8-11-2007

Effects of 6/85-strain mycoplasma gallisepticum inoculation alone at 10 weeks of age or in conjunction with fmg inoculation overlays at 22 or 45 weeks of age on the performance, egg, blood, and visceral characteristics of commercial egg laying hens

Kristin Allo Viscione

EFFECTS OF 6/85-STRAIN MYCOPLASMA GALLISEPTICUM INOCULATION ALONE AT 10 WEEKS OF AGE OR IN CONJUCTION WITH FMG INOCULATION OVERLAYS AT 22 OR 45 WEEKS OF AGE ON THE PERFORMANCE, EGG, BLOOD, AND VISCERAL CHARACTERISTICS OF COMMERCIAL EGG LAYING HENS

By

Kristin Allo Viscione

A Thesis
Submitted to the Faculty of
Mississippi State University
In Partial Fulfillment of the Requirements
For the Degree of Master of Science
in Agriculture with a Concentration in Poultry Science
in the Department of Poultry Science

Mississippi State, Mississippi

May 2007

EFFECTS OF 6/85-STRAIN MYCOPLASMA GALLISEPTICUM INOCULATION ALONE AT 10 WEEKS OF AGE OR IN CONJUCTION WITH FMG INOCULATION OVERLAYS AT 22 OR 45 WEEKS OF AGE ON THE PERFORMANCE, EGG, BLOOD, AND VISCERAL CHARACTERISTICS OF COMMERCIAL EGG LAYING HENS

By

Kristin Allo Viscione

Approved:

_____	_____
E. David Peebles (Poultry Science Major Professor and Director of Thesis)	G. Todd Pharr Assistant Professor (Committee Member)
_____	_____
Michael Kidd (Graduate Coordinator and Head of the Department of Poultry Science)	Scott L. Branton Adjunct Professor, USDA-ARS (Committee Member)
_____	_____
Timothy Cummings Assistant Professor (Committee Member)	Vance H. Watson Dean of the College of Agriculture and Life Sciences

Name: Kristin Allo Viscione

Date of Degree: May 4, 2007

Institution: Mississippi State University

Major Field: Poultry Science

Major Professor: Dr. E. David Peebles

Title of Study: EFFECTS OF 6/85-STRAIN MYCOPLASMA GALLISEPTICUM INOCULATION ALONE AT 10 WEEKS OF AGE OR IN CONJUNCTION WITH FMG INOCULATION OVERLAYS AT 22 OR 45 WEEKS OF AGE ON THE PERFORMANCE, EGG, BLOOD, AND VISCERAL CHARACTERISTICS OF COMMERCIAL EGG LAYING HENS

Pages in Study: 61

Candidate for Degree of Master of Science

The effects of 6/85-strain *M. gallisepticum* (6/85MG) inoculation alone or in conjunction with a F-strain *M. gallisepticum* (FMG) over-lay and its timing on the performance, egg, blood, and visceral characteristics of commercial egg laying hens were investigated. The applied treatments did not affect layer performance, but did affect yolk moisture and fatty acids, liver moisture, and plasma protein. The plasma protein and liver moisture changes may be indicative of the effects of the treatments on the hydration statuses of the birds during lay, whereas alterations in yolk palmitic, oleic, and linolenic acid levels with treatment may have been manifested by disturbances in the desaturation and elongation processes of fatty acid synthesis. Pre-lay 6/85MG inoculations may be a suitable substitute for pre-lay FMG inoculations and FMG overlays during lay on pre-lay 6/85MG inoculations may provide continual protection without eliciting any subsequent suppressive affects on performance.

DEDICATION

I would like to dedicate this work to my parents, Richard and Eugenia Viscione. Despite all the frustration, stress, and struggle, their faith in me never faltered. I would not have been able to continue my academic career without their help and constant support. We have been, and always will be, a team. This accomplishment is as much theirs as mine.

ACKNOWLEDGEMENTS

First and foremost, Dr. Peebles went above and beyond his role as major professor, and helped me accomplish much more than I'd ever thought possible. His faith in me and constant reassurance and support was absolutely vital. He has my deepest gratitude. Ms. Sharon Whitmarsh also went above and beyond as a first rate technician, therapist, computer whiz, and lab mom. I could not have imagined a more supportive lab to work in, or a more supportive department to work with. Dr. Morgan's faith in my typing abilities set the ball rolling, so these acknowledgements would not be complete without acknowledging him. My committee, Dr. Kidd, Dr. Branton, Dr. Cummings, and Dr. Pharr were patient and supportive through countless emails and numerous rambling voice messages. Their willingness to take this on, and have a sense of humor while doing so, is appreciated more than I could possibly express. I would also like to acknowledge Dr. Thaxton as one of the best teachers I've ever had the privilege of studying under. He pushed me to be more and always had absolute faith that I had more of myself to give. His continued encouragement has meant more than he will ever know. I'd like to acknowledge the professors, technicians, and secretaries, in the Poultry Science Department as well as the USDA scientists, technicians, and farm crew. Their generosity

is a continued surprise, and their willingness to laugh as they work makes the job so much more fun. I was exceedingly lucky to have a great support system of family and friends. My parents, Richard and Eugenia Viscione, sister and brother-in-law, Michael and Erin Fowler, and grandmothers, Loretta Gabriel and Lillian Viscione were fabulous sounding boards.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER I

INTRODUCTION

Mycoplasma gallisepticum **(MG)** is a small eubacterium that lacks a cell wall and is weakly gram-negative. Mycoplasma infections have been shown to reduce the performance of commercial laying hens, leading to significant economic losses (Yoder and Hofstad, 1964; Domermuth et al., 1967; Mohammed et al., 1987; Patterson, 1994). Mycoplasma is found in many types of birds, including chickens and turkeys. The known strains of MG include the following: S6 (**S6MG**), R, F (**FMG**), 6/85 (**6/85MG**), PG31, ts-11, 1150, MR3, A 5669, Connecticut F strain, and ATCC 15302 (McMartin, 1967; Carpenter et al., 1979; Hildebrand et al., 1983; Levinsohn et al., 1985; Yagihashi, 1988; Evans and Hafez, 1992; Sudhir et al., 1998; Branton et al., 2000; Marois et al., 2001). While vaccines are commercially available to help lower the loss of production in commercial layers, the vaccines themselves can cause drops in production. The FMG strain is known to depress the egg production (**EP**) of layers when inocula are administered at 10 or 22 weeks. When administered prelay or at lay initiation, the 6/85MG is known to disrupt layer EP less than FMG, but it does not protect as well as FMG from the more virulent strains of MG (Eleazer, 2000). There are possible benefits

of using a prelay 6/85MG inoculation in conjunction with FMG inoculations during lay to effectively protect flocks against field strain MG infections, while reducing possible subsequent depressions in EP due to a prelay FMG inoculation. Commercial operations may acquire flocks already inoculated with 6/85MG, but desire to continue using the FMG inoculation regime familiar to them. Therefore, the goal of this study was to investigate the effects of a 6/85MG inoculation prelay overlaid with FMG inoculation during lay on the performance, blood, internal egg and eggshell, and reproductive and digestive organ characteristics of commercial layers.

CHAPTER II

REVIEW OF LITERATURE

The Avian Reproductive System

Ovaries in birds are both present at all embryonic stages, but primordial germ cells to the ovaries of the bird begin to become asymmetrically distributed by the fourth day of incubation, and regression of the right oviduct begins by Day 10. Therefore, only the left ovary and oviduct are functional in the adult female except for a rare case of right oviduct development (Romanoff & Romanoff, 1949; Kinskey, 1971). The mesovarian ligament attaches the left ovary to the left kidney on the cephalic end. The number of oocytes in the chick embryo rises, falls, and usually ends with 480,000 when oogenesis is terminated. Oogenesis is terminated at hatch (roughly 21 days). Only 200-500 of these oocytes reach maturity and are ovulated in the life span of the bird. There is a follicular hierarchy in the functionally mature ovary with 4-6 large yolk filled follicles, a greater number of smaller yellow follicles, and numerous small white follicles.

The mature follicle consists of layers of tissue with concentric properties. These tissues surround the oocytes and yolk. The tissues include: (1) the oocytes plasma membrane, (2) the perivitelline membrane, (3) granulosa cells, (4) basal lamina, and (5) the theca (interna and externa). Follicles are ovulated in order of the follicular hierarchy

and any enlarged, yolk filled follicles that fail to be ovulated become atretic. The avian oviduct has five distinct regions: infundibulum, magnum, isthmus, shell gland, and vagina.

The infundibulum is a funnel-shaped section of oviduct that is not directly attached to the ovary itself. The ovum is "caught" by the infundibulum and remains there for approximately 18 minutes (Warren and Scott, 1935). This is also the site for the deposition of the first layer of albumen (Gilbert, 1979). The magnum is the largest portion of the oviduct (33 cm in the chicken) and is the site for the majority of albumen formation. The magnum is highly glandular, containing tubular glands responsible for the production of ovotransferrin and ovomucoid (Aiken, 1971 and Tuohimaa, 1975). The magnum contains the ovum for 2-3 hours and is the beginning of calcium secretion (Eastin and Spaziani, 1978a). The isthmus is the next section of oviduct and has less glandular tissue than the magnum, but more muscle mass. The isthmus has tubular gland cells overlaid with epithelial cells. The ovum remains in the isthmus one to two hours, and forms both inner and outer shell membranes.

The uterus, or "shell gland", is characterized by a prominent muscle layer lined with both tubular and unicellular goblet cells (Johnson, 1986). The egg absorbs salts and about 15g of water before calcification, this process is termed "plumping" (Wyburn et al., 1973). The egg remains in the uterus for 20-26 hours. Calcification takes about 15 hours, and pigmentation takes roughly 5 hours (Warren and Conrad, 1942). The vagina is defined from the uterus by the utervaginal sphincter muscle and ends at the cloaca. While the uterus contains many folds of mucosa lined by both ciliated and nonciliated

cells, secretory glands are absent. While the vagina and uterus work in tandem to expel the egg, the vagina plays no role in the formation of the egg.

Contractions of the oviduct transport the egg to the oviduct which functions as a stretch receptor. The ovum produces the mechanical stimulus (Arjamaa and Talo, 1983). Johnson (1986) suggests that ovum transport through the oviduct and expulsion of the egg from the shell gland may involve prostaglandin-stimulated contractility of oviduct smooth muscle.

To allow the egg to pass through the cloaca, the abdominal muscles and sphincter between the shell gland and vagina, as well as muscular contractions of the uterus must occur (Johnson, 1986).

Hormonal Influences on Avian Reproduction

The highest concentrations of the hormones progesterone and estrogen occur in the preovulatory follicles, while concentrations of testosterone decrease in the largest preovulatory follicle 6-8 hours prior to ovulation (Shahabi et al., 1975). Estrogen is produced by thecal tissue (Johnson, 1986). Lutenizing hormone plasma concentrations peak 4-6 hours prior to ovulation (Lague et al., 1975; Johnson and van Tienhoven, 1980a; Etches and Cheng, 1981). This is considered a necessary part of ovulation. This peak also coincides with the peak of progesterone (Etches and Cheng, 1981). Etches and Cheng (1981) assert that there is an additional peak of LH at 14-16 hours prior to ovulation. Testosterone shows a peak preovulatory concentration occurring 10-6 hours prior to ovulation. Testosterone is secreted from at least the four largest follicles. The highest levels of 5a-dihydrotestosterone (**DHT**) peak at 6 hours before ovulation (Etches

et al, 1981). Ovulation can occur without testosterone or DHT, indicating they are not directly involved in ovulation (Johnson, 1986). Corticosterone displays both a daily rhythm and a peak coincident with oviposition, but no increase related to ovulation (Johnson and van Tienhoven, 1981d). A peak in follicle-stimulating hormone (**FSH**) 15 hours prior to ovulation has been observed and occurs coincidentally with an increase in FSH binding in the ovarian tissues (Scanes et al., 1977a; Etches and Cheng, 1981).

