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Physiological Factors Associated With The Alteration Of Reproductive Performance Of Commercial Egg Laying Chickens Infected With F-Strain Mycoplasma Gallisepticum

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PHYSIOLOGICAL FACTORS ASSOCIATED WITH THE ALTERATION OF REPRODUCTIVE PERFORMANCE OF COMMERCIAL EGG LAYING CHICKENS INFECTED WITH F-STRAIN *MYCOPLASMA GALLISEPTICUM*

By

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The F-strain of *Mycoplasma gallisepticum* (FMG) is commonly used in vaccination programs to displace infections by more virulent natural or wild type *Mycoplasma gallisepticum* strains. However, a better understanding of the mechanisms responsible for altered egg production (EP) and egg quality in commercial layers infected with FMG is important, as these alterations can cause economic loss to the United States layer industry. This study was designed to examine potential mechanism(s) responsible for alterations in EP and egg quality by FMG-inoculation.

The effects of FMG on production parameters and physiological characteristics of commercial laying hens were evaluated. In isolation units, 12 wk FMG inoculation delayed onset of lay approximately one wk, decreased overall EP, and decreased EP 34 wk post-inoculation. A 12 wk FMG inoculation also resulted in a higher incidence of fatty liver hemorrhagic syndrome, ovarian follicular regression, and decreased isthmal

and vaginal proportions of the reproductive tract. Ovarian regression may be related to retarded production (liver), transport (blood), and/or uptake (ovary) of yolk particles. Changes in blood characteristics (i.e. lipid composition) with FMG colonization of the liver may become manifest through changes in egg constituents. As evidenced through changes in the relative weights of various reproductive organs, colonization of these organs by FMG, in addition to the liver, may also be a cause of the effects observed on EP. Increases in hematocrit, serum triglycerides, and plasma protein between 8 and 10 wk post FMG-inoculation, suggest that the initial weeks of EP are stressful. Post-peak decreases in these same variables suggest a more chronic inhibition on lipid and protein synthesis in the liver. Decreased blood lipid concentration may be directly responsible for the reductions in yolk lipid, cholesterol, and fatty acid deposition in 12 wk FMG-inoculated hens.

Dual adverse effects in the caged layer facility on feed conversion and egg mass were realized in 22 wk FMG-inoculated birds. In contrast, a 12 wk FMG inoculation delayed onset of lay without a loss in total EP or egg mass. Therefore, inoculation with FMG at 12 wk is more practical and cost effective. Higher degrees of physiological stress experienced by hens in a caged layer facility may exacerbate the effects of FMG inoculation seen in the isolation units. These data demonstrate that alterations in performance and egg characteristics of commercial layers inoculated with FMG at either 12 or 22 wk of age and housed in either isolation units or caged layer facilities are related to mutual functional disturbances in the blood, liver, ovary, and oviduct without concomitant intestinal changes.

BIOGRAPHY

Matthew Rex Burnham, the son of Mr. Buford Rex Burnham and Mrs. Linda Sue Windham Burnham, was born and raised on his parents cattle farm in Collins, Mississippi, beginning November 19, 1974 at 11:03 A.M. Pamela Sue Burnham (deceased) and Rhonda Michelle Burnham Bush are his older sisters. In high school, the author actively held offices and participated in the following organizations: Junior/Senior Beta, Student Athletes Detest Drugs (SADD), Future Business Leaders of America (FBLA), Future Farmers of America (FFA), Student Council, and the Fellowship of Christian Athletes. He also received the "Driver's Education", District V MHSAA "All District Basketball Player", and "Mr. Future Business Leader of America" awards. After graduating in the top ten of his class with special honors from Seminary Attendance Center in Seminary, Mississippi, May 29, 1993, he attended Jones County Junior College in Ellisville, Mississippi. There, Matthew received a Sanderson Farms Scholarship, was maintained on the President's list, and participated in both the intramural basketball and the livestock judging teams. He received an Associate in Arts Degree in Animal Science from Jones County Junior College on May 11, 1995.

He attended Mississippi State University in Starkville, Mississippi, and received his Bachelor of Science Degree in Animal Science on May 10, 1997. While completing his degree he was a member of both the Block & Bridle and Poultry Science clubs.

Upon graduation, he officially entered the Poultry Science Graduate Program of Mississippi State University on June 1, 1997 with a graduate teaching assistantship and was also appointed as the Graduate Student Association (GSA) Departmental Representative. He was nominated to receive the Graduate Teaching Assistant of the Year Award by the GSA in 1998, 1999, and 2000 for teaching an undergraduate course in Genetics. His thesis was titled "Effects of Incubator Humidity and Breeder Age on Yolk and Embryo Compositions in Broiler Hatching Eggs", from which two papers were published (Poultry Science 80:1444-1450 and 80:1299-1304). These publications ended a three year collaborative research project with the United States Poultry and Egg Association and North Carolina State University. A Master of Science Degree from Mississippi State University was granted to Matthew on May 13, 1999. On July 3, 1999, Matthew Rex Burnham, and his breathtaking fiancee, Dana Kay Loftin, were married in a lovely ceremony at Station Creek Baptist Church.

Matthew was accepted into the Animal Physiology Doctoral program of Mississippi State University on June 1, 1999. Course work in his Program of Study, as set forth by his committee, was completed and he passed his preliminary/comprehensive doctoral exam on May 25, 2001. At that time, he officially became a Ph.D. candidate with a major in Animal Physiology and with minors in both Statistics and Biology at Mississippi State University. During his Ph.D. program, he held a graduate research assistantship, which was funded by a grant from the Agricultural Research Service of the United States Department of Agriculture (USDA-ARS). Dr. S. L. Branton of the USDA-ARS assisted in directing his research.

