

BLACKHEAD DISEASE: VARIATION IN THE VIRULENCE OF *HISTOMONAS*  
*MELEAGRIDIS* AND ITS REDUCED SENSITIVITY TO NITARSONE

by

MATHEW ABRAHAM

(Under the Direction of Robert B. Beckstead)

ABSTRACT

*Histomonas meleagridis*, a flagellated protozoan that causes histomoniasis or blackhead disease, is a great threat to the turkey industry with its heavy mortality and morbidity. *In vivo* studies using *H. meleagridis* isolated from 3 strains collected from outbreaks in North Carolina (Strain MNC), Michigan (Strain ZM) and Georgia (strain BG) and monocultures generated from the Buford isolate show that each isolate and monoculture varied in virulence. These results suggest the variation in the virulence may contribute to the severity of the blackhead outbreak. Currently, nitarsonsone (4-nitrophenylarsonic acid) is the only approved drug available in the United States for prevention of histomoniasis. Recent blackhead disease outbreaks in turkeys fed with nitarsonsone contained feed suggest the possibility of drug resistance in certain strains of the parasites. We tested the sensitivity of MNC, ZM and BG strains to nitarsonsone using both an *in vitro* cell culture model and in turkeys. These studies reveal that strain MNC has reduced sensitivity to the nitarsonsone.

INDEX WORDS: *Histomonas meleagridis*, blackhead, virulence, nitarsonsone

BLACKHEAD DISEASE: VARIATION IN THE VIRULENCE OF *HISTOMONAS*  
*MELEAGRIDIS* AND ITS REDUCED SENSITIVITY TO NITARSONE

by

MATHEW ABRAHAM

B.V.Sc & A.H, Kerala Agricultural University, 2010

A Thesis Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfillment of  
the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2013

© 2013

Mathew Abraham

All Rights Reserved

BLACKHEAD DISEASE: VARIATION IN THE VIRULENCE OF *HISTOMONAS*  
*MELEAGRIDIS* AND ITS REDUCED SENSITIVITY TO NITARSONE

by

MATHEW ABRAHAM

Major Professor: Robert B. Beckstead

Committee: Larry R. McDougald

Andrew R. Moorhead

Electronic Version Approved:

Maureen Grasso

Dean of the Graduate School

The University of Georgia

May 2013

## DEDICATION

I would like to dedicate this thesis to my parents, Abraham Daniel and Laly Abraham. Their love, encouragement and support are always my great strength.

## ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Dr. Robert B. Beckstead, my major professor, for his guidance, support, and encouragement throughout my study. I also would like to thank my committee members, Dr. Larry R. McDougald and Dr. Andrew R. Moorhead for their guidance. I am also grateful for Dr. Lorraine Fuller for her guidance in both the laboratory and live animal experiments. Finally, I would like to thank the faculty, staff, and the graduate students in the Department of Poultry Science for helping me during my study.

## TABLE OF CONTENTS

|                                                                                                                                           | Page |
|-------------------------------------------------------------------------------------------------------------------------------------------|------|
| Acknowledgements.....                                                                                                                     | v    |
| List of Figures.....                                                                                                                      | viii |
| Chapter                                                                                                                                   |      |
| 1 Introduction .....                                                                                                                      | 1    |
| Literature cited .....                                                                                                                    | 3    |
| 2 Literature Review .....                                                                                                                 | 4    |
| Literature cited .....                                                                                                                    | 12   |
| 3 Comparative virulence of different isolates, monoculture and attenuated strains of<br><i>Histomonas meleagridis</i> .....               | 21   |
| 4 Blackhead Disease: Reduced sensitivity of <i>Histomonas meleagridis</i> to nitarsone <i>in</i><br><i>vitro</i> and <i>in vivo</i> ..... | 35   |
| 5 Conclusions.....                                                                                                                        | 49   |
| Literature cited .....                                                                                                                    | 50   |

|                                                                                                                                               |    |
|-----------------------------------------------------------------------------------------------------------------------------------------------|----|
| Appendix.....                                                                                                                                 | 52 |
| A     An Outbreak of Blackhead Disease ( <i>Histomonas meleagridis</i> ) in Farm-Reared<br>Bobwhite Quail ( <i>Colinus virginianus</i> )..... | 53 |



## LIST OF FIGURES

|                                                                                                                                                                                                                                                                                                                                                                                             | Page |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| Figure 3.1: Comparison of virulence of monocultures from the BG strain of <i>H. meleagridis</i> : Effects on Weight gain (g) and lesion scores of liver and ceca at necropsy of birds in the group UC, BG-M1 and BG-M2 at 10 dpi. <sup>a, b, c</sup> Scores within a graph with no common superscript differ significantly $P < 0.05$ .....                                                 | 31   |
| Figure 3.2: Comparison of virulence of field isolates or derived strains of <i>H. meleagridis</i> by infection of turkey poult: Effects on Weight gain (g) and lesion scores of liver and ceca at necropsy of birds in the group UC, BG-1, BG-100, ZM-1, ZM-56 and MNC at 10 dpi. <sup>a, b, c</sup> Scores within a graph with no common superscript differ significantly $P < 0.05$ ..... | 33   |
| Figure 4.1: Growth of <i>Histomonas meleagridis</i> (Strain BG, ZM and MNC) <i>in vitro</i> , as affected by addition of 100 or 400 ppm of nitarsone. <sup>a, b, c</sup> Scores within a graph with no common superscript differ significantly $P < 0.05$ .....                                                                                                                             | 44   |
| Figure 4.2: Nitarsone (187.5 ppm in feed) vs. <i>H. meleagridis</i> strain MNC in turkey poult. Weight gain (g) and lesion scores in the liver and ceca at necropsy 10 dpi with 35,000 <i>H. meleagridis</i> /bird.....                                                                                                                                                                     | 46   |
| Figure 4.3: Growth of <i>H. meleagridis</i> (Regenerated MNC) <i>in vitro</i> , as affected by addition of 100 or 400 ppm of nitarsone. Growth of the regenerated MNC treated with nitarsone at 100 ppm was                                                                                                                                                                                 |      |

more than regenerated MNC-CON (nitarsone untreated control group) during the entire period of growth. ....48

Figure A. 1. Daily mortality from blackhead disease (Histomoniasis) in a flock of 13,500 bobwhite quail (Dates are for July and August, 2011).....62

Figure A. 2. (A). Cecal lesions caused by *Histomonas meleagridis* in bobwhite quail (see arrow). Of 56 birds examined on a single day, 55 had similar *cecal* lesions characterized by thickened mucosa engorged with caseous cores. (B). Liver lesions caused by *Histomonas meleagridis* in bobwhite quail (see arrows). Of 56 birds examined, only 3 had lesions in the liver.....63

## CHAPTER 1

### INTRODUCTION

Histomoniasis or Blackhead disease is caused by the unicellular protozoan parasite *Histomonas meleagridis*, a member of the family Monocercomonadidae and class Trichomonaida (Hauck et. al., 2009). Turkeys, chickens, quail and peafowl are susceptible to this disease (Lotfi et. al., 2012), as evidenced by the presence of necrotic lesions in the liver and ulcers in ceca, with lesions also present in other tissues (Sentíes-Cué et. al., 2009). *Histomonas meleagridis* is transmitted through the intermediate host *Heterakis gallinarum*, a cecal nematode worm (Lund et. al., 1972). In turkeys this disease can also be transmitted by direct cloacal contact with contaminated liquid feces by means of a reverse peristaltic movement called cloacal drinking (Hu et. al., 2003). Blackhead disease produces mortality approaching 100% in turkeys and 10-20 % in chickens (McDougald, 2005). Heavy mortality and rapid transmission in turkey is due to their inability to mount a proper immune response and lateral transmission due to cloacal drinking.

Various drugs have been used to prevent and treat histomoniasis with limited success. Use of anthelmintics can control the transmission of cecal nematode worm, but cannot prevent lateral transmission of the disease in turkeys via the cloacal route. Use of nitroimidazole group of drugs was highly effective in treating histomoniasis, but the FDA banned these compounds in the early 1990's (McDougald, 2005) as suspected carcinogens. Arsenical compounds, such as nitarsones, have shown good prophylactic activity against histomoniasis, but are unable to treat infected birds. Currently there is not an effective chemotherapy or commercially available vaccine

against *H. meleagridis*. Lack of treatment and preventative options has resulted in an upsurge of cases of blackhead disease and increased mortality in turkeys and chickens.

There is significant variation in the percent of mortality between outbreaks of blackhead disease in turkeys, with some flocks only exhibiting only morbidity, while others exhibit 100% mortality. These differences suggest that there is a difference in the virulence of various strains of *H. meleagridis*. Studies on the comparative virulence of different isolates of *H. meleagridis* and variations in the virulence of various possible strains of the parasite within a single isolate will help determine how the pathology of blackhead disease is related to the causative strain of *H. meleagridis*. Additionally, recent outbreaks of blackhead disease in turkeys where nitarsone was included in the feed suggest that some strains of *H. meleagridis* may be acquiring resistance to this drug. No studies on the sensitivity of various strains of *H. meleagridis* to nitarsone have been published in the literature. Therefore, the protective property of nitarsone against blackhead disease needs to be tested.

The main objectives of this study are:

1. Compare the variations in the virulence of various field isolates of *H. meleagridis* and high passaged *in vitro* strains.
2. Generate monocultures of *H. meleagridis* and studies the virulence of different isolates.
3. Perform cell culture assays and *in vivo* turkey experiments to test the sensitivity of various strains of *H. meleagridis* to nitarsone.

#### Literature cited

- Hauck, R., and HM. Hafez. 2009. Partial sequence of the beta-tubulin of *Histomonas meleagridis* and the activity of benzimidazoles against *H. meleagridis in vitro*. *Parasitology Research*, 104(5): 1183-1189.
- Hu J, McDougald LR. 2003. Direct lateral transmission of *Histomonas meleagridis* in turkeys. *Avian Dis.* 47(2):489-92.
- Lotfi AR, Abdelwhab EM, Hafez HM. 2012. Persistence of *Histomonas meleagridis* in or on materials used in poultry houses. *Avian Dis.* 56(1):224-6.
- Lund EE, Chute AM. 1972. *Heterakis* and *Histomonas* infections in young peafowl, compared to such infections in pheasants, chickens, and turkeys. *J Wildl Dis.* 8(4):352-8.
- McDougald, LR. 2005. Blackhead disease (Histomoniasis) in poultry: a critical review. *Avian Dis.* 49: 462–476.
- Sentíes-Cué G, Chin RP, and Shivaprasad HL. 2009. Systemic histomoniasis associated with high mortality and unusual lesions in the bursa of Fabricius, kidneys, and lungs in commercial turkeys. *Avian Dis.* 53(2):231-8.

## CHAPTER 2

### LITERATURE REVIEW

#### History, Taxonomy and Morphology

Histomoniasis, commonly known as blackhead disease, is caused by a unicellular anaerobic protozoan parasite *Histomonas meleagridis* (Tyzzer, 1920). It causes severe mortality in turkeys, whereas chickens may be asymptomatic (McDougald, 1997). *Histomonas meleagridis* is a member of the family Dientamoebidae, order Tritrichomonadida, and class Tritrichomonadea (Cepicka et al., 2010). Members belonging to the Dientamoebidae family lack an undulating membrane, costa, suprakinetosomal body and infrakinetosomal body.

Blackhead disease was first discovered in 1893 in Rhode Island (Cushman, 1893) where it decimated the turkey industry. The introduction of ring-necked pheasant to the United States is thought to be responsible for the introduction of blackhead disease because the introduction of pheasants during the early 1890s coincided with the first outbreaks of blackhead disease in turkeys. Theobald Smith studied materials from the disease outbreak in Rhode Island and named the causative organism as *Amoeba meleagridis* (Smith, 1895). Later researchers considered the causative agent as a type of fungal infection, while other researchers believed protozoa to be a stage of *Trichomonas* (Hadley, 1920; Hadley and Amison, 1911). Tyzzer separated and named *H. meleagridis* from other related protozoans based on their flagellated and amoeboid nature (Tyzzer, 1920).

*Histomonas meleagridis* are round or amoeboid shape single cellular organisms ranging in diameter from 8-15µm (Smith, 1895). They are seen in two forms, the flagellate form in the cecal lumen and amoeboid form in the liver (Lund and Chute, 1974). Microscopically, food vacuoles are observed that contain bacteria, starch, and waste materials. Similar to *Giardia* and *Entamoeba*, *H. meleagridis* generates energy anaerobically using hydrogenosomes to convert pyruvate and malate to hydrogen, acetate, carbon dioxide and ATP (Muller, 1993, Townson et al., 1996; Brown et al., 1998, and Reeves, 1984). It has been suggested that *H. meleagridis* is able to produce cysts, but the role of this form in the transmission of this organism is unknown (Munsch et al., 2009; Zaragatzki et al., 2010).

#### Disease transmission, Pathology and Clinical Signs

*Histomonas meleagridis* requires an intermediate host for the transmission of the disease. Graybill discovered that cecal nematode worm *Heterakis gallinarum* is an important carrier of the *H. meleagridis* (Graybill, 1920). During co-infection, *H. meleagridis* infects the ova of cecal worm and together they are excreted with feces into the environment. Infected ova can survive for months to years. Earthworms act as a vector for *H. gallinarum* and thereby serve as a reservoir for *H. meleagridis* (Lund et al., 1966). Once the infected earthworm or embryonated *H. gallinarum* ova containing the infective second-stage larvae are eaten, *H. meleagridis* is liberated in the gut of the bird during the first larval molt of *H. gallinarum*. *Histomonas meleagridis* migrate to the bird's ceca and causes primary infection by invading into the mucosa resulting in thickening of cecal wall. As the disease progresses, the lumen of ceca will become occluded with cecal cores containing a caseous exudate with inflammatory cells and debris. From the ceca *H. meleagridis* reaches the blood stream and then migrates to the liver through the portal blood vessels that connects the blood supply of ceca and intestines to the liver (Clarkson, 1961; Fine,

1975). In the liver *H. meleagridis* produces necrotic foci that have raised borders with a dark, concave center. In addition, there may be large white raised necrotic areas or a green tinged liver due to the clogging of bile duct with *H. meleagridis* or inflammation. *Histomonas meleagridis* has also been found in the kidney, spleen, lungs and bursa of Fabricius using periodic acid Schiff (PAS) and Bodian staining (Malewitz and Calhoun, 1957; Malewitz et al., 1958; Senties-Cue et al., 2009). Cecal lesions of histomoniasis are highly associated with bacterial infections caused by *Escherichia coli*, *Clostridium perfringens* or *Bacillus subtilis*. However, lesions in the liver are normally bacteria free (Franker et al., 1964; Bradley and Reid., 1966).

