

Investigating Population Dynamics of *Hoplolaimus galeatus* and Select
Associated Relationships in Creeping Bentgrass Putting Greens

Matthew Aaron Tucker

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David S. McCall, Chair
Jonathan D. Eisenback
David C. Haak
William T. Crow

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Academic abstract

Hoplolaimus galeatus, a prevalent lance nematode, is increasingly problematic in U.S. turfgrass due to product restrictions and ineffectiveness. These studies aim to quantify the relationship between creeping bentgrass health and nematode populations, and to evaluate a qPCR diagnostic method. Plant growth regulators (PGRs) are used to optimize turfgrass growth and reduce management inputs, but anecdotal evidence suggests a link between increased PGR-induced phytotoxicity and high populations of lance nematodes. One study investigated the relationship between PGR usage and lance nematode populations on creeping bentgrass putting green health during summers in the Mid-Atlantic United States. Five levels of PGR and the presence or absence of fluopyram were studied across six site-years. Data suggests that elevated lance nematode populations typically do not exacerbate damage to creeping bentgrass caused by PGR applications, though injury in plots treated with prohexadione calcium was more pronounced with high lance nematode populations in some instances. Management of lance nematodes on creeping bentgrass often relies on lab assay recommendations but counts exceeding thresholds do not always coincide with visible damage. A greenhouse study examined the relationship between lance nematode populations and creeping bentgrass root biomass using inoculated nematode populations and varying levels of nitrogen to promote root growth. Increased nematode populations reduced root biomass ($r = -0.56322$), though increasing nitrogen inputs led to higher nematode counts without affecting root biomass. This relationship suggests the need for a modified root biomass-based threshold, though further evaluations and improved quantification techniques are warranted. Methods using qPCR were adapted to identify and quantify lance nematodes in golf course putting greens. Manual counts of lance nematodes from field plots were compared to qPCR cycle threshold values, showing a weak negative relationship ($r = -0.39956$) between DNA quantity and these counts. This relationship was improved by refining sources of error using handpicked samples of 20, 50, 100, and 250 lance nematodes ($r = -0.75695$). Collectively, these studies enhance our understanding of lance nematode and creeping bentgrass putting green dynamics. Lance nematodes typically did not negatively impact PGR usage, except when prohexadione calcium was used on stressed creeping bentgrass with lance populations. Our data reiterates that high lance populations negatively influence root biomass, and that adding nitrogen may increase these populations.

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General audience abstract

This dissertation investigates the complex interactions between plant growth regulators (PGRs), lance nematodes (*Hoplolaimus galeatus*), and creeping bentgrass (*Agrostis stolonifera* cv. 'L93') putting green health. The study is divided into three main chapters, each addressing different aspects of these interactions. The first chapter explores the relationship between PGR usage and lance nematode populations on creeping bentgrass putting green health during summers in the Mid-Atlantic United States. A 5x2 factorial design was employed, examining five levels of PGR and the presence or absence of fluopyram across two seasons. The results indicate that while PGR applications and elevated lance nematode populations generally do not contribute to a decline in turf quality, specific interactions between prohexadione calcium and lance nematode presence were observed, suggesting limited damage under certain conditions. The second chapter adapts quantitative polymerase chain reaction (qPCR) for the identification and quantification of lance nematodes in golf course putting greens. Comparing manually counted nematode populations with qPCR results from samples collected at Belmont Golf Course revealed a statistically significant but weak negative relationship. Refinements in the qPCR method improved accuracy, indicating its potential as a viable alternative for nematode quantification, though further refinement is necessary for broader implementation. The third chapter examines the relationship between lance nematode populations and root biomass of creeping bentgrass. A 4x5 factorial design was used to evaluate the effects of varying levels of urea nitrogen and nematode populations over 16 weeks. The findings show a significant negative linear relationship between nematode counts and root biomass, with increased nematode populations associated with reduced root biomass. Additionally, nitrogen inputs were found to increase nematode counts without affecting root biomass, highlighting the complex dynamics between nutrient supplementation and nematode activity. Overall, this dissertation provides valuable insights into the interactions between PGRs, lance nematodes, and creeping bentgrass health, offering potential strategies for turfgrass management and nematode control.

Dedication

To Hazel, Elliott, and Emilee:

This work has been a chapter of life in our journey together. I am grateful for the friends, triumphs, and struggles we have faced as a family unit over our time in Blacksburg. We have learned much about ourselves, and our time here has laid a foundation for dealing with life and its unexpected twists and turns. As we turn the page to the next chapter, let this work herein be a remembrance stone of who walks alongside us as we grow in love for one another.

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CHAPTER I

LITERATURE REVIEW

Nematodes have been identified in plants since 1743 (Eisenback, 2022). These organisms are microscopic roundworms that are aquatic, molting, unsegmented, multicellular triploblastic, bilaterally symmetric, and pseudocoelomic (Eisenback, 2022) Nematodes are generally classified into two categories: free living nematodes (entomopathogenic) or plant parasitic (Eisenback, 2022). Free living nematodes feed on bacteria, fungi, and other nematodes, whereas plant parasitic nematodes feed on plants to recover nutrients necessary for survival and reproduction (Neher, 2010). Some of the most economically important plant parasitic nematodes include *Meloidogyne* sp., *Pratylenchus* sp., and *Heterodera* sp. due to the damage that they cause in agronomic row crop systems such as cotton, soybeans, and corn (Khan, 2023). In turfgrass systems, common plant parasitic nematodes include sting (*Belonolaimus longicaudatus* Rau, 1958), stunt (*Tylenchorhynchus* spp.), spiral (*Helicotylenchus dihystra* (Cobb, 1893) Sher, 1961), ring (*Mesocriconema* spp.), stubby root (*Trichodorus* and *Paratrichodorus* spp.), dagger (*Xiphinema americanum* Cobb, 1913), root-knot (*Meloidogyne* spp.), and lance nematodes (*Hoplolaimus galeatus* (Eisenback, 2018; Khan, 2023) Another damaging plant parasitic nematode is the lance nematode (*Hoplolaimus* sp.). Lance nematodes remain worm-like (vermiform) throughout their life cycle, which can be completed in 30 days (Crow, 2021). They survive in soil as eggs and emerge in conducive conditions to feed on nearby hosts. These nematodes are more common in sandy soils and reproduce more effectively in warmer temperatures (26-30°C). Lance nematodes are classified as migratory

endoparasites, meaning that they move in and out of roots as they feed (Crow, 2021). It is also poorly understood at what stage in their life cycle will they be inside or outside roots (Settle, 2006). These nematodes are one of the larger PPNs, measuring 1.1 to 2.1 mm in length with a stylet of 30-40 μ m. The nematode's size and longer stylet complicate determining critical damage populations, as it can feed deep within roots, causing severe mechanical damage and facilitating pathogen entry. Along with multiple feeding habits, lance nematodes have a widespread distribution (Henn and Dunn, 1989; Perry et al., 1970; Heald and Perry, 1969; Giblin-Davis et al., 1995; Settle et al., 2006). Although little research has been accomplished with them, lance nematodes are important to study because they can be found in most every climate within the United States as well as inside or outside of roots of both cool-season and warm-season plants (Settle et al., 2006). Lance nematodes are pre-dominantly an issue in warm-season turfgrasses even though lance have also been studied in cool-season turfgrasses as well (Crow et al., 2017; Giblin-Davis et al., 1995; Settle et al., 2006). Warmer climates present more of a problem to lance damage due to more environmental stresses added to the plant (Crow, 2021). Very little work has been done to study the effects of lance nematodes on creeping bentgrass (Blackburn et al., 1997; Settle, 2006). Combine this with the unknown of where lance may be feeding in a plant system and lance nematodes, while slow moving, become one of the more problematic nematodes to control.

Limited turfgrass nematicides are available to control PPN in golf course putting greens due to changes in product availability for control of plant parasitic nematodes in turf. The first change came with the restriction of fenamiphos in 2008 (EPA, 2008).

Fenamiphos is a true organophosphate nematicide that has broad spectrum control of PPN

(Cáceres et al, 2010). The EPA restriction came about due to specific bacteria, fungi, and algae in soils that would degrade the active ingredient of the product requiring extreme rates to achieve desired levels of control (Cáceres et al, 2010). This information coupled with an LD₅₀ that is highly toxic made this product lethal to apply at such high rates (Singh et al, 2006; Boya and Apaydin, 2023). After the loss of fenamiphos, the chemical industry focused on turfgrass developed new products used in golf course nematode management, but have specific limitations on controlling lance populations. One example is abamectin. Abamectin has good efficacy of lance suppression and control but is limited in its efficacy by the timing of applications. This is a product that has a high binding affinity in the soil measured by a Koc value, which is the soil organic carbon-water partition coefficient that measures how strongly a chemical will adsorb to soil organic matter (Wang et al, 2015). Abamectin's Koc value is typically reported as 4000mL/g and is considered immobile in the soil (Gannon et al., 2017). This means that abamectin generally does not move much past an inch in the soil profile making application timing critical for the most coverage of bentgrass roots (Gannon et al., 2017). This lack of movement makes summer applications extremely important because that is when the best coverage potential exists with summer bentgrass roots typically being more shallow. Abamectin is also a contact nematicide and only effective when in contact with nematodes, making the use of abamectin less efficacious if lance nematodes are feeding endoparasitically (Crow et al., 2017). Another example of product limitation is fluensulfone. This product has a low Koc value (Morris et al., 2018) meaning it moves rapidly within a soil when watered. If this product is applied before a big rain event, it can be washed through a soil profile proving a wasted application. Due to its formulation, most golf course superintendents avoid using because if

improperly mixed and applied, this produce can aggregate on putting surfaces and cause phytotoxic injury (Morris et al., 2016). Finally, a recently labeled nematicide for turfgrass, fluopyram, (Crow et al., 2017) selectively targets many nematode species but fails to control lance nematodes. This nematode causes damage to a variety of turfgrasses (Martin, 2017; Settle et al., 2005). They are migratory and become semi- or endoparasitic (Martin, 2017). Research has demonstrated that fluopyram has little to no effect on suppressing lance nematodes (Crow, 2021). Besides that, its overuse may select for populations of root-knot and sting nematodes that are resistant, documenting the first case of nematicide resistance (Crow, 2024; Kammerer et al., 2023). The lack of efficacy and the development of resistance have increased interest in the management of lance nematodes.

Plant growth regulators (PGRs) are commonly used on golf course greens (Baldwin and Brede, 2011; Askew, 2017; Han et al., 2017). Two general classes of PGRs are shoot inhibitors and root inhibitors. Trinexapac-ethyl (TE) and prohexadione calcium (PC) are examples of shoot inhibitors while flurprimidol (FLUR) and paclobutrazol (PAC) are examples of root inhibitors. One of the most common PGRs used on both cool and warm-season putting greens is trinexapac-ethyl (Primo Maxx) (Baldwin and Brede, 2011). Trinexapac-ethyl is just one of a few PGRs used that inhibit gibberellin (GA) biosynthesis resulting in a reduction in plant height (Rademacher, 2016). This PGR inhibits GA biosynthesis by blocking the final step in the biosynthesis of biologically active forms of GA leading to slower shoot growth (King et al., 1997; Adams et al., 1992). Research has shown that applications of TE enhanced turf performance under heat and drought stress conditions (McCann and Huang, 2007). Another PGR that behaves similarly to TE is PC. Prohexadione calcium inhibits the hydroxylation of GA₂₀ to GA, the final step in GA

biosynthesis (Nakayama et al, 1992). It has also shown some effectiveness at *Poa annua* suppression (Beam and Askew, 2007). More importantly, studies have shown enhanced drought stress tolerance in kentucky bluegrass when applied with PC (Rezapour et al., 2015). Applications of TE and PC are common in creeping bentgrass putting greens to help superintendents reduce plant height and plant stress while maintaining healthy turfgrass during difficult summer growing periods. Even so, FLUR and PAC are two other PGRs that primarily affect the roots instead of shoots and also provide the added benefit of being classified as both PGRs and demethylation inhibiting (DMI) fungicides (Desta and Amare, 2021). The mode of action of FLUR is different than TE or PC in that it inhibits cell elongation by blocking the ent-kaurene oxidase- enzymes, which converts ent-kaurene to ent-kauronic acid, preventing GA formation (Rademacher, 2000; Beam and Askew, 2007). Flurprimidol is not always an optimal choice due to turf discoloration that can occur during heat and drought conditions (Dernoeden, 1984). Paclobutrazol is a PGR that behaves similarly to FLUR in that it inhibits GA formation (Rademacher, 2000). Likewise, PAC also has been known to discolor turf and “prune roots” (Fagerness and Yelverton, 2001). Applications of PAC may also lead to reduced turf quality during periods of heat and drought stress (Fagerness, 2000). All the above PGRs have been evaluated to some degree under periods of heat and drought stress, but there is little to no literature evaluating the effects of these PGRs under similar conditions and under the influence of damaging plant parasitic nematode populations.

Although lance nematode management has its difficulties, diagnostically it is easy to identify. Nematode diagnostics are different than most other pathogen diagnostics because nematodes are quantifiable with a microscope. Diagnostics of PPN have long included some

form of sampling, followed by some form of elutriation, followed by microscopic identification. There are two common forms of sampling, two common forms of extraction, and one common way to count nematodes using an inverted microscope.

The two forms of sampling include random sampling and diagnostic sampling. With random sampling, an area of turfgrass is selected and sampled using a soil probe taking at least 25 soil cores in a zig zag pattern from a depth of 6". Diagnostic sampling is similar in that samples are still collected with a soil probe but now they are collected from regions of susceptible damage. With diagnostic sampling, samples should be taken from within damaged areas as well as adjacent healthy areas due to the likelihood that nematodes will not be feeding in damaged areas and will likely migrate to areas where roots are present.

The two forms of extraction that are common for identifying PPN are the Jenkins method (Jenkins, 1964) and the semi-automatic elutriator (Byrd, 1974). The Jenkins method, described in his paper, generally consists of adding water in a bucket with soil and stirring for 10-30 seconds. With nematodes stirred and in solution, the water is poured into a series of mesh sieves that consist of a 25, 60, and 400 mesh. The 25 and 60 mesh sieves collect most of the soil and debris where the 400 mesh collects finer soil particles and captures PPN for sugar floatation (Jenkins, 1964). Similarly, a semi-automatic elutriator accomplishes the same goal by using air and water induction to automate the process of extraction (Byrd, 1974). While providing a level of automation to the extraction process, this method also results in loss of nematodes and relies heavily on relative estimations for accurate results (Byrd, 1974).

