ABSTRACT

CUPO, KATHERINE LYNN. Methods for Identification and Eradication of *Heterakis gallinarum* Vectors on Poultry Facilities to Prevent Blackhead Disease Outbreaks. (Under the Direction of Dr. Robert Beckstead).

Heterakis gallinarum is ubiquitous but rarely diagnosed avian parasite that poses a silent threat to poultry producers. *H. gallinarum* is a small cecal worm that thrives in a wide range of avian host species and has a large geographic distribution that mirrors that of its hosts. Infection with the nematode alone typically does not cause significant disease; however, *H. gallinarum* eggs often act as carriers of other parasites including a protozoan called *Histomonas meleagridis* which causes a potentially deadly disease in gallinaceous birds commonly referred to as blackhead disease. *H. meleagridis*, introduced to the ceca by *H. gallinarum*, multiply and penetrate the cecal mucosa where they may enter the blood stream and be transported to the liver. The protozoans accumulate in necrotic foci in the liver tissue which eventually leads to liver failure and death. The prevalence of blackhead disease has increased significantly over the past three decades due to the revocation of FDA approval for use of drugs in commercial poultry that were previously administered to prevent transmission of the protozoan and treat afflicted birds. The likelihood of developing new pharmaceuticals capable of curing the disease or inhibiting *H. meleagridis* transmission in the foreseeable future is extremely low, and poultry producers must adapt their farm management practices to mitigate the chances of transmitting the protozoan between flocks by controlling the spread of *H. gallinarum*. Preventing flock to flock transmission of *H. gallinarum* on commercial poultry facilities is made difficult by a limited understanding of reservoirs for infection and a lack of information regarding effective decontamination procedures.

The traditional method for identifying *H. gallinarum* infection is microscopic examination of bird feces. *H. gallinarum* eggs present in fecal samples are often not observed or are incorrectly identified as another nematode species using this method of detection. A molecular diagnostic technique that provides greater specificity and sensitivity is needed to investigate sources of *H. gallinarum* contamination on poultry facilities. The first objective of this thesis was to design and test a diagnostic PCR for *H. gallinarum* DNA that would enable accurate and sensitive detection of the nematode from environmental samples. Three primer sets were designed from sequenced fragments of the *H. gallinarum* genome and tested for their specificity and sensitivity to *H. gallinarum* gDNA. One of the primer sets was used to identify *H. gallinarum* DNA in tissue samples of earthworms collected from chicken and turkey farms and darkling beetles that were experimentally fed *H. gallinarum* eggs. This molecular diagnostic test is capable of specifically identifying *H. gallinarum* DNA from environmental samples containing large amounts of DNA from various other organisms and can be used for identifying *H. gallinarum* contamination on poultry facilities or identifying reservoirs and vectors of the nematode.

Decontamination of equipment and poultry houses may be important to decrease the transmission of *H. gallinarum* from contaminated farms. However, no disinfectants or chemical compounds have been assessed for their efficacy as *H. gallinarum* egg decontaminants. The second objective of this thesis addressed this issue using an *in vitro* assay that was designed to identify products that compromise the eggshell integrity and kill the larvae of *H. gallinarum* eggs. Trypan blue was used as an indicator dye to identify treatments that penetrated the eggshell and eggs were manually hatched to determine if treatment penetration also rendered the eggs non-viable. Several disinfectants and chemicals

commonly used in the industry were tested using this methodology. Only treatment with a high concentration of sodium hypochlorite (15125 ppm NaClO) for six hours rendered the *H. gallinarum* eggs non-viable. This *in vitro* assay can be used to identify compounds that may be effective for disinfecting farm equipment or poultry houses contaminated with *H. gallinarum* eggs.

Combining molecular identification of *H. gallinarum* reservoirs and chemical disinfection of these reservoirs on commercial poultry units may reduce the incidence of blackhead disease in the poultry industry.

Methods for Identification and Eradication of *Heterakis gallinarum* Vectors on Poultry
Facilities to Prevent Blackhead Disease Outbreaks.

by
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DEDICATION

To my parents, Michael Cupo and Lynn Blalock

BIOGRAPHY

Katherine Lynn Cupo, the daughter of Michael Anthony Cupo and Theresa Lynn Blalock, was born in West Chester, PA on August 31, 1993. She spent her younger years in West Chester then moved to Doylestown, PA for middle school and high school. At a young age, Katherine developed an interest in working with animals, and thanks to the support and encouragement of her parents, she worked as an animal educator and animal care intern at the Philadelphia Zoo during high school. In the fall of 2012, she enrolled at NC State to pursue a Bachelor of Science in Zoology. She began working in an innate immunology lab at NC State's vet school during her junior year and spent the next two years studying the signal transduction pathways controlling cytokine secretion. Through her experience in the lab, she developed an interest in disease and immunology research and decided to pursue a Master of Science in Poultry Science under the direction of Dr. Robert Beckstead studying nematode and protozoan parasites in poultry. Katherine will be continuing her research in Dr. Beckstead's lab as a PhD student.

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Introduction

      Heterakis gallinarum is a cecal nematode that shares the same global distribution as the poultry species it parasitizes [1]. *H. gallinarum* infection generally does not cause disease except in cases of heavy parasite burden [2]; however, the nematode egg serves as a vector for a protozoan parasite, *Histomonas meleagridis*, which causes histomoniasis (blackhead disease) in some gallinaceous birds including chickens and turkeys [3-5]. The withdrawal of anti-histomonal drugs paired with increasing consumer demand for free-range and organic poultry products led to the reemergence of histomoniasis in the poultry industry, resulting in significant loss to poultry producers in the form of bird mortality and profit loss. In the absence of pharmaceutical treatments, many turkey and chicken producers are now forced to adopt alternative strategies for preventing histomoniasis outbreaks on their farms. The simplest and perhaps most effective management protocol may be one that limits the spread of *H. gallinarum* since the nematode is a critical component of the *H. meleagridis* life cycle and plays an important role in its transmission between hosts [6]. Therefore, understanding how *H. gallinarum* and *H. meleagridis* interact in the host and are transmitted through the environment may be crucial for managing histomoniasis in the modern poultry industry.

Heterakids

Species

      The last review of the Heterakis genius was completed in 1950 by Holger Madsen. Approximately 50 species of the genius Heterakis that parasitize birds have been described, all of which reside in the ceca of their hosts [1]. The only distinguishing morphological characteristic among Heterakis species is the length and shape of the male spicules as the females of each

species in this genius are generally identical [1]. Madsen classified all Heterakis species under three groups primarily based on the morphology of the male reproductive organs: *Heterakis gallinarum, Heterakis dispar, and Heterakis isolonche* [1]. *H. gallinarum* have unequal spicules; *H. isolonche* have long, nearly equal spicules; and *H. dispar* have short, equal spicules [1]. Included in the *Heterakis gallinarum* group are species with a longer right spicule: *H. gallinarum*, *H. bosia*, *H. indica*, *H. pavonis*, and *H. valvata*, and species with a longer left spicule, *H. pusilla* and *H. vulvolabita* [1]. The *Heterakis isolonche* group consists of *H. isolonche, H. altaica, H. arquata, H. bancrofti, H. crypturi, H. fariai, H. interlabiata, H. nattereri, H. skrjabini*, and *H. tenuicauda* [1]. The *Heterakis dispar* group consists of *H. dispar*, *H. alata, H. beramporia, H. brasiliana, H. brevispiculum, H. chenonettae, H. hamulus, H. macroura, H. multidentata, H. papillosa, H. parva, H. psophiae*, and *H. silindae* [1]. While heterakids are commonly identified based on male morphology, Madsen also cautioned that this methodology is insufficient as the characteristics of the male postanal sucker, spicules and papillae are inconsistent within an individual species and between species [1]. These inconsistencies have resulted in incorrect identification of heterakids in the literature [7-9]. Furthermore, while each Heterakis species specializes in parasitizing a specific host species, some species of Heterakis may parasitize some of the same incidental host species, and mixed populations of Heterakis species have been found in the ceca of a single host [1]. The potential for discovering mixed populations of Heterakis species and the potential for false identification of these species necessitate the development of molecular diagnostics that would be more accurate in distinguishing the members of the Heterakis genus.

Distribution

      H. gallinarum has the widest geographic distribution of the heterakids that parasitize

birds and the greatest diversity of host species [1]. It thrives in the ceca of chickens, guinea fowl,

and ring-necked pheasants and has been reported in many other species of birds including ducks

and turkeys [10]. *H. gallinarum*'s broad host profile has enabled it to spread to nearly every

continent [1]. More recent surveys of helminthiasis in domestic chickens in Germany,

southeastern US, northern Italy, and northern Thailand found *H. gallinarum* to be the most

common nematode, occurring in 98%, 96%, 95.7%, and 86.7% of birds, respectively [11-14].

Others surveying chickens in Kenya, Morocco, and across 8 European countries found *H.*

gallinarum to be the second most prevalent nematode occurring in 21.33%, 10%, and 29% of

birds, respectively [12, 15, 16]. Fewer researchers have reported the prevalence of helminths in

turkeys; however, they demonstrate that *H. gallinarum* may have a relatively high prevalence

among turkeys but lower nematode burden compared to chickens. *H. gallinarum* was found in

70% of the backyard turkeys surveyed in Minas Gerais, Brazil with a mean of 26 nematodes per

bird [17]. Another study found *H. gallinarum* to be the only nematode infecting wild turkeys in

Kansas in 2003 [18]. Despite free-range poultry being at a greater risk for picking up parasites

from the environment and intermediate hosts, commercial poultry raised in close proximity

inside closed houses are at greater risk for circulating *H. gallinarum* because this species of

nematode has a short generation time and a direct life cycles not requiring an intermediate host

[19].

Lifecycle

      The *H. gallinarum* life cycle may be divided into three stages: egg, tissue-dwelling

juvenile, and luminal adult. The egg, which is deposited in the environment through the cecal

droppings of an infected bird, serves as a vessel for maintaining the larva in the environment outside a host for at least 3 years [20] and is the only stage that occurs outside a host in an aerobic environment. The egg may be consumed directly by a definitive host, such as a chicken, or an intermediate host, such as an earthworm, and hatch within the intestinal tract. Hass and Todd demonstrated that *H. gallinarum* eggs could be hatched *in vitro* by mimicking the conditions of the bird's intestine [21]. It is possible the *H. gallinarum* eggshell is digested in the intestine or environmental cues stimulate changes in the larva and eggshell that initiate hatching. In either case, the specific conditions required for hatching may indicate the *H. gallinarum* egg only hatches in a particular region of the gastrointestinal tract of specific hosts. Once the larva makes it to the ceca of the definitive host, the larva burrows into the epithelium and wrap around the lumen of the cecal crypt, where it completes maturation and grows out of the tissue into the lumen [22].

It is unclear exactly when or how the nematodes become infected with *H. meleagridis*, but the protozoan has been observed in the reproductive tracts of male and female *H. gallinarum* and in the developing ova throughout the oviduct [6]. This may suggest *H. meleagridis* is transferred between adult *H. gallinarum* as a sexually transmitted parasite. Although not every *H. gallinarum* egg contains *H. meleagridis* [23], some proportion of eggs from every population of *H. gallinarum* harbor the protozoan [10]. The proportion of *H. gallinarum* eggs that are infected with *H. meleagridis* may vary between populations and be a factor of the population size. Lund and Burtner suggest that less than half the females in a population may be infected with *H. meleagridis*, and of the infected females, two eggs per worm on average carry the protozoan [23]. However, Lund and Burtner's conclusion does not correspond to Gibbs's observation of *H. meleagridis* populating the ovaries and penetrating and multiplying within ova

at all stages in the oviduct [6]. The number of histomonads contained within a single *H. gallinarum* egg also vary; Gibbs reported numbers of *H. meleagridis* sporozoites ranging up to nine inside a single shelled egg [6].

Paratenic hosts

H. gallinarum eggs may be consumed by an intermediate host in the soil and transported to new populations of susceptible birds. Several species of earthworms (*Lumbricus terrestris*, *Allolobophora caliginosa*, and *Eiseni foetida*) were found to harbor numerous types of hatched nematode larvae in their tissues including *H. gallinarum* [24]. The *H. gallinarum* larvae can remain viable in the earthworm tissues for months arrested at an early larval stage [24] which is more vulnerable to environmental factors than the shelled egg. Transport of *H. gallinarum* by earthworms may explain why histomoniasis outbreaks in commercial poultry are more frequent in the late summer and fall and after heavy rains. Earthworms carry far more infective *H. gallinarum* larvae in the late fall than in the early spring, suggesting the larvae do not survive in the worms through the winter months [24]. Therefore, it appears that earthworms can only maintain viable *H. gallinarum* larvae in warm climates which may contribute to the seasonal spikes in histomoniasis outbreaks. Earthworms may pick up new *H. gallinarum* larvae from subsurface soil and carry them to the surface after heavy rains exposing new flocks to buried *H. gallinarum* reservoirs.

Induced histomoniasis in turkeys and chickens fed *H. gallinarum*-infected earthworms also indicates that *H. meleagridis* is transported inside the larva body rather than in the cuticle. If *H. meleagridis* were released when the larva sheds its first cuticle, *H. meleagridis* would be present within the earthworm outside of the *H. gallinarum* larva. Lund and colleagues reported no evidence of *H. meleagridis* in the earthworms although feeding the earthworms to chickens

and turkeys produced histomoniasis and *H. gallinarum* infections [23]. This corresponds to Tyzzer's observation of *H. meleagridis* inside the gut epithelium of juvenile *H. gallinarum* collected from turkey poults [22]. A study measuring the rate of *Parahistomonas wenrichi* infection via the *H. gallinarum* vector also indicates *H. gallinarum* releases the histomonads after the fourth and final molt rather than the first or second [25].

Few studies have been completed to identify other intermediate hosts for *H. gallinarum*, but some arthropod insects may carry embryonated *H. gallinarum* eggs externally. Flies have been shown to carry the *H. gallinarum* eggs mechanically, but it is unknown whether they can also carrying the eggs internally [26]. Several other species of insects have been found to ingest the *H. gallinarum* eggs. Grasshoppers fed *H. gallinarum* eggs have been shown to maintain the eggs in their digestive tracts for at least 4 hours and cause histomoniasis when fed to turkeys [26]. Because the *H. gallinarum* eggs do not hatch in the grasshopper, the eggs would likely pass out of the GI tract back into the environment. Therefore, grasshoppers are a less likely vector for the nematode. In another experiment, it was reported that the common woodlouse fed *H. gallinarum* eggs was capable of transmitting small numbers of *H. gallinarum* carrying *H. meleagridis* to turkeys; although, the birds did not develop clinical histomoniasis [27]. No studies to determine if the darkling beetle may carry *H. gallinarum* have been published, but Huber and colleagues used a diagnostic PCR for *H. meleagridis* to evaluate the mealworm's potential for carrying the protozoan. They demonstrated that *H. meleagridis* cannot survive past 4 days in the darkling beetle mealworm and the prevalence of *H. meleagridis* among mealworms on turkey and chicken facilities experiencing histomoniasis outbreaks is low [28]. Based on the limited studies for *H. gallinarum* vectors, a diversity of annelid and arthropod species can

transport *H. gallinarum*, but some species may play a more central role in spreading the nematode and therefore *H. meleagridis* than others.