Turkeys can transmit the *H. meleagridis* laterally through reverse peristalsis of infected feces through the cloaca to the ceca. This form of transmission occurs when sick turkeys huddle together and allows for rapid spread of blackhead disease in the absence of *H. gallinarum* (Hu and McDougald, 2003). Due to the absence of the huddling behavior in chickens, this mode of transmission is not observed in chicken flocks, and therefore, is not shown to be an important factor in the transmission of blackhead disease in chickens (Hu et al., 2006).

Chickens, guinea fowl, chukar partridges, and pheasants are good host for the cecal worm *H. gallinarum* and thereby act as a reservoir of infection (Lund and Chute, 1970). Since the ova of the *H. gallinarum* can survive in soil for up to 3 years, it is highly recommended to rear turkeys separate from chickens or other gallinaceous birds to avoid cross-contamination. Clinical signs of histomoniasis include lethargy, anorexia, drooping of wings and head, sulfur-colored droppings and malaise. Birds suffering from blackhead may eventually die due to liver failure (McDougald, 2003).



### In vitro culture

Optimum growth of *H. meleagridis* requires a media that supports both *H. meleagridis* and bacterial growth (McDougald, 2005; Hauck, 2010). The media needs to be slightly acidic and maintained under anaerobic conditions. Anaerobic conditions are needed for the survival of the parasite because of its dependence to hydrogenosome for energy metabolism (Muller, 1993). A carbohydrate source is also required to provide nutrition for parasites and bacteria in the media. Several methods have been employed to grow *H. meleagridis* in culture. Drbohlav (1924) successfully grew *H. meleagridis* in media composed of egg white, blood bouillon and peptone. Later Laidlaw used eight parts of modified Ringers solution, one part of horse serum and a pinch of rice powder as a medium for the growth of *H. meleagridis* (Laidlaw et al., 1928). Devolt used Locke solution, 1% serum, 10 mg of rice powder and charcoal for growing *H. meleagridis* (Devolt, 1943). Dwyer's media contains 85-95% of Medium 199, 5-10% horse serum, 5% chick embryo extract, and 1% rice powder (Dwyer, 1970). Dwyer media was modified by the removal of chick embryo extract and the rice concentration reduced to 0.8% (van der Heijden and Landman, 2007). Modified Dwyer's medium showed extensive and rapid growth of both *H. meleagridis* and bacteria and is most commonly used by current researchers.

*H. meleagridis* in modified Dwyer's medium attain a peak growth in 1-5 days. After the peak, numbers tend to decline due to death from depletion of nutrients and increase waste products. Gerhold (2010) confirmed that this media is highly successful for the transport of *H. meleagridis* samples maintained in warm temperature. Prolonged passage of *H. meleagridis in vitro* resulted in the loss of virulence of the parasite (Dwyer, 1970). This loss of virulence appears to be caused by the down regulation of virulence factors of *H. meleagridis* upon continuous passage (Wei et al., Submitted for publication). *Histomonas meleagridis* can be preserved in frozen liquid

nitrogen without the loss of virulence for many years by using 8% DMSO as cryoprotectant (Chute and Chute, 1969; Honigberg and Dwyer, 1969).

### Diagnosis

Blackhead disease can be easily diagnosed based on history of severe mortality and morbidity with gross pathology like sulfur colored droppings, necrotic lesions in liver and cecal lumen filled with an inflammatory core comprised of mucosa, blood and debris. Liver lesions of blackhead disease can be confused with avian leucosis, mycosis or other bacterial infections. Similarly, coccidiosis and some bacterial infections with *E. coli* and *Salmonellosis* can also cause cecal core in birds (McDougald, 2005). Histopathology can confirm the diagnosis of the liver section using hematoxylin and eosin, periodic acid-Schiffs or other stains (McDougald, 2005). *Histomonas meleagridis* can be isolated *in vitro* from the infected ceca and liver using a Dwyer medium (McDougald and Galloway, 1973). Molecular techniques like polymerase chain reaction (PCR) using *H. meleagridis* specific primers are widely used for the diagnosis of blackhead disease. Sequence analysis of the 5.8S rRNA genes and the internal transcribed spacer (ITS) regions of the protozoa can be used to confirm the presence of *H. meleagridis* in a tissue sample (Felleisin, 1997).

Serological tests are also available to detect histomoniasis. Detection of IgG antibodies by indirect ELISA has also been developed. This is suitable for a large-scale screening of poultry for *H. meleagridis* antibodies. These antibodies are not related to the development of protective immunity against *H. meleagridis*. However, their detection is a good diagnostic method in asymptomatic chicken by 14 days after infection (Windisch and Hess, 2009). A highly-specific blocking ELISA, based on the monoclonal antibodies, can detect the antibodies of *H.*

*meleagridis* without cross-reaction with the antibodies of other immunologically related species. These monoclonal antibodies did not bind to *Trichomonas gallinarum* antigen, and moreover, *T. gallinarum* seropositive birds showed the same inhibition percentages as negative control birds (van der Heijden et al., 2010).

#### Treatment with drugs

Many drugs were tested against *H. meleagridis* *in vitro* and *in vivo*. Nitroheterocyclic compounds showed good curative property against blackhead disease in all species (Flowers et. al, 1965). Among nitroheterocyclic compounds, enheptin was effective both in treatment and prevention, but it caused depression of growth and sexual maturity (Grumbles et. al, 1952, 1952; Hudson et. al, 1952). Nitroimidazoles like dimetridazole, ipronidazole and ronidazole were used against histomoniasis. It did not produce any side effects and was useful both as a treatment and preventive measure (Flowers et. al, 1965). Use of nitroimidazoles greatly reduced the incidence of blackhead disease. However, in the 1990's nitroimidazoles were banned in the United States by the Food and Drug Administration and in the European Union in 2003, because they were suspected carcinogens (Hafez et al., 2005; McDougald, 2005). An upsurge of blackhead disease occurred since there is no currently available treatment against blackhead disease. Arsenical compounds were reported to show very good results against histomoniasis. Nitrofurans are arsenical compounds that are very effective in reducing mortality, but allowed relapse after withdrawal (Bowen et. al, 1971; Grumbles et. al, 1952). These compounds can cause arsenic toxicity. Use of arsenical compounds was banned in European Union (Byrne, 2001). Nitarsone is a pentavalent arsenical compound and it is the only drug of choice available now against histomoniasis for preventive use in feed (McDougald, 2005)

Nitarsonsone is commercially available as Histostat-50 and approved as a feed ingredient at 187.5 ppm. Nitarsonsone was shown to be highly successful in controlling the *in vitro* growth of *H. meleagridis* and this inhibition of growth is dose dependent (Van der Heijden et. al, 2008). Nitarsonsone at 50 ppm was able to eliminate the parasite completely from the medium within 70 h. Nitarsonsone at 400 ppm was able to control the growth of parasite within 30 h. Nitarsonsone at 12.5 and 25 ppm did not show any significant effect on the growth of the parasite. *In vivo* studies showed that turkeys treated with nitarsonsone in the feed one week prior to inoculation still contracted blackhead disease (Van der Heijden et. al, 2008). Thus, the inclusion of nitarsonsone needs to be longer than 1 week to provide protection to the birds against blackhead disease. Action of nitarsonsone is prophylactic rather than curative (Hu and McDougald, 2004). Relapse of the disease was reported after the withdrawal of nitarsonsone from the feed (McGuire and Morehouse, 1952). In addition to the preventative-histomonal property, nitarsonsone is effective as an anthelmintic. The anthelmintic effect of nitarsonsone was described in turkeys against *Ascaridia galli* infection (Reynolds et al, 2009). No cases of resistance acquired by *H. meleagridis* against nitarsonsone have been reported in literature.

Anthelmintics like benzimidazoles have been widely used to prevent histomoniasis. These drugs have to be given prior to the exposure with *H. gallinarum*. This drug can control only the transmission of histomoniasis through the cecal worm by preventing the larval molting and subsequent release of *H. meleagridis*. Since anthelmintics are not targeting the protozoa, they are not useful in preventing blackhead disease transmitted through cloacal drinking (Hegnig, 1999, Hu et. al, 2004).

## Immunity and Immunization

Early research suggested that immunization could not provide complete protective immunity in turkeys against blackhead disease. Intravenous injections with liver suspension from infected birds proved to be ineffective in providing immunity (Tyzzer et. al, 1921). Antisera obtained from infected birds did not provide passive protection to naïve turkeys against infection (Clarkson, 1966). A nonpathogenic *H. meleagridis* generated by Tyzzer through continuous passage *in vitro* and used to vaccinate turkeys and chickens through the cloacal route did provide some protection (Tyzzer, 1933, 1933, 1934, 1936). Although, Lund reported that long term culturing results in the loss of immunizing ability (Lund et. al, 1966). Protection was also not developed in infected birds cured with nitroimidazole drugs (McDougald, 2005). Vaccinating with the avirulent species, *H. wenrichi* was also employed, but failed to provide immunity against *H. meleagridis* infection through *H. gallinarum* (Lund, 1963, Lund, 1959).

Immunological studies comparing chickens and turkeys suggest that turkeys fail to produce an effective innate immune response against *H. meleagridis* in the ceca resulting in greater damage and more parasites migrating into the blood. Higher antibody levels were observed in the chicken, which suggests an adaptive immune response in that species. High parasite number in turkey liver was also associated with an uncontrolled immune response due to the excess influx of cytokines. This uncontrolled immune response may lead to increased immunopathology in liver and higher mortality in turkeys (Powell et al., 2009).

Cloacal vaccination using *in vitro* passaged attenuated clonal cultures of *H. meleagridis* were also studied. Hess demonstrated that *H. meleagridis* cultures passaged *in vitro* for 95, 215 and 295 times showed severe attenuation in that none of the turkeys infected with these protozoa died upon infection through cloaca (Hess et. al, 2008). Turkeys immunized with the attenuated strains

obtained protective immunity against the original virulent strain by day 28 post-vaccination. Lesions on liver and ceca were seen only in few birds. These results suggest that the strains or species of *H. meleagridis* may have to be similar in order to acquire immunity. Liebhart and coworkers demonstrated the immunizing property of *in vitro*-attenuated parasites as an oral vaccine administered on one-day-old turkeys after the feed restriction for 5 h (Liebhart et. al, 2010). In this study no negative effects on performance were observed in vaccinated birds. These data suggest *in vitro* attenuation and the use of avirulent *H. meleagridis* is a viable tool for vaccination against histomoniasis (Liebhart et. al, 2011). Even though studies on the use of attenuated parasites to provide a protective immune response suggested convincing results, more studies need to be conducted to produce a commercial vaccine.

#### Literature Cited

- Bowen, T. E., Sullivan, T. W., and O. D. Grace.1971. Effect of cupric sulfate on prophylactic efficacy of 3 arsenic acid compounds against histomoniasis in young turkeys. Poultry Sci. 50:861-866.
- 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.
- Brown, D. M., J. A. Upcroft, M. R. Edwards, and P. Upcroft. 1998. Anaerobic bacterial metabolism in the ancient eukaryote *Giardia duodenalis*. Int. J. Parasitolol. 28:149-164.
- Byrne, D. (2001). Commission Regulation EC No. 2205/2001, amending Council Directive 70/524/EEC concerning additives in feeding stuffs as regards withdrawal of the authorization of certain additives. Official Journal of the European Communities, L297, 3\_4.

- Cepicka, I., V. Hampl, and J. Kulda. 2010. Critical Taxonomic Revision of Parabasalids with Description of one New Genus and three New Species. [Article]. Protist, 161(3), 400-433. doi: 10.1016/j.protis.2009.11.005
- Chute, M. B., and A. M. Chute. 1969. Freeze preservation of *Histomonas meleagridis*. Poult. Sci. 48:2189-2191.
- Clarkson, M. J. 1961. The blood supply of the liver of the turkey and the anatomy of the biliary tract with reference to infection with *Histomonas meleagridis*. Res. Vet. Sci. 2:259-264.
- Clarkson, M. J. 1966. Progressive serum protein changes in turkeys infected with *Histomonas meleagridis*. J. Comp. Pathol. 76:387-396.
- Cushman, S. 1893. The production of turkeys. Bulletin 25, Agricultural Experiment Station, Rhode Island College of Agriculture and Mechanical Arts, Kingston, RI. pp. 89-123.
- Devolt, H. M. 1943. A new medium for the cultivation of *Histomonas meleagridis*. J. Parasitol. 29:353-355.
- Drbohlav, J. J. 1924. The cultivation of the protozoan of blackhead. J. Med. Res. 44:677-678.
- Dwyer, D. M. 1970. An improved method for cultivating *Histomonas meleagridis*. J Parasitol, 56(1), 191-192.
- Felleisin, R. 1997. Comparative sequence analysis of 5'8S rRNA genes and internal transcribed spacer (ITS) regions of Trichomonadid protozoa. Parasitol. 115:111-119.
- Fine, P. E. M. 1975. Quantitative studies on transmission of *Parahistomonas wenrichi* by ova of *Heterakis gallinarum*. Parasitology 70:407-417.