In the end, nematodes that have been successfully extracted are counted using a counting dish placed on an inverted microscope. Using inverted light is useful for this

process since nematodes will eventually sink when in solution making magnification easier. Microscope identification of nematodes requires skillful recognition to achieve the most accurate counts of a PPN population per soil sample. This skillful recognition is also time consuming because every single nematode is counted by hand per sample and totaled for each sample. This is referred to as counting nematodes and is a critical first step to becoming a nematologist in any capacity. Even so, counting nematodes is laborious, time consuming, and subjective. Two nematologists could count the same exact sample and get different total numbers. This can create some inconsistencies in the diagnostic process. Meanwhile, in other areas of pathology, molecular methods have been more readily used to identify and quantify pathogen presence (Stackhouse et al., 2020). Genetic barcoding is a molecular technique where sections of DNA are identified within a gene of an organism that can be targeted and amplified in a random DNA samples to test for that organism's presence (Powers, 2004). Two common methods of genetic barcoding are using polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR). The PCR process typically involves 40 cycles that include denaturation, annealing, and extension of specific areas of genetic material to create billions of copies of the same genetic material (Mullis, 1990). This process is usually followed by sequencing these genetic materials to confirm identity of an organism (Bevan et al., 1992; Newton et al., 1997). The process of qPCR differs slightly in that it can also quantify the amount of DNA present using a fluorescent marker that releases as genetic material is replicated, and this release in fluorescence can help indicate the quantity of genetic material based on how concentrated the fluorescence is (Navarro et al., 2015). Primers created for PCR protocols and primers and probes created for quantitative polymerase chain reaction (qPCR) are

quite common in pathology (Bronzato-Badial et al., 2020). Most barcoding techniques that have been used in nematology have been strictly for identification purposes (Powers, 2004; Stackhouse et al., 2020). Identification on a genetic level can help to remove the subjectivity presented in traditional counting methods.

Along with counting methods, another issue that is quite relevant with lance nematodes is consistent damage threshold information. There are many factors that can affect nematode damage thresholds and the inconsistency that arises from state to state can be rather confusing for golf course superintendents. Damage thresholds are dependent on many factors but in most scenarios, is referred to as a guide with the caveat of “experiences in damage may vary”. Environmental stress, mechanical stress, root depth, soil texture, and type of nematode can play an important role in proper assessment of thresholds. Environmental stresses such as temperature and humidity play an important role in damage thresholds because plants weakened by heat and drought stress can be more at risk of damage to less nematodes feeding on roots (Biswal, 2023). Mechanical stresses are common turfgrass problems in that golf course putting greens are typically mowed daily with other cultural practices such as rolling and verticutting that can also stress plants and make more susceptible to PPN damage. Soil texture determines the mobility of PPN and how concentrated damage will be based on how much pore space a soil profile has. Plant parasitic nematodes thrive in sandy soils because sand based soils provide more pore space for nematode mobility, meaning that a problematic infestation can move more easily than in a clay soil where nematode mobility is highly restricted. Root depth is straightforward in that if there are more roots available, more nematodes can feed

before damage is apparent (Sikder and Vestergård, 2020). All these factors help make a good nematode recommendation and all must be considered to avoid turfgrass damage.

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CHAPTER II

COMBINED EFFECTS OF LANCE NEMATODE POPULATIONS AND USE OF PLANT GROWTH REGULATORS ON CREEPING BENTGRASS PUTTING GREENS.

Tucker, Matthew Aaron¹, Haak, David¹, Crow, William², Eisenback, Jon¹, McCall, David¹

Affiliations: ¹School of Plant and Environmental Science, Virginia Tech, Blacksburg, VA 24060,

²Entomology and Nematology Department, University of Florida, Gainesville, FL 32611

Abbreviations: PGR, plant growth regulator; PPN, plant-parasitic nematode; RPM, revolutions per minute; VTQ, visual turf quality

ABSTRACT

Plant growth regulators (PGRs) are used to optimize turfgrass growth, reduce management inputs, and potentially mitigate diseases. However, golf course superintendents have reported anecdotal evidence linking increased PGR-induced phytotoxicity with high populations of lance nematodes (*Hoplolaimus galeatus*) nematodes. The purpose of this study was to investigate the relationship between PGR usage and lance nematode populations on creeping bentgrass putting green health during summers in the Mid-Atlantic United States. A 5x2 factorial design with 5 levels of PGR and the presence or absence of fluopyram was studied among 5 locations across 6 site years in the field. Four PGR treatments consisted of trinexapac-ethyl (Primo Maxx) (47.7 g ai/ha, 14 days), prohexadione calcium (Anuew) (154 g ai/ha, 14 days), flurprimidol (Cutlass) (280 g ai/ha, 28 days), and paclobutrazol (Trimmit) (175 g ai/ha, 42 days) and a non-treated control. Fluopyram was applied every 28 days at 373 g ai/ha due to great control of other PPN with little efficacy against *H. galeatus* and compared against a non-treated control. Our data

suggests that the combined effects of PGR applications and elevated lance nematode populations typically do not contribute to a decline in creeping bentgrass putting green quality under the tested conditions. However, there were two instances that showed some interaction between PGR use and lance nematode presence. In 2021 at the Foundry Golf Club on September 8th, the treatment of prohexadione calcium + fluopyram resulted in the most % visible damage among all treatments, and in 2022 at this same timing, prohexadione calcium with and without fluopyram led to more visible damage than all other treatments. This suggests there may be an interaction between prohexadione calcium and lance nematode presence, but the overwhelming result of this work is limited damage resulting from a combination of PGR use and lance nematode presence.

1 INTRODUCTION

Turfgrass is a crucial component of Virginia's horticulture and recreational industries, with golf courses playing a prominent role. The Virginia Turfgrass Council (VTC, 2017) estimated that turf-related industries contribute over \$1.5 billion annually to the state's economy. Golf courses, generate revenue through tourism, golf club membership fees, and hosting tournaments, which are all dependent on high-quality, visually appealing turfgrass. Maintaining high quality turf requires significant inputs, including fertilizers, pesticides, irrigation, and mowing (Beard & Green, 1994), that are in turn, costly in terms of product, labor, and environmental impacts. Many golf greens in Virginia are surfaced with different varieties of creeping bentgrass (*Agrostis stolonifera* L.) (CBG). Managing CBG in the summer is challenging considering the physiological nature of this cool-season grass to photorespire under period of high heat and humidity (Fry and Huang, 2004). Heat stress accompanied by other pest and amendment pressures can make achieving high quality putting greens difficult in the summer months.

The use of plant growth regulators (PGRs) in golf course management has been adopted by turfgrass professionals since the 1990s (Watschke et al., 1992). These products are primarily used to slow upward shoot growth of turfgrasses, but also have intrinsic benefits, such as the production of richer color, increased turfgrass density, and enhanced root zones (Fagerness and Yelverrton, 2001). The incorporation of PGR applications into routine turfgrass management can alleviate stress and limit growth, (Kreuser and Soldat, 2011) potentially reducing disease incidence by increasing turfgrass stand density (Ervin & Zhang, 2008). Golf course superintendents have reported experiencing increased phytotoxicity of PGR applications in conjunction with high populations of lance nematode (*Hoplolaimus galeatus* (Cobb, 1913) Thorne, 1935).

Lance nematodes are part of a larger group of plant-parasitic nematodes (PPN) that are obligate parasites of plants. In turfgrass, these PPNs may reduce turf health and, in some cases, cause plant death. The most common species found on turf include sting (*Belonolaimus longicaudatus* Rau, 1958), stunt (*Tylenchorhynchus* spp.), spiral (*Helicotylenchus* spp. (Cobb, 1893) Sher, 1961), ring (*Mesocriconema* spp.), stubby root (*Trichodorus* and *Paratrichodorus* spp.), dagger (*Xiphinema* spp. Cobb, 1913), root-knot (*Meloidogyne* spp.), and lance nematodes (*Hoplolaimus galeatus* (Eisenback, 2018).

Hoplolaimus galeatus is a PPN that damages various species of turfgrass (Eisenback, 2018; Settle et al., 2005). As a migratory endoparasite, it feeds both outside and inside roots (Martin, 2017). Control options for lance nematodes are limited; for example, abamectin is an effective control option, but cannot penetrate deeper than 2.5cm into the soil even with post application irrigation. It poses a challenge for full coverage of the root system when they grow much deeper (Crow et al., 2017; Gannon et al., 2017).

Following the Environmental Protection Agency's (EPA) restrictions on the use of the popular nematicide fenamiphos (Environmental Protection Agency, 2008), fluopyram has become the most used product for nematode management in turfgrass, even though it is not effective for lance nematode control (Kammerer et al., 2023). The extensive use of this product, which is very effective on most PPNs in turf, has led to the development of very high populations of lance nematodes (Crow et al., 2017). Recently fluopyram has been approved as a nematicide to manage these pests. Unfortunately, this product has little to no effect on lance nematodes (Desaeger et al., 2020). In many golf greens where this product has been used for several years, lance populations may have increased to damaging levels (Crow et al., 2017). Repeated applications of fluopyram to reduce lance nematode populations and the long half-life of this product has helped produce the world's first case of nematicide resistance (Kammerer et al., 2023). The lack of control of lance nematode and the risk of nematicide resistance have increased interest in managing *H. galeatus* in Virginia with other potentially practical management options to alleviate turfgrass stress, including PGRs.

Anecdotally, golf course superintendents that use PGRs on their greens have seen increased phytotoxicity to turfgrass where the populations of PPNs are high. They often assumed that the exacerbation of damage caused by PGRs was because there may be an interaction with PPNs, particularly, the lance nematodes. The purpose of this research was to evaluate the interaction of PGR applications and lance nematode populations on creeping bentgrass putting green health during summer stress in the Mid-Atlantic.

2 MATERIALS AND METHODS

2.1 Research Locations

This research was conducted across six site years at five golf course locations in Virginia in 2021 and a repeated site in 2022. Studies were conducted in 2021 at the Club at Glenmore in Keswick, the Foundry Golf Club in Powhatan, the Club at Viniterra in New Kent, the Golden Horseshoe Golf Club Gold Course in Williamsburg, and the Heron Ridge Golf Club at Virginia Beach. The study was repeated at the Foundry Golf Club in 2022 over the same location used in the previous year to account for effects over time due to consistent lance nematodes pressures across all plots. All greens were built to United States Golf Association (USGA) specifications with four of six locations having established stands of 'A1/A4' creeping bentgrass (*Agrostis stolonifera* L.) (CBG), the Gold Course at the Golden Horseshoe used '007' CBG, and the Heron Ridge Golf Club grew 'L-93' CBG overseeded with '007'. Courses selected for this study include one public (Heron Ridge Golf Club), one semi-private (The Club at Viniterra), one resort (Golden Horseshoe Golf Club), and 2 private golf facilities (The Club at Glenmore and The Foundry Golf Club). Rounds of golf at each facility varied with Heron Ridge Golf Club seeing the most rounds per day (180), Golden Horseshoe (160) and The Club at Viniterra (160) having slightly less. The Foundry Golf Club staff experiences rounds in waves with 40 per day Monday through Thursday and up to 120 Friday, Saturday, and Sunday. The Club at Glenmore has the least amount of play with 70 rounds per day. Each course has slightly different management strategies but maintains their greens within a similar range of turf heights (2.54-3.175mm) and 4 of the 5 maintained nitrogen regimes of 0.454-0.90kgs of nitrogen per year. Only one course (The

Club at Glenmore) maintained their facility with less than a pound of nitrogen and focused more on micronutrient management strategies.

2.2 Experimental Design

A 5 x 2 factorial design was implemented for this study using four PGR products and a non-treated control applied at maximum labeled rates, with and without fluopyram (Table 1). Each treatment was replicated for times, with plots arranged in a randomized complete block design, and repeated over six site-years. Fluopyram was used in this study to reduce populations of most PPN and account for lance nematodes presence due to lack of efficacy against lance. The order of application of these products was critical for this research. The nematicide, fluopyram, was applied separately to replicated plots, irrigated with 2.54mm of water to move the product in the root zone for best nematode control (Crow, 2017), and dried on the surface prior to application of the PGRs. PGR applications were not followed by irrigation for consistency across all PGRs applied although recommended for root absorbed products (Kaufmann, 2020). Applications were made using a CO₂ backpack sprayer outfitted with TTI 11004 nozzles and applied at 276kPA in a spray volume of 374 L ha⁻¹.

Table 1. Plant growth regulators and fluopyram, the rates of application, and application intervals used to test the interaction with lance nematode populations on creeping bentgrass putting greens in Virginia.

Plant Growth Regulator	Rate	Application interval
trinexapac-ethyl	47.7 g ai/ha	every 14 days after initiation (DAI)
prohexadione calcium	154g ai/ha	Every 14 DAI

flurprimodol	280g ai/ha	every 28 DAI
paclobutrazol	175g ai/ha	every 42 DAI
fluopyram	373g ai/ha	every 28 DAI
None	n/a	n/a

2.3 Data Collection

Measurements collected include visual turfgrass quality (VTQ), percent phytotoxicity, and percent visible damage not associated with phytotoxicity. VTQ was assessed using a 1,9 scale, where 1 is considered dead turfgrass, 9 is considered perfect conditions, and 6 is considered minimally acceptable, based on color, density, and uniformity (Horst et al., 1984; Krans and Morris, 2007). Phytotoxicity was measured as uniform damage presumably caused by PGR applications across the entirety of a plot. These effects were assessed in the form of general discoloration of the turfgrass canopy. Visible damage was assessed by measured by non-uniform damage attributed to a decline in creeping bentgrass canopy health that may be associated with the presence of PPNs. Examples of visible damage include patches of declining or dead turf, as well as discoloration that was not uniform across the entire individual plot. All VTQ, % phytotoxicity, and % visible damage was collected at initiation of the experiment and every 14 days for a total of 9 assessments. Data were subjected to analysis of variance (ANOVA), and means were separated, when appropriate, using a Student's *t*-test for factors with 2 levels or Tukey's HSD for factors with 5 levels ($p \leq 0.05$) in JMP Pro 18 (version, SAS Institute, Cary, NC).

2.4 Nematode Sampling and Processing

Initial, midseason, and final soil samples were collected from each plot for PPN evaluation at each location between the dates of May 24 and September 8th. Each sample consisted of five soil cores taken with a 1.25 cm soil probe at a depth of 15 cm with holes backfilled with kiln-dried sand. All soil cores were measured for root length using a 22.86cm x 34.29cm tin pan with a ruler printed, laminated, and secured to the bottom left side of the pan. All soil cores were measured with verdure placed where the ruler begins and the longest root was recorded for each soil core, with the five subsamples averaged for each plot. Bulked cores containing the turfgrass roots and soil column from each plot were placed into plastic bags, sealed and labeled with the corresponding plot number and course location, and stored temporarily in a cooler until they were transferred into a walk-in cold room, maintained at 4°C, until they were processed. Nematodes were extracted from 250cm³ of soil with a North Carolina State model semi-automatic elutriator (Byrd et al., 1978). This method uses air and water induction to float nematodes in a column of water through a divider into a 400-mesh sieve and processed with a modified centrifugation and sugar floatation technique (Jenkins, 1964). Nematodes captured in the 400-mesh sieve were washed with tap water into a 100 mL Falcon® (Corning, Weston, FL) tube and centrifuged at 6,000 RPM for four minutes. After centrifugation, the water was removed, and the remaining pellet was resuspended in sugar-water solution of 454 g sugar L⁻¹ water and centrifuged at 6,000 RPM for one minute. The top 2 to 5 cm of sugar-water was poured into a 500-mesh sieve and washed with tap water for approximately 10 seconds until the sugar was removed. Nematodes captured in the 500-mesh sieve were placed into a 25mL scintillation vial for storage in a refrigerator until they were counted. The sample was

poured into a counting dish made from a 2.5 cm x 7.5 cm clear plastic pill box engraved into eight lanes, each the width of the field of view of the microscope, and one quarter of the dish was counted. The total number of nematodes counted using an Olympus® CK2 inverted microscope (Shinjuku City, Tokyo, Japan) at 40x magnification were multiplied by 20 to account for starting soil amounts of 250 cm³, nematodes diverted in the semi-automatic elutriator, and only counting one quarter of the dish to report the total estimate of nematodes per 500 mL of the initial sample. This procedure was repeated for all samples (n=720).

