BLACKHEAD DISEASE: TEST OF AN INACTIVATED VACCINE AND MINERALS FOR PROTECTION AGAINST HISTOMONAS MELEAGRIDIS

by

ANNA PALOMA KENYON

(Under the Direction of Robert B. Beckstead)

ABSTRACT

Histomonas meleagridis is an anaerobic protozoan and the causative agent of blackhead disease in gallinaceous birds. Blackhead was previously controlled with nitroimidazoles, this compound has since been deregistered and currently no approved treatment exists. A killed vaccine and experimental feed additives were tested in turkeys for possible efficacy and control of *H. meleagridis*. A vaccine was prepared with killed *H. meleagridis* cells and administrated subcutaneously with Freund's incomplete adjuvant. After challenge there was no indication that vaccination offered any protection. In the second study $ZnSO_4$ and $CuSO_4$ previously shown *in vitro* to have antihistomonal activity, were tested to determine if metals protect against a *H. meleagridis* direct challenge or decrease lateral transmission in turkeys. The addition of neither ZnSO4 nor $CuSO_4$ protected against blackhead or reduced lateral transmission in turkeys. Under the conditions of this work inactivated vaccines and $ZnSO_4$ and $CuSO_4$ used alone did not offer protection against blackhead in turkeys.

INDEX WORDS: blackhead, *Histomonas meleagridis*, vaccine, Zinc Sulfate, Copper Sulfate

BLACKHEAD DISEASE: TEST OF AN INACTIVATED VACCINE AND MINERALS FOR PROTECTION AGAINST HISTOMONAS MELEAGRIDIS

by

Anna Paloma Kenyon

B.S.A., University of Georgia, 2012

B.S.A.B., University of Georgia, 2012

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

2015

BLACKHEAD DISEASE: TEST OF AN INACTIVATED VACCINE AND MINERALS FOR PROTECTION AGAINST HISTOMONAS MELEAGRIDIS

by

ANNA PALOMA KENYON

Major Professor: Robert Beckstead
Committee: Larry McDougald
Andy Moorhead

Electronic Version Approved:

Julie Coffield
Interim Dean of the Graduate School
The University of Georgia May 2015

DEDICATION

This work is dedicated to my grandparents and parents, Vernon & Ivy Kenyon, Fernando Mandujano Trejo & Luz Vallejo Aguilar, Horace & Luz Kenyon. Thank you for providing your unconditional love and support. To my siblings Julie, Horace and Chloe Kenyon, without your presence I would not be me. I would also like to thank the Beckstead family, for providing a home and family. Robert, Roslyn, Alyssa, Natalie, Jake and Logan your love and support are more than I deserve.

ACKNOWLEDGEMENTS

Many thanks to my major advisor Dr. Beckstead, for investing the time and patience needed to mentor and educate. To my committee members Dr. Larry R. McDougald and Dr. Andy Moorhead thank you for your guidance and support. A special thank you to Miguel Barrios for pushing me and demonstrating the importance of research and the acquisition of knowledge. To all members of the Beckstead Lab—Drew Morris, Jason Payne—thank you for helping with my many questions and doubts. To the research farm team—Lindsey Racket and farm staff— your dedication, support, and flexibility are greatly appreciated. Each of the relationships we have built has pushed me to completion. I could not have completed this work without the support, guidance, and assistance of each person mentioned.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Histomoniasis, also known as blackhead disease or histomonosis, is caused by a single cell anaerobic protozoan parasite *Histomonas meleagridis*. Blackhead affects gallinaceous birds including bobwhite quail, grouse, partridge, pheasants, chickens, and turkeys (Lund & Chute 1974). Blackhead causes high mortality in turkeys, sometimes as high as 100%. Chickens are usually less severely affected and serve as carriers of blackhead. However chickens may suffer increased morbidity, culling, decreased weight gain and a decline in egg production (McDougald 2005). Affected birds show decrease in body weight gain, with ulcers seen in the ceca and necrotic foci found in the liver. Prior to the 1990's, management of blackhead was not a major concern for poultry producers, highly efficacious drugs, such as nitroimidazoles and arsenic containing compounds, were available for treatment (Berks & Neal 1952). These products have since been withdrawn from the market leaving the industry with no effective treatment for outbreaks. Nitarsone an arsenical compound used for prevention of blackhead is under review by the Food and Drug Administration and could also be banned (Nachman et al 2013a).

Given the lack of efficacious treatment drugs interest in alternative treatments for blackhead has increased. A limited number of studies suggest that immunization could be possible using live attenuated *H. meleagridis* strain (Hess et al 2008, Liebhart et al 2011, Sulejmanovic et al 2013). Attenuated vaccines however did not provide protection to birds

prior to 4 weeks of age (Hess & Liebhart 2011, Hess et al 2008, Liebhart et al 2010, Nguyen Pham et al 2013).

Conversely, vaccination with a killed version of a virulent *H. meleagridis* did not offer significant protection (Hess et al 2008). The difficulty in administering a live attenuated vaccine under field conditions, along with the delay in immune protection, makes it unlikely that this type of vaccine will be of benefit to the industry. Although an inactivated vaccine could be utilized by the industry, a product would be required to offer complete protection.

Various non-arsenic containing compounds and herbal extracts have been tested in both *in vitro* and *in vivo* systems for the ability to prevent *H. meleagridis* growth and for the treatment and prevention of blackhead disease. Many of the compounds prevent growth of *H. meleagridis* in culture although this has not been successfully translated into the treatment or prevention of the disease in turkeys. An alternative chemotherapeutic approach was suggested by previous research of *Trichomonas vaginalis* and *Tritrichomonas foetus* both closely related to *H. meleagridis*. These studies showed that the addition of $ZnSO_4$ *in vitro* resulted in damage to the hydrogenosome resulting in cell death (Benchimol et al 1993, Krieger & Rein 1982). Further work *in vitro* in our lab suggested that these metallic ions were highly toxic to *H. meleagridis*.

The aims of this work are:

1. Determine if the addition of Freund's incomplete adjuvant (FIA) to inactivated *H. meleagridis* vaccine can provide protection to turkeys against blackhead.

2. Determine if the addition of $ZnSO_4$ and $CuSO_4$ to feed or water treat or prevent the lateral transmission of blackhead in turkeys.

References

Benchimol M, Almeida JC, Lins U, Gonçalves NR, de Souza W. 1993. Electron microscopic study of the effect of zinc on Tritrichomonas foetus. Antimicrobial agents and chemotherapy 37(12):2722-2726.

Berks G, Neal RA. 1952. The effect of some drugs upon Histomonas meleagridis in vitro. *Annals of tropical medicine and parasitology* 46: 68-71

Hess M, Liebhart D, Grabensteiner E, Singh A. 2008. Cloned Histomonas meleagridis passaged in vitro resulted in reduced pathogenicity and is capable of protecting turkeys from histomonosis. Vaccine 26(33):4187-4193.

Krieger JN, Rein MF. 1982. Zinc sensitivity of Trichomonas vaginalis: in vitro studies and clinical implications. J Infect Dis 146(3):341-345.

Liebhart D, Windisch M, Hess M. 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 pathology : journal of the WVPA 39(5):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. Poultry science 90(5):996-1003.

Lund EE, Chute AM. 1974. The reproductive potential of Heterakis gallinarum in various species of galliform birds: implications for survival of H. gallinarum and Histomonas meleagridis to recent times. International journal for parasitology 4(5):455-461.

McDougald LR. 2005. Blackhead disease (histomoniasis) in poultry: a critical review. Avian diseases 49(4):462-476.

Nachman KE, Baron PA, Raber G, Francesconi KA, Navas-Acien A, Love DC. 2013. Roxarsone, inorganic arsenic, and other arsenic species in chicken: a U.S.-based market basket sample. Environ Health Perspect 121(7):818-824.

Nguyen Pham AD, De Gussem JK, Goddeeris BM. 2013. Intracloacally passaged low-virulent Histomonas meleagridis protects turkeys from histomonosis. Veterinary parasitology 196(3-4):307-313.

Sulejmanovic T, Liebhart D, Hess M. 2013. In vitro attenuated Histomonas meleagridis does not revert to virulence, following serial in vivo passages in turkeys or chickens. Vaccine 31(46):5443-5450.

CHAPTER 2

LITERATURE REVIEW

Blackhead Disease

Blackhead is a disease found in gallinaceous birds and caused by the protozoan parasite *Histomonas meleagridis.* The first documentation of blackhead occurred in 1895 in turkeys, which coincided with the arrival of ring neck pheasants from China (Cushman 1893). Variation in the virulence of blackhead has been observed between different species of gallinaceous birds and between populations of the same species (Lollis et al 2011, Lund & Chute 1972). Species that are typically asymptomatic include jungle fowl, pheasants, and domesticated chickens. Severely affected species include ruffled grouse, chucker partridges, pea fowl and turkeys (Lund & Chute 1974). For example, blackhead infections in chickens often go undetected with low rates of mortality (10-20%), while turkeys exhibit symptoms of disease 7-12 days post infection with mortality commonly in the 80-100% range (Kirkpatrick 1927, Tyzzer et al 1921). The causes of these differences in susceptibility unknown, but birds appear to differ innately to blackhead and there may be variation in the virulence of *H. meleagridis.*

Blackhead disease is clinically manifested in the turkey with languor, including drooping head and wings, prolonged standing, closed eyes, ruffled feather and emaciation. Turkeys infected with blackhead also show an increase in the huddling behavior (Kirkpatrick 1927). Clinical observations post-mortem includes necrotic lesions in the liver and ulceration of the ceca. A caseous cecal core may be present along with petechial

hemorrhaging of internal organs such as kidneys, spleen, bursa of Fabricius, and pancreas. Sulfured colored dropping may be seen as the disease progresses and liver function is compromised. Chickens infected with *H. meleagridis* may be asymptomatic or demonstrate disease symptoms similar to those seen in the turkey (Tyzzer et al 1921).