- Flowers, A. I., C. F. Hall, and L. C. Grumbles. 1965. Chemotherapy of histomoniasis of turkeys. I. Value of 1-2-dimethyl-5-nitroimidazole in treatment. *Avian Dis.* 9:394-406.
- Gerhold RW, Lollis LA, Beckstead RB, McDougald LR. 2010. Establishment of culture conditions for survival of *Histomonas meleagridis* in transit. *Avian Dis* 54(2):948-50.
- Graybill, H. W. 1921. The incidence of blackhead and occurrence of *Heterakis papillosa* in a flock of artificially reared turkeys. *J. Exp. Med.* 33:667-673.
- Grumbles, L. C., W. A. Boney, and R. D. Turk. 1952. Chemotherapy of enterohepatitis of turkeys. I. The value of 2-amino-5-nitrothiazole in prevention and treatment. *Am. J. Vet. Res.* 13:383-385.
- Grumbles, L. C., W. A. Boney, and R. D. Turk. 1952. Chemotherapy of enterohepatitis of turkeys. II. The effect of 2-amino-5-nitrothiazole and 2-acetylaminonitrothiazole on egg production, fertility and hatchability in turkey hens. *Am. J. Vet. Res.* 13:386-387.
- Hadley, P. B., and E. E. Amison. 1911. Further studies on blackhead in turkeys. *Zent. Bakt.* 58:34-41.
- Hadley, P. B. 1920. *Trichomonas* and blackhead in turkeys. *Am. Naturalist* 54:176-181.
- Hafez, H.M., Hauck, R., Lüscho, D., 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.
- Hauck, R., P. L. Armstrong, And L. R. McDougald. 2010. *Histomonas meleagridis* (Protozoa: Trichomonadidae): analysis of growth requirements *in vitro*. *J Parasitol*, 96(1), 1-7. doi: GE-1985 [pii]10.1645/GE-1985.1



- Hegngi, F.N., Doerr, J., Cummings, T.S., Schwartz, R.D., Saunders, G., Zajac, A., Larsen, C.T., 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.
- 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.
- Honigberg, B. M., and B. M. Dwyer. 1969. Maintenance of *Histomonas meleagridis* in liquid nitrogen. *J. Protozool.* 16(Suppl.):18-19.
- Hu, J., and L. R. McDougald. 2003. Direct lateral transmission of *Histomonas meleagridis* in turkeys. *Avian Dis* 47: 489-492.
- Hu, J. & McDougald, L.R. 2004. The efficacy of some drugs with known antiprotozoal activity against *Histomonas meleagridis* in chickens. *Veterinary Parasitology*, 121, 233\_238.
- Hu, J., Fuller, L., 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. Histomoniasis in chickens: attempted transmission in absence of vectors. *Avian Dis.* 50: 277-279.
- Hudson, C. B., and J. A. Pino. 1952. Physiological disturbance of the reproductive system in white leghorn cockerels following the feeding of enheptin. *Poult. Sci.* 31:1017-1022.
- Laidlaw, P. P., C. Dobell, and A. Bishop. 1928. Further experiments on the action of emetine in cultures of *Entamoeba histolytica*. *Parasitology* 20:207-220.

- Liebhart, D., Windisch, M., 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, Zahoor MA, Prokofieva I, Hess M. 2011. Safety of avirulent histomonads to be used as a vaccine determined in turkeys and chickens. Poult Sci. 90(5):996-1003.
- Lund, E. E. 1959. Immunizing action of a nonpathogenic strain of *Histomonas* against blackhead in turkeys. J Euk Microbiol 6: 182-185.
- Lund, E. E. 1963. *Histomonas wenrichi* n. sp. (mastigophora: mastigamoebidae), a nonpathogenic parasite of gallinaceous birds. J Euk Microbiol 10: 401-404.
- Lund, E .E., and A. M. Chute. 1970. Relative importance of young and mature turkeys and chickens in contaminating soil With *Histomonas*-bearing *Heterakis* eggs. Avian Dis 14: 342-348.
- Lund, E. E., and A. M. Chute. 1974. Reciprocal transfer of *Heterakis gallinarum* larvae between ring-neck pheasants and Japanese quail: effects on *H. gallinarum*, *Histomonas meleagridis*, and *Parahistomonas wenrichi*. Proc. Helm. Soc. Wash. 41:73-76.
- Lund, E. E., and A. M. Chute. 1974. The reproductive potential of *Heterakis gallinarum* in various species of galliform birds: implications for survival of *H. gallinarum* and *Histomonas meleagridis* to recent times. Int. J. Parasitol. 4:455-461.
- 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.
- Lund, E. E., Wehr, E.E., Ellis, D.J. 1966. Earthworm Transmission of *Heterakis* and *Histomonas*

- to turkeys and chickens. The Journal of Parasitology 52: 899-902.
- Malewitz, T. D., and L. Calhoun. 1957. The normal hematological picture of turkey poults and blood alterations caused by enterohepatitis. Am. J. Vet. Res. 18:396-399.
- Malewitz, T. D., R. A. Runnels, and L. Calhoun. 1958. The pathology of experimentally produced histomoniasis in turkeys. Am. J. Vet. Res. 19:181-185.
- 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. 1997. Protozoa: other protozoan diseases of the intestinal tract. In B. Calneck, H.J. Barnes, C.W. Beard, L.R. McDougald & Y.M. Saif (Eds.), Diseases of Poultry, 10th edn (pp.890\_899). Ames: Iowa State University Press.
- Mcdougald, L. R. 2003. Other protozoan diseases of the intestinal tract--histomoniasis (blakhead).
- Mcdougald, L. R. 2005. Blackhead disease (histomoniasis) in poultry: a critical review. Avian Dis, 49(4), 462-476.
- McGuire, W. C., and N. F. Morehouse. 1952. Chemotherapy studies of histomoniasis. Poult. Sci. 31:603-609.
- Muller, M. 1993.The hydrogenosome. J. Gen. Microbiol. 139:2879- 2889.
- Munsch, M., Lotfi, A., Hafez, H., Al-Quraishy, S., and H. Mehlhorn. 2009. Light and transmission electron microscopic studies on trophozoites and cyst-like stages of *Histomonas meleagridis* from cultures. Parasitol Res 104: 683-689.

- Powell, F. L., Rothwell, L., Clarkson, M. J., 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.
- Reeves, R. E. Metabolism of *Entamoeba histolytica* Schaudinn .1903. *Advances in Parasitol.* 23:105-142. 1984
- Reynolds, J., C. A. Tucker, T. A. Yazwinski, Z. Johnson, D. Clark, and S. Clark. 2009. Anthelmintic effect of nitarsonsone on *Ascaridia dissimilis* infections in the turkey. *J. Appl. Poult. Res.* 18:766–771.
- Senties-Cue, G., R. P. Chin, and H. L. Shivaprasad. 2009. Systemic Histomoniasis Associated with High Mortality and Unusual Lesions in the Bursa of Fabricius, Kidneys, and Lungs in Commercial Turkeys. [Article]. *Avian Diseases*, 53(2), 231-238.
- Smith, T. 1895. An infectious disease among turkeys caused by Protozoa (infectious enteritis). *USDA, Bur. Anim. Ind. Bull.* 8:3-27.
- Townson, S. M., J. A. Upcroft, and P. Upcroft. 1996. Characterization and purification of pyruvate: ferredoxin oxidoreductase from *Giardia duodenalis*. *Mol. Biochem. Parasitol.* 79:183-193.
- 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., Fabian, M., Foot, N.C.1921. Further Observations on "Blackhead" in Turkeys. *J Infect Dis* 29: 268-286.

- Tyzzer, E. E. 1933. Loss of virulence in the protozoon of "blackhead," a fatal disease of turkeys, and the immunizing properties of attenuated strains. *Sci* 78:522-523.
- Tyzzer, E. E. 1933. The immunizing properties of an attenuated strain of *Histomonas meleagridis*. *J Parasitol* 19:158.
- Tyzzer, E. E. 1934. Studies on Histomoniasis, or "Blackhead" Infection, in the Chicken and the Turkey. *Proc Amer Acad Art Sci* 69: 189-264.
- Tyzzer, E. E. 1936. A study of immunity produced by infection with attenuated culture strains of *Histomonas meleagridis* J . *Comp. Pathol. Ther.*49:285-303.
- Van der Heijden H.M., Landman W.J. 2008. *In vitro* effect of herbal products against *Histomonas meleagridis*. *Vet Parasitol.* 154(1-2):1-7.
- Van der Heijden H.M., Landman W.J. 2008. *In vivo* effect of herbal products against *Histomonas meleagridis* in turkeys. *Avian Pathol.* 37(1):45-50.
- 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., Stageman, A., 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(3-4):216-22
- 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.

Zaragatzki, E., Mehlhorn, H., Abdel-Ghaffar, F., Rasheid, K., Grabensteiner, E., and M. Hess.

2010. Experiments to produce cysts in cultures of *Histomonas meleagridis* the agent of histomonosis in poultry. Parasitol Res 106: 1005-1007.

## CHAPTER 3

### COMPARATIVE VIRULENCE OF DIFFERENT ISOLATES, MONOCULTURES AND ATTENUATED STRAINS OF *HISTOMONAS MELEAGRIDIS*

-----  
M. Abraham, L. R. McDougald and R. B. Beckstead\*

To be submitted to *Journal of Parasitology*

**ABSTRACT:** Blackhead disease (histomoniasis) in gallinaceous birds is caused by the anaerobic protozoan *Histomonas meleagridis*. Field observations suggest variations in virulence of strains from different locations, although this has never been confirmed by controlled experiments. In the present study, isolates from outbreaks in Michigan, North Carolina and Georgia were compared *in vivo*, several sub-strains were derived by cloning or by prolonged culture *in vitro* and propagated for study. Two-week-old turkey poults were inoculated cloacally with 35,000 *H. meleagridis* from the compared strains. Experiments were terminated 10 days post inoculation (dpi) to record weight gain and necropsy performed to compare cecal and liver lesions. Based on the lesion scores and average weight gain, BG-1, BG-M1, ZM-1 and MNC were found to be more virulent than BG-100, BG-M2 and ZM-56. These results suggest that field isolates of *H. meleagridis* may vary significantly in virulence. In addition, individuals within a cultured population may also vary in virulence.

**Keywords:** *Histomonas meleagridis*, Blackhead, Virulence

**Abbreviations:** *H. meleagridis* = *Histomonas meleagridis*; *H. gallinarum* = *Heterakis gallinarum*; dpi = days post infection; UC = Uninfected Control



## INTRODUCTION

Histomoniasis or blackhead disease is a parasitic disease of gallinaceous birds and is caused by an anaerobic, flagellated, protozoan parasite *Histomonas meleagridis* (Tyzzer and Fabyan, 1922). It causes severe mortality in turkeys and high morbidity in chickens. Histomoniasis is transmitted through an intermediate host, the cecal nematode *Heterakis gallinarum*. The disease is manifested with necrotic lesions in liver, caseous ulcerative lesions in ceca and sulfur colored droppings. Field observations suggest that there may be considerable variation in the virulence of strains causing outbreaks in turkey and chicken flocks, although these observations have not been confirmed experimentally. It is well known that *H. meleagridis* loses pathogenicity during repeated passage *in vitro* (Tyzzer, 1933, 1934, 1936), but it is not known whether this is because of some mutation within the culture, selection of a strain better adapted to culture *in vitro*, or selection of naturally existing strains with variance in virulence within the population.

The severity of blackhead disease varies considerably between species of host, with turkeys being the most severely affected (Lund, 1967; Lund and Chute, 1972). However, field observations have suggested that considerable variation may exist between strains causing outbreaks, based on the extent of mortality, morbidity, and other effects in flocks (McDougald, 1997). Many researchers have pointed out the genotypic variation in *H. meleagridis* (van der Heijden et al., 2006; Hauck et al., 2010). Lollis and coworkers found that many outbreaks are caused by different genotypes of the parasite and many other species of parasites can also cause clinical signs similar to blackhead disease (Lollis et al., 2011). However, more studies need to be conducted on the genetic variation of *H. meleagridis* on the basis of virulence and disease transmission. Concurrent outbreaks of blackhead disease in turkeys in North Carolina and Michigan allowed us to compare the isolated *H. meleagridis* from each location with a laboratory

strain from Georgia by means of experimental infections in turkeys. Further, strains were derived from each field isolate by cloning, and by prolonged culture *in vitro*. The objective of present study was to compare the virulence of *H. meleagridis* isolates collected from various outbreaks, *H. meleagridis* monocultures and *in vitro* passaged *H. meleagridis*. All these strains of *H. meleagridis* were used to experimentally infect turkeys for studying their variations in the virulence.

## **MATERIALS AND METHODS**

**Experimental Birds.** Day-old turkey poults (obtained from Butterball turkey hatchery, Goldsboro, NC) were maintained until 2 wks old in steam-sterilized colony cages, and then were moved to steam-sterilized broiler finisher cages for feeding and inoculation. Turkey starter ration and water were given *ad libitum* throughout.

**Parasites and Culture.** Isolates of *H. meleagridis* were obtained from outbreaks in Monroe, North Carolina (Strain MNC), Zeeland, Michigan (Strain ZM) and Buford, Georgia (strain BG). Strain MNC and Strain ZM from turkeys, while Strain BG is from mixed poultry reared by a bird fancier. These isolates were cultured and frozen in liquid Nitrogen. For experimental infection in birds, cultures were resuscitated and cultured in modified Dwyer's medium at 40°C. Modified Dwyer's medium consists of 0.8% (w/v) rice powder and 5% Horse serum in Medium199 with Hank's (Hauck, et al, 2010).

**Generation of monocultures and attenuated strains.** Monocultures were prepared by diluting *H. meleagridis* in media after counting the concentration using a hemocytometer. It was diluted to a concentration of 40 *H. meleagridis* cells per 200 ml of media. This suspension was divided into 200 parts and transferred into 1.5 ml micro tubes after gentle mixing (i.e. each of 200 micro

tubes containing 1 ml of suspension). These micro tubes were incubated for 5 days at 40<sup>0</sup>C. Cultures were checked for the growth of *H. meleagridis* under a light microscope at 400x. Positive cultures were marked as monocultures and transferred to 10 ml of fresh media. These monocultures were frozen and stored for future experiments. In the present study two monocultures generated from the Buford isolate designated BG-M1 & BG-M2 were compared.