Matthew was active at MSU as 1999-2000 GSA President and as 2001-2002 Student Association Senator representing the Graduate School. He received the Year 2000 GSA Outstanding Leadership Award, along with the Sigma Xi Graduate Student Excellence in Research Award. Similarly, he also received the Year 2002 GSA Outstanding Graduate Student Award, along with the Sigma Xi Graduate Student Excellence in Research Award. He was nominated to receive the Research Assistant of the Year Award by the GSA in 2000, 2001, and 2002. The Poultry Science Association (PSA) recognized Matthew with the Graduate Student Research Presentation Award in conjunction with both the XXI World's Poultry Congress and the 89th Annual PSA convention in Montreal, Canada on August 20, 2000. His presentation was entitled "Effects of F-strain *Mycoplasma gallisepticum* on hematological characteristics in commercial laying hens". Similarly, he also received the PSA Graduate Student Research Presentation Award in conjunction with both the Midwest Poultry Federation and the 90th Annual PSA convention in Indianapolis, Indiana on July 27, 2001. His presentation was entitled "Digestive and reproductive organ characteristics in commercial laying hens as affected by F-strain *Mycoplasma gallisepticum*". A manuscript was written from the awarded material and submitted in the "2002 Alltech Student Research Manuscript Award" competition. Currently, Matthew is a member of the PSA, World's Poultry Science Association, Southern Poultry Science Society, Mississippi State University GSA, Sigma Xi, Gamma Sigma Delta, Alpha Theta Chi, and Intramural Softball Association. The title of his dissertation is "Physiological factors associated with the alteration of reproductive performance of commercial egg laying chickens infected with

F-strain *Mycoplasma gallisepticum*". Matthew passed his Doctoral Defense on March 8, 2002 and graduated on May 11, 2002. His career goal is to apply the expertise he attained in Physiology, Biology, Genetics, and Statistics in either an academic, administration, extension, government, industry, or research position.

MATTHEW REX BURNHAM

Motto: "Never met a stranger"

DEDICATION

This research is dedicated to my loving wife Dana Kay Loftin Burnham, who has supported me throughout my Ph.D. program. Also, the direct descendants of my family's ancestral lineage should be acknowledged for their lifelong contributions. Ancestors included in this lineage are my paternal great grand parents Marion Rance Burnham (November 7, 1873 - July 12, 1942) and Vivia Roberta Clark Burnham (June 28, 1883 - November 7, 1938), Joe William Rutland (May 10, 1891 - January 12, 1923) and Bertie Clark Rutland Allen (May 13, 1894 - February 10, 1973); paternal grand parents Buford Novie Burnham (September 3, 1916 - June 23, 1967) and Ruby Lee Rutland Burnham (November 7, 1917 - January 25, 1969); maternal great grand parents Ben William Windham (August 17, 1901 - June 2, 1962) and Dovie Atwood Windham (March 1, 1902 - June 12, 1926), Arthur Tanner (October 12, 1901 - November 6, 1958) and Elizabell Pearson Tanner (November 23, 1905 - February 15, 1970); maternal grand parents Ben Junior Windham (February 17, 1926) and Laverne Tanner Windham (July 2, 1928 - February 7, 1998); parents Buford Rex Burnham (September 24, 1946) and Linda Sue Windham Burnham (November 18, 1947); sisters Pamela Sue Burnham (April 25, 1968 - October 23, 1974) and Rhonda Michelle Burnham Bush (September 18, 1972); father-in-law Danny Lamar Loftin (September 24, 1946); mother-in-law Brenda Kay Russell Loftin (December 6, 1947); brothers-in-law Andrew Buster Bush (August 3, 1968) and Jon S.

Neal (April 17, 1967); sister-in-law Crystal Dawn Yates Neal (December 17, 1969); nephews Dawson Ty Bush (April 6, 2000), Jon Catlin Neal (September 21, 1987), Courtney Lauren Neal (March 17, 1989), and Colby Russell Neal (April 2, 1990). I would also like to include my uncles and aunts in this dedication, and they are Michael (Mack) Lee Burnham (February 6, 1944), Sharlotte Ann Ward Burnham (June 27, 1946), Frances Hazel Burnham Herrin (July 8, 1942), Robert Glen Herrin (March 31, 1941), Joseph Stacy Burnham (December 16, 1938), Ella Ruth Dickens Burnham (May 20, 1941), Brenda Diane Windham McDonald (April 14, 1949), Larry Preston Windham (September 6, 1950), Judith Denise Anderson Windham (October 10, 1951), Terry Michael Windham (July 4, 1954 - October 2, 1976), Karry Kim Windham (June 21, 1963), and Mary Jane White Windham (August 18, 1963). In this dedication I would like to acknowledge James Michael Yawn (November 16, 1974) and Melanie Lee Pickering Yawn (January 21, 1975) as my two best friends and eternal confidants. As it is extremely difficult to include everyone, other family members and friends not mentioned are appreciated for graciously supporting my academic decisions.

Favorite Quote:

"Ontogeny Recapitulates Phylogeny" simply stated "Life Retells Ancestry"

.....Anthony B. Heckle

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I am also indebted to both Dana W. Chamblee and Jerry H. Drott for their expertise in the management of the layer facilities I used. A final acknowledgment of appreciation is extended to members of both the Poultry Science Department at Mississippi State University and the United States Department of Agriculture-Agricultural Research Service, who provided me training and who encouraged me to

strive for excellence. Other collaborative institutions include Texas A&M and Clemson University, along with the Department of Biochemistry and Molecular Biology, and the College of Veterinary Medicine at Mississippi State University. Through the support and guidance of these aforementioned individuals and our Lord and Savior Jesus Christ this project was completed on November 19, 2001.