Chickens and pheasants serve as carriers of H. gallinarum

Some bird species better serve as hosts for *H. gallinarum* than others. As Lund and his colleagues demonstrated, juvenile nematodes begin development in the ceca of ducks and geese, but development slows and eventually ceases after 10 days [29]. These species of bird have also been demonstrated to be poor hosts for *H. meleagridis* [29], indicating that they cannot spread *H. meleagridis*-harboring *H. gallinarum* eggs onto poultry facilities. Chickens and turkeys have both been found to harbor mature *H. gallinarum*; although, a larger percentage of infecting larvae reach maturity in chickens than in turkeys [30]. While turkeys may maintain small populations of mature *H. gallinarum*, they are highly susceptible to histomoniasis and typically succumb to the disease within two weeks of infection with *H. meleagridis*, preventing any *H. gallinarum* they may be carrying from reaching maturity. Therefore, turkeys are also an unlikely source of soil contamination with *H. meleagridis*-harboring *H. gallinarum* eggs. The ring-necked pheasant has been shown to maintain a larger percentage of *H. gallinarum* to maturity and produce twice as many fertile nematode eggs than the chicken [31]. However, while chickens maintain both *H. gallinarum* and *H. meleagridis* up to 35 days, the prepatent period for the nematode, only one third of ring-necked pheasants maintain *H. meleagridis* to this point [31]. Therefore, both ring-necked pheasants and chickens may serve as carriers of *H. gallinarum*, but the chicken is a more likely source of soil contamination with *H. meleagridis*-harboring *H. gallinarum* eggs.

Histomoniasis

Introduction to H. meleagridis

 H. meleagridis is a protozoan parasite typically measuring 8-12 µm in diameter [5] that causes histomoniasis in some gallinaceous birds. It possesses different forms during different phases of infection: a flagellated, amoeboid, vegetative, and resistant form [5, 32]. The flagellated form observed in the cecal lumen and grown in culture possesses at least one flagellum, several small vesicles and large food vacuoles filled with digesting bacteria, a single nucleus with one or more nucleoli, and lacks mitochondria or an endoplasmic reticulum [33]. As the parasites penetrate the tissues of the host, they lose their flagella and take on an amoeboid form possessing many pseudopodia and travel very slowly through the tissue [32]. *H. meleagridis* observed in older tissue lesions possess either a swollen, dividing vegetative form or a resistant form with a dense plasma membrane and condensed cytoplasm [32].

Etiology of the disease

 Signs of the disease in live birds include weight loss, listlessness, drooping wings, and sulfur-colored droppings. Symptoms of histomoniasis begin in the ceca where the protozoan is first introduced. As the parasite multiplies and invades the cecal tissue, the cecal wall thickens and becomes inflamed [3]. The mucosa eventually produces a fibrous exudate that begins to fill the lumen [3]. *H. meleagridis* are spread from the ceca through the hepatic portal vein directly to the liver where the protozoans are filtered out of the blood and deposited in the liver tissue [3]. The liver becomes enlarged, and as the histomonads multiply and spread through the liver tissue they produce large necrotic lesions [3]. Birds that succumb to the disease die due to the rapid loss of functional liver and cecal tissue. However, if the disease slows down or ceases, it is possible

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the birds may recover from the disease as was indicated by sclerosis in some of the birds Smith observed in 1895 [3]. Smith also reported that only the ceca and the liver were infected with the protozoan. *H. meleagridis* has since been reported in additional tissues including the spleen, kidneys, bursa of fabresious, brain, lungs, and heart via PCR detection of *H. meleagridis* DNA [34]. Detection of *H. meleagridis* DNA does not indicate whether the protozoans were alive and cannot determine whether they caused disease in these organs. Cases of systemic histomoniasis producing lesions in organs such as the kidneys, spleen, lungs, heart, pancreas, and proventriulous in addition to the liver have been reported in a few experimental infections in turkeys [35-37] and only one natural outbreak in turkeys [38].

Enterohepatitis is observed in gnotobiotic turkeys infected with *H. meleagridis* in association with *E. coli*, *Clostridium perfringens*, or *Bacillus sibtilis,* but not when they are infected with *H. meleagridis* alone or in association with *Aerobacter cloacae* and *Streptococcus fecalis* or with *Proteus mirabilis* alone [39]. These bacteria are normally part of the natural gut microflora in commercial turkeys and chickens, and while these observations suggest *H. meleagridis* requires specific species of bacteria as a food source, it has never been demonstrated. The severity of histomoniasis is exacerbated by mild cecal coccidiosis [40]. Concurrent coccidiosis infections may elevate the rate of *H. meleagridis* penetration of the cecal tissue, expediting the progression of the disease in the liver ultimately resulting in higher rates of mortality.

The role of *H. gallinarum* in the transmission of *H. meleagridis*

Transmission of H. meleagridis *by* H. gallinarum

One phase of the *H. meleagridis* life cycle that demands attention is development inside adult *H. gallinarum*. Two separate experiments have demonstrated that feeding adult male *H. gallinarum* to turkeys does not result in contraction of histomoniasis [41, 42] even though histomonads are recovered from the ceca of infected birds [42]. These limited observations suggest *H. meleagridis* transition into a new phase of the life cycle inside the male *H. gallinarum* intestinal or reproductive tract in which they become nonpathogenic to the primary host, a gallinaceous bird. This transition may be necessary to prevent form hindering the reproduction of the vector nematode; however, further study is needed to understand how the protozoan progresses from a pathogenic form in the ceca of a bird, to a non-pathogenic form in the *H. gallinarum* adult, and back to a pathogenic form when released into a new host by the larva. Development of nonpathogenic strains of *H. meleagridis* that may be used to immunize turkeys or chickens against pathogenic strains may be impeded by the changes *H. meleagridis* undergo once taken up by *H. gallinarum*. It is possible nonpathogenic vaccine strains of *H. meleagridis* may be converted back into pathogenic strains once they infect adult *H. gallinarum* also residing in the ceca.

Our understanding of the phases of the *H. meleagridis* life cycle involving the *H. gallinarum* adult and egg are based on only a few studies. It is still unknown how *H. meleagridis* come to inhabit the *H. gallinarum* adults; if the protozoan actively infects the nematode or if it is passively consumed by the *H. gallinarum* as it feeds. The stages of development the protozoan undergoes to transition from the cecal environment to the adult nematode or from the nematode to the egg are also not well characterized. How *H. meleagridis* persists inside the *H. gallinarum*

egg through temperature extremes or how long the protozoan may survive inside the egg also require further study. One researcher reports *H. meleagridis* is able to survive in the *H. gallinarum* egg for up to 3 years claiming this is the result of the protozoan entering an intermediate, more resistant stage of its life cycle in the egg [43]. Direct characterization of the form of *H. meleagridis* inside the *H. gallinarum* egg is needed.

Incorporation into the H. gallinarum *egg*

     H. gallinarum's involvement in the transmission of *H. meleagridis* was first demonstrated by Graybill and Smith who induced histomoniasis in turkey poults by feeding *H. gallinarum* (which they referred as *H. papillosa*) eggs collected from older diseased turkeys [9]. From their data it was unclear whether *H. gallinarum* acted as a secondary factor to stimulate histomoniasis or if it carried *H. meleagridis* externally or internally. Tyzzer and later Swales demonstrated that hatching of the *H. gallinarum* larvae in the bird is necessary for transmission of *H. meleagridis* [41, 44]. Tyzzer also observed *H. meleagridis* in the intestinal epithelium of juvenile *H. gallinarum* but was unsuccessful in identifying *H. meleagridis* in the *H. gallinarum* egg. It wasn't until 1962 and 1969 that *H. meleagridis* was documented in the shelled egg and reproductive tract of the female *H. gallinarum* [6, 45]. In both studies *H. meleagridis* was observed throughout the female reproductive tract. Lee observed that *H. meleagridis* progresses through several stages of development in different zones of the ovary [45]. Lee made three critical observations in his study. First, spherical or spindle shaped *H. meleagridis* that ranged in size were observed multiplying among the oogonia in the germinal zone [45]. This observation may suggest that once the reproductive tract of a female *H. gallinarum* becomes infected, the female will continually produce *H. meleagridis*-infected eggs. Another important observation was evidence of active penetration of the developing oocytes in the proximal growth zone. Lee

noted *H. meleagridis* cells inside developing oocytes with no host-derived membranes surrounding them, suggests the oocytes were not absorbing the *H. meleagridis* [45]. Stronger evidence of forced entry of the oocyte by *H. meleagridis* were invaginated tears observed in the oolema of some oocytes infected with *H. meleagridis* [45]. The third critical observation Lee made were signs of *H. gallinarum* feeding and multiplying at all stages of development, indicating *H. meleagridis* does not enter a resting state in the *H. gallinarum* reproductive tract or the shelled egg [45].

Several questions remain regarding the prevalence and distribution of *H. meleagridis*-infected eggs produced by a single nematode population. Gibbs noted the degree of infection ranged from only a few histomonads to many [6]. Lee's more detailed EM study depicted only heavy infections of the *H. gallinarum* reproductive tract. This discrepancy between the two studies may represent variation in the prevalence of *H. meleagridis* between nematode populations or between females of the same population in the case of Gibbs's study which may translate to inconsistencies in the proportion of infected eggs produced between females. The only method used to estimate the proportion of *H. gallinarum* eggs infected with *H. meleagridis* requires infecting turkeys with a known number of *H. gallinarum* eggs to calculate the number of bird infections per the number of eggs fed to each individual. Several confounding factors limit the power of this methodology. Most important, the technique used to collect the eggs for infection is not representative of a natural infection. Most researchers either select whole gravid females to feed to turkeys or dissect a known number of eggs from only a few females. If the inconsistency in the number of *H. meleagridis* infesting the reproductive tract of female nematodes reported by Gibbs is accurate, this method of egg collection automatically skews the number of *H. meleagridis*-infected eggs administered to each bird. In a natural infection, chicken

or turkeys may pick up a homogenous mixture of eggs produced by many female *H. gallinarum* when they peck at cecal droppings or scavenge through litter or soil, thus increasing the probability they will consume multiple infective eggs. Another confounding factor of this methodology is that the infective unit of *H. gallinarum* eggs is not clearly defined. Two researchers suggest the infective unit would be a single embryonated egg containing a single *H. meleagridis* cell [23], but this may be inaccurate since it is unlikely an egg would contain a single *H. meleagridis* cell as Gibbs and Lee reported evidence *H. meleagridis* continues to multiply after penetrating the ova [6, 45]. It may be necessary to determine if a single infected egg can cause clinical histomoniasis in turkey poults. Another issue is the potential for some of the larvae to perish in the bird or to never reach the ceca. In several studies it was found that there were several birds with *H. meleagridis* infections but no *H. gallinarum* remaining in the ceca as well as birds that had no *H. meleagridis* or *H. gallinarum* after inoculation [6]. Birds that had *H. meleagridis* but no *H. gallinarum* may have provided inhospitable environments for the larvae to survive beyond releasing the histomonads. No *H. gallinarum* larvae may have reached the ceca in birds that remain free of both parasites. In cases where birds are inoculated with a low concentration of *H. meleagridis*-infected eggs clinical histomoniasis may not develop due to insufficient survival rates of the infective eggs rather than low numbers of *H. meleagridis* released into the ceca. Passaging eggs through turkey poults is important for determining the pathogenicity of the *H. meleagridis* strains they carry and for defining the infective unit of *H. gallinarum* eggs, but it is an insufficient method for calculating the number of *H. gallinarum* eggs carrying *H. meleagridis* in a given nematode population. Molecular detection of *H. meleagridis* inside *H. gallinarum* eggs may prove more accurate and efficient.

The work of earlier researchers has provided invaluable information for the transmission of *H. meleagridis* by the *H. gallinarum* egg; however, there are still gaps in our understanding of this phase in the *H. meleagridis* life cycle. The mechanism by which *H. gallinarum* become infected with *H. meleagridis* is unknown. *H. meleagridis* has been observed in the intestinal wall of male *H. gallinarum* and in the lumen of the male and female reproductive tract [6]; however, it is not known how *H. meleagridis* end up in the female reproductive tract. It is assumed they enter the intestine via the oral or anal canal which is directly connected to the reproductive tract in the male. Since *H. meleagridis* has been observed among spermatocytes in the vas deference and seminal vesicles in the male and among spermatocytes in the female uterus, it has been hypothesized that the protozoan is transferred to the female reproductive tract during copulation [6]. This hypothesis does not explain why *H. meleagridis* have been observed in the ovary wall however. An alternate hypothesis is that *H. meleagridis* cells travel from the gut through the pseudocoelom to the ovary. This may account for Lee's observation of *H. meleagridis* crossing the ovary wall to lie between the oogonia [45], but would require examination of the whole female body to identify *H. meleagridis* in the pseudocoelom.

Proportion of H. gallinarum *infected with* H. meleagridis

In nearly every study where *H. gallinarum* collected from chickens were used to infect turkey poults, the turkeys developed histomoniasis [9, 20, 23, 44, 46, 47], indicating *H. meleagridis* is heavily prevalent among *H. gallinarum* populations. The proportion of eggs produced by infected *H. gallinarum* populations is not defined however. Based on one report published in 1957, at most 1 in 1000 *H. gallinarum* eggs are infected with *H. meleagridis* [46]. A second report published by another research group that same year suggests 1 in 208 to 424 eggs carry *H. meleagridis* [23]. These numbers seem very low compared to histological reports on the

prevalence of *H. meleagridis* in the *H. gallinarum* oviduct which suggest that the oviducts of most females in infected populations carry high concentrations of multiplying *H. meleagridis* throughout the reproductive tract [6, 47]. The traditional method used to determine the prevalence of *H. meleagridis* among embryonated *H. gallinarum* eggs involved feeding *H. gallinarum* eggs to turkey poults and assessing whether they develop histomoniasis. There are several confounding factors that may alter the results of this assessment. First, the methodology used for collecting *H. gallinarum* eggs varies between studies. Some researchers fed whole gravid female nematodes to individual turkeys or grouped together embryonated eggs dissected from only a few females to feed to individual poults. These strategies of egg collection do not take into consideration the possible variation in *H. meleagridis* infection between female *H. gallinarum* of a given population. Second, once the gravid *H. gallinarum* are collected from the ceca, they die, which may cut short the progression of *H. meleagridis* development and multiplication in the eggs.

Different research groups obtain different estimates of *H. meleagridis* infection rates of *H. gallinarum* eggs using distinct populations of *H. gallinarum* and variable infection methodologies. It may be that the prevalence of *H. meleagridis* differs between populations of *H. gallinarum* or the level of *H. meleagridis* infection varies between individual female nematodes. In either case, it is evident that a new methodology is needed to assess the prevalence of *H. meleagridis* among *H. gallinarum* eggs. PCR or other molecular approaches may offer a more accurate measure of the proportion of eggs per female *H. gallinarum* that are infected with *H. meleagridis* or enable comparative studies between *H. gallinarum* populations.

Lateral transmission

Experimental oral inoculation of chickens and turkeys with *H. meleagridis* cells cultured from the feces of infected birds produced very few infections presumably as a result of the low acidity of the crop [48, 49]. The fecal oral infection of free *H. meleagridis* cells in the environment is achievable in an experimental setting but may be insufficient to produce histomoniasis. Fecal-oral transmission of free *H. meleagridis* may produce half as many infections with one third the morbidity and negligible mortality [48]. Other methods of experimental inoculation of chickens and turkeys have produced histomoniasis including cloacal inoculation with free *H. meleagridis* cultures, oral and cloacal inoculation with liver lesions or cecal content from diseased birds, and feeding embryonated *H. gallinarum* eggs; however, the most consistently method for producing histomoniasis in an experimental setting is cloacal inoculation with fresh *H. meleagridis* cultures. Experimental infections with *H. meleagridis* are also produced by feeding embryonated eggs of the natural vector, *H. gallinarum*, but this varies with the survival of the *H. gallinarum* larvae in the ceca and the rate of infection of the *H. gallinarum* eggs with *H. meleagridis* (as described earlier).

