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Investigation of Histomoniasis Prevention in Poultry

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Poultry Science

by

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ABSTRACT

Histomonas meleagridis is the etiological agent of histomoniasis, also commonly known as blackhead disease. This protozoal disease of poultry is detrimental to turkeys with flock mortalities often reaching 80-100%, although other gallinaceous birds are susceptible. Since the voluntary removal of nitarsone in 2015, the poultry industry is suffering with no approved prophylactics, therapeutics, or vaccines for this disease. The objectives of this dissertation were to evaluate multiple methods for prevention or control of histomoniasis, including dietary chemoprophylaxis and vaccination. Specifically, this research evaluated quinine as a chemoprophylactic candidate (Chapter 3) or live-attenuated *H. meleagridis* as vaccine candidates (Chapter 4) in an experimental challenge model. Quinine is an antiprotozoal phytochemical that has previously shown efficacy against *Plasmodium* spp., the etiological agent of poultry malaria; therefore, quinine was hypothesized to confer antihistomonal properties. Quinine effectively reduced ($P < 0.05$) viable histomonads *in vitro* but did not mitigate histomoniasis when evaluated *in vivo,* as indicated by similar ($P > 0.05$) post-challenge body weight gain (**BWG**), mortalities, and lesion scores (**LS**) as compared to the positive-challenged control (**PC**) group. Taken together, these data suggested that phytochemicals should be further evaluated against histomoniasis but both *in vitro* and *in vivo* studies should be conducted against multiple isolates. Four experiments were conducted to evaluate age, route, and administration dose of selected live-attenuated *H. meleagridis* isolates as vaccine candidates (**Vacc**) against homologous or heterologous wild-type *H. meleagridis* (**WTH**) challenge in turkeys. Day-of-hatch administration of Vacc did not confer protection against subsequent WTH-challenge; however, WTH-challenge at day-of-hatch via the oral route induced histomoniasis whereas oral challenge at d21 did not induce disease. When administered intracloacally at d14 at a dose of 2×10^5 cells/turkey, the

Vacc isolates generally resulted in reduced ($P < 0.05$) disease severity during Challenge Phase, as indicated by lower mortalities, lower LS, and improved BWG as compared to the PC group. During Vaccination Phases, the Vacc groups resulted in lower LS and mortalities ($P < 0.05$) as compared to the PC group and were not different ($P > 0.05$) as compared to the non-challenged control group, indicating the reduced virulence and apparent safety of Vacc isolates for application to turkeys. Furthermore, d14 intracloacal vaccination offered some protection against homologous and heterologous WTH-challenge. Overall, this research demonstrated vaccination against histomoniasis to be possible although protection was not robust.

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I am especially thankful to my family for their unconditional love and support throughout this endeavor. Thank you for constantly encouraging me and always being there when I needed guidance. Finally, to summarize my collegiate journey: “For I know the plans I have for you,” declares the Lord, “plans to prosper you and not to harm you, plans to give you hope and a future.” Jeremiah 29:11

DEDICATION

Although sometimes wilting, the flower of education eventually bloomed. This dissertation is dedicated to my family and the significant people in my life – your love and encouragement through this tumultuous journey have truly been a blessing. I made it this far because of y'all.

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Chapter 3

Beer, L. C., B. M. Hargis, and C. N. Vuong. 2021. Research note: Evaluation of quinine as a chemoprophylactic candidate against histomoniasis in turkeys. (Published). Poult. Sci. 100:101469.

Chapter 4

Beer, L. C., B. D. M. Graham, T. L. Barros, J. D. Latorre, G. Tellez-Isaias, A. L. Fuller, B. M. Hargis, and C. N. Vuong. Evaluation of live-attenuated *Histomonas meleagridis* isolates as vaccine candidates against wild-type challenge. (Submitted). Poult. Sci.

CHAPTER 1: INTRODUCTION

Histomoniasis, also commonly known as blackhead disease, is a protozoal disease in poultry caused by *Histomonas meleagridis* (Clarkson, 1963; Liebhart et al., 2017). Since the voluntary removal of nitarsone in 2015, there are no effective prophylactics available to treat this disease and no vaccines have been developed for commercial application (Regmi et al., 2016; Hafez and Shehata, 2021). Phytogenic compounds are being considered for chemoprophylaxis of histomoniasis as they are often relatively cheap and could potentially be utilized in organic and traditional production (Zenner et al., 2003; Puvača et al., 2020). *In vitro* evaluations have indicated antihistomonal properties of some plant-derived compounds, but associated *in vivo* evaluations remain to be conducted (Zenner et al., 2003; Grabensteiner et al., 2007; Hauck and Hafez, 2007; van der Heijden and Landman, 2008a). Limited studies have been completed with paired *in vitro* and *in vivo* evaluation of phytochemicals against *H. meleagridis*, but results were limited or variable in protection (Duffy et al., 2004, 2005; Hafez and Hauck, 2006; Grabensteiner et al., 2008; van der Heijden and Landman, 2008b; Thøfner et al., 2012). Recently, a proprietary blend of herbal extracts was effective as a dietary supplement for prevention and treatment of histomoniasis in turkey breeders (Tykałowski et al., 2021). These successes are encouraging for the pursuit of plant-based compounds as chemoprophylactic candidates for mitigating histomoniasis.

When recovered from *H. meleagridis*-infection, turkeys and chickens exhibited a level of resistance to re-infection, but early attempts with immunization yielded inconsistent protection (Tyzzer et al., 1921; Tyzzer and Fabyan, 1922; Tyzzer, 1932, 1934, 1936; Clarkson, 1963, 1966; Joyner, 1963; Joyner et al., 1963; Cuckler, 1970). Repeated serial passage *in vitro* is an established method for attenuating *H. meleagridis* through selective pressures (Tyzzer 1934,

1936; Lund et al., 1966, 1967; Dwyer and Honigberg, 1969, 1970; Liebhart et al., 2011; Gruber et al., 2017; Wei et al., 2020). In recent years, clonal *in vitro* attenuated isolates have conferred some protection when administered as a vaccine either orally or cloacally without negatively impacting performance, although these isolates have not yet been evaluated in non-clonal challenge field conditions (Hess et al., 2008; Liebhart et al., 2010, 2013, 2017; Sulejmanovic et al., 2016; Mitra et al., 2018). Moreover, the safety and stability of attenuation was shown with *in vivo* serial back-passaging which did not revert virulence to an *in vitro* attenuated isolate (Sulejmanovic et al., 2013). Taken together, these immunological research advancements are encouraging for vaccine development, but further research is needed to evaluate the proper age, dose, and administration route for a candidate histomoniasis vaccine.

The objectives of this dissertation were to investigate multiple methods for prevention or control of histomoniasis. Specifically, research evaluated chemoprophylaxis with quinine (Chapter 3) or vaccination with live-attenuated *H. meleagridis* (Chapter 4). Early researchers hypothesized that quinine, an established antimalarial compound, could confer antihistomonal properties but results indicated that poultry were not successfully protected (Tyzzer and Fabyan, 1922; Tyzzer, 1923; Delaplane and Stuart, 1935; Farmer, 1950). The reported methodologies for administration and dose of quinine were ambiguous; therefore, quinine was further evaluated both *in vitro* and *in vivo* as a chemoprophylactic candidate for histomoniasis. Additionally, attenuated non-clonal *H. meleagridis* isolates were produced through repeated serial *in vitro* passaging and subsequently evaluated as vaccine candidates to confer protection against wild-type challenge. Previous studies have utilized clonal isolates for both vaccination and challenge, but this research was conducted with non-clonal isolates that could better resemble field conditions where turkeys are exposed to multiple isolates.

REFERENCES

Clarkson, M. J. 1963. Immunological responses to *Histomonas meleagridis* in the turkey and fowl. Immunology. 6:156-168.

Clarkson, M. J. 1966. Progressive serum protein changes in turkeys infected with *Histomonas meleagridis*. J. Comp. Path. 76:387-397.

Cuckler, A. 1970. Coccidiosis and histomoniasis in avian hosts. Pages 371-397 in Immunity to parasitic animals. GJ Jackson, R. Herman and I. Singer, eds., New York.

Delaplane, J. P., and H. O. Stuart. 1935. Blackhead (entero-hepatitis) investigations. Bulletin 247. Agricultural experiment station. RI State College.

Duffy, C. F., M. D. Sims, and R. F. Power. 2004. Preliminary evaluation of dietary Natustat™ versus Histostat® (nitarsone) for control of *Histomonas meleagridis* in broiler chickens on infected litter. Int. J. Poult. Sci. 3:753-757.

Duffy, C. F., M. D. Sims, and R. F. Power. 2005. Evaluation of dietary Natustat™ for control of *Histomonas meleagridis* in male turkeys on infected litter. Avian Dis. 49:423-425.

Dwyer, D. M., and B. M. Honigberg. 1969. Effect of *in vitro* cultivation on the virulence of a pathogenic strain of *Histomonas meleagridis*. J. Protozool. (Suppl). 16:18-19.

Dwyer, D. M., and B. M. Honigberg. 1970. Effect of certain laboratory procedures on the virulence of *Histomonas meleagridis* for turkeys and chickens. J. Parasitol. 56:694-700

Farmer, R. K. 1950. Infectious enterohepatitis (blackhead) in turkeys: A study of the prophylactic and therapeutic value of certain compounds. J. Comp. Pathol. 60:294-310.

Grabensteiner, E., N. Arshad, and M. Hess. 2007. Differences in the *in vitro* susceptibility of mono-eukaryotic cultures of *Histomonas meleagridis, Tetratrichomonas gallinarum* and *Blastocystis* sp. to natural organic compounds. Parasitol. Res. 101:193-199.

Grabensteiner, E., D. Liebhart, N. Arshad, and M. Hess. 2008. Antiprotozoal activities determined *in vitro* and *in vivo* of certain plant extracts against *Histomonas meleagridis, Tetratrichomonas gallinarum,* and *Blastocystis* sp. Parasitol Res. 103:1257-1264.

Gruber, J., P. Ganas, and M. Hess. 2017. Long-term *in vitro* cultivation of *Histomonas meleagridis* coincides with the dominance of a very distinct phenotype of the parasite exhibiting increased tenacity and improved cell yields. Parasitology. 144:1253-1263.

Hafez, H. M., and R. Hauck. 2006. Efficacy of a herbal product against *Histomonas meleagridis* after experimental infection of turkey poults. Archiv. Anim. Nutr. 60:436-442.

Hafez, H. M., and A. A. Shehata. 2021. Turkey production and health: Current challenges. Ger. J. Vet. Res. 1:3-14.

Hauck, R., and H. M. Hafez. 2007. Effect of coated plant extracts on *Histomonas meleagridis* and growth of bacteria *in vitro.* Avian Dis. 51:880-883.

van der Heijden, H. M. J. F., and W. J. M. Landman. 2008a. *In vitro* effect of herbal products against *Histomonas meleagridis* in turkeys. Vet. Parasitol. 154:1-7.

van der Heijden, H. M. J. F., and W. J. M. Landman. 2008b. *In vivo* effect of herbal products against *Histomonas meleagridis* in turkeys. Avian Pathol. 37:45-50.

Hess, M., D. Liebhart, E. Grabensteiner, and A. Singh. 2008. Cloned *Histomonas meleagridis* passaged *in vitro* resulted in reduced pathogenicity and is capable of protecting turkeys from histomonosis. Vaccine. 26:4187-4193.

Joyner, L. P. 1963. Immunity to histomoniasis in turkeys following treatment with dimetridazole. J. Comp. Pathol. Ther. 73:201-207.

Joyner, L. P., S. F. M. Davies, and S. B. Kendall. 1963. Chemotherapy of histomoniasis. Pages 333-349 in Experimental Chemotherapy. Vol. 1. Academic Press New York.

Liebhart, D., P. Ganas, T. Sulejmanovic, and M. Hess. 2017. Histomonosis in poultry: Previous and current strategies for prevention and therapy. Avian Pathol. 46:1-18.

Liebhart, D., T. Sulejmanovic, B. Grafl, A. Tichy, and M. Hess. 2013. Vaccination against histomonosis prevents a drop in egg production in layers following challenge. Avian Pathol. 42:79-84.

Liebhart, D., M. Windisch, and M. Hess. 2010. Oral vaccination of 1-day-old turkeys with *in vitro* attenuated *Histomonas meleagridis* protects against histomonosis and has no negative effect on performance. Avian Pathol. 39:399-403.

Liebhart, D., M. Zahoor, I. Prokofieva, and M. Hess. 2011. Safety of avirulent histomonads to be used as a vaccine determined in turkeys and chickens. Poult. Sci. 90:996-1003.

Lund, E. E., P. C., Augustine, and A. M. Chute. 1967. *Histomonas meleagridis* after one thousand *in vitro* passages. J. Eukaryot. Microbiol. 14:349-351.

Lund, E. E., P. C. Augustine, and D. J. Ellis. 1966. Immunizing action of *in vitro*-attenuated *Histomonas meleagridis* in chickens and turkeys. Exp. Parasitol. 18:403-407.

Mitra, T., Kidane, F. A., Hess, M., and Liebhart, D. 2018. Unravelling the immunity of poultry against the extracellular protozoan parasite *Histomonas meleagridis* is a cornerstone for vaccine development: A review. Front. Immunol. 9:2518.

Puvača, N., I. Brkić, M. Jahić, S. R. Nikolić, G. Radović, D. Ivanišević, M. Đokić, D. Bošković, D. Ilić, S. Brkanlić, and R. Prodanović. 2020. The effect of using natural or biotic dietary supplements in poultry nutrition on the effectiveness of meat production. Sustainability. 12:4373.

Regmi, P. R., A. L. Shaw, L. L. Hungerford, J. R. Messenheimer, T. Zhou, P. Pillai, A. Omer, and J. M. Gilbert. 2016. Regulatory considerations for the approval of drugs against histomoniasis (blackhead disease) in turkeys, chickens, and game birds in the United States. Avian Dis. 60:725-730.

Sulejmanovic, T., I. Bilic, M. Hess, and D. Liebhart. 2016. An *in vitro* attenuated strain of *Histomonas meleagridis* provides cross-protective immunity in turkeys against heterologous virulent isolates. Avian Pathol. 45:46-53.

Sulejmanovic, T., D. Liebhart, and M. Hess. 2013. *In vitro* attenuated *Histomonas meleagridis* does not revert to virulence, following serial *in vivo* passages in turkeys or chickens. Vaccine. 31:5443-5450.

Thøfner, I. C. N., D. Liebhart, M. Hess, T. W. Schou, C. Hess, E. Ivarsen, X. Fretté, L. P. Christensen, K. Grevsen, R. M. Engberg, and J. P. Christensen. 2012. Antihistomonal effects of artemisinin and *Artemisia annua* extracts *in vitro* could not be confirmed by *in vivo* experiments in turkeys and chickens. Avian Pathol. 41:487-496.

Tykałowski, B., M. Śmiałek, J. Kowalczyk, D. Dziewulska, T. Stenzel, and A. Koncicki. 2021. Phytoncides in the prevention and therapy of blackhead disease and their effect on the turkey immune system. J. Vet. Res. 65:79-85.

Tyzzer, E. E. 1923. Arsenical compounds in the treatment of blackhead in turkeys. J. Exp. Med. 37:851-873.

Tyzzer, E. E. 1932. Problems and observations concerning the transmission of blackhead infection in turkeys. Proc. Am. Philos. Soc. 71:407-410.

Tyzzer, E. E. 1934. Studies on histomoniasis, or "blackhead" infection, in the chicken and the turkey. Daedalus. 69:189-264.

Tyzzer, E. E. 1936. A study of immunity produced by infection with attenuated culture-strains of *Histomonas meleagridis.* J. Comp. Patho. Ther. 49:285-303.

Tyzzer, E. E., and M. Fabyan. 1922. 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

Tyzzer, E. E., M. Fabyan, and N. C. Foot. 1921. Further observations on "blackhead" in turkeys. J. Infect. Dis. 29:268-286.

Wei, Z., M. Abraham, E. V. Chadwick, and R. B. Beckstead. 2020. *Histomonas meleagridis* isolates compared by virulence and gene expression. Vet. Parasitol. 286:109233.

Zenner, L., M. P. Callait, C. Granier, and C. Chauve. 2003. *In vitro* effect of essential oils from *Cinnamomum aromaticum*, *Citrus limon* and *Allium sativum* on two intestinal flagellates of poultry, *Tetratrichomonas gallinarum* and *Histomonas meleagridis.* Parasite. 10:153-157.

CHAPTER 2: LITERATURE REVIEW

Overview of Histomoniasis

The first known histomoniasis outbreak was described by Cushman (1893) and occurred in a Rhode Island turkey flock. Smith (1895) further characterized histomoniasis and attributed it to the protozoan *Amoeba meleagridis* obtained from liver lesions. Shortly thereafter, Tyzzer (1920) more appropriately renamed this protozoon *Histomonas meleagridis*. Further studies confirmed *H. meleagridis* as the etiological agent, although the mode of cecal invasion was still uncertain (Farmer et al., 1951). Common synonyms for the disease have included blackhead disease, infectious enterohepatitis, histomonosis, and typhlohepatitis (Joyner et al., 1963; Hess et al., 2015; Clarke et al., 2017). Blackhead disease is an unfortunate misnomer as a cyanotic head is neither pathognomonic nor common; therefore, histomoniasis will be the preferred terminology used throughout this review (Davidson and Doster, 1994; Hess and McDougald, 2013). Turkeys are especially susceptible to *H. meleagridis*-infection, although other gallinaceous birds such as chickens, pheasants, and peafowl can be affected (Lund and Chute, 1972; Friend et al., 1999; Clarke et al., 2017). Annual economic losses to the turkey industry have been estimated to exceed two million USD, and a 2020 survey listed histomoniasis in position #11 of current issues facing the industry (Hess and McDougald, 2013; Clark and Froebel, 2020).

Graybill and Smith (1920) implicated *Heterakis* spp. in the role of transmitting *H. meleagridis* as they were unable to initiate the disease in absence of cecal worms. Further research showed that unprotected histomonads did not survive long periods outside the host, although duration in the environment when protected by feces or other materials was not well characterized (Tyzzer and Collier, 1925; McDougald, 2005). The separate rearing of poultry

species is critical as chickens are considered partially resistant to histomoniasis, frequently serving as asymptomatic carriers and reservoirs of *H. meleagridis*-infected heterakid eggs (Curtice, 1907; Tyzzer, 1932; Lund and Chute, 1972; Popp et al., 2011; Cupo and Beckstead, 2019a,b). Direct transmission within a flock is considered to occur through cloacal drinking which transfers materials from the vent region into the cecae through waves of reverse peristalsis (Sorvari et al., 1977; Hu et al., 2004; McDougald and Fuller, 2005; Scanes and Pierzchala-Koziec, 2014). Horizontal transmission of *H. meleagridis* has occurred by comingling and contact of infected with uninfected turkeys, regardless of floor type and in absence of *H. gallinarum* (Hu and McDougald, 2003; Armstrong and McDougald, 2011). The breed of turkeys or chickens may affect susceptibility to *H. meleagridis*-infection, although male and female turkeys appear to be similarly susceptible; however, research is limited on the possible influence on disease development (Al-Khateeb and Hansen, 1973, 1974; van der Heijden and Landman, 2008b; Liebhart et al., 2008).

Biology of *Histomonas meleagridis*

H. meleagridis is a unicellular parasite belonging to the phylum Parabasalia, class Tritrichomonadea, order Tritrichomonadida, and family *Dientamoebidae* (Cepicka et al., 2010; Lollis et al., 2011). Interestingly, the morphology can change between flagellated and amoeboid forms depending on location within the cecae or liver, respectively, with an average histomonad size of 10-14µm (Tyzzer, 1920; Bayon and Bishop, 1937; Bishop, 1938; Joyner et al., 1963; Hess and McDougald, 2013). *H. meleagridis* typically exhibits a single-flagellated form within the cecal lumen, but this flagellum is lost upon mucosal invasion with the development of pseudopods (Long et al., 1987). *H. wenrichi* (alternatively *Parahistomonas wenrichi*), a non-pathogenic but separate species, appears as 4-flagellated or amoeboid in form with a larger size

of 20-30 µm (Lund, 1963; Honigberg and Bennett, 1971; Fine, 1975; McDougald, 2005). *H. meleagridis* reproduce via binary fission; lacking mitochondria, these protozoa rely on hydrogenosomes as modified organelles for energy metabolism (Tyzzer, 1919, 1920; Mielewczik et al., 2008; Bleyen et al., 2010).

Early *in vitro* work indicated histomonads can be grown at temperatures of 36.5-37°C but not when reduced to 18-22°C for 48h or 5°C for 24h, suggesting that environmental survival of protozoa shed from infected birds is not likely to be culpable in mass infectivity (Bishop, 1938). Currently, *in vitro* propagation occurs anaerobically at 40-41°C with a Medium 199-based cell culture and bacterial co-culture to simulate the body temperature and environment of a healthy turkey (Lesser, 1960a; Bleyen et al., 2010). Dwyer's medium comprised of Medium 199, chick embryo extract, horse sera, and rice powder has been utilized, although other cell culture media such as L-15, MEM, or RPMI have been substituted effectively for Medium 199 (Hauck et al., 2010). Modified Dwyer's medium, which removes the chick embryo extract and increases rice powder from 0.096% (w/v) to 0.8% (w/v), improved histomonad growth following revival of aliquots from liquid nitrogen and serial passage (van der Heijden and Landman, 2007). Further increasing rice powder from 0.8% (w/v) to 4-8% (w/v) resulted in a nearly 10-fold growth increase, but this was not sustained longer than two days as the remaining nutrients became exhausted (van der Heijden et al., 2005). Cholesterol supplementation has improved *H. meleagridis* growth *in vitro,* even in the absence of serum, which is typically required for adequate growth (Gruber et al., 2018). Chute and Chute (1969) cryogenically preserved *H. meleagridis* isolates in combination with 8% dimethylsulfoxide for up to 345d and demonstrated viability of infection to birds following thaw. Honigberg and Dwyer (1969) demonstrated that

either 5% or 10% dimethylsulfoxide effectively preserved the protozoa in cryogenic storage as observed after seven weeks; therefore, isolates could be maintained for future studies.

Field isolates of *H. meleagridis* can be cultivated from infected carcasses, particularly cecal samples, if shipment to a laboratory occurs soon after bird mortality with greater recovery potential if temperatures are maintained above 30°C (Gerhold et al., 2010). *In vitro* growth from cecal samples can usually be confirmed between 1-4 days after inoculation into culture media; intracloacal inoculation back into live birds can be used to further diagnose *H. meleagridis* as the original cause of infection in field outbreaks (McDougald and Galloway, 1973). Histomonads have also been isolated effectively from liver lesions, but *in vitro* propagation attempts in absence of bacteria have been unsuccessful (Bayon and Bishop, 1937; McDougald and Galloway, 1973; Hirsch, 1975; McDougald, 2005). Attempts to culture the protozoa in absence of live bacteria and serum were achieved with difficulty, but supplementation of palmitic acid or cholesterol was required along with antibiotic-killed bacteria and hamster liver extract; however, these results have not been easily replicated (Lesser, 1960a,b, 1961, 1963). *In vitro* growth of *H. meleagridis* was better sustained with undefined populations of turkey cecal bacteria than with mixed chicken cecal bacteria (Lesser, 1964b). Moreover, histomonads have been grown with supplementation of single species of bacteria and monoxenic cultures have been established (Lesser 1964a; Ganas et al., 2012).

Pathogenesis

After parasitizing and degrading the cecal tissue, histomonads migrate to the liver via hepatic portal blood; the resulting pathognomonic lesions are exhibited as target-like liver lesions and caseous cecal cores (Harrison Jr. and Hansen, 1950; Berks and Neal, 1952; Bleyen et al., 2010). Histomonads have been observed in the bursa of Fabricius of 6-week-old commercial

chickens diagnosed with histomoniasis, further implicating the intracloacal route for natural infection (Cortes et al., 2004). Although less common, *H. meleagridis* has also been shown to infect the brain, heart, lungs, kidneys, and spleen (Levine, 1947; Sentíes-Cué et al., 2009; Spencer, 2010; Lotfi et al., 2014). Turkeys are especially vulnerable to histomoniasis, and chickens are less susceptible but function to serve as reservoirs (Curtice, 1907). Cloacal transmission seems less important to chickens than turkeys for transfer of histomoniasis, as horizontal transmission did not occur in the absence of vectors and was not exacerbated with *Eimeria adenoeides* challenge, which is not surprising as this *Eimeria* spp. is turkey-specific (Hu et al., 2006). While cloacal drinking is a well-known occurrence in chickens and turkeys, species differences in horizontal transmission could result from higher litter moisture and huddling behavior in turkeys than chickens, allowing greater survival and subsequent transmission of *H. meleagridis* in the absence of vectors (Sorvari et al., 1977; Hu et al., 2006). Mortalities in turkey flocks can reach 80-100%; organic farms co-rearing turkeys and broilers have struggled with series of outbreaks with broiler and turkey mortalities reaching 100% and 67.2%, respectively, possibly due to co-infection with *Eimeria* spp. (Grabensteiner et al., 2007; Bleyen et al., 2010; Popp et al., 2011). Susceptibility of different poultry species and genetic lines has only been evaluated briefly, but infection incidence and severity do appear different (Al-Khateeb and Hansen, 1973, 1974; Chute et al., 1976, AbdulRahmen and Hafez, 2009; Lotfi et al., 2014). In chickens, sex-related variations and environmental differences have influenced intestinal structure and function; therefore, it seems reasonable that these differences could factor into the incidence and severity of histomoniasis (Metzler-Zebeli et al., 2018). In addition to age and genetic line of poultry, variations in mortality rate and lesion severity could result from strain-specific differences in virulence of *H. meleagridis* or exposure dose (Lund 1955; Hu et al., 2004;

Hess et al., 2006). Interestingly, recent research has indicated that *H. meleagridis*-infection and replication are similar regardless of chicken genetic line, further suggesting that chickens may be asymptomatic or sub-clinically infected but not actually resistant to infection (Daş et al., 2021). A virulent clonal strain of *H. meleagridis* induced similar mortality and pathology in turkeys regardless of age, sex, or dose (Liebhart et al., 2008). A low dose of 3,162 histomonads induced 100% mortality to British United turkeys (**BUT-Big6**) by 2-weeks post-infection (van der Heijden and Landman, 2008b). Three different genetic lines of turkeys showed similar susceptibility to histomoniasis, although wild Canadian turkeys exhibited higher mortality rates and lower liver lesions than BUT-Big6 or Kelly-Bronze lines (AbdulRahmen and Hafez, 2009). Concurrent infection with *E. tenella* can aggravate development of histomoniasis in broiler chickens, specifically increasing liver lesions (McDougald and Hu, 2001). Conversely, turkeys co-infected with *E. adenoeides* and *H. meleagridis* resulted in significantly reduced cases of histomoniasis (McDougald and Fuller, 2005). The dosage and timing of *Eimeria* vaccination of chickens will influence the severity of aggravation due to histomoniasis, although further coinfection studies are necessary to conclude effects of combined pathogens to severity in chickens and turkeys (Chadwick et al., 2020).

Bradley and Reid (1966) inoculated gnotobiotic (bacteria-free) turkeys with *H. meleagridis* in combination with either *Escherichia coli, Bacillus subtilis,* or *Clostridium perfringens* and suggested that a combination of the protozoa and bacteria populations were required to initiate histomoniasis. Incidence of *H. meleagridis*-infection in gnotobiotic chickens and turkeys increased when concurrently challenged with a mixture of *E. coli* and *C. perfringens,* whereas histomoniasis was lessened with administration of a single bacteria species (Springer et al., 1970). Healthy turkey cecae contain predominantly (> 50%) anaerobic *Lactobacillus* spp. and

relatively low (< 1%) coliforms and *Enterococcus* spp. (Harrison Jr. and Hansen, 1950). *Salmonella typhimurium, E. coli,* and *H. meleagridis* infections have been found concurrently in broiler chicken flocks (Ganapathy et al., 2000). Cultures of *H. meleagridis* were identified to favor obligate anaerobes of the *Clostridiaceae* family, aerotolerant anaerobes of the *Bacteriodaceae* family, or facultative to obligate anaerobes of the *Baccillaceae* family (Klodnicki et al., 2013). The Proteobacteria phylum increased in relative abundance in birds with severe histomoniasis, but *E. coli* populations were maintained at the same level in turkeys regardless of the level of gut inflammation (Abdelhamid et al., 2021). *E. coli* mutually benefited histomonad growth *in vitro* and increased cecal involvement *in vivo* (Ganas et al., 2012; Abdelhamid et al., 2020). Co-infection of laying chickens with *H. meleagridis* and *E. coli* produced severe dysbiosis, increased microscopic lesions, and enhanced colonization of the cecal tissue (Abdelhamid et al., 2020). Recently however, the gastrointestinal pathology and *E. coli* load was not associated with severity of histomoniasis, while microbiota composition and dysbiosis were directly attributed with severity of inflammation (Abdelhamid et al., 2021). In addition to providing direct nutrients, bacteria appear to serve a mutualistic role with the protozoa by supplying essential proteins and metabolites during replication, as well as regulating *in vitro* environmental conditions (Bilic and Hess, 2020).

Histomoniasis has been produced in experimental settings with the intracloacal inoculation of infected liver or cecal tissues or with a suspension of *in vitro* cultivated *H. meleagridis* (Tyzzer and Collier, 1925; Berks and Neal, 1952). Variations in host resistance, challenge dose, pathogen virulence, and frequency of exposure are some factors influencing disease severity (Collett, 2013). A case reproductive rate of 8.4 was estimated in a horizontal transmission study and turkeys recovered from histomoniasis were shown to remain infectious

for 5.7 days after recovery (Landman et al., 2015). Retrospective data analysis implicated an increased relative risk of male commercial turkey grow-out flocks to contracting histomoniasis when located within one mile of a broiler breeder flock (Jones et al., 2020). Lund (1955) reported positive correlation between infective dose (10^2-10^5 histomonads/bird) and mortality; conversely, a low dose of 10 histomonads induced 100% mortality in turkeys (Liebhart et al., 2010). Liebhart and Hess (2009) administered a virulent isolate via oral administration to 1-d-old turkeys with successful initiation of histomoniasis, but the oral route of infection remains controversial. Presumably, histomonads cannot survive the low pH in the ventriculus unless protected by a vector such as *Heterakis* spp. or with a neutral to alkaline pH in the gastrointestinal tract to allow survival of the protozoa (Reid, 1967; Spencer, 2010). *H. meleagridis* has been shown to persist for up to 9h in non-chlorinated water and fecal droppings and for up to 6h on materials such as feathers and feed (Lotfi et al., 2012). Histomonads are fragile when shed unprotected into the environment, but not much is known about methods for disinfection (Daugschies et al., 2013). Consequently, the importance of *H. meleagridis*-infected water as a possible source of involvement for cloacal transmission has been suggested as an important risk factor (Lotfi et al., 2012; Badparva and Kheirandish, 2017). Although previously disregarded to form resistant structures, cyst-like forms have recently been described *in vitro,* but the importance of these structures to pathogenesis is not yet understood (Munsch et al., 2009a,b; Zaragatzki et al., 2010a,b; Daugschies et al., 2013).