Morphology

Histomonas meleagridis is a member of the family Dientamoebidae, order Tritrichomonadida, class Tritrichomonadea (Cepicka et al 2010). Characteristics of members of the Dientamoebidae family include, a single mastigont, and the absence of comb-like structure, undulating membrane, and infrakinetosomal body (Cepicka et al 2010). Members of this family rely on the hydrogenosome for production of energy. The hydrogenosome is an organelle of energy metabolism similar in function to mitochondria, and produces energy through fermentative oxidation and the conversion of pyruvate to ATP with by products of malate, acetate, CO_2, H_2, and acetyl-CoA (Muller 1988).

Histomonas meleagridis exist in two forms: flagellated and amoeboid. Flagellated *H. meleagridis* are spherical and 10– 16μm in diameter with a solitary flagellum that is 6 – 11 μm in length (Honigberg & Bennett 1971). The flagellated form of *H. meleagridis* is present in the cecal lumen of infected birds. Amoeboid *H. meleagridis* are highly pleomorphic and 10 – 17 μm in diameter (Honigberg & Bennett 1971). The amoeboid form of *H. meleagridis* is present in the liver, kidney, spleen, and cecal tissues of infected birds(Tyzzer 1920).

Microscopic visualization of *H. meleagridis* reveal food vacuoles containing bacteria and small granules of irregularly shaped material (Tyzzer 1920). Unspecified material in food vacuoles of *H. meleagridis* is thought to be bacterial excreta (Hauck et al 2010). A cyst–like state of *H. meleagridis* is producible in altered culture conditions. Authors have

proposed that cystic–like stages of *H. meleagridis* are a plausible feature of transmission, there is little evidence to support that cyst forms have a role in the transmission of blackhead (Munsch et al 2009a, Munsch et al 2009b).

Lifecycle

The life cycle of *Histomonas meleagridis* includes two known intermediate hosts—the cecal nematode worm *Heterakis gallinarum* and earthworms (Chute & Lund 1974, Lund & Chute 1973). *Histomonas meleagridis* without the aid of an intermediate host is fragile and dies soon after exposure to ambient temperature (Tyzzer & Collier 1925). Graybill was the first to determine that ova of *H. gallinarum* co-infected with *H. meleagridis* were capable of inducing blackhead in turkeys (Graybill & Smith 1920). Infection of *H. gallinarum* occurs when *H. meleagridis* penetrates the developing oocytes of *H. gallinarum* and *H. meleagridis* replicates rapidly within the oocytes (Lee 1969). Eggs of *H. gallinarum* protect anaerobic *H. meleagridis* from death due to oxygen exposure (Schwarz et al 2011). Ova of *H. meleagridis* can survive in the eggs of *H. gallinarum* for up to 3 years (Lund et al 1966). A second intermediate host, the common earthworm serves as a paratenic host for *H. gallinarum*. In the earthworm the *Heterakis* ova hatch, and the larvae survive in the coelom cavity. When earthworms are ingested by a turkey or chicken this relationship increases a source for *H. meleagridis* proliferation in the environment (Lund & Burtner 1957, Springer et al 1969).

Mechanical vectors of the ova of *H. gallinarum* and *H. meleagridis* include flies and grasshoppers (Frank 1953). Transportation of the ova of *H. gallinarum* on personnel or animals may aid in the transmission of blackhead (McDougald 2005). The possibility of *H. gallinarum* in wild gallinaceous birds transmitting *H. gallinarum* was investigated by Lund

and Chute in 1972. Susceptible galliformes could be infected with blackhead by ingestion of either *H. gallinarum* or earthworms or by directly consuming *H. gallinarum* ova contaminated with *H. meleagridis*. After ingestion of either vector *H. gallinarum* or earthworms, histomoniasis occurred upon release of *H. meleagridis* from *H. gallinarum* during the first larval molt (Lund & Chute 1972). *H. meleagridis* migrates to the ceca, the primary site of infection, and can penetrate the cecal mucosa wall (Fine 1975). Upon penetration of cecal wall *H. meleagridis* enters the blood stream through the hepatic portal system, which connects the blood supply of the ceca and intestines to the liver, a secondary site of infection (Clarkson 1961, Fine 1975, McGuire & Morehouse 1958).

Tyzzer first observed the transmission of blackhead from a directly infected turkey to un infected turkey in the absence of *H. gallinarum* (Tyzzer & Collier 1925). The mechanism by which infection was spread through a flock was not fully understood until Hu and McDougald (2003) demonstrated lateral transmission from inoculated birds to un-inoculated birds. Lateral transmission of blackhead has only been observed in turkeys and not chickens (Hu & McDougald 2003). Lateral transmission results when a bird makes cloacal contact with fresh feces infected with *H. meleagridis* (Hu & McDougald 2003). Cloacal drinking is the act of retroperistalsis—the reflexive intake of fluids through the clocal lips (McDougald & Fuller 2005). Organisms shed in feces can remain viable for 3-4 hours (Aka et al 2011). The ability of *H. meleagridis* to remain viable within the ova of *H. gallinarum* and its relationship with earthworms increases survival ability and expands its life cycle.

Immunity and Vaccination

Immune response to blackhead differs depending on the species of bird. A study of the response of various galliformes to *H. meleagridis* exposure established percent of infection based on species, possibly linked to different immune response (Lund & Chute 1972). The primary site of infection is isolated in the ceca. Chickens mount an innate immune response at the primary site of infection, effectively decreasing the number of *H. meleagridis* (Powell et al 2009). In turkeys, an innate immune response is not mounted at the primary site of infection. Proliferation of *H. meleagridis* continues in ceca, and eventually penetrates the cecal mucosa wall (Fine 1975). Upon penetration of cecal wall *H. meleagridis* enters the blood stream through the hepatic portal system, which connects the blood supply of the ceca and intestines to the liver, the secondary site of infection (Clarkson 1961, Fine 1975, McGuire & Morehouse 1958). When *H. meleagridis* enters the secondary site of infection an uncontrolled immune response occurs in the liver (Powell et al 2009). *H. meleagridis* in the liver increases mortality to 100% (McDougald 2005).

Attempts at vaccine development for blackhead included attenuated live cultures of *H. meleagridis* as well as inactivated cells. Attenuation is the reduction of pathogenic virulence and is typically preformed *in vitro* (Liebhart et al 2011, Sulejmanovic et al 2013). Inactivation is the process of destroying the biological activity of an organism (Hess et al 2008). An attenuated strain of *H. meleagridis* was produced by repeated passage *in vitro* (50 or more) and compared to the parental strain to determine its ability to cause disease in the ceca, liver, and lungs, of chicken and turkeys after challenge. Virulent *H. meleagridis* strains induced gross lesions in chickens and turkeys and infected the luman, liver, and lungs of turkeys. The attenuated *H. meleagridis* remained in the lumen of the bird and

demonstrated a decreased ability to penetrate the cecal wall and infect the liver or lungs. It showed no negative effect on overall bird health (Liebhart et al 2011). Hess *et al* investigated the effects of inactive and attenuated vaccines of *H. meleagridis* in turkeys. The inactive vaccine had no effect; the attenuated vaccine induced a protective immune response (Hess et al 2008).

Culture Media

The most common medium used for the growth of *H. meleagridis* is Dwyer's media (Dwyer 1970). The composition of this media includes: M199 with Hanks Balanced Salt Solution (HBSS), 10% horse serum, 0.035% sodium bicarbonate, and a starch source (Hauck et al 2010). Starch sources that have demonstrated efficient growth include flours prepared from rice, wheat, oats, rye, and cornstarch (Hauck et al 2010). The addition of rice flour increases the growth of *H. meleagridis* 3 to 10 fold in comparisons to other starch sources (van der Heijden & Landman 2007). It is hypothesized that rice flour acts either as a source of nourishment for accompanying bacteria or it is phagocytized by *H. meleagridis* cells for food (Hauck et al 2010).

In vitro growth of *Histomonas meleagridis* is logarithmic at optimal temperatures of 37– 40°C, the organism can reaches 5 x 10^5 cells/mL in 3 – 5 days (Hauck et al 2010). Several authors have reported that dead bacteria are nutritional sources for *H. meleagridis* (Hauck et al 2010). In order for *H. meleagridis* to grow *in vitro,* a pH of 7 and presence of bacteria are essential (Hauck et al 2010). It is standard practice to analyze treatment *in vitro* prior to *in vivo* testing. When testing alternative drug therapies *in vitro,* pH and bacteria are important contributing aspects, as the chemical composition of each treatment can alter the pH and bacterial content of the media.