Calcium in the blood is circulated in two different forms. One form is nondiffusible protein-bound calcium (unionized) and the other is diffusible ionized calcium. Plasma calcium-binding proteins, vitellogenin, and albumen bind nondiffusible calcium (Guyer et al., 1980). Estrogen increases the amount of these binding proteins, thereby increasing blood calcium levels (Bacon, et al., 1980). Layers not fed an adequate amount of calcium in their diets can experience a significant decrease or complete halt of egg production (**EP**) and regression of the ovary within 6-9 days (Taylor et al., 1962; Luck and Scanes, 1979a). Voluntary calcium consumption of layers increases when the egg enters the uterus as well as the period of eggshell calcification (Mongin and Sauveur, 1974).

Photoperiods are essential for EP in layers. Ovarian development is impacted most by 12-14 hours of light, although optimal production has been achieved by up to 18 hours of light. Hens can continue to lay in complete darkness, but production will be decreased (Wilson and Woodward, 1958; Morris, 1968).

Composition of the Avian Egg

The egg is composed of albumen, yolk, an organic matrix, and the shell. The albumen is comprised of four distinct layers: (1) a chalaziferous layer which is attached to the yolk (~2.7%); (2) an inner liquid layer (~16.8%); (3) a dense, thick layer (~25%); and (4) an outer thin, fluid layer (~50%). The organic matrix has four distinct layers as well. These consist of the shell membranes, the mammillary cores, the shell matrix, and the cuticle. The isthmus is principally responsible for producing the shell membranes, although there are still questions about whether the epithelial or tubular gland cells are responsible. These membranes contain protein fibers cross-linked by disulfide and lysine-derived bonds with small fibrous protuberances of unknown function creating a meshwork. Passage of water and crystalloids are permitted by the semipermeable membranes, and while there is no relationship between the membrane thickness and shell thickness, both decrease with age. The outer shell membrane is embedded within the mammillary core (Johnson, 1986). This has been proposed as the initial site of calcification (Stemberger et al, 1977) and represents the greatest proportion of the organic material of the eggshell. The mammillary cores are comprised of mucopolysaccharides and sulfated proteins which are thought to be formed by the isthmus epithelial cells. The shell matrix is composed of a series of layers of protein as well as acid mucopolysaccharides where calcification takes place. The shell matrix completes approximately 2% of the organic composition of the eggshell and along with calcified crystals makes up the palisade layer of the shell. This layer is deposited soon after the egg reaches the uterus. A thin waxy cuticle covers the surface of the egg. The cuticle is comprised of proteins, polysaccharides, and lipids (Johnson, 1986). The cuticle

ranges in depth of 0.5 to 12.8 µm due to an uneven distribution (Parsons, 1982). The source of the cuticle is not known, and it adds little to the structural integrity of the egg. The function of the cuticle is believed to protect the egg from evaporation and microbial invasion.

The eggs calcified portions can be divided into the mammillary knob layer, the palisade layer, and the outer surface crystal layer. The mammillary knob is formed from outward crystallization of the mammillary cores. The mammillary knob is formed in the uterus in the first 5 hours of calcification. Plumping stretches the membranes by water passage and increases distance between the tips of the mammillace. Crystals form laterally and grow to eventually coincide with crystals from other mammillae. Crystals that grow outward may extend to the shell surface (Johnson, 1986). The crystallized palisade is also termed the spongy layer, and is composed mainly of calcite and constitutes the greatest portion of the shell (~200 µm thick). Approximately 5-6 hours after the egg has entered the uterus, the calcification of this layer is initiated during a process termed "plumping".

Avian Lipid Metabolism

Fatty acids can be characterized as saturated or unsaturated as well as essential or nonessential. Avian requirements for lipids are for relatively small amounts of the few fatty acids that cannot be synthesized by the bird's body (arachidonic, linolenic, and linoleic). These fatty acids that cannot be synthesized make up the essential fatty acids. However, arachidonic acid can be procured from linoleic acid, and, therefore, a dietary supply of linoleic acid can satisfy the need for arachidonic acid. The liver and

extrahepatic tissue is the main source of fatty acid synthesis. There are two major enzyme systems involved in the synthesis of fatty acids in birds. Acetyl-CoA carboxylase (a biotin dependent enzyme) is the first, and the multienzyme system fatty acid synthetase is the second (Parkhurst and Mounthey, 1987; Kleven, 2003).

Lipase activity in the small intestine as well as in the stomach hydrolyze triglycerides to diglycerides, monoglycerides, fatty acids, and glycerol (Johnson, 1986). During this digestion, micelles are formed and eventually absorbed into the small intestine. Cholecystokinin induces pancreatic enzyme secretion in response to lipid entrance into the digestive tract (Dockray, 1975). Villi of the small intestine absorb fatty acids and the lipids are transported by the portal blood system as portomicrons after being esterified (Bensadoun and Rothfeld, 1972; Frazer et al., 1986).

Metabolism of lipids is regulated by various hormones. Glucose is the end product of carbohydrate digestion and becomes a major precursor of lipids. Glucagon and insulin are essential factors in the regulation of lipogenesis. Glucagon affects lipolytic and antilipogenic hormone while insulin stimulates lipogenesis. Avian pancreatic peptide reduces the concentration of circulating free fatty acids and partially suppresses lipolysis by glucagon. Insulin inhibits the release of glycerol and free fatty acids in adipose tissue and also stimulates the conversion of glucose to fat. By stimulating the lipoprotein lipase, incorporation of circulating triglycerides into cells increases.

Mycoplasma gallisepticum

Mycoplasmas are very small (0.25-0.50 µm) prokaryotes. They lack a cell wall and are bound by a plasma membrane which makes them resistant to antibiotics that affect cell wall synthesis or complex nutritional requirements (Kleven, 2003). Mycoplasma has a thin trilaminar cell membrane and consist of only three organelles; a cell membrane, ribosomes, and circular double-stranded DNA molecule. Mycoplasmas exploit their limited genetic material and are successful as pathogens. Mycoplasmas infect by entering the host, multiplying, evading defense mechanisms, causing damage, and escaping to infect new hosts. It targets tissues with epithelial surfaces, such as the respiratory tract, and cause lesions by stimulating the host's inflammatory and cellular responses. Mycoplasma is able to evade the host's immune system by varying their major surface antigens (Bradbury, 2005). Mycoplasmas colonize mucosal surfaces. Mycoplasmas are of the class Mollicutes, Family I *Mycoplasmataceae*, and Genus I *Mycoplasma*. Usually noninvasive, *M. gallisepticum* is now known to penetrate cells (Kleven, 2003). *M. gallisepticum* (MG) is weakly gram negative and penicillin resistant (Kleven, 2003)

Hosts of M. gallisepticum

Mycoplasma tends to be host specific and affect a broad range of animals, plants, and insects. Mycoplasma organisms have been isolated from varying species of birds such as pheasants, chukar, partridges, peafowl, as well as chickens, turkeys, duck, geese, and other processed birds. *M. gallinaeceum, M. gallinarium, M. gallisepticum, M. glycophilum, M. iners, M. lipofacies, M. pullorum,* and *M. synoviae* (**MS**) affect

commercial flocks of chicken. Turkeys alone are affected by *M. melegridis* and *M. iowae*, while MS and MG are pathogenic for both chickens and turkeys (Kleven et al., 1998). *M. imitans* was isolated from ducks, geese, partridges, and others and was found to be closely related to MG. While studying the pathogenicity of *M. imitans* in chicks no clinical signs or lesions were found with a single infection, however, sinusitis, tracheitis and airsacculitis were found in birds where infection was associated with MG and infectious bronchitis virus. Therefore, it is believed that *M. imitans* act synergistically with other pathogenic agents.

Isolation and Detection of M. gallisepticum

Every species of Mycoplasma requires the presence of cholesterol to grow in media. The growth media must be protein rich and contain 10-15% swine or horse heat-inactivated serum. A further supplication of a yeast-derived component is found to be beneficial as well. Frey's medium is a commonly used growth media for MG. *M. gallisepticum* is a slow growing organism, and is resistant to thallium acetate and penicillin, which are frequently used in the media to retard the growth of contaminant bacteria and fungi. Plates that are inoculated must be incubated in a very moist atmosphere for 3-10 d at 37-38°C. Colonies of MG are small (0.1-1.0 mm), smooth, circular, and flat with a denser, raised central elevation. Differences between colony morphology have been noted, but cannot be relied upon to differentiate between the various species. Individual colony cells can vary from 0.2-0.5 µm and are generally coccoid to coccobacilliform; however slender rods, filaments, and ring forms have been described. Mycoplasma species usually ferment glucose, hydrolyze arginine, and exhibit

phosphatase activity. This fermentation is a common trait among MG, MS, *M. gallinaeceum*, *M. glycophilum*, *M. pullorum*, and *M. gallinarium*. *M. iners* hydrolyses arginine while *M. lipofacies* ferments glucose and hydrolyses argentine (Kleven, 2003; Kleven, 1997).

Mycoplasma organisms are able to remain viable for 3 days in an outer environment, 18 weeks in egg yolk kept at 37°C, and 1-3 days in chicken feces at 20°C (Chandiramani et al., 1996). *M. gallisepticum* organisms may remain viable for 32-60 days if stored in a refrigerator, but do not remain stable at elevated temperatures. Broth MG cultures remain viable for several years if stored at -30°C (Yoder and Hofstad, 1964). After an outbreak of a MG infection, the level of infection in a flock or at a farm may decrease. This decrease results from the birds shedding the organisms into the environment, explaining the dangers in multiage farms where incoming birds are introduced to the constant source of MG in the farm environment (Butcher et al., 1998). While swabbing for MG, it has been reported that the type of swab used may influence the growth rate of MG organisms in infected birds (Shah-Majid, 1986; Shah-Majid and Nihayah, 1987). It has also been noted that the type of swab used may influence the growth rate of MG organisms in media. The effectiveness of plain/charcoal swabs on wooden or plastic sticks to rayon swabs on aluminum wire was compared. Three types of Mycoplasma were used in the comparison: S6 strain, B6/93 strain, and J35/86 strain. Plain/charcoal swabs on wooden or plastic sticks were used when better differentiated positive and negative results were found. Rayon swabs on aluminum wire inhibited Mycoplasma organism's growth during the incubation period (Zain et al., 1995).

False diagnosis results (positive or negative) can cause great economic losses, so there are several methods that may be used to detect infection. These include hemagglutination inhibition (HI), serum plate agglutination (SPA), enzyme linked immunosorbent assay (ELISA), and fluorescent antibody (FA) tests. Nonspecific reactions with MS have been reported with SPA, HI, and ELISA tests (Yoder and Hopkins, 1985; Kleven et al., 1988; Yoder, 1989; Dingfelder et al., 1991; Kempf et al., 1997).