Attenuated strain BG-100 was prepared by the continuous passage of the parental culture of strain BG in the Dwyer's medium for 99 times. Similarly, ZM-56 was prepared by the continuous passage of strain ZM in the Dwyer's medium for 55 times. Each passage into new medium was done after allowing the parasite to attain optimum growth for 1-2 days.

**Inoculation of test birds:** *H. meleagridis* were used to infect birds with 35,000 cells/bird by intra-cloacal inoculation. All birds were weighed before inoculation and at termination of studies. At 10 dpi the birds were euthanized by CO<sub>2</sub> and cervical dislocation. At necropsy, lesions of the liver and ceca were assessed and recorded. Cecal and liver lesions were scored in 0-4 scale based on the severity (Hu et al, 2004).

## **Experimental Design.**

### **Experiment 1: Comparison of virulence of monocultures of the Georgia strain (BG) of *H. meleagridis* in turkey poults**

Two- week- old turkey poults were individually weighed, banded and distributed into 9 groups of 10 birds each. Three groups each were inoculated intra-cloacally with monocultures BG-M1 or BG-M2, and the remaining 3 groups were kept uninfected (UC). At 10 days post infection birds were weighed, euthanized, necropsied, and cecal and liver lesions were scored.

## **Experiment 2: Comparison of virulence of field isolates of *H. meleagridis* by infection of turkey poults - Parent isolates vs strains derived by multiple passages *in vitro*:**

Two- week- old birds were weighed, banded, and divided into 18 groups of 10 birds each. Fifteen groups were infected with *H. meleagridis* by direct intra-cloacal inoculation and 3 groups remaining uninfected. Each of five strains of *H. meleagridis* (Buford Pass 1(BG-1), Buford Pass 100(BG-100), Michigan (ZM-1), MI pass 56 (ZM-56) and North Carolina (MNC), were used to infect 3 replicate cages of 10 birds each. At 10 days post infection birds were weighed, euthanized, necropsied and cecal and liver lesions were scored.

**Lesion scoring.** Thickening or reddening of cecal wall is scored as 1. Scores of 2 and 3 indicate increasing severity of the lesion like thickening of the cecal wall, formation of a cheesy cecal core, and inflammation of the mesenteries. Score of 4 is given when there are extensive hemorrhagic lesions in ceca with the engorgement of lumen with a cecal core. The liver lesions were scored 1-2 with only a few discrete surface lesions (score 1) or a 3 with increasing severity. A complete involvement of the liver with lesions was scored 4 (Hu *et al.*, 2004).

**Statistical Analysis.** Results of the average weight gain and average lesion scores of liver and ceca were analyzed by one-way ANOVA followed by Tukey multiple comparison test ( $P < 0.05$ ).

## **RESULTS & DISCUSSION**

### **Experiment 1: Comparison of virulence of monocultures from the BG strain of *H. meleagridis*:**

The two monocultures of *H. meleagridis* varied significantly in virulence based on cecal and liver lesions scoring in the turkey and weight gain post-inoculation. Liver lesion scores of the

groups infected with BG-M1 averaged 0.9 and those groups infected with BG-M2 averaged 0.07. Cecal lesion scores of the groups infected with BG-M1 averaged 2.5, compared with 1.0 for the groups infected with BG-M2. Similarly, the weight gain of groups UC, BG-M1 and BG-M2 averaged 615 g, 513 g and 617 g respectively (Fig. 3.1). These differences were significant at  $P < 0.001$ . These differences in lesion scores and weight gains suggest that BG-M1 appeared to be more virulent than BG-M2. Some *H. meleagridis* strains have produced high gross lesions and extra mortality while some other strains produced only mild or moderate lesions in many field outbreaks (McDougald, 1997). However, little information is available on the possible differences in virulence of individuals within a population. Results of this study imply that each isolate of *H. meleagridis* collected from an outbreak may contain many parasites with diverse virulence.

#### **Experiment 2: Comparison of virulence of field isolates of *H. meleagridis* by infection of turkey poults: Parent isolates vs strains derived by multiple passages *in vitro*:**

The tested field isolates and strains of *H. meleagridis* that have been passed multiple times in culture differed significantly in virulence when used to infect turkey poults. Average weight gain of groups UC, BG-1, BG-100, ZM-1, ZM-56 and MNC were 368.9 g, 150.6 g, 376.9 g, 175.8 g, 217.9 g and 94 g respectively (Fig. 3.2). Average liver lesion scores of groups UC, BG-1, BG-100, ZM-1, ZM-56 and MNC were 0.0, 2.21, 0.0, 2.81, 1.27 and 3.5, respectively. Average cecal lesion scores of groups UC, BG-1, BG-100, ZM-1, ZM-56 and MNC were 0.0, 3.69, 0.0, 3.24, 3.27 and 4.0, respectively (Fig. 3.2). The parent field isolates did not vary significantly in lesion scores or weight gain, with the exception of the MNC isolate, which produced significantly higher liver and cecal lesion scores than the ZM isolate. Overall, the MNC isolate appeared to be the most virulent of the three field isolates. The BG-1, ZM-1 and MNC differed in the lesion

scores and effects on weight gain suggesting that isolates of *H. meleagridis* involved in field outbreaks of blackhead may vary in virulence. Variations in virulence seen in this study may explain why there are differences in the severity of different outbreaks of blackhead disease in the field or there might be various genetically separate strains or subspecies of the *H. meleagridis*.

BG-1 and ZM-1 were found to be highly virulent compared to BG-100 and ZM-56 respectively. Lesion scores and average weight gain of BG-100 and UC were significantly different from MNC, BG-1, ZM-1 and ZM-56 ( $P < 0.001$ ). These results agree with the findings of previous studies on the effects of long-term passage *in vitro* which demonstrated that continuous *in vitro* passage of *H. meleagridis* reduces pathogenicity (Hess et al, 2008; Lund, 1969; Dwyer, 1970, Tyzzer, 1933, 1933, 1934). The mechanisms by which cultures become avirulent during passage *in vitro* are not known, but may involve selection of those individuals better able to survive *in vitro*. Our data suggest *H. meleagridis* exist within a population that varies in virulence. It is possible that highly virulent strains may require host tissue for their survival or expression of virulence factors. Prolonged culturing may cause the eventual loss of these virulent strains leaving behind the less virulent parasites in culture or decrease in the expression of virulence genes. More studies on the variation in the virulence of different strains and their genotypes are needed to confirm these observations.

## REFERENCES

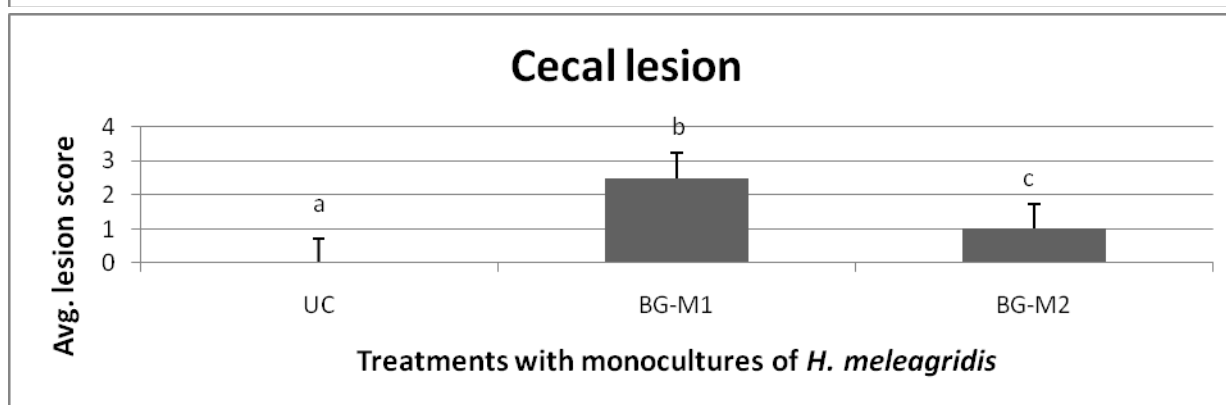
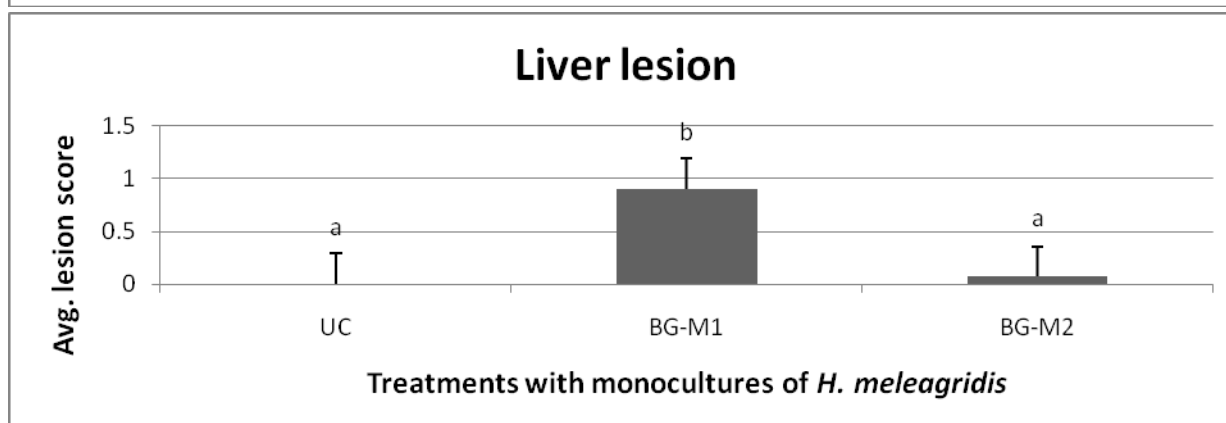
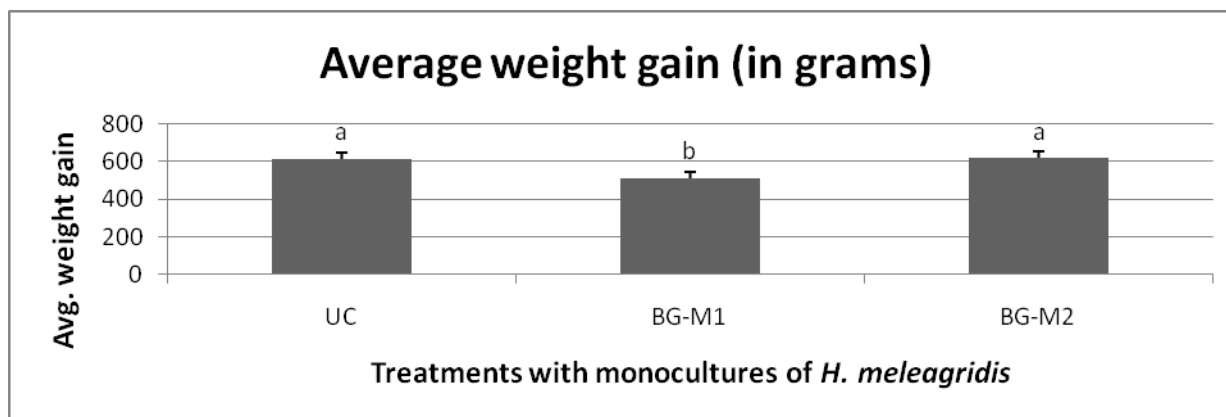
1. Franker, C. K., and J. P. Doll. Experimental histomoniasis in gnotobiotic turkeys. II. Effects of some cecal bacteria on pathogenesis. *J. Parasitol.* 50:636-640. 1964.

2. Hauck, R., Balczulat, S., and M. Hafez. Detection of DNA of *Histomonas meleagridis* and *Tetratrichomonas gallinarum* in German Poultry Flocks Between 2004 and 2008. *Avian Diseases* 54: 1021-1025. 2010.
3. Hess, M., D. Liebhart, E. Grabensteiner, and A. Singh. Cloned *Histomonas meleagridis* passaged in vitro resulted in reduced pathogenicity and is capable of protecting turkeys from histomonosis. *Vaccine* 26:4187–4193. 2008.
4. Hu, J., and L. R. McDougald. Direct lateral transmission of *Histomonas meleagridis* in turkeys. *Avian Dis* 47: 489-492. 2003.
5. Hu, J., Fuller, L, and L. R. McDougald. Infection of turkeys with *Histomonas meleagridis* by the cloacal drop method. *Avian Dis.* 48: 746-750. 2004.
6. Lollis L, Gerhold R, McDougald L, Beckstead R. 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(4):610-5. 2011.
7. Lund, E. E. Response of four breeds of chickens and one breed of turkeys to experimental *Heterakis* and *Histomonas* infections. *Avian Dis.* 11:491-502. 1967.
8. Lund, E. E., and A. M. Chute. Reciprocal responses of eight species of galliform birds and three parasites: *Heterakis gallinarum*, *Histomonas meleagridis*, and *Parahistomonas wenrichi*. *J Parasitol* 58: 940-945. 1972.
9. McDougald, L.R. Blackhead disease (histomoniasis) in chickens. *Poult. Digest* 56(9):8, 10-11. 1997.