Natural transmission of *H. meleagridis* is generally accepted to occur through the *H. gallinarum* egg [50]; however, inconsistencies in the pattern of histomoniasis outbreaks between commercial chickens and turkeys implies the mode of transmission of *H. meleagridis* may differ between the two species. Some researchers propose other vectors may be responsible for transmission of *H. meleagridis* in cases where *H. gallinarum* cannot be identified [51], but transmission by a vector does not explain the rapid spread of histomoniasis seen in turkeys but not in chickens.

Helminthiasis prevention and treatment strategies

Dewormers

Piperazine is the only anthelmintic FDA-approved for use in chickens to treat ascarids. Several benzamidizole drugs are commonly used off-label in chickens and turkeys to treat a wider variety of helminths in the US. Fenbendazole is the only anthelmintic that has been shown to reduce the number of *H. gallinarum* [52]. The percent reduction of *H. gallinarum* is less than 90% which is insufficient to define the treatment as an effective dewormer [53]. Aside from the low availability of FDA-approved anthelmintics for use in poultry, a looming concern for deworming commercial poultry is the potential for nematodes to become resistant to the anthelmintics. Use of one drug class to treat helminth infection has produced drug resistance in a number of agriculture species and drug resistance has become a significant problem for grazing animals especially small ruminants [54]. So far, *A. galli* has not demonstrated drug resistance to benzamidizoles through repeated use in commercial layer flocks [55]; however, the potential for poultry nematodes to build resistance, especially as consumers push for organic or free-range chicken, necessitates early detection and appropriate administration of anthelmintics in commercial flocks. Research for combination drug therapy as well as nutraceutical and medicinal plant extracts are being conducted to define alternative anthelmintic strategies to control helminthiasis in poultry. Several plant extracts have been shown to have significant anthelmintic activity against *A. galli in vitro* and *in vivo*. According to a review of medicinal plant extracts used to treat ascariasis in poultry, extracts from *Psorelia corylifolia*, *Piper betle*, *Pilostigma thonningi*, *Caesalpinia crista*, *Ocimum gratissimum* and *Anacardium occidentale* were effective for reducing *A. galli* burden in chickens in a dose-dependent manner [56]. *Aloe ferox*, *Agave sisalana*, and *Gunnera perpensa* have all been demonstrated to act as anthelmintics

against *H. gallinarum in vivo* [57]; although, the percent reduction in fecal egg counts were all less than 90%. More work needs to be done to identify effective plant extracts for acting as anthelmintics for *H. gallinarum* in vivo before they can be used in conjunction with or in place of pharmaceutical anthelmintics in commercial poultry.

Treatment of nematodes with disinfectants

In many, cases birds become repeatedly infected with nematodes regardless if a dewormer is used regularly because they consume embryonated eggs in the poultry house and repeatedly reinfect themselves. To reduce the dependence on anthelmintic pharmaceuticals, decontamination of the poultry house may be used to reduce exposure of birds to infective nematode eggs. Chemical disinfectants including fumigants, carbomates, and fertilizers have been used in agriculture to control soil nematodes that parasitize crops [58]. Various chemical disinfectants and compounds are used on poultry facilities; however, the efficacy of these products against *H. gallinarum* eggs has not been reported. Materials commonly used in attempts to kill the *H. gallinarum* eggs in poultry houses include salt and feed-grade limestone. Researchers investigating the effects of limestone of the eggs of small ruminant gastrointestinal nematodes determined that this compound was incapable of rendering the nematode egg nonviable [59]. It is reasonable to predict a similar outcome for *H. gallinarum* eggs. High concentrations of chlorine have been demonstrated to have deleterious effects on the eggs and larvae of a diversity of nematode genera including root knot nematodes, mammalian gastrointestinal nematodes, and fish-parasitic nematodes [59-61]. However, this same compound has been demonstrated to exacerbate the parasite burden at low concentrations by stimulating a larger portion of fish-parasitic eggs to larvate [60] and causing greater numbers of root knot nematodes to hatch [61, 62]. Using high concentrations of chlorine to disinfect poultry houses

may damage materials in the building and create and environmental pollution concern. Studies using other disinfectant compounds or a combination of disinfectants to damage or kill *H. gallinarum* eggs are needed to establish practical decontamination protocols that can be used in commercial poultry houses.

Molecular analysis

No molecular protocols have been published for identifying *H. gallinarum*. One research team developed a nested PCR assay to distinguish *H. isolonche* from a genius of treamatodes using partial sequences of the Cytochrome C oxidase subunit 1 gene from *H. isolonche* and *Glaphyrostomum* sp. [63]. The primers have not been tested for specificity with *H. gallinarum* and were designed with the intention of distinguishing *H. isolonche* from a specific genus of treamatodes; however, the nested PCR did prove to be a more sensitive test compared to microscopic examination of feces. Development of a PCR test using primers specific to *H. gallinarum* would provide a more efficient and highly sensitive diagnostic test using fecal samples that may negate the need to euthanize and necropsy birds to confirm *H. gallinarum* infection.

Conclusions

H. gallinarum is a critical part of the *H. meleagridis* life cycle and understanding how it interacts with *H. meleagridis* in the host and is carried through the environment are essential to control the spread of *H. meleagridis* on poultry facilities. *H. gallinarum* is a poorly studied nematode and large deficits in knowledge of the nematode life cycle and biology hinder progress toward managing the parasite in commercial poultry flocks. Most of the *H. gallinarum* genome is not sequenced which impedes molecular studies and development of highly sensitive diagnostic

tests that may be used to gather information on the prevalence and distribution of *H. gallinarum* among poultry producers. Development of a molecular diagnostic test would enable poultry producers to identify reservoirs of *H. gallinarum* on their properties and enable researchers to investigate potential vectors of the nematode.

Determining the range and transmission of *H. gallinarum* is an important first step in managing this parasite in commercial poultry, but research for identifying effective disinfection treatments are also needed to reduce the prevalence of the parasite on farms that are heavily contaminated. Many poultry producers implement a program for deworming their birds but do not remove the reservoir for the parasite which is usually the litter. Therefore, poultry that are given anthelmintics immediately become reinfected. The producer not only wastes money treating the birds in this cycle but also creates the potential for the nematode to develop resistance to the anthelmintic. Nematode management strategies that combine decontamination of the reservoirs of the nematode with deworming programs are needed to effectively diminish the presence of *H. gallinarum* on commercial facilities. No studies for determining the efficacy of approved anthelmintics for eliminating *H. gallinarum* infection in poultry have been published and is important for identifying which dewormers may be prescribed specifically for treating *H. gallinarum*. Similarly, many poultry producers apply litter treatments in attempts to remove the *H. gallinarum* egg from the facility, but no studies have been conducted to determine the effects of these treatments on the *H. gallinarum* egg. Studies to characterize the effects of commercial disinfectants on the integrity and viability of the *H. gallinarum* egg are critical to being able to eradicate *H. gallinarum* reservoirs. Research for understanding the mechanism by which the *H. gallinarum* egg hatches may also be necessary for identifying classes of compounds that may be effective for decontaminating the *H. gallinarum* egg in poultry houses.

The *H. gallinarum* nematode is a small parasite that largely goes unnoticed but may have a substantial impact on the poultry industry. The role *H. gallinarum* plays as the vector for *H. meleagridis* means prevention of blackhead disease in commercial poultry is dependent upon the regulation of the nematode. Despite there being limited information available for the lifecycle of *H. gallinarum*, the data presented will serve as the foundation for new investigations into its distribution, biology, and transmission of *H. meleagridis*.

REFERENCES

1. Madsen, H. 1950. Studies on species of Heterakis (Nematodes) in birds. Danish Review of Game Biology. 1(3):1-43.

2. Kaushik, R. K. and V. P. Sharma Deorani. 1969. Studies on Tissue Responses in Primary and Subsequent Infections with Heterakis gallinae in Chickens and on the Process of Formation of Caecal Nodules. Journal of Helminthology. 43(1-2):69-78. doi:10.1017/S0022149X0000393X.

3. Smith, T. 1895. An infectious disease among turkeys caused by protozoa (infectious entero-hepatitis). U. S. Department of Agriculture Bulletin. 8:7-15.

4. Tyzzer, E. E. 1926. Heterakis vesicularis Frölich 1791. Proceedings of the Society for Experimental Biology and Medicine. 23(8):708-709. doi:10.3181/00379727-23-3129.

5. Tyzzer, E. E. and M. Fabyan. 1920. Further Studies on "Blackhead" in Turkeys, with Special Reference to Transmission by Inoculation. The Journal of Infectious Diseases. 27(3):207-239.

6. Gibbs, B. J. 1962. The occurrence of the protozoan parasite Histomonas meleagridis in the adults and eggs of the cecal worm Heterakis gallinae. The Journal of Protozoology. 9(3):288-293. doi:10.1111/j.1550-7408.1962.tb02622.x.

7. Walton, A. C. 1924. Studies in nematode gametogenesis. Zeitschrift für Zellforschung und Mikroskopische Anatomie. 1(2):167-239.

8. Graybill, H. W. 1921. DATA ON THE DEVELOPMENT OF HETERAKIS PAPILLOSA IN THE FOWL. The Journal of experimental medicine. 34(3):259-270. doi:10.1084/jem.34.3.259.

9. Graybill, H. W. and T. Smith. 1920. Production of fatal blackhead in turkeys by feeding embryonated eggs of Heterakis papillosa. The Journal of experimental medicine. 31(5):647-655. doi:10.1084/jem.31.5.647.

10. Lund, E. E. and A. M. Chute. 1974. The reproductive potential of Heterakis gallinarum in various species of galliform birds: implications for survival of H. gallinarum and Histomonas meleagridis to recent times. International journal of parasitology. 4:455-461.

11. Butboonchoo, P. and C. Wongsawad. 2017. Occurrence and HAT-RAPD analysis of gastrointestinal helminths in domestic chickens (Gallus gallus domesticus) in Phayao province, northern Thailand. Saudi Journal of Biological Sciences. 24(1):30-35. doi:10.1016/j.sjbs.2015.09.002.

12. Yazwinski, T., C. Tucker, E. Wray, L. Jones, Z. Johnson, S. Steinlage and J. Bridges. 2013. A survey on the incidence and magnitude of intestinal helminthiasis in broiler breeders originating from the southeastern United States. J Appl Poult Res. 22(4):942-947. doi:10.3382/japr.2013-00776.

13. Wuthijaree, K., C. Lambertz and M. Gauly. 2017. Prevalence of gastrointestinal helminth infections in free-range laying hens under mountain farming production conditions. British Poultry Science. 58(6):649-655. doi:10.1080/00071668.2017.1379049.

14. Kaufmann, F., G. Das, B. Sohnrey and M. Gauly. 2011. Helminth infections in laying hens kept in organic free range systems in Germany. Livestock Science. 141(2-3):182-187. doi:10.1016/j.livsci.2011.05.015.

15. Irungu, L. W., R. N. Kimani and S. M. Kisia. 2004. Helminth parasites in the intestinal tract of indigenous poultry in parts of Kenya: short communication. Journal of the South African Veterinary Association. 75(1):58-59.

16. Hassouni, T. and D. Belghyti. 2006. Distribution of gastrointestinal helminths in chicken farms in the Gharb region—Morocco. Parasitol Res. 99(2):181-183. doi:10.1007/s00436-006-0145-8.

17. Brener, B., R. Tortelly, R. C. Menezes, L. C. Muniz-Pereira and R. M. Pinto. 2006. Prevalence and pathology of the nematode Heterakis gallinarum , the trematode Paratanaisia bragai , and the protozoan Histomonas meleagridis in the turkey, Meleagris gallopavo. Memórias do Instituto Oswaldo Cruz. 101(6):677-681. doi:10.1590/S0074-02762006000600017.

18. McJunkin, J. W., R. D. Applegate and D. A. Zelmer. 2003. Enteric Helminths of Juvenile and Adult Wild Turkeys (Meleagris gallopavo) in Eastern Kansas. Avian Diseases. 47(4):1481-1485. doi:10.1637/7055.

19. Ruff, M. D. 1999. Important parasites in poultry production systems. Veterinary Parasitology. 84(3):337-347. doi:10.1016/S0304-4017(99)00076-X.

20. Farr, M. M. 1961. Further observations on survival of the protozoan parasite, Histomonas meleagridis, and eggs of poultry nematodes in feces of infected birds. The Cornell veterinarian. 51(1):3-13.

21. HASS, D. K. and A. C. TODD. 1962. Extension of a technique for hatching ascarid eggs in vitro. Am.J.Vet.Res. 23(92):170 pp.

22. Tyzzer, E. E. 1934. Studies on Histomoniasis, or "Blackhead" Infection, in the Chicken and the Turkey. Proceedings of the American Academy of Arts and Sciences. 69(5):189-264. doi:10.2307/20023041.

23. Lund, E. E. and R. H. Burtner. 1957. Infectivity of Heterakis gallinae eggs with Histomonas meleagridis. Experimental Parasitology. 6(2):189-193. doi:10.1016/0014-4894(57)90014-0.

24. Lund, E. E., E. E. Wehr and D. J. Ellis. 1966. Earthworm Transmission of Heterakis and Histomonas to Turkeys and Chickens. The Journal of Parasitology. 52(5):899-902. doi:10.2307/3276528.

25. Fine, P. E. M. 1975. Quantitative studies on the transmission of Parahistomonas wenrichi by ova of Heterakis gallinarum. Parasitology. 70(3):407-417. doi:10.1017/S0031182000052185.

26. Frank, J. F. 1953. A Note on the Experimental Transmission of Enterohepatitis of Turkeys by Arthropods. Can J Comp Med Vet Sci. 17(5):230-231.

27. Spindler, L. A. 1967. Experimental transmission of Histomonas meleagridis and Heterakis gallinarum by the sow-bug, Porcellioscaber, and its implications for further research. Proceedings of the Helminthological Society of Washington. 34(1):26-29.

28. Huber, K., L. Gouilloud and L. Zenner. 2007. A preliminary study of natural and experimental infection of the lesser mealworm Alphitobius diaperinus (Coleoptera: Tenebrionidae) with Histomonas meleagridis (Protozoa: Sarcomastigophora). Avian Pathology. 36(4):279-282. doi:10.1080/03079450701447317.

29. Lund, E. E., A. M. Chute and M. E. L. Vernon. 1974. Experimental Infections with Histomonas meleagridis and Heterakis gallinarum in Ducks and Geese. The Journal of Parasitology. 60(4):683-686. doi:10.2307/3278739.

30. Lund, E. E. 1967. Response of Four Breeds of Chickens and One Breed of Turkeys to Experimental Heterakis and Histomonas Infections. Avian Diseases. 11(3):491-502. doi:10.2307/1588197.

31. Everett E. Lund and Anne M. Chute. 1972. The Ring-Necked Pheasant (Phasianus colchicus torquatus) as a Host for Heterakis gallinarum and Histomonas meleagridis. The American Midland Naturalist. 87(1):1-7. doi:10.2307/2423877.

32. Tyzzer, E. E. 1919. Developmental Phases of the Protozoon of "Blackhead" in Turkeys. J Med Res. 40(1):30.3.

33. Schuster, F. L. 1968. Ultrastructure of Histomonas meleagridis (Smith) Tyzzer, a Parasitic Amebo-Flagellate. The Journal of Parasitology. 54(4):725-737. doi:10.2307/3277030.