3 RESULTS AND DISCUSSION

3.1 Results

3.1.1 Effectiveness of Fluopyram

To confirm the effectiveness of fluopyram, a full factorial regression analysis of total PPN w/o lance nematodes for all factor combinations of fluopyram, location, and assessment timing (initial, midseason, and final sampling dates) ($p < 0.0001$) was conducted. The factor combination of fluopyram x location x assessment timing was significant ($p < .0001$) and within this factor combination, no significant effects of fluopyram were observed of total PPN excluding lance across locations at the initial sampling date (Table 2). Two of six midseason sampling locations and four of six final sampling locations led to a reduction in PPN without lance due to fluopyram (Table 2).

Table 2. Summary data of factor combination of fluopyram x location x assessment timing. Significance is noted with bold lettering ($\alpha = 0.05$).

	Total plant parasitic nematode without <i>Hoplolaimus galeatus</i>
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Location	Initial sampling Pvalue	Midseason sampling Pvalue	Final sampling Pvalue
The Foundry Golf Club (2021)	NA	0.0071	0.0691
The Foundry Golf Club (2022)	0.3925	0.5975	0.0073
Golden Horseshoe Golf Club	0.2408	0.1170	0.0077
The Club at Glenmore	NA	0.8312	0.4073
Heron Ridge Golf Club	0.6103	0.9389	0.0072
The Club at Viniterra	0.9066	<0.0001	<0.0001

A full factorial regression analysis of lance nematodes for all factor combinations of fluopyram, location, and assessment timing was evaluated (Table 3). The factor combination of fluopyram by location resulted in no change in lance population at four of five locations ($p > 0.0866$) but fluopyram treatments at Heron Ridge Golf Club resulted in more lance nematode populations ($p=0.0163$) (Figure 1).

Table 3. Summary of a full factorial regression analysis effects test of lance nematode counts

Factor combinations	Lance nematodes
Fluopyram	0.1134
Location	<0.0001
Fluopyram*location	0.0012
Assessment timing	<0.0001
Fluopyram*assessment timing	0.9199
Location*assessment timing	<0.0001
Fluopyram*location*assessment timing	0.9989

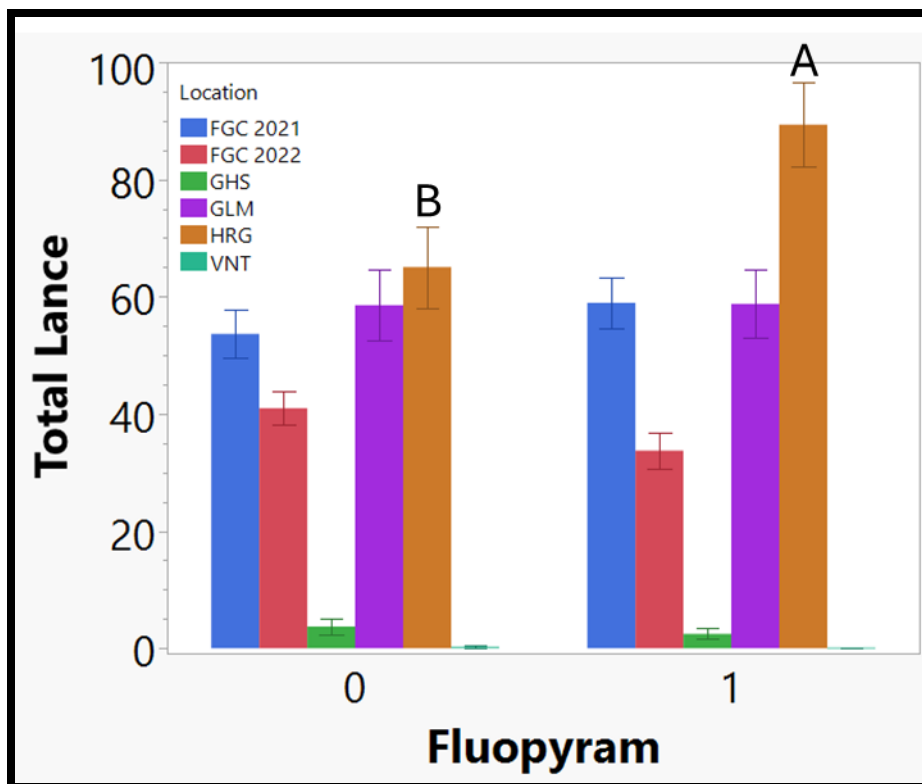


Figure 1. An analysis of variance of fluopyram effects on total lance nematode counts across all locations. Means were separated using Tukey’s HSD ($\alpha=0.05$) by the presence (1) or absence (0) of fluopyram within location. Difference in capital lettering indicates differences of fluopyram within location.

3.1.2 Summary of Visual Assessment Data

A full factorial regression analysis of Visual assessment data was conducted for factor combinations of PGR, fluopyram, location, and assessment date. No differences in TQ were observed for any factor combination (Table 4). Differences in percent phytotoxicity were noted for 9 of 15 factor combinations and differences in percent visible damage was observed for 7 of 15 factor combinations (Table 4).

Table 4. Summary of a full factorial regression analysis effects test of all assessment data collected for this research. Significant data is bold ($\alpha=0.05$).

Factor combinations	Turf Quality (TQ)	% phytotoxicity	% visible damage
PGR	0.6296	<0.0001	<0.0001
fluopyram	0.1558	0.5973	0.6768
PGR*fluopyram	0.5747	0.0949	0.0576
location	0.2465	<0.0001	<0.0001
PGR*location	0.7319	<0.0001	<0.0001
fluopyram*location	0.1288	0.1114	0.3927
PGR*fluopyram*location	0.7082	0.8814	0.6581
assessment date	0.3525	<0.0001	<0.0001
PGR*assessment date	0.4416	<0.0001	<0.0001
fluopyram*assessment date	0.5159	0.0258	0.2061
PGR*fluopyram*assessment date	0.4662	0.8551	0.3421
location*assessment date	0.3381	<0.0001	<0.0001
PGR*location*assessment date	0.3709	<0.0001	<0.0001
fluopyram*location*assessment date	0.6694	0.0051	0.6063
PGR*fluopyram*location*assessment date	0.4562	0.9974	0.5512

Two of five locations resulted in differences in percent visible damage by PGR over six assessment dates where one date was observed at Golden Horseshoe Golf Club and the remaining five were observed at The Foundry Golf Club. On September 8th, 2021 at Golden Horseshoe Golf Club, PGR treatments of flurprimidol and paclobutrazol resulted in more visible damage; however, lance populations at this site were low throughout the study and not correlated with visible damage (Figure 2).

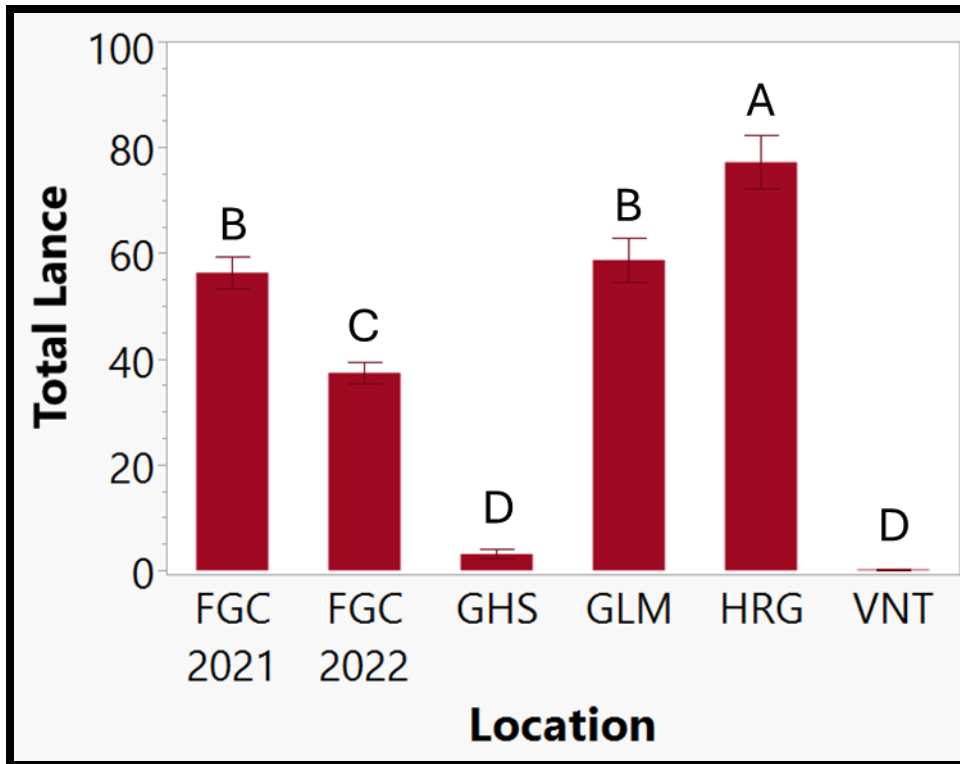


Figure 2. An analysis of variance of location by total lance counts. Means were separated using Tukey's HSD ($\alpha=0.05$). Significance is indicated by different capital letters.

Difference in visible damage by PGR effects were noted on six assessment dates at The Foundry Golf Club across 2021 and 2022 (Figure 3). On July 7th, 2021, flurprimidol led to an increase in visible damage with an average damage rating of 4%. All other dates show prohexadione calcium leading to an increase in visible damage with an average visible damage range of 5.125% to 24.375% among these dates (Figure 3).

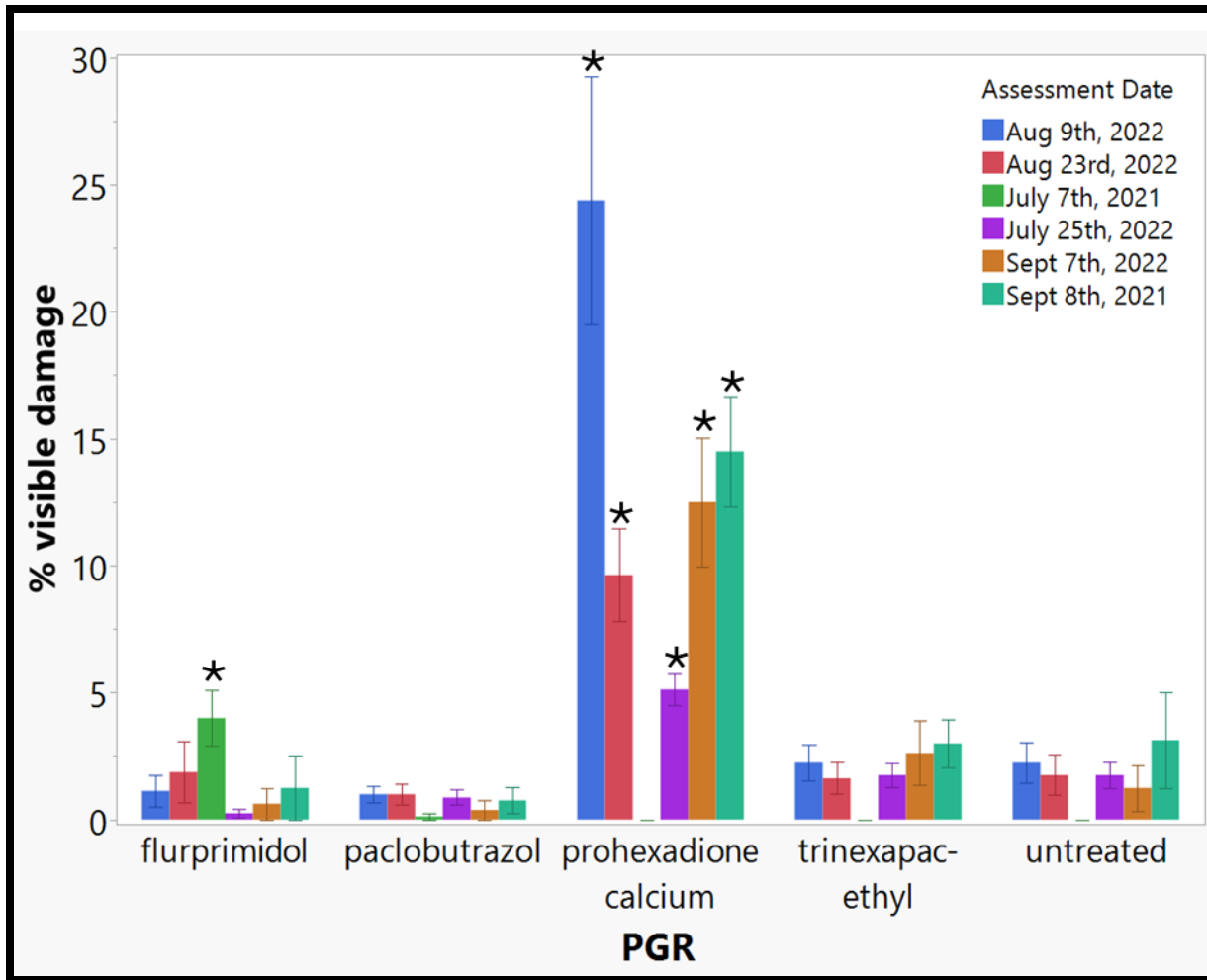


Figure 3. An analysis of variance of Plant Growth Regulator (PGR) effects on percent visible damage by assessment date. Means were separated using Tukey’s HSD ($\alpha=0.05$). Asterisks indicate a single PGR difference at each assessment date where that PGR resulted in increased visible damage.

3.2 Discussion

3.2.1 Effectiveness of Fluopyram

Results indicate that after two maximum label rate applications of fluopyram, two of six locations had reduced PPN counts excluding lance. Four of six locations resulted in a reduction of PPN nematode excluding lance after 4 max rate application of fluopyram

(Table 2). These results support the idea that fluopyram has efficacy of PPN outside of lance. Even so, when we evaluate the effects of a full factorial on lance nematode presence, we discover a significant effect of fluopyram by location (Table 3). Figure 1 shows us the effects fluopyram has on lance nematodes at different locations selected in this study and ultimately shows that fluopyram had no effect on lance populations at four of five locations. It also shows that fluopyram treated plots led to an increase in lance nematode population at Heron Ridge Golf Club (Figure 1). Review of the literature indicated that fluopyram, a 3-F nematicide (containing a trifluoro group), has broad spectrum activity of PPN (Moreira and Desaeger, 2019; Desaeger et al., 2020). Even so, it is also understood that fluopyram had little efficacy against *H. galeatus* for reasons that are not well understood (Crow et al., 2017). This study was consistent with the literature on fluopyram's efficacy in that it suppressed most of the PPNs in this study, but not *H. galeatus*, allowing us to directly access the relationship between lance nematodes and the use of PGR at various locations within Virginia (Crow et al., 2020; Kammerer et al., 2023).