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CHAPTER I

INTRODUCTION

Mycoplasmas are often referred to as bacteria because they are the smallest of the free living organisms. In actuality, they are not true bacteria or viruses. Most mycoplasma related infections are the cause of significant health problems in commercial and experimental birds. Although significant advances in disease prevention technology have been made, reasons for the effects of both *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* in birds remain perplexing to scientists and researchers.

Mycoplasma gallisepticum-free layers still produce the highest percentage of eggs. However, vaccinations with live F-strain MG (FMG) are known to strategically reduce the percentage of egg production (EP) losses caused by natural wild strains of MG. During the past several years, vaccination of commercial layers with live FMG of low to moderate virulence have become available to protect flocks against natural MG infections (Branton and Deaton, 1985; Branton, *et al.*, 1988; Branton, *et al.*, 1997; Branton, *et al.*, 1999), and FMG is the most commonly used live vaccine in the United States (Barbour *et al*., 2000). The vaccine strain displaces natural field strain infections and FMG has a relatively poor transmissibility (Kleven *et al.*, 1990). Attempts to alleviate or reduce MG infection and improve EP and overall performance through live FMG vaccination programs are currently being pursued. However, no single anatomical or physiological

model exists for the relationship between FMG infection and altered performance in layers. Monitoring FMG vaccinations given at different dosages and points of time and under different conditions during the laying cycle is a key element in understanding how FMG affects layer performance. To-date, research concerning the effects of MG on avian reproduction has been limited to its effects on feed utilization, EP and egg quality parameters. The goals of this research include the identification and evaluation of possible physiological mechanism(s) by which FMG affects layer performance. More specifically, this research was conducted to determine the effects of FMG on physiological factors which control EP and egg characteristics in commercial layers. Examination of these mechanisms were made while making further repeated observations of their associated effects on various EP and egg quality parameters of flocks within sequential trials, under different housing conditions. Tissues which were examined in these birds included the liver, blood, intestines, oviduct, and ovary.

This dissertation contains eight chapters, five of which specifically describe different assessments of the effects and modes of action of FMG on the reproductive performance of commercial layers when inoculated at 12 wk while housed in biological isolation units, and when inoculated at both 12 and 22 wk while housed in a caged layer facility. Chapter III (Manuscript 1) describes the effects of a 12 wk FMG inoculation on EP, egg characteristics, feed consumption, and fecal contents of layers housed in negative pressure fiberglass isolation units throughout production. Chapter IV (Manuscript 2) describes the influence of a 12 wk FMG inoculation on digestive and reproductive organ characteristics of layers housed in isolation units throughout production. Chapters V

(Manuscript 3) and VI (Manuscript 4) more specifically describe the influences of FMG on the blood and yolk constituents of these birds, respectively. In addition, the effects of FMG inoculation on serum vitellogenin and lipoprotein profiles were assessed in Chapter V. Chapter VII details similar characteristics as in the previous manuscripts, but in layers inoculated with FMG at 12 and 22 wk and housed in a commercial caged layer facility. Chapter VII further describes the feed consumption, nutrient utilization, and feed conversion of these layers when provided a normal basal diet.

CHAPTER II

LITERATURE REVIEW

Digestion and Absorption of Lipids

Lipids in a diet eventually undergo emulsification, digestion, micellar solubilization, cell permeation, and intracellular esterification via hydrolysis in the intestines. These lipids are broken down into triglycerides, which are then converted into diglycerides, monoglycerides, glycerol, and fatty acids prior to absorption (Krogahl, 1985). Bile salts, which emulsify fats, enter the duodenum directly through the hepatic duct via the gall bladder. A majority of lipid is absorbed in the jejunum of chickens (Hurwitz *et al.,* 1973). Whitehead and Fisher (1975) confirmed the importance of fatty acid absorption in the jejunum; whereas, the ileum was specifically instrumental in the absorption of only a few saturated fatty acids, namely linoleic, stearic, and palmitic acids. Lipids pass through the intestinal brush border membrane in a passive manner and are absorbed according to chain length and degree of saturation (Garrett and Young, 1975; Bierbach *et al*., 1979). Polyunsaturated fatty acids are absorbed better than saturated fatty acids in chicks as described by Sklan (1978). Sklan (1978) also stated that absorption of all fatty acids is higher when chickens are fed triglycerides and lower when chickens are fed free fatty acids, demonstrating that glycerol is instrumental for fatty acid absorption in the intestinal lumen. Glycerol has to be present in order for complete micellular

solubilization of free fatty acids to occur. A fatty acid-binding protein influences fatty acid transport and has a greater affinity for unsaturated fatty acids than saturated fatty acids (Ockner *et al*., 1972; Ockner and Manning, 1974). Fatty-acid binding proteins are maximal in the proximal portion of the chicken intestinal tract (Katongale and March, 1979). Emulsified lipid along with low pH bile salts lead to the formation of a micelle. Lack and Weiner (1961) suggested that the disruption of micelle formation is necessary for the absorption of lipids in chickens.