HI titers are strongly positive at 1:80, strongly suspected at 1:40, and negative for MG negative at 1:20. The effectiveness of HI and ELISA tests in the detection of a laboratory and field strain of MG was tested by Czifra et al. (1995). A5969 strain was used as a hemagglutinating antigen. Of inoculated birds, 62.7% were detected to be MG positive by the HI test, whereas the same birds were found to be 83% positive by ELISA. False negative results with birds infected with the K703-strain of MG were reported in commercial ELISA kits. False negative results in ELISA could be explained by the inability of some strains of Mycoplasma to inhibit the activity of the A5969 strain used in the ELISA (Kempf et al., 1997). A more reliable technique than ELISA is the FA technique (May et al., 1997). Mycoplasma species can be differentiated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SAS-PAGE), Southern blot, or by restriction endonuclease analysis (RFLP) (Kleven et al., 1988).

The Virulence and Transmission of M. gallisepticum

Strains of MG vary in virulence, tissue tropism, antigenic makeup, and are capable of altering the expression of major surface proteins (Garcia, et al., 1994). The

following strains of MG are those known to date: F (FMG), 6/85 (6/85MG), K503, K530, R, S6, PG-31, TS-11, 1150, K1453, K1501, MR3, M1148, A5969, Connecticut F strain, and ATCC 15302 (McMartin, 1967; Carpenter et al., 1979; Hildebrand et al., 1983; Levinsohn et al., 1985;Yagihashi, 1988; Evans and Hafez, 1992; Sudhir et al., 1998; Branton et al., 2000; Marois et al., 2001). RMG strain MG appears to be more virulent than FMG, which is considered to be moderately virulent. The 6/85 strain MG is considered low virulence (Kleven, 2003).

It is thought that phenotypic switching could provide a specific adaptation mechanism to the surrounding environment. Phenotypic switching is believed to be the differential expression of pMGA genes. This effect was discovered *in vitro*, and it is suggested that such an event enables the MG to better adhere to its host (Athamna et al., 1997).

It has been shown that MG organisms are able to adhere to the epithelial surface of the respiratory tract of chickens by terminal bleb structures (Maniloff and Quinlan, 1973). It's been suggested that while the primary location of MG infection colonization is the respiratory system, the invasion of other organs is possible. Migration of MG through the blood stream from the mucosal membrane of the respiratory system to other organs may occur via attachment to erythrocytes (Winner et al., 2000).

Economic Impacts of M. gallisepticum

In 1994, economic losses due to MG infection of commercial laying flocks in the US were up to $118 and $150 million annually. California lost approximately $127 million due to a MG outbreak in 1984 and $6.5 million due to decreased EP and disease

prevention programs (Patterson, 1994). At least 75% of multi-age layer farms in the industry are positive for MG. This is the most pathogenic and economically significant pathogen in poultry. Air sac infections cause significant economic decreases by causing condemned carcasses in processing plants. This, along with reduced feed and EP efficiency, as well as increased medication costs, combine to make this one of the costliest diseases confronting commercial poultry production. In addition to the costs of the disease, costs are further increased by prevention and control methods, such as vaccinations (Kleven, 2003). Despite short survival times in the environment, MG is able to successfully transmit to new hosts horizontally and vertically (hen to egg) in flocks. The horizontal transmission is accentuated by intensive husbandry practices as well as stress factors. Although costly, vaccination programs help prevent the high morbidity and mortality is usually attributed to a second infection (Newcastle, bronchitis, E. Coli, etc).

Pathological Symptoms of M. gallisepticum Infections

M. gallisepticum infections are characterized by respiratory symptoms such as respiratory rales, coughing, nasal discharge, and conjunctivitis (Klevens, 2003). These signs vary from bird to bird, and between species. The severity of symptoms depends on the strain of MG (Klevens, 2003), environmental conditions, ammonia concentration in the air, stress, and the presence of other pathogenic factors (Sato et al., 1973; Zander, 1984; Mohammed et al., 1987b). For example, MG infection of embryos can subsequently result in inflammatory processes and changes in the joints of young chicks (Chute and Cole, 1954). While symptoms may be slow to develop, the infection may

have a long course. *M. gallisepticum* is able to pass through mucosal barriers to cause systemic infections, and has been isolated from the respiratory system, brain, periovarian region, oviduct (including uterus and vagina), liver, spleen, and cloaca (Kleven, 2003).

Treatment of M. gallisepticum Infected Birds

Mycoplasma is susceptible to a number of antibiotics: tylosin, tiamulin, gentamicin, tetracycline, streptomycin, erythromycin, spiromycin, valnemulin, enrofloxacin, lincomycin, and spectinomycin (Ose et al., 1979; Jordan et al., 1998; Timms et al., 1989). Enrofloxacin administered to infected layers through drinking water normalized EP in 3 weeks, and also reduced level of egg transmissions of MG. Only 0.05% of live embryos were shown to be MG positive in antibiotic-treated groups when cultured, whereas 12.8% of all fertile eggs in control birds were MG positive. However, lincomycin and spectinomycin were less successful in preventing MG transmission through the egg (Ortiz, 1995; Jordan et al., 1998). Tilmicosin was shown to reduce the incidence and severity of airsacculitis caused by MG infection in birds when administered for 3-5 days via drinking water (Charleston, 1998). *M. gallisepticum* is able to induce a cross-resistance to antibiotics over several *in vitro* passages. High cross resistance was demonstrated for streptomycin, erythromycin, spiromycin, tylosin, and enrofloxacin. Little induced resistance was demonstrated for streptomycin, chlortetracycline, or enrofloxacin.

Prevention of M. gallisepticum Infections

Mycoplasma organisms are very susceptible to disinfection, so biosecurity is a crucial factor in preventing MG infections. However, this problem is complex as many farms contain flocks composed of many different ages. This cycling of birds makes eliminating an infection extremely difficult, if not impossible.

Protection against the highly pathogenic field strains of MG involves vaccination with live-attenuated and killed (bacterins) vaccines. Vaccination programs also defend flocks from drastic production drops. The bacterins of MG with oil-emulsion adjuvant protected young flocks from intrasinus challenge with virulent MG and commercial layers from the drastic EP drops mentioned above. While bacterins have proven effective in reducing colonization by MG, it usually does not eliminate the organisms completely. Because of this, and the cost of two vaccinations and handling individual birds, bacterins are not considered useful in long term control of MG.

Live FMG strain vaccines have been used in live culture pullet vaccination programs and extensively in multiple-age laying complexes to prevent or reduce MG-caused EP losses (Carpenter, 1981; Glisson, 1984; Rodriguez, 1980; Kleven, 2003) and provided protection against airsacculitis following aerosol challenge with RMG. The biologic mechanism underlying the protection offered by FMG vaccinations was found not to involve competition for adherence sites or blockage by prior colonization, furthermore, the FMG vaccination did not prevent colonization by the challenge strain of MG (Levisohn et al., 1987). FMG can be transmitted through the egg (Lin et al., 1982) as well as among penmates (Evans et al., 92; Kleven, 1981). This transmission is altered by administration techniques. Administering the vaccine through a right-eye drop

prevents transmission from bird to bird if an empty pen or aisle is between the birds (Kleven, 1981). This type of administration also produced birds with higher EP as compared to unvaccinated MG infected flocks, but not as high as control birds (MG-clean flocks) (Carpenter et al., 1981; Mohammed et al., 1987). Flocks vaccinated with FMG maintain the organism in the upper respiratory tract for life (Kleven, 1981). In studies FMG vaccines reduced the population of a challenge or field strain in the upper respiratory tract and in pen trials F strain vaccination effectively displaced challenging infections (Cummings et al., 1986; Levisohn et al., 1987; Kleven et al., 1998). F-strain MG vaccination programs managed to successfully displace field-strain MG from multiple-age layer complex using 2 years of continuous vaccination for replacement pullets (Kleven et al., 1990). F-strain MG vaccine can be administered by several methods including eye-drop, intranasal, and coarse spray (Levisohn et al., 2000). Administration of the vaccine can begin as early as 2 weeks or less if risk of exposure is great, but is usually administered at 8-14 weeks of age (Levisohn et al., 2000).

The 6/85-strain of MG originated in the United States, and its development and vaccination characteristics are described by Evans et al, 1992. (Evans et al., 1992). When 6/85MG vaccines were studied, minimal virulence, rare to no transmissibility, and resistance to challenge with virulent MG were noted (Abd, 1993; Evans, 1992; Evans et al., 1992). The 6/85MG vaccine is administered by spray and while it results in little or no detectable serologic response, it can be detected in the upper respiratory tract for 4-8 weeks after administration of vaccine (Abd, 1993; Evans, 1992; Ley, 1997). 6/85-strain MG vaccines have been used in the United States for prevention of EP losses in the commercial table egg layers. The vaccine (Noblis MG 6/85, Intervet Inc., Millsboro,

Delaware, USA) is presented to the birds in a freeze-dried pellet and administered as a single dose to pullets 6 weeks of age or older; however, to be fully effective, the vaccine must be administered by the aerosol route (Kleven, 2003). Due to its safety comparable to the FMG, 6/85MG vaccination is a desirable alternative when vaccinating susceptible flocks (Levisohn et al., 2000). While 6/85MG affects EP less than FMG, it is not able to displace field strain infections equally (Levisohn et al., 2000).

Effects of M. gallisepticum Infection on Layer Performance

M. gallisepticum causes a decrease in EP along with respiratory disease (Patterson, 1994) and feed consumption in laying hens (Yoder and Hofstad, 1964; Domermuth et al., 1967; Mohammed et al., 1987a; Patterson, 1994). Patterson (1994) also reported that the reduced EP and egg quality in laying hens is a result of the spread of infection from the respiratory system to the oviduct. In a separate report hens inoculated with FMG at 12 weeks of age experienced a delay in EP compared to control birds. This delay was approximately 1 week. There was also a significant decrease in EP after week 42, and FMG-treated birds produced 19 fewer total eggs than control birds (Burnham et al., 2002a). *M. gallisepticum* has also been shown to reduce the overall growth rate of chickens (Butcher, 1998) as well as affecting the eggshell forming mechanisms (Domermuth et al., 1967). Burnham et al (2003c) also asserted that FMG infection affects yolk lipid (significantly higher at weeks 32 and 44, and lower at week 48) and cholesterol concentrations (decreased at week 22) as well as fatty acid composition. Yolk linoleic, stearic and arachidonic acids were increased, while concentrations of palmitoleic and oleic acids were decreased. These yolk lipid

concentration differences may be due to adaptations to infection, where the yolk fatty acid concentrations are indicative of changes in liver lipid synthesis. While the effects of FMG on performance are noted by Burnham (Burnham et al., 2002a) among others, 6/85MG is lacking in literature documenting its effect on performance.

Effects of F and 6/85-strain M. gallisepticum on the Blood Characteristics of Laying Hens

There is little literature that reports the effects of MG on the blood characteristics (such as HCT, STRIG, SCHOL, SCA, and PP). Nevertheless Burnham et al. (2003a) reported levels of HCT, STRIG and PP were affected by FMG inoculations. The HCT levels of birds inoculated with FMG at 12 weeks of age was lower 8 weeks post inoculation when compared to Control birds. *M. gallisepticum*-inoculated hens had increased levels of STRIG and PP during pre-peak EP, but decreased levels in post-peak EP. It has been suggested that increased HCT, STRIG, and PP levels were due to an adaptive immunological response toward the infection. It has been reported that the higher levels of STRIG are associated with an immunological response to infection (Guyton and Hall, 1996). The increased levels of STRIG and PP between 20 and 22 weeks suggest the birds are stressed, further decreasing their immunity toward the FMG. Lower levels of STRIG and PP post-peak EP may accelerate follicular regression, increasing the incidence of fatty livers, and inhibiting liver lipid and protein synthesis. Decreased blood lipid levels may also lead to changes in the deposition of yolk lipid and cholesterol (Burnham et al., 2003a).