Products Tested on *H. meleagridis*

A wide spectrum of products including, herbal extracts, essential oils, and heavy metals have been tested against *H. meleagridis in vitro* and *in vivo* with wide-ranging results (Grabensteiner et al 2007, Hafez & Hauck 2006, Hafez et al 2010, Hu & McDougald 2004, van der Heijden et al 2011, van der Heijden & Landman 2008a, van der Heijden & Landman 2008b). A comparison of tests *in vitro* and *in vivo* are listed in **(Table 2.1)**. Products with confounding results include Protophyt^{SP} (feed additive) and Paromomycin as noted in the **(Table 2.1)**. (Hafez & Hauck 2006, Hafez et al 2010, Hu & McDougald 2004, van der Heijden et al 2011, van der Heijden & Landman 2008a, van der Heijden & Landman 2008b). Products that are effective *in vitro* but not effective *in vivo* include Enteroguard and Carbadox (van der Heijden et al 2011). The products, dimetridazole, Nitarsone Protophyt ^{B} metronidazole, ornidazole, tinidazole show a reduction for both *in vivo* and *in vitro* (Hafez & Hauck 2006, Hafez et al 2010, Hu & McDougald 2004, van der Heijden & Landman 2008b). However, the majority of products listed are arsenic containing compounds or nitroimidazoles and will not be available for use *in vivo* (Nachman et al 2013a). Recent *in vitro* and *in vivo* studies on turkeys, indicate that tested strains of *H. meleagridis* have developed a resistance to Nitarsone (Abraham et al 2014). Many of the products tested *in vitro* have not been tested *in vivo.* Although many of the tested compounds decrease the growth of *H. meleagridis in vitro* it this does not guarantee effective *in vivo* protection against *H. meleagridis*.

Personal communication with the Beckstead lab indicated that *in vitro* test of the metals Zinc Sulfate and Copper Sulfate decreased the number of *H. meleagridis* present in a controlled experiment. *H. meleagridis* growth was measured in the presence of $ZnSO_4$ and

$CuSO_4$ at four concentrations: 50, 100, 500, and 1000 ppm. Growth of *H. meleagridis* was significantly reduced in the cultures treated with $ZnSO_4$ at 500 and 1000 ppm ($p<0.05$) and $CuSO_4$ 100pmm, 500ppm and 1000ppm, as compared to the control. These *in vitro* tests demonstrate that $ZnSO_4$ and $CuSo_4$ were toxic to *H. meleagridis* at levels that are tolerated by turkeys and chickens in the diet, suggesting that dietary zinc and copper might provide a new therapeutic alternative to treating this disease. This information is not listed in Table 2.2.

Table 2.1. Recent publications of products tested *in vitro* and *in vivo*.

Product	In vitro ppm or (%)	Reduction	In vivo ppm or (%)	Disease Prevention
Dimetridazole	50-400	GI	200	+
Histostat-50	200-400	GI	375	+
Enteroguard	200-400	GI	500	–
Protophyt SP-Feed additive	400	*	3000:002 %	– / +
Protophyt B-Water additive	.12-.24%	GI	0.03%	+
Aromabiotic	400	*	0	–
Paromomycin	10-100	GI	100,200,400:200-400	+ / –
Metronidazole	10-100	GI	200,400	+
Ornidazole	10-100	GI	200,400	+
Tinidazole	10-100	GI	200,400	+
Mebendazole	100	GI	0	NT
Albendazole	100	GI	0	NT
Diloxanide furoate	100	GI	0	NT
5,7-diiodo-8-hydroxy-quinoline	100	GI	0	NT
Carbadox	100	GI	50,100	*
Carvacrol	0.6	GI	0	NT
Cassia oil	0.3	GI	0	NT
Thyme/Rosemary oil	0.55	GI	0	NT
Quillaja saponaria	0.8	GI	0	NT

saponin				

[1](NT)—Not Tested (GI)—Growth Inhibited (+)—prevention of disease (-)—no prevention of disease.

Table 2.2. References for products in Table 2.1.

Product	Reference
Dimetridazole	(van der Heijden and Landman, 2008a)
Histostat-50	(van der Heijden and Landman, 2008a)
Enteroguard	(van der Heijden and Landman, 2008a), (van der Heijden and Landman, 2008b)
Protophyt SP-Feed additive	(van der Heijden and Landman, 2008a), (van der Heijden and Landman, 2008b), (Hafez and Hauck, 2006)
Protophyt B-Water additive	(van der Heijden and Landman, 2008a), (Hafez and Hauck, 2006)
Aromabiotic	(van der Heijden and Landman, 2008a), (van der Heijden and Landman, 2008b)
Paromomycin	(Hafez and others, 2010), (Hu and McDougald, 2004)
Metronidazole	(Hu and McDougald, 2004)
Ornidazole	(Hu and McDougald, 2004)
Tinidazole	(Hu and McDougald, 2004)
Mebendazole	(Hu and McDougald, 2004)
Albendazole	(Hu and McDougald, 2004)
Diloxanide furoate	(Hu and McDougald, 2004)
5,7-diiodo-8-hydroxy-quinoline	(Hu and McDougald, 2004)
Carbadox	(Hu and McDougald, 2004)
Carvacrol	(Grabensteiner and others, 2007)
Cassia oil	(Grabensteiner and others, 2007)
Thyme/Rosemary oil	(Grabensteiner and others, 2007)
Quillaja saponaria saponin	(Grabensteiner and others, 2007)

References

1994. *Nutrient Requirements of Poultry: Ninth Revised Edition, 1994*. Washington, DC: The National Academies Press. 176 pp.

Abraham M, McDougald LR, Beckstead RB. 2014. Blackhead disease: reduced sensitivity of Histomonas meleagridis to nitarsone in vitro and in vivo. *Avian diseases* 58: 60-3

Aka J, Hauck R, Blankenstein P, Balczulat S, Hafez HM. 2011. [Reoccurrence of histomonosis in turkey breeder farm]. *Berl Munch Tierarztl Wochenschr* 124: 2-7

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

Benchimol M, Almeida JC, Lins U, Gonçalves NR, de Souza W. 1993. Electron microscopic study of the effect of zinc on Tritrichomonas foetus. *Antimicrobial agents and chemotherapy* 37: 2722-26

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

Bradley RE, Reid WM. 1966. Histomonas meleagridis and several bacteria as agents of infectious enterohepatitis in gnotobiotic turkeys. *Experimental parasitology* 19: 91-101

Brener B, Tortelly R, Menezes RC, Muniz-Pereira LC, Pinto RM. 2006. Prevalence and pathology of the nematode Heterakis gallinarum, the trematode Paratanaisia bragai, and the protozoan Histomonas meleagridis in the turkey, Meleagris gallopavo. *Mem Inst Oswaldo Cruz* 101: 677-81

Cepicka I, Hampl V, Kulda J. 2010. Critical taxonomic revision of Parabasalids with description of one new genus and three new species. *Protist* 161: 400-33

Chute AM, Lund EE. 1974. Heterakis gallinarum in the guniea fowl, Numida meleagris: survival and comparative potential for transmitting Histomonas meleagridis. *Exp Parasitol* 35: 102-9

Clark DS. 2014. Turkey Industry Annual Report - Current Health and Industry Issues Facing the US Turkey Industry, Durham, North Carolina

Clarkson M. 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: i0005-2086

Cushman. 1893. The production of Turkeys. Agricultural Experiment Station, Bulletin 25.

Dwyer DM. 1970. An improved method for cultivating Histomonas meleagridis. *J Parasitol* 56: 191-2

EB L. 2000. Methods in molecular medicine: Vaccine adjuvents - preparation methods and resreach protocols., ed. OH DT: Humana Press

Edward T. 1921. Further Observations on "Blackhead" in Turkeys.

Fine PEM. 1975. Quantitative studies on the transmission of Parahistomonas wenrichi by ova of Heterakis gallinarum. *Parasitology* 70: 407-17

Frank JF. 1953. A Note on the Experimental Transmission of Enterohepatitis of Turkeys by Arthropoids. *Canadian journal of comparative medicine and veterinary science* 17: 230-31

Grabensteiner E, Arshad N, Hess M. 2007. Differences in the in vitro susceptibility of mono-eukaryotic cultures of Histomonas meleagridis, Tetratrichomonas gallinarum and Blastocystis sp. to natural organic compounds. *Parasitol Res* 101: 193-9

Graybill HW, Smith T. 1920. Production of Fatal Blackhead in Turkeys by Feeding Embryonated Eggs of Heterakis Papillosa *The Journal of experimental medicine* 31: 647-55

Hafez HM, Hauck R. 2006. Efficacy of a herbal product against Histomonas meleagridis after experimental infection of turkey poults. *Arch Anim Nutr* 60: 436-42

Hafez HM, Hauck R, Gad W, De Gussem K, Lotfi A. 2010. Pilot study on the efficacy of paromomycin as a histomonostatic feed additive in turkey poults experimentally infected with Histomonas meleagridis. *Arch Anim Nutr* 64: 77-84

Hauck R, Armstrong PL, McDougald LR. 2010. Histomonas meleagridis (Protozoa: Trichomonadidae): analysis of growth requirements in vitro. *J Parasitol* 96: 1-7

Hauck R, Lüschow D, Hafez HM. 2006. Detection of Histomonas meleagridis DNA in Different Organs after Natural and Experimental Infections of Meat Turkeys. *Avian diseases* 50: 35-38

Hess M, Grabensteiner E, Liebhart D. 2006. Rapid transmission of the protozoan parasite Histomonas meleagridis in turkeys and specific pathogen free chickens following cloacal infection with a mono-eukaryotic culture. *Avian Pathol* 35: 280-5

Hess M, Liebhart D. 2011. A live vaccine is safe and efficient to protect poultry against histomonosis. *Procedia in Vaccinology* 4: 100-03

Hess M, Liebhart D, Grabensteiner E, Singh A. 2008. Cloned Histomonas meleagridis passaged in vitro resulted in reduced pathogenicity and is capable of protecting turkeys from histomonosis. *Vaccine* 26: 4187-93

Honigberg BM, Bennett CJ. 1971. Lightmicroscopic observations on structure and division of Histomonas meleagridis (Smith). *J Protozool* 18: 687-700

Hu J, Fuller L, McDougald LR. 2004. Infection of turkeys with Histomonas meleagridis by the cloacal drop method. *Avian Dis* 48: 746-50

Hu J, McDougald LR. 2003. Direct lateral transmission of Histomonas meleagridis in turkeys. *Avian Dis* 47: 489-92

Hu J, McDougald LR. 2004. The efficacy of some drugs with known antiprotozoal activity against Histomonas meleagridis in chickens. *Vet Parasitol* 121: 233-8

Jiao XD, Cheng S, Hu YH, Sun L. 2010. Comparative study of the effects of aluminum adjuvants and Freund's incomplete adjuvant on the immune response to an Edwardsiella tarda major antigen. *Vaccine* 28: 1832-7

Kirkpatrick LFRaWMF. 1927. An Epidemiological Study of Blackhead in Turkeys.

