected at 30 days. Avicta also had no effect on the number of adult females or eggs produced on plants grown for 30 and 60 days in our experiments. The 30- and 60-day time frames were enough time for one and two generations of nematodes to occur, respectively. Given the short half-life and limited mobility of abamectin, it is possible that Avicta did not protect larger root systems of plants 30 days or older under our experimental conditions.

We found Avicta to have no effect on hatching of *H. glycines*. That is, hatching in leachates of soil planted with Avicta-treated seed was not significantly different than hatching in the water control. The egg shell of *H. glycines* may act as a barrier, protecting developing juveniles from the effects of abamectin, or perhaps the developing juveniles in the eggs may not
yet have fully developed nervous systems for the abamectin to affect. Also, perhaps the leachates we used were too dilute, leading to no observed effects.

Clariva contains spores of *P. nishizawai* that attach to the cuticle of the nematode J2, so it was not unexpected that we saw no effect of Clariva seed treatment on hatching. Like Avicta, Clariva seed treatment significantly affected *H. glycines* J2 movement, penetration success of J2 near or 3 and 5 cm below the treated seeds, and development in young plants 10 and 20 days after planting. The observed decrease in J2 movement after incubation in leachate of soil in which Clariva-treated seeds were planted implies that spore attachment may interfere with nematode mobility. A decrease in J2 mobility also may explain why significantly fewer nematodes penetrated roots grown from Clariva-treated seeds compared to roots from untreated Williams 82 seeds at all three depths studied (0, 3, and 5 cm below the planted seed). The fact that Clariva reduced nematode penetration at 5 cm below where the treated seed was planted in the soil suggests that the active ingredient may be mobile in the soil. If so, *P. nishizawai* would encounter more nematodes to infect than if it were immobile. The observed decrease in rate of development of *H. glycines* in young plants 10 and 20 days old is most likely due to the penetrated J2s being infected with *P. nishizawai*.

There was no difference in the rate of development of *H. glycines* in roots of plants grown from Clariva-treated seeds compared to the untreated control at 30 days after planting or later. Likewise, there was no observed difference in the total number of penetrated juveniles on whole plants, number of adult females, or number of eggs in these older plants. The lack of effects of Clariva seed treatment in root systems of older plants in our experiments may be attributed to several factors. Environmental conditions in our experiments may have affected the development of *P. nishizawai* within the nematode. Also, not all *H. glycines* hatch at the same
time (Niblack et al. 2006), so perhaps a disproportionately high number of Clariva spores attached to the first hatched J2s entering roots, leaving less of an impact on J2s that did not hatch right away. These later-hatching J2s may have been successful at parasitizing the roots.

Antagonistic effects of nematicidal seed treatments that repel nematodes from roots could add another layer of protection. In experiments using microfluidic chemotaxis chips, we found that Avicta and Clariva seed treatments neither attracted or repelled *H. glycines* J2s. This was lower overall movement of the J2s in our attraction experiments (8 to 13 percent) than Beeman et al. (2016) reported (32 to 52 percent). So it is possible some treatment effects may have been missed due to lower movement of the J2s in our own studies. Still, J2s in the control treatments in our experiments were attracted to KNO₃ and repelled from CaCl₂ just as reported by Beeman et al. (2016), indicating that our assays were valid. It is likely that Avicta may not diffuse through the microfluidic filters into the lanes of the chemotaxis chips given the insolubility of abamectin in water, resulting in no effect. And spores of *P. nishizawai* are on average 1.9 μm wide (Atibalentija et al. 2004) and most likely did not pass through the 1 μm width of the filters from the treatment ports into the resting chambers of the microfluidic chips to affect the J2. Additionally, it is unknown if spores of *P. nishizawai* secrete any exudates that may have been able to diffuse into the lanes of the chemotaxis chip.