Tyzzer (1934) indicated the survival of *H. meleagridis* within heterakid ova for two months during winter temperatures. Heterakids can thereby serve as primary transmitters for initial introduction of disease due to infected ova withstanding environmental conditions for long durations (Tyzzer and Fabyan, 1922; Tyzzer, 1932; Connell, 1950; McDougald, 1998).

Histomonads are released when the infected *Heterakis* spp. larvae hatch in poultry (Lee, 1969; Waters et al., 1994). Lifetime fecundity of *H. gallinarum* is regulated by both inverse density and density-dependent mechanisms (Daş and Gauly, 2014). *Alphitobius diaperinus* (darkling beetle or lesser mealworm) function as environmental contaminants for accidental introduction of *H. meleagridis* into a flock rather than serving as a primary transmitter like *Heterakis* spp. (Huber et al., 2007). The importance of *A. diaperinus* as reservoirs is uncertain due to the persistence of *H. gallinarum* and *H. meleagridis* DNA within dead beetles and litter from depopulated houses even after long time periods (Huber et al., 2007; Beckmann et al., 2021). *Lumbricus* spp. (earthworms) are not required for completion of the heterakid larvae or histomonad life cycles, serving rather as paratenic hosts and mechanical vectors if consumed by poultry (Lund et al., 1966b; Friend et al., 1999; Cupo and Beckstead, 2019a,b).

Phylogenetic and Molecular Characterizations

Indirect and blocking ELISAs have been developed for detection of *H. meleagridis* but have not yet been rigorously tested for specificity or cross-reactivity to other related protozoa commonly found in field isolates (Windisch and Hess, 2009; van der Heijden et al., 2010). Other parasites such as *Tetratrichomonas gallinarum* and *Blastocystis* spp. may be present in field outbreaks and potentially confused with *H. meleagridis* (Tyzzer, 1919; Sulejmanovic et al., 2019). PCR has been successfully utilized to detect *H. meleagridis* in samples and infected birds as well as to differentiate from *T. gallinarum* and *Blastocystis* spp. (Hafez et al., 2005; Huber et al., 2005; Grabensteiner and Hess, 2006; Reis et al., 2009; Liu et al., 2011; Landman et al., 2015). DNA presence does not necessarily indicate active infection; therefore, diagnosis of histomoniasis is recommended to include microscopy to confirm presence of the protozoa (Sulejmanovic et al., 2019; Beckmann et al., 2021; Daş et al., 2021). *H. meleagridis* conforms

similarly to other trichomonad parasites in structure and division; close phylogenetic relationships to *D. fragilis* and *Tritrichomonas foetus* were identified based on gene sequencing analysis of β-tubulin and small subunit rRNA genes (Honigberg and Bennett, 1971; Gerbod et al., 2001; Hauck and Hafez, 2009; Lynn and Beckstead, 2012). Analysis of 18S rRNA and internal transcribed spacer (**ITS**)-1 sequences has demonstrated a clear distinction between *H. meleagridis* isolates and other trichomonads such as *D. fragilis* (Munsch et al., 2009b). Genetic sequencing and phylogenetic analysis of 5.8S rRNA and the flanking ITS-1 and ITS-2 regions revealed marked genetic diversity of *H. meleagridis* isolates (Lollis et al., 2011).

Analysis of 18S rRNA, α-actinin1, and *rpb1* genetic loci revealed two different phylogenetic clusters of *H. meleagridis* isolates in Europe and further identified two genotypes; in contrast, probed sequence and partial 18S rRNA have displayed genetic similarity of six purportedly different isolates (Bilic et al., 2014; Landman et al., 2019). Biological relevance and incidence of these two distinct genotypes have not yet been elucidated (Popp et al., 2011; Bilic et al., 2014). Using micromanipulation, clonal cultures of *H. meleagridis* and other protozoa have been established which enable researchers to better understand pathogenicity, morphology, and genetic differences between species (Hess et al., 2006). Mono-eukaryotic cultures have also been established from mixed field samples containing *H. meleagridis, T. gallinarum*, and *Blastocystis* spp., and these monocultures could potentially better mimic field strains as opposed to clonal cultures while removing the interference of other protozoa (Pham et al., 2013b). Thirty-seven unique surface and intracellular antigens were identified through analysis of a cDNA library generated from a monoculture and screened against polyclonal anti-*H. meleagridis* rabbit sera (Bilic et al., 2009). A cDNA library generated from a non-clonal culture resulted in the identification of 3,425 putative genes belonging to *H. meleagridis* (Klodnicki et al., 2013).

Hydrogenosome protein coding sequences and three different α-actinin proteins (α-actinin1, α-actinin2, α-actinin3) were identified and shown to be immunogenic to turkeys and chickens (Bilic et al., 2009; Leberl et al., 2010). Humoral immune response to *H. meleagridis* α-actinin1 and α-actinin3 was higher and induced sooner in specific-pathogen-free layer-type chickens as compared to meat-type chickens (Lotfi et al., 2014). Shotgun proteomics has been utilized to compare virulent and attenuated mono-eukaryotic monoxenic *H. meleagridis*; cysteine proteases were the predominant lytic molecules in the virulent exoproteome as compared to the attenuated isolate (Mazumdar et al., 2019; Wei et al., 2020).

Chemotherapy and Prophylaxis

Tyzzer (1923) tested several trivalent arsenicals (including arsenious acid, atoxyl, neoarsphenamine, and tryparsamide) as chemotherapeutics against histomoniasis, but with inconsistent results. Pentavalent arsenicals such as nitarsone (4-nitrophenyl-arsonic acid; Histostat-50^{TM}), carbasone (4-carbamylamino-phenylarsonic acid), and roxarsone (3-nitro-phenylarsonic acid) offered less toxicity concerns than the trivalent compounds for poultry but also exhibited a narrower chemotherapeutic index (McDougald, 1979, 2005). Carbasone was highly effective in prevention of a field isolate of *H. meleagridis* (McDougald, 1979). Nitroimidazole compounds (including dimetridazole, metronidazole, ornidazole, and tinidazole) were effective *in vitro* at concentrations of ≥ 10 µg/mL and *in vivo* at 200ppm in the feed, but were toxic if overdosed (Lindquist et al., 1962; McGuire et al., 1964; Mitrovic et al., 1968; Doneley, 2004; Hu and McDougald, 2004). Dimetridazole was highly effective for treating histomoniasis and was rapidly metabolized and eliminated by turkeys with no detectable tissue residue (< 0.02ppm) following 3d post-administration (Law et al., 1963). Enheptin-T (2-amino 5-nitrothiazole) was used at 0.05% in the feed with effective prophylaxis against histomoniasis, but

average weights of turkeys were suppressed in direct proportion to drug inclusion (Seeger et al., 1950). Nithiazide [1-ethyl-3-(5-nitro-2-thiazolyl) urea] was an effective therapeutic in turkeys when administered at 3d post-infection and was somewhat better tolerated than enheptin-T (Cuckler and Malanga, 1956). Benzimidazole compounds, such as albendazole and fenbendazole, were effective *in vivo* when provided prophylactically and mechanism of action was attributed to damage of heterakid larvae or histomonads residing in the cecal lumen (Hegngi et al., 1999).

Research with *H. meleagridis* waned around the 1970s, partly due to effective antihistomonal compounds alleviating disease outbreak, but research increased again in the early 2000s following the removal of effective drugs and feed additives from poultry production in the European Union and the United States which resulted in a re-emergence of disease due to lack of treatment options (Spencer, 2010; Hauck and Hafez, 2013; Regmi et al., 2016). The nitroimidazoles and nitrofurans were banned in the United States in 1987 and 1991, respectively (Clark and Baily, 2015; Jones et al., 2020). Nitarsone was the last-remaining prophylactic drug for the treatment of histomoniasis until the voluntary removal from the U.S. market in late 2015 because of consumer carcinogenic concerns (Grabensteiner et al., 2008; Clark and Bailey, 2015; Regmi et al., 2016; Hafez and Shehata, 2021). Despite occasional success with antihistomonal candidates *in vitro,* subsequent *in vivo* evaluations have failed to conclusively prevent or treat histomoniasis (Grabensteiner et al., 2008; Thøfner et al., 2012; Clark and Kimminau, 2017; Barros et al., 2020; Beer et al., 2020a,b). Boric acid, deoxycholic acid, sodium chlorate, and sodium nitrate are among just a few chemoprophylaxis candidates with antimicrobial or antifungal properties that have been recently tested with *in vitro* evaluation showing significant antihistomonal properties but with no effective prophylaxis *in vivo* (Barros et al., 2020; Beer et

al., 2020a,b). The antibacterial properties of some candidate antihistomonal compounds are known to impact effectiveness *in vitro*, but histomonads can survive 48h after destruction of xenic bacterial populations (Berks and Neal, 1952; Callait et al., 2002; McDougald, 2005; Grabensteiner et al., 2007). Further complicating the problem, *H. meleagridis* isolates have varied in susceptibility to candidate compounds *in vitro* and *in vivo* (Berks and Neal, 1952; Grabensteiner et al., 2007; van der Heijden and Landman, 2008a,b; Thøfner et al., 2012). Drug resistance was not previously known to occur with *H. meleagridis*; however, some isolates have developed partial resistance to nitarsone and metronidazole, further emphasizing the necessity of new solutions to prevent histomoniasis and supporting the likelihood of different populations of protozoa and corresponding drug-susceptibility (Long et al., 1987; Abraham et al., 2014; Umar et al., 2016). A comparatively reliable compound to replace the previously used dimetridazole and nitarsone drugs is critically needed, but mitigation of histomoniasis remains elusive and inconsistent (McDougald, 2005; Hess et al., 2015). Adaptations likely need to occur for concentration and administration of compounds for *in vivo* protection, but effective *in vitro* evaluation is the initial key step to determining whether to devote resources towards a live animal study (Zenner et al., 2003; Grabensteiner et al., 2008). *In vitro* methods are useful for initially evaluating candidate chemoprophylactics, but emphasis is placed on *in vivo* evaluation against more than one isolate of *H. meleagridis* before concluding effectiveness.

In absence of approved effective drugs or vaccines for histomoniasis, the prevailing measure for disease prevention is to minimize exposure to *H. meleagridis.* Worm treatment programs and flock management to prevent *H. gallinarum* and accessory hosts such as earthworms and darkling beetles will help to reduce histomoniasis incidence, since histomonads cannot survive for long durations if shed unprotected directly into the environment (Tyzzer and

Collier, 1925; McDougald, 2005). Limiting exposure to mechanical vectors such as rodents, insects, or contaminated litter is critical to reducing potential contamination. Prompt removal of infected birds and utilization of migration barriers are additional control strategies to prevent rapid horizontal transmission in turkey flocks, while de-worming options would be more appropriate to control histomoniasis in chickens based on the differences in bird-to-bird transmission (Fine et al., 1975; Hu and McDougald, 2003; Hu et al., 2006).

Phytochemicals for Prevention of Histomoniasis

Phytogenic compounds offer great potential as alternatives to mitigate histomoniasis and improve poultry health since the exclusion of antibiotics (Puvača et al., 2020). Herbal products have received much interest for antihistomonal properties, but *in vitro* results are often encouraging while *in vivo* trials yield unsuccessful protection (van der Heijden and Landman, 2008a,b; Thøfner et al., 2012). Protophyt™ and Natustat™, plant-derived proprietary combinations of herbal extracts, were successful antihistomonal products *in vitro* but generated only limited success in field trials when provided prophylactically (Duffy et al., 2004, 2005; Hafez and Hauck, 2006; van der Heijden and Landman, 2008a,b). Further complicating the search and development of antihistomonal drugs, different monoculture strains of *H. meleagridis* have exhibited varied susceptibilities to natural organic compounds (Grabensteiner et al., 2007). Two proprietary blends of plant extract products containing unspecified amounts of *Capsicum* essential oils exhibited antihistomonal and antibacterial effects after only 48h *in vitro*; furthermore, mode of action was suggested as cell membrane disruption directly on the histomonads rather than attributed to indirect effects of antibacterial reduction, but *in vivo* studies have not yet been conducted (Hauck and Hafez, 2007). Recently, a dietary supplement (adiCox^{SOL}PF) comprised of a proprietary mixture of herbal extracts was effective

prophylactically and therapeutically against histomoniasis in a turkey breeder flock (Tykałowski et al., 2021). With increasing demand for organic-raised poultry, naturally derived plant compounds offer a certain attraction as they could potentially be utilized in both organic and traditional production facilities. Plant-based compounds are often relatively cheap to produce, leading to a greater likelihood for industry application (Zenner et al., 2003).

Anti-Protozoal Effects of Quinine

Quinine, an alkaloid obtained from *Cinchona* tree bark, was the first successful antimalarial drug for humans (Uzor, 2020). *Cinchona* alkaloids (including quinine, quinidine, cinchonine, and cinchonidine) exhibited antimalarial activity when administered orally to *Plasmodium iophurae*-infected Pekin ducks (Seeler et al., 1943). Quinine was effective for 12h post-injection to ducks with rapid blood clearance following 8h, but the exact mode of action whereby quinine invoked plasmocidal effects was inconclusive (Waletzky and Brown, 1943). Quinine was later determined to inhibit reproduction of *Plasmodium* spp., the etiological agent of malaria, effectively reducing infections in poultry (Taliaferro et al., 1944; Taliaferro and Taliaferro, 1948). The degradation rate of quinine varies between poultry species, with chickens and ducks exhibiting qualitatively similar degradation products but with the duck having quantitatively more than the chicken (Dearborn and Marshall, 1945). Quinine acted directly upon the carbohydrate metabolism of *P. gallinaceum* with irreversible inhibitory effects to the parasite's metabolism observed within 24h following intravenous injection of 20 mg quinine/kg BW to chickens (Moulder, 1948). Quinine specifically induced the following changes to *P. gallinaceum* metabolism: increased rate of glucose use, decreased rate of pyruvate use, lowered ratio of oxygen to glucose, and lowered oxygen uptake - presumably due to aerobic phase inhibition by quinine via the pyruvate oxidation step in the TCA cycle (Moulder, 1948).

High concentrations of quinine can produce feed avoidance and malaise in birds (Alcock, 1970). Chickens can detect and avoid bitter substances such as quinine, and this taste detection can be a method for preventing potential toxicity (Skelhorn and Rowe, 2010; Roura et al., 2013; Niknafs and Roura, 2018). Layer chickens and broiler chickens differed in sensitivity to quinine with detection levels as low as 2.0 mM/L and 0.5 mM/L, respectively (Kudo et al., 2010). The bitter taste of quinine has been utilized to reduce feather pecking and aggression in laying hens; a 4% quinine sulfate solution induced learned behavior with no long-lasting avoidance while maintaining normal preening behavior (Harlander-Matauschek et al., 2009). Application of 2% and 4% solutions of quinine sulfate effectively reduced feather plucking, pecking, and consumption, further suggesting that sensitivity to this bitter alkaloid could occur with detection by taste buds in the oral cavity or negative gastrointestinal consequences leading to learned behavior (Harlander-Matauschek and Rodenburg, 2011). Dietary concentrations of 0.5% or 1% quinine sulfate decreased feed intake in chicks and caused rejection of these diets when birds were allowed free access of two dietary choices of quinine-supplemented or basal diets (Ueda et al., 2002). Interestingly, feed intake of chicks was significantly reduced with dietary levels higher than 0.2% quinine but not at levels less than 0.1% (Ueda et al., 2002, 2005).

Quinine has been successfully utilized to combat malaria, leading early researchers to postulate its potential for treating histomoniasis; however, researchers hypothesized that an antihistomonal compound would have to be active more than just locally within the intestines because *H. meleagridis* embeds within the cecal lining and migrates to hepatic tissue (Smith, 1895; Tyzzer and Fabyan, 1922). Tyzzer (1923) observed no reduction in histomoniasis following injection of unspecified levels of quinine into the veins or muscles of turkeys. Delaplane and Stuart (1935) reported quinine sulfate to be ineffective against *H. meleagridis*-

infection but did not specify dose or route of administration. Farmer (1950) injected 0.1 mL of 10% quinine iodobismuthate with no apparent protection against histomoniasis. Tyzzer and Fabyan (1922) suggested a possible reason for the failure of compounds utilized in human amebic infections to protect poultry from histomoniasis could be due to histomonads exhibiting a predominantly flagellated form rather than solely an amoebic form, leading to some products being amebicidal but not antihistomonal. Other antimalarial compounds such as the herb *Artemisia annua* and plant extracts have been tested against *H. meleagridis* with limited success *in vitro* but no protection transferred to birds when tested *in vivo* (Hauck and Hafez, 2007; Thøfner et al., 2012). Despite the success with quinine as an antiprotozoal, no recent studies have been conducted to evaluate quinine against histomoniasis; hence, the rationale for completing *in vitro* and *in vivo* evaluations as described in Chapter 3 of this dissertation.

Immune Response to *H. meleagridis* Infection

Turkeys and chickens recovered from *H. meleagridis*-infection have shown a degree of natural resistance, although both species may retain histomonads sub-clinically and thereby serve as carriers (Tyzzer et al., 1921; Joyner et al., 1963). Joyner (1963) administered 0.05% dimetridazole in the water to *H. meleagridis*-infected turkeys, and the recovered turkeys were resistant to re-infection which suggested a level of acquired immunity. Protective immunity was observed in birds that recovered from histomoniasis and were then subsequently re-infected with *H. meleagridis*, but further attempts with immunization have been inconsistent (Tyzzer et al., 1921; Tyzzer and Fabyan, 1922; Tyzzer, 1932, 1934, 1936; Clarkson, 1963). Sera recovered from immune birds failed to confer robust protection to histomoniasis when injected into the peritoneum of naïve poultry that were subsequently challenged intracloacally with *H. meleagridis*-infected liver homogenate (Clarkson, 1963, 1966; Cuckler, 1970). Passive immunity

(via peritoneal injection of antisera) or active immunity (via intramuscular or intraperitoneal injection of lysed clonal *H. meleagridis*) failed to protect against wild-type challenge (Hess et al., 2008; Bleyen et al., 2009). Turkeys surviving *H. meleagridis*-infection have exhibited resistance to re-infection while still maintaining populations of the protozoa within the ceca (Cuckler, 1970). Humoral immunity does not seem to be the primary component of protective immunity to histomoniasis, although antibodies may work in combination with local immunity initiated by leukocytes in the cecae (Cuckler, 1970).

Clarkson (1966) reported that turkeys exhibited decreased albumin and elevated globulin concentrations at 12d post-infection as compared to the non-challenged controls. Similarly, albumin concentrations greatly decreased by 9d post-infection in chickens subjected to *H. meleagridis*-infection, with normal levels of albumin and globulin fractions restored by 12d post-infection, suggesting disease recovery (McDougald and Hansen, 1969). The immune barrier in purportedly histomoniasis-resistant chickens was suggested to be limited to cecal epithelial tissue as *H. gallinarum* could disrupt and overcome any developed immunity (Lund, 1955). Natural and experimental *H. meleagridis*-infection produced antibodies in both chickens and turkeys but transfer of antibodies to naïve birds did not successfully confer protection (Clarkson, 1963; Bleyen et al., 2009). Subsequently, Clarkson (1963) suggested that antibody production alone was not a good indicator of histomoniasis recovery or immunity to re-infection. Antibody titers of passively immunized birds were increased compared to pre-immunized groups; however, no protection was induced against intracloacal infection with 3×10^5 *H. meleagridis*, possibly due to the experimental challenge dose not accurately mimicking a natural challenge, antibodies levels lower than needed for protection, or more likely, serum antibodies not primarily responsible for protection against *H. meleagridis*-infection (Bleyen et al., 2009). IgA levels have been shown to

increase throughout the intestine, while IgG levels particularly increased in the cecae following infection with an established clonal *H. meleagridis* isolate (Windisch and Hess, 2010).

Heterophils begin to accumulate around histomonads following initial infection, but the protozoa secrete tissue-degrading enzymes to phagocytose leukocytes (Bleyen et al., 2010). Total numbers of heterophils increase throughout the body as *H. meleagridis* migrates to parasitize other tissues; other leukocytes involved include macrophages, giant cells, and plasma cells (Levine, 1947; McGuire and Cavett, 1952; Reis et al., 2009; Bleyen et al., 2010). Once the histomonads invade the cecal submucosa or enter the portal blood, degenerating *H. meleagridis* can be observed within the gut-associated lymphoid tissue (Bleyen et al., 2010). Plasma levels of glutamic oxaloacetic transaminase can indicate cellular damage, and this enzyme can increase in turkeys with liver and cecal damage from histomoniasis (Al-Khateeb and Hansen, 1973, 1974). CD4+ and CD8α+ T cells have been implicated in immune response to histomoniasis (Powell et al., 2009; Mitra et al., 2017; Lagler et al., 2019). Recently, populations of CD4+, CD8α+ and non-CD4+CD8α+ T-cells in the liver and spleen of turkeys were induced following administration of attenuated *H. meleagridis* as a putative vaccine and subsequent virulent infection (Lagler et al., 2021). Comparative study of chickens and turkeys indicated that vaccination with a monoxenic, clonal culture of live-attenuated *H. meleagridis* resulted in higher systemic immune response in turkeys as compared to chickens, with increased levels of IFN-γ producing CD4+ T cells confirmed in the spleens of infected chickens as compared to turkeys (Lagler et al., 2021). Increased T-helper cell type-1 (**Th1**) and type-2 (**Th2**) cytokine responses of IFN-γ and IL-13 occurred in chickens that were co-infected with *H. gallinarum* and *H. meleagridis* (Schwarz et al., 2011). Chickens developed a stronger pro-inflammatory innate immune response than turkeys, along with higher antibody levels, with specific increase in Th2

response in cecal and liver tissues to mitigate infection (Powell et al., 2009). Despite the extracellular nature of *H. meleagridis* which would be expected to stimulate differentiation of Th2 cells, immune response to this pathogen was suggested to be dominated by Th1 rather than Th2 cells (Schwarz et al., 2011; Sharma and Rautenschlein, 2013; Lagler et al, 2019, 2021). Turkeys appeared to have a delayed and uncontrolled immune response as compared to chickens when infected with *H. meleagridis,* allowing greater tissue destruction and ultimately higher mortality in turkeys (Mitra et al., 2018).

Attempted Vaccination with Attenuated Isolates

Tyzzer (1934) evaluated avirulent field strains of *H. meleagridis* for immunization against histomoniasis, but inoculation of turkeys was required at a young age and constant reinfection was necessary to maintain a level of effective protection. Partial protection was conferred with an attenuated isolate against subsequent cloacal challenge with a virulent isolate; however, administration of histomonads as an immunization incorporated into *Heterakis* spp. ova and likewise challenged did not satisfactorily confer protection (Lund, 1959; Lund et al., 1966a). The resulting conclusion was that the low-virulent histomonads were not introduced in sufficient numbers via heterakid ova to successfully initiate immune response to protect against virulent challenge (Lund, 1959; Lund et al., 1966a). Tyzzer (1934, 1936) reported attenuation of *H. meleagridis* following repeated passage *in vitro* but attempts with immunization did not produce consistent protection. An isolate repeatedly passaged *in vitro* for six years resulted in loss of immunizing ability to chickens and turkeys (Lund et al., 1966a). Further study observed a steady decline of immunizing ability of attenuated histomonads after 730, 766, and 1000 passages *in vitro* (Lund et al., 1967). Specifically, passage 1000 was nonpathogenic and had lost

nearly all ability to confer protection to either chickens or turkeys against virulent challenge (Lund et al., 1967).

Long-term serial passaging *in vitro* places selective pressures on *H. meleagridis* and co-cultured bacterial populations. Freshly obtained field samples of histomonads could not grow in the limited bacterial populations of attenuated culture media; similarly, the attenuated protozoa were unable to survive with the field isolates of cecal bacteria (Lund et al., 1966a). Importantly, *in vitro* attenuation of *H. meleagridis* occurred independently of bacterial populations in culture media (Ganas et al., 2012). *In vitro* growth of *H. meleagridis* Hm-L1 strain at 41.5°C for nine weeks resulted in low pathogenicity while histomonads stored in liquid nitrogen maintained their original virulence (Dwyer and Honigberg, 1969, 1970). Serial *in vivo* passaging of the Hm-L1 attenuated strain from chicken-to-chicken or turkey-to-turkey restored the strain to original virulence (Dwyer and Honigberg, 1969, 1970). Differences in virulence have been found within *H. meleagridis* isolates obtained from different geographical location, in addition to varied loss of pathogenicity following repeated passaging (Wei et al., 2020). Furthermore, subpopulations of serially passaged monocultures originating from the same parental isolate have shown marked difference in virulence, supporting the idea of genetic mutation through repeated serial passaging *in vitro* (Wei et al., 2020). Long-term passaging *in vitro* (>290 serial passages) resulted in a phenotype shift towards greater tenacity of histomonad survival at lower temperatures and improved growth rates (Gruber et al., 2017). Gross lesion scoring and histology samples have demonstrated the lowered pathogenicity and reduced ability of attenuated isolates to invade host tissues (Liebhart et al., 2011). After 295 serial passages *in vitro*, an avirulent strain of *H. meleagridis* parasitized only the cecal region with no translocation to other tissue in chickens or

turkeys, while a virulent strain could be identified in cecal, hepatic, and lung tissues (Liebhart et al., 2011).

Vaccination attempts for histomoniasis have yielded variable but limited success in controlled experimental conditions, and a histomoniasis vaccine has not been developed for commercial application (Hess et al., 2008; EFSA, 2013; McAllister, 2014; Liebhart et al., 2010, 2013, 2017; Mitra et al., 2018). A clonal *in vitro* attenuated strain of *H. meleagridis* administered cloacally as a vaccine at d14 protected turkeys that were subsequently challenged on d42 with a virulent strain; in-contact turkeys from the vaccination were also resistant to subsequent infection (Hess et al., 2008). Furthermore, birds that were administered an attenuated clonal strain as a vaccine were negative for *H. meleagridis* DNA in the liver (Hess et al., 2008). Oral administration of *in vitro* attenuated *H. meleagridis* to turkeys at day-of-hatch has protected against subsequent wild-type challenge with no adverse effects to performance data during vaccination phase; the oral route would be a preferable administration route for the poultry industry (Liebhart et al., 2010). Under experimental conditions, vaccination of layer chickens with attenuated histomonads prevented a drop in egg production upon virulent challenge and pathological histomoniasis lesions were also reduced (Liebhart et al., 2013). *In vivo* serial passaging five times in chickens and turkeys did not revert virulence to an *in vitro* attenuated strain, demonstrating stability and safety of attenuated histomonads as vaccine candidates (Sulejmanovic et al., 2013). An attenuated clonal strain (passage 295) induced cross-protective immunity in turkeys against subsequent challenge with heterologous virulent isolates; however, vaccination occurred at 1d-of-age and a booster vaccination occurred at d14, with challenge administration at 6-weeks-of-age (Sulejmanovic et al., 2016). Repeated intracloacal passaging of *H. meleagridis* in turkeys produced an isolate of low virulence which was successfully used to

induce protection against a virulent strain (Pham et al., 2013a). More research is necessary for histomoniasis vaccine development and to elucidate practical methods for industry application.

Research Objectives

The objectives of this dissertation were to investigate multiple methods for preventing or controlling histomoniasis in poultry. Specifically, this research evaluated a chemoprophylactic candidate (Chapter 3) or vaccination (Chapter 4) for mitigating histomoniasis in an experimental challenge model. Phytochemicals are of great interest as antihistomonal compounds, and quinine was evaluated *in vitro* and *in vivo* as a chemoprophylactic candidate. Additionally, live-attenuated *H. meleagridis* isolates were developed and evaluated as vaccine candidates for administration at different ages, routes, and doses in turkeys. The overall goal of this research was to address a critical need for histomoniasis prevention in the poultry industry.

REFERENCES

Abdelhamid, M. K, I. Rychlik, C. Hess, T. Hatfaludi, M. Crhanova, D. Karasova, J. Lagler, D. Liebhart, M. Hess, and S. Paudel. 2021. Typhlitis induced by *Histomonas meleagridis* affects relative but not the absolute *Escherechia coli* counts and invasion in the gut in turkeys. Vet. Res. 52:1-12.

Abdelhamid, M. K., N. M. Quijada, M. Dzieciol, T. Hatfaludi, I. Bilic, E. Selberherr, D. Liebhart, C. Hess, M. Hess, and S. Paudel. 2020. Co-infection of chicken layers with *Histomonas meleagridis* and avian pathogenic *Escherichia coli* is associated with dysbiosis, cecal colonization and translocation of the bacteria from the gut lumen. Front. Microbiol. 11:586437.

AbdulRahman, L., and H. M. Hafez. 2009. Susceptibility of different turkey lines to *Histomonas meleagridis* after experimental infection. Parasitol. Res. 105:113-116.

Abraham, M., L. R. McDougald, and R. B. Beckstead. 2014. Blackhead disease: Reduced sensitivity of *Histomonas meleagridis* to nitarsone *in vitro* and *in vivo*. 58:60-63.

Alcock, J., 1970. Punishment levels and the response of black-capped chickadees (*Parus atricapillus*) to three kinds of artificial seeds. Anim. Behav. 18:592–599.

Al-Khateeb, G. H., and M. F. Hansen. 1973. Plasma glutamic oxalacetic transaminase as related to liver lesions from histomoniasis in turkeys. Avian Dis. 17:269-273.

Al-Khateeb, G. H., and M. F. Hansen. 1974. Plasma enzymes as a measure of susceptibility of chickens and turkeys to infection with *Histomonas meleagridis.* Avian Dis. 18:507-514.

Armstrong, P.L., and L. R. McDougald. 2011. The infection of turkey poults with *Histomonas meleagridis* by contact with infected birds or contaminated cages. Avian Dis. 55:48-50.

Badparva, E., and F. Kheirandish. 2017. Epidemiology of pathogenic parasite *Histomonas meleagridis* in poultry in Lorestan province, western Iran. J. Parasit. Dis. 41:1040-1043.

Bayon, H. P., and A. Bishop. 1937. Cultivation of *Histomonas meleagridis* from the liver lesions of a hen. Nature. 139:370-371.

Beckmann, J. F., T. Dormitorio, S. O. Oladipupo, M. T. B. Terra, K. Lawrence, K. S. Macklin, and R. Hauck. 2021. *Heterakis gallinarum* and *Histomonas meleagridis* DNA persists in chicken houses years after depopulation. Vet. Parasitol. 298:109536.