Krieger JN, Rein MF. 1982. Zinc sensitivity of Trichomonas vaginalis: in vitro studies and clinical implications. *J Infect Dis* 146: 341-5

Lee DL. 1969. The structure and development of Histomonas meleagridis (Mastigamoebidae: Protozoa) in the female reproductive tract of its intermediate host, Heterakis gallinarum (Nematoda). *Parasitology* 59: 877-84

Liebhart D, Windisch M, Hess M. 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. *Poultry science* 90: 996-1003

Lollis L, Gerhold R, McDougald L, Beckstead R. 2011. Molecular characterization of Histomonas meleagridis and other parabasalids in the United States using the 5.8S, ITS-1, and ITS-2 rRNA regions. *J Parasitol* 97: 610-5

Lund EE, Burtner RH, Jr. 1957. Infectivity of Heterakis gallinae eggs with Histomonas meleagridis. *Exp Parasitol* 6: 189-93

Lund EE, Chute AM. 1972. Reciprocal responses of eight species of galliform birds and three parasites: Heterakis gallinarum, Histomonas meleagridis, and Parahistomonas wenrichi. *J Parasitol* 58: 940-5

Lund EE, Chute AM. 1973. Means of acquisition of Histomonas meleagridis by eggs of Heterakis gallinarum. *Parasitology* 66: 335-42

Lund EE, Chute AM. 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: 455-61

Lund EE, Wehr EE, Ellis DJ. 1966. Earthworm Transmission of Heterakis and Histomonas to Turkeys and Chickens. *The Journal of parasitology* 52: 899-902

McDougald LR. 2005. Blackhead disease (histomoniasis) in poultry: a critical review. *Avian Dis* 49: 462-76

McDougald LR, Fuller L. 2005. Blackhead disease in turkeys: direct transmission of Histomonas meleagridis from bird to bird in a laboratory model. *Avian Dis* 49: 328-31

McGuire WC, Morehouse NF. 1958. Blood-Induced Blackhead. *The Journal of parasitology* 44: 292-96

Muller M. 1988. Energy metabolism of protozoa without mitochondria. *Annual review of microbiology* 42: 465-88

Munsch M, Lotfi A, Hafez HM, Al-Quraishy S, Mehlhorn H. 2009a. Light and transmission electron microscopic studies on trophozoites and cyst-like stages of Histomonas meleagridis from cultures. *Parasitol Res* 104: 683-9

Munsch M, Mehlhorn H, Al-Quraishy S, Lotfi AR, Hafez HM. 2009b. Molecular biological features of strains of Histomonas meleagridis. *Parasitol Res* 104: 1137-40

Nachman KE, Baron PA, Raber G, Francesconi KA, Navas-Acien A, Love DC. 2013a. Roxarsone, inorganic arsenic, and other arsenic species in chicken: a U.S.-based market basket sample. *Environ Health Perspect* 121: 818-24

Nachman KE, Baron PA, Raber G, Francesconi KA, Navas-Acien A, Love DC. 2013b. Roxarsone, inorganic arsenic, and other arsenic species in chicken: a US-based market basket sample. *Environ Health Perspect* 121: 818-24

National Research Council (U.S.). Subcommittee on Poultry Nutrition. 1994. *Nutrient requirements of poultry*. Washington, D.C.: National Academy Press. xiii, 155p. pp.

Nguyen Pham AD, De Gussem JK, Goddeeris BM. 2013. Intracloacally passaged low-virulent Histomonas meleagridis protects turkeys from histomonosis. *Veterinary parasitology* 196: 307-13

Powell FL, Rothwell L, Clarkson MJ, Kaiser P. 2009. The turkey, compared to the chicken, fails to mount an effective early immune response to Histomonas meleagridis in the gut. *Parasite Immunol* 31: 312-27

Qureshi MA. 2003. Avian macrophage and immune response: an overview. *Poultry science* 82: 691-8

Schwarz A, Gauly M, Abel H, Das G, Humburg J, et al. 2011. Pathobiology of Heterakis gallinarum mono-infection and co-infection with Histomonas meleagridis in layer chickens. *Avian Pathol* 40: 277-87

Seto F. 1981. Early development of the avian immune system. *Poultry science* 60: 1981-95

Sharma JM. 1990. *Avian Cellular Immunology*. CRC Press 160-61 pp.

Sorvari R, Naukkarinen A, Sorvari T. 1977. Anal sucking-like movements in the chicken and chick embryo followed by the transportation of environmental material to the bursa of Fabricius, caeca and caecal tonsils. *Poultry science* 56: 1426-29

Springer WT, Johnson J, Reid WM. 1969. Transmission of histomoniasis with male Heterakis gallinarum (Nematoda). *Parasitology* 59: 401-05

Sulejmanovic T, Liebhart D, Hess M. 2013. In vitro attenuated Histomonas meleagridis does not revert to virulence, following serial in vivo passages in turkeys or chickens. *Vaccine* 31: 5443-50

Tyzzer EE. 1920. The Flagellate Character and Reclassification of the Parasite Producing "Blackhead" in Turkeys: Histomonas (Gen. nov.) meleagridis (Smith). *The Journal of parasitology* 6: 124-31

Tyzzer EE, Collier J. 1925. Induced and Natural Transmission of Black-Head in the Absence of Heterakis. *The Journal of Infectious Diseases* 37: 265-76

Tyzzer EE, Fabyan M, Foot NC. 1921. Further Observations on "Blackhead" in Turkeys. *The Journal of Infectious Diseases* 29: 268-86

van der Heijden HM, De Gussem K, Landman WJ. 2011. Assessment of the antihistomonal effect of paromomycin and tiamulin. *Tijdschr Diergeneeskd* 136: 410-6

van der Heijden HM, Landman WJ. 2008a. In vitro effect of herbal products against Histomonas meleagridis. *Vet Parasitol* 154: 1-7

van der Heijden HM, Landman WJ. 2008b. In vivo effect of herbal products against Histomonas meleagridis in turkeys. *Avian Pathol* 37: 45-50

van der Heijden HMJF, Landman WJM. 2007. Improved Culture of Histomonas Meleagridis in a Modification of Dwyer Medium. *Avian diseases* 51: 986-88

CHAPTER 3

INACTIVATED HISTOMONAS MELEAGRIDIS VACCINE IN COMBINATION WITH FREUND'S INCOMPLETE ADJUVANT DOES NOT OFFER PROTECTION AGAINST BLACKHEAD IN TURKEYS[1]

[1] A. Kenyon, L. R. McDougald and R. B. Beckstead. To be submitted to *Avian Diseases*

ABSTRACT

Histomonas meleagridis is an anaerobic protozoan parasite and the causative agent of blackhead disease in gallinaceous birds. Several studies have reported test with attenuated and inactivated vaccines against blackhead in turkeys with confounding results (Hess et al 2008, Liebhart et al 2011). The present study tested the efficacy of a vaccine developed with Freund's incomplete adjuvant (FIA) and killed cells of virulent *H. meleagridis* at high and low cell concentrations. Treatment profiles were—control uninfected (CUI), control infected (CI), High with adjuvant, Low with adjuvant, High without adjuvant, Low without adjuvant. 10-day-old poults were vaccinated subcutaneous and administered a booster vaccination at 24-days-old. Poults challenged 14 days post vaccination (p.v) and terminated 10 days post challenge (p.c). Individual body weights, ceca and liver lesions scores, and blood samples were collected at termination. There was no significant difference ($P>0.05$) in final body weight of each treatment in comparison to control infected (CI) and there was no significant difference ($P > 0.05$) in ceca or liver scores in each treatment group in comparison to infected control (CI). Under the conditions of this study an inactivated vaccine developed from killed *H. meleagridis* with or without Freund's incomplete adjuvant did not protect against direct *H. meleagridis* challenge.

Keyword: Vaccination, Blackhead, *Histomonas meleagridis,* Freunds incomplete adjuvant

Abbreviations: p.c = post challenge; p.v = post vaccination; CI= Control Infected; CUI=Control Uninfected; FIA= Freund's Incomplete Adjuvant

INTRODUCTION

Blackhead severely affects turkeys with a high rate of mortality (100%) (McDougald 2005). In the 1990's nitroimidazoles, including dimetridazole, ipronidazole and ronidazole were used to treat blackhead. Due to increased consumer concern of suspected carcinogens nitroimidazoles have been restricted from sale in the United States and Europe (Nachman et al 2013b). Currently the only preventative drug available for blackhead is nitarsone. As nitarsone is an arsenical compound it is presently under review by the Food and Drug Administration (Nachman et al 2013b).