After fatty acids are used to synthesize triglycerides (Mathews and Van Holde, 1990), newly synthesized triglycerides are then incorporated into lipoproteins in the intestinal wall before being drained from the intestine by portal blood (Robins *et al*., 1971). A lipase inhibitor is probably used to aid in the transport of fatty acids in the form of triglycerides. It has also been suggested that fatty acids are transported via the portal system in birds in the form of portomicrons (Conrad and Scott, 1942). Portomicrons and very low density lipoproteins (VLDL-transport triglycerides, phospholipids, and cholesterol) form micelle structures with an inner core consisting of triacylglycerol and cholesterol esters and an outer monolayer of phospholipids, unesterified cholesterol and proteins (Bradley and Gotto, 1978). Portomicrons have a short half-life in avian blood and are likely metabolized similarly to chylomicrons in mammals (Bensadoun and Rothfield, 1972). Circulating portomicrons and VLDL assist in transporting fatty acid rich triacylglycerols into tissues via hydrolysis of the triacylglycerol core in lipoprotein particles by lipoprotein lipase (LPL) (Nilsson-Ehle *et al*., 1980). Brown and Goldstein (1991) proposed that low density lipoprotein (LDL) metabolism occurs after absorption

of exogenous dietary fat and cholesterol through the intestinal wall and is packaged with triglycerides to form portomicrons in birds and chylomicrons in mammals.

Normally in the intestines, 50% of the cholesterol can be absorbed, but rate of absorption is lower when high dietary levels of cholesterol are present (Janacek *et al*., 1959). Cholesterol is an essential structural component of cell membranes and lipoproteins and serves as the main precursor for steroid hormones and bile acids (Yeagle, 1988). There is an association between blood levels of cholesterol and the risk of coronary heart disease in humans (Stamler *et al*., 1986) and premature development of atherosclerosis (Oliver, 1990). Griminger (1986) reported that exogenous cholesterol increases serum cholesterol in birds. The main dietary modifications needed to reduce plasma LDL-cholesterol concentration involve decreased intake of dietary cholesterol and total fat (Expert Panel, 1988; USDHHS, 1988).

Relation of Diseased or Altered States of the Digestive System and Intestinal Lipid Absorption

More than 36 nutrients are essential and must be in the diet in appropriate concentration and balance in order to maximize the ability of poultry to express their genetic potential to be productive (Austic and Scott, 1997). Nutritional balance is necessary to sustain symmetrical growth, proper feathering, and adequate egg production (EP). Non-specific signs of deficiency may be brought about by a number of causes, including infectious diseases and toxicants. Quantitative nutrient requirements of the young growing chick and for laying hens are well established (Agricultural research council, 1975; Austic, 1981). Food substances of importance in nutrition of poultry are

proteins and amino acids, carbohydrates, fats, vitamins, essential inorganic elements, and water (Austic and Scott, 1997). Fats are important as concentrated sources of energy and essential linoleic and arachidonic acids. Lack of these fatty acids in the diet of young chicks results in suboptimal growth and enlarged fatty livers (Hopkins and Nesheim, 1967). Ultimately, deficiencies of essential fatty acids in laying hens results in lowered EP and egg size (Menge *et al*., 1965). Reduced concentration of arachidonic acid and increased concentration of eicosatrienoic acid in tissue and egg lipids are characteristic of essential fatty acid deficiency (Austic and Scott, 1997).

Poultry production is affected by interactions, synergisms, and antagonisms between nutrition and immunity. Klasing (1997) reported that nutrition impacts a birds immunocompetence, which alters growth, reproduction, metabolism, resistance to infectious disease, and immune responses due to infectious challenges, especially during immune maturation. Opportunistic infections and poor production can result from inadequate nutrition or control of diseases, causing the immune system and nutrient status to deteriorate simultaneously (Klasing, 1997). Performance and EP data provide indirect measurements of dietary needs. An immune system has a high priority for circulating nutrients and is able to compete favorably with many other tissues for these nutrients. In this regard, leukocytes have a high position in the hierarchy of competition for nutrient use. During an immune response, leukocytes release cytokines such as interleukins 1 and 6, which act systemically to mobilize large quantities of nutrients from other tissues (Klasing, 1997). Hill and Garren (1961) demonstrated that decreasing the protein level in the diet from 30% to 20% to 10% of the diet resulted in progressively decreasing rates of

mortality of chicks from *Salmonella gallinarum* infection. Boyd and Edwards (1963) observed decreased mortality with protein deficient diets following challenges with either *S. gallinarum* or Newcastle disease virus. Protein deficient diets also reduced the severity of disease and the mortality level from *E. tenella* infection due to the lack of trypsin activity in the intestinal tract and a decrease in excystation of the oocysts (Britton *et al.*, 1964). In growing white leghorn chicks, feeding dietary n-3 fatty acids from fish oil enhances the antibody response of chicks to sheep red blood cells but suppresses rates of lymphocyte proliferation after mitogen stimulation (Fritsche *et al.*, 1991). Macrophage function and the regulatory roles of macrophages are also sensitive to dietary fat sources. The release of interleukin 1 by *Staphylococcus aureus* stimulated macrophages from broiler chicks fed high levels of n-6 fatty acids was markedly increased compared with that seen with chicks fed diets high in n-3 fatty acids (Korver and Klasing, 1995). Practical feed ingredients high in n-6 fatty acids include corn, vegetable oils, restaurant grease, and poultry fat (Klasing 1997), but translation of the regulatory shifts due to these nutrients into disease resistance has not been well studied. Excess vitamin E in broiler chick diets stimulates humoral immunity and reduces the severity of an *Escherichia coli* infection as indicated by mortality and weight gain (Nockels, 1988). This excess vitamin E also decreases the morbidity of subclinical infectious bursal disease when compared with those as a result of reduced vitamin E diets (McIlroy *et al.*, 1993). Anti-inflammatory effects of n-3 fatty acids reduce intestinal pathology associated with *E. tenella* in broilers (Korver and Klasing, 1995).