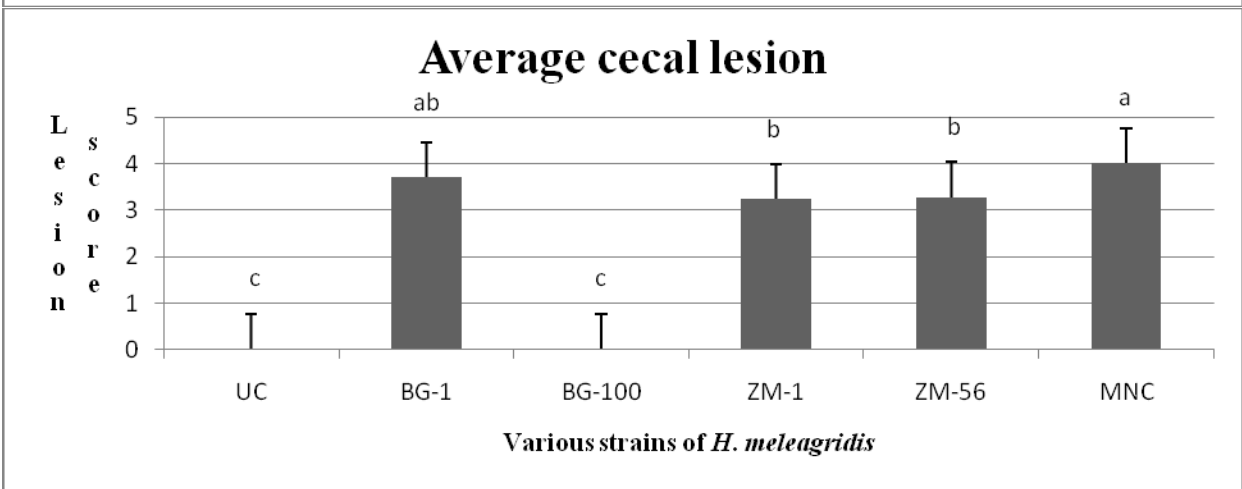
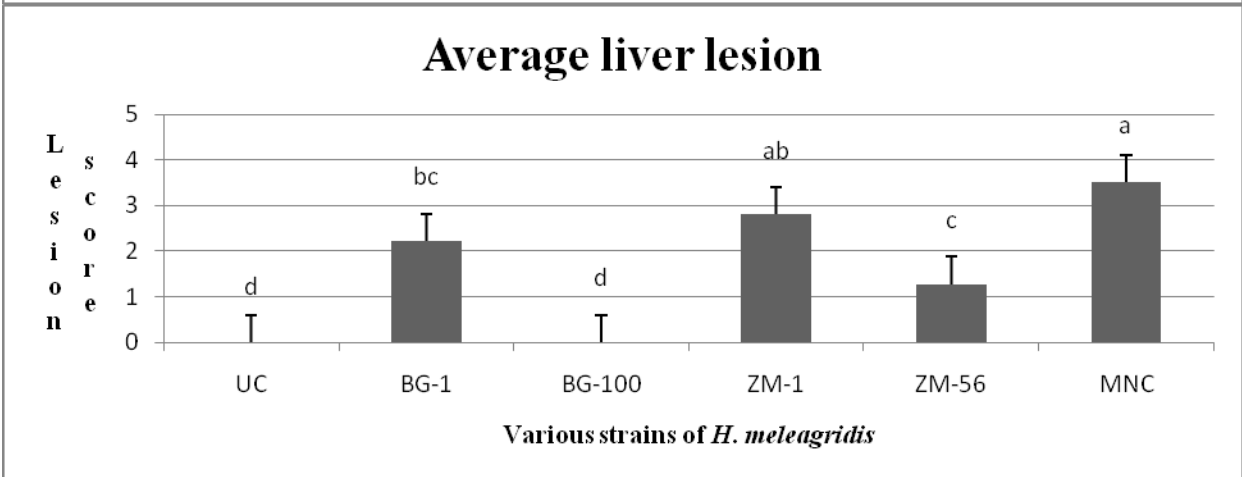
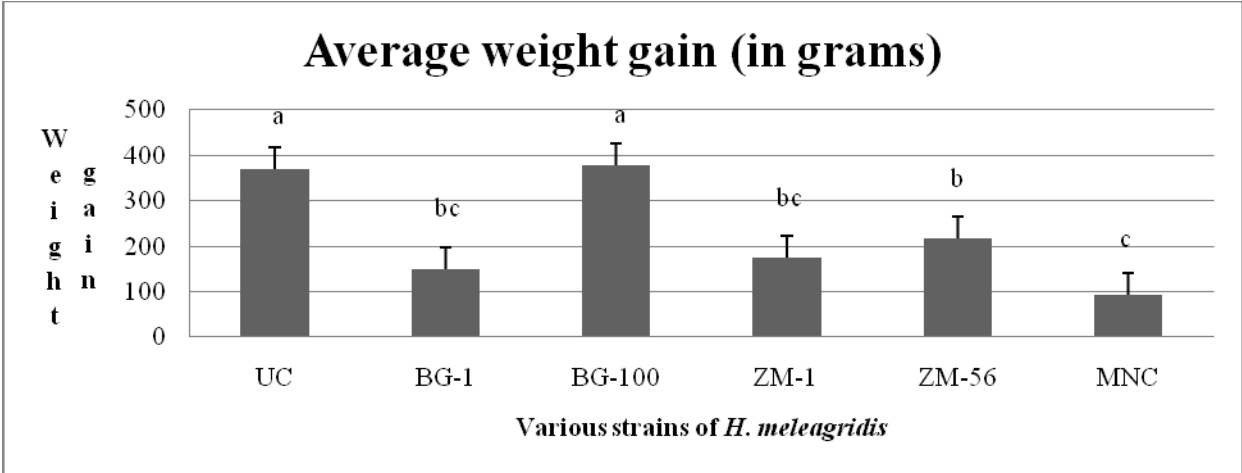
10. McDougald, L.R. Blackhead disease (Histomoniasis) in poultry: a critical review. *Avian Dis.* 49: 462–476. 2005.
11. van der Heijden, H.M.J.F., Landman, W.J.M., Greve, S., and R. Peek. Genotyping of *Histomonas meleagridis* isolates based on Internal Transcribed Spacer-1 sequences. *Avian Pathology* 35:330-334. 2006.
12. van der Heijden H.M., Landman W.J. Improved culture of *Histomonas meleagridis* in a modification of Dwyer medium. *Avian Dis.* 51: 986–988. 2007.
13. van der Heijden H.M., Landman W.J. *In vivo* effect of herbal products against *Histomonas meleagridis* in turkeys. *Avian Pathol.* 37(1):45-50. 2008.



**Figure 3.1.** Comparison of virulence of monocultures from the BG strain of *H. meleagridis*: Effects on Weight gain (g) and lesion scores of liver and ceca at necropsy of birds in the group UC, BG-M1 and BG-M2 at 10 dpi. <sup>a, b, c</sup> Scores within a graph with no common superscript differ significantly P<0.05



**Figure 3.2.** Comparison of virulence of field isolates or derived strains of *H. meleagridis* by infection of turkey poults: Effects on Weight gain (g) and lesion scores of liver and ceca at necropsy of birds in the group UC, BG-1, BG-100, ZM-1, ZM-56 and MNC at 10 dpi. <sup>a, b, c</sup>  
Scores within a graph with no common superscript differ significantly  $P < 0.05$ .



## CHAPTER 4

### BLACKHEAD DISEASE: REDUCED SENSITIVITY OF *HISTOMONAS MELEAGRIDIS* TO NITARSONE *IN VITRO* AND *IN VIVO*.

---

M. Abraham, L. R. McDougald and R. B. Beckstead\*

To be submitted to *Avian diseases*

**ABSTRACT:** *Histomonas meleagridis*, a flagellated protozoan parasite, is the causative agent of blackhead disease in gallinaceous birds. Currently, nitarsone (4-nitrophenylarsonic acid) is the only approved preventative-histomonal drug available in the United States. Initially we tested the sensitivity of 3 different isolates of *H. meleagridis* collected from outbreaks in North Carolina (Strain MNC), Michigan (Strain ZM) and Georgia (strain BG) to nitarsone using *in vitro* conditions. Strain ZM and Strain BG at both 100 and 400 mcg/mL showed a diminished growth in comparison with their respective control groups. However, strain MNC treated with nitarsone at 100 mcg/mL did not show any effect and their growth pattern was almost similar to that of control group. Secondly, in the *in vivo* study, turkey poults were inoculated cloacally with *H. meleagridis* (Strain MNC) at a dose of 35,000 cells/bird. Nitarsone treated group of birds (INT) did not show any significant improvement in the average weight gain compared to that of infected control group (IC). There were no significant difference in the liver and cecal lesions of IC and INT. In addition, *H. meleagridis* isolated from the INT group of *in vivo* study were also subjected to the nitarsone *in vitro* and those regenerated *H. meleagridis* treated with nitarsone at 100 ppm showed more growth rate than untreated control. This study suggests that strain MNC has acquired resistance to nitarsone. The necessity of alternative chemotherapeutics or immunoprophylaxis against the blackhead disease is more imperative due to the development of nitarsone resistance by certain strains.

**Keywords:** *Histomonas meleagridis*, Blackhead, Nitarsone, Histostat-50

**Abbreviations:** *H. meleagridis* = *Histomonas meleagridis*; *H. gallinarum* = *Heterakis gallinarum*; dpi = days post infection; UC = Uninfected Control; IC = Infected Control; INT = Infected Nitarsone Treated

## INTRODUCTION

*Histomonas meleagridis*, a protozoan parasite of the family Monocercomonadidae, class Trichomonada (Hauck *et al.*, 2009) is the causative agent of blackhead disease in gallinaceous birds. It is transmitted horizontally through a cecal nematode, *Heterakis gallinarum*, or through direct contact (only in turkeys) between infected and uninfected birds (McDougald, 2005). Affected birds show reduced body weight, morbidity, and sometimes high mortality (especially in turkeys) with necrotic foci in the liver and ulcers in ceca. Lesions may also be seen in other tissues (Sentíes-Cué *et al.*, 2009).

Historically, blackhead disease could be readily treated or prevented by the use of nitroimidazole drugs. However, the use of these products has been disallowed in United States and Europe, resulting in an upsurge of blackhead disease. There is no approved vaccine or treatment for blackhead, although nitarsone (4-nitrophenylarsonic acid) may be used for prevention (McDougald, 2005). There is no previous report of reduced sensitivity of *H. meleagridis* to nitarsone. However, recent histomoniasis outbreak in nitarsone-fed turkeys suggested that the strains of *H. meleagridis* might be resistant to the drug. This raises concerns for the future of blackhead prevention and management in commercial poultry in the United States. In the present work we test strains of *H. meleagridis* obtained from outbreaks of blackhead disease in turkeys and broilers for sensitivity to nitarsone.

## MATERIALS AND METHODS

**Nitarsone.** Purified nitarsone was obtained from Sigma-Aldrich, Inc. (St. Louis, Missouri) for use *in vitro*. The compound was suspended in dimethyl-formamide and diluted to working concentrations of 100 ppm and 400 ppm in Hank's balanced salt solution. For *in vivo* studies

Histostat-50<sup>TM</sup> (Pfizer Animal Health, Inc., Fort Washington, New Jersey, USA) was used in turkey starter ration at 0.0187% (187 ppm).

**Parasites and Culture.** Virulent isolates of *H. meleagridis* were obtained from outbreaks in Monroe, North Carolina (Strain MNC), Zeeland, Michigan (Strain ZM) and Buford, Georgia (strain BG). These isolates were cultured and frozen to -175<sup>0</sup>C in liquid Nitrogen. For use *in vitro* or to inoculate birds, cultures were resuscitated and cultured at 40<sup>0</sup>C in modified Dwyer's medium consisting of 0.8% (w/v) rice powder and 5% Horse serum in Medium199 with Hank's salts (van der Heijden, et al, 2007).

***In vitro* study to test the sensitivity of nitarsone to *H. meleagridis***

After 48 h of growth in media, cultures were counted using a hemocytometer (Hausser Scientific, Horsham, PA) and diluted to 40,000 cells/ mL in fresh culture medium and transferred to 50 ml culture flasks (10 ml/flask). For each strain, nitarsone was tested at 0 (BG-CON, ZM-CON, MNC-CON), 100 (BG-100, ZM-100, MNC-100), or 400 (BG-400, ZM-400, MNC-400) mcg/mL in 3 replicate cultures. *Histomonas meleagridis* in each flask were counted after 12, 24, 36 and 48 h of growth and average count of each time was calculated.

***In vivo* study to test the sensitivity of nitarsone to *H. meleagridis***

**Experimental Birds.** Day-old turkey poults (obtained from Butterball turkey hatchery, Goldsboro NC) were maintained until 2 wks old in steam-sterilized colony cages, and then were moved to steam-sterilized broiler finisher cages for feeding and inoculation.

**Experiment Design.** Two week old poults were individually weighed, banded and distributed into 9 groups of 10 birds each. Three groups were inoculated cloacally with *H. meleagridis*



(Strain MNC) by means of direct cloacal inoculation (IC), 3 groups infected with *H. meleagridis* were fed with nitarsone (187.5 ppm) from the first day of life (INT) and other 3 groups were remained uninfected (UC).

Poults were inoculated intracloacal using a blunt-tipped pipette inserted about 3 cm into the cloaca. A dose of 35,000 cells/bird was given in 1 ml. of culture medium. Unmedicated feed and water were provided ad-libitum (3 groups were fed with feed mixed with Histostat-50 premix for a nitarsone content of 187.5 ppm of feed). At 10 days post infection (dpi) birds were weighed, euthanized with CO<sub>2</sub> and cervical dislocation, necropsied to determine cecal and liver lesions scores.

**Lesion scoring.** Thickening or reddening of cecal wall is scored as 1. Scores of 2 and 3 indicate increasing severity of the lesion like thickening of the cecal wall, formation of a cheesy cecal core, and inflammation of the mesenteries. Extreme lesions like complete involvement of the ceca and the engorgement of lumen with cecal cores was scored as 4. The liver lesions were scored 1-2 with only a few discrete surface lesions (score 1) or a 3 with increasing severity. A complete involvement of the liver with lesions was scored 4 (Hu *et al.*, 2004).