34. Hauck, R., D. Lüschow and H. M. Hafez. 2006. Detection of Histomonas meleagridis DNA in Different Organs After Natural and Experimental Infections of Meat Turkeys. Avian Diseases. 50(1):35-38. doi:10.1637/7421-081505R.1.

35. Clarkson, M. J. 1962. The progressive pathology of Heterakis-produced histomoniasis in turkeys. Research Veterinary Science. 3(4):443-448.

36. Malewitz, T. D., R. A. Runnells and M. L. Calhoun. 1958. The pathology of experimentally produced histomoniasis in turkeys. American Journal of Veterinary Research. 19:181-185.

37. Pierson, F. W. and S. D. Fitzgerald. 2003. Hemorrhagic enteritis and related infections. Pages 237-247 in Diseases of Poultry. Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadley, L. R. McDougald and D. E. Swayne eds. Iowa State University Press, Ames, IA.

38. Sentíes-Cué, G., R. P. Chin and H. L. Shivaprasad. 2009. Systemic Histomoniasis Associated with High Mortality and Unusual Lesions in the Bursa of Fabricius, Kidneys, and Lungs

in Commercial Turkeys. Avian Diseases. 53(2):231-238. doi:10.1637/8363-051508-Reg.1.

39. Bradley, R. E. and W. M. Reid. 1966. Histomonas meleagridis and several bacteria as agents of infectious enterohepatitis in gnotobiotic turkeys. Experimental Parasitology. 19(1):91-101. doi:10.1016/0014-4894(66)90057-9.

40. McDougald, L. R. and J. Hu. 2001. Blackhead Disease (Histomonas meleagridis) Aggravated in Broiler Chickens by Concurrent Infection with Cecal Coccidiosis (Eimeria tenella). Avian Diseases. 45(2):307-312. doi:10.2307/1592969.

41. Tyzzer, E. E. 1934. Studies on histomoniasis, or "blackhead" infection, in the chicken and the turkey. Proceedings of the American Academy of Arts and Sciences. 69(5):189-264.

42. Springer, W. T., J. Johnson and W. M. Reid. 1969. Transmission of histomoniasis with male Heterakis gallinarum (Nematoda). Parasitology. 59:401-405.

43. Long, P. L. 1966. Transmission and experimental infection of Histomoniasis. Poult.Rev. 6:7-11.

44. Swales, W. E. 1948. Enterohepatitis (blackhead) in turkeys, II. Observations on transmission by the cecal worm (Heterakis gallinae). Canadian Journal of Comparative Medicine. 12:97-111.

45. Lee, D. L. 1969. The structure and development of Histomonas meleagridis (Mastigamoebidae: Protozoa) in the female reproductive tract of its intermediate host, Heterakis gallinarum (Nematoda). Parasitology. 59(4):877-884.

46. Kendall, S. B. 1957. Some Factors Influencing Resistance to Histomoniasis in Turkeys. British Veterinary Journal. 113(10):435-439. doi:10.1016/S0007-1935(17)45805-2.

47. Kendall, S. B. 1959. The occurrence of Histomonas meleagridis in Heterakis gallinae. Parasitology. 49(1-2):169-172. doi:10.1017/S0031182000026809.

48. Lund, E. E. 1956. Oral Transmission of Histomonas in Turkeys. Poult Sci. 35(4):900-904. doi:10.3382/ps.0350900.

49. Horton-Smith, C. and P. I. Long. 1956. The infection of chickens (gallus gallus) with histomonad suspensions. Parasitology. 46:79-90.

50. Hu, J., L. Fuller and L. R. McDougald. 2004. Infection of Turkeys with Histomonas meleagridis by the Cloacal Drop Method. Avian Diseases. 48(4):746-750. doi:10.1637/7152.

51. Norton, R. A., F. D. Clark and J. N. Beasley. 1999. An Outbreak of Histomoniasis in Turkeys Infected with a Moderate Level of Ascaridia dissimilis but No Heterakis gallinarum. Avian Diseases. 43(2):342-348. doi:10.2307/1592628.

52. Yazwinski, T. A., C. A. Tucker, E. Wray, L. Jones and F. D. Clark. 2013. Observations of benzimidazole efficacies against Ascaridia dissimilis, Ascaridia galli, and Heterakis gallinarum in naturally infected poultry. J Appl Poult Res. 22(1):75-79. doi:10.3382/japr.2012-00606.

53. Coles, G. C., C. Bauer, F. H. M. Borgsteede, S. Geerts, T. R. Klei, M. A. Taylor and P. J. Waller. 1992. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Veterinary Parasitology. 44(1):35-44. doi:10.1016/0304-4017(92)90141-U.

54. Kaplan, R. M. and A. N. Vidyashankar. 2012. An inconvenient truth: Global worming and anthelmintic resistance. Veterinary Parasitology. 186(1-2):70-78. doi:10.1016/j.vetpar.2011.11.048.

55. Tarbiat, B., D. S. Jansson, E. Tyden and J. Hoglund. 2017. Evaluation of benzimidazole resistance status in Ascaridia galli. Parasitology. 144(10):1338-1345.

56. Raza, A., F. Muhammad, S. Bashir, B. Aslam, M. I. Aswar and M. U. Naseer. 2016. In-vitro and in-vivo anthelmintic potential of different medicinal plants against Ascaridia galli infection in poultry birds - ProQuest. World's Poultry Science Journal. 72(1):115-124.

57. Mwale, M. and P. Masika. 2015. In vivo anthelmintic efficacy of Aloe ferox, Agave sisalana, and Gunnera perpensa in village chickens naturally infected with Heterakis gallinarum. Trop Anim Health Prod. 47(1):131-138. doi:10.1007/s11250-014-0696-0.

58. Hussain, M., M. Zouhar and P. Rysanek. 2017. Comparison between biological and chemical management of root knot nematode, Meloidogyne hapla. Pakistan Journal of Zoology. 49(1):205-210.

59. Howell, J. M., J. M. Luginbuhl, M. J. Grice, K. L. Anderson, P. Arasu and J. R. Flowers. 1999. Control of gastrointestinal parasite larvae of ruminant using nitrogen fertilizer, limestone and sodium hypochlorite solutions. Small Ruminant Research. 32(3):197-204. doi:10.1016/S0921-4488(98)00186-2.

60. Martins, M. L., V. Watral, J. P. Rodrigues-Soares and M. L. Kent. 2017. A method for collecting eggs of Pseudocapillaria tomentosa (Nematoda: Capillariidae) from zebrafish Danio rerio and efficacy of heat and chlorine for killing the nematode's eggs. Journal of Fish Diseases. 40(2):169-182. doi:10.1111/jfd.12501.

61. Stanton, J. M. and W. E. O'Donnell. 1994. Hatching, motility, and infectivity of root-knot nematode (Meloidogyne javanica) following exposure to sodium hypochlorite. Australian Journal of Experimental Agriculture. 34(1):105-108. doi:10.1071/EA9940105.

62. Kanwar, R. S., R. K. Jain, I. J. Paruthi and D. C. Gupta. 1991. Effects of sodium hypochlorite on egg liberation from egg masses, hatching and infectivity of Meloidogyne javanica juveniles. Nematologica. 37:236-240.

63. Zhao, C., M. Onuma, M. Asakawa, T. Nagamine and T. Kuwana. 2009. Preliminary studies on developing a nested PCR assay for molecular diagnosis and identification of nematode (Heterakis isolonche) and trematode (Glaphyrostomum sp.) in Okinawa rail (Gallirallus okinawae). Veterinary Parasitology. 163(1-2):156-160. doi:10.1016/j.vetpar.2009.03.038.

Chapter 1

A Diagnostic PCR Protocol for Identifying *Heterakis gallinarum*

in Environmental Samples

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Summary

Heterakis gallinarum is a widely distributed cecal nematode that parasitizes many gallinaceous birds including chickens and turkeys. The nematode rarely causes disease and goes undiagnosed in most poultry flocks. *H. gallinarum* infection poses a problem for the poultry industry as the nematode eggs may serve as a vector for a protozoan parasite called *Histomonas meleagridis* which causes blackhead disease, a potentially deadly disease that results in necrosis of the ceca and liver. The only method for detecting the nematode in the environment is to identify the *H. gallinarum* egg. This method is prone to misidentification of the nematode as the *H. gallinarum* egg is almost identical to the *Ascaridia galli* egg. A diagnostic PCR capable of identifying *H. gallinarum* DNA from environmental samples would provide a more sensitive and specific diagnostic test that may be used with a variety of sample types. Three primer sets were designed from sequences cloned from the *H. gallinarum* genome. PCR reactions performed with these primers set amplified a product from *H. gallinarum*, while no product was produced using DNA isolated from *H. meleagridis*, *Ascaridia galli*, *cestode* spp. *H. gallinarum* sequence was PCR amplified from earthworms and *Alphitobius diaperinus* (darkling beetles), demonstrating that the diagnostic PCR is sensitive enough to be used for identifying the presence of *H. gallinarum* in environmental samples.

Introduction

Heterakis gallinarum is a parasitic nematode that thrives in the ceca of gallinaceous birds including chickens and turkeys (10). Unlike *Ascaridia galli*, and other intestinal parasites common to domestic poultry, *H. gallinarum* does not normally cause disease and, as a result, normally goes undetected in commercial flocks (9, 18). However, *H. gallinarum* is the vector for

Histomonas meleagridis (17-19), a protozoan parasite that causes blackhead disease (also known as histomoniasis, histomonosis, or infectious enterohepatitis) in gallinaceous birds (15). The withdrawal of the anti-histomonal drugs resulted in a surge of blackhead disease among poultry, especially turkeys. In a single year, the number of blackhead outbreaks increased from 55 to 101 following the withdrawal of Nitarsone from the US market (2). Poultry producers must adapt their management practices to prevent spreading *H. gallinarum* between their flocks to inhibit the transmission of *H. meleagridis*.

Very little is understood about the transmission of *H. gallinarum* or its interactions with *H. meleagridis*. *H. gallinarum* eggs have been demonstrated to remain viable in the environment and capable of transmitting *H. meleagridis* to poultry for 3 years (3). Therefore, the land a poultry facility is built on may act as its own reservoir for *H. gallinarum* carrying *H. meleagridis*. Lund and colleagues demonstrated that earthworms are capable of carrying *H. gallinarum* larvae and can transmit the nematode and *H. meleagridis* to turkeys and chickens (12). It is reasonable to assume earthworms infected with *H. gallinarum* carry the infective larvae into the poultry houses where they are consumed by the birds. Transport of the *H. gallinarum* larvae by earthworms may result in the seasonal spikes in blackhead outbreaks in the poultry industry that occur in late summer and early fall. Earthworms have been noted to carry heavier loads of *H. gallinarum* in late fall than they do in early spring (12). Therefore, the nematodes may not survive the winter months hatched inside the earthworm and the potential for earthworms to carry *H. meleagridis*-infected *H. gallinarum* larvae is greatest in the fall. However, no studies have been conducted to determine if *H. gallinarum* eggs are present in the soil or if earthworms are infected with the larvae on poultry facilities that experience repeated

blackhead outbreaks. It is also unclear how *H. gallinarum* carrying *H. meleagridis* may be transmitted between poultry facilities.

Development of a highly sensitive polymerase chain reaction (PCR) diagnostic test that is specific to *H. gallinarum* DNA may be used to identify *H. gallinarum* in environmental samples. This type of diagnostic tool would enable experimental investigations for potential vectors for *H. gallinarum* or identify areas of a poultry facility that are contaminated with the nematode egg. The objective of this research was to design primer sets from sequenced fragments of the *H. gallinarum* genome that were specific to *H. gallinarum*, were conserved between *H. gallinarum* populations, and were capable of amplifying *H. gallinarum* sequence from environmental samples containing larger concentrations of DNA from other organisms.

Materials and Methods

Specimen collection

Mature *H. gallinarum* were collected from the ceca of chickens sampled from 6 different commercial and research flocks (two flocks in Raleigh, NC; 1 flock in Kinston, NC; and three flocks from a processing plant in Marietta, GA. Mature *A. galli* and *Cestode* spp. were collected from one of the chicken flocks sampled in Raleigh, NC. *H. meleagridis* were isolated from a chicken sampled from a flock in Buford, GA. Seven earthworms were collected from a backyard chicken flock in Raleigh, NC, and five earthworms were collected from a commercial turkey facility in Clinton, NC. Darkling beetles (*Alphitobius diaperinus*) were collected from the North Carolina State University Talley Turkey Education Unit.

Experimental infection of darkling beetles with H. gallinarum *eggs*

Ten darkling beetles were placed in a petri dish for 1 week with 0.5 g of turkey feed containing approximately 350 embryonated *H. gallinarum* eggs. The 5 surviving beetles were euthanized and placed into individual microcentrifuge tubes. The 5 beetles that died during the week were collected into a single microcentrifuge tube.

Genomic DNA isolation, DNA cloning and primer design

Genomic DNA (gDNA) from the first *H. gallinarum* population sampled from Marietta, GA was isolated using the Quick-gDNA MiniPrep kit (Genesee Scientific, San Diego, CA). All other gDNA isolation was accomplished using the PureLink Genomic DNA kit (Thermo Fisher Scientific, Waltham, MA).

H. gallinarum gDNA isolated from the first Marietta, GA population and pBluescript plasmid were digested with FastDigest EcoRI and XbaI restriction endonucleases (Thermo Fisher Scientific, Waltham, MA) at 37 degrees Celsius for 1 hour. One hundred fifty nanograms of digested *H. gallinarum* gDNA and 50 ng of digested plasmid were incubated with T4 DNA Ligase (Thermo Fisher Scientific, Waltham, MA) at room temperature for two hours. The ligation products were transformed into GC5 competent cells according to manufacturer's protocol (Genesee Scientific, San Diego, CA). Fifty-four white colonies were transferred from sample plates to separate test tubes containing LB-amp with 100 µg/ml ampicillin and incubated at 37 degrees Celsius for 16 hours. Plasmid DNA was isolated from each bacterial culture using the GeneJet Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol.

Plasmid DNA was sequenced using Sanger sequencing at the Georgia Genomics and Bioinformatics Core at the University of Georgia, Athens, GA. A nucleotide BLAST search was conducted for each unknown sequence to identify those that shared homology to other sequences in the database. Primers were designed for 3 sequence using the IDT PrimerQuest Tool (https://www.idtdna.com). One primer set was selected for each sequence that would amplify a region between 75 and 200 bp long. The T_m of the forward and reverse primers for each sequence was 62°C. Primer sequences are given in Table 1: HG1 (MK122633), HG2 (MK122634), and HG3 (MK122635).

PCR protocol

PCR reactions were prepared for each test using Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's recommendations. The gradient PCR samples contained 10 ng of *H. gallinarum* gDNA isolated from chickens in Raleigh, NC. The thermal profile for the gradient PCR was as follows: initial denaturation step at 98 degrees Celsius for 1 minute; followed by 35 cycles of 98 degrees Celsius for 5 seconds; 55, 56, 57, 58, 59, or 60 degrees Celsius for 5 seconds; 72 degrees Celsius for 5 seconds; and final elongation at 72 degrees Celsius for 1 minute. The thermal profile for all subsequent PCR tests was the same using 60 degrees Celsius as the annealing temperature. PCR samples containing DNA isolated from *A. galli*, *Cestode* spp, *H. meleagridis* and 5 populations of *H. gallinarum* used 10 ng of gDNA. The diagnostic PCR samples prepared with DNA isolated from earthworms collected from the backyard chicken pen or darkling beetles used 100 ng of gDNA, and the diagnostic PCR samples prepared with DNA isolated from earthworms collected from a commercial turkey facility used 200 ng of gDNA. The PCR products of all PCR tests were

separated on agarose gels prepared with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA) at 100 V for 45 to 60 minutes. The gels were viewed under a blue light to observe the amplicon bands in each PCR sample.