Vaccination against blackhead is a possible alternative treatment option. Hess first determined that an inactivated vaccine in combination with Gerbu LQ adjuvant, failed to offer protection against a severe challenge of *H. meleagridis* (Hess et al 2008). An adjuvant enhances the immune response of an antigen, typically in combination with a vaccine (Jiao et al 2010). Freund's Incomplete adjuvant (FIA) is a water-in-mineral oil emulsion, without the addition of heat killed *Mycobacterium tuberculosis* and was selected due to its efficiency in raising high antibody titers and its ability to stimulate a humoral immune response (EB 2000). Although Hess determined that inactive vaccine did not offer protection, alternative compositions of different adjuvants and a gradient of high and low cell concentrations were not tested. An inactivated vaccine with (FIA) was developed from a virulent strain of *Histomonas meleagridis* and tested at a high and low cell concentration. Ten-day-old turkeys were vaccinated and administered booster 14 days post vaccination (p.v). Challenge with a virulent strain of *H. meleagridis* was administered 14 days after the booster vaccination.

In the present work a vaccine was constructed of killed cells of *H. meleagridis* and Freund's incomplete adjuvant, administered subcutaneously for potential protection against *H. meleagridis*.

MATERIAL AND METHODS

Experimental Animal and Room Design

Day old toms (Butterball Turkey Hatchery, Goldsboro NC) were housed in floor pens (5 x 6ft) on fresh wood pine shavings at The University of Georgia Poultry Research Farm (Athens, GA). There were of six treatments with 10 poults per pen, and three replications for a total of 180 birds. Pens were randomly sorted using Excel (Version 14.4.4 for Mac 2011) Figure 3.1. Birds were individually tagged for identification using tab end poultry bands (National Band and Tag Co., Newport, KY). One plastic hanging feeder was placed in each pen. Poults were fed non-medicated turkey starter diets to meet or exceed 1994 NRC requirements (1994). Access to nipple water drinking system was *ad libitum.* Poults were kept on a 24-hour lighting schedule for the duration of the experiment.

Histomonas meleagridis – Buford

Buford— a virulent strain of *Histomonas meleagridis* was collected from an outbreak of blackhead in a backyard flock in a farm in North Georgia. Collections of a half inch tissue samples of infected ceca including liquid cecal contacts were inoculated into 10 ml of Dwyer's medium as modified by Hauck with Hanks Balanced Salt Solution (HBSS), 10% horse serum, .035% sodium bicarbonate, at 42°C (Hauck et al 2010). The sample was incubated for 24hrs at 42°C. 1ml of the cultured sample was passed into 10ml fresh media and cells were allowed to proliferate for 48 hrs. Once cell concentration reached 5 x 10^5 cells/ml cell sample cryogenic preserved with 10% dimethyl sulfoxide (DMSO) and stored

at −180°C in cryogen tubes until needed. Cultures of *H. meleagridis* – Buford were resuscitated from −180°C and cultured in 10ml of Dwyer's media.

Vaccine Development

1 ml of *H. meleagridis* – Buford cell culture was added to twenty flasks with 10 mL of Dwyer's media. The twenty flasks were incubated at 42°C for 72hrs in order to reach maximum cell concentration. Each flask was pooled to combine *H. meleagridis* – Buford cells. Pooled *H. meleagridis* – Buford cells were counted using a neubuer hemocytometer. *H. meleagridis* – Buford cells were diluted to with Dwyer's media to cell concentrations of High – 10^6 and Low – 10^4 per 0.05 ml. Cells were exposed to oxygen to inactivate. Oxygen exposed cells were then centrifuged at 200 x *g* for 5 minutes. Pellets were suspended in 1X Phosphate Buffered Saline (PBS) and placed at -80°C for 24 hours. Cell solution was then removed from −80°C and placed at 23°C for 24 hours. Cell solution was lysed by 5X sonication for 20 seconds at 85% power. Sonication was used to completely inactivate *H. meleagridis*-Buford cells. Cells were visualized under inverted light microscope to determine complete inactivation of cell culture. Inactive cell culture was combined 1:1 with Freund's Incomplete Adjuvant — .85% paraffin oil 15% mannide monooleate.

Challenge of Experiential Animal

Histomonas meleagridis – Buford cells were used to inoculate birds with 100,000 cells/birds by intra-cloacal inoculation. Birds were euthanized by cervical dislocation. Lesions of the liver and ceca were evaluated and recorded at necropsy. Liver and cecal lesions were scored on a 0 – 4 scale based on severity of lesion pattern (Hu et al 2004).

Dietary treatments

Diet formulated to meet or exceed nutrient concentrations given by the Nutrient Requirement of Poultry NRC (1994)

Experimental Design

10-day-old poults were vaccinated with predetermined treatment subcutaneously at the base of the neck. 14 days later 24-day-old poults were given a booster vaccination subcutaneously at the base of the neck. 14 days post vaccination poults were challenged via cloaca inculcation with 100,000 cells/ 0.05 ml of virulent *H. meleagridis*- Buford. Blood collected 5 days after challenge. The study was terminated 10 days post challenge. Individual lesion scores, final body weights, and blood smears were collected at termination.

Lesion Scoring

Scores were determined using a 0 – 4 lesion scoring system for both the ceca and liver. Normal tissue was assigned a score of 0. Scores of 1 is determined as the thickening and reddening of the cecal wall. Scores of 2 and 3 have an intermediate severity of lesions inflammation and the thickening of cecal walls. Scores of 4 are assigned to ceca when the formation of caseous cheese like cecal core is present. Liver lesions assigned a score of 1–2 have only a few necrotic lesions. A liver score of a 3 included increase necrotic lesions and increased severity of lesions. Scores of 4 are reserved for the complete involvement of the liver and lesions present throughout liver tissue (Hu et al 2004). For the purpose of this study, a score of 0 was indicative of negative for disease, and any score determined to be a 1 or more in the liver was indicative of positive disease progression and inevitable death of the bird.

Blood Samples

Whole blood samples were collected from each treatment group at the termination of the study. Blood samples were collected using 25 *G* x 1-inch needle from the left brachial vein of each bird. Samples were analyzed by Dr. Paul Cotter (Cotter Laboratory, Arlington, MA, USA, 02476-7209).

Statistical Analysis

The experimental data was analyzed using the GLIMMIX procedure in SAS (9.4 for Windows, SAS Institute, Cary, NC). Least square mean were calculated, and interactions between treatments were determined an alpha of .05 was used to establish significance. A binomial response distribution was used for liver and ceca data.

RESULTS

Least square means of each experimental treatment groups final body weights were determined for each treatment group (Table 3.1). There was a significant difference in body weights across infected treatment groups in comparison to the CUI group ($P < 0.05$). There was no statistical significance difference in final body weight in comparison to the control infected group ($P > 0.05$).

The average cecal score for each experimental treatment group were 0.03 in the control infected, 3.46 in the control uninfected, 2.90 High without an adjuvant, 2.76 in Low without an adjuvant, 3.23 in High with an adjuvant, and 3.16 in Low with an adjuvant (Table 3.2). All experimental treatment groups excluding the control uninfected (CUI) group had a combined average ceca score of 3.10. The average cecal score of the control uninfected (CUI) was .366 (Table 3.3). There was a significant difference in body weights across infected treatment groups in comparison to the CUI group ($P < 0.05$). There was no

statistical significance in cecal scores ($P > 0.05$) across all experimental treatment groups in comparison to the control infected group.

The average liver scores each experimental treatment group were 0.06 in the control uninfected, 2.40 in the control uninfected, 1.86 in High without an adjuvant, 1.76 in Low without an adjuvant, 2.66 in High with an adjuvant, and 2.13 in Low with an adjuvant (Table 3.2). Liver lesion scores for experimental treatment groups excluding the control uninfected (CUI) had a combined average liver score of 2.16. The average liver score of the control uninfected (CUI) was .06 (Table 3.2). The percentages of total liver lesions were 0.06% in the control uninfected, 80% in the control infected, 80% in High without an adjuvant, 73% in Low without an adjuvant, 70% in High with an adjuvant and 56% in Low with an adjuvant (Table 3.3). The rates of infection for each experimental treatment group were 23% in the control uninfected, 93% in the control infected, 90% in High without an adjuvant, 86% in Low without an adjuvant, 96% in High with an adjuvant and 93% in Low with an adjuvant (Table 3.4).

Hematology reports were collected for the experimental treatment groups: control infected, control uninfected, High with adjuvant, and High without adjuvant. Hematology in percentage for the treatments control uninfected, control infected, High with adjuvant, treatment High without adjuvant are reported in (Table 3.5). Blood samples collected and reviewed reviled a combination of fungemia and bacteremia as a response to *H. meleagridis* infection. Treatments Low with adjuvant and Low without adjuvant were not analyzed due to time and cost constraints. All hematology and blood count data conducted by Paul Cotter, Cotter Laboratory (Arlington, MA, 02476).