Barros, T. L., L. C. Beer, G. Tellez, A. L. Fuller, B. M. Hargis, and C. N. Vuong. 2020. Research Note: Evaluation of dietary administration of sodium chlorate and sodium nitrate for *Histomonas meleagridis* prophylaxis in turkeys. Poult. Sci. 99:1983-1987.

Beer, L. C., J. D. Latorre, S. J. Rochell, X. Sun, G. Tellez, A. L. Fuller, B. M. Hargis, and C. N. Vuong. 2020a. Research note: Evaluation of deoxycholic acid for antihistomonal activity. Poult Sci. 99:3481-3486.

Beer, L. C., C. N. Vuong, T. L. Barros, J. D. Latorre, G. Tellez, A. L. Fuller, and B. M. Hargis. 2020b. Research Note: Evaluation of boric acid as a chemoprophylaxis candidate to prevent histomoniasis. Poult. Sci. 99:1978-1982.

Berks, G., and R. Neal. 1952. The effect of some drugs upon *Histomonas meleagridis in vitro*. Ann. Trop. Med. Parasitol. 46:68-71.

Bilic, I, and M. Hess. 2020. Interplay between *Histomonas meleagridis* and bacteria: Mutualistic or predator-prey? Trends Parasitol. 36:232-235.

Bilic, I., B. Jaskulska, R. Souillard, D. Liebhart, and M. Hess. 2014. Multi-locus typing of *Histomonas meleagridis* isolates demonstrates the existence of two different genotypes. PLoS One. 9:e92438.

Bilic, I., M. Leberl, and M. Hess. 2009. Identification and molecular characterization of numerous *Histomonas meleagridis* proteins using a cDNA library. Parasitology. 136:379-391.

Bishop, A. 1938. *Histomonas meleagridis* in domestic fowls (*Gallus gallus*). Cultivation and experimental infection. Parasitology. 30:181-194.

Bleyen, N., J. Mast, K. De Gussem, J. De Guseem, M. De Gussemand, and B. M. Godderis. 2010. *Histomonas meleagridis:* A new focus on a re-emerging protozoan parasite. Pages 1-47 in Veterinary Parasitology. G. V. LaMann. Nova Science Publishers, New York, NY.

Bleyen, N., E. Ons, M. De Gussem, and B. M. Goddeeris. 2009. Passive immunization against *Histomonas meleagridis* does not protect turkeys from an experimental infection. Avian Pathol. 38:71-76.

Bradley, R. E., and W. M. Reid. 1966. *Histomonas meleagridis* and several bacteria as agents of infectious enterohepatitis in gnotobiotic turkeys. Exp. Parasitol. 19:91-101.

Callait, M. P., C. Granier, C. Chauve, and L. Zenner. 2002. *In vitro* activity of therapeutic drugs against *Histomonas meleagridis* (Smith, 1895). Poult Sci. 81:1122-1127.

Cepicka, I., V. Hampl, and J. Kulda. 2010. Critical taxonomic revision of parabasalids with description of one new genus and three new species. Protistology 161:400-433.

Chadwick, E., R. Malheiros, E. Oviedo, H. A. C. Noboa, G. A. Q. Ospina, M. C. A. Wisaquillo, C. Sigmon, and R. Beckstead. 2020. Early infection with *Histomonas meleagridis* has limited effects on broiler breeder hens' growth and egg production and quality. Poult. Sci. 99:4242-4248.

Chute M. B., and A. M. Chute. 1969. Freeze preservation of *Histomonas meleagridis.* Poult. Sci. 48:2189-2191.

Chute A. M., E. E. Lund, and G. C. Wilkins. 1976. Comparative responses of white leghorn and New Hampshire chickens to experimental infections with *Histomonas meleagridis* and *Heterakis gallinarum*. Poult. Sci. 55:710-713.

Clarke, L. L., R. B. Beckstead, J. R. Hayes, and D. R. Rissi. 2017. Pathologic and molecular characterization of histomoniasis in peafowl (*Pavo cristatus*). J. Vet. Diagn. Invest. 29:237-241.

Clark, S., and A. Bailey. 2015. Turkey industry annual report – Current health and industry issues facing the US turkey industry. Proc. 119th Annual Meeting of the United States Animal Health Assoc. Providence, Rhode Island. Pg 433-442.

Clark, S. R., and L. Froebel. 2020. Turkey industry annual report – Current health and industry issues facing the US turkey industry. Retrieved from https://huvepharma.com/news/article/2020-turkey-industry-annual-report-current-health-and-industry-issues-facing-the-us-turkey-industry/

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

Clarkson, M. J. 1963. Immunological responses to *Histomonas meleagridis* in the turkey and fowl. Immunology. 6:156-168.

Clarkson, M. J. 1966. Progressive serum protein changes in turkeys infected with *Histomonas meleagridis*. J. Comp. Path. 76:387-397.

Collett, S. R. 2013. Principles of disease prevention, diagnosis, and control. Pages 3-60 in Diseases of Poultry. 13th ed. E. Swayne, J. R. Glisson, L. R. McDougald, L. K. Nolan, D. L. Suarez, and V. L. Nair, eds., Wiley-Blackwell, Ames, IA.

Connell, R. 1950. Enterohepatitis (blackhead) in turkeys. Can. J. Comp. Med. 14:331-337.

Cortes, P. L., R. P. Chin, M. C. Bland, R. Crespo, and H. L. Shivaprasad. 2004. Histomoniasis in the bursa of Fabricius of chickens. Avian Dis. 48:711-715.

Cuckler, A. 1970. Coccidiosis and histomoniasis in avian hosts. Pages 371-397 in Immunity to parasitic animals. GJ Jackson, R. Herman and I. Singer, eds., New York.

Cuckler, A. C., and C. M. Malanga. 1956. Nithiazide II. Effect on enterohepatitis in turkeys. Proc. Soc. Exp. Biol. Med. 92:485-488.

Cupo, K. L., and R. B. Beckstead. 2019a. *Heterakis gallinarum,* the cecal nematode of gallinaceous birds: A critical review. Avian Dis. 63:381-388.

Cupo, K. L., and R. B. Beckstead. 2019b. PCR detection of *Heterakis gallinarum* in environmental samples. Vet. Parasitol. 271:1-6.

Curtice, C. 1907. Further experiments in connection with the blackhead disease of turkeys. Agricultural Experiment Station of the Rhode Island College of Agriculture and Mechanic Arts. Vol. 124.

Cushman, S. 1893. The production of turkeys. Agricultural Experiment Station, Rhode Island College of Agriculture and Mechanical Arts, Kingston, RI. 25:89-123.

Daş, G., and M. Gauly. 2014. Density related effects on lifetime fecundity of *Heterakis gallinarum* in chickens. Parasit. Vectors. 7:1-9.

Daş, G., L. Wachter, M. Stehr, I. Bilic, B. Grafl, P. Wernsdorf, C. C. Metges, M. Hess, and D. Liebhart. 2021. Excretion of *Histomonas meleagridis* following experimentall co-infection of distinct chicken lines with *Heterakis gallinarum* and *Ascaridia galli.* 14:1-15.

Daugschies, A., B. Bangoura, and M. Lendner. 2013. Inactivation of exogenous endoparasite stages by chemical disinfectants: Current state and perspectives. Parasitol. Res. 112:917-932.

Davidson, W. R., and G. L. Doster. 1994. Blackhead disease does not really cause black heads. National Wild Turkey Federation. Bulletin 25.

Dearborn, E. H., and E.K. Marshall. 1945. The degradation of quinine in the duck, chicken, and dog. J. Pharmacol. Exp. Ther. 85:202-205.

Delaplane, J. P., and H. O. Stuart. 1935. Blackhead (entero-hepatitis) investigations. Bulletin 247. Agricultural experiment station. RI State College.

Doneley, B. 2004. Treating liver disease in the avian patient. In seminars in avian and exotic pet medicine. W. B. Saunders. 13:8-15.

Duffy, C. F., M. D. Sims, and R. F. Power. 2004. Preliminary evaluation of dietary Natustat™ versus Histostat® (nitarsone) for control of *Histomonas meleagridis* in broiler chickens on infected litter. Int. J. Poult. Sci. 3:753-757.

Duffy, C. F., M. D. Sims, and R. F. Power. 2005. Evaluation of dietary Natustat™ for control of *Histomonas meleagridis* in male turkeys on infected litter. Avian Dis. 49:423-425.

Dwyer, D. M., and B. M. Honigberg. 1969. Effect of *in vitro* cultivation on the virulence of a pathogenic strain of *Histomonas meleagridis*. J. Protozool. (Suppl). 16:18-19.

Dwyer, D. M., and B. M. Honigberg. 1970. Effect of certain laboratory procedures on the virulence of *Histomonas meleagridis* for turkeys and chickens. J. Parasitol. 56:694-700.

EFSA. 2013. Event report: Technical meeting on histomonosis in turkeys. EFSA Supporting Pub. 10: 464, pg 1-12.

Farmer, R. K. 1950. Infectious enterohepatitis (blackhead) in turkeys: A study of the prophylactic and therapeutic value of certain compounds. J. Comp. Pathol. 60:294-310.

Farmer, R. K., D. L. Hughes, and G. Whiting. 1951. Infectious enterohepatitis (blackhead) in turkeys: A study of the pathology of the artificially induced disease. J. Comp. Path. 61:251-262.

Fine, P. E. M. 1975. Quantitative studies on the transmission of *Parahistomonas wenrichi* by ova of *Heterakis gallinarum.* Parasitology. 70:407-417.

Friend, M., J. C. Franson, and E. A. Ciganovich. 1999. Field manual of wildlife diseases: General field procedures and diseases of birds. USGS. Madison, WI.

Ganapathy, K., M. H. Salamat, C. C. Lee, and M. Y. Johara. 2000. Concurrent occurrence of salmonellosis, colibaccillosis and histomoniasis in a broiler flock fed with antibiotic-free commercial feed. Avian Pathol. 29:639-642.

Ganas, P, D. Liebhart, M. Glösmann, C. Hess, and M. Hess. 2012. *Escherichia coli* strongly supports the growth of *Histomonas meleagridis,* in a monoxenic culture, without influence on its pathogenicity. Int. J. Parasitol. 42:893-901.

Gerbod, D., V. P. Edgcomb, C. Noel, L. Zenner, R. Wintjens, P. Delgado-Viscogliosi, M. E. Holder, M. L. Sogin, and E. Viscogliosi. 2001. Phylogenetic position of the trichomonad parasite of turkeys *Histomonas meleagridis* (Smith) Tyzzer, inferred from small subunit rRNA sequence. J. Eukaryot. Microbiol. 48:498-504.

Gerhold, R. W., L. A. Lollis, R. B. Beckstead, and L. R. McDougald. 2010. Establishment of culture conditions for survival of *Histomonas meleagridis* in transit. Avian Dis. 54:948-950.

Grabensteiner, E., and M. Hess. 2006. PCR for identification and differentiation of *Histomonas meleagridis, Tetratrichomonas gallinarum* and *Blastocystis* spp. Vet. Parasitol. 142:223-230.

Grabensteiner, E., N. Arshad, and M. Hess. 2007. Differences in the *in vitro* susceptibility of mono-eukaryotic cultures of *Histomonas meleagridis, Tetratrichomonas gallinarum* and *Blastocystis* sp. to natural organic compounds. Parasitol. Res. 101:193-199.

Grabensteiner, E., D. Liebhart, N. Arshad, and M. Hess. 2008. Antiprotozoal activities determined *in vitro* and *in vivo* of certain plant extracts against *Histomonas meleagridis, Tetratrichomonas gallinarum,* and *Blastocystis* sp. Parasitol Res. 103:1257-1264.

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

Gruber, J., P. Ganas, and M. Hess. 2017. Long-term *in vitro* cultivation of *Histomonas meleagridis* coincides with the dominance of a very distinct phenotype of the parasite exhibiting increased tenacity and improved cell yields. Parasitology. 144:1253-1263.

Gruber, J, A. Pletzer, and M. Hess. 2018. Cholesterol supplementation improves growth rates of *Histomonas meleagridis in vitro.* Exp. Parasitol. 185:53-61.

Hafez, H. M., and R. Hauck. 2006. Efficacy of a herbal product against *Histomonas meleagridis* after experimental infection of turkey poults. Archiv. Anim. Nutr. 60:436-442.

Hafez, H. M., R. Hauck, D. Lüschow, and L. R. McDougald. 2005. Comparison of the specificity and sensitivity of PCR, nested PCR, and real-time PCR for the diagnosis of histomoniasis. Avian Dis. 49:366-370.

Hafez, H. M., and A. A. Shehata. 2021. Turkey production and health: Current challenges. Ger. J. Vet. Res. 1:3-14.

Harlander-Matauschek, A., P. Beck, and H. Piepho. 2009. Taste aversion learning to eliminate feather pecking in laying hens, Gallus gallus domesticus. Anim. Behav. 78:485–490.

Harlander-Matauschek, A. and T. Rodenburg. 2011. Applying chemical stimuli on feathers to reduce feather pecking in laying hens. Appl. Anim. Behav. Sci. 132:146–151.

Harrison Jr., A. P., and P. A. Hansen. 1950. The bacterial flora of the cecal feces of healthy turkeys. J. Bacteriol. 59:197-210.

Hauck, R., P. L. Armstrong, and L. R. McDougald. 2010. *Histomonas meleagridis* (Protozoa: Trichomonadidae): Analysis of growth requirements *in vitro*. J. Parasitol. 96:1-7.

Hauck, R., and H. M. Hafez. 2007. Effect of coated plant extracts on *Histomonas meleagridis* and growth of bacteria *in vitro.* Avian Dis. 51:880-883.

Hauck, R., and H. M. Hafez. 2009. Partial sequence of the beta-tubulin of *Histomonas meleagridis* and the activity of benzimidazoles against *H. meleagridis in vitro*. Parasitol. Res. 104:1183-1189.

Hauck, R., and H. M. Hafez. 2013. Experimental infections with the protozoan parasite *Histomonas meleagridis:* A review. Parasitol. Res. 112:19-34.

Hegngi, F. N., J. Doerr, T. S. Cummings, R. D. Schwartz, G. Saunders, A. Zajac, C. T. Larsen, and F. W. Pierson. 1999. The effectiveness of benzimidazole derivatives for the treatment and prevention of histomonosis (blackhead) in turkeys. Vet. Parasitol. 81:29-37.

van der Heijden, H. M. J. F., and W. J. M. Landman. 2007. Improved culture of *Histomonas meleagridis* in a modification of Dwyer medium. Avian Dis. 51:986-988.

van der Heijden, H. M. J. F., and W. J. M. Landman. 2008a. *In vitro* effect of herbal products against *Histomonas meleagridis* in turkeys. Vet. Parasitol. 154:1-7.

van der Heijden, H. M. J. F., and W. J. M. Landman. 2008b. *In vivo* effect of herbal products against *Histomonas meleagridis* in turkeys. Avian Pathol. 37:45-50.

van der Heijden, H. M. J. F., L. R. McDougald, and W. J. M. Landman. 2005. High yield of parasites and prolonged *in vitro* culture of *Histomonas meleagridis*. Avian Pathol. 34:505-508.

van der Heijden, H. M. J. F., A. Stegeman, and W. J. M. Landman. 2010. Development of a blocking-ELISA for the detection of antibodies against *Histomonas meleagridis* in chickens and turkeys. Vet. Parasitol. 171:216-22.

Hess, M., D. Liebhart, I. Bilic, and P. Ganas. 2015. *Histomonas meleagridis*—New insights into an old pathogen. Vet. Parasitol. 208:67-76.

Hess, M., T. Kolbe, E. Grabensteiner, and H. Prosl. 2006. Clonal cultures of *Histomonas meleagridis, Tetratrichomonas gallinarum* and a *Blastocystis* sp. established through micromanipulation. Parasitology. 133:547-554.

Hess, M., D. Liebhart, E. Grabensteiner, and A. Singh. 2008. Cloned *Histomonas meleagridis* passaged *in vitro* resulted in reduced pathogenicity and is capable of protecting turkeys from histomonosis. Vaccine. 26:4187-4193.

Hess, M., and L. McDougald. 2013. Histomoniasis (blackhead) and other protozoan diseases of the intestinal tract. Pages 1172-1179 in Diseases of Poultry.13th ed. E. Swayne, J. R. Glisson, L. R. McDougald, L. K. Nolan, D. L. Suarez, and V. L. Nair, eds., Wiley-Blackwell, Ames, IA.

Hirsch, D. S. 1975. Attempts at axenic cultivation of *Histomonas meleagridis.* M. S. Thesis. Kansas State Univ., Manhattan, KS.

Honigberg, B. M., and C. J. Bennett. 1971. Lightmicroscopic observations on structure and division of *Histomonas-meleagridis* (Smith). 18:687-697.

Honigberg, B. M., and D. M. Dwyer. 1969. Maintenance of *Histomonas meleagridis* in liquid nitrogen. J. Protozool (Suppl). 16:18-19.

Huber, K., C. Chauve, and L. Zenner. 2005. Detection of *Histomonas meleagridis* in turkeys cecal droppings by PCR amplification of the small subunit ribosomal DNA sequence. Vet. Parasitol. 131:311-316.

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 Pathol. 36:279-282.

Hu, J., L. Fuller, and L. R. McDougald. 2004. Infection of turkeys with *Histomonas meleagridis* by the cloacal drop method. Avian Dis. 48:746-750.

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

Hu, J., and L. R. McDougald. 2003. Direct lateral transmission of *Histomonas meleagridis* in turkeys. Avian Dis. 47:489-492.

Hu, J., and L. R. McDougald. 2004. The efficacy of some drugs with known antiprotozoal activity against *Histomonas meleagridis* in chickens. Vet. Parasitol. 121:233-238.

Jones, R. E., D. V. Rives, O. J. Fletcher, and M. P. Martin. 2020. Histomoniasis outbreaks in commercial turkeys in the southeastern United States: Proximity of broiler breeder farms as a potential risk factor in disease development. J. Appl. Poult. Res. 29:496-501.

Joyner, L. P. 1963. Immunity to histomoniasis in turkeys following treatment with dimetridazole. J. Comp. Pathol. Ther. 73:201-207.

Joyner, L. P., S. F. M. Davies, and S. B. Kendall. 1963. Chemotherapy of histomoniasis. Pages 333-349 in Experimental Chemotherapy. Vol. 1. Academic Press New York.

Klodnicki, M. E., L. R. McDougald, and R. B. Beckstead. 2013. A genomic analysis of *Histomonas meleagridis* through sequencing of a cDNA library. J. Parasitol. 99:264-269.

Kudo, K., J. Shiraishi, S. Nishimura, T. Bungo, and S. Tabata. 2010. The number of taste buds is related to bitter taste sensitivity in layer and broiler chickens. Anim. Sci. J. 81:240-244.

Lagler, J., T. Mitra, S. Schmidt, A. Pierron, E. Vatzia, M. Stadler, S. E. Hammer, K. H. Mair, B. Grafl, P. Wernsdorf, F. Rauw, B. Lambrecht, D. Liebhart, and W. Gerner. 2019. Cytokine production and phenotype of *Histomonas meleagridis*-specific T cells in the chicken. Vet. Res. 50:107.

Lagler, J., S. Schmidt, T. Mitra, M. Stadler, P. Wernsdorf, B. Grafl, T. Hatfaludi, M. Hess, W. Gerner, and D. Liebhart. 2021. Comparative investigation of IFN-γ-producing T cells in chickens and turkeys following vaccination and infection with the extracellular parasite *Histomonas meleagridis*. Dev. Comp. Immunol. 116:103949.

Landman, W. J. M., N. Gantois, J. H. H. van Eck, H. M. J. F van der Heijden, and E. Viscogliosis. 2019. *Tetratrichomonas gallinarum* granuloma disease in a flock of free range layers. Vet. Q. 39:153-160.

Landman, W. J. M., C. ter Veen, H. M. J. F. van der Heijden, and D. Klinkenberg. 2015. Quantification of parasite shedding and horizontal transmission parameters in *Histomonas meleagridis*-infected turkeys determined by real-time quantitative PCR. Avian Pathol. 44:358-365.

Law, G. L., G. P. Mansfield, D. F. Muggleton, and E. W. Parnell. 1963. Dimetridazole: Absorption, excretion and metabolism in turkeys. Nature. 197:1024-1025.

Leberl, M., M. Hess, and I. Bilic. 2010. *Histomonas meleagridis* possesses three alpha-actinins immunogenic to its hosts. Mol. Biochem. Parasitol. 169:101-107.

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. 59:877-884.

Lesser, E. 1960a. Cultivation of *Histomonas meleagridis* in a modified tissue culture medium. J. Parasitol. 6:686.

Lesser, E. 1960b. Replacement of serum in the cultivation of *Histomonas meleagridis.* J. Parasitol. 46:271.

Lesser, E. 1961. *In vitro* cultivation of *Histomonas meleagridis* free of demonstrable bacteria. J. Protozool. 8:228-230.

Lesser, E. 1963. Effect of amphotericin-B on *in vitro* growth of *Histomonas meleagridis*. J. Parasitol. 49:329.

Lesser, E. 1964a. Studies on the *in vitro* growth of *Histomonas meleagridis* with single species of bacteria. Proc. Helm. Soc. Wash. 31:365-366.

Lesser, E. 1964b. Studies on the *in vitro* growth of *Histomonas meleagridis* with three different mixed bacterial flora. Proc. Helm. Soc. Wash. 31:263-264.

Levine, P. P. 1947. Histomoniasis in a kidney of a turkey. Cornell Vet. 37:269-270.

Liebhart, D., P. Ganas, T. Sulejmanovic, and M. Hess. 2017. Histomonosis in poultry: Previous and current strategies for prevention and therapy. Avian Pathol. 46:1-18.

Liebhart, D., E. Grabensteiner, and M. Hess. 2008. A virulent mono-eukaryotic culture of *Histomonas meleagridis* is capable of inducing fatal histomonosis in different aged turkeys of both sexes, regardless of the infective dose. Avian Dis. 52:168-172.

Liebhart, D., and M. Hess. 2009. Oral infection of turkeys with *in vitro*-cultured *Histomonas meleagridis* results in high mortality. Avian Pathol. 38:223-227.

Liebhart, D., T. Sulejmanovic, B. Grafl, A. Tichy, and M. Hess. 2013. Vaccination against histomonosis prevents a drop in egg production in layers following challenge. Avian Pathol. 42:79-84.

Liebhart, D., M. Windisch, and M. Hess. 2010. Oral vaccination of 1-day-old turkeys with *in vitro* attenuated *Histomonas meleagridis* protects against histomonosis and has no negative effect on performance. Avian Pathol. 39:399-403.

Liebhart, D., M. Zahoor, I. Prokofieva, and M. Hess. 2011. Safety of avirulent histomonads to be used as a vaccine determined in turkeys and chickens. Poult. Sci. 90:996-1003.

Lindquist, W. D. 1962. Some effects of paromomycin sulfate on blackhead in turkeys. Am. J. Vet. Res. 23:1053-1056.

Liu, W., J. Peng, F. Li, H. Sun, Y. Ding, J. He, and Y. Liu. 2011. Identification of *Histomonas meleagridis* by *in vitro* microculture and polymerase chain reaction. Rep. Parasitol. 1:1-6.

Lollis, L., R. Gerhold, L. McDougald, and R. Beckstead. 2011. Molecular characterization of *Histomonas meleagridis* and other parabasalids in the United States using the 5.8S, ITS-1, and ITS-2 rRNA regions. J. Parasitol. 97:610-615.

Long, P. L., W. L. Current, and G. P. Noble. 1987. Parasites of the Christmas turkey. Parasitol. Today. 3:360-366.

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

Lotfi, A., R. Hauck, P. Olias, and H. M. Hafez. 2014. Pathogenesis of histomonosis in experimentally infected specific-pathogen-free (SPF) layer-type chickens and SPF meat-type chickens. Avian Dis. 58:427-432.

Lund., E. E. 1955. The progress of histomoniasis (blackhead) in turkeys as related to the size of the infective dose. Poult. Sci. 34:127-130.

Lund, E. E. 1959. Immunizing action of a nonpathogenic strain of *Histomonas* against blackhead in turkeys. J. Protozool. 6:182-185.

Lund, E. E. 1963. *Histomonas wenrichi* n. sp. (Madtigophora: Mastigamoebidae), a nonpathogenic parasite of gallinaceous birds. J. Protzool. 10:401-404.

Lund, E. E., and A. M. Chute. 1972. *Heterakis* and *Histomonas* infections in young peafowl, compared to such infections in pheasants, chickens, and turkeys. J. Wildl. Dis. 8:352-358.

Lund, E. E., P. C., Augustine, and A. M. Chute. 1967. *Histomonas meleagridis* after one thousand *in vitro* passages. J. Eukaryot. Microbiol. 14:349-351.

Lund, E. E., P. C. Augustine, and D. J. Ellis. 1966a. Immunizing action of *in vitro*-attenuated *Histomonas meleagridis* in chickens and turkeys. Exp. Parasitol. 18:403-407.

Lund, E. E., E. E. Wehr, and D. J. Ellis. 1966b. Earthworm transmission of *Heterakis* and *Histomonas* to turkeys and chickens. J. Parasitol. 52:899-902.

Lynn, E. C., and R. B. Beckstead. 2012. Identification of gene expression elements in *Histomonas meleagridis* using splinkerette PCR, a variation of ligated adaptor PCR. J. Parasitol. 98:135-141.

Mazumdar, K. Nöbauer, K. Hummel, M. Hess, and I. Bilic. 2019. Molecular characterization of *Histomonas meleagridis* exoproteome with emphasis on protease secretion and parasite-bacteria interaction. PLoS One. 14:e0212429.

McAllister, M. M. 2014. Successful vaccines for naturally occurring protozoal diseases of animals should guide human vaccine research: A review of protozoal vaccines and their designs. Parasitology. 141:624-640.

McDougald, L. R. 1979. Efficacy and compatibility of amprolium and carbarsone against coccidiosis and blackhead in turkeys. Poult. Sci. 58:76-80.

McDougald, L. R. 1998. Intestinal protozoa important to poultry. Poult. Sci. 77:1156-1158.

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

McDougald, L. R., and L. Fuller. 2005. Blackhead disease in turkeys: Direct transmission of *Histomonas meleagridis* from bird to bird in a laboratory model. Avian Dis. 49:328-331.

McDougald, L. R., and R. B. Galloway. 1973. Blackhead disease: *In vitro* isolation of *Histomonas meleagridis* as a potentially useful diagnostic aid. Avian Dis. 17:847-850.

McDougald, L. R., and M. F. Hansen. 1969. Research note: Serum protein changes in chickens subsequent to infection with *Histomonas meleagridis.* Avian Dis. 13:673-677.

McDougald, L. R., and J. Hu. 2001. Blackhead disease (*Histomonas meleagridis*) aggravated in broiler chickens by concurrent infection with cecal coccidiosis (*Eimeria tenella*). Avian Dis. 45:307-312.

McGuire, W. C., and J. W. Cavett. 1952. Blood studies on histomoniasis in turkeys. Poult. Sci. 31:610-617.

McGuire, W. C., M. W. Moeller, and N. F. Morehouse. 1964. The effect of dimetridazole on the growth and prevention of histomoniasis in poultry. Poult. Sci. 43:864-871.

Metzler-Zebeli, B. U., E. Magowan, M. Hollmann, M. E. E. Ball, A. Molnár, K. Witter, R. Ertl, R. J. Hawken, P. G. Lawlor, N. E. O'Connell, J. Aschenbach, and Q. Zebeli. 2018. Differences in intestinal size, structure, and function contributing to feed efficiency in broiler chickens reared at geographically distant locations. Poult. Sci. 97:578-591.

Mielewczik, M., H. Mehlhorn, S. Al-Quraishy, E. Grabensteiner, and M. Hess. 2008. Transmission electron microscopic studies of stages of *Histomonas meleagridis* from clonal cultures. Parasitol. Res. 103:745-750.

Mitra, T., W. Gerner, F. A. Kidane, P. Wernsdorf, M. Hess, A. Saalmüller, and D. Liebhart. 2017. Vaccination against histomonosis limits pronounced changes of B cells and T-cell subsets in turkeys and chickens. Vaccine. 35:4184-4196.

Mitra, T., Kidane, F. A., Hess, M., and Liebhart, D. 2018. Unravelling the immunity of poultry against the extracellular protozoan parasite *Histomonas meleagridis* is a cornerstone for vaccine development: A review. Front. Immunol. 9:2518.

Mitrovic, M., M. Hoffer, and E. G. Schilknecht. 1968. Antihistomonal activity of 1,2-disubstituted 5-nitroimidazoles. Antimicrob. Agents Chemother. 8:445-448.

Moulder, J. W. 1948. Effect of quinine treatment of the host upon the carbohydrate metabolism of the malarial parasite *Plasmodium gallinaceum*. The journal of infectious diseases. 83:262-270.

Munsch, M., A. Lotfi, H. M. Hafez, S. Al-Quraishy, and H. Mehlhorn. 2009a. Light and transmission electron microscopic studies on trophozoites and cyst-like stages of *Histomonas meleagridis* from cultures. Parasitol. Res. 104:683-689.

Munsch, M., H. Mehlhorn, S. Al-Quraishy, A. R. Lotfi, and H. M. Hafez. 2009b. Molecular biological features of strains of *Histomonas meleagridis*. Parasitol. Res. 104:1137-1140.

Niknafs, S., and E. Roura. 2018. Nutrient sensing, taste and feed intake in avian species. Nutr. Res. Rev. 31:256-266.

Pham, A. D. N. , J. K. De Gussem, and B. M. Goddeeris. 2013a. Intracloacally passaged low-virulent *Histomonas meleagridis* protects turkeys from histomonosis. Vet. Parasitol. 196:307-313.

Pham, A. D. N., J. Mast, J. K. De Gussem, L. R. McDougald, and B. M. Goddeeris. 2013b. Establishing mono-eukaryotic *Histomonas meleagridis* cultures from *in vivo* infection contaminated with *Tetratrichomonas gallinarum* and *Blastocystis* spp. Parasitology. 140:1266-1274.

Popp, C., R. Hauck, S. Balczulat, and H. M. Hafez. 2011. Recurring histomonosis on an organic farm. Avian Dis. 55:328-330.

Powell, F. L., L. Rothwell, M. J. Clarkson, and P. Kaiser. 2009. 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.

Puvača, N., I. Brkić, M. Jahić, S. R. Nikolić, G. Radović, D. Ivanišević, M. Đokić, D. Bošković, D. Ilić, S. Brkanlić, and R. Prodanović. 2020. The effect of using natural or biotic dietary supplements in poultry nutrition on the effectiveness of meat production. Sustainability. 12:4373.