Cloning and sequencing of PCR amplicons

HG1, HG2, and HG3 PCR amplicons produced from the combined Georgia populations were excised from an agarose gel and purified using the PureLink Quick Gel Extraction and PCR Purification Combo kit (Thermo Fisher Scientific, Waltham, MN) and ligated into the pJET1.2 blunt Cloning Vector (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's protocols. Ligation products were transformed and plasmid DNA isolated as above. Plasmid containing amplicons from HG1, HG2, and HG3 were Sanger sequenced at the Genomic Sciences Laboratory at North Carolina State University, Raleigh, NC. The sequences of the inserts were compared to the sequence used to design the primers in A Plasmid Editor (http://jorgensen.biology.utah.edu/wayned/ape/).

Results

Sequence analysis of cloned H. gallinarum *gDNA*

Limited sequence information is available for *H. gallinarum* (4, 5, 8, 16, 20). Fragments of the *H. gallinarum* genome were cloned into pBluescript vector and sequenced. Three sequences which shared low identity to other nematode species were selected for designing primers. Eight percent of the 327 bp HG1 (MK122633) sequence shared the greatest sequence identity, 96%, with *Dicrocoelium dendriticum*, a liver fluke. Five percent of the 872 bp HG2 (MK122634) sequence shared 86% identity with *Caenorhabditis remanei*; however, HG2 shared

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the greatest sequence identity (94%, covering 3% of the HG2 sequence) with *Trichechus manatus*, the West Indian manatee. The 632 bp HG3 (MK122635) sequences shared no sequence identity with any helminth sequences from Genbank; HG3 shared the greatest sequence identity (100 %, covering 3% of the HG3 sequence) with *Solanum lycopersicum*, a tomato, or *Tetrapisispora blattae*, a fungus.

Primer specificity and sensitivity evaluation

A gradient PCR was conducted to test the amplification efficiency of each primer set at annealing temperatures between 55 degrees Celsius and 60 degrees Celsius to select the PCR annealing temperature that would increase primer specificity without compromising binding affinity for the target sequences. All 3 primer sets amplified the target sequence at all tested annealing temperatures in the gradient PCR (Figure 1). The fluorescence intensity for HG1 decreased mildly between 56 degrees Celsius and 57 degrees Celsius. The fluorescence intensity of HG2 bands were the faintest between the primer sets; no discernable variation in fluorescence intensity was observed between HG2 samples. The fluorescence intensity in the HG3 bands did not vary between annealing temperatures; these bands were the brightest among the 3 primer sets. Sixty degrees Celsius was selected as the annealing temperature for all subsequent PCR tests.

The species specificity of the primer sets and conservation of the target sequences were tested by PCR amplification of DNA isolates from single populations of *H. meleagridis*, *A. galli,* and *Cestode* spp. and 5 populations of *H. gallinarum* collected from chicken flocks from Raleigh, NC; Kinston, NC; and Marietta, GA. All 3 primer sets amplified a single amplicon in all 5 *H. gallinarum* isolates and were unable to amplify any sequence from the *H. meleagridis*, *A. galli*, or *Cestode* spp. samples (Figure 2). The fluorescence intensities of the amplicon bands

appeared equal between *H. gallinarum* samples for each primer set based on visual examination of the gel. Sequencing of HG1, HG2, and HG3 amplicons PCR purified from a mixed sample of DNA from two Georgia *H. gallinarum* populations showed all 3 amplicon sequences exactly matched the sequences used to design the primers (data not shown).

The sensitivity of the primer sets to target DNA sequences was evaluated by preparing a set of 5-fold serial dilution standards of pure *H. gallinarum* gDNA isolated from the chicken flock in Kinston, NC. Triplicate PCR reactions were prepared for each primer set containing 5 ng, 1 ng, 200 pg, 40 pg, 8 pg, 1.6 pg, and 320 fg of *H. gallinarum* gDNA. The threshold for detection of pure *H. gallinarum* gDNA was determined for each primer set based on the decline in consistent amplification of target in all 3 replicates of a given gDNA standard. HG1 primers amplified target in all 3 replicates of the 5 ng, 1ng, and 200 pg samples, but failed to amplify target in any of the more dilute PCR samples (Figure 3A). The florescence intensity of the amplicon bands steadily declined from the 5 ng samples to the 200 pg samples, but fluorescence intensity was consistent between replicates for these three gDNA standards. HG2 primers amplified target in all 3 replicates of the 5 ng, 1 ng, and 200 pg, but only amplified target in 2 of the 40 pg samples, 1 of the 8 pg samples, 1 of the 1.6 pg samples, and all 3 of the 320 fg samples (Figure 3B). The fluorescence intensity of the amplicon bands steadily decreased from the 5 ng to the 200 pg samples, but successful amplification of target and the fluorescence intensity of amplicons in the more dilute samples was variable; all but one of the amplicon bands in the 1.6 pg and 320 fg samples were very faint. HG3 primers amplified target in all 3 replicates of the 5 ng, 1 ng, 200 pg, and 40 pg samples, but only amplified target in 2 of the 8 pg samples, 2 of the 1.6 pg samples, and all three of the 320 fg samples (Figure 3C). The fluorescence intensity of the amplicon bands steadily decreased from the 5 ng to the 40 pg samples. The fluorescence

intensity of amplicons between replicates of each standard was consistent for the 5 ng, 1 ng, and 200 pg samples, but became more variable in the 40 pg samples. Fluorescence intensity of the amplicon bands was inconsistent between replicates of the 1.6 pg and 320 fg samples. The HG3 primer set demonstrated consistent amplification of *H. gallinarum* sequence at lower concentrations of gDNA template than HG1 or HG2 primer sets, and therefore was selected for running the diagnostic PCR on earthworm and darkling beetle environmental samples.

H. gallinarum *detection in earthworms* and *darkling beetles*

To assess the feasibility of using the *H. gallinarum* diagnostic PCR with environmental samples from poultry facilities, the diagnostic PCR protocol was tested with DNA isolated from 7 earthworms that were collected from a backyard chicken flock. This flock was previously determined to be infected with *H. gallinarum* based on earlier coprologic examinations (data not shown). PCR amplification of a single band at approximately 138 bp was detected from Worms 1, 2, 3, 5, and 7 (Figure 4).

To test whether *H. gallinarum* DNA could be amplified from earthworms isolated from a turkey facility experience a blackhead outbreak, DNA isolated from 5 earthworms collected from the property were subjected to PCR amplification by HG3 primers. The second isolate from Worm 1 and the fourth isolate from Worm 2 produced an HG3 amplicon band (Figure 5). All other worm isolates were negative.

To determine if the darkling beetle may act as a carrier for *H. gallinarum*, a diagnostic PCR test was run with DNA isolated from darkling beetles that had been fed embryonated *H. gallinarum* eggs. No eggs were observed attached to the exterior of the darkling beetles prior to

DNA isolation. Three samples prepared from live beetles (Beetle 1, 2, and 5) amplified the HG3 target (Figure 6). All other samples, including the dead beetles, were negative.

Discussion

To develop a molecular diagnostic test for the detection of *H. gallinarum* in environmental samples, genomic sequences were identified and utilized to design 3 PCR primer sets. The use of a molecular diagnostic test provides increased specificity for *H. gallinarum* in the environment where misdiagnosis is common due to the similarity in morphology of *H. gallinarum* and its eggs to other nematodes. The use of molecular detection also allows for increased sensitivity, enabling the detection of *H. gallinarum* in potential vectors.

There is limited genomic information available for *H. gallinarum*, with only mitochondrial and ribosomal sequences known (4, 5, 8, 16, 20). Designing primers against these conserved sequences often results in amplification that is not species-specific and requires sequencing of the amplicon for proper diagnosis (1, 7). To generate sequence information, short fragments of the genome were cloned and sequenced from a population of *H. gallinarum*. Three sequences were chosen to design primers against because each of these sequences had very low identity to any sequences in Genbank, included sequences from other nematodes. Sequencing of HG 1, HG2 and HG3 amplicons produced the same sequence obtained from the cloned *H. gallinarum* genomic sequences, demonstrating that the primers were specific to those sequences. There is no *H. gallinarum* reference genome to compare the sequences obtained from the cloned fragments of gDNA to; therefore, it is possible these sequences are not from *H. gallinarum*. To test the specificity of the primers to *H. gallinarum*, PCR was run using gDNA isolated from 5 different populations of *H. gallinarum* and other gastrointestinal parasites common to poultry including *H. meleagridis*, *Cestode* spp and a closely related species of nematode, *A. galli*. DNA

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samples from all 5 *H. gallinarum* populations produced an amplicon of the predicted size while none of the primers amplified DNA from the samples of *H. meleagridis*, *A. galli*, or *Cestode* spp. The *H. meleagridis* used in the study was obtained from an *in vitro* culture where it was co-cultured with cecal bacteria. The observation that gDNA isolated from this culture did not yield an amplicon, also demonstrates that the primer sets are most likely not amplifying cecal bacteria. Based on this PCR data, it is likely the cloned gDNA of all 3 primer sets specifically amplifies *H. gallinarum* sequences.

The PCR primer sequences must be conserved between populations of *H. gallinarum* to ensure the diagnostic PCR can identify *H. gallinarum* DNA in environmental samples collected from different geographic regions. All *H. gallinarum* isolated gDNA in this study was amplified with each primer set, suggesting that the target sequence of each primer set is conserved in the *H. gallinarum* genome. To assess the degree of sequence conservation of the amplified regions between *H. gallinarum* populations, HG1, HG2, and HG3 amplicons produced by amplification of a pooled sample of gDNA from the *H. gallinarum* populations collected from the Marietta, GA processing plant were cloned and sequenced. The sequences of these amplicons shared 100% identity to the sequences obtained from the cloned gDNA fragments, which further suggests the sequences of the genomic regions amplified by each primer set are highly conserved between distinct populations of *H. gallinarum*. However, it is possible populations of *H. gallinarum* not tested in this study have mutations in one or more of the sequences amplified by these primer sets. Using one of the primer sets to test samples containing *H. gallinarum* from unknown populations may result in failed detection of *H. gallinarum* DNA. The use of a combination of these primer sets would further increase the accuracy of the diagnostic test for detecting *H. gallinarum*. For this study, the HG3 primer set was selected for running the diagnostic protocol

with earthworm and darkling beetle samples because this primer set has the lowest threshold for detection of pure *H. gallinarum* gDNA, approximately 40 pg, compared to approximately 200 pg for HG1 and HG2 and consistently gave a brighter band on the agarose gel due to the higher molecular weight of the amplicon.

Earthworms were selected as the first environmental samples to test the optimized *H. gallinarum* diagnostic PCR for two reasons: *H. gallinarum* larvae have been shown to hatch inside the earthworm's intestines and burrow into the worm's tissues (12), and earthworms continually pick up *H. gallinarum* eggs from the soil thereby concentrate the larvae in their bodies. Isolating DNA from *H. gallinarum* eggs is made difficult by the thick chitinous layer of the eggshell which is difficult to degrade in most DNA isolation protocols. Since the earthworm is a known paratenic host that carries hatched larvae, adapting a DNA isolation protocol to degrade the chitinous eggshells is not necessary. Earthworms also accumulate *H. gallinarum* larvae over the course of the summer as they continue to ingest eggs from the soil. Therefore, earthworms collected from an area known to be contaminated with *H. gallinarum* eggs provide a more concentrated, accessible source of *H. gallinarum* DNA than soil or fecal samples.

Backyard chickens are more prone to parasitic infections than commercial poultry because they have access to outdoor pen areas (13). As a result, backyard poultry more commonly suffer from parasitic infection than commercial poultry housed indoors. Earthworms collected from a backyard chicken pen were used as the first PCR test subjects because previous microscopic examination of chicken feces and soil from the pen indicated the area was contaminated with *H. gallinarum* eggs. Of the 7 earthworms sampled, 5 were positive for the HG3 target indicating the PCR protocol is capable of positively identifying *H. gallinarum* from environmental samples. While it was not the aim of this experiment to survey the number of

earthworms that may be carrying *H. gallinarum*, based on these results, most earthworms from backyard chicken flocks may be infected with the *H. gallinarum* larvae, a demographic than may warrant further investigation.

Turkeys are a less suitable host for *H. gallinarum* than chickens (11) and generally harbor lower numbers of adult nematodes capable of producing embryonated eggs. However, *H. gallinarum* do not need to reach maturity to transmit *H. meleagridis* to their host. Chickens are capable of harboring both *H. gallinarum* and *H. meleagridis* in the absence of disease (14), and act as carriers producing large numbers of *H. meleagridis*-infected *H. gallinarum* eggs which can survive in the environment for 3 years (3). When a commercial turkey facility (which had been converted from a chicken facility two years earlier) contracted blackhead disease, earthworms were collected from the property to test for *H. gallinarum* using the diagnostic PCR to investigate the possibility the earthworms ingested *H. gallinarum* eggs left behind by the chickens. Based on the results of this test, it is possible the blackhead outbreak was initiated by ingestion of earthworms carrying *H. meleagridis*-infected *H. gallinarum*. The data presented for both diagnostic tests performed with earthworm gDNA isolates demonstrates that environmental contamination with *H. gallinarum* eggs may be diagnosed on a poultry facility by identifying *H. gallinarum* DNA in earthworms collected from the property.

An experimental protocol for detecting *H. gallinarum* DNA in darkling beetles exposed to embryonated *H. gallinarum* eggs was designed to determine whether the darkling beetle may act as an intermediate host for *H. gallinarum*. Darkling beetles have been speculated as vectors of *H. gallinarum* or *H. meleagridis*, but no studies have confirmed their role in the transmission of either parasite. Huber and colleagues identified *H. meleagridis* DNA via PCR in only a small percentage of darkling beetle mealworms from one chicken and one turkey facility experiencing

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blackhead outbreaks (6). No data has been published demonstrating darkling beetles as carriers of *H. gallinarum* prior to the current study. It is unlikely *H. gallinarum* DNA amplified from these samples came from unhatched eggs in the beetles because the DNA digestion protocol was not adapted to degrade the chitinous eggshell. Three of the live darkling beetles were positive for *H. gallinarum* DNA which indicates darkling beetles are capable of ingesting *H. gallinarum* eggs; however, this does not demonstrate whether the *H. gallinarum* larvae are alive and able to be transmitted by the darkling beetles. Live darkling beetles that have been fed embryonated *H. gallinarum* eggs and cleansed of any *H. gallinarum* eggs attached to their exteriors would need to be fed to naïve chickens to determine if they can transmit viable *H. gallinarum* to the birds. This data represents the first molecular evidence that darkling beetles may carry *H. gallinarum* and demonstrates the potential of using the *H. gallinarum* diagnostic PCR to investigate reservoirs and vectors for transmitting *H. gallinarum* between poultry facilities.

Conclusion and applications

Three PCR primer sets were designed and tested for specificity to *H. gallinarum* and were found to be suitable for use in an *H. gallinarum* diagnostic PCR as the amplified sequence was specific to *H. gallinarum* and conserved between populations. Earthworms sampled from a backyard chicken pen and a commercial turkey facility were both found to carry *H. gallinarum* DNA using the standardized PCR protocol which demonstrates the potential for using the diagnostic PCR to investigate sources of *H. gallinarum* on poultry facilities. For the first time, *H. gallinarum* DNA was amplified from experimentally infected darkling beetles, indicating further research in needed to evaluate the capability of the darkling beetle to act as a vector or carrier for *H. gallinarum*. Therefore, the PCR test may also be used as a research tool to design experiments

for identifying potential *H. gallinarum* vectors and investigating alternate modes of transmission of the nematode.