DISCUSSION

The average of the final body weight of each experimental treatment group was 500g less, in comparison to the control uninfected group. Decrease in body weight gain is a typical clinical sign of blackhead in turkeys (Edward 1921). The average ceca and liver scores of each experimental treatment groups excluding the control uninfected were 3.10 for the ceca and 2.16 for the liver. A score of 1 or higher in the ceca the primary site of infection, indicates the increase proliferation of *H. meleagridis*. Increased proliferation of *H. meleagridis* in the ceca leads to degradation of the ceca and the subsequent travel of *H. meleagridis* to the liver, the secondary site of infection (Bradley & Reid 1966). The average liver score of 2.16 in this study is indicative of the transport of *H. meleagridis* to the secondary site of infection. The high percentage of birds with liver lesions of a 1 or greater was used as an indication of failed protective immune response in the primary site of infection the ceca.

There was a 23% rate of infection in the control uninfected experimental group, 93% in the control infected, 90% in High without an adjuvant, 86% in Low without adjuvant, 96% in High with adjuvant, and 93% in Low with an adjuvant (Table 3.4). The 23% rate of infection in the control uninfected treatment group is likely due to failure of experimental design to account for the proximity of the control uninfected to directly infected birds of each experimental treatment groups. It is the believed that the infection in the control uninfected experimental group is due to lateral transmission of *H. meleagridis* and pen-to-pen contact of birds, as the only barrier between pens was a thin sheet of plastic netting.

The hematology data (Table 3.5) indicated the percentage of various blood cells that were present in the control infected, control uninfected, High with adjuvant and High without adjuvant treatment groups. The type of blood cell included in this table were typical and variant heterophils, small and medium lymphocytes, Natural Killer (NK) cells, gramuloblast, monocytes, basophlilic granulocytes, eosinophils, reactive thrombocytes (RT) and red blood cells (RBC). Heterophils are the avian equivalent to neutrophils in mammalian species and are characterized as granules in avian species and are the most abundant in avian blood (Seto 1981). Lymphocytes account for a third of all circulating leukocytes and are formed in a variety of lymphoid tissues. They are functionally divided into T-cells, B-cells and Natural Killer (NK) cells. Lymphocytes vary in size (6-30um) and are classified as small, medium or large. Lymphocytes, along with the associated supporting cells, form the immune system and recognize antigens, produce antibodies and destroy pathogens (Qureshi 2003).

Noteworthy is the 40% natural killer (NK) cell count in the control uninfected group, the 46% NK cell count in the control infected group, the 0% NK cell count in the Hig with no adjuvant and 12% NK cell count in the High with an adjuvant. Natural Killer cells are associated with the innate immune response and are classified as lymphocytes that are capable of recognizing antigens (Sharma 1990). This is indicative of the turkey's innate response to a direct of infection of *H. meleagridis* as discussed by Powell (Powell et al 2009).

Under our conditions an inactivated vaccine developed from a virulent strain of *H. meleagridis* at High and Low cell concentrations with or without the addition of Freund's incomplete adjuvant does not offer protection to turkeys challenged with the same virulent

strain of *H. meleagridis*. Vaccination failed to protect challenged birds from secondary infection of blackhead in the liver. The result of this study supports the results of the inability of inactive vaccination to protect turkeys challenged with *H. meleagridis* determined by Hess in 2008 (Hess & Liebhart 2011).

Table 3.1 – Final body weight for each experimental treatment group.

Treatment	Body Weight (Kg)
Control Uninfected	2.95[a]
Control Infected	2.56[b]
High without adjuvant	2.84[b]
Low without adjuvant	2.59[b]
High with adjuvant	2.63[b]
Low with adjuvant	2.73[b]

[a,b] Means within rows with no common superscripts differ significantly (P<.05).

Table 3.2 – Average ceca and liver score for each experimental treatment group.

Treatment	Cell Concentration	Ceca	Liver
Control Uninfected	–	0.03[a]	0.06[a]
Control Infected	–	3.46[b]	2.40[b]
High without adjuvant	1×10^6	2.90[b]	1.86[b]
Low without adjuvant	1×10^4	2.76[b]	1.76[b]
High with adjuvant	1×10^6	3.23[b]	2.66[b]
Low with adjuvant	1×10^4	3.16[b]	2.13[b]

[a,b] Means within rows with no common superscripts differ significantly (P<.05).

Table 3.3 - Percentage of birds with a score of 1 or greater in the liver for each experimental treatment group.

Treatment	Liver (%)
Control Uninfected	0.06[a]
Control Infected	80[b]
High without adjuvant	80[b]
Low without adjuvant	73[b]
High with adjuvant	70[b]
Low with adjuvant	56[b]

[a,b] Means within rows with no common superscripts differ significantly (P<.05).

Table 3.4 – Rate of birds infected with blackhead for each experimental treatment group.

Treatment	Birds with blackhead (%)
Control Uninfected	23[a]
Control Infected	93[b]
High without adjuvant	90[b]
Low without adjuvant	86[b]
High with adjuvant	96[b]
Low with adjuvant	93[b]

[a,b] Means within rows with no common superscripts differ significantly (P<.05).

Table 3.5 – Hematology by percentage.

Treatment	Typ Het[1]	Var Het	Lymp (sm)	Lymp (med)	NK	Mono	Baso	Eosin	RT	RBC	Total (%)
Control Uninfected	-	20	34	1	40	0	2	1	-	-	98
Control Infected	-	0	32	15	46	0	0	3	0	2	98
High with adjuvant	66	0	5	25	0	2	1	1	-	-	100
High without adjuvant	-	33	0	45	12	0	0	9	1	0	100

[1]Abbreviations: Typ, Var Het – typical, variant heterophil, Lymp – lymphocyte, NK –natural killer, Mono – monocyte, Baso – basophilic granulocyte, Eosin – eosinophil, RT – reactive thrombocyte, RBC – red blood cell.

Experimental treatment group pen placement

Empty	High without adjuvant	10^4 cells with adjuvant	Low with adjuvant	Low without adjuvant
Control Uninfected	High without adjuvant	Control Uninfected	High without adjuvant	High with adjuvant
Aisle				
Control Uninfected	Control Infected	High with adjuvant	Low without adjuvant	Low without adjuvant
Empty	Control Infected	10^4 cells with adjuvant	Control Infected	High with adjuvant

Figure 3.1 – Individual experimental groups pen placement. All pen locations were determined by randomization using Excel for mac 2011.

References

1994. *Nutrient Requirements of Poultry: Ninth Revised Edition, 1994*. Washington, DC: The National Academies Press. 176 pp.

Bradley RE, Reid WM. 1966. Histomonas meleagridis and several bacteria as agents of infectious enterohepatitis in gnotobiotic turkeys. *Experimental parasitology* 19: 91-101

EB L. 2000. Methods in molecular medicine: Vaccine adjuvents - preparation methods and resreach protocols., ed. OH DT: Humana Press

Edward T. 1921. Further Observations on "Blackhead" in Turkeys.

Hauck R, Armstrong PL, McDougald LR. 2010. Histomonas meleagridis (Protozoa: Trichomonadidae): analysis of growth requirements in vitro. *The Journal of parasitology* 96: 1-7

Hess M, Liebhart D, Grabensteiner E, Singh A. 2008. Cloned Histomonas meleagridis passaged in vitro resulted in reduced pathogenicity and is capable of protecting turkeys from histomonosis. *Vaccine* 26: 4187-93

Hess M, Liebhart D. 2011. A live vaccine is safe and efficient to protect poultry against histomonosis. *Procedia in Vaccinology* 4: 100-03

Hu J, McDougald LR. 2004. The efficacy of some drugs with known antiprotozoal activity against Histomonas meleagridis in chickens. *Veterinary parasitology* 121: 233-8(Hess et al 2008, Liebhart et al 2011)

McDougald LR. 2005. Blackhead disease (histomoniasis) in poultry: a critical review. *Avian diseases* 49: 462-76

Nachman KE, Baron PA, Raber G, Francesconi KA, Navas-Acien A, Love DC. 2013b. Roxarsone, inorganic arsenic, and other arsenic species in chicken: a US-based market basket sample. *Environ Health Perspect* 121: 818-24

Powell FL, Rothwell L, Clarkson MJ, Kaiser P. 2009. The turkey, compared to the chicken, fails to mount an effective early immune response to Histomonas meleagridis in the gut. *Parasite immunology* 31: 312-27

Qureshi MA. 2003. Avian macrophage and immune response: an overview. *Poultry science* 82: 691-8

Seto F. 1981. Early development of the avian immune system. *Poultry science* 60: 1981-95

Sharma JM. 1990. *Avian Cellular Immunology*. CRC Press 160-61 pp.

CHAPTER 4

ZINC SULFATE OR COPPER SULFATE FAILS TO TREAT OR PROTECT AGAINST HISTOMONAS MELEAGRIDIS IN TURKEYS.[2]

[2] A. Kenyon, L. R. McDougald and R. B. Beckstead. To be submitted to *Avian Diseases*

ABSTRACT

Blackhead is a disease of gallinaceous birds, caused by the protozoan parasite *Histomonas meleagridis*. Once controlled by nitroimidazoles and arsenical drugs, there is currently no effective treatment or preventative. Test *in vitro* suggested that Zinc or Copper salts were toxic to *H. meleagridis* and might be candidates for chemotherapy. In this study we tested $ZnSO_4$ and $CuSO_4$ added to feed or water for possible chemo preventative or treatment of direct infection and lateral transmission of *H. meleagridis* in turkeys. Six treatments were replicated in 3 pens of 20 poults in each of two experiments. Four poults in each pen were directly inoculated with *H. meleagridis* from culture, and commingled with the 16 un-inoculated poults to test the effects of the compound on direct infection and lateral transmission of blackhead. The combined mortality due to *H. meleagridis* in experiment one was 97% and 32% in experiment two. In experiment one neither $ZnSO_4$ nor $CuSO_4$ effectively treated or provided complete protection against the lateral transmission of blackhead in turkeys. In experiment two $ZnSO_4$ did not treat or provide protection against *H. meleagridis* in direct or lateral transmission. However in experiment two treatments of $CuSO_4$ in the diet and water decreased mortality due to the lateral transmission of blackhead.