Regmi, P. R., A. L. Shaw, L. L. Hungerford, J. R. Messenheimer, T. Zhou, P. Pillai, A. Omer, and J. M. Gilbert. 2016. Regulatory considerations for the approval of drugs against histomoniasis (blackhead disease) in turkeys, chickens, and game birds in the United States. Avian Dis. 60:725-730.

Reid, W. M. 1967. Etiology and dissemination of the blackhead disease syndrome in turkeys and chickens. Exp. Parasitol. 21:249-275.

Reis Jr., J. L., R. B. Beckstead, C. C. Brown, and R. W. Gerhold. 2009. *Histomonas meleagridis* and capillarid infection in a captive chukar (*Alectoris chukar*). Avian Dis. 53:637-639.

Roura, E., M. W. Baldwin, and K.C. Klasing. 2013. The avian taste system: Potential implications in poultry nutrition. Anim. Feed Sci. Technol. 180:1- 9.

Scanes, C. G., and K. Pierzchala-Koziec. 2014. Biology of the gastro-intestinal tract in poultry. Avian Biol. Res. 7:193-222.

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

Seeger, K. C., W. C. Lucas, and A. E. Tomhave. 1950. The use of enheptin-T in the control of enterohepatitis in turkeys. Poult. Sci. 29:610-611.

Seeler, A. O., E. Dusenbery, and C. Malanga. 1943. The comparative activity of quinine, quinidine, cinchonine, cinehonidine and quinoidine against *Plasmodium iophurae* infection of Pekin ducklings. J. Pharmacol. Exp. Ther. 78:159-163.

Sentíes-Cué, G., R. 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 Dis. 53:231-238.

Sharma, J. M., and S. Rautenschlein. 2013. Host factors for disease resistance. Pages 47-74 in Diseases of Poultry. 13th ed. E. Swayne, J. R. Glisson, L. R. McDougald, L. K. Nolan, D. L. Suarez, and V. L. Nair, eds., Wiley-Blackwell, Ames, IA.

Skelhorn J., and C. Rowe. 2010. Birds learn to use distastefulness as a signal of toxicity. Proc Biol Sci. 277:1729-1734.

Smith, T. 1895. An infectious disease among turkeys caused by protozoa (infectious entero-hepatitis). USDA, Bur. Anim. Ind. Bull. 8:3-27.

Sorvari, R., A. Naukkarinen, and T. E. Sorvari. 1977. Anal sucking-like movements in the chicken and chick embryo followed by the transportation of environmental material to the bursa of Fabricius, caeca, and caecal tonsils. Poult. Sci. 56:1426-1429.

Spencer, T. 2010. Parasite management for natural and organic poultry: Blackhead in turkeys. ATTRA.

Springer, W. T., J. Johnson, and W. M. Reid. 1970. Histomoniasis in gnotobiotic chickens and turkeys: Biological aspects of the role of bacteria in the etiology. Exp. Parasitol. 28:383-392.

Sulejmanovic, T., I. Bilic, M. Hess, and D. Liebhart. 2016. An *in vitro* attenuated strain of *Histomonas meleagridis* provides cross-protective immunity in turkeys against heterologous virulent isolates. Avian Pathol. 45:46-53.

Sulejmanovic, T., D. Liebhart, and M. Hess. 2013. *In vitro* attenuated *Histomonas meleagridis* does not revert to virulence, following serial *in vivo* passages in turkeys or chickens. Vaccine. 31:5443-5450.

Sulejmanovic, T., V. Turblin, I. Bilic, B. Jaskulska, and M. Hess. 2019. Detection of *Histomonas meleagridis* DNA in dust samples obtained from apparently healthy meat turkey flocks without effect on performance. Avian Pathol. 48:329-333.

Taliaferro, L. G., F. Coulston, and M. Silverman. 1944. The antimalarial activity of tyrothricin against *Plasmodium gallinaceum*. J. Inf. Dis. 75:179-210.

Taliaferro, W. H., and L. G. Talliaferro. 1948. Reduction in immunity in chicken malaria following treatment with nitrogen mustard. J. Inf. Dis. 1:5-30.

Thøfner, I. C. N., D. Liebhart, M. Hess, T. W. Schou, C. Hess, E. Ivarsen, X. Fretté, L. P. Christensen, K. Grevsen, R. M. Engberg, and J. P. Christensen. 2012. Antihistomonal effects of artemisinin and *Artemisia annua* extracts *in vitro* could not be confirmed by *in vivo* experiments in turkeys and chickens. Avian Pathol. 41:487-496.

Tykałowski, B., M. Śmiałek, J. Kowalczyk, D. Dziewulska, T. Stenzel, and A. Koncicki. 2021. Phytoncides in the prevention and therapy of blackhead disease and their effect on the turkey immune system. J. Vet. Res. 65:79-85.

Tyzzer, E.E. 1919. Developmental phases of the protozoon of “blackhead” in turkeys. J. Med. Res. 40:1-30.

Tyzzer, E. E. 1920. The flagellate character and reclassification of the parasite producing “Blackhead” in turkeys: *Histomonas* (gen. nov.) *meleagridis* (Smith). J. Parasitol. 6:124-131.

Tyzzer, E. E. 1923. Arsenical compounds in the treatment of blackhead in turkeys. J. Exp. Med. 37:851-873.

Tyzzer, E. E. 1932. Problems and observations concerning the transmission of blackhead infection in turkeys. Proc. Am. Philos. Soc. 71:407-410.

Tyzzer, E. E. 1934. Studies on histomoniasis, or “blackhead” infection, in the chicken and the turkey. Daedalus. 69:189-264.

Tyzzer, E. E. 1936. A study of immunity produced by infection with attenuated culture-strains of *Histomonas meleagridis.* J. Comp. Patho. Ther. 49:285-303.

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

Tyzzer, E. E., and M. Fabyan. 1922. 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

Tyzzer, E. E., M. Fabyan, and N. C. Foot. 1921. Further observations on “blackhead” in turkeys. J. Infect. Dis. 29:268-286.

Ueda, H., and S. Kaidou. 2005. Aversion to quinine is associated with taste sensation in chicks. J. Poult. Sci. 42:254-262.

Ueda, H., A. Takagi, K. Katou, and S. Matsumoto. 2002. Feeding behavior in chicks fed tea saponin and quinine sulfate. J. Poult. Sci. 39:34-41.

Umar S., M. I. Khan, S. Ahmed, M. Usman, M. Younus, F. Sarwar, and A. Shahzad. 2016. *In vitro* and *in vivo* sensitivity of a flagellated protozoan, *Histomonas meleagridis*, to metronidazole and nitarsone. Pakistan J. Zool. 48:209-214.

Uzor, P. E. 2020. Alkaloids from plants with antimalarial activity: A review of recent studies. Evid. Based Complement. Alternat. Med. 2020:1-17.

Waletzky, E., and H. W. Brown. 1943. Studies on the mode of action of quinine in avian malaria. Malar J. 2:53-62.

Waters III, C. V., L. D. Hall, W. R. Davidson, E. A. Rollor III, and K. A. Lee. 1994. Status of commercial and noncommercial chickens as potential sources of histomoniasis among wild turkeys. Wildl. Soc. Bull. 22:43-49.

Wei, Z., M. Abraham, E. V. Chadwick, and R. B. Beckstead. 2020. *Histomonas meleagridis* isolates compared by virulence and gene expression. Vet. Parasitol. 286:109233.

Windisch, M., and M. Hess. 2009. Establishing an indirect sandwich enzyme-linked-immunosorbent-assay (ELISA) for the detection of antibodies against *Histomonas meleagridis* from experimentally infected specific pathogen-free chickens and turkeys. Vet. Parasitol. 161:25-30.

Windisch, M., and M. Hess. 2010. Experimental infection of chickens with *Histomonas meleagridis* confirms the presence of antibodies in different parts of the intestine. Parasite Immunol. 32:29-35.

Zaragatzki, E., M. Hess, E. Grabensteiner, F. Abdel-Ghaffar, K. A. S. Al-Rasheid, and H. Mehlhorn. 2010a. Light transmission electron microscopic studies on the encystation of *Histomonas meleagridis.* Parasitol. Res. 106:977-983.

Zaragatzki, E., H. Mehlhorn, F. Abdel-Ghaffar, K. S. Rasheid, E. Grabensteiner, and M. Hess. 2010b. Experiments to produce cysts in cultures of *Histomonas meleagridis* – The agent of histomonosis in poultry. Parasitol. Res. 106:1005-1007.

Zenner, L., M. P. Callait, C. Granier, and C. Chauve. 2003. *In vitro* effect of essential oils from *Cinnamomum aromaticum*, *Citrus limon* and *Allium sativum* on two intestinal flagellates of poultry, *Tetratrichomonas gallinarum* and *Histomonas meleagridis.* Parasite. 10:153-157.

CHAPTER 3: EVALUATION OF QUININE AS A CHEMOPROPHYLACTIC CANDIDATE AGAINST HISTOMONIASIS IN TURKEYS

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ABSTRACT

Histomoniasis, also commonly referred to as blackhead disease, is caused by the protozoan parasite *Histomonas meleagridis.* Since the removal of nitarsone in 2015, no approved prophylactics are available for mitigating histomoniasis. Disease incidence and high mortalities are frequently associated with turkey flocks, although infection of broiler breeders also occurs. Quinine is a naturally occurring alkaloid with antimalarial properties. *In vitro* assays have shown strong antihistomonal properties of quinine, leading to our hypothesis that quinine inclusion within the feed could prevent histomoniasis in turkeys. Selected concentrations of quinine were included within a turkey starter diet to evaluate effects on body weight gain (BWG), liver lesions, cecal lesions, and mortality of *H. meleagridis*-challenged turkeys. On day-of-hatch, poults were randomly assigned to either the basal diet or a quinine diet. Groups consisted of a non-challenged control (NC; basal diet), 0.022% quinine + challenge, 0.067% quinine + challenge, 0.2% quinine + challenge, or a positive-challenged control (PC; basal diet). On d10, challenged groups were intracloacally inoculated with 10^5 *H. meleagridis* cells/turkey, and lesions were evaluated on d21 post-infection. Individual body weights were recorded on d0, d10, and d31 to calculate the pre-challenge and post-challenge BWG. No significant differences ($P >$ 0.05) were observed between the d0-10 pre-challenged BWG between quinine treatment diets and the basal diet. Similarly, no differences ($P > 0.05$) were observed in post-challenge d10-31 BWG of the quinine dietary treatments as compared to the PC. Cumulative mortalities, liver lesions, and cecal lesions related to histomoniasis were not reduced ($P > 0.05$) in any of the quinine treatment groups as compared to the PC. Although quinine successfully reduced *H. meleagridis* cells *in vitro*, results from the *in vivo* experiment indicated no reduction in histomoniasis severity as evidenced by similar lesions and mortality as the PC. Taken together,

these data indicate that quinine inclusion within the feed at these concentrations and under these experimental conditions was not efficacious in the prevention or treatment of histomoniasis.

Key Words: histomoniasis; *Histomonas meleagridis*; quinine; turkey; blackhead

INTRODUCTION

Histomonas meleagridis, the etiological agent of histomoniasis, is a protozoan disease primarily affecting turkeys and commonly resulting in high mortalities with no prophylactic drugs commercially available to mitigate outbreaks (Liebhart et al., 2017). Quinine is a naturally occurring cinchona alkaloid that has previously been shown to impair carbohydrate metabolism of the parasite *Plasmodium gallinaceum*, etiological agent of malaria in poultry, both *in vitro* and *in vivo* (Moulder, 1948). Following an intravenous injection, chickens exhibited quinine-free blood within 4 to 5 hours; however, the inhibitory effects of quinine incurred by *P. gallinaceum* lasted for 24 hours post-injection (Moulder, 1948). This continued inhibition of the parasite's carbohydrate metabolism following blood clearance of quinine would further suggest that quinine acts directly upon *P. gallinaceum,* causing irreversible inhibition to the parasite rather than indirectly impacting phagocytic mechanisms (Moulder, 1948). Dietary concentrations of 0.5% or 1% quinine sulfate have resulted in decreased feed intake and diet rejection when chicks were allowed free access of two dietary choices of quinine-supplemented diet or basal diet (Ueda et al., 2002). Previous studies suggest that chicks are able to perceive the bitter taste of quinine similar to human perception, resulting in the decreased feed intake, especially at dietary concentrations higher than 0.2% quinine (Ueda and Kainou, 2005). Feed intake of chicks was not significantly reduced when chicks were supplied with a 0.1% quinine diet (Ueda et al., 2002).

Tyzzer (1923) evaluated large unspecified doses of quinine HCl injected intramuscularly or intravenously into turkeys but observed no mitigation of histomoniasis. No further evaluations of quinine against histomoniasis appear to have been conducted despite this alkaloid's marked antimalarial effect which we hypothesized could transfer antihistomonal properties to turkeys. Studies in chickens indicate taste aversion at high quinine concentrations, but the maximum

tolerance in turkeys has not been established (Ueda et al., 2002; Ueda and Kainou, 2005). *H. meleagridis* adapts to flagellated or amoeboid form depending on cecal lumen location or tissue invasion; therefore, quinine could potentially impair *Histomonas* metabolism before the parasite enters the liver via the hepatic portal vein following cecal degradation and translocation. As a chemoprophylactic compound, quinine is rapidly cleared in chickens with accompanying strong antimalarial properties; therefore, quinine should be further evaluated against other protozoal diseases, such as histomoniasis. The purpose of the present study was to evaluate quinine as a chemoprophylactic administered in feed against histomoniasis in turkeys at specified dietary concentrations.

MATERIALS AND METHODS

Histomonas *Isolates and Culture*

Isolates of *H. meleagridis* were obtained from field outbreaks in the southern United States (Buford, Georgia; **Bu** strain; isolated from infected chickens) and Northwest Arkansas (**PHL2017** strain; isolated from infected turkeys). Histomonads were grown according to previously described methods (van der Heijden and Landman, 2007; Beer et al., 2020). In brief, Modified Dwyer's Media (**MDM**) was comprised of Medium 199 (Product #12-118F, Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated horse serum (Product #26050-088, Gibco, Life Technologies Corporation, Waltham, MA) and 1.6mg/mL white rice flour (Arrowhead Mills, Boulder, CO) with an undefined bacterial population. Subculture occurred every 48-72 hours into 25cm^2 tissue culture flasks (Product #10062-874, VWR International, Radnor, PA) containing fresh, supplemented MDM. Incubation occurred anaerobically at 40°C.

In vitro ***Assessment of Quinine***

Three *in vitro* assays per *H. meleagridis* strain (Bu and PHL2017) were independently completed to evaluate selected concentrations of food-grade quinine HCl dihydrate (Product #W297607; Sigma-Aldrich, St. Louis, MO) on histomonad viability. Histomonads were revived from aliquots and propagated as described above with passages occurring fewer than ten times prior to each assay. Each tube contained a ratio of 250µL histomonads + 50µL of treatment + 700µL of MDM into sterile microcentrifuge snap-cap tubes (Product #20170-333; VWR). Initial histomonad seeding density for each treatment was 1.5 x 10^5 cells/tube. Treatments included the negative treatment control or final concentrations of either 0.022%, 0.067%, or 0.2% quinine reconstituted in sterile H_2O. Each treatment was performed in triplicate. Incubation occurred at 40°C under anaerobic conditions for 20h. Viable histomonads/mL were enumerated with a hemocytometer using Trypan blue dye exclusion (Product #15250-061, Gibco). A series of 10-fold dilutions of each treatment were subsequently plated on tryptic soy agar (**TSA**, Product #211822, Becton Dickinson, Sparks, MD) at 0h and 20h time-points to enumerate bacterial colony forming units (**CFU**). Plates were incubated anaerobically at 40°C. Additional assay treatment controls for bacterial enumeration included: MDM control or final concentrations of either 0.022%, 0.067%, or 0.2% quinine in MDM to ensure no bacteria were introduced other than the deliberate seeding of bacteria from the *Histomonas* culture in treatment groups.

Animal Trial and Diet

On day-of-hatch, a total of 200 female turkey poults were obtained from a local commercial hatchery, wing-tagged, and randomly allocated to battery cages at the University of Arkansas Poultry Health Laboratory. All animal handling procedures were in compliance with regulations of the University of Arkansas Institutional Animal Care and Use Committee (IACUC

protocol #21094). A corn soy-based starter feed meeting NRC requirements (1994) and water were provided *ad libitum.* Quinine was incorporated into the basal diet at concentrations of 0.022%, 0.067%, or 0.2%. Groups consisted of a non-challenged control (**NC**; basal diet), 0.022% quinine + challenge, 0.067% quinine + challenge, 0.2% quinine + challenge, or a positive-challenged control (**PC**; basal diet). From d0 to d13, each group was allocated to 4 replicate cages of 10 turkeys. From d14 to d31, NC and PC groups each consisted of 8 replicate cages of 4 turkeys, while quinine feed treatment groups each consisted of 6 replicate cages of 4 turkeys.

H. meleagridis *Challenge*

The PHL2017 strain of *H. meleagridis* was selected to be utilized for experimental challenge. Viable histomonads/mL were enumerated with a hemocytometer as described above, and dilutions for the challenge inoculum were prepared with fresh MDM. On d10, each poult in a challenged group received a total of 10^5 histomonads administered intracloacally with an animal gavage needle. The NC group received a sham-inoculation consisting of MDM.

Lesion Scores and Body Weight Gain

All poults were individually weighed on days 0, 10, and 31 for calculation of pre-challenge and post-challenge body weight gain (**BWG**). Liver and cecal lesions were recorded from all mortalities following challenge. On d31, all remaining poults were humanely euthanized and lesion-scored according to previously established methods (Beer et al., 2020). In brief, liver and cecal lesions were scored separately on a scale of “0” to “3” where a score of “0” indicates a healthy organ; “1” indicates the beginning of detectible lesions; “2” indicates intermediate histomoniasis lesions; and “3” indicates classical histomoniasis lesions. Individuals determining lesion scores were blinded to treatment groups.

Statistical Analysis

Cell viability, bacterial growth, and BWG data were analyzed using JMP Pro 15 software (SAS Institute Inc., Cary, NC) with significant differences between treatment groups determined using ANOVA. Where applicable, means were further separated using Tukey's multiple range test. Mortalities related to histomoniasis were analyzed using a chi-square test. Lesion score data were analyzed using the Proc Mixed Procedure in SAS 9.4 software with significance between mean lesion score values considered in comparison to the PC group. Using a chi-square test, each subgrouping of scores ("0" to "3") were further compared between quinine feed treatment groups and the PC. Significance for all statistical analyses was set at $P < 0.05$.

RESULTS AND DISCUSSION

In vitro *Cell Viability Assessment*

Addition of 0.2% quinine to *in vitro* cultures of Bu strain and PHL2017 strain significantly reduced ($P < 0.05$) the growth of histomonads after 20h of incubation as compared to the negative control (**Table 1**). The 0.067% quinine treatment significantly reduced ($P < 0.05$) histomonads in all PHL2017 strain assays and in two of the Bu strain assays. The 0.022% quinine only reduced ($P < 0.05$) histomonads in the third Bu strain assay, with no significant reduction of histomonads ($P > 0.05$) in any other assays. Following 20h of incubation, the 0.2% quinine significantly reduced ($P < 0.05$) recoverable bacterial CFU/mL in all assays as compared to the negative control. Recoverable bacterial CFU/mL was significantly reduced ($P < 0.05$) in the 0.067% quinine group for one Bu strain assay and two PHL2017 strain assays as compared to the negative control. There was no reduction ($P > 0.05$) in recoverable bacterial CFU/mL in the 0.022% quinine group as compared to the negative control. These data suggest that the

antihistomonal impact of quinine is not contributed solely to destruction of accompanying bacteria which is well known to impair *H. meleagridis* growth *in vitro* (Lesser, 1964).

Performance, Mortalities, and Lesions

No significant differences ($P > 0.05$) were observed in pre-challenge BWG between quinine treatments and the basal diet from d0 to d10 of age. Previous research indicates that chicks can perceive the bitter taste of quinine, resulting in decreased feed intake when dietary concentrations are higher than 0.2% (Ueda and Kainou, 2005). Similarly, in this current study, the comparable BWG between groups suggests that turkeys did not have an aversion to the highest feed inclusion rate of 0.2% quinine. The post-challenge BWG from d10 to d31 of age in quinine feed treatments was not significantly different ($P > 0.05$) as compared to the PC group. The PC group and all quinine feed treatments resulted in lower post-challenge BWG ($P < 0.05$) than the NC group. Taken together, these data indicate that quinine inclusion in the feed at concentrations as high as 0.2% was not detrimental to pre-challenge BWG, suggesting that turkeys do not have taste aversion to this cinchona alkaloid at these tested levels. Levels higher than 0.2% quinine are known to reduce feed intake in chickens, but the specific threshold for dietary quinine inclusion has not been evaluated in turkeys (Ueda and Kainou, 2005). Given this information, a higher inclusion rate of quinine could potentially be included within the feed but the impact on feed intake, growth, and histomoniasis severity in turkeys is unknown until further tests are conducted.

None of the quinine feed treatments effectively reduced ($P > 0.05$) mortalities related to histomoniasis when compared to the PC group. Moreover, there was no reduction ($P > 0.05$) in liver or cecal lesions in the quinine feed treatments, regardless of inclusion, as compared to the PC group. Interestingly, the 0.022% quinine feed treatment actually resulted in a higher average

liver lesion as compared to the PC group ($P < 0.05$). Although quinine exhibited antihistomonal activity *in vitro* against two different *Histomonas* strains*,* quinine was not effective for preventing histomoniasis when evaluated *in vivo* at the selected concentrations and experimental conditions, which is consistent with previous research evaluating antihistomonal candidates (Thøfner et al., 2012).

Interestingly, *H. meleagridis* exhibits a flagellate nature rather than a solely amoebic form, which could be a possible reason antimalarial drugs, such as quinine, are ineffective at reducing severity of histomoniasis (Tyzzer and Fabyan, 1922; Tyzzer 1923). In 1948, Moulder showed that intravenous injection of 20mg/kg BW to *P. gallinaceum*-infected chickens resulted in parasite metabolism changes including: increased rate of glucose use, decreased rate of pyruvate use, lowered ratio of oxygen to glucose, and lowered oxygen uptake, presumably due to aerobic phase inhibition by quinine via the pyruvate oxidation step in the TCA cycle. Although *H. meleagridis* can adopt either a flagellated or amoeboid form, this parasite is cultured *in vitro* under anaerobic conditions. The primary mode of action of quinine against *P. gallinaceum* appears to be via inhibition of the parasite's aerobic pathway, which could explain the lack of ameliorative effects when provided to *H. meleagridis*-infected turkeys. Further research with quinine as an antihistomonal would be discouraged unless delivery to the cecae could be ensured and mode of action further elucidated. This research note emphasizes the importance of *in vivo* evaluation for verifying *in vitro* results and emphasizes that dietary inclusion of quinine at the levels evaluated in this study were not efficacious for preventing histomoniasis in turkeys.

REFERENCES

Beer, L. C., C. N. Vuong, T. L. Barros, J. D. Latorre, G. Tellez, A. L. Fuller, and B. M. Hargis. 2020. Research Note: Evaluation of boric acid as a chemoprophylaxis candidate to prevent histomoniasis. Poult. Sci. 99:1978-1982.

van der Heijden, H. M. J. F., and W. J. M. Landman. 2007. Improved culture of *Histomonas meleagridis* in a modification of Dwyer medium. Avian Dis. 51:986-988.

Lesser, E. 1964. Studies on the *in vitro* cultivation of *Histomonas meleagridis* with three different mixed bacterial flora. Proc. Helm. Soc. Wash. 31:263-264.

Liebhart, D., P. Ganas, T. Sulejmanovic, and M. Hess. 2017. Histomonosis in poultry: Previous and current strategies for prevention and therapy. Avian Pathol. 46:1-18.

Moulder, J. W. 1948. Effect of quinine treatment of the host upon the carbohydrate metabolism of the malarial parasite *Plasmodium gallinaceum*. J. Infect. Dis. 83:262-270.

Thøfner, I. C. N., D. Liebhart, M. Hess, T. W. Schou, C. Hess, E. Ivarsen, X. C. Fretté, L. P. Christensen, K. Grevsen, R. M. Engberg, and J. P. Christensen. 2012. Antihistomonal effects of artemisinin and Artemisia annua extracts *in vitro* could not be confirmed by *in vivo* experiments in turkeys and chicken. Avian Pathol. 41:487-496.

Tyzzer, E. E. 1923. Arsenical compounds in the treatment of blackhead in turkeys. J. Exp. Med. 37:851-873.

Tyzzer, E. E., and M. Fabyan. 1922. An 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.

Ueda, H., A. Takagi, K. Katou, and S. Matsumoto. 2002. Feeding behavior in chicks fed tea saponin and quinine sulfate. J. Poult. Sci. 39:34-41.

Ueda, H., and S. Kaidou. 2005. Aversion to quinine is associated with taste sensation in chicks. J. Poult. Sci. 42:254-262.

TABLES AND FIGURES

Table 1. *In vitro* viability assays evaluating selected concentrations of food-grade quinine for antihistomonal properties against two different strains of *Histomonas meleagridis*.[1,2]

Treatment	**Viable *Histomonas* cells/mL (Log_{10})**	**Bacterial CFU/mL (Log_{10})**	
Buford strain	20h	0h	20h
Assay 1			
Negative Control	5.44 ± 0.02^{a}	7.69 ± 0.12^{a}	8.72 ± 0.21^{a}
0.022% Quinine	5.24 ± 0.05^{a}	7.52 ± 0.14^{a}	8.65 ± 0.09^{a}
0.067% Quinine	4.13 ± 0.09^{ab}	7.46 ± 0.24^{a}	8.10 ± 0.10^{b}
0.2% Quinine	1.45 ± 1.45^{b}	7.46 ± 0.09^{a}	6.52 ± 0.04^{c}
Assay 2			
Negative Control	5.30 ± 0.05^{a}	7.64 ± 0.11^{b}	8.37 ± 0.11^{a}
0.022% Quinine	5.19 ± 0.08^{a}	7.93 ± 0.02^{a}	8.20 ± 0.20^{a}
0.067% Quinine	4.57 ± 0.10^{b}	7.83 ± 0.12^{ab}	8.07 ± 0.07^{a}
0.2% Quinine	0.00 ± 0.00^{c}	7.95 ± 0.03^{a}	6.79 ± 0.26^{b}
Assay 3			
Negative Control	5.29 ± 0.05^{a}	7.77 ± 0.12^{a}	8.43 ± 0.13^{a}
0.022% Quinine	4.91 ± 0.04^{b}	7.78 ± 0.04^{a}	8.16 ± 0.16^{a}
0.067% Quinine	3.45 ± 0.10^{c}	7.66 ± 0.06^{a}	8.40 ± 0.10^{a}
0.2% Quinine	0.00 ± 0.00^{d}	7.66 ± 0.12^{a}	6.30 ± 0.00^{b}
PHL2017 strain			
Assay 1			
Negative Control	5.36 ± 0.05^{a}	8.27 ± 0.20^{a}	8.60 ± 0.00^{a}
0.022% Quinine	5.14 ± 0.06^{a}	7.91 ± 0.07^{ab}	8.83 ± 0.02^{a}
0.067% Quinine	4.08 ± 0.09^{b}	7.96 ± 0.06^{ab}	8.06 ± 0.06^{b}
0.2% Quinine	0.00 ± 0.00^{c}	7.87 ± 0.02^{b}	6.33 ± 0.20^{c}
Assay 2			
Negative Control	5.38 ± 0.05^{a}	8.46 ± 0.09^{a}	8.77 ± 0.08^{a}
0.022% Quinine	5.39 ± 0.04^{a}	7.97 ± 0.03^{b}	8.94 ± 0.07^{a}
0.067% Quinine	4.79 ± 0.06^{b}	8.04 ± 0.15^{ab}	8.18 ± 0.09^{b}
0.2% Quinine	0.00 ± 0.00^{c}	8.05 ± 0.18^{ab}	6.20 ± 0.20^{c}
Assay 3			
Negative Control	5.46 ± 0.01^{a}	7.40 ± 0.20^{a}	8.77 ± 0.07^{a}
0.022% Quinine	5.28 ± 0.03^{a}	7.73 ± 0.19^{a}	8.70 ± 0.10^{a}
0.067% Quinine	2.39 ± 1.20^{b}	7.46 ± 0.16^{a}	8.48 ± 0.18^{a}
0.2% Quinine	0.00 ± 0.00^{b}	7.58 ± 0.19^{a}	6.54 ± 0.16^{b}

[a-d]Data expressed as mean ± SE. Statistical evaluation using ANOVA followed by post hoc Tukey's range test. No common superscripts within a column indicate means differ significantly ($P < 0.05$).
[1]Quinine HCl dihydrate (Product #W297607, Sigma-Aldrich, St. Louis, MO) utilized in *in vitro* assays; n=3 replicates/treatment.
[2]Assay seeding density was 5.18 *H. meleagridis* cells/mL (Log_{10}). Media controls were included to verify no initial bacterial contamination was introduced.