Conceptually there are at least three possible scenarios that explain how nematode-protectant seed treatments may adversely affect nematode biology: 1) they may reduce the number of nematodes that enter a root but not affect their development after infection, 2) they may affect nematode development within a root but not the number of nematodes that enter a root, or 3) they may affect both nematode penetration and development. Our experiments suggest that Avicta was most effective at reducing the number of nematodes that penetrated roots of
younger plants, but had no effect on development (scenario 1). Clariva reduced the number of nematodes that entered into the roots and also affected development up to 20 days after planting (scenario 3). Neither seed treatment, however, had effects on the nematode in older plants with larger root systems. To fully understand the effectiveness of Avicta and Clariva seed treatments, more research is needed on the interactions with different environments and the presence of other pathogens.

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Figure 1. Hatching of *Heterodera glycines* in leachates of soil planted with Avicta- and Clariva-treated and untreated Williams 82 (W82) soybean seeds, leachate of unplanted soil, and control solutions. The number of second-stage juveniles hatched was counted at 3, 7, and 14 days (A),
and cumulative percentage hatch was calculated at day 14 (B). Letters above each bar indicates differences in cumulative percentage hatch according to Tukey’s honestly significant difference test (α=0.05).
Figure 2. Movement of *H. glycines* second-stage juveniles (J2s) after exposure to leachates of soil planted with nematicide-treated soybean seeds was measured by speed (black bars) and change in curvature (white bars) over 60 seconds of observation. The speed was analyzed separate from the change in curvature. Letters above each bar indicate differences among speed (ABC) or change in curvature (XYZ) according to Tukey’s honestly significant difference test ($\alpha=0.05$).
Figure 3. Percentage of *H. glycines* second-stage juveniles (J2) that moved towards (attraction) or away from (repulsion) leachates of soil planted with nematicide-treated soybean seeds. Experiments in chips were validated by observing movement in response to leachates of soil planted with untreated seed (W82) and unplanted soil (fallow), KNO$_3$ (positive control), and CaCl$_2$ (negative control). Black bars pointing right indicate the percentage of nematodes in a population that were attracted to a given treatment. Grey bars pointing left indicate the percentage of nematodes in a population that were repelled by that treatment. An asterisk next to a bar indicates significant attraction to or repulsion from the treatment according to a paired T-test ($\alpha$=0.05).
Figure 4. The number of penetrated *H. glycines* second-stage juveniles (J2s) on seedlings grown from a nematicide-treated seed, was counted 24 hours after inoculation at depths of 2, 5, and 7 cm below the soil line. Treatments tested were Avicta- and Clariva-treated Williams 82 seeds denoted as W81 + Avicta, W82 + Clariva, respectively. Control treatments included seeds of untreated Williams 82 (W82 untreated) and Jack (Jack untreated). This number was then compared to the average number of penetrated J2s on seedlings of untreated Williams 82, yielding a proportion of penetrated J2s. The proportion of J2s at each depth was calculated by dividing the number of J2s per treatment, per gram of root by the average number of penetrated J2s per gram of root of the untreated Williams 82 control. Asterisks indicate a treatment is significantly different from the null hypothesis that the proportion is equal to 1 (the same as the untreated Williams 82 control) according to a T-test with α=0.05.
Figure 5. The proportion of *H. glycines* in each developmental stage in roots of plants grown from nematicide-treated and untreated seeds at 10 (A), 20 (B), and 30 (C) days after planting.
The developmental stages identified were second-stage juveniles (J2), third-stage juveniles (J3), fourth-stage juveniles (J4), and adult females. The number of nematodes in each developmental stage was compared to the untreated Williams 82 control, yielding a proportion of nematodes in that stage, for that treatment. The proportion was calculated by dividing the percentage of nematodes in a given developmental stage by the average percentage of nematodes in that same stage on the untreated Williams 82 control. Asterisks indicate a treatment is significantly different from the null hypothesis that the proportion is equal to 1 (the same as the untreated Williams 82 control) according to a T-test with $\alpha=0.05$. 
CHAPTER 4. GENERAL SUMMARY

The focus of this doctoral research was twofold. The first was to develop new methods to study the biology of *Heterodera glycines*. The second was to use these new methods and traditional methods in laboratory and greenhouse assays to study the effects of Avicta® and Clariva® nematicidal seed treatments on the biology of *H. glycines*. In the first research chapter (dissertation chapter two), two new methods to quantitatively measure multiple behavioral parameters of individuals and populations of *H. glycines* are described. In the second research chapter (dissertation chapter three), the effects of Avicta and Clariva seed treatments on individual life stages and processes of *H. glycines* were studied.