Inadequate feedstuffs can result in an increased incidence of infectious disease in the intestine. Components of the feedstuffs that are not enzymatically digested in the upper gastrointestinal tract provide nutrients to micro flora in the ileum, cecum, and large intestine, influencing the ecology within these organs (Klasing, 1997). The presence of unstabilized, rancid fat in the diet increases numbers of *E. coli* and lowers numbers of lactobacilli in the small intestine. Fat sources with high levels of free fatty acids, polyunsaturated fatty acids, and low levels of antioxidants are susceptible to oxidative rancidity. During many infectious agent challenges, monocytes and macrophages recognize foreign organisms and release interleukin 1, 6, and tumor necrosis factor. Each of these monokines has a specific role in the regulation of the immune response by acting in the local area of challenge. They all act systemically by binding to receptors on cells of all tissues. Lastly, they can have indirect systemic effects by altering levels of hormones such as insulin, glucagon, and corticosterone. Through their systematic actions, leukocytic cytokines orchestrate metabolic changes that underlie the classic acute phase symptoms (Klasing, 1988; Klasing and Johnstone, 1991). Partitioning of dietary nutrients should be balanced between production aspects and immunocompetence. Decreased feed intake accounts for about 70% of reductions in performance, while the remainder is due to metabolic inefficiencies caused by the immune response (Klasing *et al.*, 1987). There is a plethora of information on nutrition in the healthy birds and little information on unhealthy diseased birds (Klasing, 1988). In fact, there is a general reluctance to publish the results of experiments designed to determine nutrient requirements if a disease situation develops (Klasing, 1997).

Usually a disease challenge reduces growth and reproduction due to decreased feed intake, therefore, nutritional manipulations need to be investigated. Increasing the energy density of a ration with carbohydrates while keeping required nutrients at a constant percent of the energy improved energy intake and the rate of gain of broiler chicks challenged with *S. typhimurium* lipopolysaccharide (Benson *et al.*, 1993).

A diseased state may cause additional changes in nutrient requirements, especially when they affect the gastrointestinal tract and impair nutrient absorption (Sell and Angel, 1990). Nutrient absorption during coccidiosis is related to the nutrient studied, the stage of infection, and the severity of infection (Turk, 1974). Each species of *Eimeria* tends to localize in a specific region of the intestinal tract resulting in disruptions of digestive, absorptive, and secretory functions specific for that region. Compensatory changes occurring in uninfected regions mitigate some of the negative impacts (Ruff and Wilkins, 1980). Changes in intestinal morphology, including shortening of villi, loss of micro villi, decrease in villous surface area, and reduction of mucosal thickness are probably related to impaired digestive and absorptive capacities. A decreased rate of passage of feed through the gastrointestinal tract and retention of food into the crop and gizzard probably also contribute to lower digestibility of feed (McKenzie *et al.*, 1987). The leakage of plasma proteins into the intestinal tract is also a major factor affecting the apparent digestibility of protein (Joyner *et al.*, 1975).

Chickens housed in germ-free environments grow 15% faster than those raised in conventional environments where they are continuously exposed to micro flora (Klasing, 1997). Antibiotics may act by limiting the number of times, and the vigor with which, the

immune system must respond to dispose of frequent microbial challenges in the intestines (Roura *et al.*, 1992).

Lipid Transport to the Liver and Liver Function

Concurrently, in the capillaries of fat and muscle tissue, the ester bonds in triglycerides are cleaved by LPL and the fatty acids are liberated from portomicrons. Next, the cholesterol-rich remnants reach the liver, bind to specialized receptors and are taken up by liver cells. A dramatic increase in VLDL and decrease in high density lipoprotein (HDL) and LDL after feeding a high cholesterol diet supports the idea that blood cholesterol level is significantly involved in VLDL production by the liver (Khan *et al*., 1990; Castillo *et al*., 1992). Hermier and Dillon (1992) hypothesized that this may be due to reduced production of phospholipids needed in HDL and LDL formation. The cholesterol can then be secreted into the intestine or packaged with triglycerides into VLDL particles and released into the circulation. Portomicrons following starvation may be synthesized in the intestine from exogenous fat and subsequently transported into liver for VLDL synthesis, thereby allowing the preparation of lipoproteins rich in a particular monoacid triacylglycerol. When triglycerides are removed from VLDL particles in fat or muscle tissue, each particle is transformed to a cholesterol-rich intermediate-density-lipoprotein (IDL). Some of the IDL binds to LDL receptors in the liver and are rapidly taken up by liver cells. The remaining IDL stays in circulation and is converted into LDL. Benson *et al*., (1975) suggested that LPL are responsible for the conversion; that is, once the IDL is acted upon by a lipase, the density will change due to liberation of triglycerides and cholesterol. Most of the LDL binds to LDL receptors on liver or other

cells and is removed from circulation. Cholesterol binds to HDL particles, is esterfied, and transformed to IDL, while the LDL complexes are taken up by cells.

Fatty Acid Synthesis in the Liver

Mammalian and avian liver's are quite rich in lipid and the process of fatty acid synthesis in each have many common features (Poulose *et al*., 1981; Girard and Ferre, 1982; Wakil *et al*., 1983; Naggert *et al*., 1988; Gurr, 1992). A chicken's liver is responsible for synthesis of fatty acids *de novo* (O'Hea and Leveille, 1969; Donaldson, 1990). *De novo* synthesis of fatty acids in the liver requires biotin-dependent acetyl-CoA (coenzyme A) carboxylase and fatty acid synthetase (De Titta *et al*., 1980; Wada and Tanabe, 1983; Wakil *et al*., 1983; Takai *et al*., 1987, 1988; Knowles, 1989; Goodridge, 1991; Lehninger, 1993; Harwood, 1988, 1994). Acetyl-CoA carboxylase converts acetyl-CoA to malonyl-CoA and appears to be the rate-limiting step in maximal lipogenesis and fatty acid synthetase elongates an acyl-enzyme complex until a saturated fatty acid is formed (Ganguly, 1960; Donaldson, 1979). Fatty acid synthetase can be regulated by adaptive changes in enzyme content (Wakil *et al*., 1983). Several elongases used in the fatty acid elongation system have been detected (Goldberg *et al*., 1973). Cook (1991) detected the four reaction intermediates of elongation. The animal microsomal system contains at least two condensing enzymes - one for saturated and one for unsaturated primers. Unsaturated fatty acids can be produced by anaerobic or aerobic pathways (Harwood, 1994). Aerobic desaturases have been extensively studied with only a few successful purifications (Gurr, 1974).