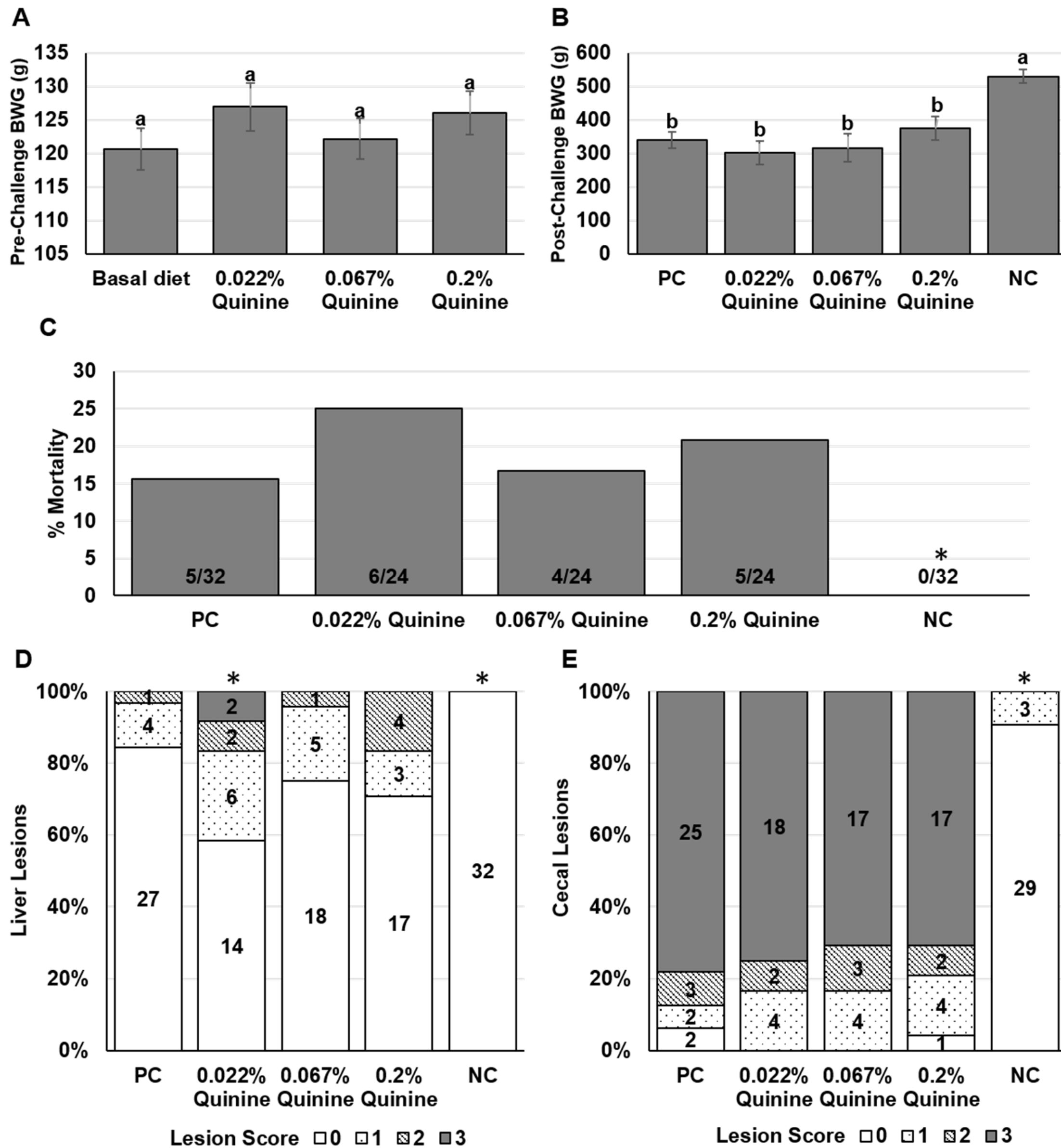

Figure 1. Effect of quinine on body weight gain (**BWG**) at **A**) pre-challenge from d0 to d10 and **B**) post-challenge from d10 to d31. BWG data expressed as mean ± SE and analyzed using ANOVA in JMP Pro 15. Different superscripts denote significance ($P < 0.05$). **C)** Percentage of mortalities associated with histomoniasis. No difference was detected between quinine treatments as compared to the positive-challenged control (**PC**) with a chi-square test. Cumulative lesion scores associated with histomoniasis from d9 to d21 post-challenge for **D)** liver and **E)** cecae. A lesion score of "0" indicates a healthy organ whereas a score of "3" indicates severe histomoniasis. Numbers within columns indicate the number of turkeys per evaluated lesion score. Statistical difference was detected by SAS Proc Mixed Procedure between mean lesion scores as compared to the PC ($^*P < 0.05$). PC and quinine groups were intracloacally challenged with 10^5 histomonads/turkey of *Histomonas meleagridis* (PHL2017 strain) on d10. Negative control (**NC**) received sham challenge. Quinine obtained as quinine HCl dihydrate (Product #W297607; Sigma-Aldrich, St. Louis, MO).

CHAPTER 4: EVALUATION OF LIVE-ATTENUATED *HISTOMONAS MELEAGRIDIS* ISOLATES AS VACCINE CANDIDATES AGAINST WILD-TYPE CHALLENGE[1]

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[1]Portions of mortalities and lesion frequency data (Experiments 1-3) were previously included in Beer's M. S. thesis (2019). These data were used in the current manuscript following re-analysis and with approval from the Doctoral Committee.

Beer, L. C. 2019. Development and evaluation of selected chemoprophylaxis candidates and a candidate live-attenuated vaccine for prevention of histomoniasis in turkeys. Graduate Theses and Dissertations. Retrieved from https://scholarworks.uark.edu/etd/3147.

ABSTRACT

Repeated serial *in vitro* passage of *Histomonas meleagridis*, the etiological agent of histomoniasis (blackhead) of turkeys, was demonstrated to markedly achieve attenuation and reduction of virulence as compared to the original wild-type isolate. Four experiments were performed to evaluate attenuated *H. meleagridis* isolates as vaccine candidates against wild-type challenge. As has been demonstrated previously, wild-type *H. meleagridis* cultures administered orally after one day of age were not infective in these studies, but infection with wild-type cultures could be induced orally at day-of-hatch. Intracloacal inoculation of turkeys with the attenuated passaged isolates as vaccine candidates at d14 was shown to produce significant ($P < 0.05$) protection from mortality, reduction in body weight gain, as well as reduction in hepatic and cecal lesions in these experiments following challenge with either the homologous wild-type isolate or from a wild-type strain obtained years later from a geographically disparate area of the United States. Inoculation with the attenuated *H. meleagridis* isolates at day-of-hatch, either orally or cloacally, did not produce significant protection against subsequent wild-type challenge. While offering significant protection with minimal vaccine-related negative effects, the protection from cloacal vaccine administration was neither significantly robust nor encouraging for industry application using the methods evaluated in the present manuscript.

Key Words: histomoniasis; *Histomonas meleagridis*; live-attenuated; turkey; vaccine

INTRODUCTION

Histomoniasis (synonyms: blackhead disease, infectious enterohepatitis, and histomonosis) is an intestinal protozoal disease of gallinaceous birds with particularly deleterious impact to turkeys (Clarkson, 1963; Hess et al., 2015). Initial infection with the trichomonad parasite *Histomonas meleagridis*, the etiological agent of histomoniasis, is thought to occur via infected *Heterakis gallinarum* cecal worms (Tyzzer, 1934; Lund et al., 1966b; Cupo and Beckstead, 2019). Disease transmission can occur rapidly in turkeys via cloacal drinking, whereby reverse peristalsis quickly uptakes materials into the cloaca and transfers to the cecae (Sorvari et al., 1977; Hu and McDougald, 2003; Hu et al., 2004; McDougald and Fuller, 2005). *H. meleagridis* has been characterized as an extracellular parasite that reproduces through binary fission and can exhibit either amoeboid or flagellated morphology (Tyzzer, 1920; Bayon and Bishop, 1937; Cuckler, 1970). Turkeys should be reared separately from chickens due to the propensity of chickens to serve as reservoirs of histomonads and heterakids (Joyner et al., 1963; Lund et al., 1966b; McDougald, 1998). Since the voluntary removal of nitarsone in 2015, no approved therapeutics or prophylactics are available to treat histomoniasis, leaving turkey flocks to suffer morbidities and mortalities often reaching 80-100% (McDougald, 2005; Hess and McDougald, 2013; Regmi et al., 2016). *In vitro* and *in vivo* studies have yielded inconsistent results for antihistomonal candidate compounds, and no effective alternatives have been introduced to replace the previously used nitroimidazoles, nitrofurans, and arsenical compounds (Grabensteiner et al., 2008; van der Heijden and Landman, 2008a,b; Thøfner et al., 2012). Further complicating this problem, *H. meleagridis* isolates may vary in susceptibility to chemotherapeutics *in vitro* and *in vivo* (Berks and Neal, 1952; Grabensteiner et al., 2007; van der Heijden and Landman, 2008a,b). Without viable substitute treatment options, producers are

suffering losses of turkeys and decreased performance of broiler breeders and layer pullets (Hu and McDougald, 2004; Popp et al., 2011). Although vaccinations are important for the induction of a host-immune response to protect against disease, early immunological studies were discouraging towards successful vaccine development for histomoniasis (Tyzzer, 1934, 1936; Clarkson, 1963; Lund et al., 1966a). Some success with immunization of histomoniasis has been reported experimentally in recent years, but a vaccine has not yet been developed for industry application (McAllister, 2014; Liebhart et al., 2017; Mitra et al., 2018).

Cloacal administration of infected liver or cecal tissue or suspensions of *H. meleagridis* culture has reproduced histomoniasis within experimental settings; however, direct oral ingestion of unprotected histomonads has not reliably induced disease presumably due to adverse acidity and mechanical action within the crop and ventriculus (Berks and Neal, 1952; Hu et al., 2004). Liebhart and Hess (2009) induced histomoniasis with *in vitro* cultivated clonal *H. meleagridis* administered orally to 1-day-old turkeys followed by 5h feed withdrawal. A putative cyst-like structure of *H. meleagridis* was identified *in vitro*; therefore, the oral transmission route for poultry in contact with large amounts of contaminated excreta or litter in the absence of *H. gallinarum* should not be disregarded (Munsch et al., 2009a,b; Zaragatzki et al., 2010a,b). Turkeys recovered from histomoniasis had a semblance of protection upon maturity, but re-infection occurred when birds were kept on infected soil, suggesting only temporary immunity or overload of pathogen exposure (Tyzzer and Fabyan, 1922). Dimetridazole treatment of *H. meleagridis*-infected turkeys resulted in resistance to subsequent infection in the recovered turkeys, further suggesting that acquired protective immunity is possible (Joyner, 1963). Additionally, Cuckler (1970) reported turkeys recovered from histomoniasis were resistant to subsequent challenge, even with maintained presence of histomonads within the cecae, further

supporting the idea of protective immune response development that prevented migration of the protozoa to hepatic tissue.

Tyzzer (1932, 1934, 1936) observed inconsistent reduction in virulence of *H. meleagridis* serially passaged for extended periods of time *in vitro,* and immunization results yielded conflicting success. Following two years of *in vitro* propagation, an isolate originally pathogenic to chickens had lost pathogenicity but was able to induce protection against virulent strains only when allowed to propagate within the chicken's cecae (Tyzzer, 1932). Attenuated isolates were later found to confer immunity against virulent challenge only when the histomonads were administered cloacally and not incorporated into heterakid eggs (Lund, 1959; Lund et al., 1966a). *In vitro* passaging more than 1000 times over a period of 7 years resulted in loss of pathogenicity and efficacy as an immunizing strain for protection against pathogenic *H. meleagridis* (Lund et al., 1967). Prolonged *in vitro* passaging has been reported to decrease vaccination efficacy, and the attenuated histomonads could be restored to original virulence with serial passage in poultry (Dwyer and Honigberg, 1970). *In vitro* attenuation is variable, and susceptibility of chickens and turkeys to histomoniasis varies based on breed (Al-Khateeb et al., 1974; Lotfi et al., 2014). In experimental settings, the oral or cloacal administration of clonal *in vitro* attenuated *H. meleagridis* and subsequent challenge with a virulent isolate has conferred some protection (Hess et al., 2008; Liebhart et al., 2010, 2013; Sulejmanovic et al., 2016). Liver and cecal lesions were reduced in chickens and turkeys following intracloacal administration of attenuated histomonads utilized as a vaccine strain (Hess et al., 2008; Liebhart et al., 2013). Liebhart et al. (2010) demonstrated a protective effect of *in vitro* attenuated *H. meleagridis* administered orally to 1-day-old turkeys, suggesting the necessity to further evaluate this age and route. With prolonged *in vitro* passaging, *H. meleagridis* adapt to cell culture and lose the ability to invade host tissue

as seen in a recent study where an attenuated isolate was observed only in cecal tissue and presumably unable to parasitize other regions (Liebhart et al., 2011). *In vitro* attenuated histomonads have induced protection against virulent challenge without reducing performance (Liebhart et al., 2010, 2013). Pullets vaccinated at 18-weeks-of-age with an attenuated isolate exhibited reduced pathology and prevented the severe drop in egg production observed in unvaccinated pullets (Liebhart et al., 2013). Furthermore, cross-protection against heterologous virulent isolates was demonstrated by vaccinating with an attenuated clonal strain of *H. meleagridis* developed through prolonged *in vitro* propagation (Sulejmanovic et al., 2016). Although known to rely on bacteria for cultivation, the *in vitro* attenuation of *H. meleagridis* occurs independently of culture media bacterial load (Ganas et al., 2012). A low-virulent isolate obtained after intracloacal serial back-passaging in turkeys protected against subsequent virulent challenge (Pham et al., 2013). Stable attenuation of *H. meleagridis* with no reversion to virulence was demonstrated after 295 serial passages *in vitro* and 5 subsequent back-passages *in vivo* (Sulejmanovic et al., 2013).

Intravenous injection of *H. meleagridis*-infected liver tissue did not protect turkeys from subsequent subcutaneous challenge (Tyzzer et al., 1921). Attempts with inactivated vaccine for inducing a humoral response either passively (by intraperitoneal injection of antisera from immune into naïve poultry) or actively (by intramuscular injection of lysed *H. meleagridis* fragments) have also failed to confer protection against virulent challenge (Clarkson, 1963; Hess et al., 2008; Bleyen et al., 2009). IgG increased following administration of attenuated *H. meleagridis* and subsequent virulent challenge, although antibodies do not seem to serve a substantial role in development of protective immunity (Windisch and Hess, 2009, 2010). T and B cell subset deviations were reduced with an attenuated isolate in chickens and turkeys, while

histomoniasis-related mortality in turkeys was associated with higher cellular immune response as compared to chickens (Mitra et al., 2017). An increase of CD4+ T cells occurred in chickens that were challenged with a virulent monoxenic culture of *H. meleagridis* (Lagler et al., 2019). Acquired immunity for histomoniasis may be primarily cell-mediated rather than antibody-based humoral.

Although the feasibility of administering live-attenuated *H. meleagridis* to confer immunity is questionable for meeting industry demand, attenuated histomonads appear to be somewhat efficacious for initiating an immune response to subsequent wild-type challenge (Hess and McDougald, 2013; Hess et al., 2015). Taken together, these data suggest protective immune response against histomoniasis may be possible with administration of live-attenuated *H. meleagridis* isolates. Development of a histomoniasis vaccine would be beneficial to the poultry industry and is encouraged by these immunological research advances. The objectives of this study were to evaluate highly *in vitro*-passaged *H. meleagridis* isolates for protection of turkeys in an experimental challenge model and to further elucidate the possible routes and age for administration of vaccine candidates.

MATERIALS AND METHODS

Animal Source

On day-of-hatch, female poults were obtained from a local commercial hatchery (Cargill, Gentry, AR), individually tagged, and randomly allocated to floor pens at the University of Arkansas Poultry Health Laboratory. All animal handling procedures complied with regulations of the University of Arkansas Institutional Animal Care and Use Committee (IACUC protocols #18113 and #19032). A corn-soy based starter feed meeting the nutrient requirements of poultry

(NRC, 1994) and water were provided *ad libitum*. Mortalities unrelated to histomoniasis were recorded and the altered group numbers are reported.

Histomonas meleagridis *Isolates and Culture*

Two field isolates of *H. meleagridis* were obtained from histomoniasis outbreaks in Georgia (**Buford** strain; isolated from infected chickens) and Arkansas (**PHL2017** strain; isolated from infected turkeys). These wild-type field isolates were serially passaged up to 200 times *in vitro* and selected for evaluation as live-attenuated *H. meleagridis* vaccine candidates (**Vacc**). The strain and passage indicator of Vacc isolates are listed below within corresponding experiments. Challenge in all experiments occurred with wild-type *H. meleagridis* (**WTH**) consisting of low-passaged (< 10 serial passages) Buford strain. According to previously published methods, histomonads were grown in 25cm^2 tissue culture flasks (Product #10062-874, VWR International, Radnor, PA) containing Modified Dwyer's Media (**MDM**) comprised of Medium 199 (Product #12-118F, Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated horse serum (Product #26050-088, Gibco, Life Technologies Corporation, Waltham, MA), 1.6mg/mL organic white rice flour (Arrowhead Mills, Boulder, CO), and an undefined bacterial population from the original field cecal isolate (van der Heijden and Landman, 2005, 2007; Beer et al., 2020). Culture flasks were incubated anaerobically at 40°C for 48-72h before 1mL was sub-cultured into 12.5mL of fresh, supplemented MDM. For long-term preservation of *H. meleagridis*, 10% dimethylsulfoxide (OmniSolv, MilliporeSigma, Burlington, MA) was added as a cryoprotectant, and aliquots were cryogenically stored. Viable *H. meleagridis* cells/mL were enumerated using Trypan blue dye exclusion (Product #15250-061, Gibco) and a hemocytometer. MDM was utilized as the diluent to prepare the proper *H. meleagridis* dosage concentration within all experiments.

Lesion Scoring System

According to previously described methods, liver and cecal lesions were separately scored on a scale of "0" to "3", with "3" indicating the most severe lesion (Beer et al., 2020). Described briefly, healthy liver or cecae received a score of "0"; detectible yet not clinically relevant lesions received a score of "1"; intermediate lesions related to *H. meleagridis*-infection received a score of "2"; and classically confluent lesions related to *H. meleagridis*-infection received a score of "3". Individuals assigning lesion scores (**LS**) were blinded to the treatment groups. All mortalities were evaluated for liver and cecal LS pertaining to histomoniasis.

Experiment 1

The objective of Experiment 1 was to evaluate the efficacy of Vacc administered intracloacally at d14 for protection against subsequent cloacal WTH-challenge.

Vaccination Phase (d14-29). Groups included a non-challenged control (**NC**; n = 59), Vacc (n = 39), and positive-challenged control (**PC**; n = 20). On d14, the Vacc group received a total dose of 2 x 10^5 Vacc Buford P80a cells/turkey, and the PC group received a total dose of 2 x 10^5 Buford WTH cells/turkey (**Figure 1A**). Intracloacal administration occurred with an animal gavage needle and occurred twice at half dosage with 1h between each inoculation. On d27, the PC group was humanely euthanized to evaluate characteristic disease lesions and compared against the Vacc group. On d28, a subset of n = 5 turkeys/group was sampled from the NC and Vacc groups to evaluate for lesions. Individual body weights were recorded from the Vacc and NC groups on d14.

Challenge Phase (d29-40). On d29, individual body weights were recorded from all groups, and all groups except for the NC were intracloacally challenged with 2 x 10^5 Buford WTH cells/turkey using the procedure described above. A newly introduced PC group (n = 27)

was created from a subset of the NC group (n = 27 remaining) to serve as concurrent reference against the Vacc group (n = 34 remaining). On d40, individual body weights were recorded, and all remaining poults were humanely euthanized and subsequently evaluated for liver and cecal LS.

Experiment 2

The objective of Experiment 2 was to evaluate different doses and routes of Vacc administered at day-of-hatch to protect against subsequent cloacal WTH-challenge.

Vaccination Phase (d0-21). Groups included NC (n = 34), Vacc Oral 2 x 10^3 (n = 36; **Vacc Oral 2k**), Vacc Oral 2 x 10^4 (n = 38; **Vacc Oral 20k**), Vacc Cloacal 2 x 10^3 (n = 36; **Vacc Cloacal 2k**), Vacc Cloacal 2 x 10^4 (n = 37; **Vacc Cloacal 20k**), Vacc Cloacal 2 x 10^5 (n = 30; **Vacc Cloacal 200k**), and PC Cloacal 2 x 10^5 (n = 36; **PC Cloacal 200k**). On day-of-hatch and prior to feeding, Vacc group turkeys were either orally or intracloacally administered with respective dose of Vacc Buford P80a cells/turkey (**Figure 1B**). The PC Cloacal received 2 x 10^5 Buford WTH cells/turkey administered intracloacally. A total of 30 day-of-hatch poults were humanely euthanized prior to feeding, and the pH of the combined proventriculus-ventriculus region was measured of each poult using pH indicator strips (Sigma-Aldrich, St. Louis, MO). On d15, a subset from each group was evaluated for liver and cecal LS to compare the Vacc groups to the PC Cloacal group, leaving a remaining subset of n = 20 from each Vacc group for the Challenge Phase. Individual body weights were recorded on d0, d7, d14, and d21, except for the PC Cloacal group which was terminated on d15 for liver and cecal LS.

Challenge Phase (d21-35). On d21, the NC poults were reallocated into new groups consisting of PC Oral (n = 14) and PC Cloacal (n = 20). On d21, all groups received intracloacal challenge of 2 x 10^5 total Buford WTH cells/turkey in a pair of inoculations, except for the PC

Oral group which received this dose orally in a single administration. On d35, individual body weights were recorded, and all remaining poults were humanely euthanized and evaluated for liver and cecal LS.

Experiment 3

The objective of Experiment 3 was to further evaluate the Vacc administered at day-of-hatch or d14 to protect against subsequent cloacal WTH-challenge. Additionally, the oral and cloacal administration routes at day-of-hatch were compared between Vacc and WTH isolates.

Vaccination Phase 1 (d0-14). Groups included NC (n = 216), d0 Vacc Oral (n = 53), d0 Vacc Cloacal (n = 52), d0 Oral PC (n = 60), and d0 Cloacal PC (n = 60). On day-of-hatch and prior to feeding, turkeys were either orally or intracloacally administered 2 x 10^5 cells/turkey of either Vacc Buford P80a or Buford WTH (**Figure 1C**). A total of 60 day-of-hatch poults were humanely euthanized prior to feeding, and the pH of the combined proventriculus-ventriculus region was measured of each poult using pH indicator strips (Sigma-Aldrich). On d14, all turkeys from the PC Oral and PC Cloacal groups were humanely euthanized and evaluated for liver and cecal LS. A total of n = 10 turkeys/group were likewise evaluated from the NC, d0 Vacc Oral, and d0 Vacc Cloacal groups to compare to the PC groups. Individual body weights were recorded on d0 and d14.

Vaccination Phase 2 (d14-28). On d14, the newly introduced PC Cloacal (n = 43) and Vacc Cloacal (n = 55) groups were created from subsets of the NC group (n = 108 remaining). The PC Cloacal and d14 Vacc Cloacal groups were intracloacally administered 2 x 10^5 cells/turkey of either Buford WTH or Vacc Buford P80a, respectively. On d28, all poults from the PC Cloacal were humanely euthanized and evaluated for liver and cecal LS. A total of n = 10 turkeys/group were likewise evaluated from the NC and d14 Vacc Cloacal groups; n = 5

turkeys/group were evaluated from the d0 Vacc Oral and d0 Vacc Cloacal groups. Individual body weights were recorded on d28.

Challenge Phase (d28-42). On d28, the newly introduced PC Cloacal (n = 45) was created from a subset of the NC group (n = 53 remaining). On d28, all turkeys except for the NC group were intracloacally challenged with 2 x 10^5 Buford WTH cells/turkey. On d42, individual body weights were recorded, and all remaining turkeys were humanely euthanized and evaluated for liver and cecal LS.

Experiment 4

The objective of Experiment 4 was to evaluate Buford and PHL Vacc isolates intracloacally administered at d14 to protect against subsequent cloacal Buford WTH-challenge; therefore, to compare efficacy of Vacc isolates to homologous and heterologous WTH-challenge.

Vaccination Phase (d14-35). Groups included NC (n = 69), PC Buford (n = 60), Vacc PHL P67 (n = 59), Vacc PHL P129 (n = 60), Vacc Buford P80a (n = 60), Vacc Buford P200a (n = 60), Vacc Buford P138b (n = 59), and Vacc Buford P198c (n = 60). On d14, the Vacc groups were intracloacally administered 2 x 10^5 cells/turkey of the respective Vacc isolate, and the PC Buford group received a total dose of 2 x 10^5 Buford WTH cells/turkey (**Figure 1D**). On d28, subsets of n = 10 turkeys/group were evaluated from the Vacc groups to compare liver and cecal LS to subsets of the NC (n = 5) and PC Buford (n = 34). On d35, subsets of n = 10 turkeys/group were likewise evaluated from the Vacc groups to compare LS to subsets of the NC (n = 5) and the remainder of PC Buford (n = 26). Individual body weights were recorded on d14, d28, and d34.

Challenge Phase (d35-49). On d35, the newly introduced PC Buford group (n = 39) was created from a subset of the NC group (n = 20 remaining). On d35, all groups except for the NC

group were intracloacally challenged with a total of 2 x 10^5 Buford WTH cells/turkey. On d49, individual body weights were recorded, and all remaining turkeys were humanely euthanized and evaluated for liver and cecal LS.

Statistical Analysis

Differences in mortalities and each subgrouping of LS were compared to the PC group using the chi-square test. BWG data were analyzed using JMP Pro 16 software (SAS Institute Inc., Cary, NC) with significant differences between treatment groups determined using ANOVA. Tukey's multiple range test was used to further separate the means, where applicable. LS data were analyzed using the Proc Mixed Procedure in SAS 9.4 software (SAS Institute). Statistical significance for all analyses was set at $P < 0.05$.

RESULTS

Experiment 1: d14 Vacc Administration

Vaccination Phase (d14-29). Histomoniasis-related mortalities in the PC, Vacc, and NC groups were 30.0, 0.00, and 0.00%, respectively (**Table 1**). The Vacc and NC group mortalities were significantly different ($P < 0.05$) as compared to the PC group. No difference ($P > 0.05$) in d14-29 BWG was observed between the Vacc group as compared to the NC group. On d27, cumulative lesions of the PC group revealed 75% liver and 80% cecal lesions characteristic of histomoniasis (data not shown). On d28 among the Vacc subset (n = 5) examined, one turkey exhibited normal liver and cecae under macroscopic examination. Two turkeys exhibited normal livers and relatively normal cecae except for small, button-like lesions. One turkey had target-like liver lesions with the cecae feeling hard, thickened, and exhibiting larger bumps and scalloping. The fifth turkey exhibited pale liver edges with narrow and thin margins and was possibly beginning to develop liver lesions; in addition, the cecae were large, with the presence

of thickened walls and scalloping. No histomoniasis-related lesions were observed in the NC group at any time.

Challenge Phase (d29-40). Histomoniasis-related mortalities in the PC, Vacc, and NC groups were 22.2, 2.94, and 0.00%, respectively (**Table 1**). The Vacc and NC group mortalities were different ($P < 0.05$) as compared to the PC group. The d29-40 BWG for Vacc and PC groups were significantly lower ($P < 0.05$) as compared to the NC group; however, the Vacc group BWG was significantly higher ($P < 0.05$) than the PC group. The Vacc group resulted in lowered ($P < 0.05$) mean liver and cecal LS as compared to the PC group (**Table 2**). From all turkeys evaluated, those with a positive liver LS for the PC, Vacc, and NC groups were 95.7, 29.4, and 3.70% while those with a positive cecal LS were 95.7, 70.6, and 7.41%, respectively. From all turkeys evaluated, those with a positive LS of "1-3" were further considered with the breakdown as follows: positive liver LS and positive cecal LS for the PC, Vacc, and NC groups were 100, 41.7, and 0.00%; negative liver LS and positive cecal LS were 0.00, 58.3, and 66.7%; positive liver LS and negative cecal LS were 0.00, 0.00, and 33.3%, respectively. Within the NC group, LS were only a score of "1" and were not considered to be related to histomoniasis according to the scoring system. Frequencies of liver and cecal LS for each group are shown in **Figure 2A and 2B**.

Experiment 2: Day-of-Hatch Vacc Administration

Vaccination Phase (d0-21). A mean pH of 4.4 was determined from the proventriculus-ventriculus region from the day-of-hatch poult subset prior to feeding. Histomoniasis-related mortalities in the PC Cloacal 200k, Vacc Oral 2k, Vacc Oral 20k, Vacc Cloacal 2k, Vacc Cloacal 20k, Vacc Cloacal 200k, and NC groups were 22.2, 2.78, 2.63, 0.00, 5.41, 3.33, and 0.00%, respectively (**Table 3**). The Vacc and NC group mortalities were lower ($P < 0.05$) as compared

to the PC Cloacal 200k group. The Vacc Cloacal 20k group had higher ($P < 0.05$) d0-7 BWG as compared to the PC Cloacal 200k and was not different ($P > 0.05$) from the NC group. The Vacc 200k group had lower ($P < 0.05$) d0-7 BWG as compared to the NC group, but the groups were similar ($P > 0.05$) for d7-14, d14-21, and d0-21 BWG. The d7-14 BWG for Vacc Oral 20k and Cloacal 20k groups were higher ($P < 0.05$) as compared to the PC Cloacal 200k and were not different from the NC group. The d14-21 BWG and d0-21 BWG for the Vacc Cloacal 20k group were higher ($P < 0.05$) as compared to the Vacc Cloacal 200k and NC groups. On d15, the mean liver and cecal LS were lower in all Vacc groups as compared to the PC Cloacal 200k (**Table 4**). From all turkeys evaluated, those with a positive liver LS were lower ($P < 0.05$) in all Vacc groups as compared to the PC Cloacal 200k; those with a positive cecal LS were lower ($P < 0.05$) in all Vacc groups, except for the Vacc Oral 20k group, as compared to the PC Cloacal 200k. Further comparisons of turkeys with a positive LS of "1-3" are shown in **Table 4**; frequencies of liver and cecal LS for each group are shown in **Figure 3A and 3B**.

Challenge Phase (d21-35). Histomoniasis-related mortalities in the PC Cloacal 200k, Vacc Oral 2k, Vacc Oral 20k, Vacc Cloacal 2k, Vacc Cloacal 20k, Vacc Cloacal 200k, and PC Oral 200k were 55.0, 50.0, 50.0, 60.0, 55.0, 35.0, and 0.00% respectively (**Table 5**). The PC Oral 200k group mortalities were lower ($P < 0.05$) as compared to the PC Cloacal 200k group. Mortalities in the Vacc groups were not different ($P > 0.05$) as compared to the PC Cloacal 200k group. The d21-35 BWG was similar ($P > 0.05$) for all groups. On d35, the mean liver and cecal LS were similar ($P > 0.05$) for all Vacc groups as compared to the PC Cloacal 200k group (**Table 4**). The PC Oral group received LS of only "0", indicating no detectable lesions associated with histomoniasis. From all turkeys evaluated, those with a positive liver or cecal LS were similar ($P > 0.05$) in all Vacc groups as compared to the PC Cloacal 200k. Further

comparisons of turkeys with a positive LS of "1-3" are shown in **Table 4**; frequencies of liver and cecal LS for each group are shown in **Figure 3C and 3D**.

Experiment 3: Day-of-Hatch vs. d14 Vacc Administration

Vaccination Phase 1 (d0-14). A mean pH of 5.0 was determined from the proventriculus-ventriculus region from the day-of-hatch poult subset prior to feeding. Histomoniasis-related mortalities in the PC Cloacal, PC Oral, d0 Vacc Oral, d0 Vacc Cloacal, and NC groups were 15.0, 16.7, 0.00, 0.00, and 0.00%, respectively (**Table 6**). The Vacc and NC groups mortalities were lower ($P < 0.05$) as compared to the PC Cloacal group. The d0 Vacc Cloacal group had higher ($P < 0.05$) d0-14 BWG than the PC Cloacal and PC Oral groups and was not different ($P > 0.05$) from the NC group. The mean liver and cecal LS were lower ($P < 0.05$) in the Vacc and NC groups as compared to the PC Cloacal and PC Oral groups (**Table 7**). The PC Oral group had lower ($P < 0.05$) mean liver LS as compared to the PC Cloacal group. From all turkeys evaluated, those with a positive liver LS were lower ($P < 0.05$) in all groups as compared to the PC Cloacal; those with a positive cecal LS were lower ($P < 0.05$) in the Vacc and NC groups as compared to the PC Cloacal. Further comparisons of turkeys with a positive LS of "1-3" are shown in **Table 7**; frequency of liver and cecal LS for each group are shown in **Figure 4A and 4B**.