Effects of M. gallisepticum Infection on Layer Necropsy Results

Significant effects due to FMG were reported by Burnham et al. (2002b) on magnum length, isthmal and vaginal weights, and the number of mature ovarian follicles. The reduced magnum length of FMG-infected hens is thought to alter albumen deposits. The decreases in isthmal and vaginal weights are thought to impact EP.

CHAPTER III

MATERIALS AND METHODS

Pullet Housing and Management

In both trials, 1-d-old Hy-Line w-36 Leghorn pullets were obtained from a commercial hatchery certified free for MG and MS (USDA-APHIS-VS, 2003). Until 10 weeks of age, birds were raised on clean dry litter, in a 5.5 x 6.1 m section of a conventional house, on a USDA facility. Initial flock density was 0.034 m^2 / bird. In the conventional house, a daily artificial lighting schedule followed a 13 L : 11 D cycle. One 75-Watt light bulb was used to illuminate each 8.4 cm^2 of floor space, providing a 35.5 lx intensity at bird level.

At 10 day of age, all pullets were vaccinated for infectious bursal disease. At 12 days and at 4 weeks of age, they were vaccinated against Newcastle Disease and infectious bronchitis virus via drinking water. At 5 week of age, choanal cleft swabs were taken (Branton et al., 1984), and chicks were bled from a wing vein and tested for MG/MS antibodies using serum plate agglutination (**SPA**) and hemagglutinin inhibition (**HI**) tests (Yoder., 1975). Choanal cleft swabs were placed into tubes containing Frey's broth medium (Frey et al., 1968) supplemented with thallium acetate and 10^6 IU

penicillin-G/mL. Tubes were incubated at 37°C. Colonies with morphology suggestive of Mycoplasma species were examined by an agar plate fluorescent antibody (**FA**) method (Bass and Jasper, 1972).

Layer Housing and Management

At 10 week of age, eleven pullets were randomly assigned to each of sixteen negative pressure biological units. The units were located in a previously described USDA poultry disease isolation facility (Branton and Simmons, 1992). All birds were wing-banded for purposes of identification and individual data collection. At initiation of lay, the number of hens in each treatment unit was reduced to 10, resulting in a bird density of 0.116m^2/bird. The temperatures inside each biological unit were maintained 25°C. Four negative pressure units were assigned to each of four treatments, and each treatment was represented by one row of the four units. In trial II, the location of treatments within the isolation facility was different from that in trial I to assure environmental randomization between trials. At 18 week of age, duration of the artificial lighting schedule was increased 15 min L : h 45 min D cycle was achieved in Trial 1, and a 17 h 15 min L : 6 h 45 min D cycle was achieved in Trial II. These artificial lighting programs were maintained until the end of both trials.

Pullet and Layer Diets

For the duration of each trial, chickens had ad libitum access to feed and water. Birds received standard diets that met or exceeded National Research Council (1994) recommendations. Diets were formulated according to the age of the birds and included

the following: starter (0-6 weeks), grower (7-12 weeks), developer (13-18 weeks), pre-lay (18-19 weeks), and layer (20-60 weeks). Ingredient percentages, calculated, and determined analysis of these diets were described by Burnham et al. (2002a).

6/85 MG and FMG Inoculation

Control birds (**Control**) received sham inoculations in the right eye with 0.04 mL of sterile Frey's broth media at 10 weeks of age. A second treated group of birds were inoculated with a 24-h broth culture of 6/85MG at 10 weeks of age (**6/85MG-10**). In a third treatment group, birds inoculated with 6/85 MG at 10 weeks received an overlay of FMG at 22 weeks (**6/85MG-10, FMG-22**); and a fourth treatment group were inoculated with 6/85MG at 10 weeks followed by a 45 weeks overlay inoculation of FMG (**6/85MG-10, FMG-45)**. *Mycoplasma gallisepticum* organisms were advanced after being received from S. H. Kleven (University of Georgia, Athens, GA) at the 212th passage. Titers and passages of the inocula are presented in Table 1.

Mycoplasma Detection

At 58 weeks of age in both trials, 3 randomly selected hens from each replicate unit in each of the 4 treatment groups (sham inoculated or inoculated with 6/85MG alone, or 6/85MG with FMG overlays at 22 or 45 weeks of age during lay) were bled from a wing vein and were swabbed to test for the presence of MG. Testing procedures at this time were described for those at 5 weeks of age.

Data Collection

In both trials individual BW were recorded at 10, 20, 24, 32, 43, 47, and 58 weeks. Weekly EP was recorded daily and analyzed weekly from weeks 22-58 and was expressed as hen-day production. Furthermore, the total number of eggs produced per hen (total hen EP) from weeks 22-58 was determined. Total hen EP was calculated as total daily numbers of eggs produced as a percentage of total daily numbers of hens for each replicate group over the entire 22-58 weeks production period. Mortality was recorded daily and was expressed as a percentage of the total number of birds in each unit.

In both trials, egg weight (**EW**) was determined weekly from 23 through 55 weeks and at weeks 24, 32, 43, 47, and 58, eggshell weight per unit of surface area (**SWUSA**), and percentage yolk weight (**PY**), albumen weight (**PA**), eggshell weight (**PSW**), yolk moisture (**YM**), and yolk lipid (**YL**) were determined. For determination of the above mentioned internal egg and eggshell quality parameters (SWUSA, PY, PA, PSW, YM, and YL) a total of 10 eggs were collected and used from each replicate unit. If less than 10 eggs were collected on a given day, the rest were collected the following day of the same week. Nevertheless, determinations were made on the same day that eggs were collected. Eggshell weight per unit of eggshell surface area was determined using the following formula: SWUSA = [eggshell weight (mg)/ SA (cm^2)], where SA = $3.9782*W^{0.37056}$] and where W is EW (g) (carter, 1975). Eggshell weight was determined according to Brake et al (1984). Relative egg yolk, albumen, and shell weights were expressed as percentages of total EW.

At the end of each trial (weeks 58), 2 birds from each replicate unit were euthanized by cervical dislocation, their organs were removed, and the following parameters were determined: liver weight, color, and moisture and lipid content; ovary weight, mature follicle number (those that are = 12 mm in diameter); total oviduct weight and length, total small intestine weight and length; oviduct segments (infundibulum, magnum, isthmus, and vagina) weights and lengths, and small intestine segments (duodenum, jejunum, and ileum), weights and lengths; total oviduct and small intestine weights were calculated as percentages of BW. Furthermore, oviduct and small intestine segment weights were calculated as percentages of BW and total organ weight and oviduct and small intestine segment lengths were calculated as percentages of total organ length. The number of mature follicles in an ovary was assigned a category from zero to six, where zero indicates the absence of mature follicles, and where six is the number of maximum follicles recorded. The percentage of birds in each unit possessing zero, one, two, three, four, five, or six follicles was calculated. Livers were examined for color and for incidence of FLHS. Three categories were used for liver color and for incidence of FLHS. Three categories were used for liver color assignment: neutral, red, and yellow. The number of birds in each unit possessing a particular liver color was expressed as a percentage of total number of birds examined. Also, birds with normal livers or those exhibiting FLHS were calculated as percentages of the total number of birds in each unit.

In both trials, blood samples from 2 tagged birds from each replicate unit were collected at 24, 32, and 43 weeks of age for determination of whole blood hematocrit (**HCT**), and serum calcium (**SCA**), triglycerides (**STRIG**), cholesterol (**SCHOL**), and

plasma total protein (**PP**) concentrations. All data collected prior to weeks 22 were designated as belonging to age interval I; all data collected from weeks 22 through 44 was designated as belonging to age internal II; and all data collected from weeks 45 through 58 were designated as belonging to internal III.

Quantitation of Yolk Moisture and Lipid Content

Yolk moisture concentration determination, yolk samples were dried according to the procedure of Peebles et al. (1999) by using 2 g of yolk sample in a commercial oven (Model EL20, General electric CO., Chicago Heights, IL 60411). After samples were removed from the oven, they were allowed 30 min to cool and the dry weights were recorded. Yolk moisture was calculated as the difference between the wet and dry weight of the sample and was expressed as a percentage of wet sample weight. Yolk lipid was extracted according to the procedure described by Bligh and Dryer (1959), and as modified by Latour et al. (1998). A 3 g sample of fresh yolk was thoroughly mixed with 10 mL of methanol and 10 mL of chloroform in a 100 mL beaker. Mixing was done by hand until the mixture was a pasty homogenate. Water (5 mL) was added to the homogenate and the mixture was filtered through Whatman No. 1 filter paper in a Coors Buchner funnel. The filtered mixture was pulled under vacuum suction into 50 mL centrifuge tubes. Additionally, the beaker that contained the original 3 g sample was rinsed with 3 mL of chloroform to extract any additional yolk, and that liquid was also poured through filter paper as described above. Centrifuge tubes were spun in a Beckman JB-6 centrifuge (Beckman Instruments, Inc., Fullerton, CA 93634) for 15 mins at 3000 rpm in order to separate the alcohol and lipid. The centrifuged samples contained

two well-defined layers (lower chloroform-lipid layer and upper methanol-water layer). The upper layer was discarded and the lower chloroform-lipid layer was filtered through a column of anahydrous sodium sulfate into a glass tube. The chloroform containing lipid extract was placed into a V-Evap apparatus (Organomantion, Shrewsbury, MA 01545) and dried with a stream of air. Dried samples were weighed and the expression of YL concentration was dry lipid sample weight as a percentage of total wet yolk sample weight.

Liver Moisture and Lipid Content

Liver moisture analysis of a 2 g homogenized sample was performed according to the procedure previously described for yolk moisture analysis. The expression of liver moisture was the difference between wet and dry sample weight as a percentage of wet sample weight. A homogenized liver sample was used for liver lipid analysis. Liver lipid extraction was accomplished according to the procedure previously described for YL extraction. The expression of liver lipid concentration was dry lipid weight as a percentage of total wet sample weight.

Blood and Serum Constituents

Two tagged birds from each unit were selected and bled from the *cutanea ulnae* wing vein. Blood (4-5 mL) was collected into tubes designed for either serum or plasma collection. Two heparinized capillary tubes (75 mm long) were used on each blood sample for HCT determination. Serum and plasma tubes were centrifuged at 4000 rpm for 20 min. Then, serum and plasma samples were collected and stored at -20°C for later

analysis. A Kodak Ektachem DT-60 analyzer (Eastman Kodak CO., Rochester, NY 14619) was used to determine levels of SCHOL and PP. A Kodak Ektachem DTSC analyzer was used to assay serum samples for SCA. Hematocrit was expressed as percentage blood packed cell volume. Concentrations of SCHOL, STRIG, and SCA were expressed as mg/dL, and PP in g/dL.

Statistical Analysis

A completely randomized experimental design, with trial as a block, was employed. Data prior to weeks 22 (age interval I), from weeks 22 through 44 (age interval II), and from weeks 45 through 58 (age interval III) were analyzed separately. The data of both trials were pooled then analyzed together. Therefore, the results from both trials were not reported independently but were reported over both trials. Trial was considered as a random effect. All data within each age interval were subjected to a repeated measures analysis if parameters were examined at multiple age periods in an age interval. Otherwise, data obtained at only one age period within an age interval was subjected to one-way ANOVA.