3.2.2 Variability in Visual Assessment Data

In 57 assessments, out of a total of 63 assessments across six site-years, there were no negative interactions between applications of PGRs and lance nematode populations on the health of creeping bentgrass putting greens throughout the summertime in Virginia. Applications of trinexapac ethyl did not induce non-uniform visible damage on any assessment date. Applications of flurprimidol led to differences in non-uniform damage on two assessment dates at two locations, and paclobutrazol led to differences in non-uniform damage on one assessment date at one location. Prohexadione calcium induced damage only on 5 dates, with no visible damage in plots on any other date.

Non- uniform damage occurred at 2 of 6 locations in this study. Damage at Golden Horseshoe Golf Club was observed on September 8th (final rating date) for treatments of flurprimidol and paclobutrazol ($p = <0.0001$). Higher phytotoxic effects were observed at this location; however, the lance populations were not consistently high enough throughout the season to adequately explain negative correlation with any significant metric (Figure 2). Instead, high numbers of stubby root nematodes (*Trichodorus* spp.) may have influenced the observed negative effects of PGRs. It is possible that this species is also unaffected by fluopyram, but confirming this hypothesis was beyond the scope of this study.

Damage in connection with applications of prohexadione calcium at the Foundry Golf Club occurred on September 8th in 2021 and July 25th, August 9th, August 23rd, and September 7th in 2022. The weather throughout most of the summer of 2021 was relatively mild shown by only one assessment date of visible damage. The weather in 2022 was noticeably more hot and humid to the point that the golf course superintendent left fans on for 24 hours a day from July until September to alleviate heat stress. Most reports of damage caused by prohexadione calcium occurred during periods of heat and drought stress. However, damage associated with CBG in this study was non-uniform rather than general phytotoxicity across all plots. This non-uniform injury suggests that there are likely biotic factors impacting injury beyond the general abiotic stresses of heat and humidity.

Our data showed a strong positive relationship between visible damage and lance nematode counts, but only on assessments where there was visible injury to the CBG. These data suggest that increasing numbers of lance nematodes that can feed on CBG roots may be predisposing the affected areas to additional stress.

4 CONCLUSIONS

The primary objective of this research was to evaluate how the relationship between PGR applications and the presence of lance nematodes impacts the health of creeping bentgrass golf putting greens. Studies were conducted on five golf courses across Virginia in 2021 with an additional year of data collection on one golf course in 2022. Applications of four common PGRs were made throughout the summer, with and without applications of fluopyram. Turfgrass quality, non-uniform damage, and uniform phytotoxicity were visually assessed 63 times across six site-years between May and September. There were no visible differences on 57 of the 63 assessments across locations, suggesting that most PGR applications are generally safe with the presence of some lance nematodes on golf course putting greens.

Non-uniform damage was present in prohexadione calcium-treated plots on 5 dates at one location. The damage that occurred during these assessments coincided with heat and humidity stress for creeping bentgrass putting greens, though the non-uniform injury suggested a biological stressor as well. Further analysis of these data in prohexadione calcium-treated plots suggested a positive linear relationship between damage to the creeping bentgrass and lance nematode counts. Collectively, these comprehensive datapoints provide some evidence that there may be an increased likelihood of creeping bentgrass damage when prohexadione calcium applications coincide with high lance nematode counts and environmental stresses, though more research is needed to validate these suggestions.

A secondary objective of this study was to determine whether applications of fluopyram impacted populations of lance and other plant-parasitic nematodes. Our data

suggests that lance nematodes were not impacted by fluopyram while all other PPN present in this study were suppressed. This is consistent with reports by Crow (2017) even though the current Indemnify nematicide label (Bayer CropScience, 2016) suggests suppression of *Hoplolaimus* sp. To our knowledge, this is the first report in scientific literature that fluopyram does not suppress lance nematodes when applied to creeping bentgrass putting greens.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Please list any author ORCID iDs here.

David Haak: 0000-0002-3692-3152

Jon Eisenback: 0000-0002-2102-4867

William Crow: 0009-0000-4902-6680

David McCall: 0000-0002-7113-9486

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CHAPTER III

INFLUENCE OF NITROGEN FERTILIZATION ON THE RELATIONSHIP BETWEEN LANCE NEMATODES (*HOPLOLAIMUS GALEATUS*) AND THE ROOT BIOMASS OF CREEPING BENTGRASS (*AGROSTIS STOLONIFERA*)

M.A. Tucker, D. Haak, W. T. Crow, J. D. Eisenback, and D. S. McCall

KEYWORDS

hyperspectral radiometry, root biomass threshold, plant-parasitic nematodes, nematode population dynamics, plant-nematode interactions, turfgrass management, turfgrass root health

ABSTRACT

Management of lance nematodes on creeping bentgrass putting greens are often reliant on action threshold recommendations from a lab assay. However, counts exceeding these thresholds often do not coincide with visible damage to creeping bentgrass. The goal of this work was to determine the relationship between lance nematode (*Hoplolaimus galeatus*) populations and root biomass of creeping bentgrass (CBG) (*Agrostis stolonifera* cv. 'L-93'). To understand these effects, a 4x5 factorial design was arranged with 4 levels of urea (46-0-0) nitrogen (0, .00118, .00237, and .00484kgs/m² applied over 16 weeks) and 5 levels of *H. galeatus* nematode populations (0, 100, 500, 1500, and 3000) evaluated over 16 weeks. Mature 'L-93' CBG plugs from an established putting green were inoculated with the five *H. galeatus* population sizes and allowed to acclimate for 18 weeks. Nitrogen treatments were applied bi-weekly and hyperspectral radiometry measurements were collected at experiment termination to evaluate changes in visible and spectral plant health

indices. Destructive sampling was performed every 4 weeks, with one representative of each factorial combination removed, nematodes extracted, verdure discarded, and root biomass was collected by drying samples in a muffle furnace. At week 12, a significant negative linear relationship was found between *H. galeatus* counts and root biomass, indicating that increased nematode populations were associated with reduced root biomass. Also, for 12 weeks, an analysis of variance showed that nitrogen inputs led to higher *H. galeatus* nematodes counts without an effect on root biomass. Destructive samplings on 4, 8, and 16 weeks resulted in statistically insignificant data without any observable trends. Overall, *H. galeatus* caused a moderate decrease on root biomass of 'L93' CBG, whereas supplementing nitrogen increased *H. galeatus* population rather than strengthening CBG to overcome nematode feeding.

INTRODUCTION

Golf course putting green management requires meticulous attention to detail to produce a desirable, playable surface. Management practices vary depending on the type of turfgrass, which can be categorized into cool-season (C3) and warm-season (C4) grasses (Fry and Huang, 2004, Nu et al., 2008). Creeping bentgrass (*Agrostis stolonifera* L.) (CBG) and ultradwarf bermudagrass (*Cynodon dactylon* (L.) Pers. × *Cynodon transvaalensis* Burt Davy) (UDB) are the primary representatives of these categories, respectively. The management of these grasses is closely linked to their development of roots (Nu et al., 2008).

Creeping bentgrass root development begins with spring green-up, during which roots grow vigorously, penetrating deeply into the soil (Fry and Huang, 2004). As summer heat approaches, roots become shallower and more susceptible to biotic and abiotic stresses, with pest pressures peaking during this period. Cooler night temperatures in the fall provide relief from photorespiration loss and allow for root recovery before winter dormancy. This root development pattern can be visualized as a bell curve, with roots deepening in spring, shallowing in summer, and recovering in fall before dormancy (Nu et al., 2008).

Conversely, UDB root development follows an inverted pattern compared to CBG. During spring green-up, UDB root development begins slowly. As temperatures rise, the Calvin Benson cycle in the bundle sheath cells of bermudagrasses allows photosynthesis to continue efficiently with minimal respiration loss (Fry and Huang, 2004). Cooler fall temperatures reduce photosynthetic activity, leading to shallower root systems. Pest management for UDB is most critical in the spring, when roots are developing slowly, and in the fall, when root production has significantly decreased. A 2-D graphical representation of UDB root development resembles an inverse bell curve, opposite that of CBG. Understanding these opposing root development patterns is crucial for making effective pest management decisions. Another critical aspect of turfgrass biology is recognizing root damage. For pests like plant-parasitic nematodes (PPN), damage varies depending on the turf type. In UDB greens, PPNs can cause more damage because rooting is typically shallow with higher accumulations of thatch (McCarty and Canegallo, 2005). Warm-season plants, like UDB, are adapted to adverse heat conditions, making PPN feeding potentially devastating on developing root systems. Conversely, cool-season plants are less

efficient at managing heat stress, but breeding efforts are more common to help develop resistant varieties to pest pressures (Bonos et al., 2006). Damage to cool-season greens usually appear as a gradient rather than a binary state due to more robust root systems (Lehman and Engelke, 1991). Golf course superintendents often mistake soil-borne fungal diseases for PPN damage, as both can appear similar without diagnostic testing (Tredway et al., 2023). Fertilizer supplementation is often used to alleviate pest pressures in greens management. In disease management, increased nitrogen can either suppress dollar spot incidence or increase soil-borne disease incidence (Golembiewski and Danneberger, 1998; Liu et al., 2021). The primary macronutrients used are nitrogen (N), phosphorus (P) in the form of phosphate (P₂O₅), and potassium (K) in the form of potash (K₂O). Phosphorus aids in root development and can bolster roots affected by stress, but it is not readily available to plants. Restrictions on phosphorus exist in some areas due to its potential to leach into groundwater (Waschbusch et al., 1999). The effectiveness of phosphorus depends on soil uptake capacity (Frank and Guertal, 2013). Potassium influences plant defense responses, potentially reducing pest impact, but its effects are harder to measure scientifically (Frank and Guertal, 2013). Nitrogen is the most limiting nutrient in turfgrass management for plant survival and directly impacts shoot and root growth. Different rates of nitrogen have been used to measure its effects on plant growth and development (Liu et al., 2021).

Hoplolaimus galeatus (Cobb, 1913 Thorne, 1935) is one of the larger (1.1 to 2.1 mm x 30 to 40µm) plant-parasitic nematodes (PPN) that feed on turfgrasses. This migratory endoparasite's habits in or outside of turf roots are not well understood (Settle et al., 2006). Its size and longer stylet (30-40 µm) complicate determining critical damage

populations, causing severe mechanical damage, facilitating pathogen entry, and directly contributing to reduced biomass.

Recently, *H. galeatus* populations have risen in creeping bentgrass (CBG) putting greens across Virginia, attributed to two events: 1.) the restriction of fenamiphos, a long-used nematicide with excellent PPN control, led to the development of new nematicide solutions, and 2.) shortly after, fluopyram, a succinate dehydrogenase inhibiting fungicide, was introduced as an alternative for PPN control that promoted root health and was nematostatic for most PPN, although it was suggested to have no efficacy against *H. galeatus* (Crow et al., 2017; Crow 2021; Kammerer et al., 2023). Consequently, golf courses using fluopyram with initial lance populations have seen an increase in these nematodes on CBG.

Previous research suggests that environmental factors can play a role in what determines actionable PPN populations (Todd and Tisserat, 1990; Settle et al., 2007). When PPN are feeding on turfgrass roots, nutrients are being lost to the nematodes as well as other fates (Luc, 2004). Loss of nutrients can lead to a decline in turfgrass health even when not visible (Luc, 2004; Settle et al., 2007). Other studies have shown that by lowering soil temperatures through artificial means under periods of high temperature can improve creeping bentgrass resilience to environmental pressures (Xu and Huang, 2001). These former works suggest that a singular action threshold is not sufficient for a blanket assessment but should be viewed along with other turf health metrics to better predict PPN damage.

Understanding the gradient of damage on CBG putting greens raises two key questions for golf course superintendents: 1) Will increased lance nematode populations

decrease root biomass? 2) Can urea nitrogen (46-0-0) help CBG roots withstand more nematode feeding? The objective of this work was to determine the relationship between *H. galeatus* populations and CBG root biomass, as influenced by nitrogen inputs.

MATERIALS AND METHODS

Plant materials for this work were obtained from an 'L-93' CBG putting green at the Virginia Tech Turfgrass Research Center in Blacksburg, VA during the winter with limited growth. Fifty 1.3 cm soil cores were collected randomly, homogenized, and extracted by semi-automatic elutriation and modified sugar floatation to test for plant-parasitic nematode presence. After confirming little to no *H. galeatus* presence (± 5 lance nematodes), 160, 5 cm diameter soil cores were removed and placed in 5 cm diameter Cone-tainers® (Stuewe and Sons Inc., Tangent, OR) in a 90:10 sand to peat moss soil mixture and grown in a greenhouse for five days with daily watering. Standard coffee filters were placed at the bottom of each Cone-tainer to keep soil from escaping. The actively growing CBG plugs were inoculated with five concentrations of *H. galeatus*; 0, 100, 500, 1500, and 3000 nematodes per Cone-tainer. Inoculated nematodes were recovered from pure cultures of lance nematodes established on St. Augustinegrass (*Stenotaphrum secundatum* (Walter) Kuntze) that had been maintained in the greenhouse. The infested St. Augustinegrass plants were destructively sampled and placed in Baermann traps for 72 hours and sieved with a 400-mesh sieve to extract sufficient nematode populations for inoculation adapted from procedures by Whitehead and Hemming (1965). Inoculation amounts were determined by estimating the population per one mL, five mLs, and 20 mLs and these numbers averaged for a total estimation. Amounts were then diluted to 100 nematodes per 1ml of water for inoculation of the 100 level and 500 nematodes per 1ml of

water for levels of 500, 1500, and 3000. Samples inoculated with 500, 1500, and 3000 nematodes received 1mL, 3mLs, and 6mLs respectively. Inoculated CBG samples acclimated in the greenhouse for 18 weeks to allow time for lance populations to reproduce to induce plant damage (Willut,1983).

This experiment was a randomized complete block design in a 5x4 factorial arrangement with four replications repeated once. The five levels of *H. galeatus* were evaluated across 4 levels of urea (Nutrien Ag, Saskatoon, CA) (46-0-0) nitrogen applied at four rates: 0, .00118, .00237, and .00484kgs/m² over 16 weeks. An acclimation period of 18 weeks was allowed after inoculation prior to nitrogen applications. Urea was broadcast in solution at the mentioned rates every 2 weeks over corresponding Cone-tainers using a CO₂ backpack sprayer calibrated at 276 kPa over 374 L ha⁻¹ through VisiFlow® Even Flat Fan 8004 nozzle tips (TeeJet Technologies, Wheaton, IL). Cone-tainers were hand-watered daily and mowed 5x a week using an adjustable trimmer (HomeCut Combo, Wahl, Sterling, IL) set at its lowest setting (3.175 mm).

Every 4 weeks (4, 8, 12, and 16) from the initial nitrogen applications, a destructive sampling of one rep from each factor combination (n=20) was implemented and *H. galeatus* nematodes were extracted from each Cone-tainer using two methodologies. For samples removed at weeks 4 and 8, nematodes were extracted using Baermann trays (Hooper, 1986), and total *H. galeatus* recovered through a 400-mesh sieve were determined using an Olympus® CK2 inverted compound microscope. Baermann trays were initially used to ensure less disturbance to sample root material for measuring root biomass. For destructive samplings at weeks 12 and 16, nematodes were extracted using two methods

(Jenkins, 1974) to increase recovery efficiency. After 72 hours in Baermann trays, nematodes were counted using this method, then each soil sample was subjected to a modified Jenkins method and combined totals from both methods were compared against root biomass recovered.