Vaccination Phase 2 (d14-28). No histomoniasis-related mortalities occurred in the d0 Vacc Oral, d0 Vacc Cloacal, d14 Vacc Cloacal, or NC groups by d28, whereas the PC Cloacal group reached 48.8% (**Table 6**). All Vacc groups had higher ($P < 0.05$) d14-28 BWG than the PC Cloacal group and were not different ($P > 0.05$) from the NC group. The d0 Vacc Oral, d0 Vacc Cloacal, d14 Vacc Cloacal, and NC groups were lower in mean liver and cecal LS as compared to the PC Cloacal group (**Table 7**). From all turkeys evaluated, those with a positive

liver or cecal LS were lower ($P < 0.05$) in the Vacc Cloacal and NC groups as compared to the PC Cloacal. Further comparisons of turkeys with a positive LS of "1-3" are shown in **Table 7**; frequencies of liver and cecal LS for each group are shown in **Figure 4C and 4D**.

Challenge Phase (d28-42). Histomoniasis-related mortalities in the PC Cloacal, d0 Vacc Oral, d0 Vacc Cloacal, d14 Vacc Cloacal, and NC groups were 42.2, 44.7, 32.4, 22.2, and 0.00%, respectively (**Table 6**). The Vacc groups were similar ($P > 0.05$) for d28-42 BWG as compared to the PC Cloacal group; however, the d14 Vacc Cloacal group was also not different ($P > 0.05$) for d28-42 BWG as compared to the NC group. The d14 Vacc Cloacal group had lower ($P < 0.05$) mean liver LS than the PC Cloacal group (**Table 7**). From all turkeys evaluated, those with a positive liver LS were lower ($P < 0.05$) in d14 Vacc Cloacal and NC groups as compared to the PC Cloacal. Further comparisons of turkeys with a positive LS of "1-3" are shown in **Table 7**; frequencies of liver and cecal LS for each group are shown in **Figure 4E and 4F**.

Experiment 4: d14 Vacc Administration with Homologous or Heterologous WTH-challenge

Vaccination Phase (d14-35). No histomoniasis-related mortalities occurred in the Vacc PHL P67, Vacc PHL P129, Vacc Buford P80a, Vacc Buford P200a, Vacc Buford P138b, Vacc Buford P198c, or NC groups whereas the PC Buford group reached 41.7% by d28 (**Table 8**). All Vacc groups had higher ($P < 0.05$) d13-34 BWG than the PC Buford group and were not different ($P > 0.05$) than the NC group. All Vacc groups had lower ($P < 0.05$) liver and cecal LS for d28 and d35 as compared to the PC Buford group (**Table 9**). From all turkeys evaluated on d28, those with a positive liver LS were lower ($P < 0.05$) in the Vacc and NC groups as compared to the PC Cloacal; those with a positive cecal LS were lower ($P < 0.05$) in the Vacc Buford P80a, Vacc Buford P200a, and NC groups as compared to the PC Cloacal. From all

turkeys evaluated on d35, those with a positive liver LS were lower ($P < 0.05$) in all Vacc and NC groups, except for the Vacc Buford P200a group, as compared to the PC Cloacal; those with a positive cecal LS were lower ($P < 0.05$) in all Vacc and NC groups, except for the Vacc PHL P129 and Vacc Buford P198c, as compared to the PC Cloacal. Further comparisons of turkeys with a positive LS of “1-3” are shown in **Table 9**; frequencies of liver and cecal LS for each group are shown in **Figure 5A-D**.

Challenge Phase (d35-49). Histomoniasis-related mortalities in the PC Buford, Vacc PHL P67, Vacc PHL P129, Vacc Buford P80a, Vacc Buford P200a, Vacc Buford P138b, Vacc Buford P198c, and NC groups were 61.5, 20.5, 22.5, 17.5, 7.50, 30.8, 17.5, and 0.00%, respectively (**Table 8**). The Vacc PHL P67 group had higher ($P < 0.05$) d34-49 BWG as compared to the PC Buford group and was not different ($P > 0.05$) than the NC group. All Vacc groups, except for the Vacc PHL P129 group, had significantly lower ($P < 0.05$) mean liver LS as compared to the PC Buford group (**Table 9**). The Vacc PHL P67, Vacc Buford P80a, Vacc Buford P138b, and Vacc Buford P198c groups had significantly lower ($P < 0.05$) mean cecal LS as compared to the PC Buford group. From all turkeys evaluated, positive liver LS were lower ($P < 0.05$) in all the Vacc groups and the NC group as compared to the PC Buford group; positive cecal LS were lower in the Vacc Buford P80a and NC groups as compared to the PC Buford group. Further comparisons of turkeys with a positive LS of “1-3” are shown in **Table 9**; frequencies of liver and cecal LS for each group are shown in **Figure 5E and 5F**.

DISCUSSION

During the Vaccination Phase of all experiments, mortalities and mean LS were lower ($P < 0.05$) in the Vacc groups regardless of dose, route, or attenuated isolate (Vacc Buford or Vacc PHL) when compared to the WTH PC Cloacal group. Additionally, there was no difference ($P >$

0.05) in BWG with d14 cloacal administration of the Vacc Buford P80a isolate as compared to the NC group, indicating that the Vacc administered alone did not harm performance. Moreover, BWG was improved ($P < 0.05$) with d14 cloacal administration of the Vacc Buford P80a isolate as compared to the WTH PC Cloacal group during the Vaccination Phases (Experiments 1, 3, and 4). These results are consistent with previous research indicating the safety of attenuated *H. meleagridis* administration (Hess et al., 2008; Liebhart et al., 2010, 2011, 2013). During the Challenge Phase, the d14 cloacally administered Vacc Buford P80a group resulted in lowered mortalities and liver LS ($P < 0.05$) than the WTH PC Cloacal group (Experiments 1, 3, and 4), suggesting that this might be an efficacious option to prevent histomoniasis.

Long-term *in vitro* passaging of *H. meleagridis* can eventually reduce the ability to parasitize host tissue or to confer an immune response; however, studies have reported stable attenuation of histomonads without reversion to virulence upon serial back-passage in the bird (Tyzzer, 1936; Lund et al., 1966a, 1967; Sulejmanovic et al., 2013). Meanwhile, successful vaccination with *in vitro* attenuated (passage 295) clonal *H. meleagridis* induced protection in turkeys subsequently challenged with a virulent isolate (passage 21); the attenuated histomonads were restricted to the cecae with reduced pathogenicity (Liebhart et al., 2011). Although histomoniasis was not completely prevented in our study, the lowered LS and decreased mortalities during the Challenge Phase suggest the Vacc Buford P80a isolate is sufficiently attenuated to stimulate the turkey's immune response without resulting in Vacc-related lethality or rampant disease (Experiments 1, 3, and 4). A similar response was observed with the Vacc PHL P67 isolate (Experiment 4). Taken together, these data suggest intracloacal administration of live-attenuated *H. meleagridis* at d14 to turkeys appears to induce acquired immunity, which aligns with previous research (Lund et al., 1967; Hess et al., 2008; Pham et al., 2013). Incidence

of cecal LS of "2" and "3" occurring in the Vacc Buford P80a group following Buford WTH-challenge (**Figures 2B**, **4F**, and **5F)** suggests robust immunity did not occur; therefore, the case reproductive rate is unlikely to decrease, which potentially allows horizontal transmission to occur due to residual cecal infection and shedding. This lack of robust immunity to completely protect against LS or mortalities following WTH-challenge suggests more research is necessary before live-attenuated *H. meleagridis* can be recommended as an industry relevant vaccination option for histomoniasis. Complete protection against disease was not conferred and intracloacal administration of live-attenuated *H. meleagridis* would be both labor-intensive and economically unfeasible for commercial application at a large industry level. Lund (1959) experimented with heterakid eggs to deliver attenuated histomonads, but immunization via this method was not protective. Even if it were possible to incorporate the Vacc isolates into a heterakid delivery system to provide a possible method of mass-scale administration, the variation in protection and inconsistency of response to WTH-challenge is concerning.

Liebhart et al. (2010) reported that oral vaccination of turkeys at day-of-hatch with clonal live-attenuated *H. meleagridis* effectively protected against subsequent intracloacal challenge with clonal WTH. Conversely, Experiments 2 and 3 indicated day-of-hatch administration of the Buford Vacc P80a isolate either orally or cloacally was not effective ($P > 0.05$) in reducing mortalities or LS upon subsequent challenge with Buford WTH, and BWG was not improved ($P > 0.05$) as compared to the PC Cloacal group. Within the current experiments, only the cloacal route at d14 appeared to be efficacious for inducing protection with *H. meleagridis* Vacc as compared to the d0 oral administration route. Previous research by Sulejmanovic et al. (2016) suggested cloacal booster administrations would be needed at d14 if attenuated *H. meleagridis* are administered orally as a vaccine at day-of-hatch, which is further discouraging for relevance

and practicality to the turkey industry. The optimum vaccination window was possibly missed with the day-of-hatch vaccination in our experiments; but if so, the method of boosters would still not seem promising as a commercial-scale application for industry.

In the Vaccination Phase of Experiment 2, low Vacc-related mortalities and cecal LS occurred in the Vacc Oral (2k and 20k doses) and Vacc Cloacal (20k and 200k doses) groups. The d0 Vacc Oral (200k dose) group in Experiment 3 also exhibited low Vacc-related liver and cecal LS during Vaccination Phases 1 and 2. Since the *H. meleagridis* Vacc isolate was not an established clonal population, low levels of virulent histomonads potentially remaining in the culture could have contributed to low LS and mortalities. Alternatively, turkeys could have greater susceptibility to infection at day-of-hatch prior to feeding, even with apparently live-attenuated *H. meleagridis*. The more likely hypothesis would be that the variation in mortalities and LS frequency could be a result of population differences within the Vacc isolate. The high levels of *in vitro* propagation and replication by binary fission could lead towards a consistent population of histomonads adapted for an *in vitro* environment and thereby a relatively homogenous live-attenuated culture with low-virulent properties (Lund et al., 1966a, 1967). The Vacc isolates in these experiments remain a potential mixture of genotypes since they were not single-cell cloned; the Buford WTH-challenge also originated from a field outbreak potentially containing multiple genotypes and better simulating realistic challenge conditions. The lack of complete protection against either homologous or heterologous WTH-challenge would suggest that the use of Vacc isolates may not be efficacious for conferring robust immune protection. Although the Vacc isolates used in these experiments are not conclusively clonal populations and potentially contain a greater diversity of genotypes, this diversity would be expected to better mimic a real-world scenario where turkeys are not exposed to a single isolate at any given time.

The possible incidence of different genotypes remaining in either the WTH or Vacc isolates could arguably be considered more efficacious for inducing broad protection against re-infection, but regardless, only partial immunity seemed to be imparted with this methodology.

In Experiment 4, the Vacc PHL P67 offered some protection against heterologous challenge with Buford WTH, as indicated by lowered liver and cecal LS ($P < 0.05$) similar to the Vacc Buford P80a group response (Experiments 1, 3, and 4) as compared to the WTH PC Buford. The Vacc Buford P138b and Vacc Buford P198c groups also resulted in lowered lesions ($P < 0.05$) following challenge with Buford WTH. Since *H. meleagridis* reproduces by binary fission, the Buford and PHL isolates are likely to be genetically different from each other due to the temporal and geographical differences from when these isolates were obtained. Recent research indicates variation in virulence factors and pathogenicity of *H. meleagridis* isolates obtained from different geographical sources (Wei et al., 2020). Interestingly, the similar efficacy of protection of the Vacc PHL (particularly P67) and Vacc Buford (particularly P80a) isolates following challenge with Buford WTH in Experiment 4 suggest no serotype differences between isolates. The similarity in response of Vacc PHL P67 as the Vacc Buford P80a isolate for immunoprophylaxis against heterologous Buford WTH-challenge is encouraging and consistent with previous research showing that attenuated *H. meleagridis* can induce cross-protective immunity to heterologous isolates (Sulejmanovic et al., 2016).

Absence of detectable lesions, as indicated by LS of “0” within a subset of the PC group, could have resulted from a difference of susceptibility to Buford WTH-challenge, variation in cecal retrograde of the inoculum, or expulsion of the inoculum before cloacal uptake in some turkeys. Although passages of the Buford WTH isolate were reduced to prevent *in vitro* attenuation, changes in virulence or population are possible with each propagation depending on

spontaneous mutation occurrences and media adaptation. Nevertheless, since field isolates of *H. meleagridis* are certainly of varied genotype and virulence, turkeys would be exposed to more than one strain in an industry setting (Bilic et al., 2014). The consideration remains that the non-clonal yet attenuated Vacc strains reported above did not induce vigorous protection to histomoniasis; therefore, immunization with live-attenuated *H. meleagridis* remains doubtful for industry purposes. Although if future studies were to be conducted, WTH and Vacc isolates should be single-cell cloned to ensure the same genetic population is being evaluated in subsequent experimental situations. Efficacy of clonal vaccination for non-clonal field challenge conditions remains to be evaluated.

Interestingly, the PC Oral 200k group (Experiment 2) resulted in no mortalities or LS following oral challenge with Buford WTH on d21, which is consistent with the prevailing understanding that unprotected *H. meleagridis* do not survive the low pH within the proventriculus-ventriculus region. Conversely, the PC Oral group (Experiment 3) challenged with Buford WTH on d0 was similar in mortalities and cecal LS as compared to the PC Cloacal group, indicating susceptibility of turkeys at early age to *H. meleagridis*-infection. Previous studies with chickens have demonstrated that feed deprivation and an alkaline pH prior to oral challenge resulted in the development of lesions characteristic with histomoniasis; therefore, potential oral transfer of *H. meleagridis* should not be discounted (Cuckler, 1970). An average pH of 3.5 has been reported in the proventriculus-ventriculus region of broiler chickens following feed ingestion with variability between a pH of 1.9 and 4.5 (Svihus, 2011). The susceptibility of day-of-hatch turkeys to oral *H. meleagridis*-infection prior to feeding could be potentially explained by pH closer to neutral (measured as 4.4 and 5.0 in Experiments 2 and 3, respectively) within the proventriculus-ventriculus region. Environmental pH could have allowed

the histomonads to survive long enough to reach the cecae and parasitize the tissue. If repeated in future studies, pH should also be measured in a subset of turkeys post-feeding to compare to pre-feeding measurements. Recently, a purported cyst-like stage of *H. meleagridis* has been observed *in vitro* which could function in oral transmission but has not been elucidated *in vivo* (Munsch et al., 2009a,b; Zaragatzki et al, 2010a,b; Gruber et al., 2017).

Intracloacal administration of attenuated histomonads has previously provided some immunoprophylaxis against virulent isolates, but further research is warranted to elucidate the most efficacious administration route, dose, and age for the inoculation procedure since the current methods do not induce robust immunity and are not an applicable industry solution (Pham et al., 2013; Sulejmanovic et al., 2016). Furthermore, although male and female turkeys have similar susceptibility to infection with *H. meleagridis,* variation occurs between genetic lines (van der Heijden and Landman, 2008; Liebhart et al., 2008; Abdul-Rahman and Hafez, 2009). A major limiting factor to large-scale production of Vacc isolates is the requirement for cell culture which is impractical for mass production because histomonads grow at varied rates and culture media is relatively costly. Efficient methods to feasibly propagate *H. meleagridis* to meet commercial production needs would be challenging, and the d14 intracloacal administration would not be practical for large-scale application to the turkey industry. Vaccine administration at day-of-hatch via the oral route would be ideal for incorporation within the hatchery but results are conflicting. Our data indicate only the cloacal route at d14 to be an effective administration method for Vacc but with only partial protection to subsequent WTH-challenge. Within these experiments, the NC group did not exhibit mortalities or LS from histomoniasis, further confirming that management and absence of exposure are crucial to preventing this disease. In conclusion, considering the research completed previously and as reported above, vaccination

seems possible yet impractical for industry application with the current methods. Acquired immunity appears achievable but is not robust using the current methodology.

REFERENCES

Al-Khateeb, G. H., and M. F. Hansen. 1974. Plasma enzymes as a measure of susceptibility of chickens and turkeys to infection with *Histomonas meleagridis.* Avian Dis. 18:507-514.

Bayon, H. P., and A. Bishop. 1937. Cultivation of *Histomonas meleagridis* from the liver lesions of a hen. Nature. 139:370-371.

Beer, L. C., C. N. Vuong, T. L. Barros, J. D. Latorre, G. Tellez, A. L. Fuller, and B. M. Hargis. 2020. Research Note: Evaluation of boric acid as a chemoprophylaxis candidate to prevent histomoniasis. Poult. Sci. 99:1978-1982.

Berks, G., and R. Neal. 1952. The effect of some drugs upon *Histomonas meleagridis in vitro*. Ann. Trop. Med. Parasitol. 46:68-71.

Bilic, I., B. Jaskulska, R. Souillard, D. Liebhart, and M. Hess. 2014. Multi-locus typing of *Histomonas meleagridis* isolates demonstrates the existence of two different genotypes. PLoS One. 9:e92438.

Bleyen, N., E. Ons, M. De Gussem, and B. M. Goddeeris. 2009. Passive immunization against *Histomonas meleagridis* does not protect turkeys from an experimental infection. Avian Pathol. 38:71-76.

Clarkson, M. 1963. Immunological responses to *Histomonas meleagridis* in the turkey and fowl. Immunology. 6:156-168.

Cuckler, A. 1970. Coccidiosis and histomoniasis in avian hosts. Pages 371-397 in Immunity to parasitic animals. G. J. Jackson, R. Herman and I. Singer, eds., New York.

Cupo, K. L., and R. B. Beckstead. 2019. *Heterakis gallinarum,* the cecal nematode of gallinaceous birds: A critical review. Avian Dis. 63:381-388.

Dwyer, D. M., and B. M. Honigberg. 1970. Effect of certain laboratory procedures on the virulence of *Histomonas meleagridis* for turkeys and chickens. J. Parasitol. 56:694-700.

Ganas, P, D. Liebhart, M. Glösmann, C. Hess, and M. Hess. 2012. *Escherichia coli* strongly supports the growth of *Histomonas meleagridis,* in a monoxenic culture, without influence on its pathogenicity. Int. J. Parasitol. 42:893-901.

Grabensteiner, E., N. Arshad, and M. Hess. 2007. Differences in the *in vitro* susceptibility of mono-eukaryotic cultures of *Histomonas meleagridis, Tetratrichomonas gallinarum* and *Blastocystis* sp. to natural organic compounds. Parasitol. Res. 101:193-199.

Grabensteiner, E., D. Liebhart, N. Arshad, and M. Hess. 2008. Antiprotozoal activities determined *in vitro* and *in vivo* of certain plant extracts against *Histomonas meleagridis, Tetratrichomonas gallinarum,* and *Blastocystis* sp. Parasitol Res. 103:1257-1264.

Gruber, J., P. Ganas, and M. Hess. 2017. Long-term *in vitro* cultivation of *Histomonas meleagridis* coincides with the dominance of a very distinct phenotype of the parasite exhibiting increased tenacity and improved cell yields. Parasitology. 144:1253-1263.

van der Heijden, H. M. J. F., and W. J. M. Landman. 2007. Improved culture of *Histomonas meleagridis* in a modification of Dwyer medium. Avian Dis. 51:986-988.

van der Heijden, H. M. J. F., and W. J. M. Landman. 2008a. *In vitro* effect of herbal products against *Histomonas meleagridis* in turkeys. Vet. Parasitol. 154:1-7.

van der Heijden, H. M. J. F., and W. J. M. Landman. 2008b. *In vivo* effect of herbal products against *Histomonas meleagridis* in turkeys. Avian Pathol. 37:45-50.

van der Heijden, H. M. J. F., L. R. McDougald, and W. J. M. Landman. 2005. High yield of parasites and prolonged *in vitro* culture of *Histomonas meleagridis*. Avian Pathol. 34:505-508.

Hess, M., D. Liebhart, I. Bilic, and P. Ganas. 2015. *Histomonas meleagridis*—New insights into an old pathogen. Vet. Parasitol. 208:67-76.

Hess, M., D. Liebhart, E. Grabensteiner, and A. Singh. 2008. Cloned *Histomonas meleagridis* passaged *in vitro* resulted in reduced pathogenicity and is capable of protecting turkeys from histomonosis. Vaccine. 26:4187-4193.

Hess, M., and L. McDougald. 2013. Histomoniasis (blackhead) and other protozoan diseases of the intestinal tract. Pages 1172-1178 in Diseases of Poultry.13th ed. E. Swayne, J. R. Glisson, L. R. McDougald, L. K. Nolan, D. L. Suarez, and V. L. Nair, eds., Wiley-Blackwell, Ames, IA.

Hu, J., L. Fuller, and L. R. McDougald. 2004. Infection of turkeys with *Histomonas meleagridis* by the cloacal drop method. Avian Dis. 48:746-750.

Hu, J., and L. R. McDougald. 2003. Direct lateral transmission of *Histomonas meleagridis* in turkeys. Avian Dis. 47:489-492.

Hu, J., and L. R. McDougald. 2004. The efficacy of some drugs with known antiprotozoal activity against *Histomonas meleagridis* in chickens. Vet. Parasitol. 121:233-238.

Joyner, L. P. 1963. Immunity to histomoniasis in turkeys following treatment with dimetridazole. J. Comp. Pathol. Ther. 73:201-207.

Joyner, L. P., S. F. M. Davies, and S. B. Kendall. 1963. Chemotherapy of histomoniasis. Pages 333-349 in Experimental Chemotherapy. Vol. 1. Academic Press New York.

Lagler, J., T. Mitra, S. Schmidt, A. Pierron, E. Vatzia, M. Stadler, S. E. Hammer, K. H. Mair, B. Grafl, P. Wernsdorf, F. Rauw, B. Lambrecht, D. Liebhart, and W. Gerner. 2019. Cytokine production and phenotype of *Histomonas meleagridis*-specific T cells in the chicken. Vet. Res. 50:107.

Liebhart, D., P. Ganas, T. Sulejmanovic, and M. Hess. 2017. Histomonosis in poultry: Previous and current strategies for prevention and therapy. Avian Pathol. 46:1-18.

Liebhart, D., E. Grabensteiner, and M. Hess. 2008. A virulent mono-eukaryotic culture of *Histomonas meleagridis* is capable of inducing fatal histomonosis in different aged turkeys of both sexes, regardless of the infective dose. Avian Dis. 52:168-172.

Liebhart, D., and M. Hess. 2009. Oral infection of turkeys with *in vitro*-cultured *Histomonas meleagridis* results in high mortality. Avian Pathol. 38:223-227.

Liebhart, D., T. Sulejmanovic, B. Grafl, A. Tichy, and M. Hess. 2013. Vaccination against histomonosis prevents a drop in egg production in layers following challenge. Avian Pathol. 42:79-84.

Liebhart, D., M. Windisch, and M. Hess. 2010. Oral vaccination of 1-day-old turkeys with *in vitro* attenuated *Histomonas meleagridis* protects against histomonosis and has no negative effect on performance. Avian Pathol. 39:399-403.

Liebhart, D., M. Zahoor, I. Prokofieva, and M. Hess. 2011. Safety of avirulent histomonads to be used as a vaccine determined in turkeys and chickens. Poult. Sci. 90:996-1003.

Lotfi, A., R. Hauck, P. Olias, and H. M. Hafez. 2014. Pathogenesis of histomonosis in experimentally infected specific-pathogen-free (SPF) layer-type chickens and SPF meat-type chickens. Avian Dis. 58:427-432.

Lund, E. E. 1959. Immunizing action of a nonpathogenic strain of *Histomonas* against blackhead in turkeys. J. Protozool. 6:182-185.

Lund, E. E., P. C., Augustine, and A. M. Chute. 1967. *Histomonas meleagridis* after one thousand *in vitro* passages. J. Eukaryot. Microbiol. 14:349-351.

Lund, E. E., P. C. Augustine, and D. J. Ellis. 1966a. Immunizing action of *in vitro*-attenuated *Histomonas meleagridis* in chickens and turkeys. Exp. Parasitol. 18:403-407.

Lund, E. E., E. E. Wehr, and D. J. Ellis. 1966b. Earthworm transmission of *Heterakis* and *Histomonas* to turkeys and chickens. J. Parasitol. 52:899-902.

McAllister, M. M. 2014. Successful vaccines for naturally occurring protozoal diseases of animals should guide human vaccine research: A review of protozoal vaccines and their designs. Parasitology. 141:624-640.

McDougald, L. R. 1998. Intestinal protozoa important to poultry. Poult. Sci. 77:1156-1158.

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

McDougald, L., and L. Fuller. 2005. Blackhead disease in turkeys: Direct transmission of *Histomonas meleagridis* from bird to bird in a laboratory model. Avian Dis. 49:328-331.

Mitra, T., W. Gerner, F. A. Kidane, P. Wernsdorf, M. Hess, A. Saalmüller, and D. Liebhart. 2017. Vaccination against histomonosis limits pronounced changes of B cells and T-cell subsets in turkeys and chickens. Vaccine. 35:4184-4196.

Mitra, T., Kidane, F. A., Hess, M., and Liebhart, D. 2018. Unravelling the immunity of poultry against the extracellular protozoan parasite *Histomonas meleagridis* is a cornerstone for vaccine development: A review. Front. Immunol. 9:2518.

Munsch, M., A. Lotfi, H. M. Hafez, S. Al-Quraishy, and H. Mehlhorn. 2009a. Light and transmission electron microscopic studies on trophozoites and cyst-like stages of *Histomonas meleagridis* from cultures. Parasitol. Res. 104:683-689.

Munsch, M., H. Mehlhorn, S. Al-Quraishy, A. R. Lotfi, and H. M. Hafez. 2009b. Molecular biological features of strains of *Histomonas meleagridis*. Parasitol. Res. 104:1137-1140.

NRC. 1994. Nutrient Requirements of Poultry. 9th rev. ed. Natl. Acad. Press, Washington, DC.

Pham, A. D. N. , J. K. De Gussem, and B. M. Goddeeris. 2013. Intracloacally passaged low-virulent *Histomonas meleagridis* protects turkeys from histomonosis. Vet. Parasitol. 196:307-313.

Popp, C., R. Hauck, S. Balczulat, and H. M. Hafez. 2011. Recurring histomonosis on an organic farm. Avian Dis. 55:328-330.

Regmi, P. R., A. L. Shaw, L. L. Hungerford, J. R. Messenheimer, T. Zhou, P. Pillai, A. Omer, and J. M. Gilbert. 2016. Regulatory considerations for the approval of drugs against histomoniasis (blackhead disease) in turkeys, chickens, and game birds in the United States. Avian Dis. 60:725-730.

Sorvari, R., A. Naukkarinen, and T. E. Sorvari. 1977. Anal sucking-like movements in the chicken and chick embryo followed by the transportation of environmental material to the bursa of Fabricius, caeca, and caecal tonsils. Poult. Sci. 56:1426-1429.

Sulejmanovic, T., I. Bilic, M. Hess, and D. Liebhart. 2016. An *in vitro* attenuated strain of *Histomonas meleagridis* provides cross-protective immunity in turkeys against heterologous virulent isolates. Avian Pathol. 45:46-53.

Sulejmanovic, T., D. Liebhart, and M. Hess. 2013. *In vitro* attenuated *Histomonas meleagridis* does not revert to virulence, following serial *in vivo* passages in turkeys or chickens. Vaccine. 31:5443-5450.

Svihus, B. 2011. The gizzard: Function, influence of diet structure and effects on nutrient availability. Worlds Poult. Sci. J. 67:207-224.

Thøfner, I. C. N., D. Liebhart, M. Hess, T. W. Schou, C. Hess, E. Ivarsen, X. Fretté, L. P. Christensen, K. Grevsen, R. M. Engberg, and J. P. Christensen. 2012. Antihistomonal effects of artemisinin and *Artemisia annua* extracts *in vitro* could not be confirmed by *in vivo* experiments in turkeys and chickens. Avian Pathol. 41:487-496.

Tyzzer, E. E. 1920. The flagellate character and reclassification of the parasite producing "Blackhead" in turkeys: *Histomonas* (gen. nov.) *meleagridis* (Smith). J. Parasitol. 6:124-131.

Tyzzer, E. E. 1932. Problems and observations concerning the transmission of blackhead infection in turkeys. Proc. Am. Philos. Soc. 71:407-410.

Tyzzer, E. E. 1934. Studies on histomoniasis, or "blackhead" infection, in the chicken and the turkey. Daedalus. 69:189-264.

Tyzzer, E. E. 1936. A study of immunity produced by infection with attenuated culture-strains of *Histomonas meleagridis.* J. Comp. Patho. Ther. 49:285-303.

Tyzzer, E. E., and M. Fabyan. 1922. 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

Tyzzer, E. E., M. Fabyan, and N. C. Foot. 1921. Further observations on "blackhead" in turkeys. J. Infect. Dis. 29:268-286.

Wei, Z., M. Abraham, E. V. Chadwick, and R. B. Beckstead. 2020. *Histomonas meleagridis* isolates compared by virulence and gene expression. Vet. Parasitol. 286:109233.

Windisch, M., and M. Hess. 2009. Establishing an indirect sandwich enzyme-linked-immunosorbent-assay (ELISA) for the detection of antibodies against *Histomonas meleagridis* from experimentally infected specific pathogen-free chickens and turkeys. Vet. Parasitol. 161:25-30.

Windisch, M., and M. Hess. 2010. Experimental infection of chickens with *Histomonas meleagridis* confirms the presence of antibodies in different parts of the intestine. Parasite Immunol. 32:29-35.

Zaragatzki, E., M. Hess, E. Grabensteiner, F. Abdel-Ghaffar, K. A. S. Al-Rasheid, and H. Mehlhorn. 2010a. Light transmission electron microscopic studies on the encystation of *Histomonas meleagridis.* Parasitol. Res. 106:977-983.

Zaragatzki, E., H. Mehlhorn, F. Abdel-Ghaffar, K. S. Rasheid, E. Grabensteiner, and M. Hess. 2010b. Experiments to produce cysts in cultures of *Histomonas meleagridis* – The agent of histomonosis in poultry. Parasitol. Res. 106:1005-1007.