***In vitro* study to test the sensitivity of nitarsone to *H. meleagridis* regenerated from the *in vivo* study.**

Ceca of birds in the INT group (which shows high lesions of Histomoniasis) of the *in vivo* study were transferred to media and allowed *H. meleagridis* to grow for 72 h. After attaining optimum growth in media, regenerated *H. meleagridis* (MNC) were transferred to fresh culture media and allowed to grow for 48 h. These 48 h cultures were counted using a hemocytometer (Hausser Scientific, Horsham, PA). Cultures were diluted to 40,000 cells/ mL in fresh culture medium,

then mixed and transferred to 50 ml culture flasks (10 ml/flask). For each strain, nitarsone was tested at 0 (Regenerated MNC-CON), 100 (Regenerated MNC-100), or 400 (Regenerated MNC-400) mcg/mL in 3 replicate cultures. *Histomonas meleagridis* in each flask were counted after 12, 24, 36 and 48 h of growth.

**Statistical Analysis.** Results of the *in vitro* study were analyzed statistically by a two-way ANOVA (time and concentrations) using General Linear Model and three duplicates for each treatment. In the live-bird study, the average weight gain and average lesion scores of liver and ceca were analyzed by one-way ANOVA followed by Tukey multiple comparison test ( $P < 0.05$ ).

## RESULTS

### *In vitro* study to test the sensitivity of nitarsone to *H. meleagridis*

In the *in vitro* study to test the sensitivity of nitarsone, BG-CON grew rapidly for 24 h to 161,666/ml. In cultures treated with nitarsone (BG-100 and BG-400) count of *H. meleagridis* was diminished within 12 h post-inoculation (Fig.1). The ZM-CON peaked at 55,000/ml after 24 h. With the addition of nitarsone (ZM-100 or ZM-400), counts decreased at each observation after inoculation (Fig. 4. 1). Both BG-CON and ZM-CON showed a significant difference from their respective nitarsone treatments at both 100 ppm and 400 ppm ( $P < 0.0001$ ). MNC-CON grew rapidly through 12 h post-inoculation to 146,667/ml and it remained stable up to 24<sup>th</sup> h, and then declined (Fig. 4. 1). MNC-100 had no apparent difference on count for the first 12 h and the growth pattern was similar to that of MNC-CON (143,333/ml). Both MNC-CON and MNC-100 showed higher count than MNC-400. MNC-400 had only a small increase in the number of *H. meleagridis* at 12 h, with a decline thereafter (Fig. 1). MNC-CON and MNC-100 showed a significant difference from MNC-400 ( $P < 0.0001$ ).

### ***In vivo* study to test the sensitivity of nitarsone to *H. meleagridis***

In the *in vivo* study conducted with the strain MNC. INT didn't show any significant improvement in the average weight gain compared to that of IC ( $p = 0.066$ ). Average weight gains of the UC, IC and INT were 191.97g, 117.71g or 83.91, respectively (Fig. 4. 2). Average liver lesion scores of IC and INT were 2.00 and 2.38, respectively. Similarly, average cecal lesion scores were 2.94 and 3.52, respectively, for IC and INT (Fig. 5). There were no significant difference in the liver lesions ( $p = 0.486$ ) and cecal lesions ( $p = 0.108$ ) of IC and INT.

### ***In vitro* study to test the sensitivity of nitarsone to *H. meleagridis* regenerated from the *in vivo* study.**

Count of regenerated *H. meleagridis* was higher than the *in vitro* growth of other isolates we used in our *in vitro* study. Regenerated MNC treated with nitarsone at 100 ppm showed a more average count than the untreated control group during all measured times. At 400 ppm regenerated strain showed diminished growth. (Fig.4.3). Regenerated MNC-CON and regenerated MNC-100 showed a significant difference from regenerated MNC-400 ( $P < 0.0001$ ).

## **DISCUSSION**

Nitarsone is the only drug approved in the United States for the prevention of Histomoniasis (McDougald, 2005). Previously, there has been no documentation of drug resistance with this compound. However, recent field experience suggested that some strains of *H. meleagridis* might be developing tolerance. In the present study, a comparison *in vitro* of 3 strains suggested that prior exposure to nitarsone in turkeys was related to diminished effectiveness in turkeys. The Buford strain (BG), which does not have a history of exposure to nitarsone, responded as expected to 100 or 400 ppm *in vitro*. Similarly, the strain from Michigan (ZM) was controlled

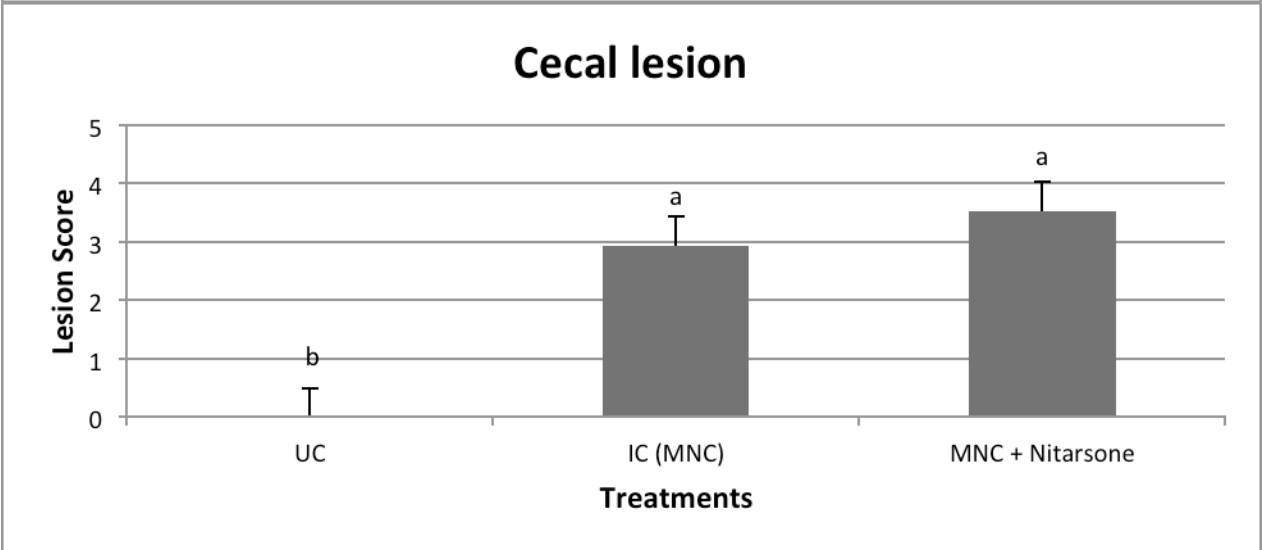
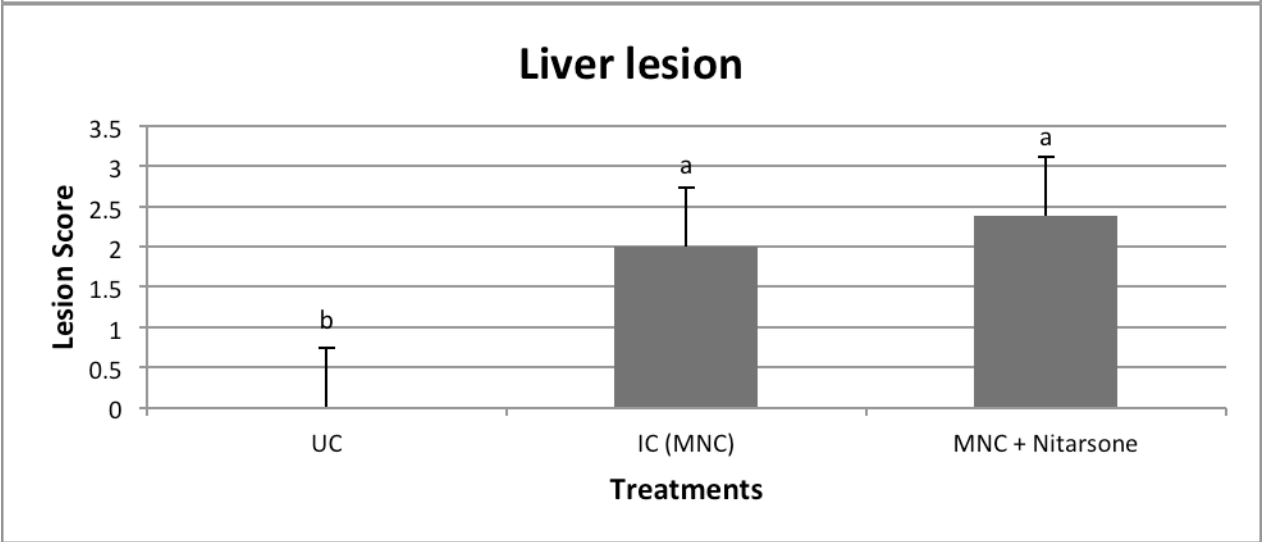
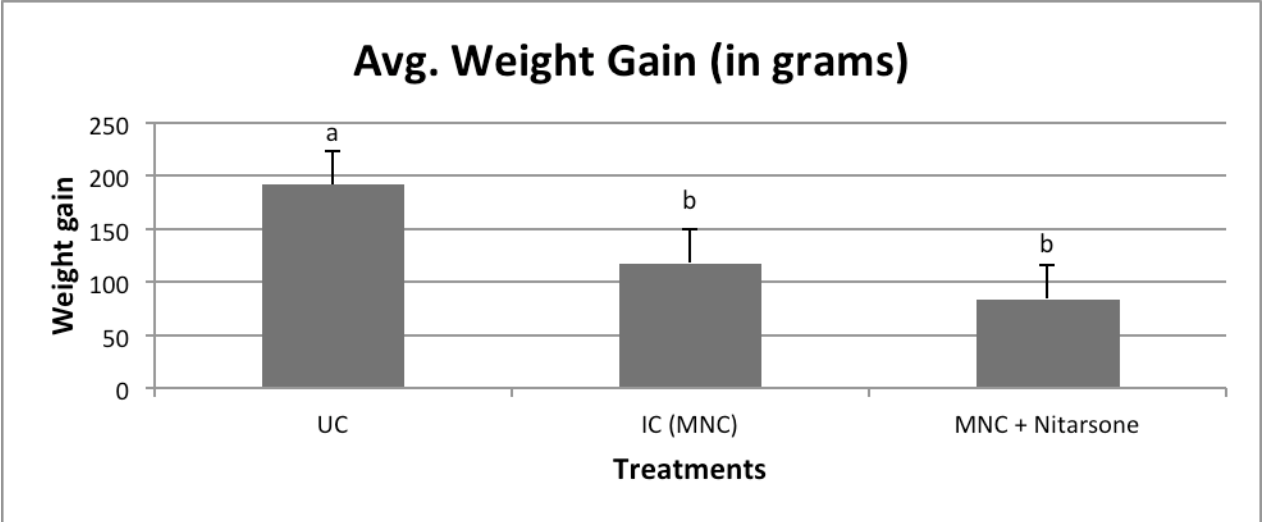
by nitarsone *in vitro*. However, the North Carolina strain (MNC), which was isolated from a flock experiencing an outbreak of blackhead while being medicated with nitarsone in the feed, was not affected by nitarsone at 100 ppm. Treatment with 400 ppm resulted in much reduced count of parasites, suggesting that the parasites were responsive to the drug. However, when tested *in vivo* at the recommended level in feed (187.5 ppm), nitarsone failed to reduce liver or cecal lesions or to improve weight gain, in comparison with infected and uninfected controls. Van der Heiden (2008b) reported similar results with a strain of *H. meleagridis* of Dutch origin, although it is not known whether the strain had been exposed previously to nitarsone. In a previous unpublished study in our lab, nitarsone administered to the diet after inoculating with 10,000 *H. meleagridis* per bird (strain ZM) imparted full protection with no mortality and absence of both liver and cecal lesions in turkeys (data not shown). *Histomonas meleagridis* regenerated from *in vivo* studies showed higher growth *in vitro* than *H. meleagridis* resuscitated from frozen condition. In our *in vitro* study to test the nitarsone sensitivity of regenerated strain MNC suggests that *H. meleagridis* maintains this property of nitarsone resistance even after successive infection. Due to the unavailability of effective treatment or vaccine against the blackhead disease, nitarsone resistance is a potential threat to the turkey industry. Cell culture method can be used as a rapid test for assessing the nitarsone sensitivity of field isolates.

## REFERENCES

1. Hauck, R., and H. M. Hafez. Partial sequence of the beta-tubulin of *Histomonas meleagridis* and the activity of benzimidazoles against *H-meleagridis* in vitro. Parasitology Research, 104(5): 1183-1189. 2009.