Root biomass was captured at 4, 8, 12, and 16 weeks by drying root material at 60°C for at least 24 hours then sieving soil from root material using a 60-mesh sieve before measuring dry weights. Dry weights of each sample were subjected to a muffle furnace at 500°C for 8 hours before being measured for ash weights. Root biomass was determined by subtracting ashed weights from dry weights and recorded in grams.

Two models were assessed for response variables of lance nematode counts and root biomass in grams (Table 1). The first model included inoculation level, nitrogen level, and the interaction between these factors, while the second model included nitrogen level, sampling time, and the interaction between these factors. Factors were subjected to an analysis of variance (ANOVA) displaying significant data and means were compared with each ANOVA using Tukey's HSD ($\alpha=0.05$).

RESULTS

The overall range of lance nematodes recovered per Cone-tainer across all destructive sampling times was 0-1139. Inoculation levels of 0, 100, 500, 1500, and 3000 resulted in average nematode counts of 7.44, 153.69, 235.69, 326.21, and 248.31, respectively. Final nematode counts at the time of destructive sampling from inoculation levels of 500, 1500, and 3000 were different than 0, but all were similar to 100 ($p < 0.0001$) (Table 1). Linear regression across all sampling times showed a significant

negative relationship between *H. galeatus* and root biomass, despite high variability (Figure 1). Nitrogen treatments did not significantly impact *H. galeatus* counts ($p=0.0550$) or root biomass ($p=0.3658$) when subjected to ANOVA ($\alpha=.05$) across all sampling dates. Data for each destructive sampling time is outlined below.

The range of *H. galeatus* recovered for all inoculation levels at the 4-week destructive sampling was 3-409. Mean of lance nematodes per inoculation level in ascending order was 4.75, 36.25, 237.35, 210.5, and 202.5, respectively. The inoculation level of 500 was different than 0 after destructive sampling but like all other inoculation levels at this time. A negative linear regression was present 12 weeks after initial application (WAIA) but not at other destructive samplings. Significant differences were observed in an ANOVA of nitrogen effects on lance nematode counts or root biomass (Figure 2).

Hoplolaimus galeatus recovered 8 weeks after initial nitrogen application ranged from 1-305 across all inoculation levels. Means at 8 weeks were similar to the 4-week samplings with averages of 2, 92.95, 166.75, 214.25, and 122.5 respective to inoculation levels in ascending order. Inoculation levels of 1500 and 500 resulted in more lance nematodes compared to no nematodes inoculated but were statistically the same as the other inoculation levels. A negative trend was noted between *H. galeatus* counts and root biomass; however, this relationship was not statistically significant ($p= 0.1282$) (Figure 2). An ANOVA of nitrogen levels by lance nematode counts and nitrogen levels by root-biomass in grams resulted in no differences at the 8-week interval.

The range of nematodes recovered among all inoculation levels at week 12 using the Baermann tray method alone was 0-490. Average counts of 3, 47.5, 57.5, 207, and 78 were observed for inoculation levels of 0, 100, 500, 1500, and 3000 respectively. No differences were observed between inoculation levels using the Baermann tray method alone. When supplemented with the modified Jenkins method, the range of nematodes recovered expanded to 0-573 for all inoculation levels and means for each inoculation levels increased. With combined methodologies of extraction, the inoculation level of 1500 resulted in more lance nematodes compared with no inoculated nematodes while also being like other inoculation levels. No significant differences were observed when evaluating a linear regression of *H. galeatus* counts by root biomass for the Baermann extraction alone; however, a significant negative linear regression was observed when the modified Jenkins methodology was included (Figure 2). A root biomass threshold ratio was created by the following equation:

$$\text{Root biomass threshold ratio} = \frac{\text{H. galeatus counts}}{\text{root biomass (g)}}$$

Root biomass threshold ratios showed a significant negative relationship with normalized difference vegetation index (NDVI) using linear regression (Figure 3) (Xue and Su, 2017). The nitrogen level of .00484kgs/m² resulted in more *H. galeatus* nematodes than levels of 0.11kgs and 0kgs (Figure 4). Root biomass was numerically lower at the .00484kgs/m² nitrogen level although not significant (Figure 5).

The Baermann tray methodology alone resulted in a range of lance nematodes from 0-484 across all inoculation levels with means of 4.75, 137.25, 59, 149.75, and 119.5 respective of inoculation levels in ascending order. No differences were observed between

inoculation levels at this time using this methodology. The range and means of each inoculation level increased when supplementing extraction by using the additional modified Jenkins extraction methodology. Even with this increase, no differences were observed across inoculation levels. Regardless of extraction methodology, no differences were observed by linear regression of *H. galeatus* counts by root biomass or by an ANOVA using Tukey's HSD test for nitrogen levels by either lance counts or root biomass (Figure 2). Likewise, no differences were observed when evaluating an ANOVA of nitrogen level by either *H. galeatus* counts or root biomass.

DISCUSSION

The 12-week timing interval is the primary window where differences are observed in this study. Combined with an 18-week inoculation acclimation period, this totals 30 weeks for *H. galeatus* nematodes to reproduce and feed, covering approximately seven life cycles from J1 to adult. Previous research with *Hoplolaimus* species indicates that longer feeding periods and opportunities for nematode population growth can lead to more observable damage in turfgrass systems.

With significant variability in the study (Figure 1), time plays an important role in evaluating plant health effects caused by *H. galeatus*. Feeding from these populations led to regression differences between lance counts and root biomass, directly influenced by nitrogen inputs. We applied minute rates of nitrogen to determine if small doses would impact nematode counts and/or root biomass. While nitrogen affected nematode counts, it did not significantly impact root biomass, likely due to the low nitrogen inputs.

Typically, golf course superintendents apply around .00484kgs/m² of nitrogen per growing season on creeping bentgrass (CBG) putting greens, equating to 0.045 kg weekly during active growth. In this study, we applied 0.045 kg every two weeks at the highest rate, potentially creating a constant struggle between *H. galeatus* population support and root uptake. Future experiments with higher nitrogen rates at consistent intervals could provide more informative data.

Despite the significant differences observed at 12 weeks, none were noted at 4, 8, and 16 weeks. At 4 weeks, plants had 22 weeks of *H. galeatus* acclimation, with 0.11 kg of nitrogen applied at the highest rate. This was expected, as limited time and nitrogen inputs should not show significant differences. Nitrogen applications were foliar and not watered in, potentially affecting root development.

At 8 weeks, *H. galeatus* recovery increased, resulting in a shift in regression analysis. The negative regression indicated that nitrogen inputs were approaching significance, but due to low inputs, no significance was observed. The lack of support from the modified Jenkins extraction method may have diluted the data, leading to non-significance.

A similar trend was present at 12 weeks without the secondary modified Jenkins extraction method. The Baermann method alone led to non-significance, but *H. galeatus* recovered from both methodologies showed significant data. At 16 weeks, inoculation similarities in recovered *H. galeatus* were observed, suggesting that *H. galeatus* populations and root biomass damage plateaued.

By week 34 (18-week acclimation plus 16 weeks post-nitrogen application), approximately eight generations of *H. galeatus* may have occurred. Two possibilities arise:

1) nitrogen effects had little influence as nematode populations increased, or 2) inoculation levels were skewed by inoculation methods. The second idea is more plausible, as the variability of inoculation increased as the water/nematode solution dwindled, affecting inoculation levels and root biomass similarities at this sampling time.

Ultimately, what do we learn from this work? First, we learn about the variability in studying *H. galeatus* nematode effects on CBG roots. Three out of four destructive samplings showed no differences; however, where differences were observed, valuable insights were gained on how *H. galeatus* affects root biomass. Nitrogen inputs increased lance nematode counts at specific intervals but had no effect on root biomass. If higher nitrogen levels had been used, they may have potentially increased nematode counts and decreased root biomass, suggesting that nitrogen inputs may increase nematode populations rather than bolster roots against feeding.

The need for more effective options for controlling *H. galeatus* are evident through this work. Since nematodes are more closely related to humans than plants, developing true nematicides is riskier. The use of nematostatic pesticides has led to resistance issues, which were historically non-existent in nematology (Kammerer et al., 2023). Finally, this work emphasizes the importance of practical turfgrass nematode discovery. Although nematodes represent a smaller portion of pest management costs, they can be crucial when damage is difficult to control or leads to turfgrass replacement.

TABLES AND FIGURES

Table 1. Effects test of two models for response variables of lance nematode counts and root biomass in grams. Significant data is bold ($\alpha=0.05$).

	Lance nematode counts	Root biomass (g)
Inoculation level	<0.0001	0.3734
Nitrogen level	0.0550	0.2636
Inoculation level*Nitrogen level	0.3658	0.9149
Nitrogen level	0.6022	0.0159
Sampling time	0.8032	<0.0001
Nitrogen level*Sampling time	0.0187	0.0392

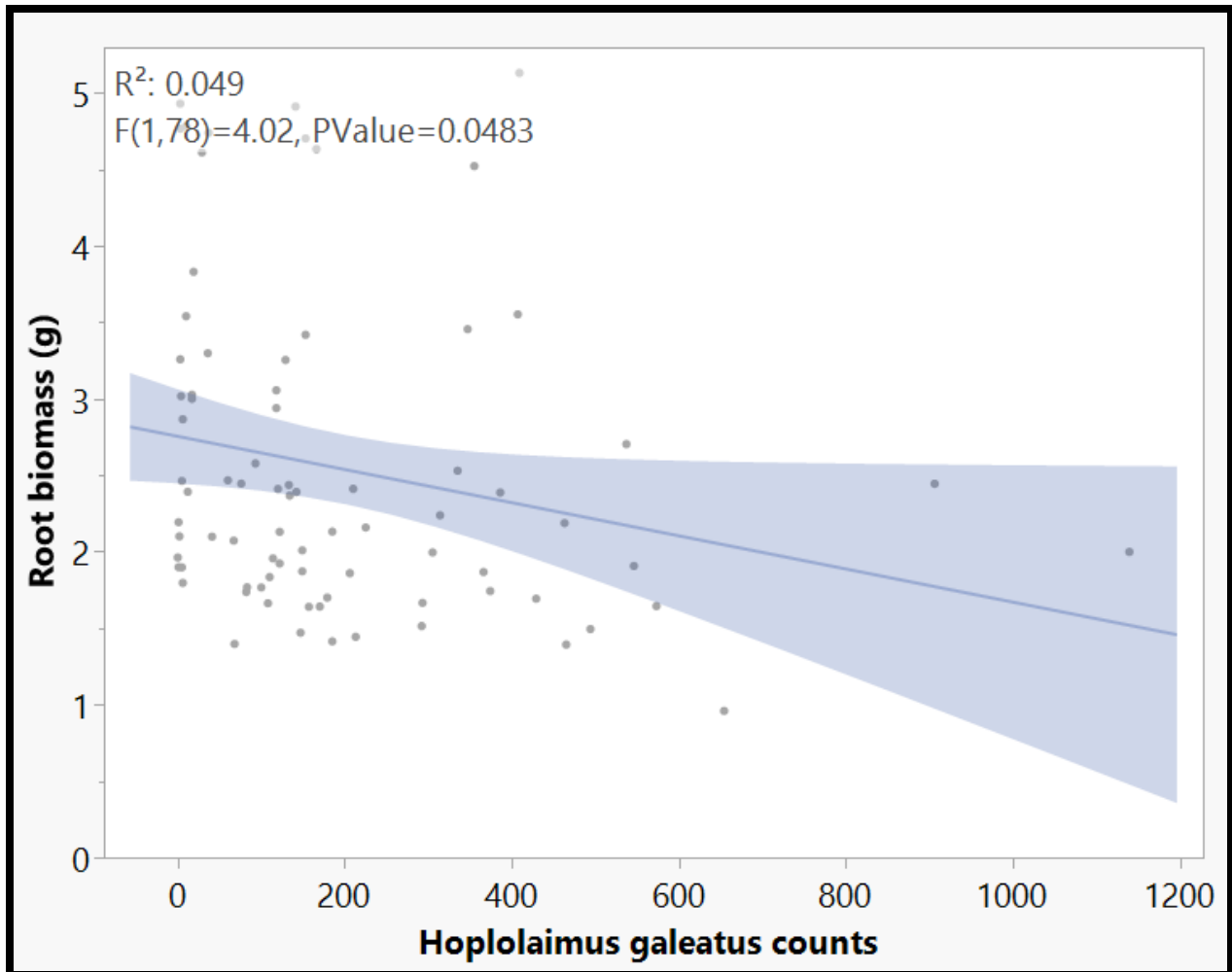


Figure 4. Linear regression of *Hoplolaimus galeatus* (Cobb, 1913) Thorne, 1935 counts by root biomass of creeping bentgrass (*Agrostis stolonifera* cv. 'L93'). ($R^2= 0.049$).