In the first age interval, Controls and the 6/85MG-10 treatment group were compared. In the second age interval, Controls and those having had 6/85MG-10, and 6/85MG-10, FMG-22 inoculations were compared. In the third age interval, Control, 6/85MG-10, 6/85MG-10, FMG-22, and 6/85MG-10, FMG-45 groups were compared. Individual sample data within each of these replicate units were averaged prior to analysis. Least-squares means were compared in the event of significant global effects (Steel and Torrie, 1980). Global effects and differences among least-squared means were

considered significant at P = .05. All data were analyzed using the MIXED procedure of SAS software (SAS Institute, 2003).

CHAPTER IV

RESULTS

All initial cultures of Mycoplasma as well as HI and SPA test results were obtained from pullets at 5 wk of age and were negative for MG and MS. Samples of serum obtained from control birds at 58 wk of age were SPA and HI negative for MG, whereas the same tests were positive for MG in the 6/85MG and FMG inoculated hens. Hens were considered to be free of MG when no detectable HI titers were exhibited, and FA culture results showed no MG growth. All MG inoculated hens showed HI titers = 1:80 (geometric mean of 80.0), and were MG-positive (positive for MG fluorescence) and MS-negative (negative for MS fluorescence) using FA tests.

No significant age or treatment main effects or age by treatment interactions were found for bird mortality and BW in any of the 3 age intervals examined. However, mean BW values at the various age periods examined in each age interval are provided in Table 2 for reference. There were significant age main effects for weekly EP in intervals II ($P = 0.001$) and III ($P = 0.001$) as shown in Table 3. Weekly EP was significantly increased between wk 22 and 23 and between wk 23 and 25 in interval II. In that interval EP did not change significantly between wk 25 and 44. Furthermore, weekly EP significantly decreased at wk 47, increased at wk 49, and then decreased again at wk 50 and 54 in

Interval III. In intervals II and III there was no significant treatment or age by treatment interaction for weekly EP. There were no significant main effects due to treatment or age, and there were no age by treatment interactions for weekly EP in interval I. Furthermore, total EP across lay (interval II and III) was not significantly affected by treatment.

Significant age main effects were found for EW during intervals II (P = 0.0001) and III (P= 0.003) as shown in Table 4. Egg weight increased as birds progressively increased in age from 23 to 44 wk (interval II), and again from 45 to 55 wk (interval III). Significant increases in EW in interval II occurred between wk 25 and 26, 29 and 31, 33 and 34, 36 and 37, 37 and 40, and 39 and 44. Also, significant increases in EW occurred in interval III between wk 49 and 50. During intervals II and III, no significant treatment main effects or age by treatment interactions were found, and in interval I no significant effects of any kind were noted. There were also no significant effects of any kind for relative eggshell conductance or SWUSA in intervals II and III or for egg shape index in interval II. However, there was a significant age main effect (P = 0.04) for egg shape index in interval III. Egg shape index decreased between wk 49 and 53. Egg shape index at wk 49 and 53 was 76.0 and 75.3, respectively (pooled SEM = 0.36). There was no significant treatment main effect or age by treatment interaction for egg shape index in interval III.

There were no significant effects of any kind for yolk lipid concentration in age intervals II and III or for YM content in interval III. However, there was a significant treatment main effect (P = 0.03) for YM in interval II (Table 6). Yolk moisture content was higher in birds inoculated with 6/85MG-10, FMG-22 compared to those inoculated with 6/85MG at 10 wk alone and was also higher in birds inoculated with 6/85MG at 10

wk alone compared to that in control birds. No significant age main effect or age by treatment interactions were found for YM in interval II. Significant treatment main effects were found for yolk palmitic (P = 0.02), oleic (P = 0.03), and linolenic (P = 0.04) concentrations in interval III (Table 7). Palmitic acid concentrations were higher in the 6/85MG-10, FMG-22 inoculation group in comparison to the 6/85MG-10 and 6/85-10, FMG-45 treatment groups, with the control group intermediate. Oleic acid concentrations were lower in 6/85MG-10, FMG-22 and control groups in comparison to the 6/85MG-10 group, with the 6/85MG-10, FMG-45 treatment group intermediate. Linolenic acid concentrations in control birds were higher than those in the 6/85MG-10 birds with the 6/85MG-10, FMG-45 group intermediate. Furthermore, the 6/85MG-10, FMG-45 birds had a higher yolk linolenic acid concentration than did the 6/85MG-10, FMG-22 group, with the 6/85MG-10 group intermediate.

A significant treatment main effect (P = 0.0004) was found for liver moisture in age interval III (Table 8). Liver moisture concentrations were higher in 6/85MG-10 and 6/85MG-10, FMG-45 treatment groups than in Control and 6/85MG-10, FMG-22 groups. There was no treatment main effect for the following parameters in interval III: liver weight as a percentage of BW; liver color and FLHS scores; follicular hierarchy; ovary, total oviduct, infundibulum, magnum, uterus, vagina, and isthmus weights as percentages of BW; infundibulum, magnum, uterus, vagina, and isthmus weights as percentages of total oviduct weight; infundibulum, magnum, uterus, vagina, and isthmus lengths as percentages of total oviduct length; total small intestine, duodenum, ileum, and jejunum weights as percentages of BW; duodenum, jejunum, and ileum weights as percentages of

total small intestine weight; and duodenum, jejunum, and ileum lengths as percentages of total small intestine length.

There was a significant age by treatment interaction (P = 0.03) for PP concentration (Table 9) in age interval II, and there was a significant treatment main effect (P = 0.04) for SCA at week 47 (interval III). Treatment differences in PP concentration were found only at wk 32 in interval II. At wk 32 PP concentrations was significantly lower for birds in the 6/85MG-10 treatment group in comparison to the Control and 6/85MG-10, FMG-22 treatment groups. At week 47, SCA was higher in the 6/85MG-10, FMG-45 group compared to the Control and 6/85MG-10, FMG-22 groups. Mean SCA in the Control, 6/85MG-10, FMG-45 treatment groups were 29.0, 34.5, 29.4, and 36.9%, respectively (pooled SEM = 1.96%). No significant effects of any kind were noted for SCHOL, STRIG, SCA, or HCT in interval II, or for SCHOL, STRIG, PP, or HCT in interval III.

CHAPTER V

DISCUSSION

At the end (58 weeks of age in both trials) of this study, SPA, HI, and FA tests verified systemic infections in 6/85MG and FMG inoculated hens. Conversely, sham-inoculated birds remained free of *M. gallisepticum* throughout the study. *M. gallisepticum* manifestations generally occur in the respiratory system and lesions can become extensive when complicated by other bacteria. Also, environmental factors such as dust and ammonia, in conjunction with intensive rearing or stress, crowding, cold weather, live virus vaccination, or natural virus infection may be important in the incidence and severity of lesions (Jordan, 1972; Springer et al., 1974; Jordan, 1985). However, mycoplasma infection is often subclinical or mild when there are no secondary infections (Kerr and Olson, 1967). Birds in both trials were housed in biological isolation units from 10 weeks of age through the remainder of the study, where natural infections and other environmental stressors common in commercial operations were eliminated. As a result, the 6/85MG and FMG inoculated hens exhibited no outward pathological symptoms.

In a previous study by Burnham et al. (2002a), weekly EP was delayed 1 week in each of 2 experimental trials, whereas total EP was reduced in only 1 of the 2 trials in layers inoculated with the F-strain of MG at 12 weeks of age. The decrease in total EP in that trial became apparent at each week after week 42. Burnham et al. (2002b) later suggested that alterations in the performance and egg characteristics of the layers examined were related to mutual functional disturbances in the liver, ovary, and oviduct, without any noticeable sign of intestinal changes. This suggestion was supported by results showing a reduction in ripe ovarian follicle numbers as well as ovarian follicle size, and increased incidences of FLHS in birds that demonstrated depressed performance subsequent to FMG inoculation at 12 weeks of age. However, previous findings by Evans and Hafez (1992) have indicated that while providing protection against field strain MG (i.e. R-strain) infections, 6/85MG inoculations at 2 weeks of age did not suppress growth in broiler chickens. Furthermore, Branton et al. (2002) showed that 6/85MG had no impact on commercial layer performance or egg quality when inoculated pre-lay (10 weeks of age). These results suggest that using 6/85MG rather than FMG pre-lay may achieve the same level of protection against field strain MG infections, while eliminating the noted negative effects of pre-lay FMG vaccines on EP (weekly and total).

In accordance with the results reported by Branton et al. (2002), inoculation of commercial layers with 6/85MG at 10 weeks had no significant effect on weekly or total EP in the current study. Furthermore, F-strain MG overlays at 22 and 45 week of age on the 10 week 6/85MG inoculation had no significant effect on weekly or total EP. These results confirm the hypothesis that pre-lay 6/85MG inoculations may be a suitable substitute for pre-lay FMG inoculations, and that FMG overlays during lay on pre-lay

6/85MG inoculations may also provide continual protection without eliciting any subsequent suppressive affects on performance.

M. gallisepticum has shown the ability to invade cells (Winner et al., 2000), thereby suggesting that MG may be capable of interfering with liver lipid metabolism. Furthermore, MG is capable of passing through the mucosal barrier to cause systemic infections, and it is possible to culture MG from the avian liver (Winner et al., 2000; Sahu et al., 1976). Colonization of hepatic tissue by MG would be expected to alter liver lipid metabolism with associated effects on liver weight, histology, and lipid and moisture content. Further, reverse trends in liver moisture and lipid concentrations would be expected. In this study, however, liver moisture concentration was significantly higher in the 6/85MG-10 and 6/85MG-10, FMG-45 treatment groups in comparison to 6/85MG-10, FMG-22 and control treatment groups, while liver lipid content, histology, FLHS incidence, and relative weight were not significantly affected by treatment. Apparently, a significant alteration in the moisture content of the liver occurred without an opposite significant effect on lipid content, and without subsequent affects on liver weight and histology. The reason for this singular effect is not clear. Nevertheless, it is important to note that the treatment effect on liver moisture had no impact on layer performance.

In a companion report by Burnham et al. (2003b), in which egg yolk composition was altered by 12 week FMG inoculations, it was also concluded that the alterations in EP were associated with colonization of the liver by FMG with subsequent functional disturbances in the metabolism of liver lipids and the deposition of circulating lipids in the ovarian follicles. Furthermore, Burnham et al. (2003b) more specifically reported

that yolk total lipid and cholesterol were decreased, and that linoleic, stearic, and arachidonic acid concentrations were increased, and that yolk myristic, palmitoleic, and oleic acid concentrations were decreased by 12 week FMG inoculations.

Similar to the results of Burnham et al. (2003b), treatment effects on yolk fatty acid profile were found in this study. However, the specific changes in the fatty acid profile of the yolk were different in response to treatment. The yolk concentrations of palmitic, oleic, and linolenic acids were affected by the 10 week 6/85MG inoculation and the FMG overlays in this study. Taken together, these results suggest that the inoculation regimen used in this study may have affected fatty acid elongation and desaturation processes in the hens' liver endoplasmic reticula. Included in fatty acid synthesis, palmitic acid is elongated and desaturated to form oleic acid. Oleic acid then undergoes further desaturation to form linolenic acid (Lehninger, 1975). Palmitic acid concentrations were significantly higher in 6/85MG-10, FMG-22 birds compared to 6/85MG-10 and 6/85MG-10, FMG-45 birds, with Control birds intermediate. Oleic acid concentrations were significantly lower for the 6/85MG-10, FMG-22 and Control birds when compared to the 6/85MG-10 birds, with the 6/85MG-10, FMG-45 birds intermediate. Linolenic acid concentrations were higher in Control birds when compared to 6/85MG-10 and6/85MG-10, FMG-22 treatment groups, with the 6/85MG-10, FMG-45 group intermediate. In general, the 6/85MG-10 inoculation may have blocked or interfered with the desaturation of oleic to linolenic acid. A depression in the desaturation process without a concomitant increase in oleic acid may have likewise occurred when the 6/85MG-10 inoculation was given in conjunction with the FMG overlay at 22 weeks. Also, the FMG overlay at 22 weeks in birds given a pre-lay

6/85MG inoculation at 10 weeks may have interfered with the elongation of palmitic to oleic acid, leading to an associated increase of palmitic acid in the 6/85MG-10, FMG-22 treated birds. However, as in liver moisture, these fatty acid differences had no affect or relation to performance.