Table 1. HG1, HG2, and HG3 forward and reverse primer sequences, GC content, T_m and amplicon length.

Figure 1. Gradient PCR products of HG1, HG2, and HG3 primer sets run at 1 (55°C), 2 (56°C), 3 (57°C), 4 (58°C), 5 (59°C) and 6 (60°C) separated on a 1.5% agarose gel prepared with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, Waltham, MN) run at 100 V for 45 minutes. L=1 kb plus DNA Ladder (Thermo Fisher Scientific, Waltham, MN).

Figure 2. PCR products of gDNA isolates from *H. meleagridis* collected from culture (1), *Cestode* spp. collected from a chicken (2), *A. galli* collected from a chicken (3), and 5 populations of *H. gallinarum* collected from distinct chicken flocks (4-8) separated on a 1.5% agarose gel prepared with SYBR Safe DNA Gel Stain run at 100 V for 45 minutes, demonstrating species specificity and conservation of the amplified sequence between *H. gallinarum* populations for A) HG1, B) HG2, and C) HG3 primer sets. L=1 kb plus DNA Ladder.

Figure 3. PCR products of 5-fold *H. gallinarum* gDNA serial dilutions ranging from 5 ng to 320 fg separated on a 1.5% agarose gel prepared with SYBR Safe DNA Gel Stain run at 100 V for 45 minutes, demonstrating the sensitivity of the primer sets. L=1 kb plus DNA Ladder.

Figure 4. Diagnostics PCR products of gDNA isolates from 7 earthworms collected from a backyard chicken pen separated on a 1.5% agarose gel prepared with SYBR Safe DNA Gel Stain run at 100 V for 45 minutes. Each number on the gel corresponds to a different earthworm. L=1 kb plus DNA Ladder.

Figure 5. Diagnostic PCR products of the 4 pieces of 5 earthworms collected from a commercial

turkey facility separated on a 1.5% agarose gel prepared with SYBR Safe DNA Gel

Stain run at 100 V for 45 minutes. L=1 kb plus DNA Ladder.

Figure 6. Diagnostic PCR products of gDNA isolates from darkling beetles 1 week after feeding

them *H. gallinarum* eggs separated on a 1.5% agarose gel prepared with SYBR Safe

DNA Gel Stain run at 100 V for 45 minutes. The 3 replicate PCR reactions for each

darkling beetle isolate are grouped under the corresponding DNA isolate: live darkling

beetle isolates (Beetle 1-5), and the isolate consisting of 5 dead darkling beetles (Dead

Beetles). L=GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, Waltham, MN).

REFERENCES

1. Boubaker, G., I. Marinova, F. Gori, A. Hizem, N. Müller, A. Casulli, L. Puebla, H. Babba, B. Gottstein, and M. Spiliotis. A dual PCR-based sequencing approach for the identification and discrimination of Echinococcus and Taenia taxa. 30:211-217. 2016.

2. Clark, S., and E. Kimminau. Critical review: future control of blackhead disease (histomoniasis) in poultry. 61:281-288. 2017.

3. Farr, M. M. Further observations on survival of the protozoan parasite, Histomonas meleagridis, and eggs of poultry nematodes in feces of infected birds. 51:3-13. 1961.

4. Gu, X. B., B. J. Wang, X. B. Zhao, Y. F. Li, G. Y. Yang, W. M. Lai, Z. J. Zhong, and G. N. Peng. Genetic variation in mitochondrial cox2 of Heterakis gallinarum from poultry in Sichuan, China. Mitochondrial DNA A DNA Mapp Seq Anal 29:629-634. 2018.

5. Gu, X., J. Y. Zhu, K. L. Jian, B. J. Wang, X. R. Peng, G. Y. Yang, T. Wang, Z. J. Zhong, and K. Y. Peng. Absence of population genetic structure in Heterakis gallinarum of chicken from Sichuan, inferred from mitochondrial cytochrome c oxidase subunit I gene. Mitochondrial DNA A DNA Mapp Seq Anal 27:3612-3617. 2016.

6. Huber, K., L. Gouilloud, and L. Zenner. A preliminary study of natural and experimental infection of the lesser mealworm Alphitobius diaperinus (Coleoptera: Tenebrionidae) with Histomonas meleagridis (Protozoa: Sarcomastigophora). Avian Pathology 36:279-282. 2007.

7. Ibáñez-Escribano, A., J. J. Nogal-Ruiz, V. J. Arán, J. A. Escario, A. Gómez-Barrio, and J. F. Alderete. Determination of internal transcribed spacer regions (ITS) in Trichomonas vaginalis isolates and differentiation among Trichomonas species. 63:427-431. 2014.

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8. Jiménez, F. A., S. L. Gardner, G. Navone, and G. Ortí. Four Events of Host Switching in Aspidoderidae (Nematoda) Involve Convergent Lineages of Mammals. Journal of Parasitology 98:1166-1175. 2012.

9. Kaushik, R. K., and V. P. Sharma Deorani. Studies on Tissue Responses in Primary and Subsequent Infections with Heterakis gallinae in Chickens and on the Process of Formation of Caecal Nodules. 43:69-78. 1969.

10. Lund, E. E., and A. M. Chute. The reproductive potential of Heterakis gallinarum in various species of galliform birds: Implications for survival of H. gallinarum and Histomonas meleagridis to recent times. International Journal for Parasitology 4:455-461. 1974.

11. Lund, E. E., and A. M. Chute. The reproductive potential of Heterakis gallinarum in various species of galliform birds: implications for survival of H. gallinarum and Histomonas meleagridis to recent times. 4:455-461. 1974.

12. Lund, E. E., E. E. Wehr, and D. J. Ellis. Earthworm Transmission of Heterakis and Histomonas to Turkeys and Chickens. 52:899-902. 1966.

13. Permin, A., M. Bisgaard, F. Frandsen, M. Pearman, J. Kold, and P. Nansen. Prevalence of gastrointestinal helminths in different poultry production systems. 40:439-443. 1999.

14. Powell, F. L., L. Rothwell, M. J. Clarkson, and P. Kaiser. The turkey, compared to the chicken, fails to mount an effective early immune response to Histomonas meleagridis in the gut. Parasite Immunol. 31:312-327. 2009.

15. Smith, T. An infectious disease among turkeys caused by protozoa (infectious entero-hepatitis). 8:7-15. 1895.

16. Smythe, A. B., M. J. Sanderson, and S. A. Nadler. Nematode small subunit phylogeny correlates with alignment parameters. Syst. Biol. 55:972-992. 2006.

17. Tyzzer, E. E., and M. Fabyan. Further Studies on "Blackhead" in Turkeys, with Special Reference to Transmission by Inoculation. 27:207-239. 1920.

18. Tyzzer, E. E. Studies on Histomoniasis, or "Blackhead" Infection, in the Chicken and the Turkey. 69:189-264. 1934.

19. Tyzzer, E. E. Heterakis vesicularis Frölich 1791. 23:708-709. 1926.

20. Wang, B. J., X. B. Gu, G. Y. Yang, T. Wang, W. M. Lai, Z. J. Zhong, and LH. Mitochondrial genomes of Heterakis gallinae and Heterakis beramporia support that they belong to the infraorder Ascaridomorpha. Infect. Genet. Evol. 40:228-235. 2016.

Table 1: Sequence information for HG1, HG2, and HG3 primers.

DNA fragment	Primer	Primer sequence	GC%	Primer length	Amplicon
HG1	Forward	GCGCCTGACTCTCGAATTTA	50	20	118 bp
	Reverse	ACTGTATTCCTTAACCGCACTC	45.5	22	
HG2	Forward	GACCGGCGAATTTAGCATATTG	45.5	22	99 bp
	Reverse	AGGAGGGCTGGTAACTATGTA	47.6	21	
HG3	Forward	GCATCTTGGCATGCGTTAAG	50	20	138 bp
	Reverse	ACGCTACCGTAGTCCATACT	50	20	
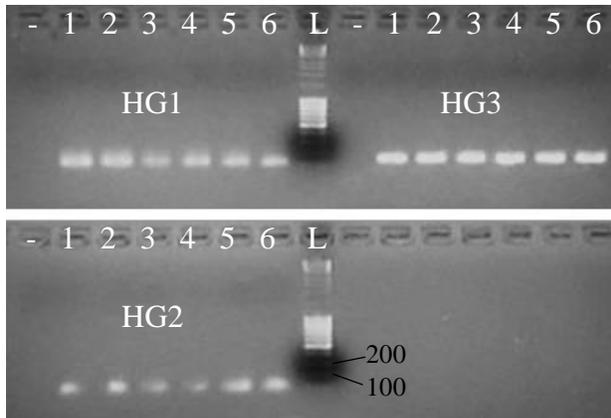
Figure 1: Gradient PCR for all 3 primer sets.
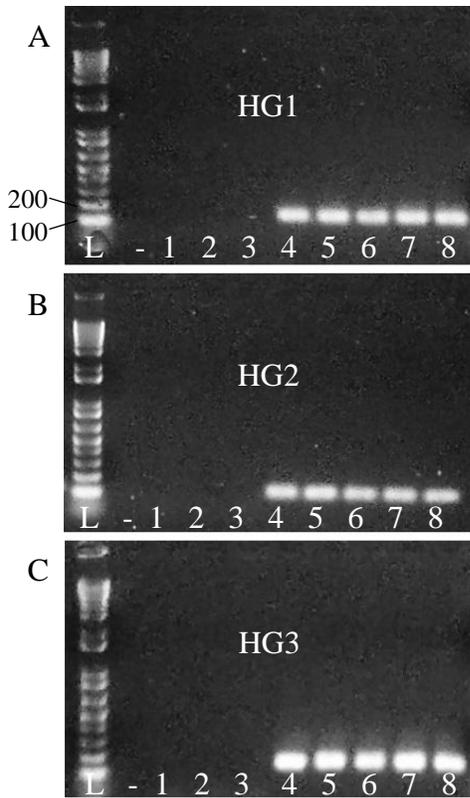
Figure 2: PCR demonstrating species specificity and sequence conservation of each primer set.
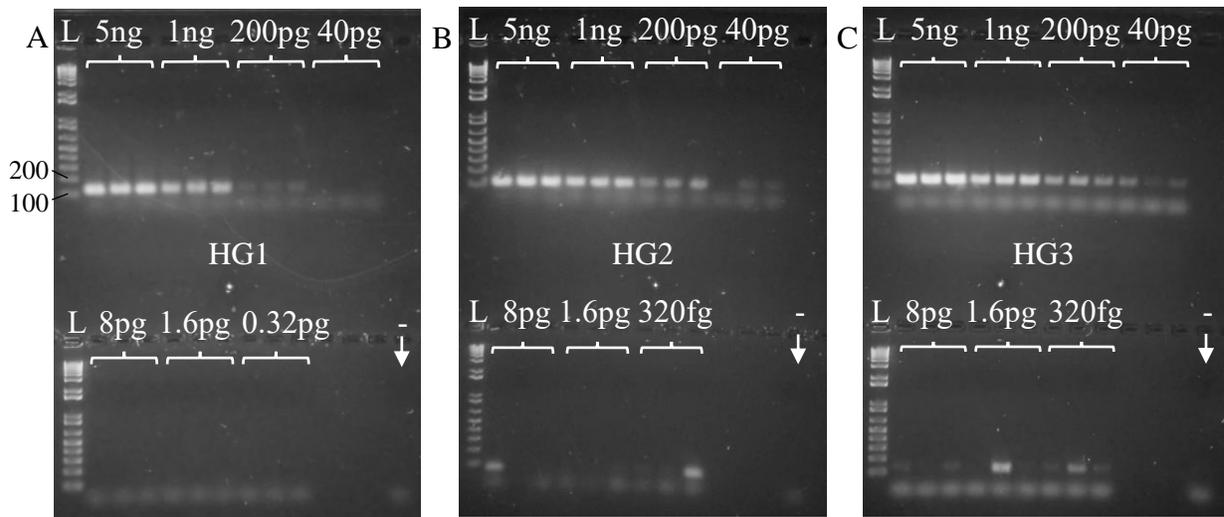
Figure 3: PCRs for each primer set using 5-fold serial dilution standards of pure *H. gallinarum* gDNA, demonstrating the level of sensitivity of each primer set.

Figure 4: HG3 diagnostic PCR for gDNA isolates of earthworms collected from a backyard chicken pen to detect the presence of *H. gallinarum* DNA.
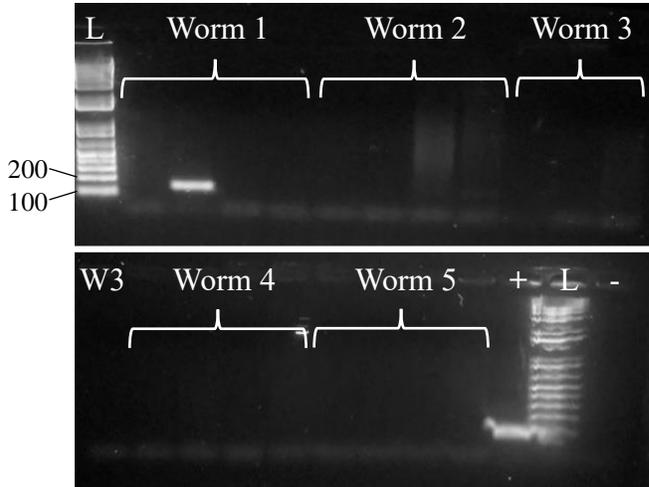
Figure 5: HG3 diagnostic PCR for gDNA isolates of earthworms collected from a commercial turkey facility to detect the presence of *H. gallinarum* DNA.
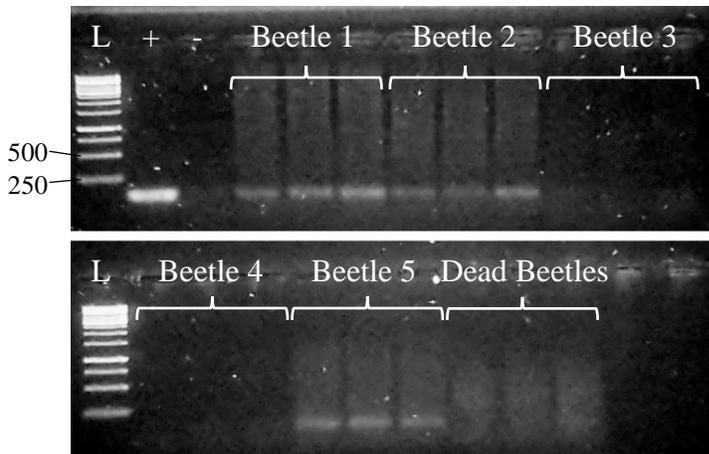
Figure 6: Experimental HG3 diagnostic PCR for gDNA isolates of darkling beetles fed *H. gallinarum* eggs to determine the potential for darkling beetles to carry the nematode.