TABLES AND FIGURES

Table 1. Body weight gain (BWG) and histomoniasis-related mortalities during Vaccination and Challenge Phases (Experiment 1).[1]

	Vaccination Phase		Challenge Phase	
Group[2]	**Mortality**	**d14-29 BWG (g)**[3]	**Mortality**	**d29-40 BWG (g)**
PC	6/20 (30.0%)	-	6/27 (22.2%)	230 ± 41.4^{c}
Vacc	0/39 (0.00%)*	621 ± 16.5^{a}	1/34 (2.94%)*	435 ± 45.8^{b}
NC	0/59 (0.00%)*	630 ± 12.4^{a}	0/27 (0.00%)*	732 ± 18.7^{a}

$^{a\text{-}c}$BWG data are expressed as mean ± SE; Values within a column with no common superscript differ significantly ($P < 0.05$). Data were analyzed using JMP Pro 16 ANOVA, further separated by Tukey's HSD; *indicates significant difference in mortalities ($P < 0.05$) as compared to PC with chi-square test.

[1]Vaccination Phase consisted of intracloacal administration of either 2×10^5 Vacc Buford P80a or Buford WTH cells/turkey on d14; Challenge Phase began on d29 when the Vacc and a newly introduced PC group (formed from a subset of NC) were intracloacally challenged with 2×10^5 Buford WTH cells/turkey.

[2]PC = positive-challenged control, Vacc = live-attenuated *Histomonas meleagridis*; NC = non-challenged control; WTH = wild-type *H. meleagridis.*

[3]The PC was terminated on d27 due to mortality percentage; No BWG data were collected for PC at this time-point.

Table 2. Liver and cecal lesion scores (LS) for histomoniasis during Challenge Phase (Experiment 1).[1]

	out of total n scored[2,3]				out of positive for n w/1-3 LS		
Group[4]	**Mean Liver LS**	**Mean Cecal LS**	**+Liver LS**	**+Cecal LS**	**+Liver LS; +Cecal LS**	**- Liver LS; +Cecal LS**	**+Liver LS; - Cecal LS**
PC	2.78 ± 0.14^{a}	2.61 ± 0.15^{a}	22/23 (95.7%)	22/23 (95.7%)	22/22 (100%)	0/22 (0.00%)	0/22 (0.00%)
Vacc	0.76 ± 0.21^{b}	1.78 ± 0.22^{b}	10/34 (29.4%)*	24/34 (70.6%)*	10/24 (41.7%)*	14/24 (58.3%)*	0/24 (0.00%)
NC	0.04 ± 0.04^{c}	0.07 ± 0.05^{c}	1/27 (3.70%)*	2/27 (7.41%)*	0/3 (0.00%)*	2/3 (66.7%)*	1/3 (33.3%)*

[a-c]LS data are expressed as mean ± SE; Values within a column with no common superscript differ significantly ($P < 0.05$). LS were based on a scale of “0” to “3” and were analyzed using the Proc Mixed Procedure in SAS 9.4 software; *indicates significant difference of categorical LS classifications ($P < 0.05$) compared to PC with chi-square test.

[1]Vaccination Phase consisted of intracloacal administration of either 2 x 10^5 Buford P80a Vacc or Buford WTH cells/turkey on d14; Challenge Phase began on d29 when the Vacc and a newly introduced PC group (formed from a subset of NC) were intracloacally challenged with 2 x 10^5 Buford WTH cells/turkey.

[2]Scores in the NC were only “1” on the LS scale of “0-3”.

[3]Experimental error resulted in 4 of the PC turkeys not being evaluated for LS; hence, the difference in total n for Challenge Phase mortality and LS values in Table 1.

[4]PC = positive-challenged control, Vacc = live-attenuated *Histomonas meleagridis*; NC = non-challenged control; WTH = wild-type *H. meleagridis*.

Table 3. Body weight gain (BWG) and histomoniasis-related mortalities during Vaccination Phase (Experiment 2).[1,2]

Group[3]	Mortality	BWG (g)			
		d0-7	d7-14	d14-21	d0-21
PC Cloacal 200k	8/36 (22.2%)	67 ± 3.94^{bc}	99 ± 7.17^{b}	-	-
Vacc Oral 2k	1/36 (2.78%)*	70 ± 3.61^{abc}	112 ± 6.09^{ab}	202 ± 6.60^{abc}	401 ± 14.7^{abc}
Vacc Oral 20k	1/38 (2.63%)*	81 ± 2.61^{ab}	133 ± 4.05^{a}	210 ± 6.86^{ab}	430 ± 7.73^{ab}
Vacc Cloacal 2k	0/36 (0.00%)*	81 ± 2.38^{ab}	116 ± 5.51^{ab}	195 ± 7.27^{abc}	397 ± 14.6^{abc}
Vacc Cloacal 20k	2/37 (5.41%)*	84 ± 3.73^{a}	133 ± 4.26^{a}	213 ± 6.05^{a}	440 ± 11.4^{a}
Vacc Cloacal 200k	1/30 (3.33%)*	58 ± 3.61^{c}	119 ± 5.76^{ab}	181 ± 7.35^{bc}	373 ± 14.1^{bc}
NC	0/34 (0.00%)*	76 ± 3.80^{ab}	110 ± 7.27^{ab}	181 ± 6.77^{c}	366 ± 14.6^{c}

$^{a-c}$BWG data are expressed as mean ± SE; Values within a column with no common superscript differ significantly ($P < 0.05$). Data were analyzed using JMP Pro 16 ANOVA, further separated by Tukey's HSD; *indicates significant difference in mortalities ($P < 0.05$) as compared to PC Cloacal 200k with chi-square test.

[1]Vaccination Phase began on d0 with administration of respective dose and route of either Vacc Buford P80a or Buford WTH cells/turkey.

[2]The PC was terminated on d15 for lesion scores.

[3]PC = positive-challenged control, Vacc = live-attenuated *Histomonas meleagridis*; NC = non-challenged control; WTH = wild-type *H. meleagridis.*

Table 4. Liver and cecal lesion scores (LS) for histomoniasis during Vaccination and Challenge Phases (Experiment 2).[1,2]

Group[3]	**out of total n**[4]				**out of positive for n w/1-3 LS**		
Vaccination Phase (d15)	**Mean Liver LS**	**Mean Cecal LS**	**+Liver LS**	**+Cecal LS**	**+Liver LS; +Cecal LS**	**- Liver LS; +Cecal LS**	**+Liver LS; - Cecal LS**
PC Cloacal 200k	1.17 ± 0.20^{a}	1.67 ± 0.21^{a}	18/30 (60.0%)	26/30 (86.7%)	17/26 (65.4%)	9/26 (34.6%)	1/26 (3.85%)
Vacc Oral 2k	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0/15 (0.00%)*	0/15 (0.00%)*	0/0 (0.00%)	0/0 (0.00%)	0/0 (0.00%)
Vacc Oral 20k	0.00 ± 0.00^{b}	0.75 ± 0.11^{b}	0/16 (0.00%)*	12/16 (75.0%)	0/12 (0.00%)*	12/12 (100%)*	0/12 (0.00%)
Vacc Cloacal 2k	0.00 ± 0.00^{b}	0.50 ± 0.13^{b}	0/16 (0.00%)*	8/16 (50.0%)*	0/8 (0.00%)*	8/8 (100%)*	0/8 (0.00%)
Vacc Cloacal 20k	0.00 ± 0.00^{b}	0.27 ± 0.15^{b}	0/15 (0.00%)*	3/15 (20.0%)*	0/3 (0.00%)*	3/3 (100%)*	0/3 (0.00%)
Vacc Cloacal 200k	0.22 ± 0.22^{b}	0.56 ± 0.34^{b}	1/9 (11.1%)*	3/9 (33.3%)*	1/3 (33.3%)	2/3 (66.7%)	0/3 (0.00%)
Challenge Phase (d35)							
PC Cloacal 200k	2.00 ± 0.31^{ab}	1.93 ± 0.28^{a}	14/20 (70.0%)	15/20 (75.0%)	14/15 (93.3%)	1/15 (6.67%)	0/15 (0.00%)
Vacc Oral 2k	1.90 ± 0.30^{ab}	1.88 ± 0.25^{a}	14/20 (70.0%)	16/20 (80.0%)	14/16 (87.5%)	2/16 (12.5%)	0/16 (0.00%)
Vacc Oral 20k	2.05 ± 0.27^{ab}	2.15 ± 0.24^{a}	16/20 (80.0%)	17/20 (85.0%)	16/17 (94.1%)	1/17 (5.88%)	0/17 (0.00%)
Vacc Cloacal 2k	1.90 ± 0.30^{ab}	1.85 ± 0.26^{a}	14/20 (70.0%)	16/20 (80.0%)	14/16 (87.5%)	2/16 (12.5%)	0/16 (0.00%)
Vacc Cloacal 20k	2.25 ± 0.27^{a}	2.30 ± 0.25^{a}	16/20 (80.0%)	17/20 (85.0%)	16/17 (94.1%)	1/17 (5.88%)	0/17 (0.00%)
Vacc Cloacal 200k	1.45 ± 0.30^{b}	1.80 ± 0.30^{a}	12/20 (60.0%)	14/20 (70.0%)	12/14 (85.7%)	2/14 (14.3%)	0/14 (0.00%)
PC Oral 200k	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0/14 (0.00%)*	0/14 (0.00%)*	0/0 (0.00%)	0/0 (0.00%)	0/0 (0.00%)

[a-c]LS data are expressed as mean ± SE; Values within a column with no common superscript differ significantly ($P < 0.05$). LS were based on a scale of "0" to "3" and were analyzed using the Proc Mixed Procedure in SAS 9.4 software; *indicates significant difference of categorical LS classifications ($P < 0.05$) compared to PC Cloacal 200k with chi-square test.

[1]Vaccination Phase began on d0 with administration of respective dose and route of either Vacc Buford P80a or Buford WTH cells/turkey; Challenge Phase began on d21 with the intracloacal administration of 2×10^5 Buford WTH cells/turkey to all groups, except for PC Oral 200k which received the dose orally. Turkeys from the NC group were redistributed to form the new PC groups for the Challenge Phase.

[2]Scores in the NC were only "1" on the LS scale of "0-3".

[3]PC = positive-challenged control, Vacc = live-attenuated *Histomonas meleagridis*; NC = non-challenged control; WTH = wild-type *H. meleagridis.*

[4]Experimental error resulted in the LS not being recorded from turkey subsets during the Vaccination Phase as follows: PC Cloacal 200k (n = 6), Vacc Oral 2k (n = 1), Vacc Oral 20k (n = 2), Vacc Cloacal 20k (n = 2), and Vacc Cloacal 200k (n = 1); hence, the difference in total n between Vaccination Phase mortality and LS values in Table 3.

Table 5. Body weight gain (BWG) and histomoniasis-related mortalities during Challenge Phase (Experiment 2).[1]

Group[2]	Mortality	d21-35 BWG (g)
PC Cloacal 200k	11/20 (55.0%)	531 ± 61.9[a]
Vacc Oral 2k	10/20 (50.0%)	546 ± 84.1[a]
Vacc Oral 20k	10/20 (50.0%)	497 ± 74.2[a]
Vacc Cloacal 2k	12/20 (60.0%)	511 ± 71.4[a]
Vacc Cloacal 20k	11/20 (55.0%)	487 ± 68.2[a]
Vacc Cloacal 200k	7/20 (35.0%)	527 ± 51.0[a]
PC Oral 200k	0/14 (0.00%)*	596 ± 34.7[a]

[a]BWG data are expressed as mean ± SE; Values within a column with no common superscript differ significantly ($P < 0.05$). BWG data were analyzed using JMP Pro 16 ANOVA, with no difference detected; *indicates significant difference in mortalities ($P < 0.05$) as compared to PC Cloacal 200k with chi-square test.

[1]Challenge Phase began on d21 with the intracloacal administration of 2×10^5 Buford WTH cells/turkey to all groups, except for PC Oral 200k which received the dose orally. Turkeys from the NC group were redistributed to form the new PC groups for the Challenge Phase.

[2]PC = positive-challenged control, Vacc = live-attenuated *Histomonas meleagridis*; NC = non-challenged control; WTH = wild-type *H. meleagridis.*

Table 6. Body weight gain (BWG) and histomoniasis-related mortalities during Vaccination and Challenge Phases (Experiment 3).[1,2]

Vaccination Phase 1	**Mortality**	**d0-14 BWG (g)**
PC Cloacal	9/60 (15.0%)	136 ± 5.87^{c}
PC Oral	10/60 (16.7%)	146 ± 6.81^{c}
d0 Vacc Oral	0/53 (0.00%)*	154 ± 4.39^{bc}
d0 Vacc Cloacal	0/52 (0.00%)*	166 ± 3.96^{ab}
NC	0/216 (0.00%)*	172 ± 2.41^{a}
Vaccination Phase 2	**Mortality**	**d14-28 BWG (g)**
PC Cloacal	21/43 (48.8%)	250 ± 26.4^{c}
d0 Vacc Oral	0/43 (0.00%)*	400 ± 8.48^{ab}
d0 Vacc Cloacal	0/42 (0.00%)*	423 ± 8.86^{a}
d14 Vacc Cloacal	0/55 (0.00%)*	380 ± 8.00^{b}
NC	0/108 (0.00%)*	393 ± 5.05^{ab}
Challenge Phase	**Mortality**	**d28-42 BWG (g)**
PC Cloacal	19/45 (42.2%)	521 ± 40^{bc}
d0 Vacc Oral	17/38 (44.7%)	431 ± 58^{c}
d0 Vacc Cloacal	12/37 (32.4%)	548 ± 42^{bc}
d14 Vacc Cloacal	10/45 (22.2%)*	642 ± 28^{ab}
NC	0/53 (0.00%)*	719 ± 16^{a}

[a-c]BWG data are expressed as mean ± SE; Values within a column with no common superscript differ significantly ($P < 0.05$). Data were analyzed using JMP Pro 16 ANOVA, further separated by Tukey's HSD; *indicates significant difference in mortalities ($P < 0.05$) as compared to PC Cloacal with chi-square test.

[1]Vaccination Phase 1 began on d0 with administration of 2×10^5 cells/turkey of either Vacc Buford P80a or Buford WTH cells/turkey via respective route; Vaccination Phase 2 began on d14 with the introduction of a d14 Vacc group and new PC (formed from subsets of the NC) which received intracloacal administration of 2×10^5 either Vacc Buford P80a or Buford WTH cells/turkey, respectively; Challenge Phase began on d28 with the intracloacal administration of 2×10^5 Buford WTH cells/turkey.

[2]PC = positive-challenged control, Vacc = live-attenuated *Histomonas meleagridis*; NC = non-challenged control; WTH = wild-type *H. meleagridis.*

Table 7. Liver and cecal lesion scores (LS) for histomoniasis during Vaccination and Challenge Phases (Experiment 3).[1,2]

Group[3]	out of total n				out of positive for n w/1-3 LS		
Vaccination Phase 1 (d14)	**Mean Liver LS**	**Mean Cecal LS**	**+Liver LS**	**+Cecal LS**	**+Liver LS; +Cecal LS**	**- Liver LS; +Cecal LS**	**+Liver LS; -Cecal LS**
PC Cloacal	1.65 ± 0.18^{a}	1.93 ± 0.17^{a}	38/60 (63.3%)	44/60 (73.3%)	38/44 (86.4%)	6/44 (13.6%)	0/44 (0.00%)
PC Oral	1.25 ± 0.19^{b}	1.67 ± 0.17^{a}	27/60 (45.0%)*	44/60 (73.3%)	27/44 (61.4%)*	17/44 (38.6%)*	0/44 (0.00%)
d0 Vacc Oral	0.20 ± 0.20^{c}	0.30 ± 0.30^{b}	1/10 (10.0%)*	1/10 (10.0%)*	1/1 (100%)	0/1 (0.00%)	0/1 (0.00%)
d0 Vacc Cloacal	0.20 ± 0.13^{c}	0.40 ± 0.31^{b}	2/10 (20.0%)*	2/10 (20.0%)*	2/2 (100%)	0/2 (0.00%)	0/2 (0.00%)
NC	0.00 ± 0.00^{c}	0.10 ± 0.10^{b}	0/10 (0.00%)*	1/10 (10.0%)*	0/1 (0.00%)*	1/1 (100%)*	0/1 (0.00%)
Vaccination Phase 2 (d28)							
PC Cloacal	2.26 ± 0.18^{a}	2.33 ± 0.16^{a}	35/43 (81.4%)	38/43 (88.4%)	35/38 (92.1%)	3/38 (7.89%)	0/38 (0.00%)
d0 Vacc Oral	1.00 ± 0.55^{b}	1.40 ± 0.51^{b}	3/5 (60.0%)	4/5 (80.0%)	3/4 (75.0%)	1/4 (25.0%)	0/4 (0.00%)
d0 Vacc Cloacal	0.00 ± 0.00^{b}	0.40 ± 0.24^{b}	0/5 (0.00%)*	2/5 (40.0%)*	0/2 (0.00%)*	2/2 (100%)*	0/2 (0.00%)
d14 Vacc Cloacal	0.30 ± 0.15^{b}	1.20 ± 0.39^{b}	3/10 (30.0%)*	6/10 (60.0%)*	3/6 (50.0%)*	3/6 (50.0%)*	0/6 (0.00%)
NC	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0/10 (0.00%)*	0/10 (0.00%)*	0/0 (0.00%)	0/0 (0.00%)	0/0 (0.00%)
Challenge Phase (d42)							
PC Cloacal	1.84 ± 0.21^{a}	1.71 ± 0.19^{ab}	29/45 (64.4%)	30/45 (66.7%)	29/30 (96.7%)	1/30 (3.33%)	0/30 (0.00%)
d0 Vacc Oral	1.79 ± 0.23^{a}	2.11 ± 0.21^{a}	24/38 (63.2%)	29/38 (76.3%)	24/29 (82.8%)	5/29 (17.2%)	0/29 (0.00%)
d0 Vacc Cloacal	1.62 ± 0.25^{a}	1.70 ± 0.21^{ab}	20/37 (54.1%)	25/37 (67.6%)	20/25 (80.0%)*	5/25 (20.0%)*	0/25 (0.00%)
d14 Vacc Cloacal	0.73 ± 0.19^{b}	1.31 ± 0.19^{b}	13/45 (28.9%)*	28/45 (62.2%)	13/28 (46.4%)*	15/28 (53.6%)*	0/28 (0.00%)
NC	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0/53 (0.00%)*	0/53 (0.00%)*	0/0 (0.00%)	0/0 (0.00%)	0/0 (0.00%)

[a-c]LS data are expressed as mean ± SE; Values within a column with no common superscript differ significantly ($P < 0.05$). LS were based on a scale of "0" to "3" and were analyzed using the Proc Mixed Procedure in SAS 9.4 software; *indicates significant difference of categorical LS classifications ($P < 0.05$) compared to PC with chi-square test.

[1]Vaccination Phase 1 began on d0 with administration of 2 x 10^5 cells/turkey of either Vacc Buford P80a or Buford WTH cells/turkey via respective route; Vaccination Phase 2 began on d14 with the introduction of a d14 Vacc group and new PC (formed from subsets of the NC) which received intracloacal administration of 2 x 10^5 either Vacc Buford P80a or Buford WTH cells/turkey, respectively; Challenge Phase began on d28 with the intracloacal administration of 2 x 10^5 Buford WTH cells/turkey.

[2]Scores in the NC were only "1" on the LS scale of "0-3".

[3]PC = positive-challenged control, Vacc = live-attenuated *Histomonas meleagridis*; NC = non-challenged control; WTH = wild-type *H. meleagridis.*

Table 8. Body weight gain (BWG) and histomoniasis-related mortalities during Vaccination and Challenge Phases (Experiment 4).[1]

Group[2,3]	Vaccination Phase			Challenge Phase	
	d14-28 Mortality	**d28-35 Mortality[4]**	**d13-34 BWG (g)**	**d35-49 Mortality**	**d34-49 BWG (g)**
PC Buford	25/60 (41.7%)	14/35 (40.0%)	682 ± 86.4^{b}	24/39 (61.5%)	647 ± 112^{c}
Vacc PHL P67	0/59 (0.00%)*	0/49 (0.00%)*	901 ± 15.6^{a}	8/39 (20.5%)*	1090 ± 76.7^{ab}
Vacc PHL P129	0/60 (0.00%)*	0/50 (0.00%)*	917 ± 16.8^{a}	9/40 (22.5%)*	893 ± 85.6^{bc}
Vacc Buford P80a	0/60 (0.00%)*	0/50 (0.00%)*	861 ± 16.0^{a}	7/40 (17.5%)*	937 ± 88.4^{bc}
Vacc Buford P200a	0/60 (0.00%)*	0/50 (0.00%)*	898 ± 15.1^{a}	3/40 (7.50%)*	931 ± 62.0^{bc}
Vacc Buford P138b	0/59 (0.00%)*	0/49 (0.00%)*	909 ± 13.4^{a}	12/39 (30.8%)*	899 ± 68.5^{bc}
Vacc Buford P198c	0/60 (0.00%)*	0/50 (0.00%)*	874 ± 18.0^{a}	7/40 (17.5%)*	1000 ± 76.5^{abc}
NC	0/69 (0.00%)*	0/59 (0.00%)*	888 ± 13.8^{a}	0/20 (0.00%)*	1359 ± 33.5^{a}

[a-c]BWG data are expressed as mean ± SE; Values within a column with no common superscript differ significantly ($P < 0.05$). Data were analyzed using JMP Pro 16 ANOVA, further separated by Tukey's HSD; *indicates significant difference in mortalities ($P < 0.05$) as compared to PC Buford with chi-square test.

[1]Vaccination Phase consisted of intracloacal administration of 2 x 10^5 cells/turkey of either Vacc (PHL2017 or Buford isolates of passage indicated) or Buford WTH cells/turkey on d14; Challenge Phase began on d35 with the intracloacal administration of 2 x 10^5 Buford WTH cells/turkey to the Vacc and PC groups (new PC Buford formed from subset of the NC).

[2]PC = positive-challenged control, Vacc = live-attenuated *Histomonas meleagridis*; NC = non-challenged control; WTH = wild-type *H. meleagridis.*

[3]Isolates of *H. meleagridis:* Buford strain (isolated from infected chickens in Georgia); PHL2017 strain (isolated from infected turkeys in Arkansas); Passage number and isolate indicator follow each group name.

[4]Adjusted for remaining n/group after d28 lesion score subset.

Table 9. Liver and cecal lesion scores (LS) for histomoniasis during Vaccination and Challenge Phases (Experiment 4).[1,2]

Group[3,4]	out of total n				out of positive for n w/1-3 LS		
Vaccination Phase (d28)	**Mean Liver LS**	**Mean Cecal LS**	**+Liver LS**	**+Cecal LS**	**+Liver LS; +Cecal LS**	**- Liver LS; +Cecal LS**	**+Liver LS; - Cecal LS**
PC Buford	2.82 ± 0.12^{a}	2.62 ± 0.10^{a}	32/34 (94.1%)	34/34 (100%)	32/34 (94.1%)	2/34 (5.88%)	0/34 (0.00%)
Vacc PHL P67	0.10 ± 0.10^{b}	0.90 ± 0.10^{b}	1/10 (10.0%)*	9/10 (90.0%)	1/9 (11.1%)*	8/9 (88.9%)*	0/9 (0.00%)
Vacc PHL P129	0.20 ± 0.13^{b}	1.00 ± 0.00^{b}	2/10 (20.0%)*	10/10 (100%)	2/10 (20.0%)*	8/10 (80.0%)*	0/10 (0.00%)
Vacc Buford P80a	0.30 ± 0.21^{b}	1.00 ± 0.30^{b}	2/10 (20.0%)*	6/10 (60.0%)*	2/6 (33.3%)*	4/6 (66.7%)*	0/6 (0.00%)
Vacc Buford P200a	0.10 ± 0.10^{b}	0.90 ± 0.23^{b}	1/10 (10.0%)*	7/10 (70.0%)*	1/7 (14.3%)*	6/7 (85.7%)*	0/7 (0.00%)
Vacc Buford P138b	0.20 ± 0.13^{b}	1.20 ± 0.13^{b}	2/10 (20.0%)*	10/10 (100%)	2/10 (20.0%)*	8/10 (80.0%)*	0/10 (0.00%)
Vacc Buford P198c	0.00 ± 0.00^{b}	1.10 ± 0.18^{b}	0/10 (0.00%)*	9/10 (90.0%)	0/9 (0.00%)*	9/9 (100%)*	0/9 (0.00%)
NC	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}	0/5 (0.00%)*	0/5 (0.00%)*	0/0 (0.00%)	0/0 (0.00%)	0/0 (0.00%)
Vaccination Phase (d35)							
PC Buford	2.04 ± 0.26^{a}	2.27 ± 0.19^{a}	19/26 (73.1%)	25/26 (96.2%)	19/25 (76.0%)	6/25 (24.0%)	0/25 (0.00%)
Vacc PHL P67	0.10 ± 0.10^{b}	0.70 ± 0.15^{bc}	1/10 (10.0%)*	7/10 (70.0%)*	1/7 (14.3%)*	6/7 (85.7%)*	0/7 (0.00%)
Vacc PHL P129	0.10 ± 0.10^{b}	0.80 ± 0.13^{bc}	1/10 (10.0%)*	8/10 (80.0%)	1/8 (12.5%)*	7/8 (87.5%)*	0/8 (0.00%)
Vacc Buford P80a	0.00 ± 0.00^{b}	0.80 ± 0.20^{bc}	0/10 (0.00%)*	7/10 (70.0%)*	0/7 (0.00%)*	7/7 (100%)*	0/7 (0.00%)
Vacc Buford P200a	0.40 ± 0.16^{b}	0.80 ± 0.20^{bc}	4/10 (40.0%)	7/10 (70.0%)*	4/7 (57.1%)	3/7 (42.9%)	0/7 (0.00%)
Vacc Buford P138b	0.20 ± 0.13^{b}	0.80 ± 0.20^{bc}	2/10 (20.0%)*	7/10 (70.0%)*	2/7 (28.6%)*	5/7 (71.4%)*	0/7 (0.00%)
Vacc Buford P198c	0.20 ± 0.13^{b}	1.30 ± 0.33^{b}	2/10 (20.0%)*	8/10 (80.0%)	2/8 (25.0%)*	6/8 (75.0%)*	0/8 (0.00%)
NC	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}	0/5 (0.00%)*	0/5 (0.00%)*	0/0 (0.00%)	0/0 (0.00%)	0/0 (0.00%)
Challenge Phase (d49)							
PC Buford	2.62 ± 0.15^{a}	2.72 ± 0.10^{a}	36/39 (92.3%)	39/39 (100%)	36/39 (92.3%)	3/39 (7.69%)	0/39 (0.00%)
Vacc PHL P67	1.79 ± 0.22^{bd}	2.10 ± 0.16^{bcd}	27/39 (69.2%)*	37/39 (94.9%)	27/37 (73.0%)*	10/37 (27.0%)*	0/37 (0.00%)
Vacc PHL P129	2.18 ± 0.21^{ab}	2.35 ± 0.14^{ac}	30/40 (75.0%)*	39/40 (97.5%)	30/39 (76.9%)	9/39 (23.1%)	0/39 (0.00%)
Vacc Buford P80a	1.10 ± 0.21^{ef}	1.85 ± 0.18^{d}	19/40 (47.5%)*	34/40 (85.0%)*	19/34 (55.9%)*	15/34 (44.1%)*	0/34 (0.00%)
Vacc Buford P200a	1.50 ± 0.19^{cde}	2.45 ± 0.14^{abc}	30/40 (75.0%)*	38/40 (95.0%)	30/38 (78.9%)	8/38 (21.1%)	0/38 (0.00%)
Vacc Buford P138b	1.85 ± 0.23^{bc}	2.23 ± 0.16^{bcd}	26/39 (66.7%)*	37/39 (94.9%)	26/37 (70.3%)*	11/37 (29.7%)*	0/37 (0.00%)
Vacc Buford P198c	1.28 ± 0.22^{df}	2.08 ± 0.17^{bcd}	21/40 (52.5%)*	37/40 (92.5%)	21/37 (56.8%)*	16/37 (43.2%)*	0/37 (0.00%)
NC	0.00 ± 0.00^{g}	0.60 ± 0.11^{f}	0/20 (0.00%)*	12/20 (60.0%)*	0/12 (0.00%)*	12/12 (100%)*	0/12 (0.00%)

[a-g]LS data are expressed as mean ± SE; Values within a column with no common superscript differ significantly ($P < 0.05$). LS were based on a scale of "0" to "3" and were analyzed using the Proc Mixed Procedure in SAS 9.4 software; *indicates significant difference of categorical LS classifications ($P < 0.05$) compared to PC Buford with chi-square test.

[1]Vaccination Phase consisted of intracloacal administration of 2 x 10^5 cells/turkey of either Vacc (PHL2017 or Buford isolates of passage indicated) or Buford WTH cells/turkey on d14; Challenge Phase began on d35 with the intracloacal administration of 2 x 10^5 Buford WTH cells/turkey to the Vacc and PC groups (new PC Buford formed from subset of the NC).

[2]Scores in the NC were only "1" on the LS scale of "0-3".

[3]PC = positive-challenged control, Vacc = live-attenuated *Histomonas meleagridis*; NC = non-challenged control; WTH = wild-type *H. meleagridis.*

[4]Isolates of *H. meleagridis:* Buford strain (isolated from infected chickens in Georgia); PHL2017 strain (isolated from infected turkeys in Arkansas); Passage number and isolate indicator follow each group name.