Burnham et al. (2003a) concluded that decreased concentrations of lipids (STRIG) in the blood during lay may be directly responsible for reductions in YL, yolk cholesterol, and changes in yolk fatty acid profile in birds having been inoculated with FMG at 12 weeks of age. Conversely, there were no significant changes in any of the blood lipids examined in this study due to treatment with 6/85MG at 10 weeks alone or in combination with FMG overlays at 22 or 45 weeks of age. Therefore, the treatment effects observed on the yolk lipids examined in this study were limited to yolk fatty acid profile, and changes in the yolk fatty acid profile occurred without any blood lipid changes. The bases for the differences in the relationship between the lipids in the blood and yolk in response to the inoculation regimes used in the reports by Burnham et al. (2003a,b) and in this study are not evident, but these results do indicate that the strain of MG and the timing of inoculation can lead to changes in yolk lipid characteristics without related changes in blood lipids and without having an effect on EP.

Unlike the results of Burnham et al. (2003b), who found no affect of a 12 week FMG inoculation on yolk moisture content, in this study, yolk moisture was significantly different between 6/85MG-10, FMG-22 and the Control birds, with 6/85MG-10 intermediate in Interval II. Furthermore, PP was found to be reduced in age interval II (32 weeks of age) in birds given a 6/85MG-10 inoculation, and was subsequently increased to control values when the 6/85MG-10 inoculation was given in conjunction

with a 22 week FMG inoculation overlay. Burnham et al. (2003a) suggested that post-peak decreases in PP after a 12 week FMG inoculation may be associated with ovarian follicular regression, reproductive tissue atrophy, and the onset of FLHS incidence. However, no association between PP and incidences of follicular regression, FLHS, or reproductive tissue regression were noted in this study. Warriss et al. (1003) has also suggested that significant increases in PP and numerical increases in blood osmolaity were indicative of dehydration in broilers during prolonged transport at slaughter age. It is, therefore, suggested that associated changes in PP, yolk moisture, and liver moisture reflect changes in the hydration statuses of the birds exposed to the inoculation regimens in this study. These data also indicate that the inoculation treatments used influenced the hydration status of the layers without impacting overall performance. It is noteworthy that the 6/85MG-10 treatment alone led to concomitant increases in blood dilution (decreased PP) and liver moisture content. This would suggest that the pre-lay inoculation of 6/85MG may provide a hydration affect, which was manifested through increased moisture levels in both the liver and plasma of the birds. Nevertheless, changes in the moisture content of the yolk with treatment were not associated with those in the liver and plasma, and the moisture content changes in the plasma and liver had no impact on performance.

In conclusion, despite possible 6/85MG and FMG inoculation treatment and timing effects on the liver, yolk, and PP of birds housed in environmentally controlled conditions, these changes exerted no overall impact on layer performance. These results establish that pre-lay 6/85MG inoculations may be a suitable substitute for pre-lay FMG inoculations, and that FMG overlays during lay on pre-lay 6/85MG inoculations may also

provide continual protection without eliciting any subsequent suppressive affects on performance. Because these birds were housed in isolation units, these results do not preclude the possibility that different or greater performance and physiological effects may occur in birds infected pre-lay, at lay onset, or late in lay with 6/85MG or FMG alone or in combination, when housed in facilities where there are increased levels of environmental stress.

CHAPTER VI

SUMMARY

The primary goal of the present study was to investigate the effects of a 6/85-strain M. *gallisepticum* inoculation alone, and in conjunction with F-strain *M. gallisepticum* overlays at 22 or 45 weeks of age during lay. F-strain MG has previously been shown to negatively affect laying hens' performances when given pre-lay or during post-peak lay, whereas 6/85MG administered pre-lay has previously been shown to not exert these negative effects.

In this study, yolk moisture and fatty acids, liver moisture, and PP were affected by the inoculation regimes used. The PP and liver moisture changes may be indicative of the hydration statuses of the birds during lay. Alterations in yolk palmitic, oleic, and linolenic acid levels with treatment may have manifested disturbances in the desaturation and elongation processes of fatty acid synthesis; however, as for liver and yolk moisture concentration changes, these also did not effect EP at a significant level.

These results confirm that pre-lay 6/85G inoculations may be a suitable substitute for pre-lay FMG inoculations, and that FMG overlays during lay on pre-lay 6/85MG inoculations may also provide continual protection without eliciting any subsequent

suppressive affects on performance. The data from this study indicates that using 6/85MG in conjunction with FMG does not negatively affect laying hen performance. Furthermore, this inoculation combination offers benefits above the use of those achieved through either inoculation alone. Also, because FMG is a more virulent strain, FMG may offer stronger protection during lay than 6/85MG. These results would be of particular interest in an industry setting in which flocks previously inoculated with 6/85MG are acquired by a company comfortable with a traditionally used FMG inoculation regimen.

TABLE 1. Titers used in 6/85MG @ 10 wk, FMG @ 22 wk, and FMG @ 43 wk of age inocula in Trials 1 and 2.

	Trial 1	Trial 2
Inoculum	----------(cfu/mL)----------	
6/85MG @10 wk	1.0×10^{7}	1.0×10^{6}
FMG @ 22 wk	1.3×10^{10}	1.0×10^{10}
FMG @ 45 wk	7.5×10^{11}	7.0×10^{11}

TABLE 2. Body weight in age intervals I (20 wk), II (24, 32, and 43 wk), and III (47 wk)[1]

Age Interval				
I[2]	II[3]			III[4]
20	24	32	43	47
----------(kg)----------				
1.27	1.39	1.41	1.44	1.47

[1]n = 16 replicate units for calculation of mean at each age period within each hen age interval.
[2]n = SEM based on pooled estimate of variance = 0.043.
[3]n = SEM based on pooled estimate of variance = 0.027.
[4]n = SEM based on pooled estimate of variance = 0.017.

TABLE 3. Weekly egg production (EP; % hen day) in hen age intervals II (23-44 wk) and III (45-54 wk)[1]

Age Interval			
II[2]		III[3]	
Wk of Age	EP	Wk of Age	EP
----------(%)----------		----------(%)----------	
22	42.3[d]	45	81.1[a]
23	68.1[c]	46	77.1[a]
24	79.6[abc]	47	75.6[b]
25	83.8[ab]	48	72.7[b]
26	85.4[a]	49	78.2[a]
27	85.0[a]	50	75.8[b]
28	82.9[ab]	51	70.4[b]
29	83.3[ab]	52	73.3[b]
30	82.5[ab]	53	72.1[b]
31	77.8[abc]	54	62.4[c]
32	82.1[ab]	-	-
33	84.7[ab]	-	-
34	79.7[abc]	-	-
35	74.1[abc]	-	-
36	81.4[ab]	-	-
37	73.0[abc]	-	-
38	81.5[ab]	-	-
39	75.8[abc]	-	-
40	74.9[abc]	-	-
41	75.6[abc]	-	-
42	75.7[abc]	-	-
43	71.9[bc]	-	-
44	74.2[abc]	-	-

[a-c] = Means among weeks within age interval with no common superscript differ significantly ($P \leq 0.05$).

[1]n = 16 replicate units for calculation of means at each age period within each hen age group.

[2]n = SEM based on pooled estimate of variance = 5.27.

[3]n = SEM based on pooled estimate of variance = 3.76.

TABLE 4. Weekly egg weight (EW) in hen age intervals II (23-44 wk) and III (45-55 wk)[1]

Age Interval			
II[2]		IIII[3]	
Week of Age	EW	Week of Age	EW
----------(g)----------		----------(g)----------	
23	47.2^{h}	45	58.5^{b}
24	48.5^{h}	46	59.0^{b}
25	49.8^{h}	47	59.0^{b}
26	50.9^{g}	48	59.2^{b}
27	51.8^{g}	49	59.6^{b}
28	52.4^{g}	50	60.0^{a}
29	53.2^{g}	51	60.0^{a}
30	53.6^{fg}	52	60.6^{a}
31	54.1^{f}	53	60.1^{a}
32	54.5^{f}	54	60.0^{a}
33	55.1^{f}	55	60.6^{a}
34	55.6^{e}	-	-
35	55.9^{e}	-	-
36	55.1^{e}	-	-
37	57.0^{d}	-	-
38	57.2^{cd}	-	-
39	57.7^{bcd}	-	-
40	57.9^{abc}	-	-
41	57.8^{abc}	-	-
42	57.9^{abc}	-	-
43	58.1^{ab}	-	-
44	58.5^{a}	-	-

$^{a-h}$ = Means among weeks with no common superscript differ significantly ($P \leq 0.05$).
[1]n = 16 replicate units for calculation of means at each age period within each hen age group.
[2]n = SEM based on pooled estimate of variance = 1.06.
[3]n = SEM based on pooled estimate of variance = 0.75.

TABLE 5. Percentage yolk weight (% of egg weight) in age interval II (24, 32, and 43 wk)[1]

Week of Age			
24	32	43	Pooled SEM
----------(%)----------			
24.3[c]	26.1[b]	27.8[a]	0.51

[a-c] = Means among weeks with no common superscript differ significantly ($P \leq 0.05$).
[1]n = 16 replicate units used for calculation of means within each column (wk).

TABLE 6. Egg yolk moisture concentration in control, 6/85MG at 10 wk (6/85MG-10), and 6/85MG at 10 wk and FMG at 22 wk (6/85MG-10, FMG-22) treatment groups in age interval II (across 24, 32, and 43 wk)[1]

Control	6/85MG-10	6/85MG-10, FMG-22	Pooled SEM
----------(%)----------			
50.4[b]	51.2[ab]	51.7[a]	0.28

[a,b] = Means among treatments with no common superscript differ significantly ($P \leq 0.05$).
[1]n = 12 replicate units used for calculation of means within each column (treatment).

TABLE 7. Yolk Palmitic, oleic, and linolenic acid concentrations, (% of total yolk fatty acids) in control, 6/85MG at 10 wk (6/85MG-10), 6/85MG at 10 wk and FMG at 22 wk(6/85MG-10, FMG-22), and 6/85MG at 10 wk and FMG at 45 wk(6/85MG-10, FMG-45) treatment groups at age interval II (58 wk of age)[1]

Treatment	Palmitic acid	Oleic acid	Linolenic acid
	----------(%)----------		
Control	31.7[ab]	31.2[b]	15.0[a]
6/85MG-10	31.3[b]	32.2[a]	11.3[bc]
6/85MG-10, FMG-22	32.1[a]	31.0[b]	9.7[c]
6/85MG-10, FMG-45	31.4[b]	31.6[ab]	13.8[ab]
SEM	0.28	0.43	0.54

[a-c] = Means among column with no common superscript differ significantly ($P \leq 0.05$).
[1]n = 4 replicate units used for calculation of means within each treatment group.