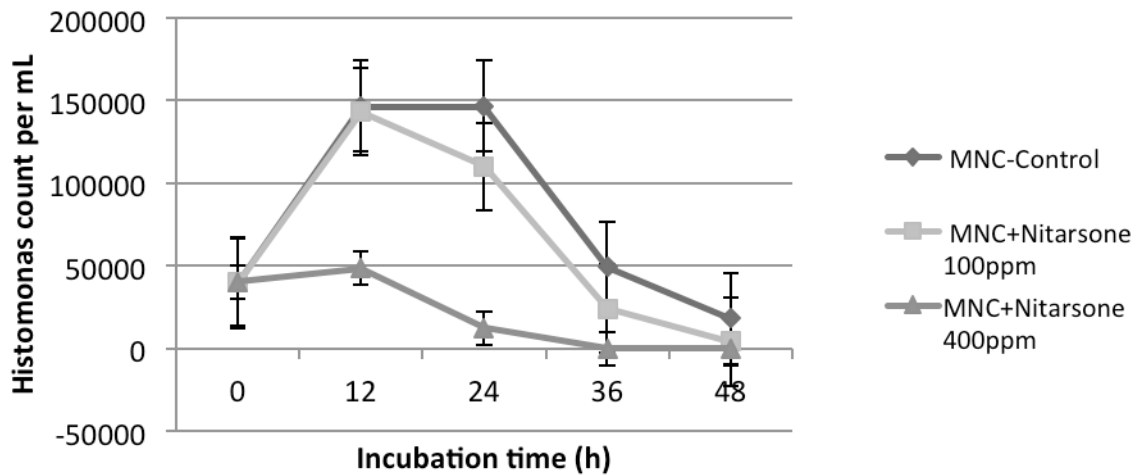
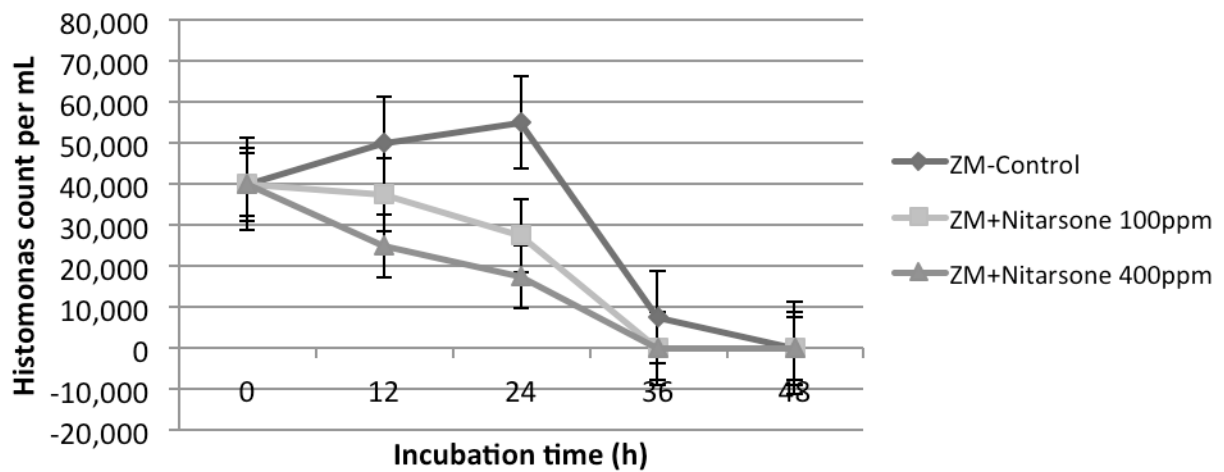
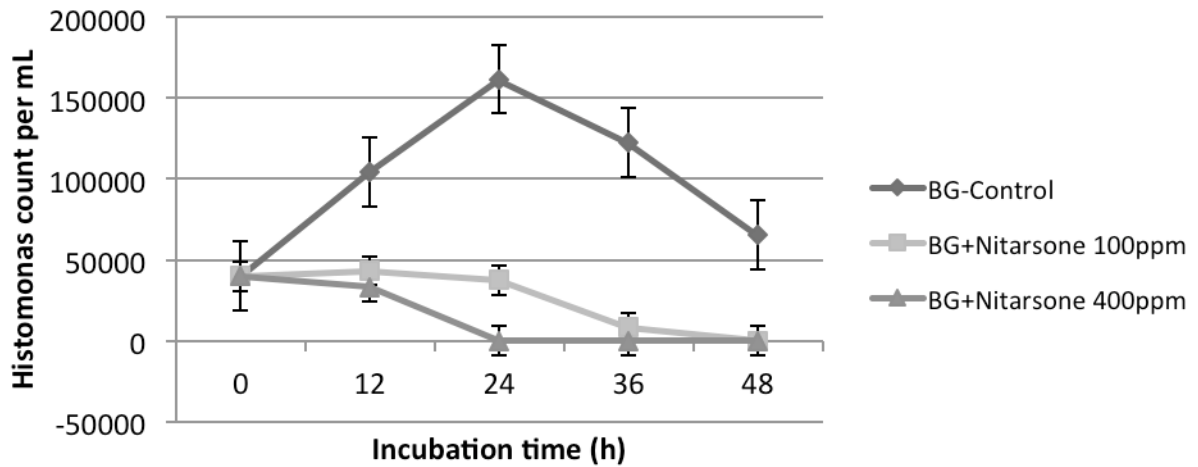
2. Hu, J., Fuller, L, and L. R. McDougald. Infection of turkeys with *Histomonas meleagridis* by the cloacal drop method. *Avian Dis.* 48: 746-750. 2004.
3. Hu, J., and McDougald, L.R. The efficacy of some drugs with known antiprotozoal activity against *Histomonas meleagridis* in chickens. *Veterinary Parasitology*, 121, 233-238. 2004.
4. Mathis, G. F. Efficacy of arsenical feed additives against pathogenic turkey coccidia. *Poult. Sci.* 77 (Suppl.):156. (Abstr.). 1998.
5. McDougald, L.R. Blackhead disease (Histomoniasis) in poultry: a critical review. *Avian Dis.* 49: 462–476. 2005.
6. Reynolds, J., C. A. Tucker, T. A. Yazwinski, Z. Johnson, D. Clark., and S. Clark.. Anthelmintic effect of nitarsone on *Ascaridia dissimilis* infections in the turkey. *J.Appl. Poult. Res.* 18:766–771. 2009.
7. Senties-Cué G., Chin R.P, and Shivaprasad HL. Systemic histomoniasis associated with high mortality and unusual lesions in the bursa of Fabricius, kidneys, and lungs in commercial turkeys. *Avian Dis.* 53(2):231-8. 2009.
8. Van der Heijden H.M., Landman W.J. Improved culture of *Histomonas meleagridis* in a modification of Dwyer medium. *Avian Dis.* 51: 986–988. 2007.
9. Van der Heijden H.M., Landman W.J. *In vitro* effect of herbal products against *Histomonas meleagridis*. *Vet Parasitol.* 154(1-2):1-7. 2008.
10. Van der Heijden H.M., Landman W.J. *In vivo* effect of herbal products against *Histomonas meleagridis* in turkeys. *Avian Pathol.* 37(1):45-50. 2008.

**Figure 4.1.** Growth of *Histomonas meleagridis* (Strain BG, ZM and MNC) *in vitro*, as affected by addition of 100 or 400 ppm of nitarsone. . <sup>a, b, c</sup> Scores within a graph with no common superscript differ significantly  $P < 0.05$ .

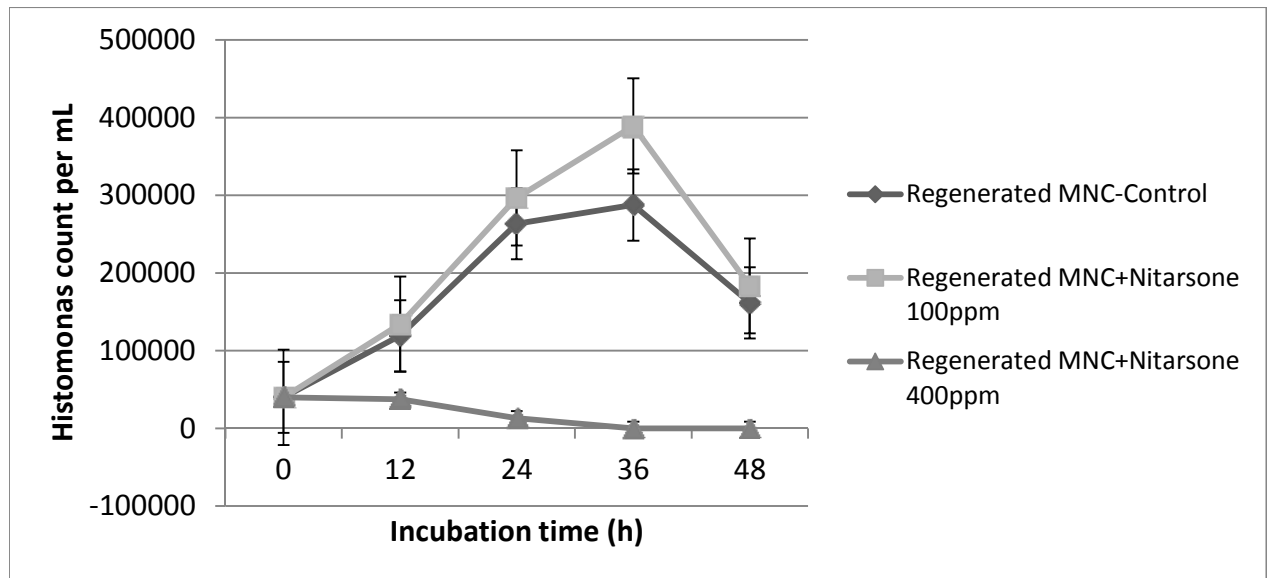


**Figure 4. 2.** Nitarsone (187.5 ppm in feed) vs. *H. meleagridis* strain MNC in turkey poults.  
Weight gain (g) and lesion scores in the liver and ceca at necropsy 10 dpi with 35,000 *H.*  
*meleagridis* /bird.





**Figure 4. 3.** Growth of *H. meleagridis* (Regenerated MNC) *in vitro*, as affected by addition of 100 or 400 ppm of nitarsone. Growth of the regenerated MNC treated with nitarsone at 100 ppm was more than regenerated MNC-CON (nitarsone untreated control group) during the entire period of growth.



## CHAPTER 5

### CONCLUSIONS

Certain blackhead disease outbreaks result in severe mortality and lesions, while moderate or mild disease is also seen in field. Our study to test the variations in the virulence of various isolates of *H. meleagridis* suggests a possible explanation to why these differences in the severity of blackhead disease outbreaks are observed. Variations in the virulence of two monocultures derived from a single isolate suggest the presence of various strains of the *H. meleagridis*. Isolate of *H. meleagridis* collected from an outbreak may be a mixture of various strains with wide range of virulence. Many researchers pointed out the reduced pathogenicity of *in vitro* passaged strains and the scope of these attenuated strains to use as a vaccine against histomoniasis (Tyzzer 1933, 1934, 1936; Hess et al, 2008; Liebhart et al, 2010, 2011). A scientific explanation to this attenuation is not available. Presence of various strains of *H. meleagridis* within an isolate put forward the possibility of selection between strains to survive in the media. Highly virulent strains may not be able to survive in the *in vitro* condition for a long time. Availability of host tissue may be needed for many strains of *H. meleagridis* to maintain their ability to survive and also to express its virulence factors. Our *in vitro* study on the regenerated *H. meleagridis* (Fig 4. 3) shows an increase in the growth rate of *H. meleagridis* collected immediately from an infection. These results explain the possible reason of attenuation of parasites *in vitro*.

Nitarsonsone is the only antihistomonal drug available in United States to use as a preventive measure in feed (0.0187 % in feed). Study to test the sensitivity of various isolates of *H.*

*meleagridis* to nitarsone suggests the possibility of the resistance acquired by certain strains of parasites to the drug. This resistance would explain the recent blackhead disease outbreaks in turkey flocks fed with nitarsone containing feed. *In vivo* studies to test the protective ability of nitarsone against the *H. meleagridis* isolate collected from the North Carolina outbreaks showed the drug gave no protection to the birds. *In vitro* studies to find the drug sensitivity of *H. meleagridis* collected from nitarsone treated birds showed no sensitivity to the drug as well. Similar results seen *in vitro* and *in vivo* suggest that the use of a cell culture based system could be used to rapidly test nitarsone sensitivity of field isolates. The ability of *H. meleagridis* to maintain drug resistance after passage in the turkey suggests that the strain is genetically altered and this resistance is fixed. Nitarsone resistance is a potential threat in turkey industry due to the sole dependence on this drug as a preventive therapy. Development of alternate treatment or vaccine against the blackhead disease is needed for the effective control of histomoniasis.

#### Literature Cited

- 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.
- Liebhart, D., Windisch, M., 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, Zahoor MA, Prokofieva I, Hess M. 2011. Safety of avirulent *H. meleagridis* to be used as a vaccine determined in turkeys and chickens. *Poult Sci.* 90(5):996-1003.

- Tyzzar, E. E. 1933. The immunizing properties of an attenuated strain of *Histomonas meleagridis*. J Parasitol 19:158.
- Tyzzar, E. E. 1934. Studies on Histomoniasis, or "Blackhead" Infection, in the Chicken and the Turkey. Proc Amer Acad Art Sci 69: 189-264.
- Tyzzar, E. E. 1936. A study of immunity produced by infection with attenuated culture strains of *Histomonas meleagridis* J . Comp. Pathol. Ther.49:285-303.

## APPENDIX

***CASE REPORT:***

AN OUTBREAK OF BLACKHEAD DISEASE (*HISTOMONAS MELEAGRIDIS*) IN FARM-  
REARED BOBWHITE QUAIL (*COLINUS VIRGINIANUS*)

-----

L.R. McDougald, M. Abraham, and R.B. Beckstead, 2012, *Avian Diseases*, Vol. 56 (4): 754-756

Reprinted here with permission of Publisher: Accepted by *Avian Diseases*

**ABSTRACT:** An outbreak of blackhead disease (*Histomonas meleagridis*) in farm-reared flock of 13,500 bobwhite quail resulted in mortality totaling approximately 1500 in 4 weeks.

Necropsy of 56 dead birds at mid-outbreak (from a total that day of 131) revealed that 55 had severe cecal lesions typical of blackhead, and only 3 had visible lesions in the liver. Necropsy of apparently healthy birds failed to detect any signs of infection. Presence of *H. meleagridis* in affected ceca was proved by culture *in vitro* and PCR tests.

**Keywords:** Bobwhite quail, *Colinus virginianus*, *Histomonas meleagridis*, blackhead disease, gamebirds

**Abbreviations:** PCR = polymerase chain reaction; *H. meleagridis* = *Histomonas meleagridis*; *H. gallinarum* = *Heterakis gallinarum*; *spp.* = species.