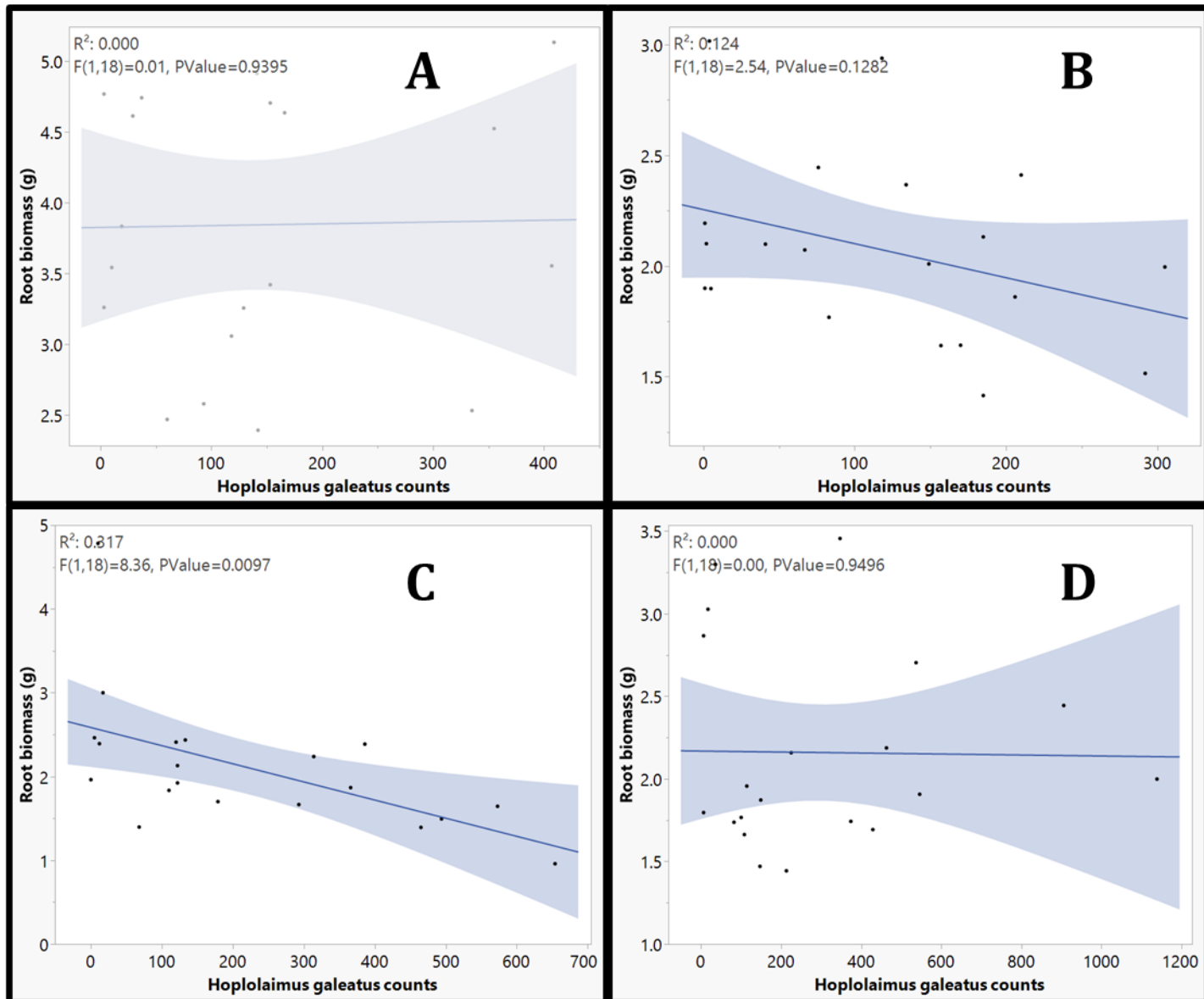


Figure 5. Linear regression of *Hoplolaimus galeatus* (Cobb, 1913) Thorne, 1935 counts by root biomass of creeping bentgrass (*Agrostis stolonifera* cv. ‘L93’) at four destructive sampling dates. Destructive sampling dates were split into four, 4-week timings of 4 (A), 8 (B), 12 (C), and 16 (D) weeks after initial urea nitrogen applications.

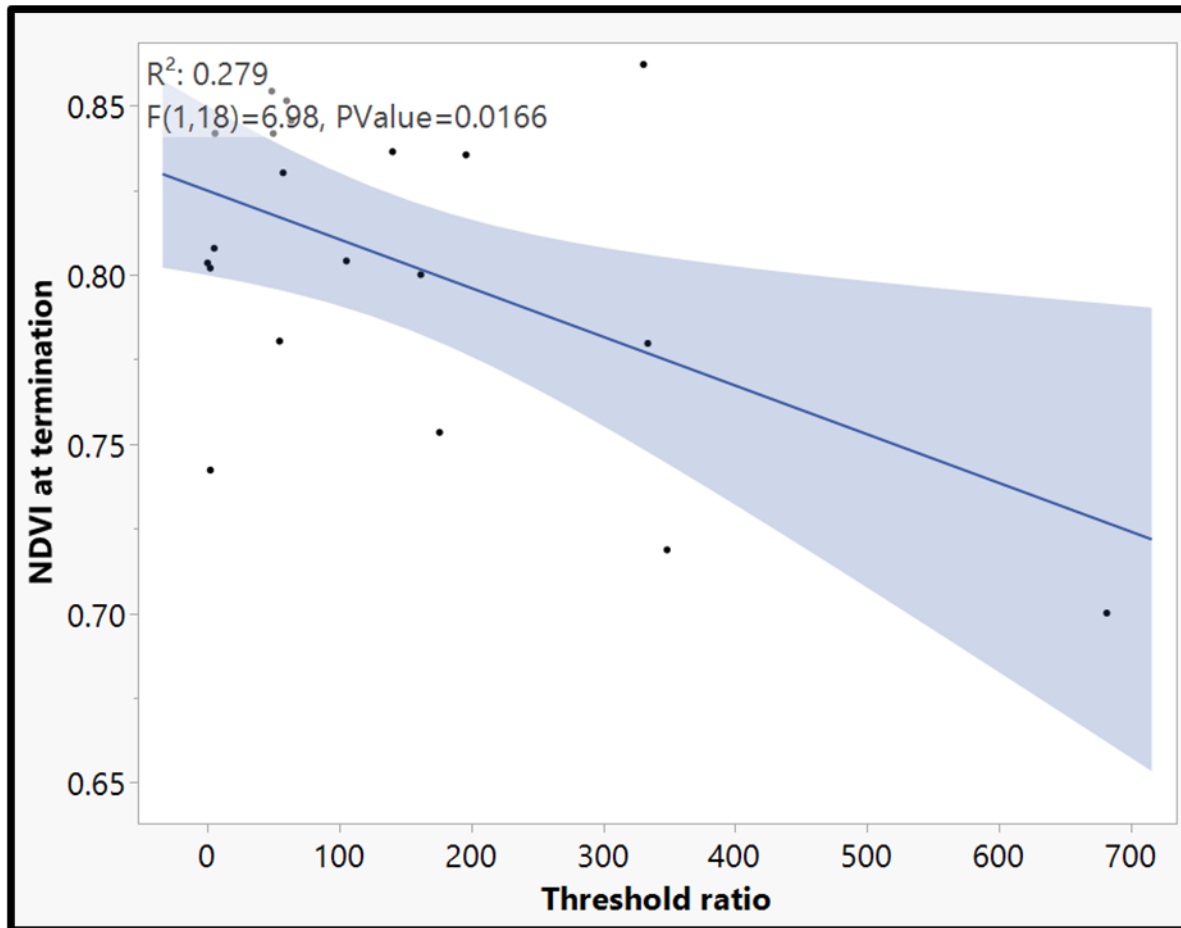


Figure 6. Linear regression of root biomass threshold ratio by normalized difference vegetation index (NDVI) at week 12 termination of creeping bentgrass (*Agrostis stolonifera* cv. ‘L93’). Root biomass threshold ratio was determined by evaluating *Hoplolaimus galeatus* (Cobb, 1913) Thorne, 1935 counts/root biomass (g).

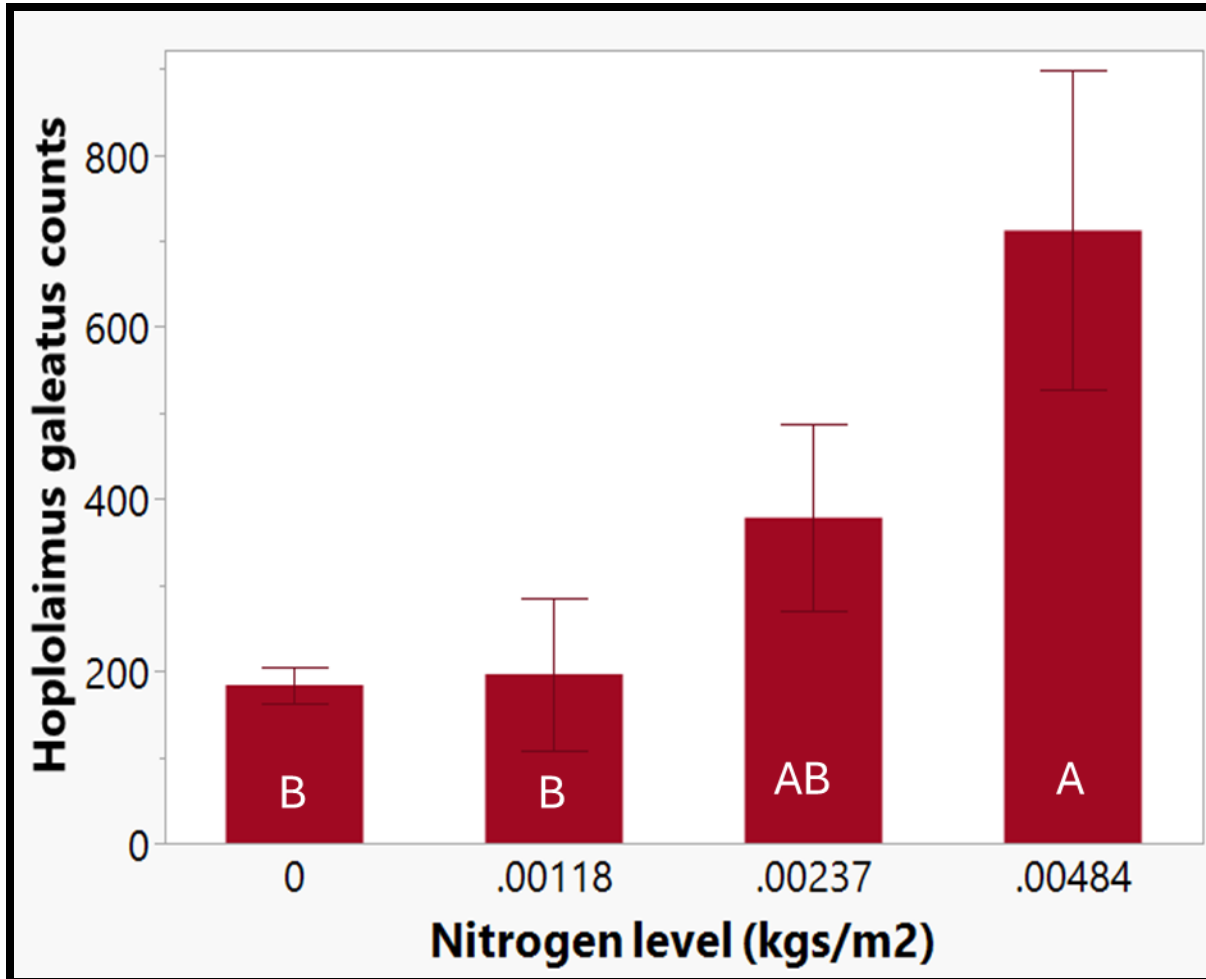


Figure 7. An analysis of variance (ANOVA) for nitrogen level on creeping bentgrass (*Agrostis stolonifera* cv. ‘L93’) by *Hoplolaimus galeatus* (Cobb, 1913) Thorne, 1935 counts 12 weeks after initial nitrogen applications. Bars with the same letter are not statistically significant according to a Tukey’s HSD ($\alpha=0.05$).

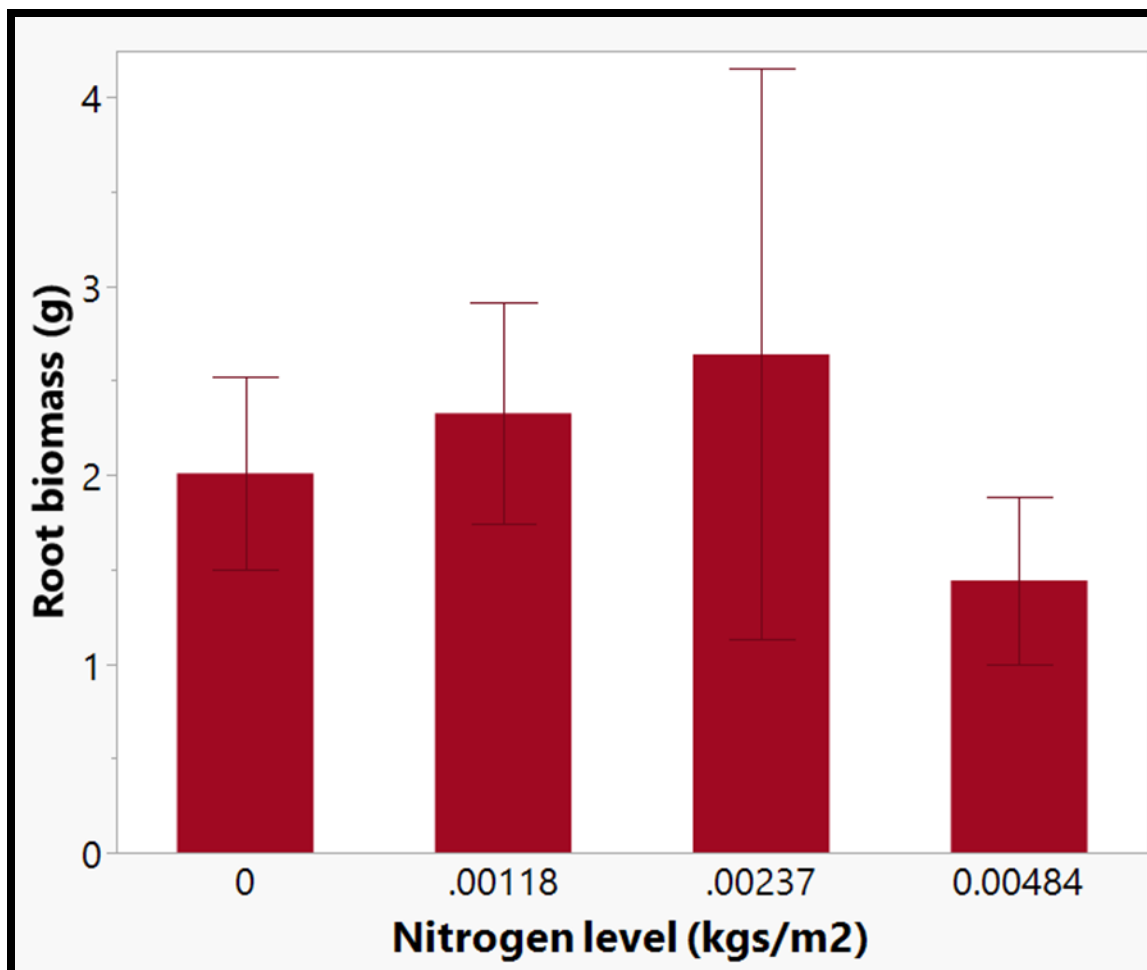


Figure 8. An analysis of variance (ANOVA) for nitrogen level by root biomass in grams 12 weeks after initial nitrogen applications on creeping bentgrass (*Agrostis stolonifera* cv. ‘L93’). Means were separated using a Tukey’s HSD ($\alpha=0.05$) (p value = 0.0869).

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CHAPTER IV

COMPARING TRADITIONAL AND QPCR METHODS FOR IDENTIFICATION AND POPULATION ESTIMATION OF HOPLOLAIMUS GALEATUS IN TURFGRASS

M.A. Tucker, Henderson, C.A, Haak, D, Crow, W.T., Eisenback, J.D., and McCall, D.S.

KEYWORDS

cycle threshold (Ct), molecular diagnostics, quantitative polymerase chain reaction (qPCR), soil health, turfgrass management

ABBREVIATIONS

PPN, DNA, Ct, qPCR, BGC, EPA, GNSS, RTK, FAM, SYBR, MGBNFQ, min, sec, bp

ABSTRACT

Quantitative polymerase chain reaction (qPCR) is widely used for organism identification but has been underutilized for turfgrass nematode estimation. This study adapted a qPCR approach for the identification and quantification of lance nematodes (*Hoplolaimus galeatus*) in golf course putting greens. Lance nematode populations from 28 plots collected at Belmont Golf Course (BGC) were manually counted and compared to qPCR results, yielding a cycle threshold range of 20.949-25.686. A negative relationship using linear regression between DNA quantity and manually counted nematodes was statistically significant ($p=0.0352$) but weak ($r^2=0.16$). To improve accuracy, DNA was extracted from handpicked samples of 250, 100, 50, and 20 lance nematodes and suspected degrees of human error were accounted for. These improvements produced a stronger negative relationship between variables ($r^2= 0.573$, $p=0.0002$). These results indicate that

qPCR may be a viable alternative for nematode quantification, though further refinement is needed for broader implementation.