TABLE 8. Hen liver moisture concentration in control, 6/85MG at 10 wk (6/85MG-10), 6/85MG at 10 wk and FMG at 22 wk(6/85MG-10, FMG-22), and 6/85MG at 10 wk and FMG at 45 wk(6/85MG-10, FMG-45) treatment groups in age Interval III (58 wk of age)[1]

Control	6/85MG-10	6/85MG-10, FMG-22	6/85MG-10, FMG-45	Pooled SEM
----------(%)----------				
42.5[b]	47.1[a]	40.6[b]	46.7[a]	10.2

[a,b] = Means among column with no common superscript differ significantly ($P \leq 0.05$).
[1]n = 4 replicate units used for calculation of means within each column (treatment).

TABLE 9. Plasma total protein concentration in control, 6/85MG at 10 wk (6/85MG-10), and 6/85MG at 10 wk and FMG at 22 wk (6/85MG-10, FMG-22) treatment groups in age interval II(24, 32, and 43 wk)[1]

	Week of Age		
Treatment	24	32	43
	----------(g/dL)----------		
Control	3.87	4.10[a]	4.89
6/85MG-10	4.01	3.85[b]	4.56
6/85MG-10, FMG-22	4.10	4.43[a]	4.44

[a,b] = Means within weeks with no common superscript differ significantly ($P \leq 0.05$).
[1]n = 4 replicate units used for calculation of treatment means within each column (wk).
[2]n = SEM based on pooled estimate of variance = 0.344.

REFERENCES

Abd el Motelib, T.Y and S.H Kleven. 1993. A comparative study of *Mycoplasma gallisepticum* vaccines in young chickens. Avian Dis. 37:981-987.

Aitken, R. N. C. 1971. Physiology and Biochemistry of the Domestic Fowl. Vol 3. Bell, J. and Freeman, B. M. ed. London and New York: Academic Press, Chapter 53.

Athamna, A., R. Rosengarten, S. Levinsohn, I. Kahane, and D. Yogev. 1997. Adherence of *Mycoplasma gallisepticum* involves variable surface membrane proteins. Infect. Immun. 65(6):2468-2471.

Arjamaa, O., and A Talo. 1983. The membrane potential of the Japanese quail's oviductal smooth muscle during ovum transport. Acta Physiol. Scand., 118:349.

Baas, E. J., and D. E. Jasper. 1972. Agar block technique for identification of mycoplasmas by use of fluorescent antibody. Appl. Microbiol. 23:1097-1100.

Bacon, W. L., K. I. Brown, and M. A. Musser. 1980. Changes in the plasma calcium, phosphorus, lipids, and estrogens in turkey hens with reproductive status. Poult. Sci. 59:444.

Bensadoun, A., and A. Rothfeld. 1972. The form of absorption of lipids in the chicken, *Gallus domesticus*. Proc. Soc. Exp. Biol. Med. 141(3):814-817.

Bligh, E. G., and W. J. Dryer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911-917.

Bradbury, J. M. 2005. Poultry mycoplasmas: sophisticated pathogens in simple guise. Br. Poult. Sci. 46(2):125-136.

Brake, J., J. F. Ort, T. A. Carter, and W. R. Campbell. 1984. Effect of insect growth regulator CGA-72662 (Larvadex®) on broiler breeder production, hatchability, and subsequent chick performance. Poult. Sci. 63:910-916.

Branton, S. L., S. M. D. Bearson, B. Bearson, B. D. Lott, W. R. Maslin, S. D. Collier, G. T. Pharr, and D. L. Boykin. 2002. The effects of 6/85 live *Mycoplasma gallisepticum* vaccine in commercial layer hens over a 43-week laying cycle on egg production, selected egg quality parameters, and egg size distribution when challenged before beginning of lay. Avian Dis. 46(2):423-428.

Branton, S. L., H. Gerlach, and S. H. Kleven. 1984. *Mycoplasma gallisepticum* isolation in layers. Poult. Sci. 63:1917-1919.

Branton, S. L., B. D. Lott, J. D. May, W. R. Maslin, G. T. Pharr, J.E. Bearson, S. D. Collier, D. L. Boykin, 2000. The effects of ts-11 strain *Mycoplasma gallisepticum* vaccination in commercial layers on egg production and selected egg quality parameters. Avian Dis. 44:618-623.

Branton, S. L., and J. D. Simmons, 1992. Design of a poultry disease isolation facility with programmable environmental control. Appl. Eng. Agric. 8:695-699.

Burnham, M. R., S. L. Branton, E. D. Peebles, M. S. Jones, P.D. Gerard, and W. R. Maslin, 2002b. Effects of F-strain *Mycoplasma gallisepticum* inoculation at twelve weeks of age on digestive and reproductive organ characteristics of commercial egg laying hens. Poultry Sci. 81:1884-1891.

Burnham, M. R., S. L. Branton, E. D. Peebles, B. D. Lott, and P. D. Gerard. 2002a. Effects of F-strain *Mycoplasma gallisepticum* inoculation at twelve weeks of age on performance and egg characteristics of commercial egg laying hens. Poult. Sci. 81:1478-1485.

Burnham, M. R., E. D. Peebles, S. L. Branton, and S. K. Whitmarsh. 2003a. Effects of F-strain *Mycoplasma gallisepticum* inoculation at twelve weeks of age on the blood characteristics of commercial egg laying hens. Poult. Sci. 82:1397-1402.

Burnham, M. R., E. D. Peebles, S. L. Branton, and S. K. Whitmarsh. 2003b. Effects of F-strain *Mycoplasma gallisepticum* inoculation at twelve weeks of age on the egg yolk composition in commercial egg laying hens. Poult. Sci. 82(4):577-584.

Butcher, F. G., R. D. Miles, and J. P. Jacobs. 1998. MG in commercial layers still a worldwide problem. World Poult. (special issue). Page 27.

Carpenter, T.E., E.T. Mallinson, K.F. Miller, R.F. Gentry and L.D. Schwartz. 1981. Vaccination with F-strain *Mycoplasma gallisepticum* to reduce production losses in layer chickens. Avian Dis. 25:404-9.

Carpenter, T. E., K. F. Miller, R. F. Gentry, L. D. Schwartz, and E. T. Mallinson. 1979. Control of *Mycoplasma gallisepticum* in commercial laying chickens using

artificial exposure to Connecticut F-strain *Mycoplasma gallisepticum*. Proc. M. S. Anim. Health Assoc. 83:364-370.

Chandiramani, N.K., H. Van Roekel, and O. M. Olesiuk. 1996. Viability studies with *Mycoplasma gallisepticum* under different environmental conditions. Poult. Sci. 45(5):1029-1044.

Charleston, V., J. J. Gate, I. A. Aitken, and J. L. Reeve. 1998. Assessment of the efficacy of Tilmicosin as a treatment for *Mycoplasma gallisepticum* infections in chickens. Avian Pathol. 27(2):190-195.

Chute, H. L., and C. R. Cole. 1975. Lesions in chicken embryos produced by pleuropneumonia-like organisms from chronic respiratory disease in chickens and infectious sinusitis of turkeys. A. J. Vet. Res. 15:108-118.

Czifra, F., S. H. Kleven, B. Engstrom, and B. L. Stipkovits. 1995. Detection of specific antibodies directed against a consistently expressed surface antigen of *Mycoplasma gallisepticum* using a monoclonal blocking enzyme-linked immunosorbent assay. Avian Dis. 39(1):28-31.

Dingfelder, R. S., D. H. Ley, J. M. McLaren, and C. Brownie. 1991. Experimental infection of turkeys with *Mycoplasma gallisepticum* of low virulence, transmissibility, and immunogenicity. Avian Dis. 35:910-919.

Dockray, G. J. 1975. Comparison of the actions of porcine secretion and extracts of chicken duodenum on pancreatic exocrine secretion in the cat and turkey. J. Physiol. 244:625-637.

Domermuth, C. J., W. B. Gross, and R. T. Dubose. 1967. Mycoplasmal salpingitis of chickens and turkeys. Avian Dis. 11:393-398.

Eastin, W. C. and E. Spaziani. 1978. Control of calcium secretion in the avian shell gland (uterus). Biol. Reprod. 19:493.

Etches, R. J. and K. W. Cheng. 1981. Changes in the plasma concentrations of lutenizing hormone, progesterone, oestradiol and testosterone and in the binding of follicle-stimulating hormone to the theca of follicles during the ovulation cycle of the hen (*Gallus domesticus*). J. Endocrinol. 91:11.

Evans, R.D., Y.S. Hafez, and C.S. Schreurs. 1992. Demonstration of the genetic stability of a *Mycoplasma gallisepticum* strain following in vivo passage. Avian Dis. 36:554-60.

Evans, R.D. and Y.S. Hafez. 1992. Evaluation of a *Mycoplasma gallisepticum* strain exhibiting reduced virulence for prevention and control of poultry mycoplasmosis. Avian Dis. 36:197-201.

Frazer, R., V. R. Heslop, F. E. Murray, and W. A. Day, 1986. Ultrastructural studies of the portal transport of fat in chicken. Br. Exp. Pathol. 67(6):783-791.

Frey, M. C., R. P Hanson, and D. P Anderson, 1968. A medium for the isolation of avian Mycoplasma. Am. J. Vet. Res. 29:2164-2171.

Garcia, M., M. G. Elfaki, and S. H. Kleven. 1994. Analysis of the variability in expression of *Mycoplasma gallisepticum* surface antigens. Vet. Microbiol. 42:147-158.

Gilbert, A. B. 1979. Female genital organs in: Form and Function in Birds. Vol. 1 (A. S. King and J. McLelland, ed.) London and New York Academic Press, Ch. 5.

Glisson, J. R., S. H. Kleven. 1984. *Mycoplasma gallisepticum* vaccination: Effects on egg transmission and egg production. Avian Dis. 28(2):406-415.

Guyer, R. V., A. A. Grunder, E. G. Buss, and C. O. Clagett. 1980. Calcium-binding proteins in serum of chickens: Vitellogenin and albumin. Poult. Sci. 59:874.

Guyton, A. C., and J. E. Hall. 1996. The adrenocortical hormones in: Textbook of Medical Physiology. 9th ed. Pages 957-969. W. B. Saunders Co. Philadelphia, PA.

Hildebrand, D. G., D. E. Page, and J. R. Berg. 1983. *Mycoplasma gallisepticum* (MG)-Laboratory and Field Studies Evaluating the Safety and Efficacy of an Inactivated MG Bacterin. Avian Dis. 27(3):792-802.

Johnson, A. L., and A. van Tienhoven. 1980. Plasma concentrations of six steroids and LH during the ovulatory cycle of the hen, *Gallus domesticus*. Biol. Reprod. 23:386.

Johnson, A. L. 1986. Reproduction in the Female in: Avian Physiology. 4th ed. Pages 403-425. Springer-Verlag. New York, New York.

Jordan, F. T. 1972. The epidemiology of disease of multiple aetiology: The avian respiratory disease complex. Vet. Rec. 90:556-562.

Jordan, F. T. 1985. Gordon memorial lecture: People, poultry and pathogenic mycoplasmas. Br. Poult. Sci. 26:1-15.

Jordan, F. T., C. A. Forrester, P. H. Ripley, and D. G. S. Burch. 1998. *In vitro* and *in vivo* comparisons of valnemulin, tiamulin, tylosin, enrofloxacin, and lincomycin/spectinomycin against Mycoplasma gallisepticum. Avian Dis. 42(4):738-745.

Kempf, I., F. Gesbert, and M. Guittet. 1997. Experimental infection of chickens with an atypical Mycoplasma *gallisepticum* strain: comparison of diagnostic methods. Res. Vet. Sci. 63(3):211-213.