The bobwhite quail is a popular sporting bird throughout the Eastern USA, although wild populations are low in many areas because of changes in land use, increased pressure from predators, and interference with nesting by fire ants. However, the bobwhite is also produced on farms for release on hunting plantations. It is estimated that 30-40 million birds are produced annually for this trade, providing substantial income for farmers (Pers. Comm., North American Gamebird Association). There is no typical farm for this type of production; as the flock size may vary from a few dozen kept in a backyard pen to many thousands kept in buildings similar to broiler houses. Common disease problems include ulcerative enteritis (2), coccidiosis (6), and viral diseases. As early as 1919, Tyzzer (18) reported seeing histomoniasis in ‘a number of bobwhite quail’, but there are few reports in the literature. In the present study, we report a severe outbreak of blackhead disease in bobwhite quail on a large farm in Northeast Georgia.

#### **CASE REPORT:**

A flock of 15,000 bobwhite chicks were placed on May 28, 2011, in a 34 x 200 foot building on a 4-house farm. There was significant mortality during the first week, apparently because of overheating during transport from the hatchery caused by malfunction of equipment. At 3-4 weeks, there were some losses from bacterial enteritis, which was successfully treated with chlortetracycline, leaving approximately 13,500 surviving birds. On June 28 (about 4 weeks of age), a new surge of mortality was recognized by the farm owner as characterized by lesions typical of blackhead disease. In the following 4 weeks, the mortality peaked at more than 100/day (Fig. 1). We viewed the flock at the height of the mortality and necropsied dead birds. In general, the flock appeared healthy and the birds were feeding well. Only occasional sick birds or runts were seen. Of 56 dead birds examined (from a day total of 135), 55 had died with severe lesions in the ceca and associated tissues. The ceca were greatly distended and engorged

with a firm yellowish caseous core, as is typical of this disease, and the associated mesenteries and cecal tissues were inflamed (Fig. 2A). The ceca were so enlarged and firm that they were easily palpable from the exterior in live birds. Only 3 of the birds had lesions in the liver (Fig. 2B); all other livers were normal. Several runts removed at the same time all had severe cecal lesions. Necropsy of 6 apparently healthy birds from the flock revealed no lesions of histomoniasis. Samples of cecal material taken to the laboratory in warmed insulated packs (5) were successfully cultured and frozen in liquid nitrogen for future comparisons. Tissue from liver lesions was taken for PCR and sequence analysis (8). Analysis of the sequence revealed 100% sequence identity to *H. meleagridis* isolates obtained from samples taken from a infected turkey in Arkansas (accession number HQ334189) and in Georgia (accession number HQ334190) (14).

Overall, the outbreak lasted about 4 weeks and resulted in a total mortality of approximately 1500 birds (11 % of the flock). Subsequently, blackhead mortality was seen in flock in the adjacent house. At that time, both flocks were given medicated feed containing nitarsone (187.5 ppm) by prescription. Mortality in both flocks continued at a low level of 0-5 birds/day for the following month, although unaffected birds in the flock were robust and healthy.

The producer reported that a few birds had died of blackhead disease the previous year, but in a different building than those that broke this year. Farm preparation included complete clean-out between flocks, application of disinfectants to the bare soil, then installing about 4 inches (10 cm) of fresh wood shavings. Chicks were brooded within a cardboard circle then given access to the entire house. Feed was purchased from a commercial poultry feed mill, and contained monensin as an anticoccidial. There was no previous history of other poultry

(chickens) on the farm. Other poultry are produced in the area, including broiler breeder replacements, located within 2.5-3 miles, and litter is spread on fields in the area.

## **DISCUSSION:**

Reports of severe blackhead disease in bobwhite quail are rare. However, in 1980 it was reported that an outbreak in a flock of 850 bobwhites mortality was 95% (3). Extensive work was presented to show that extensive lesions of the liver and ceca were in fact caused by *H. meleagridis*, and cultured organisms were used to infect chickens. More recently, Radi (17) reported a mixed outbreak of histomoniasis and other parasites and bacteria in a flock of bobwhites, resulting in mortality near 10%.

While losses in the present case were not as dramatic, the overall mortality was costly. Several aspects of the case are unusual and not easily understood. Apparently, most if not all of the death losses were due to the cecal lesions. In other birds, mortality usually accompanies liver failure after severe lesions develop in that organ. There are no detailed studies of the contribution of septicemia or other potential causes of mortality associated with blackhead other than the report of Radi (17), where *Escherichia coli* was isolated from the liver, spleen and intestine, and concurrent colonization of the intestine by *Clostridium perfringens* and *Capillaria* spp. worms. The next major question is the source of the outbreak. Prior studies have shown that turkeys can become infected by direct contact with infected birds, providing a means for rapid spread of infections through a flock (10) and even by oral infection (13). In contrast, chickens are less likely to become infected by this means, suggesting that blackhead in chicken flocks results mostly from direct ingestion of cecal worm ova (*Heterakis gallinarum*) which are known to serve as a vector by harboring the infective *H. meleagridis* (11). Previous studies suggest that

bobwhite quail are not good hosts for *H. gallinarum* (15). This would seem to preclude the possibility that a heavy concentration of cecal worm ova in the facility could account for the present outbreak. Even though there has been no indication of a worm problem, the producer uses a program of regular deworming with a benzimidazole-type dewormer. It was previously shown that deworming reduced the challenge of blackhead in turkeys, probably by destroying the larvae before they can release the *H. meleagridis* as they molt to the adult stage (4,9). Finally, can it be assumed that the outbreak in the second house had a common origin with the first? While this is a tempting conclusion it is by no means certain, as it is not known what would serve as the carrier for such transmission.

The importance of chickens as a reservoir for blackhead was emphasized by the results of recent surveys. The seroprevalence of *H. meleagridis* in chicken layer flocks in a recent survey of 116 flocks in Holland was 100% (19), and it is assumed that the prevalence is similar in the USA (9). Another study in Austria showed a 37% prevalence (7). While it is generally assumed that chickens, particularly broiler breeder replacements, are the main reservoir of blackhead disease for other susceptible birds, it is not clear how infection is transported from the poultry flock to the target flock. The possibility of additional species of worms serving as vectors has been disproved (16), and the possibility of the darkling beetles or its larvae (*Alphitobius spp.*) serving as a mechanical transporter has also been discounted (12). Other insects are being investigated as possible mechanical agents of transmission.

It is not unusual for flocks of turkeys, chickens, or other birds to break with histomoniasis and suffer extensive mortality and morbidity, while in other cases the outbreak might be mild. While this would suggest differences in virulence of the strains, laboratory tests of *H. meleagridis* isolates from severe outbreaks have rarely shown any unusual virulence. It is

possible that the culture methods used for this work fail to propagate all of the variant organisms, and that culture-adapted organisms could be less virulent. This hypothesis is being tested by using freeze-preserved strains nearer to the actual outbreak, rather than after prolonged culture.

It is important to mention that there is no approved drug for treatment or prevention of blackhead in bobwhite quail. An arsenical compound, nitarsone (Histostat®, Alpharma, Inc., Fort Washington, New Jersey, USA), is approved for preventive use in feed (at 170.1 g/ton or 187 ppm), but only for turkeys and chickens (1). The only approval for this product is in turkeys; Bacitracin MD at 4-50 g/ton (5-55 ppm).

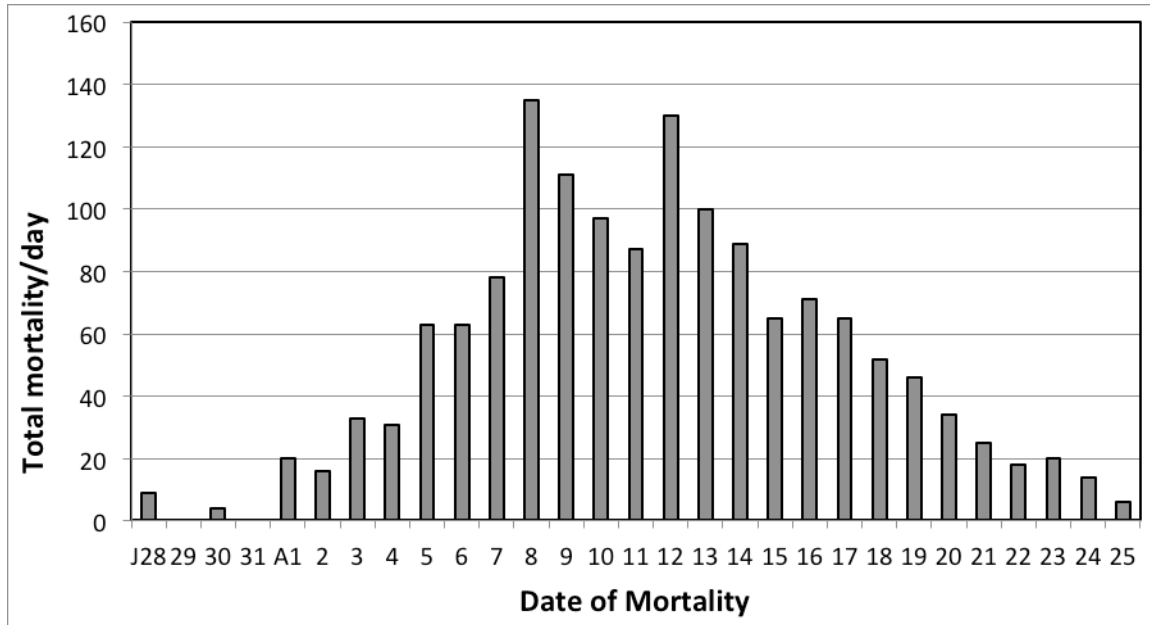
## REFERENCES

1. Anonymous. Feed Additives Compendium, Miller Publishing Company, Minnetonka, Minnesota, 55343, USA. Pp. 339-340. 2007.
2. Brown, J., D. L. Dawe, R. Killingsworth, and R. B. Davis. Antibiotic treatment of ulcerative enteritis of bobwhite quail. *J. Wildlife Diseases*, 6:8-12. 1970.
3. Dhillon, A. S., R. W. Winterfield, H. L. Thacker, K. R. Kazacos, and L. J. Alby. Case Report—Atypical histomoniasis in bobwhite quail. *Avian Dis.* 24:510-516. 1980.
4. Fine, P. E. M. Quantitative studies on transmission of *Parahistomonas wenrichi* by ova of *Heterakis gallinarum*. *Parasitology* 70:407-417. 1975.
5. Gerhold, R. W., L. A. Lollis, R. B. Beckstead, and L. R. McDougald. Establishment of culture conditions for survival of *Histomonas meleagridis* in transit. *Avian Dis.* 54:948-950. 2010.

6. Gerhold, R. W., L. R. McDougald, and R. B. Beckstead. Construction of PCR primers to detect and distinguish *Eimeria* spp. In Northern bobwhites and a survey of *Eimeria* on gamebird farms in the United States. J. Parasitol. 97:892-895. 2011.
7. Grafl, B., D. Liebhart, M. Windisch, C. Ibesich, and M. Hess. Seroprevalence of *Histomonas meleagridis* in pullets and laying hens determined by ELISA. Vet. Rec., 168:160-163. 2011.
8. Hafez, H. M., R. Hauck, D. Luschow, and L. McDougald. Comparison of the specificity and sensitivity of PCR, nested PCR, and real-time PCR for the diagnosis of Histomoniasis. Avian Dis. 49:366-370.
9. Hegngi, F. N., J. Doperr, T. S. Cummings, R. D. Schwartz, G. Saunders, A. Zajac, C. T. Larsen, and F. W. Pierson. The effectiveness of benzimidazole derivatives for the treatment and prevention of histomoniasis (blackhead) in turkeys. Vet. Parasitol. 81:29-37. 1999.
10. Hu, J. and L. R. McDougald. Direct lateral transmission of *Histomonas meleagridis* in turkeys. Avian Dis. 47:489-492. 2003.
11. Hu, J., L. Fuller, P. Armstrong, and L. R. McDougald. Histomoniasis in chickens: Attempted transmission in absence of vectors. Avian Dis. 50:277-279. 2006.
12. Jung, A. M. Ryll, G. Glunder, and S. Rautenschlein. Course of infection with *Histomonas meleagridis* in a turkey flock. Deutsche Tierärztliche Wochenschrift, 116:392-397. 2009.
13. Liebhart, D., and M. Hess. Oral infection of turkeys with in vitro-cultured *Histomonas meleagridis* results in high mortality. Avian Pathology, 38:223-227.

14. Lollis, L., R. Gerhold, L. McDougald, and R. Beckstead. 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. 2011.
15. Lund, E. E., and A. M. Chute. Bobwhite, *Colinus virginianus*, as host for *Heterakis* and *Histomonas*. *J. Wildlife Diseases*, 7:70-75. 1971.
16. Norton, R. A., F. D. Clark, and J. N. Beasley. An outbreak of Histomoniasis in turkeys infected with a moderate level of *Ascaridia dissimilis* but no *Heterakis gallinarum*. *Avian Dis.* 43:342-348. 1999.
17. Radi, Z. A. An epizootic of combined *Clostridium perfringens*, *Eimeria* sp. And *Capillaria* sp. enteritis and *Histomonas* sp. Hepatitis with *Escherichia coli* septicaemia in bobwhite quails (*Colinus virginianus*). *Int. J. Poultry Science*, 3:438-441. 2004.
18. Tyzzer, E. E. Developmental phases of the protozoan of “blackhead” disease in turkeys. *J. Med. Res.* 40:1-30. 1919.
19. Van der Heiden, H. M. J. F., and W. J. M. Landman. Seroprevalence of *Histomonas meleagridis* in Dutch layer chickens. *Avian Dis.* 55:324-327. 2011.

**Figure A. 1.** Daily mortality from blackhead disease (Histomoniasis) in a flock of 13,500 bobwhite quail (Dates are for July and August, 2011).





**Figure A. 2. A.** Cecal lesions caused by *Histomonas meleagridis* in bobwhite quail (see arrow).

Of 56 birds examined on a single day, 55 had similar *cecal* lesions characterized by thickened mucosa engorged with caseous cores.

**B.** Liver lesions caused by *Histomonas meleagridis* in bobwhite quail (see arrows). Of 56 birds examined, only 3 had lesions in the liver.