Stearoyl-CoA ? 9-desaturase was first purified by Strittmatter *et al*., (1974). Strittmatter *et al*. (1988) also determined the bacterial synthesis of active liver desaturase. Palmitoyl CoA is the principle product of fatty acid synthesis and is also a feedback inhibitor. The conversion of this palmitate to palmitoleate occurs when a double bond is introduced into the fatty acid chain by an oxidative reaction catalyzed by ? 9-fatty acyl-CoA desaturase (Lehninger, 1993; Cook, 1991). Palmitate can also be elongated by further additions of acetyl groups from the smooth endoplasmic reticulum and mitochondria to form Stearoyl CoA (Stearate), which can further be desaturated by ? 9-desaturase to produce oleate (Cook, 1991). Most mammalian tissues can modify acyl chain composition by introducing more than one bond. Like ? 9-desaturation, further desaturation requires molecular oxygen and an associated electron transport system. Animal systems cannot generally insert double bonds beyond the ? 9 position as in plants, however, double bonds are inserted at the ? 6, ? 5, and ? 4 positions (Harwood, 1994). Oleic acid, which is a precursor to linoleic acid, is derived from stearic acid through appreciable ? 9-desaturation activity (Cook, 1991). Ding and Lilburn (1996) reported that oleic acid comprised the largest proportion of total yolk fatty acids. Linoleate cannot be synthesized from oleate by animals, but plants can synthesize this acid. In the endoplasmic reticulum of plants, ? 6-desaturation of oleate to linoleate uses phospholipids and phosphatidylcholine, containing at least one oleate linked to glycerol (Lehninger, 1993; Harwood, 1994). Linolenic acid is derived from linoleic acid through appreciable ? 6-desaturation activity (Noble and Cocchi, 1990; Noble and Shand, 1985). Eicosatrienoate acid is derived from the elongation of linolenic acid, which employs

malonyl CoA and thus is dependent on the activity of acytel-CoA carboxylase (Lehninger, 1993; Cook, 1991). Arachidonic acid is then derived from eicosatrienoate acid through appreciable ? 5-desaturation activity (Lehninger, 1993; Cook, 1991).

Alterations in Lipid Transport to the Liver and in Liver Function

Isaacks *et al.* (1964) and Raju and Reiser (1967) stated that fatty acid synthesis was depressed to a greater extent during periods of unsaturated fatty acid ingestion. The fatty acid composition of yolk lipid (YL) is dependent on the hen's diet, but dietary fat has little effect on YL or cholesterol content (Kuksis, 1992). Bouziane *et al*. (1994) observed that feeding growing rats a diet of salmon oil raised the n-3 fatty acid level in serum and VLDL triacylglycerol fractions. Suarez *et al*. (1996) reported that in weaning rats fed a diet with high oleic acid, ~30% of the total fatty acids in VLDL was comprised of oleic acid. Nir *et al*. (1973) reported that the re-feeding of a diet following a period of food deprivation increased plasma triacylglycerol in geese. Each of these findings is suggestive of the fact, that fatty acid composition of triacylglycerol in portomicrons/chilomicrons and VLDL could be changed by dietary fat.

Lipid Transport from the Liver to the Ovary

The circulatory system is responsible for the transport of protein and lipid particles from the liver to the ovary. Triglycerides are transported to the yolk in the form of ß-lipoproteins. The distribution of fatty acids of various chain lengths is relatively constant in YL and is related to their synthesis in the hens' liver (Watkins and Kratzer, 1987; Watkins, 1995; Walzem, 1996; Speake and Thompson, 1999). After the formation of VLDL and vitellogenin (VTG; a phosphoglycoprotein) occurs in the liver, they are

transported in via the circulatory system. Although yolk LDL constitutes the largest portion of avian egg yolk solids, its synthesis has received less attention than VLDL (Burley and Vadehra, 1989). Hillyard *et al*., (1972) reported that VLDL in the blood of laying birds is the same as the major LDL in yolk. Burley and Vadehra (1989) state that blood VLDL is an unprocessed precursor of yolk lipoprotein in much the same way as VTG is the precursor of the yolk granules.

Acetyl-CoA molecules can serve as building blocks for yolk cholesterol. Cholesterol biosynthesis occurs in many areas of the body of birds, including the liver, carcass, intestine, and skin. Biosynthesis in the thoracic and abdominal aorta also occurs in young birds (Yeh and Leveille, 1973; Griminger, 1986). In the endoplasmic reticulum acetyl-CoA produces HMG-CoA, which reacts with a reductase to yield mevalonate and is the rate-limiting step in cholesterol biosynthesis (Mathews and Van Holde, 1990).