Chapter 2

In vitro characterization of the effects of disinfectants on eggs

of the nematode *Heterakis gallinarum*

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Primary Audience: Poultry Producers, Veterinarians, Parasitologists

Keywords: *Heterakis gallinarum*, *Histomonas meleagridis*, embryonated eggs, trypan blue, decontamination

Summary

Heterakis gallinarum is a common, globally distributed cecal nematode infecting many gallinaceous birds. *H. gallinarum* is the vector for the protozoan parasite, *Histomonas meleagridis*, which is the causative agent of histomoniasis (blackhead) disease. Chickens can spread the disease to turkeys by acting as asymptomatic carriers of both the nematode and protozoan. Because *H. meleagridis* is transmitted through the nematode egg, destruction of *H. gallinarum* eggs in poultry houses that have suffered a histomoniasis outbreak may prevent transmission of *H. meleagridis* to subsequent flocks. Herein is described a standardized *in vitro* assay to characterize the effects of chemical disinfectants on *H. gallinarum* eggshell integrity and embryo viability. Six chemical compounds (sodium hypochlorite in Clorox Bleach; sodium dichloroisocyanurate; a combination of hydrogen peroxide, N-Alkyl-N,N-dimethyl-N-benzylammonium chloride, and diacetin; a combination of propionic acid, acetic acid, ammonium hydroxide, sodium hydroxide, and iodine; sodium bisulfate; and a combination of potassium peroxymonosulfate and sodium chloride) in addition to salt, limestone, and diesel fuel were tested. Both sodium hypochlorite and sodium dichloroisocyanurate damaged the eggshell, but only 15125 ppm of NaClO was capable of rendering the egg nonviable.

Introduction

Heterakis gallinarum is a 4-15 mm long cecal nematode that thrives in several species of galliformes (11). Nearly all chickens harbor some degree of *H. gallinarum* infection (4). In low numbers *H. gallinarum* is non-pathogenic; however, damage to the cecal mucosa is observed in cases of heavy infection (1). Although mono-infections with *H. gallinarum* do not significantly impact growth performance of commercial poultry, the nematode serves as the vector for

Histomonas meleagridis, the protozoan parasite that causes histomoniasis (blackhead disease), a fatal disease for turkeys that also causes morbidity in chickens (5, 19, 22) *H. meleagridis* is only capable of surviving free in the environment for a short period (10, 20, 21), necessitating a vector for transmission between flocks.

Chickens elicit an immune response to *H. meleagridis* infection and typically recover from histomoniasis (16). These asymptomatic chickens can act as reservoirs for *H. meleagridis* (20) capable of transmitting the protozoan to other susceptible populations when co-infected with *H. gallinarum*. Turkeys are less suitable hosts for strains of *H. gallinarum* adapted to chickens (12); however, turkeys can contract histomoniasis by ingesting embryonated *H. gallinarum* eggs that contain *H. meleagridis* (18). Turkeys mount an insufficient immune response to *H. meleagridis* infection and transmit the protozoan directly between birds resulting in flock mortality up to 100% (7, 14, 15).

The prevalence of histomoniasis in US poultry production doubled in 2016 following the revocation of the FDA approved use of Nitarsone, the last remaining available drug effective for preventing histomoniasis in turkeys and chickens (2). The increasing rates of histomoniasis in commercial poultry production systems warrant the development of new strategies to limit transmission of *H. meleagridis*. Poultry facilities that have repeated outbreaks of histomoniasis may be contaminated with *H. gallinarum* eggs harboring *H. meleagridis*. Decontamination of infective *H. gallinarum* eggs in poultry houses between flocks may aid in preventing future histomoniasis outbreaks. Chemicals e.g., fumigants, carbomates, and fertilizers such as oxamyl, dazomet, and calcium cyanamide respectively have been used to control soil nematodes that are crop pests (8). Various chemical disinfectants and compounds are used on poultry facilities; however, the efficacy of these products against *H. gallinarum* eggs has not been reported.

The infectivity of *H. gallinarum* eggs and parasite burden in the chicken is measured by counting mature worms in the ceca (12) of necropsied birds or isolating eggs from feces (3). The first method is impractical because it requires euthanasia of birds and the second method is prone to misdiagnosis. Infectivity of *H. gallinarum* eggs in turkeys has been assessed by observing histomoniasis that occurs as a result of eggs becoming infected with *H. meleagridis* (22). This method is insufficient to determine the successful establishment of *H. gallinarum* in the birds as the number of eggs infected with *H. meleagridis* is not well defined and may vary between bird populations. Compounds that may be capable of rendering *H. gallinarum* eggs non-infective can be identified based on their ability to damage the *H. gallinarum* eggshell and render the larva non-viable in an *in vitro* assay. The objective of this research was to describe a standardized *in vitro* assay for *H. gallinarum* eggshell integrity and larva viability and to use the assay to test several compounds. Two studies were conducted, in the first study the feasibility of the methodology of the *in vitro* assay was determined, and the second study used the assay to compare the effects of several chemicals and compounds used in the poultry industry on the *H. gallinarum* egg.

Materials and Methods

Specimen collection

Ceca were collected from broiler breeder hens at the end of lay obtained from a Georgia processing plant. *H. gallinarum* were removed from the ceca and transferred to a petri dish. Gravid females were isolated and stored in 3% formalin at room temperature for three weeks to allow embryonation. Embryonated eggs were extracted from the oviducts of females and approximately 250-350 eggs were pipetted into each well of a 24-well culture plate. Only

embryonated eggs that adhered to the plate could be included in the results as non-adherent eggs were lost during media changes.

Eggshell integrity test

In study 1, a 3 by 3 factorial design with treatment type and exposure time was used with single replicates for each treatment consisting of 250-350 eggs tested at 2, 4, and 24 hours. Treatments tested were: 6050 ppm of sodium hypochlorite (NaClO) from Clorox Bleach (Clorox, Oakland, CA) consisting of 6.05% NaClO and 93.95% inert ingredients; 833 ppm sodium dichloroisocyanurate (SDIC) from dissolved Green Klean chlorinated disinfecting tablets (Green Klean, Cary, IL) consisting of 48.21% SDIC and 51.79% other ingredients; and tap water as a negative control. In study 2, 9 chemicals and a tap water control were tested with 6 hours of contact and 3 replicates of 250-350 eggs per treatment: NaClO at 6050 ppm and 15125 ppm; SDIC at 833 ppm and 7488 ppm; a combination of 39591 ppm hydrogen peroxide (H_2O_2), 1550 ppm N-Alkyl-N,N-dimethyl-N-benzylammonium chloride, and 19186 ppm diacetin and other inert ingredients supplied in a product called Decon7 (Decon7 Systems, Scottsdale, AZ); 329670 ppm iodine mixed with a product called Kem San (Kemin, Des Moines, IA) containing a combination of 20604 ppm propionic acid, 4121 ppm acetic acid, 1099 ppm ammonium hydroxide, 549 ppm sodium hydroxide, and other inert ingredients; a dry and 500000 ppm solution of sodium bisulfate ($NaHSO_4$) supplied in a product called Poultry Litter Treatment (Jones-Hamilton, Walbridge, OH); a combination of 53525 ppm potassium peroxymonosulfate ($KHSO_5$), 3750 ppm sodium chloride, and other inert ingredients supplied in a product called Virkon S (Du Pont, Wilmington, DE); dry and saturated salt (NaCl); dry feed-grade limestone ($CaCO_3$); and diesel fuel (Exxon Mobile, Irving, TX). In both studies, stock solutions of each

treatment were prepared immediately prior to egg challenge. Replicate wells were covered with either 1 ml of liquid treatment or approximately 2 g of solid treatment. Eggs were incubated with treatments at room temperature for exposure times as noted above. At the end of exposure, eggs were washed with 1 ml of tap water and incubated with 250 µl of 0.4% trypan blue (Sigma, St. Louis, MO) at room temperature for 2 min. After replacing the trypan blue with 250 µl of water, eggshell integrity was determined by counting the number of blue (compromised) and clear (intact) eggs in each well under a dissecting microscope. Eggs that were damaged during egg collection and unembryonated eggs were excluded from the counts.

Embryo viability test

In study 2, eggs from treatments that produced blue-stained eggs were desiccated for 2 days after determining eggshell integrity. Eggs were then rehydrated with 1 ml of water, and the replicates for each treatment were combined. Approximately 5 eggs from each treatment were transferred to a clean petri dish. The eggs were manually hatched using forceps to release the embryos. Each embryo was observed for signs of controlled movement to assess viability. Treatments that produced only motile, living embryos were deemed ineffective for rendering *H. gallinarum* eggs nonviable.

Statistics

For study 2, the proportion of blue eggs in each treatment, except 39591 ppm H_2O_2, was compared to that in the water control using Dunnets HSD test in JMP Pro 13.2 (SAS, Cary, NC).

Results/Discussion

A pilot study (study 1) was carried out to evaluate the feasibility of using trypan blue to detect eggshell damage. Trypan blue is an impermeable dye routinely used in cell culture to distinguish dead cells which absorb the dye from living cells which exclude it. The eggshell integrity assay is based on the same principle; eggs with compromised eggshells will accumulate the dye while eggs with intact eggshells will exclude it (Fig. 1). In study 1, embryonated eggs were treated for 2, 4, or 24 hours with water, 6050 ppm NaClO, or 833 ppm SDIC (Fig. 2). Eggs in the water control from this study remained clear whereas all the eggs treated with NaClO stained blue after 2, 4, or 24 hours of exposure indicating that NaClO compromised eggshell integrity. The proportion of eggs that absorbed trypan blue when treated with 833 ppm SDIC increased from 0% to 6% between 2 and 24 hours of exposure, respectively, but was still 20x less effective than 6050 ppm NaClO. The increased efficacy of SDIC with increased exposure may suggest a mild additive effect of SDIC on eggshell integrity over time. Six hours was selected as the contact time to compare the effects of different compounds on *H. gallinarum* eggs. Although this contact time is longer than is practical for industry use, it allows the detection of eggshell damage caused by treatments that have some activity while excluding those that have limited to no activity. In study 2, all the eggs treated with 6050 ppm or 15125 ppm NaClO, 7488 ppm SDIC, or 39591 ppm H_2O_2 appeared blue, and some of the eggs in each replicate of the dry $NaHSO_4$ appeared blue. The proportion of blue eggs in each of these treatments, except 39591 ppm H_2O_2, was statistically different from that in the water control. Statistics could not be calculated for 39591 ppm H_2O_2 treatment because all the eggs from one of the replicates were lost. Eggs treated with 833 ppm SDIC, iodine, dry $NaHSO_4$, $KHSO_5$, dry and

saturated NaCl, dry CaCO$_3$, or diesel fuel excluded the dye (Fig. 3); therefore, they did not compromise eggshell integrity.

Chlorine has been shown to deleteriously affect: the eggs of root knot nematodes at concentrations of 50000 to 125000 ppm chlorine after 1 and 24-hour exposure (17); the larvae of mammalian gastrointestinal nematodes at 50000 ppm NaClO for 4-hour exposure (6); and the eggs of fish-parasitic nematodes at 3000 ppm and 6000 ppm chlorine for 10-minute exposure (13). By contrast, lower concentrations of chlorine (10-100 ppm NaClO for up to 15 days or 5-50 ppm chlorine for 1 week) have been observed to increase the rate of hatch for root knot nematodes (9, 17). Exposure of fish-parasitic nematodes to 500 ppm and 1000 ppm chlorine for up to 10 days increase the rate of larvation (13). The two chlorinated disinfectants in this study, bleach (NaClO) and SDIC (Sodium dichloroisocyanurate), produced detectable damage to 100% of the eggshells of the *H. gallinarum* eggs as all the eggs in these treatments absorbed trypan blue (Figure 3). It was noted in this study that *H. gallinarum* eggs treated with 15125 ppm NaClO or 7488 ppm SDIC were easier to manually hatch compared to all other treatments. Based on these observations, exposure of *H. gallinarum* eggs to concentrations of chlorine up to 15125 ppm may weaken the *H. gallinarum* eggshell but not harm the embryo, potentially improving the probability of infection in a host or making the embryos susceptible to treatment with other products. Killing of *H. gallinarum* larvae inside the eggs was only observed at 15125 ppm NaClO. The concentration and contact time required to render *H. gallinarum* eggs nonviable in the poultry house environment requires further study.

Although H$_2$O$_2$ eggs appeared blue, two observations suggest trypan blue was unable to penetrate the eggshell and remained on the surface. First, some of the eggs in all replicates for all treatments were washed out during solution changes with the majority retaining an average of 43

to 156 eggs between all three replicates, which is greater than the average retained from the water control, 43. H_2O_2, 833 pm SDIC, and 7488 ppm SDIC retained fewer eggs than the water control, averaging 17, 18, and 21 recovered eggs, respectively. All the eggs in H_2O_2 replicate 1 were washed out of the well while only 4 and 47 eggs remained in the other two replicates. No statistical comparison of lost eggs can be made between treatments because the initial number of eggs placed in each well was not counted; however, based on this observation, H_2O_2 and SDIC may alter the exterior of the eggs reducing their adhesive properties. If this is the case, these compounds may be used as cleaning agents rather than disinfecting agents to wash *H. gallinarum* eggs off equipment. Second, upon closer observation, most H_2O_2-treated eggs appeared to have clear centers while the rest of the eggs' surfaces stained blue. Washing trypan blue-stained H_2O_2 eggs with a dilute soap solution (60 µl of dish soap/ml of water) instantly removed all the trypan blue from the eggs while similar treatment of 6050 ppm NaClO eggs resulted in lighter blue staining over time, but the dye never washed out completely (data not shown). Therefore, the H_2O_2 treatment altered the surface of the *H. gallinarum* egg but did not degrade the eggshell causing the trypan blue to cling to the exterior of the eggshell rather than penetrate the egg.

Aqueous solutions of limestone have proven ineffective for lysing the larvae of small ruminants parasitic nematodes (6). Based on the results of this study, dry $CaCO_3$ has no effect on *H. gallinarum* eggs under the conditions of the current study. Similarly, dry and aqueous NaCl did not significantly damage *H. gallinarum* eggs. Therefore, $CaCO_3$ and NaCl, which routinely are dumped onto poultry house floors, do not have nematicidal properties. It was observed that replicates of dry NaCl and one replicate of $CaCO_3$ contained blue eggs. These replicates did not significantly differ from the water control. The proportion of dry $NaHSO_4$-treated blue eggs was statistically greater than the water control; however, there was a substantial amount of variation

between the replicates (Fig. 3). Based on this data, blue eggs in dry substance treatments were damaged by mechanical abrasion when the treatments were removed from the wells rather than chemical degradation of the eggshell. Diesel fuel, 329670 ppm iodine, and 53525 ppm $KHSO_5$ did not damage the eggs under these experimental conditions; while these compounds may be caustic to other organisms, they are not effective for disinfecting *H. gallinarum* eggs.

Conclusion

Two chlorinated disinfectants, sodium hypochlorite and sodium dichloroisocyanurate, can damage the eggshells of *H. gallinarum*; however, relatively low concentrations of these compounds are insufficient to render the *H. gallinarum* eggs non-viable. In this study, the only successful treatment capable of killing the larvae within the eggshells after 6 hours of exposure was NaClO at a concentration of 15125 ppm. Other compounds may be tested on *H. gallinarum* eggs using this *in vitro* assay without resorting directly to feeding treated eggs to birds thereby minimizing cost, time, and bird suffering in establishing effective treatment protocols that may be effective in a poultry house. Evaluating potential *H. gallinarum* egg decontamination protocols starting with an *in vitro* test and ending with live bird trials can identify effective disinfection protocols that may be deployed in commercial poultry houses in the most cost and time effective manner.

REFERENCES

1. Butt, Z., S. A. Memon, and A. A. Shaikh. Pathology of *Heterakis gallinarum* in the ceca of naturally infected chicken (*Gallus domesticus*). Pure and Appl. Biol. 5:815-821. 2016.

2. Clark, S., and E. Kimminau. Critical Review: Future control of blackhead disease (Histomoniasis) in poultry. Avian Dis. 61:281-288. 2017.