Kerr, K. M., and N. O. Olsen. 1967. Pathology in chickens experimentally inoculated or contract-infected with *Mycoplasma gallisepticum*. Avian Dis. 11:559-578.

Kinsky, F. C. 1971. The consistent presence of paired ovaries in the Kiwi (*Aptryx*) with some discussion of this condition in other birds. J. Ornithol. 112:334.

Kleven, S. H. 1985. Transmissability of the F strain of *Mycoplasma gallisepticum* in leghorn chickens. Avian Dis. 25:1005-18.

Kleven S. H. 1997. Mycoplasmosis. 10th Ed. Pages191-193 in: Diseases of Poultry. B. W. Calneck ed. Iowa State University Press. Ames, Iowa.

Kleven S. H. 1998. Mycoplasmas in the etiology of multifactorial respiratory disease. Poult. Sci. 77:1146-1148.

Kleven, S. H. 2003. Mycoplasmosis. 11th Ed. Pages 719-735 in : Diseases of Poultry. Y. M. Saif ed. Iowa State University Press. Ames, Iowa.

Kleven, S.H., H.H. Fan, and K.S. Turner. 1998. Pen trial studies on the use of live vaccines to displace virulent *Mycoplasma gallisepticum* in chickens. Avian Dis. 42:300-6.

Kleven, S.H.., M.I. Khan, and R. Yamamoto. 1990. Fingerprinting of *Mycoplasma gallisepticum* strains isolated from multiple-age layers vaccinated with live F-strain. Avian Dis. 34:984-90.

Kleven, S. H., C. J. Morrow, and K. G. Whithear. 1988. Comparison of *Mycoplasma gallisepticum* strains by hemagglutination-inhibition and restriction endonuclease analysis. Avian Dis. 32:731-741.

Lague, P. CA. van Tienhoven, and F. J. Cunningham. 1975. Concentrations of estrogens, progesterone, and LH during the ovulatory cycle of the laying chicken (*Gallus domesticus*). Biol. Reprod., 12:590.

Latour, M. A., E. D. Peebles, S. M Doyle, T. M. Smith, and C. R. Boyle, 1998. Broiler breeder age and dietary fat influence the yolk fatty acid profiles of fresh eggs and newly hatched chicks. Poult. Sci. 77:47-53.

Lehninger, A. C. 1975. The Biosynthesis of Lipids. 2nd edition. Pages 659-691 in: Biochemistry. Worth Publishers, Inc. New York, New York.

Levisohn, S. and M.J. Dykstra. 1987. A quantitative study of single and mixed infection of the chicken trachea by *Mycoplasma gallisepticum*. Avian Dis. 31:1-12.

Levinsohn, S., J. R. Glisson, and S. H. Kleven, 1985. In ovo pathogenicity of *Mycoplasma gallisepticum* strains in the presence and absence of maternal antibody. Avian Dis. 29(1):188-197.

Levisohn, S. and S.H. Kleven. 2000. Avian Mycoplasmosis (*Mycoplasma gallisepticum*). Rev. Sci. Tech. 19:425-42.

Ley D.H., J.M. McLaren, A.M. Miles, H.J. Barnes, S.H. Miller, and G. Franz. 1997. Transmissibility of live *Mycoplasma gallisepticum* vaccine strains ts-11 and 6/85 from vaccinated layer pullets to sentinel poultry. Avian Dis. 41: 187-94.

Lin, M.Y., and S.H. Kleven. 1982. Egg transmission of the strains of *Mycoplasma gallisepticum* in chickens. Avian Dis. 26:487-95.

Luck, M. R., and C. G. Scanes. 1979. Plasma levels of ionized calcium in the laying hen (*Gallus domesticus*). Comp. Biochem. Physiol. A. 63:177.

Maniloff, J., and D. C. Quinlan. 1973. Biosynthesis and subcellular organization of nucleic acids in *Mycoplasma gallisepticum* strain A5969. Ann. NY Acad. Sci. 225:181-189.

Marois, C., F. Dufour-Gesbert, and I. Kempf. 2001. Molecular Differentiation of *Mycoplasma gallisepticum* and *Mycoplasma imitans* strains by pulsed-fields gel electrophoresis and random amplified polymorphic DNA. J. Vet. Med. B. 48:693-703.

May, J. D., S. L. Branton. 1997. Identification of mycoplasma isolates by ELISA. Avian Dis. 41(1):93-96.

McMartin, D. A. 1967. *Mycoplasma gallisepticum* in the respiratory tract of the fowl. Acta. Cytsol. 11(4):317-320.

Mohammed, H.O., T.E. Carpenter, and R. Yamamoto. 1987. Economic impact of MG and *M. synoviae* in commercial layer flocks. Avian Dis. 31:477-82.

Mohammed, H.O., T.E. Carpenter, and R. Yamamoto. 1987. Evaluation of factors associated with infection of commercial layers with *Mycoplasma gallisepticum* and *M. Synoviae*. Avian Dis. 31:470-476.

Mongin, P. and B. Sauveur. 1974. Voluntary food and calcium intake by the laying hen. Br. Poult. Sci. 15:349.

Morris, T. R. 1968. Light requirements of the fowl. Page 15 in: Environmental Control in Poultry Production. T. C. Carter ed. Endinburgh: Oliver and Boyd.

National Research Council. 1994. Nutrient Requirements of Poultry. 9th Rev. ed. National Academy Press, Washington, DC.

Ortiz, A., R. Froyman, and S. H. Kleven, 1995. Evaluation of enrofloxacin against egg transmission of *Mycoplasma gallisepticum*. Avian Dis. 39(4):830-836.

Ose, E., R. H. Wellenreiter, and L. V. Tonkinson. 1979. Effects of feeding tylosin to layers exposed to *Mycoplasma gallisepticum*. Poult. Sci. 58:42-49.

Parkhurst, C. R. and G. J. Mountney. 1987. Poultry Nutrition. Poult M. E. Prod. 110-125. Chapman and Hall, New York, NY.

Parsons, A. H. 1982. Structure of the eggshell. Poult. Sci. 61(10):2013-2021.

Patterson, P.H. 1994. Coping with Mycoplasma gallisepticum. Internews. 7(2).

Peebles, E. D., Lumu Li, S. Miller, T. Pansky, S. Whitmarsh, M. A. Latour, and P. D. Gerard. 1999. Embryo and yolk compositional relationships in broiler hatching eggs during incubation. Poult. Sci. 78:1435-1442.

Rodriguez, R. and S. H. Kleven. 1980. Pathogenicity of two strains of *Mycoplasma gallisepticum* in broilers. Avian Dis. 24(4):800-806.

Romanoff, A. L. and A. J. Romanoff. 1949. The Avian Egg. Wiley and Sons Printing. New York, New York.

Sahu, S. P., and N. O. Olsen. 1976. Use of the agar-gel precipitin test to evaluate broiler breeder and commercial layer flocks for *Mycoplasma gallisepticum* infection. Avian Dis. 20:563-573.

Scanes, C. G., P. M. M. Godden, and P. J. Sharp. 1977. An homologous radioimmunoassay for chicken follicle-stimulating hormone: observations on the ovulatory cycle. J. Endocrinol. 73:473.

Shahabi, N. A, J. M. Bahr, and A. V. Nalvandov. 1975. Effect of LH injection on plasma and follicular steroids in the chicken. Endocrinol. 96:969.

Shah-Majid, M. 1986. An attempt to isolate avian mycoplasmas by inoculating directly on to mycoplasma agar plates. Vet. Rec. 118:177-178.

Shah-Majid, M. and M. Ninayah. 1987. Choanal cleft as alternative site for the isolation of avian mycoplasmas. Vet. Rec. 121:153.

Shizuo, S., Sgigemi, S., Hiroyuki, K. 1972. Effect of ammonia on *Mycoplasma* gall*isepticum* infection in chickens. Nat. Inst. Anim. Hlth. Quart. 13:45-53.

Springer, E. T., C. Luskus, and S. S. Pourciau. 1974. Infectious bronchitis and mixed infections of *Mycoplasma synoviae* and *Escherichia coli* in gnotobiotic chickens. Synergistic role in the airsacculitis syndrome. Infect. Immun. 10:578-589.

Steel, R. G. D. and J. H. Torrie. 1980. Principles and Procedures of Statistics. A Biometrical Approach. 2nd Ed. McGraw-Hill, New York, NY.

Stemberger, B. H., W. J. Mueller, and R. M. Leach. 1977. Microscopic study of the initial stages of eggshell calcification. Poult. Sci. 56:537.

Sudhir, K. R., P. Singh, A. Silim, J. A. Newman. 1998. Lymphoproliferative responses of specific-pathogen free chickens to *Mycoplasma gallisepticum* strain PG31. Avian Pathol. 27:277-283.

Taylor, T. G., T. R. Morris, and F. Hertelendy. 1962. The effect of pituitary hormones on ovulation in calcium deficient pullets. Vet. Rec. 74:123.

Timms, L. M., R. N. Marshall, and M. F. Breslin. 1989. Evaluation of the efficacy of chlortetracycline for the control of chronic respiratory disease caused by Escherichia coli and *Mycoplasma gallisepticum*. Res. Vet. Sci. 47:377-382.

Tuohimaa, P. 1975. Immunofluorescence demonstration of avidin in the immature chick oviduct epithelium after progesterone. Histochem. 44:95.

USDA-APHIS-VS. 2003. National poultry improvement plan and auxiliary provisions. Fed. Regist. 68:28169-28175

Warren, D. C. and H. M. Scott. 1935. The time factor in egg formation. Poult. Sci. 14:195.

Warren, D. C. and R. M. Conrad. 1942. Time of pigment deposition in brown-shelled hen eggs and in turkey eggs. Poult. Sci. 21:515.

Wilson, W. O. and A. E. Woodward. 1958. Egg production of chickens kept in darkness. Poult. Sci. 37:1054.

Winner, F., R Rosengarten, and C. Citti. 2000. In vitro cell invasion of *Mycoplasma gallisepticum*. Infect. Immun. 68:4238-4244.

Wyburn, G. M., H. S. Johnston, M. H. Draper, and M. F. Davidson. 1973. The ultrastructure of the shell-forming region of the oviduct and the development of the shell of *Gallus domesticus*. J. Exp. Physiol. 58:143.

Yagihashi, T., T. Nunoya, and M. Tajima. 1988. Pathogenicity for chickens of six strains of *Mycoplasma gallisepticum* isolated from various birds. Avian Pathol. 17:725-729.

Yoder, H. W. 1975. Mycoplasmosis: Isolation and identification of avian pathogens. Pages 109-116 in: American Assoc. of Avian Path. College Station, TX.

Yoder, H. W. Jr. 1989. Nonspecific reactions to Mycoplasma serum plated antigens induced by inactivated poultry disease vaccines. Avian Dis. 33:60-68.

Yoder, H. W. Jr. and M. S. Hofstad. 1964. Characterization of avian Mycoplasma. Avian Dis. 8:481-512.

Yoder, H. W. Jr. and S. R. Hopkins. 1985. Efficacy of experimental inactivated *Mycoplasma gallisepticum* oil-emulsion bacterin in egg layer chickens. Avian Dis. 29:332-334.

Zain, Z. M., J. M. Bradbury. 1995. The influence of type of swab and laboratory method on the recovery of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in broth medium. Avian Pathol. 24:707-716.

Zander, D. V. 1984. Principles of disease prevention. Pages 1-37 in: Diseases of Poultry. 8th ed. M. S. Hofstad, H. J. Barnes, B. W. Calnek, W. M. Reid, and H. W. Yoder, Jr., ed. Iowa State University Press, Ames, IA.