Lipoproteins

Biochemical studies indicate VLDL of laying hens to be small-diameter triacylglycerol-rich particles (Chapman *et al*., 1977), and there is a remarkable reduction in mean direct comparisons between VLDL diameter distributions of roosters and laying hens (Walzem *et al*., 1994, 1999). Studies of large numbers of hens showed that VLDL with particle diameters of 25-44 nm were highly correlated with successful EP (Walzem, 1996). Hens with decreased or increased amounts of VLDL particles <25 nm or >50 nm were poor egg producers (Walzem *et al*., 1994, 1999). Size-exclusion chromatography of plasma VLDL in laying hens with mean particle diameter of 30 nm was indicative of a higher content of triacylglycerol than any other particles, indicating that small diameter

triacylglycerol-rich VLDL (VLDLy) is "targeted" for yolk deposition (Walzem *et al*., 1994, 1999). Impaired VLDLy assembly may be indicative of reproductive dysfunction in hens (Walzem, 1996).

Laying hen VLDLy resists the lipolytic activity of LPL both *in vitro* (Griffin *et al*., 1982) and *in vivo* (Bacon *et al*., 1978). This LPL resistance prevents IDL or LDL formation from VLDLy, thereby securing a high triacylglycerol content for the egg yolk (Walzem *et al*., 1994, 1999). The degree of LPL-resistance appears to vary somewhat among individual birds, and may lead to subsequent reproductive disturbance (Walzem *et al*., 1994). Specific VLDLy properties are associated with particle diameter, high apolipoprotein VLDL-II (apoVLDL-II) to apolipoprotein B (apoB) ratio, resistance to LPL, and high triacylglycerol to very low cholesterol ester content (Walzem *et al*., 1999). Concentration of VLDLy is influenced by genetic background and energy intake (Walzem *et al*., 1994; Chen *et al*., 1997).

Although the molecular mechanisms are unknown, apoVLDL-II confers LPL resistance to VLDLy (Griffin *et al*., 1982; Schneider *et al*., 1990; Schneider, 1996), perhaps by competitively excluding other exchangeable apoproteins from the surface of VLDLy (Miller and Lane, 1984). Similar to apoB, molecules of apoVLDL-II do not exchange between lipoprotein surfaces, but the presence of apoVLDL-II on nascent VLDLy may limit the expansion of the VLDL particle during the assembly process, giving rise to the smaller VLDLy (Walzem *et al*., 1994, 1999). Particle diameter of VLDLy is a highly regulated physical property in hens sufficiently overfed to produce a fatty liver (Walzem *et al*., 1993). Overfed egg-laying hens allowed only a 22% increase

in VLDLy particle diameter, from 27 to 33 nm (Walzem *et al*., 1994). Matthews and Van Holde (1990) reported that particles of avian HDL may supply adequate cholesterol to the liver for bile production, remove excess cholesterol from the body, and lower LDL levels.

A divergent selection of Japanese quail lines for total plasma phosphorus at the onset of lay was initiated by Nestor *et al*. (1982) to study the inheritance of plasma yolk precursor and the genetic relationship of yolk precursor to growth and reproduction traits. Concentrations of phosphorus were demonstrated to be highly correlated to total lipids in plasma lipoproteins of density <1.006 g/ml (VLDL) in laying quail (Bacon *et al*., 1982). Most (>90%) of the increased plasma lipid is triglyceride and can be isolated in plasma lipoproteins that float after ultracentrifugation at a background density of 1.006 g/ml (Kudzma *et al*., 1975, 1979; Griffin *et al*., 1982; Hermier *et al*., 1985, 1989).

Stress Effects on Circulating Lipids

Numerous animal studies have provided empirical support for a relationship between stressors and blood lipid concentration. Layer triglycerides are high due to EP (Lorentz *et al*., 1938) and a stressor may elevate this to compensate for infection, which is considered a natural endocrine response. Environmental heat stress and diseases may lead to the development of Fatty Liver Hemorrhagic Syndrome in birds (Couch 1956; Riddell, 1997), which may disrupt lipid and lipoprotein assembly. It is believed that epinephrine administration stimulates hepatic HMG-CoA reductase activity in normally fed and cholesterol-fed rabbits, which in turn may lead to greater hepatic cholesterol synthesis (Deverey *et al*., 1986). Siegel (1960) increased adrenocortical activity and

stimulated the effects of increased glucocorticoid levels in broilers by repeated injections of adrenocorticotropin. Continuous infusion of adrenocorticotropin has also been found to increase plasma glucose, cholesterol, HDL and corticosterone, while suppressing body weights in 3-wk old broilers (Latour *et al*., 1996).

Estrogenic Stimulation of Yolk Precursors

Onset of lay in hens and estrogen treatment in chicks are both accompanied by large increases in plasma lipids (Kudzma *et al*., 1975). Formation of yolk protein mainly occurs in the liver and is primarily regulated by gonadotropic and steroid hormones. There is an excess of lipoproteins in the blood of laying birds (Lorentz *et al*., 1938), and this production is prompted by estrogenic stimulation (Hillyard *et al*., 1956). These lipoproteins are clearly destined for receptor-mediated endocytosis (Nimph and Schneider, 1991) in oocytes for eventual YL deposition (Luskey *et al*., 1974). Several decades ago, Luskey *et al*. (1974) reported that hepatic VLDL synthesis was stimulated about fourfold 16 h after estrogen was administered to roosters. As with VTG, administration of estradiol in immature hens will induce a 400-fold increase in the concentration of blood VLDL (Kudzma *et al*., 1975). Estrogen stimulates bird hepatic triacylglycerol and phospholipid biosynthetic rates several-fold, whereas cholesterol synthesis remarkably remains unchanged (Kudzma *et al*., 1975; Dashti *et al*., 1983). Estrogen (17ß-estradiol) can stimulate the liver to produce VTG as a polypeptide with a molecular weight of approximately 240,000 daltons, which is necessary for vitellogenesis (Bergink *et al*., 1974; Deeley *et al*., 1975; Christmann *et al*., 1977). Initially, it is phosphorylated, glycosylated, associated with lipid, and secreted into the blood where it