INTRODUCTION

The turfgrass industry in Virginia is valued at over a billion-dollars (National Agricultural Statistics Service Virginia Field Office, 2006 (NASSVFO). Golf course maintenance, which covers only 2.2% of the commonwealth's managed turfgrass acreage, accounted for nearly \$100 million in expenditures in 2004 (NASSVFO, 2006). Approximately 10% of this budget was allocated to crop protectants for managing weeds, insects, and diseases (NASSVFO, 2006). Around 150 acres of turfgrass are maintained on an average 18-hole facility with roughs comprising approximately 33%, fairways around 20%, and tees and greens less than 5% (Gelernter and Stowell, 2017). Despite relatively small acreage of golf courses, putting greens require significant financial investment due to the high cost of maintenance with pest management being a primary factor.

Managing nematodes in turfgrass has become increasingly challenging. The use of abamectin can be effective at suppressing plant parasitic nematodes, but it does not move down in the soil profile making full coverage of the root system a problem if root depths are greater than 2.5 cm (Crow et al., 2017). Environmental Protection Agency (EPA) restrictions on the organophosphate fenamiphos and the limited effectiveness of newer products reduce the number of effective management options (Crow et al. 2017). Because of the EPA's fenamiphos restriction, fluopyram has become the most commonly used chemical for nematode management (Kammerer et al., 2023).

A recently labeled nematicide for turfgrass, fluopyram, (Crow et al., 2017) selectively targets many nematode species but fails to control *H. galeatus*. This nematode causes damage to a variety of turfgrasses (Martin, 2017; Settle et al., 2005). They are migratory and become semi- or endoparasitic (Martin, 2017). Research has demonstrated that fluopyram has little to no effect on suppressing lance nematodes. Besides that, its overuse may select for populations of root-knot and sting nematodes that are resistant, documenting the first case of nematicide resistance (Crow, 2024; Kammerer et al., 2023). The lack of efficacy and the development of resistance have increased interest in the management of lance nematodes in Virginia, making their identification and quantification more important.

Molecular methods have been successful at identifying plant pathogens in turfgrass (Stackhouse et al., 2020). Currently, methods such as quantitative polymerase chain reaction (qPCR) are used to identify pathogens such as ectotrophic root-infecting fungi of ultradwarf bermudagrass greens with success (Bronzato Badial et al., 2020). Even so, traditional methods of nematode identification and quantification appears straight forward and not necessary to pursue a molecular approach. Unpublished data (Eisenback, unpublished) shows that the current methods of extraction, identification, and quantification of supposed similar samples sent to ten different state labs breaks down when a level of automation is not involved. The objective of this work is to test the viability of a qPCR method for lance nematodes at identification and quantification using an objective approach.

MATERIALS AND METHODS

Sampling:

Soil and turfgrass system samples were collected from Belmont Golf Course in Richmond, VA on a United States Golf Association specification (Murphy, 2007) '777' creeping bentgrass (*Agrostis stolonifera* L.) putting green with a history of high lance nematode (*Hoplolaimus galeatus* (Cobb, 1913) Thorne, 1935) populations. Selection of sample areas from within this green was based on green size and shape where 28 samples were collected from centroids spaced 3 meters apart in a gridded design. Geospatial coordinates for each ground control point were collected using a pair of Emlid rs+ (Emlid, Budapest, Hungary) Global Navigation Satellite System (GNSS) receivers configured as a rover and base station for RTK correction with 30-second location averaging per point allowing for theoretical corrections < 14 mm. Each sample consisted of five 1.27 cm soil and turfgrass system cores within 1 meter of designated X, Y coordinates. Cores were extracted to a depth of 15 cm and verdure was kept intact for consistency. Root lengths were measured using a 22.86cm x 34.29cm tin pan with a printable ruler secured to the bottom left-hand side. Each soil core was placed with verdure flush on the left side and the longest root was measured and averaged among 5 representative soil cores for each sample area. Nematodes were extracted from 250 cm³ of soil using a semi-automatic elutriator (Byrd et al., 1976) and further processed with sugar-floatation/centrifugation (Jenkins, 1964). Samples included populations of *H. galeatus*, *Paratrichodorus* sp., *Mesocriconema* sp., *Tylenchorynchus* sp., *Meloidogyne* sp., and *Helicotylenchus* sp. Total lance nematode counts were recorded from subsamples of each plot for comparison against qPCR assay estimates. Handpicked lance nematode counts of 20, 50, 100, and 250

were processed with qPCR to establish a correlation with DNA extracted from known numbers for the analysis of BGC samples. Each hand-picked count was replicated five times.

Primer and Probe design:

A complete sequence of the internal transcribed spacer (ITS) region of *H. galeatus* was selected from GenBank (EU515322) (Bae et al., 2008). Primers were designed from this sequence using Primer3plus (Untergasser et al., 2012) with a coverage of 574 base pairs (bp). The forward primer was 20 bp in length containing 50% GC content, and the reverse primer was 20 bp with a 55% GC content. Both forward and reverse primers had secondary hairpin structures. The forward and reverse primers were named EU515322 *H. gal* F (5'-ACC TGG TGT GGG TTT TGC TT-3') and EU515322 *H. gal* R (5'-GCC GAG TGA TCC ACC GAT AA-3'), respectively. A TaqMan® (Thermo Fisher, Carlsbad, CA) probe was also designed for this region of interest to target amplification of *H. galeatus* to avoid false positives during amplification. This probe had 23bp and was named Lance probe with a 6-FAM reporter on the 5' end and MGBNFQ quencher on the 3' end.

DNA extraction:

DNA was extracted from two types of samples, samples from BGC and handpicked quantities of 250, 100, 50, and 20 lance nematodes. Samples of lance nematodes from BGC were extracted using a Thomas Scientific® 500-mesh sieve (Chads Ford Township, PA) by suspending nematodes captured in the sieve using 250µL of deionized water dispensed from a micro pipette. The captured water nematode solution was dispensed into 1.5 ml micro centrifuge tubes to equal 250µl of water/nematode solution. These lance nematode samples were subjected to DNA extraction using the standard protocol with a Qiagen®

Powersoil® Pro DNA extraction kit (Hilden, Germany). Handpicked samples of 250, 100, 50, and 20 lance nematodes were placed into 10µl of water and micro pipetted into a clean micro centrifuge tube to a total volume of 250µl. To ensure complete nematode removal, handpicked samples were transferred from the original 1.5µl microcentrifuge tube and all remaining nematodes in the pipette tip were washed into a clean 1.5µl microcentrifuge tube before DNA was extracted. DNA extraction for handpicked samples followed the same protocol as for BGC samples.

qPCR protocol:

Thermo Fisher Scientific QuantStudio™ 3 (Waltham, MA) was used for qPCR assays. Each assay included 1µl of each primer, 0.5µl of TaqMan probe, 1µl of template DNA, 9µl of distilled water, and 12.5µl of SYBR™ Green Master Mix. The qPCR program included an initial denaturation at 95°C for 2 min., followed by 40 cycles of 95°C for 45 sec, 57°C for 90 sec, and 72°C for 2 min, with a final extension at 72°C for 10 min following procedures described by Bae et al., (2008). Each qPCR run contained a negative control of distilled water in place of the template DNA.

RESULTS

Sample estimations from Belmont Golf Course were made for *Hoplolaimus* sp., *Tylenchorynchus* sp., *Paratrichodorus* sp., *Helicotylenchus* sp., and *Mesocriconema* sp. using extraction methods as described. According to estimations, 18 of the 28 samples were above economic thresholds for *H. galeatus* (500 nematodes per 500cm³ of soil) according to guidelines used by the Virginia Tech Nematode Assay Lab. The total number of *H.*

galeatus per sample was categorized as follows: 12 samples contained 26-100 nematodes, 6 had 101-200, 4 had 201-300, 4 had 301-400, and 2 had 401-488 (Table 1). A linear regression analysis between *H. galeatus* counts and average root length (mm) showed a negative relationship ($R^2 = 0.531$, $F = 29.43$, $p < 0.0001$) (Fig. 1). A non-significant linear regression trend exists between Ct values of lance nematodes from BGC samples and average root length (mm) ($p = 0.0952$, $R^2 = 0.103$). Total nematode counts were directly compared to cycle threshold (Ct) values from qPCR assays.

Complete and partial sequences of six plant parasitic nematodes and the model organism *Caenorhabditis elegans* (Maupas, 1900) Dougherty, 1955 (NC_003280) were aligned using Geneious® gene alignment software (Biomatters® Ltd., Auckland, New Zealand). The following nematode sequences from the National Center for Biotechnology Information (NCBI) database were included: *H. galeatus* (EU515322), *Tylenchorynchus claytoni* Steiner, 1937 (KJ934130), *Paratrichodorus minor* (Colbran, 1956) Siddiqi, 1974 (MN97051), *Belonolaimus longicaudatus* Rau, 1958 (AB602616), *Helicotylenchus dihystera* (Cobb, 1893) Sher, 1961 (LC030375), *Mesocriconema xenoplax* (Raski, 1952) Loof and de Grisse 1989 (FN600588), and *Caenorhabditis elegans*. These sequences were included because they are plant parasitic nematodes commonly found damaging turfgrass. Small sections of these sequences (<100 bp) overlapped among these accessions, but not between these selections and *H. galeatus* (EU515322) in a section of sequences about 800 bp in length. Primers and probes were selected from within this section of sequence data.

Primer specificity was tested for lance against ring, spiral and sheath nematodes (Table 2). These three genera were selected because that samples used in testing included only these four PPN. Ten of each nematode genus were handpicked for each of the seven

reactions to test specificity and only reactions that included lance nematodes led to an amplification curve (Table 2).

Cycle threshold (Ct) values from 28 samples at Belmont Golf Course confirmed positive amplifications for *H. galeatus* in all samples, with Ct values ranging from 20.931-25.686 (Table 1). The average Ct values for samples containing 26-100, 101-200, 201-300, 301-400, and 401-488 were 24.107, 23.2025, 23.116, 23.6065, and 21.8255, respectively. Linear regression analysis, using hand-counted *H. galeatus* of these BGC samples as the independent variable and Ct value as the dependent variable, revealed a weak negative correlation ($R^2 = 0.160$, $p = 0.0352$, $\alpha = 0.05$) (Fig. 2).

Handpicked samples of 250, 100, 50, and 20 *H. galeatus* nematodes yielded average Ct values of 29.39, 30.77, 32.01, and 33.59, respectively. The corresponding range of Ct values for these sample sizes were 28.555-29.94 (250 nematodes), 29.80-31.85 (100 nematodes), 30.97-32.98 (50 nematodes), and 32.00-37.00 (20 nematodes) (Table 3). Linear regression analysis, using the handpicked lance nematode count as the independent variable and Ct values as the dependent variable, showed a moderate negative correlation ($R^2 = 0.573$, $F = 22.81$, $p = 0.0002$, $\alpha = 0.05$) (Fig. 3).

DISCUSSION

Nematode assays can vary significantly due to multiple factors, including (1) sample collection and handling, (2) sample processing, (3) extraction method, and (4) the skill and experience of the technician. These variables can lead to inconsistencies in nematode identification and quantification. To improve accuracy and reproducibility, a molecular

approach such as quantitative PCR (qPCR) offers a promising alternative by reducing variability and increasing objectivity.

Numerous studies have demonstrated the effectiveness of molecular diagnostic techniques in turfgrass pathology for detection of pathogens that cause economically important diseases, such as dollar spot, brown patch, summer patch, other several others (Stackhouse et al., 2020). For example, qPCR has successfully been used to identify and quantify ectotrophic root-infecting fungal root pathogens in Ultradwarf hybrid bermudagrass (*Cynodon dactylon* (L.) Pers. × *Cynodon transvaalensis* Davy) (Bronzato-Badial et al., 2020). Similarly, qPCR has proven useful in detecting and quantifying various plant-parasitic nematodes (PPN) (Braun-Kiewick & Braun-Kiewick, 2018). Kawanobe et al. (2015) developed a qPCR-based method for identifying PPN in sugarcane (*Saccharum officinarum* L.), particularly *Hoplolaimus columbus* Sher, 1963, and *H. seinhorsti* Luc, 1958. However, limited research has been conducted on *H. galeatus* (Bae et al., 2008). This study aims to develop a qPCR-based approach for identifying and quantifying *H. galeatus* in turfgrass, providing a more precise alternative to traditional hand-counting methods.

While qPCR shows strong potential for nematode identification and quantification, cost and technical requirements currently limit its widespread adoption. Unlike traditional methods that rely on inexpensive materials such as sugar, water, sieves, and centrifugation, qPCR requires specialized reagents, a thermocycler, and DNA isolation kits. Future advancements in soil-based DNA extraction may help make qPCR more practical and cost-effective for routine diagnostics.

Skill and Experience in Molecular vs. Traditional Assays:

The effectiveness of both molecular and traditional nematode assays depends on the expertise of the scientist conducting the analysis. Trained nematologists are required to extract, identify, and count specific PPN genera, whereas molecular biologists must be skilled in designing primers and probes to ensure accurate results. Although technicians with basic molecular training can perform qPCR by following standardized protocols, classical morphological nematode identification requires extensive training and experience to ensure accuracy. In modern scientific research, molecular techniques have become more widely adopted, yet fewer specialists are trained in traditional nematode morphology-based identification. Since traditional nematode assays require expertise in morphology, the accuracy of results often depends on the analyst's experience.

Precision vs. Accuracy: Morphological vs. Molecular Assays:

This study highlights the trade-off between precision and accuracy when comparing morphological and molecular assays. The linear regression analysis of *H. galeatus* counts and Ct values from Belmont Golf Club confirmed a statistically significant relationship between variables, although variance was high. The handpicked assay was more precise than qPCR due to potential sources of error in the qPCR method. Two key error sources were identified and corrected in the handpicked assays: 1. Ensuring no nematodes remained in the microcentrifuge tube when transferring via pipette. 2. Ensuring all nematodes were successfully dispensed from the pipette tip for DNA extraction.

Addressing these issues improved the model's accuracy at estimating lance counts. Although precision was improved, the overall conclusion remained unchanged: both BGC sample qPCR analysis and handpicked assays produced significant results. This confirms

that qPCR is a viable diagnostic tool for identifying and quantifying *H. galeatus*, though further refinements are needed to enhance reproducibility.

qPCR Methodological Challenges: Off-Target Effects:

One challenge in developing qPCR methodologies is the potential for off-target effects, particularly primer dimerization. Both qPCR assays in this study exhibited subtle initial amplification curves that did not cross the reaction threshold, indicating primer dimer formation. This occurs when forward and reverse primers form secondary hairpin structures and bind to each other instead of the target DNA. Primer dimerization can lead to false signals and inaccurate quantification if amplification extends beyond the reaction threshold. However, in this study, dimerization effects remained minimal, as reactions were consistent and did not cross the detection threshold. Direct interpretation of the qPCR data was also challenging. For example, a strong relationship between lance nematode counts and root lengths exists (Fig. 1). When Ct values interpreted through qPCR are substituted for actual lance counts by average root lengths, the data is a non-significant trend ($p=0.0952$, $R^2=0.103$) suggesting that as Ct values decrease, root lengths decrease. If significant, this Ct value by root length relationship would strengthen the current molecular methodology as having a practical field application. In reality, it shows that more work is necessary to improve these methods for practical use.