3. Das, G., T. Savas, F. Kaufmann, A. Idris, H. Abel, and M. Gauly. Precision, repeatability and representative ability of faecal egg counts in *Heterakis gallinarum* infected chickens. Vet. Parasitol. 183:87-94. 2011.

4. Gibbs, B. J. The occurrence of the protozoan parasite *Histomonas meleagridis* in the adults and eggs of the cecal worm *Heterakis gallinae*. J. Protozool. 9:288-293. 1962.

5. Graybill, H. W., and T. Smith. Production of fatal blackhead in turkeys by feeding embryonated eggs of *Heterakis papillosa*. J. Exp. Med. 31:647-655. 1920.

6. Howell, J. M., J. M. Luginbuhl, M. J. Grice, K. L. Anderson, P. Arasu, and J. R. Flowers. Control of gastrointestinal parasite larvae of ruminant using nitrogen fertilizer, limestone and sodium hypochlorite solutions. Small Ruminant Res. 32:197-204. 1999.

7. Hu, J., L. Fuller, P. L. Armstrong, and L. R. McDougald. *Histomonas meleagridis* in chickens: Attempted transmission in the absence of vectors. Avian Dis. 50:277-279. 2006.

8. Hussain, M., M. Zouhar, and P. Rysanek. Comparison between biological and chemical management of root knot nematode, *Meloidogyne hapla*. Pakistan J. of Zool. 49:205-210. 2017.

9. Kanwar, R. S., R. K. Jain, I. J. Paruthi, and D. C. Gupta. Effects of sodium-hypochlorite on egg liberation from egg masses, hatching and infectivity of *Meloidogyne-javanica* juveniles. Nematologica 37:236-240. 1991.

10. Lotfi, A. R., E. M. Abdelwhab, and H. M. Hafez. Persistence of *Histomonas meleagridis* in or on materials used in poultry houses. Avian Dis. 56:224-226. 2012.

11. Lund, E. E., and A. M. Chute. The reproductive potential of Heterakis gallinarum in various species of galliform birds: implications for survival of *H. gallinarum* and *Histomonas meleagridis* to recent times. Int. J. Parasitol. 4:455-461. 1974.

12. Lund, E. E., A. M. Chute, and S. L. Myers. Performance in chickens and turkeys of chicken-adapted *Heterakis gallinarum*. J. Helminthol. 44:97-106. 1970.

13. Martins, M. L., V. Watral, J. P. Rodrigues-Soares, and M. L. Kent. A method for collecting eggs of Pseudocapillaria tomentosa (Nematoda: *Capillariidae*) from zebrafish *Danio rerio* and efficacy of heat and chlorine for killing the nematode's eggs. J. Fish Dis. 40:169-182. 2017.

14. McDougald, L. R. Blackhead disease (histomoniasis) in poultry: a critical review. Avian Dis. 49:462-476. 2005.

15. Powell, F. L., L. Rothwell, M. J. Clarkson, and P. Kaiser. The turkey, compared to the chicken, fails to mount an effective early immune response to *Histomonas meleagridis* in the gut. Parasite Immunol. 31:312-327. 2009.

16. Schwarz, A., M. Gauly, H. Abel, G. Das, J. Humburg, A. T. Weiss, G. Breves, and S. Rautenschlein. Pathobiology of *Heterakis gallinarum* mono-infection and co-infection with *Histomonas meleagridis* in layer chickens. Avian Pathol. 40:277-287. 2011.

17. Stanton, J. M., and W. E. O'Donnell. Hatching, motility, and infectivity of root-knot nematode (*Meloidogyne javanica*) following exposure to sodium hypochlorite. Aust. J. Exp. Agric. 34:105-108. 1994.

18. Swales, W. E. Enterohepatitis (blackhead) in turkeys; observations on transmission by the caecal worm (*Heterakis gallinae*). Can. J. Comp. Med. Vet. Sci. 12:97-100. 1948.

19. Tyzzer, E. E. *Heterakis vesicularis* Frölich 1791:A vector of an infectious disease. Proc. Soc. Exp. Biol. Med. 23:708-709. 1926.

20. Tyzzer, E. E. Studies on histomoniasis, or "blackhead" Infection, in the chicken and the turkey. Proc. Am. Acad. Arts Sci. 69:189-264. 1934.

21. Tyzzer, E. E., and J. Collier. Induced and natural transmission of blackhead in the absence of Heterakis. J. Infect. Dis. 37:265-276. 1925.

22. Tyzzer, E. E., and M. Fabyan. A further inquiry into the source of the virus in blackhead of turkeys, together with observations on the administration of ipecac and of sulfur. J. Exp. Med. 35:791-812. 1922.

Figure 1. *Heterakis gallinarum* eggs treated for 6 hours then covered with trypan blue and

washed with water observed at 100x. A) a clear, unstained water control egg. B) a

light blue-stained 6050 ppm NaClO egg.

Figure 2. Proportion of eggs treated with 6050 ppm NaClO, 833 ppm SDIC, and water for 2, 4,

and 24 hours that absorbed trypan blue in study 1.

Figure 3. A) Proportion of eggs per replicate in each treatment that absorbed trypan blue

following 6 hours of treatment in study 2. Asterisk indicates significant difference

from water control. **p-value <0.0001, *p<0.01. [+]Also contains 1550 ppm N-Alkyl-

N,N-dimethyl-N-benzylammonium chloride and 19186 ppm diacetin. [T]Also contains

20604 ppm propionic acid, 4121 ppm acetic acid, 1099 ppm ammonium hydroxide,

and 549 ppm sodium hydroxide. B) Proportion of eggs from the water control and

treatments that produced blue-stained eggs that were determined to be non-viable by

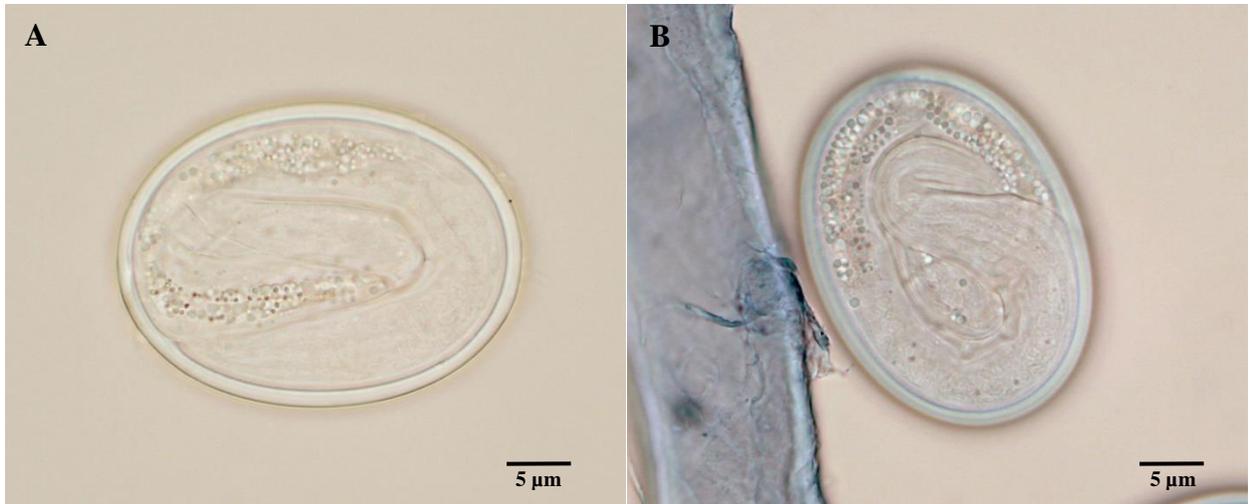manually releasing the larvae from the eggshells.

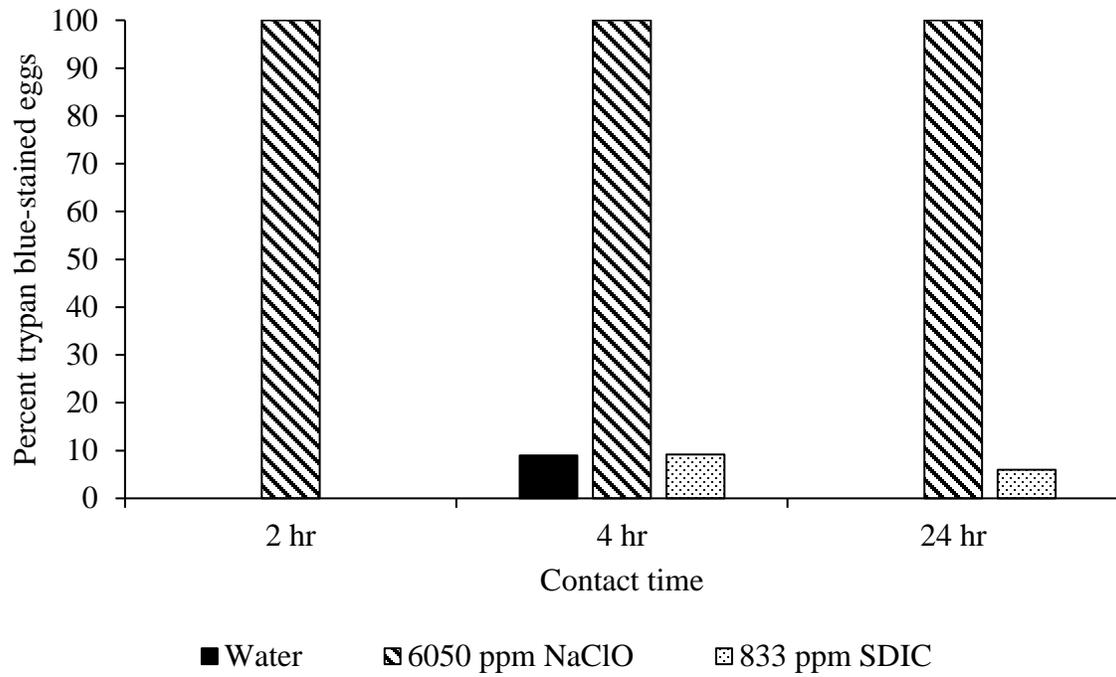Figure 1: An unstained *H. gallinarum* egg (A), and a blue-stained *H. gallinarum* egg (B).

Figure 2: The proportion of eggs that stained blue after treatment with 6050 ppm NaClO or 833 ppm SDIC for 2, 4, and 24 hours.
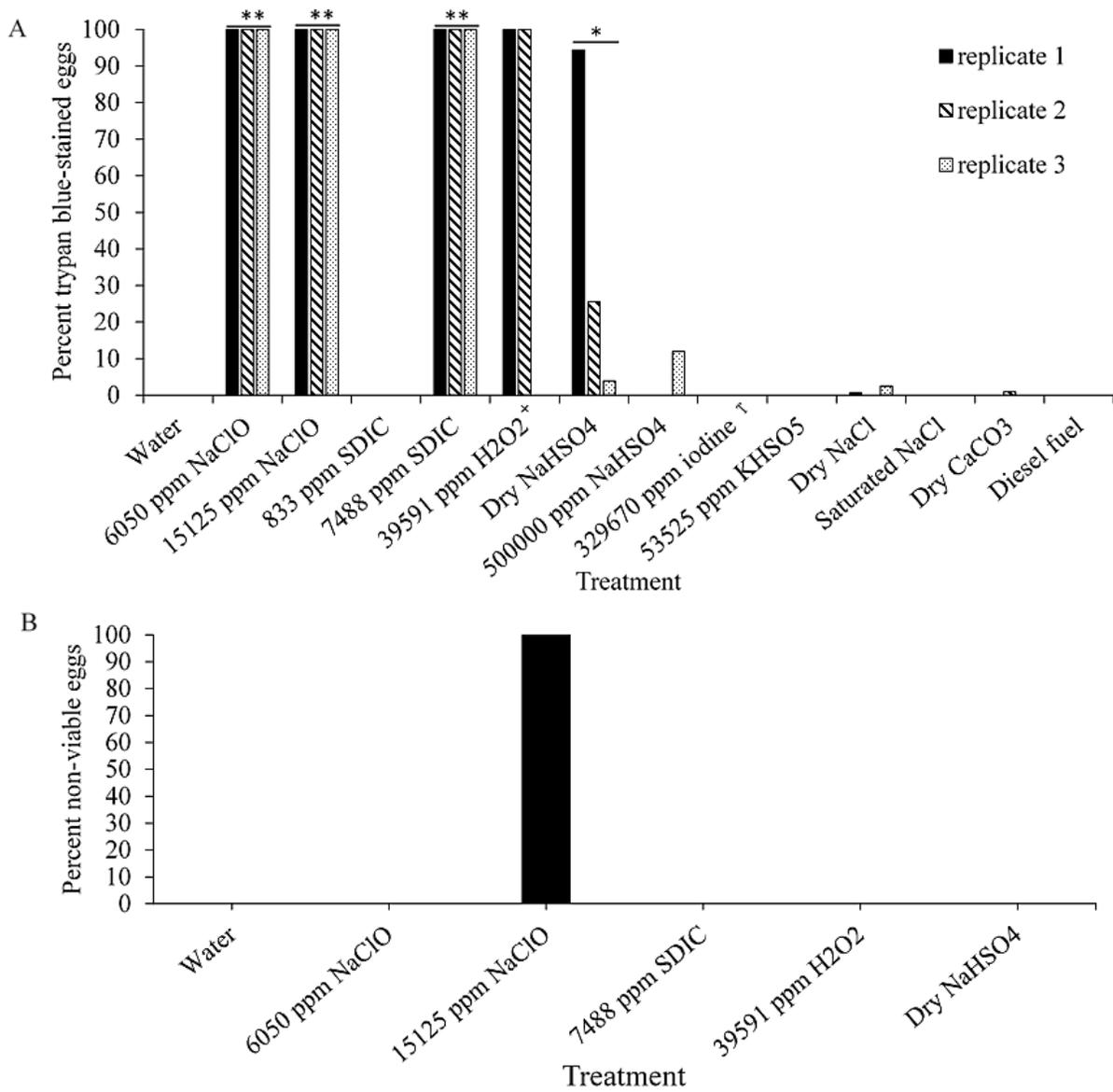
Figure 3: The proportion of eggs that stained blue after chemical treatment (A), and the proportion of eggs rendered non-viable following treatment with chemicals that produced blue-stained eggs (B).

Acknowledgements

CONCLUSIONS

The purpose of this research was to design a molecular diagnostic test that could be used to identify DNA samples containing *H. gallinarum* and an *in vitro* egg degradation assay for comparing the effects of disinfectants on the eggshells and larva viability of *H. gallinarum* eggs. The following conclusions were supported by the research presented herein:

1. The diagnostic PCR using HG3 primers is capable of identify the presence of *H. gallinarum* DNA in environmental samples.

2. Earthworms from backyard poultry and commercial farms have been shown to be contaminated with *H. gallinarum* DNA via diagnostic PCR.

3. Adult darkling beetles have been shown to carry *H. gallinarum* DNA for at least a week after exposure to the nematode eggs, indicating further investigation is needed to determine if the beetles may be contributing to the transmission of the nematode.

4. Chlorinated disinfectants, 6050 ppm to 15125 ppm NaClO and 833 ppm to 7488 ppm Sodium dichloroisocyanurate used to treat *H. gallinarum* eggs for 6 hours are sufficient for compromising eggshell integrity, but only concentrations of 15125 ppm NaClO were capable of rendering the larvae within the eggs non-viable *in vitro*. Further testing is needed to determine the efficacy of these compounds for decontaminating eggs in a more complex substrate such as a soil matrix.