Keywords: Blackhead disease, Zinc Sulfate, Copper Sulfate, histomonosis, *Histomonas meleagridis*, turkeys

INTRODUCTION

Blackhead disease is a growing concern for poultry producers in the United States and Europe because of a lack of effective treatment products, and an increase in the number of reported cases (Clark 2014). New prevention and treatment products are needed, prompting investigation of alternative treatment and prevention methods. Studies by Benchimol suggested Zinc salts were toxic for *Tritrichomonas foetus*, a close relative of *Histomonas meleagridis,* because of effects on the hydrogenosome (a substitute for mitochondria in anaerobic protozoa bacteria)(Benchimol et al 1993). Test *in vitro* in our lab confirmed that zinc sulfate and related metallic elements had similar effects on *H. meleagridis*. (Beckstead, et al, pers. comm, 2015). Blackhead disease is normally initiated in a turkey flock by introduction of cecal worm eggs (the vector of *H. meleagridis*). However, the disease spreads rapidly through a flock by contact between sick birds and uninfected birds (Hu & McDougald 2003) (Hess et al 2006). It is thought that such lateral transmission could occur via reflexive intake of fluids through the cloaca (SORVARI et al 1977). This process is called clocal drinking (McDougald & Fuller 2005). This suggests two test models: (1) direct inoculation of test birds, and (2) exposure by commingling of directly- inoculated and uninoculated test birds. To test the effects of Zinc and Copper on direct and indirect infections of *H. meleagridis* in turkey poults, these salts were added to feed or water of birds inoculated with parasites or commingled with infected birds.

MATERIAL AND METHODS

Experimental Animal

Two experiments were conducted at The University of Georgia Poultry Research Farm (Athens, GA). Turkey poults for experiment one were obtained from a commercial hatchery in North Carolina (Butterball), poults for experiment two were obtained from a commercial hatchery in South Carolina (Aviagen). Poults were individually tagged using tab end poultry bands (National Band and Tag Co., Newport, KY) for identification. Male poults were used in experiment one and female poults in experiment two. Poults were housed in colony cages in a room used only for brooding and given non-medicated turkey starter feed and water *ad libitum*. At 13-days of age for experiment one and 10–days of age for experiment two, birds were placed in floor pens (5 x 6ft) on fresh wood pine shavings and started on treatment diets. Each of the 6 treatments was replicated in 3 pens of 20 poults. Four poults in each pen were inoculated with *H. meleagridis* from culture, and isolated in separate rooms, and feed non-medicated diets for 72 hours. The remaining 16-poults per pen were started on assigned treatment diets. The 4 directly infected poults and 16 un- infected poults were commingled and given the assigned treatment diets and given access to nipple water drinking system *ad libitum* until the termination of studies.

Experimental Design

Experiment One: All diets were formulated to meet or exceed NRC requirements (National Research Council (U.S.). Subcommittee on Poultry Nutrition. 1994). Treatment diets were $ZnSO_4$ (1500ppm), $ZnSO_4$ (750ppm), $CuSO_4$ (1000ppm), and CuSO4 (500ppm). In addition, a control uninfected treatment (CUI) and a control inoculated (CI) were included. Four 13-

day-old seeder poults were inoculated via cloaca with 100,000-cells/ml of virulent *H. meleagridis* - Buford, and maintained separately for 72 hours on non-medicated diets. On day 13 the four directly inoculated poults and sixteen un-inoculated poults were commingled. Seeder poults were identified with a spray on dye. The study was terminated 20 days after commingling of infected seeder birds with non-infected birds. Individual body weight, ceca and liver scores were recorded at termination.

Experiment Two: 10-day-old poults were placed in floor pens and given one of six treatments: Nitarsone (187ppm feed), $ZnSO_4$ (1500ppm feed), $CuSO_4$ (1000ppm feed), and $CuSO_4$ (250 ppm water). Medicated water was prepared by the addition of pulverized $CuSO_4$ added to water unit drinking system with daily mixing. In addition, a control uninfected treatment (CUI) and a control inoculated (CI) were included. Each treatment was replicated in 3 pens. Similar to experiment one, seeder poults were inoculated at day 10 via cloaca inculcation with 100,000-cells/ml of virulent *H. meleagridis* and maintained separately for 72 hours on non-medicated diets. Four seeder poults and sixteen un-inoculated poults were combined into a single floor pen for a total of twenty poults per treatment. The study was terminated 28 days after commingling of seeder birds with un-inoculated birds. Individual body weight, cecal and liver lesion scores were recorded at termination.

Inoculation with Histomonas Meleagridis

The Buford strain of *H. meleagridis* isolated from an outbreak in turkeys in Buford, GA, was cultured in Dwyer's medium and diluted for use in Dwyer's medium. Birds were inoculated per cloaca with 100,000 cells/ml by means of repeater pipette.

Lesion Scoring

Lesion scores were determined using a 0 - 4 system. Ceca and livers scored separately, normal tissue was assigned a score of 0. Scores of 1 and 2 have few necrotic lesions. Scores of 3 have an increased severity of necrotic lesions. Scores of 4 in the ceca have a caseous cecal core. Scores of 4 in the liver have lesions that are present throughout infected tissue and complete involvement of the liver (Hu et al 2004). For the purpose of this study a score of 1 or more in the ceca was indication of successful direct inoculation and lateral transmission. A score of 1 or more of the liver indicated positive disease progression and potential death of the bird from blackhead.

RESULTS

Experiment one: Severe blackhead infection developed in all directly inoculated birds in all treatment groups, with (91-100%) mortality (Table 4.1). Lateral transmission from directly inoculated birds to in- contact birds presented a high mortality in all groups (93-97%) (Table 4.1). The overall rate of infection combined direct and lateral transmission rates for each treatment group was 0% in the uninfected control and 94-100% in all other treatment groups (Table 4.2). Accordingly, weight gains were depressed in all treatments; in comparison to the control infected there is no significant difference ($P < .05$) in final body weights for each infected treatment group (Table 4.3).

Experiment two: Mortality in directly infected birds in experiment two was 0% in the CUI, 91% in the CI, 41% in nitarsone 187 ppm, 31% in ZnSO4 1500ppm, 83% in $CuSO_4$ 1000ppm, and 83% in $CuSO_4$ 250ppm in water (Table 4.4). Mortality among commingled in –contact birds was 0% in the CUI, 46% in CI, 0% in $ZnSO_4$ 1500 ppm, 35% in $ZnSO_4$ 750

ppm, 22% in $CuSO_4$ 1000 ppm and 2% in $CuSO_4$ 250 ppm in water (Table 4.4). The standard deviation (SD) for directly infected birds was 0 in the CUI, .58 in the CI, 1.41 in nitarsone 187 ppm, 1.41 in ZnSO4 1500ppm, 0 in $CuSO_4$ 1000ppm, and .71 in $CuSO_4$ 250ppm in water (Table 4.4). The standard deviation (SD) for in-contact birds was 0 in the CUI, 7.57 in the CI, 0 in nitarsone 187 ppm, 0 in ZnSO4 1500ppm, 0 in $CuSO_4$ 1000ppm, and .71 in $CuSO_4$ 250ppm in water (Table 4.4). The overall combined rate of infection in the directly infected birds and the in-contact birds was 0% in the CUI, 55% in the CI, 8.3% in nitarsone 187 ppm, 45% in ZnSO4 1500ppm, 35% in $CuSO_4$ 1000ppm, and 18.3% in $CuSO_4$ 250ppm in water (Table 4.5).

DISCUSSION

The model for lateral transmission of *H. meleagridis* from bird to bird was successful in producing a high rate of infection in in-contact birds in experiment one (Armstrong & McDougald 2011, McDougald & Fuller 2005). The high mortality in experiment one in directly inoculated birds (100%), and among in-contact birds (93-97%), indicated a highly virulent infection. In this experiment, the addition of $ZnSO_4$ or $CuSO_4$ to the feed did not reduce direct or indirect infection of blackhead in turkeys. It is not surprising that neither mineral did not reduced direct infection mortality because the birds were infected and maintained on non-medicated feed for 3 days prior to being moved to the medicated diets to allow the infection to be established. Infection rates of directly inoculated birds in experiment two were lower, probably accounting for low infection and mortality percentages in the in-contact birds. However, mortality in birds given $CuSO_4$ 1000ppm in feed and $CuSO_4$ 250ppm in water was lower than that of the directly or indirectly exposed control birds, despite a high rate of direct infection (83%). Although there was a decrease

in the percentage of mortality due to lateral transmission in the treatment groups treated with $CuSO_4$ there was great variance among replication of each treatment group. The standard deviation of in-contact birds in the CI group was 47%, this high SD is indicative of variance in lateral transmission of the three-replication pens. Even though $CuSO_4$ 250ppm in water had only 1 in-contact bird was infected with blackhead, because of the wide variation observed between pens, there was no statically significant difference ($P < .05$) in any of the treatment groups in comparison to the CI. Experiment two will need to be repeated in order to determine if $CuSO_4$ 250ppm in water is a viable preventative.