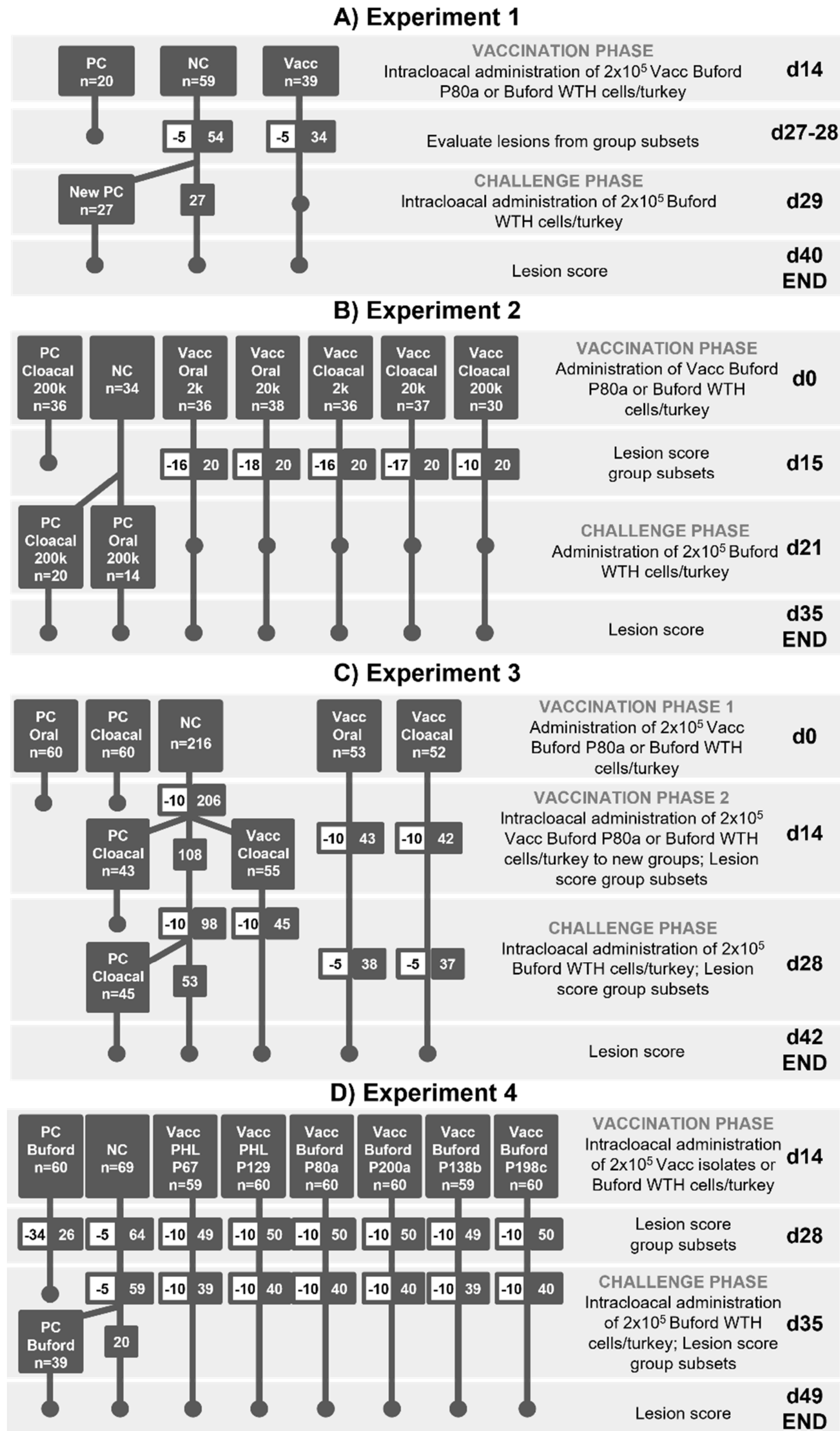


Figure 1. Experimental timelines for administration of live-attenuated vaccine candidate (Vacc) *Histomonas meleagridis* and subsequent challenge with Buford strain wild-type *H. meleagridis* (WTH). The Buford strain was isolated from infected chickens in Georgia; PHL2017 strain was isolated from infected turkeys in Arkansas. Vacc passage number and isolate indicator are included in each group name, where applicable. PC = positive-challenged control; NC = non-challenged control.

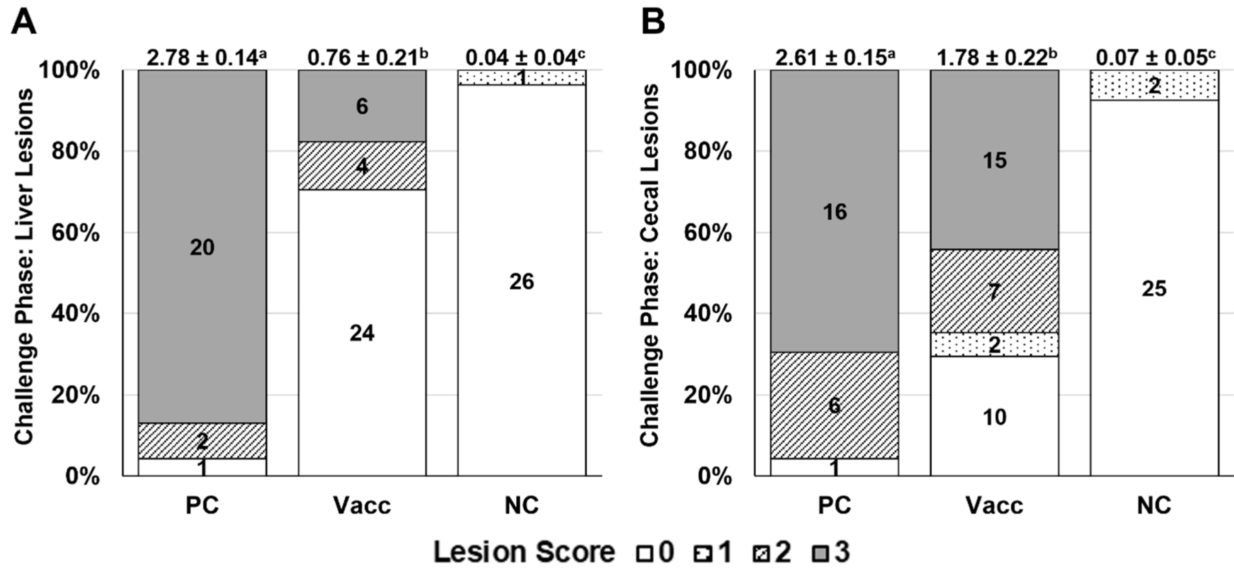


Figure 2. Experiment 1 frequency of lesion scores during Challenge Phase for **A)** liver and **B)** cecae. Numbers within columns indicate the number of turkeys per evaluated lesion score. Numbers at the top of each column indicate the lesion score mean ± SE for that group with different superscripts denoting significance ($P < 0.05$). Lesion scores were based on a scale of "0" to "3" and were analyzed using the Proc Mixed Procedure in SAS 9.4 software. Challenge Phase began on d29 when the Vacc and PC group were intracloacally challenged with 2 x 10^5 Buford WTH cells/turkey. PC = positive-challenged control; Vacc = live-attenuated *Histomonas meleagridis;* NC = non-challenged control; WTH = wild-type *H. meleagridis.*

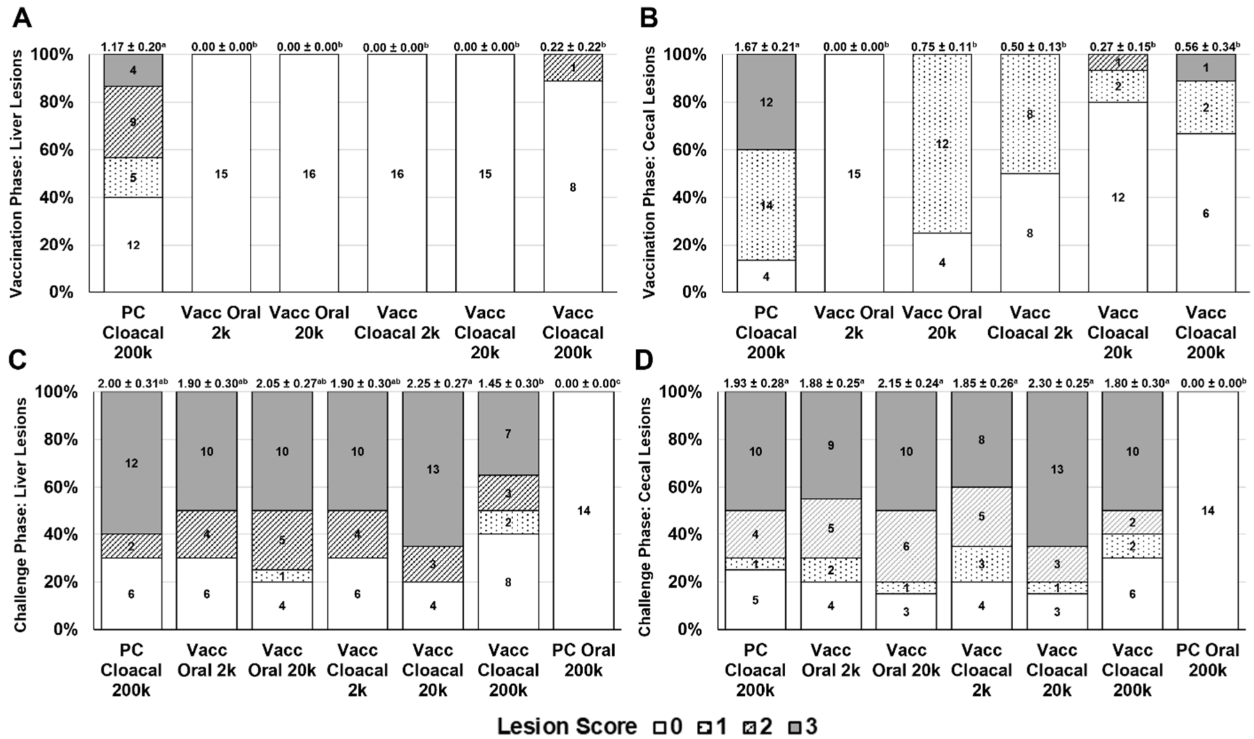


Figure 3. Experiment 2 frequency of lesion scores during Vaccination Phase for **A)** liver and **B)** cecae and during Challenge Phase for **C)** liver and **D)** cecae. Numbers within columns indicate the number of turkeys per evaluated lesion score. Numbers at the top of each column indicate the lesion score mean ± SE for that group with different superscripts denoting significance ($P < 0.05$). Lesion scores were based on a scale of "0" to "3" and were analyzed using the Proc Mixed Procedure in SAS 9.4 software. Vaccination Phase began on d0 with administration of respective dose and route of either Vacc Buford P80a or Buford WTH cells/turkey; Challenge Phase began on d21 with the intracloacal administration of 2×10^5 Buford WTH cells/turkey to all groups, except for PC Oral 200k which received the dose orally. Turkeys from the NC group were redistributed to form the new PC groups for the Challenge Phase. PC = positive-challenged control, Vacc = live-attenuated *Histomonas meleagridis*; WTH = wild-type *H. meleagridis.*

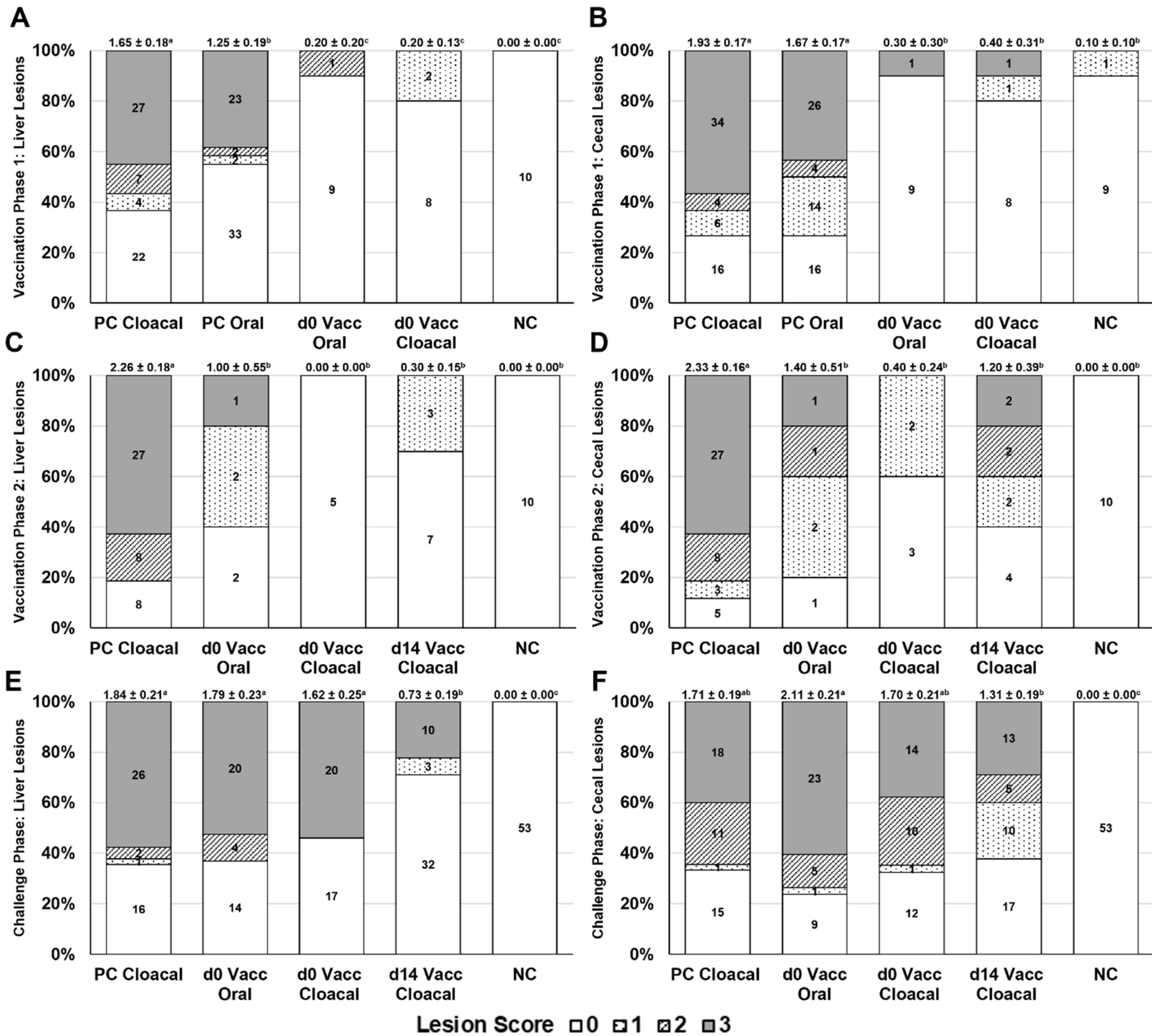


Figure 4. Experiment 3 frequency of lesion scores during Vaccination Phase 1 for **A)** liver and **B)** cecae; Vaccination Phase 2 for **C)** liver and **D)** cecae; Challenge Phase for **E)** liver and **F)** cecae. Numbers within columns indicate the number of turkeys per evaluated lesion score. Numbers at the top of each column indicate the lesion score mean ± SE for that group with different superscripts denoting significance ($P < 0.05$). Lesion scores were based on a scale of "0" to "3" and were analyzed using the Proc Mixed Procedure in SAS 9.4 software. Vaccination Phase 1 began on d0 with administration of 2 x 10^5 cells/turkey of either Vacc Buford P80a or Buford WTH cells/turkey via respective route; Vaccination Phase 2 began on d14 with the introduction of a d14 Vacc group and new PC (formed from subsets of the NC) which received intracloacal administration of 2 x 10^5 either Vacc Buford P80a or Buford WTH cells/turkey, respectively; Challenge Phase began on d28 with the intracloacal administration of 2 x 10^5 Buford WTH cells/turkey. PC = positive-challenged control, Vacc = live-attenuated *Histomonas meleagridis*; NC = non-challenged control; WTH = wild-type *H. meleagridis.*

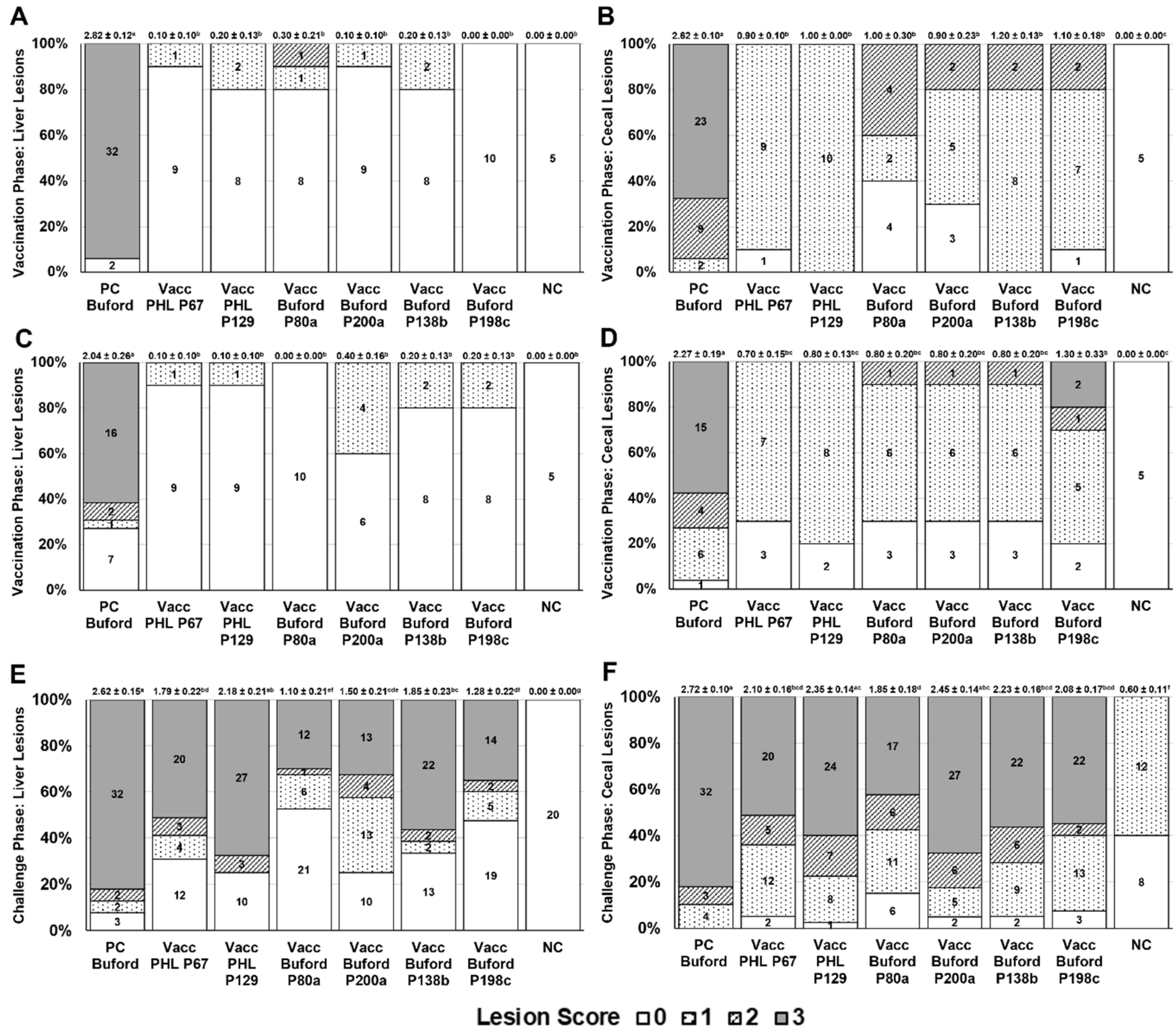


Figure 5. Experiment 4 frequency of lesion scores during Vaccination Phase for **A)** liver and **B)** cecae on d28; Vaccination Phase for **C)** liver and **D)** cecae on d35; Challenge Phase for **E)** liver and **F)** cecae. Numbers within columns indicate the number of turkeys per evaluated lesion score. Numbers at the top of each column indicate the lesion score mean ± SE for that group with different superscripts denoting significance ($P < 0.05$). Lesion scores were based on a scale of "0" to "3" and were analyzed using the Proc Mixed Procedure in SAS 9.4 software. Vaccination Phase began on d14 with the intracloacal administration of 2 x 10^5 cells/turkey of the respective Vacc isolate while the PC Buford received the same dose of Buford WTH; Challenge Phase began on d35 with the intracloacal administration of 2 x 10^5 Buford WTH cells/turkey. PC = positive-challenged control; Vacc = live-attenuated *Histomonas meleagridis;* NC = non-challenged control; WTH = wild-type *H. meleagridis.*

CHAPTER 5: CONCLUSIONS

The prevalence of histomoniasis has increased in the poultry industry for the past 30 years following the removal of effective prophylactics, and turkeys are especially susceptible to high mortalities which subsequently results in large economic losses to producers (McDougald, 2005; Hess and McDougald, 2013; Regmi et al., 2016). Biosecurity measures to prevent exposure to *Histomonas meleagridis* or vectors of this protozoa are important to reduce histomoniasis incidence in the absence of vaccines or approved drugs. Data from this dissertation indicated the non-challenged control did not contract histomoniasis, further supporting that proper management practices are critical to reducing disease incidence. Although separate rearing of poultry can reduce disease incidence by limiting contact between asymptomatic carriers and susceptible hosts, an effective prophylactic or vaccination program is still greatly needed. This dissertation evaluated feed supplementation of quinine as a chemoprophylactic candidate (Chapter 3) and live-attenuated *H. meleagridis* isolates as vaccine candidates (**Vacc**; Chapter 4) for prevention of histomoniasis in turkeys.

Quinine is an effective antimalarial drug, leading to the hypothesis that these antiprotozoal properties might also be effective against *H. meleagridis.* Data indicated quinine was efficacious as an antihistomonal *in vitro,* but dietary inclusion of quinine at the tested concentrations of 0.022%, 0.067%, or 0.2% did not reduce disease in turkeys challenged with wild-type *H. meleagridis* (**WTH**). Ensuring delivery of chemoprophylactic candidates directly to the cecae is a challenge, and quinine may not have reached the cecae in sufficient concentration to impair the protozoa. Previously, chickens recognized the bitter taste of quinine and reduced feed intake of diets containing more than 0.2% quinine, but threshold levels have not been established for turkeys (Ueda and Kainou, 2005). The 0.2% dietary inclusion of quinine was

hypothesized to be maximum for turkeys as well; however, the d0-10 body weight gain (**BWG**) in the quinine diets was not different ($P > 0.05$) as compared to the basal diet. Turkeys may perceive the bitter taste of quinine differently from chickens and subsequently have higher threshold levels than 0.2%, but the impact to performance at higher inclusion levels is unknown. This study demonstrated the necessity of pairing *in vitro* and *in vivo* experiments to ensure effectiveness of candidate antihistomonal compounds; furthermore, novel information was contributed on acceptable tolerance levels of quinine dietary inclusion to turkeys.

Oral challenge with virulent histomonads on day-of-hatch has previously induced histomoniasis in turkeys, although the oral route in absence of vectors remains somewhat controversial (Liebhart et al., 2008). Data from this dissertation demonstrated that WTH-challenge prior to feeding on day-of-hatch induced disease regardless of oral or cloacal route, presumably due to the near-neutral pH in the proventriculus-ventriculus region allowing the histomonads to survive and parasitize the cecae. Interestingly, oral WTH-challenge at d21 did not induce histomoniasis, further suggesting that the cloacal route rather than the oral route is the primary method for transmitting unprotected histomonads in older birds; however, the oral route should not be disregarded for young birds.

Despite immunological research advancements, a histomoniasis vaccine has not been developed for commercial application (McAllister, 2014; Liebhart et al., 2017; Mitra et al., 2018). Clonal *in vitro* attenuated histomonads have been administered orally or cloacally with some protection in experimental settings against virulent challenge without negative performance impacts; however, evaluations have not occurred in field conditions against heterologous, multi-isolate challenge (Hess et al., 2008; Liebhart et al., 2010, 2013). Day-of-hatch oral vaccination with live-attenuated histomonads was previously reported as effective, but booster vaccination

was recommended at d14 for established protection (Liebhart et al., 2010; Sulejmanovic et al., 2016). Data from vaccination experiments in this dissertation demonstrated that day-of-hatch administration of Vacc isolates either orally or cloacally did not protect turkeys against subsequent WTH-challenge, contrary to previously reported success with oral vaccination at this age (Liebhart et al., 2010; Sulejmanovic et al., 2016). Importantly, administration of non-clonal Vacc isolates on d14 conferred protection against challenge with homologous and heterologous WTH isolates; moreover, these conditions potentially better portrayed the field environment where turkeys are exposed to multiple isolates.

The Vacc isolates were distinctly attenuated as indicated by lowered mortalities ($P < 0.05$), lowered lesion scores ($P < 0.05$), and similar BWG ($P > 0.05$) as the non-challenged controls during the Vaccination Phases. This information is consistent with previous research indicating attenuation of *H. meleagridis* following repeated *in vitro* passage (Tyzzer, 1934, 1936; Lund et al., 1966; Wei et al., 2020). Unfortunately, utilizing live histomonads would be difficult for industry application due to the required intracloacal administration, as well as the additional concerns of attenuation stability and inconsistent protective immunity (Hess and McDougald, 2013; Hess et al., 2015). In practicality, the administration of live-attenuated histomonads on a commercial scale seems unlikely due to the high cost of cell culture propagation and application complexities, although the benefit to further develop a histomoniasis vaccine would be tremendous (Joyner et al., 1963; McAllister, 2014).

The overall data presented in this dissertation further reflect the difficulties in mitigating histomoniasis. Dietary inclusion of quinine alone was not encouraging for prevention of *H. meleagridis*-infection in turkeys, but vaccination appeared somewhat efficacious when live-attenuated histomonads were administered at d14 via the cloacal route. Unfortunately, the

protection against subsequent WTH-challenge of vaccinated turkeys was neither consistent nor robust. Further research should be conducted with phytochemicals as these compounds may offer a natural remedy for histomoniasis that could also be economical for the industry and acceptable to the consumer. Vaccination should be further pursued, especially to elucidate the administration route, dose, and age of bird. Taken together, these data are encouraging for immunity to histomoniasis, but the administration of a vaccine and the possible requirement for booster vaccination needed with the live-attenuated method is more experimentally interesting rather than practical for industry application.

REFERENCES

Hess, M., D. Liebhart, I. Bilic, and P. Ganas. 2015. *Histomonas meleagridis*—New insights into an old pathogen. Vet. Parasitol. 208:67-76.

Hess, M., D. Liebhart, E. Grabensteiner, and A. Singh. 2008. Cloned *Histomonas meleagridis* passaged *in vitro* resulted in reduced pathogenicity and is capable of protecting turkeys from histomonosis. Vaccine. 26:4187-4193.

Hess, M., and L. McDougald. 2013. Histomoniasis (blackhead) and other protozoan diseases of the intestinal tract. Pages 1172-1179 in Diseases of Poultry.13th ed. E. Swayne, J. R. Glisson, L. R. McDougald, L. K. Nolan, D. L. Suarez, and V. L. Nair, eds., Wiley-Blackwell, Ames, IA.

Joyner, L. P., S. F. M. Davies, and S. B. Kendall. 1963. Chemotherapy of histomoniasis. Pages 333-349 in Experimental Chemotherapy. Vol. 1. Academic Press New York.

Liebhart, D., P. Ganas, T. Sulejmanovic, and M. Hess. 2017. Histomonosis in poultry: Previous and current strategies for prevention and therapy. Avian Pathol. 46:1-18.

Liebhart, D., E. Grabensteiner, and M. Hess. 2008. A virulent mono-eukaryotic culture of *Histomonas meleagridis* is capable of inducing fatal histomonosis in different aged turkeys of both sexes, regardless of the infective dose. Avian Dis. 52:168-172.

Liebhart, D., T. Sulejmanovic, B. Grafl, A. Tichy, and M. Hess. 2013. Vaccination against histomonosis prevents a drop in egg production in layers following challenge. Avian Pathol. 42:79-84.

Liebhart, D., M. Windisch, and M. Hess. 2010. Oral vaccination of 1-day-old turkeys with *in vitro* attenuated *Histomonas meleagridis* protects against histomonosis and has no negative effect on performance. Avian Pathol. 39:399-403.

Lund, E. E., P. C. Augustine, and D. J. Ellis. 1966. Immunizing action of *in vitro*-attenuated *Histomonas meleagridis* in chickens and turkeys. Exp. Parasitol. 18:403-407.

McAllister, M. M. 2014. Successful vaccines for naturally occurring protozoal diseases of animals should guide human vaccine research: A review of protozoal vaccines and their designs. Parasitology. 141:624-640.

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

Mitra, T., Kidane, F. A., Hess, M., and Liebhart, D. 2018. Unravelling the immunity of poultry against the extracellular protozoan parasite *Histomonas meleagridis* is a cornerstone for vaccine development: A review. Front. Immunol. 9:2518.

Regmi, P. R., A. L. Shaw, L. L. Hungerford, J. R. Messenheimer, T. Zhou, P. Pillai, A. Omer, and J. M. Gilbert. 2016. Regulatory considerations for the approval of drugs against histomoniasis (blackhead disease) in turkeys, chickens, and game birds in the United States. Avian Dis. 60:725-730.

Sulejmanovic, T., I. Bilic, M. Hess, and D. Liebhart. 2016. An *in vitro* attenuated strain of *Histomonas meleagridis* provides cross-protective immunity in turkeys against heterologous virulent isolates. Avian Pathol. 45:46-53.

Tyzzer, E. E. 1934. Studies on histomoniasis, or "blackhead" infection, in the chicken and the turkey. Daedalus. 69:189-264.

Tyzzer, E. E. 1936. A study of immunity produced by infection with attenuated culture-strains of *Histomonas meleagridis.* J. Comp. Patho. Ther. 49:285-303.

Ueda, H., and S. Kaidou. 2005. Aversion to quinine is associated with taste sensation in chicks. J. Poult. Sci. 42:254-262.

Wei, Z., M. Abraham, E. V. Chadwick, and R. B. Beckstead. 2020. *Histomonas meleagridis* isolates compared by virulence and gene expression. Vet. Parasitol. 286:109233.

APPENDIX

UNIVERSITY OF ARKANSAS

Office of Research Compliance

To: Billy Hargis
Fr: Craig Coon
Date: April 12th, 2018
Subject: IACUC Approval
Expiration Date: April 5th, 2021

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **18113:** *Development and evaluation of prophylactic treatments for Histomoniasis in turkeys*.

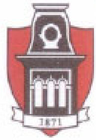
In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond April 5th, 2021 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Billy Hargis, Guillermo Tellez, Lesleigh Beer, and Cheryl Lester. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp

18113



UNIVERSITY OF
ARKANSAS

Office of Research Compliance

To:	Billy Hargis
Fr:	Craig Coon
Date:	October 8th, 2018
Subject:	IACUC Approval
Expiration Date:	October 4th, 2021

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **19032:** *Development and evaluation of prophylactic treatments, vaccinations, and routes of administration on the prevention of Histomoniasis in turkeys*.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond October 4th, 2021 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Billy Hargis, Guillermo Tellez, Lesleigh Beer, Cheryl Lester, Thaina Barros, and Christine Vuong. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp

19032

DIVISION OF AGRICULTURE
RESEARCH & EXTENSION
University of Arkansas System

To:	Billy Hargis
Fr:	Beth Kegley - Ag-IACUC co-Chair
Date:	February 22nd, 2021
Subject:	IACUC Approval
Expiration Date:	February 18th, 2024

The Division of Agriculture Institutional Animal Care and Use Committee (Ag-IACUC) has APPROVED your protocol # 21094 *Development and evaluation of prophylactic treatments for histomoniasis in turkeys - II.*

In granting its approval, the Ag-IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the Ag-IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond February 18th, 2024 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy, the Ag-IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Billy Hargis, Guillermo Tellez-Isaias, Christine Vuong, Danielle Graham, Callie McCreery Selby, Makenly Coles, Cheryl Lester, Roberto Senas Cuesta, Aaron Forga, Lesleigh Beer, Thaina Barros, and Lucas Graham. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The Ag-IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

BMH/tmp

21094