CONCLUSION

This goal of this work was to develop a qPCR-based approach for identifying and quantifying *H. galeatus* in turfgrass, providing a more precise alternative to traditional hand-counting methods. This study demonstrates that qPCR may become a viable

alternative for identifying and quantifying *H. galeatus* in turfgrass systems with further development. However, methodological refinements are necessary to improve consistency, reduce variability, and optimize precision. Future research should focus on enhancing DNA extraction techniques, minimizing off-target effects, and improving cost-efficiency to make qPCR a more accessible and practical diagnostic tool for nematode quantification in turfgrass management.

TABLES AND FIGURES

Table 1. *Hoplolaimus galeatus* estimates from samples collected at Belmont Golf Course, Richmond, obtained using estimation by traditional methods (second column) and the actual counts from five subsamples within the plot (third column), compared against cycle threshold values from qPCR (fourth column).

Sample	<i>Hoplolaimus galeatus</i> estimation by traditional methods	<i>Hoplolaimus galeatus</i> total counts by traditional methods	Cycle threshold values
1	620	107	22.844
2	360	51	23.209
3	220	26	23.128
4	3280	470	21.493
5	1520	222	23.437
6	2320	288	20.931
7	1220	173	24.721
8	300	41	25.519
9	2120	243	24.424
10	380	50	24.039
11	1700	240	23.672

12	620	74	23.081
13	220	42	24.452
14	280	43	23.769
15	2640	351	22.533
16	1960	118	20.949
17	1200	176	22.865
18	3460	391	23.695
19	1320	180	23.075
20	860	135	24.761
21	3160	349	25.008
22	420	65	24.845
23	420	52	23.595
24	580	79	25.686
25	160	35	24.407
26	480	58	23.552
27	2600	394	23.190
28	3340	488	22.158

Table 2. Primer specificity test with selected genera of nematodes.

Nematodes present (10 per species)	+/- Identification
Lance	Positive (Ct= 36.11)
Ring	Negative
Spiral	Negative
Sheath	Negative

Lance + Ring	Positive (Ct= 37.31)
Lance + Spiral	Positive (Ct= 35.73)
Lance + Sheath	Positive (Ct= 37.12)
Water	Negative

Table 3. Summary of cycle threshold (Ct) values qPCR analysis of handpicked lance nematode samples of 250, 100, 50, and 20 specimens.

	Ct values			
	250	100	50	20
Rep 1	29.262	29.806	30.973	32.986
Rep 2	29.94	30.746	31.756	32.385
Rep 3	29.66	30.854	32.354	31.997
Rep 4	28.555	31.854	31.961	36.999
Rep 5	29.54	30.607	32.964	Undetermined
Avg.	29.391	30.773	32.002	33.592

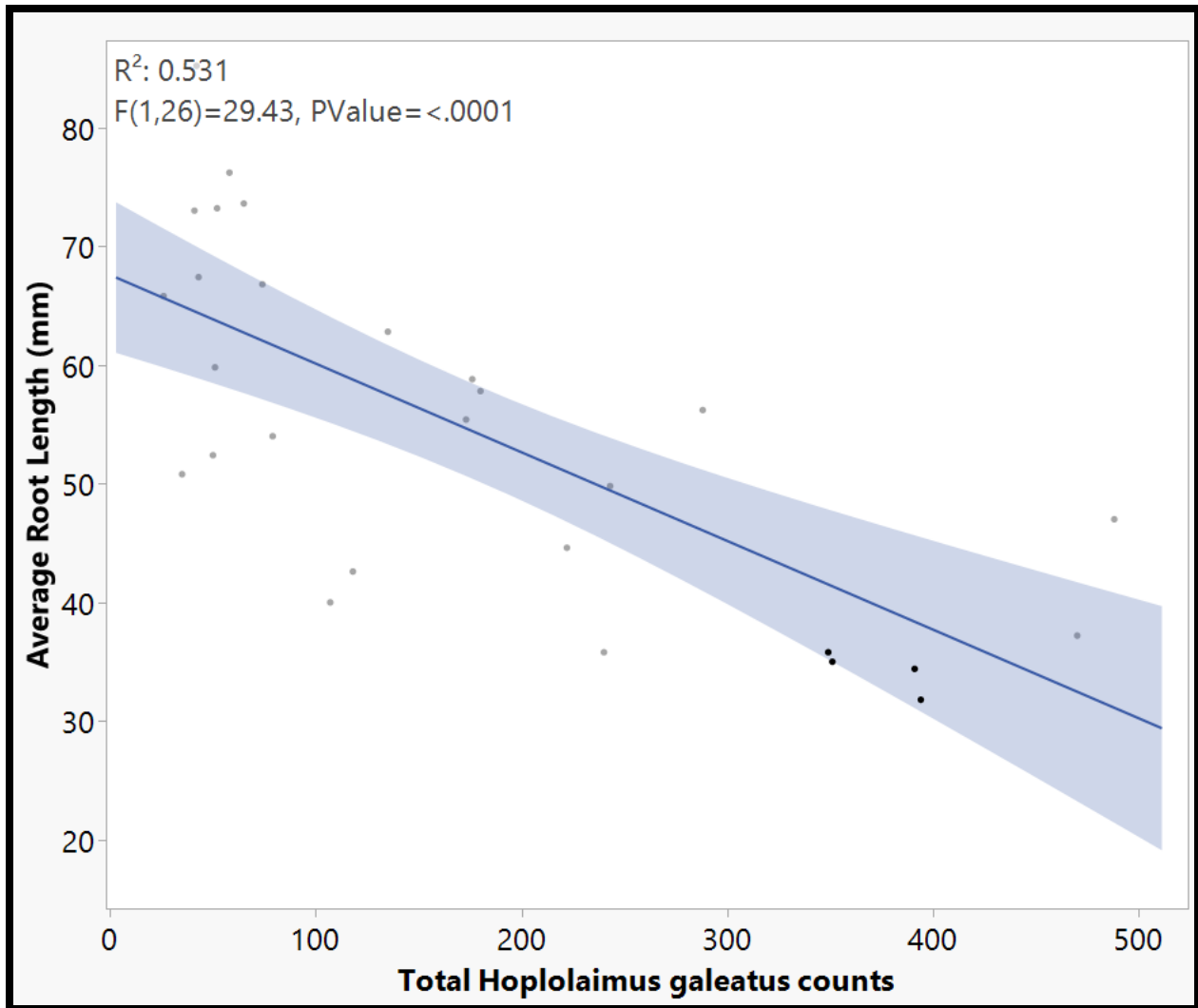


Figure 9. Negative relationship between *Hoplolaimus galeatus* counts from traditional diagnostic methods and creeping bentgrass root length (mm) from samples collected at Belmont Golf Course, Richmond, VA.

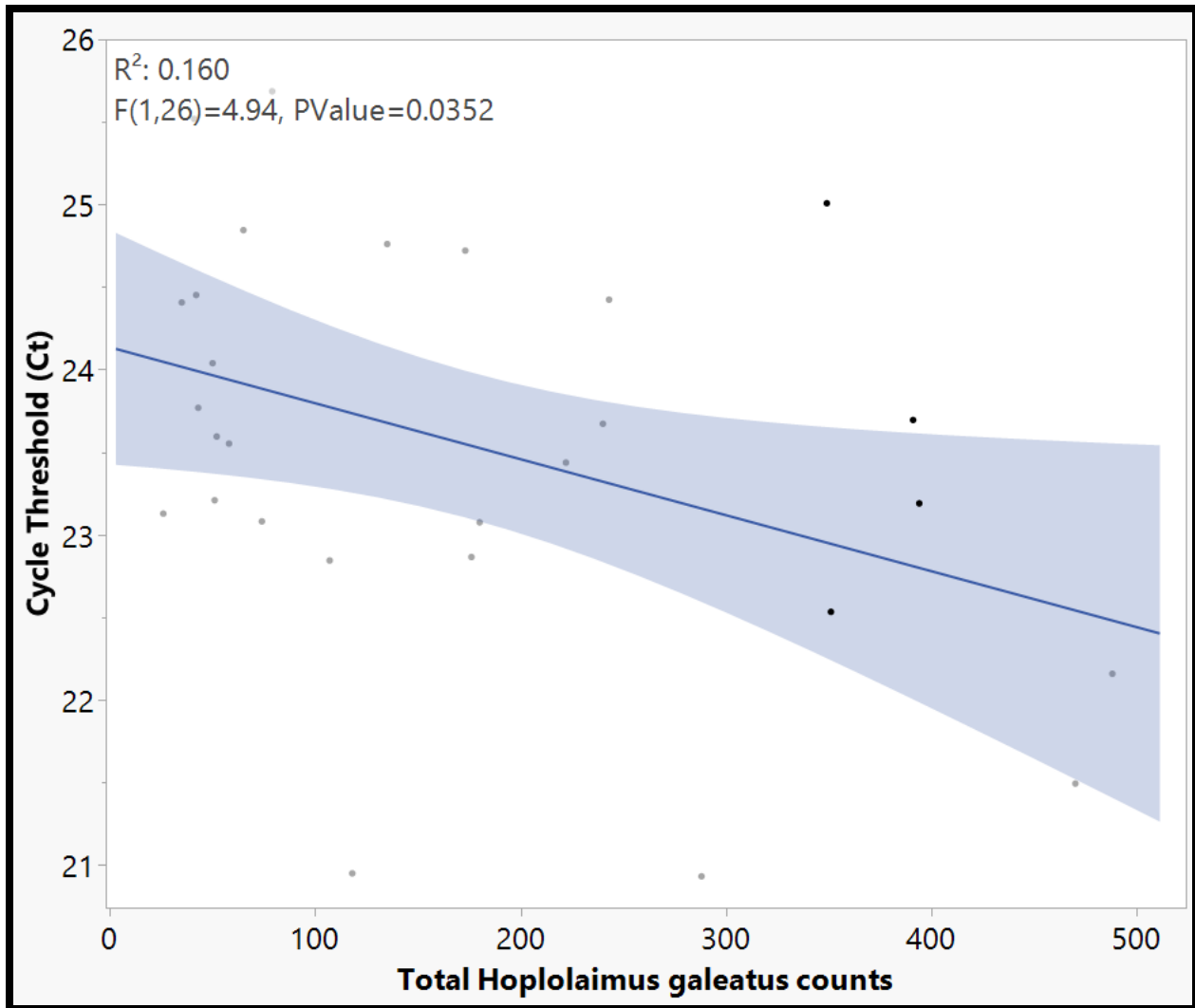


Figure 2. Linear relationship between total lance nematode counts obtained through traditional extraction methods and cycle threshold (Ct) values from samples collected at Belmont Golf Course, Richmond, VA.

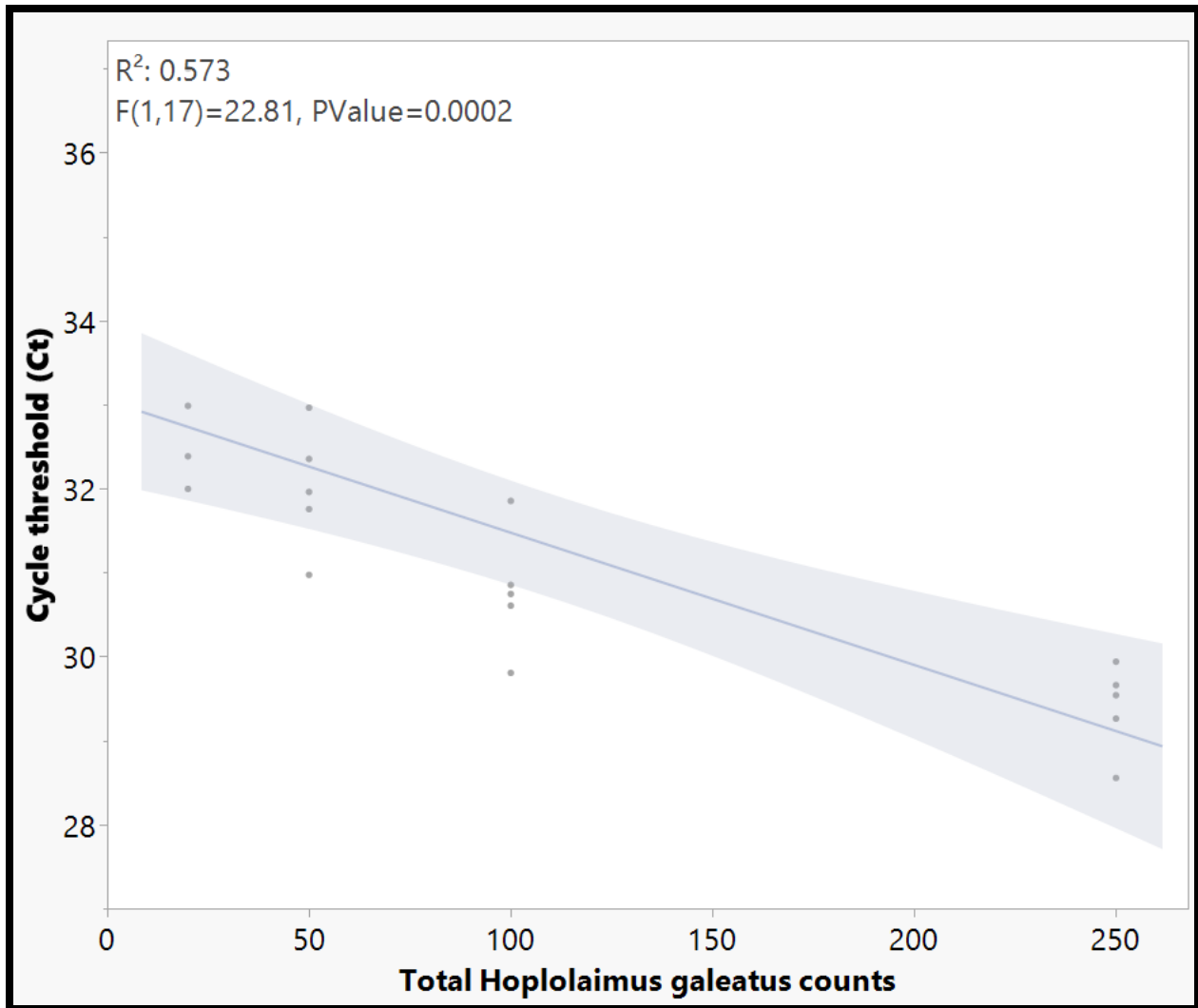


Figure 3. Linear regression analysis comparing handpicked *Hoplolaimus galeatus* (Cobb, 1913) Thorne, 1935 samples of 250, 100, 50, and 20 specimens and cycle threshold (Ct) values from a qPCR assays.

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