One theory for the high variability in rate of infection between experiment one and experiment two is environmental temperature. Experiment one was conducted in the winter and experiment two was conducted in late spring. Even though heating was thermostatically controlled, variation in temperature was inevitable. We believe that birds in experiment one huddled more during decreasing outdoor ambient temperatures. Providing more opportunity for transfer of infection. Birds in experiment two likely huddled less as outdoor temperatures were higher. Another possible explanation for difference between experiments one and two is the sex of birds. Male poults were used in experiment one and female poults in experiment two. While previous works has shown no difference in susceptibility of male and female (Brener et al 2006, Hauck et al 2006) some case reports suggested unusual differences in losses in a sex-separated farm.

$ZnSO_4$ and $CuSO_4$ added to diets in experiment one did not protect against direct infection of *H. Meleagridis* in turkeys and did not prevent lateral transfer. However in experiment two there was some suggestion that $CuSO_4$, given in feed or water, reduced the

overall rate of lateral transmission and mortality. This could be due to less intensive infection or environmental effects of huddling behavior. Further work is needed to resolve this difference.

Table 4.1- Mortality for each experimental group in experiment one due to direct and in-contact transmission of *Histomonas meleagridis*.

Treatment	Direct (%)	In-Contact (%)
Control Uninfected	0[a]	0[a]
Control Infected	100[b]	93.75[b]
ZnSO4 1500	100[b]	93.75[b]
ZnSO4 750	91.67[b]	97.92[b]
CuSO4 1000	91.67[b]	93.75[b]
CuSO4 500	100[b]	97.92[b]

1 High rate of mortality in all infected treatments.

Table 4.2 – Overall rate of infection for each experimental group in experiment one due to combined direct and lateral transmission of *Histomonas meleagridis*.

Treatment	Concentration (ppm)	Rate of Combined Direct and In-contact Infection (%)
Control Uninfected	–	0
Control Infected	–	91.31
High $ZnSO_4$	1500	98.33
Low $ZnSO_4$	750	96.66
High $CuSO_4$	1000	96.66
Low $CuSO_4$	500	98.24

[1] High rate of infection in all infected treatment groups

Table 4.3 – Final body weight average in experiment one for each experimental group.

Treatment	Concentration (ppm)	Body Weight (Kg)
Control Uninfected	–	1.11[a]
Control Infected	–	0.594[b]
High $ZnSO_4$	1500	0.574[b]
Low $ZnSO_4$	750	0.449[b]
High $CuSO_4$	1000	0.504[b]
Low $CuSO_4$	500	0.495[b]

[a,b] Means within rows with no common superscripts differ significantly (P<.05).

Table 4.4 – Mortality for each experimental group in experiment two due to direct and in-contact transmission of *Histomonas meleagridis*.

Treatment	Concentration (ppm)	Direct (%)	In-contact (%)	Direct (SD%)	In-Contact (SD%)
Control Uninfected	–	0^{a}	0^{a}	0	0
Control Infected	–	91.6^{b}	45^{b}	14.2	47
Nitarsone	187	41.6^{b}	0^{a}	38	0
ZnSO4	1500	31.2^{b}	35.4^{b}	28.7	50
CuSO4	1000	83.3^{b}	22.9^{b}	14.2	40
CuSO4 H2O	250	83.3^{b}	2.08^{b}	14.2	14.2

a,b Means within rows with no common superscripts differ significantly (P<.05). Standard deviation (SD%).

Table 4.5 – Overall rate of infection for each experimental group in experiment two due to combined direct and lateral transmission of *Histomonas meleagridis*.

Treatment	Concentration (ppm)	Rate of Combined Direct and In-contact Infection (%)
Control Uninfected	–	0
Control Infected	–	55
Nitarsone	187	8.3
$ZnSO_4$	1500	45
$CuSO_4$	1000	35
$CuSO_4$ H_2O	250	18.3

[1]Rate of infection of combined direct and in-contact birds highly variable for each treatment group.

References

1994. *Nutrient Requirements of Poultry: Ninth Revised Edition, 1994*. Washington, DC: The National Academies Press. 176 pp.

Abraham M, McDougald LR, Beckstead RB. 2014. Blackhead disease: reduced sensitivity of Histomonas meleagridis to nitarsone in vitro and in vivo. *Avian diseases* 58: 60-3

Aka J, Hauck R, Blankenstein P, Balczulat S, Hafez HM. 2011. [Reoccurrence of histomonosis in turkey breeder farm]. *Berliner und Munchener tierarztliche Wochenschrift* 124: 2-7

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

Benchimol M, Almeida JC, Lins U, Gonçalves NR, de Souza W. 1993. Electron microscopic study of the effect of zinc on Tritrichomonas foetus. *Antimicrobial agents and chemotherapy* 37: 2722-26

Berks G, Neal RA. 1952. The effect of some drugs upon Histomonas meleagridis in vitro. *Annals of tropical medicine and parasitology* 46: 68-71

Bradley RE, Reid WM. 1966. Histomonas meleagridis and several bacteria as agents of infectious enterohepatitis in gnotobiotic turkeys. *Experimental parasitology* 19: 91-101

Brener B, Tortelly R, Menezes RC, Muniz-Pereira LC, Pinto RM. 2006. Prevalence and pathology of the nematode Heterakis gallinarum, the trematode Paratanaisia bragai, and the protozoan Histomonas meleagridis in the turkey, Meleagris gallopavo. *Memorias do Instituto Oswaldo Cruz* 101: 677-81

Cepicka I, Hampl V, Kulda J. 2010. Critical taxonomic revision of Parabasalids with description of one new genus and three new species. *Protist* 161: 400-33

Chute AM, Lund EE. 1974. Heterakis gallinarum in the guniea fowl, Numida meleagris: survival and comparative potential for transmitting Histomonas meleagridis. *Experimental parasitology* 35: 102-9

Clark DS. 2014. Turkey Industry Annual Report - Current Health and Industry Issues Facing the US Turkey Industry, Durham, North Carolina

Clarkson M. 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: i0005-2086

Cushman. 1893. The production of Turkeys. Agricultural Experiment Station, Bulletin 25.

Dwyer DM. 1970. An improved method for cultivating Histomonas meleagridis. *The Journal of parasitology* 56: 191-2

EB L. 2000. Methods in molecular medicine: Vaccine adjuvents - preparation methods and resreach protocols., ed. OH DT: Humana Press

Edward T. 1921. Further Observations on "Blackhead" in Turkeys.

Fine PEM. 1975. Quantitative studies on the transmission of Parahistomonas wenrichi by ova of Heterakis gallinarum. *Parasitology* 70: 407-17

Frank JF. 1953. A Note on the Experimental Transmission of Enterohepatitis of Turkeys by Arthropoids. *Canadian journal of comparative medicine and veterinary science* 17: 230-31

Grabensteiner E, Arshad N, Hess M. 2007. Differences in the in vitro susceptibility of mono-eukaryotic cultures of Histomonas meleagridis, Tetratrichomonas gallinarum and Blastocystis sp. to natural organic compounds. *Parasitology research* 101: 193-9

CHAPTER 5

CONCLUSIONS

The goal of the present study was to test alternative treatment and preventative methods against blackhead disease in turkeys. It was the aim of this work to develop an inactivated vaccine that would provide complete protection against blackhead and test if the addition of $ZnSO_4$ and $CuSO_4$ to feed and water were efficacious in treatment and prevention. All experimental treatment methods were tested in turkey poults.

Inactivated vaccines composed of killed *Histomonas meleagridis* cells at 10^6 and 10^4 with and without Freund's Incomplete Adjuvant were tested to determine the ability of each to provide immune protection to turkey's challenged with a virulent strain of *H. meleagridis.* Under the conditions of this study the use of an inactivated vaccine composed with *H. meleagridis* failed to provide complete protection against a sever challenge of blackhead. A similar inactivated vaccine composition was tested by Hess and the results of this work supports the conclusions that an inactivated vaccine fails to provide immune protection against blackhead (Hess et al 2008).

The secondary objective of this study was to determine if the addition of $ZnSO_4$ and $CuSO_4$ to feed and water of poults would aid in treatment or protection against the lateral transmission of blackhead in turkeys. The additions of these compounds were based on *in vitro* data collected in the Beckstead lab that showed a decline in *H. meleagridis* growth (data not published). Under the conditions of experiment one, addition of $ZnSO_4$ or $CuSO_4$ to feed did not treat or provide protection against the lateral transmission of blackhead in

turkeys. Under the conditions of experiment two $ZnSO_4$ failed to treat or prevent the lateral transmission of blackhead in turkeys. The addition of $CuSO_4$ in feed and water presented a reduction in mortality of in contact birds, however a large variance in the lateral transmission of blackhead in the control infected group, calls for further investigation.

Future work to determine efficacious treatment and preventative methods are needed. There are currently no treatment or preventive methods available in the United States. Continued testing of metals and compounds with structures similar to arsenic show promise. The synergistic effects of $ZnSO_4$ and $CuSO_4$ at various concentrations will be tested in an *in vitro* assay.

References

Hess M, Liebhart D, Grabensteiner E, Singh A. 2008. Cloned Histomonas meleagridis passaged in vitro resulted in reduced pathogenicity and is capable of protecting turkeys from histomonosis. *Vaccine* 26: 4187-93