Historically, nematode movement in response to stimuli has been analyzed by human observation under light microscopy and lack of movement is assumed to reflect nematode mortality. Such studies can yield only a single, qualitative observation: movement or lack thereof. In contrast sequential digital imaging of nematodes has the ability to provide data-intensive, quantitative measurements of nematode behavior in a semi-automated manner.

Two movement analysis methods were developed to study the effects of abamectin on *H. glycines*. The first method employed a high-resolution flatbed scanner that automatically recorded images of populations of second-stage *H. glycines* juveniles (J2s) every hour for 24 hours. Comparing the locations of nematodes from one image to the next determined the percentage of the population that had moved. Using this method, significant inhibition of movement of populations of *H. glycines* by three concentrations of the nematicide abamectin (1, 10, and 100 μg/ml) was detected. In the second method, videos of individual *H. glycines* J2s incubated in a range of concentrations of abamectin were recorded with overhead-light
microscopy. Computer analysis of the videos measured movement parameters for 13 evenly spaced tracking points along the nematode body. Analysis of selected points near the head, middle, and tail of the juvenile worms revealed that speed and changed in curvature were significantly reduced in 10 and 100 μg/ml of abamectin. Both analysis methods had sufficient sensitivity to measure subtle differences in nematode movement, allowing for assessment of both lethal and sub-lethal effects of various concentrations of abamectin. The validation of the two methods allowed for their use to study the effects of the nematicidal seed treatments Avicta and Clariva on nematode behavior.

Large-scale, field studies of nematicidal seed treatments primarily measure the effects on yield and season-long nematode reproduction. This information is critical to help farmers make decisions on what products to use in nematode management programs, but such research does not determine how and when nematicidal seed treatments affect H. glycines. Greenhouse and laboratory studies are a necessary complement to determine the effects of seed treatments on nematode biology. Such controlled experiments were conducted to determine the effects of Avicta and Clariva nematicidal seed treatments on specific life stages and processes of H. glycines. The stages and processes studied included juvenile hatching, juvenile movement, chemotaxis, penetration, development, and female reproduction.

Avicta seed treatment, with active ingredient abamectin, inhibited nematode movement and root penetration. Clariva seed treatment, with spores of Pasteuria nishizawae, inhibited juvenile movement, root penetration, and development in younger plants. Both treatments were most effective at affecting H. glycines outside the root and protecting young plants (10 to 20 days). Older plants (30 to 60 days) had little to no nematode protection from either seed treatment. The methods used in these studies controlled outside influences (to the extent
possible) to study the interaction of the seed treatments with *H. glycines*. Such control allowed for the study of how the seed treatments may work against the nematode, but may not directly translate to how they function in the field setting.

The emergence of nematicidal seed treatments has opened new opportunities for the economically feasible use of nematicides in large-scale row-crop production fields in the Midwest. The addition of a nematicidal seed treatment to current nematode management practices may help slow down *H. glycines* adaptation to PI 88788 source of resistance in soybeans. Controlled-environment greenhouse and laboratory studies are a necessary complement to field-based experiments that reveal the specific effects of the nematicide on nematode biology and the efficacy of the active ingredient in the soil. Although the effects of either Avicta or Clariva seed treatment were not long-lasting through the nematode life cycle, the results from these studies provide evidence for early-season protection from *H. glycines*. Such limited protection may still be enough to allow strong stand establishment and higher yield for the Iowa farmer.