exists as a dimer with a molecular weight of approximately 500,000 daltons (Deeley *et al*., 1975). Vitellogenin synthesis is a remarkable process not only in females, but also in males, because estrogen increases the concentration of VTG over 150 fold in roosters (Clegg *et al*., 1951; Hillyard *et al*., 1956; Greengard *et al*., 1964; Bos *et al*., 1972; Bergink *et al*., 1973; Goldstein and Hasty, 1973; Talwar *et al*., 1973). In 1945, McDonald and Riddle injected estradiol into male birds to increase the amount of VTG in males to that of a female. Since then, Blue and Williams (1981) determined that without estradiol, the level of VTG in roosters was 4-8 ng/ml, while in laying hens, VTG levels were 10-25 mg/ml. Following a second injection of estrogen in roosters, long term effects are evident and the magnitude of response is greater (Bergink *et al*., 1973; Talwar *et al*., 1973). Also, 17ß-estradiol secretion by the thecal cells of hens has been demonstrated (Bahr *et al*., 1983), and depends upon luteinizing hormone in a dose-dependent manner (Robinson and Etches, 1986). This suggests a vital role of luteinizing hormone in stimulating steroidogenesis in both nonhierarchical and hierarchal follicles in birds, and in the regulation of ovarian follicular growth (Chen *et al*., 1999). Estrogen induced responses occur in differentiated tissues without DNA synthesis (Jost *et al*., 1973, 1978; Wangh and Knowland, 1975; Green and Tata, 1976; Deeley *et al*., 1977).

Medullary bone develops under the influence of the ovarian hormones estrogen and testosterone (Bloom *et al*., 1941), and it can be readily induced in intact male birds by administration of estrogen (Landauer and Zondek, 1944) or in castrated males by estrogen and testosterone (Common *et al*., 1948). Gonadal steroids act directly on the cells in the medullary cavity (Benoit and Clavert, 1945), and independently of calcium intake.

As with other substances such as VLDL (Luskey *et al.*, 1974) and triacylglycerol and phospholipid (Kudzma *et al.*, 1975; Dashti *et al.*, 1983), estrogen dramatically stimulates hepatic synthesis of apoB in birds four to six fold (Williams, 1979; Blue *et al*., 1980; Capony and Williams, 1980; Kirchgessner *et al*., 1987) and induces the *de novo* synthesis of apoVLDL-II (Chan *et al*., 1976; Williams, 1979). Exogenous estrogen in the rooster liver induces apoVLDL-II and apoB, which accumulates in VLDL (Williams, 1979). Gene expression of apoVLDL-II and apoB are estrogen-responsive in bird livers, but apoB is only made in the adult kidney and there it is not responsive to estrogen (Lazier *et al*., 1994). Treatment with estrogen in males has been shown to decrease LDL, increase HDL, and reduce LPL (Hiraga *et al*., 1993). Kudzma *et al.* (1975) experimentally showed that estrogen-induced hypertriglyceridemia in immature hens was accompanied by a greatly increased production of triglycerides by the liver. Plasma neutral lipids increase drastically in chickens (Heald and Badman, 1963) and turkeys (Bacon *et al*., 1974) during

initiation of EP. This increase in neutral lipid concentration is inducible by estrogen administration (Lusky *et al*., 1974).

Lipid Uptake and Deposition in the Ovary

Triglycerides in the form of ß-lipoproteins are incorporated into yolk as lipid globules. In the ovary, VTG is cleaved into lipovitellin and phosvitin, which are two major yolk phosphoproteins (Tata, 1976), and deposited into the developing oocyte. Phosvitin is a glycophosphoprotein of highly unusual amino acid composition (Meecham and Olcott, 1949; Allerton and Perlmann, 1965; Shainkin and Perlmann, 1971).

Lipovitellin consists of two yolk proteins called a- and ß-lipovitellin (Bernardi and Cook, 1960). Each of these are lipophosphoproteins comprised of 80% protein and 20% phosphorylated lipid. These yolk proteins are then released from capillaries, where they can pass across the basement membrane and through gaps between granulosa cells to the plasma membrane of the oocyte (Walzem *et al*., 1999). Translocation of VTG and VLDL across the oocyte plasma membrane is a receptor-mediated event, and the receptor for both VTG and VLDL is localized within coated vesicles of the oocyte plasma membrane (Shen *et al*., 1993). Vitellogenin and VLDL are highly correlated with egg yolk formation. Following transport across the plasma membrane, VTG and VLDL become localized to yolk spheres where proteolytic processing of these yolk precursors to phosvitin, lipovitellin, triglycerides, cholesterol, and phospholipids occurs by cathepsin D (Retzek *et al*., 1992). Lipids and protein are deposited into growing ovarian follicles at about the same ratio for most of the growth phase, but during the final rapid growth phase, relatively more lipid is incorporated. Several reports accessing the ultrastructure of developing follicles have suggested that deposition of yolk into the maturing follicle is terminated by 24 hr before ovulation (Wyburn *et al*., 1965; Rothwell and Solomon, 1977; Perry *et al*., 1978; Gilbert *et al*., 1980).

Perry *et al*. (1978) used ultrastructural studies to document that intact lipoprotein particles could be endocytosed via coated pits located on the oolemma of the oocyte of developing yolk follicles. Nimpf *et al*. (1988) were the first group to publish evidence of avian oocyte receptors. One decade later, Bujo *et al.*, (1997) reported the molecular mechanisms of oocyte triacylglycerol deposition as being receptor-mediated endocytosis

of intact VLDL particles via the ligand apolipoprotein. Interestingly, ultrastructural studies of the granulosa basal lamina surrounding the oocyte indicate that this membrane acts as a selective mechanical sieve to circulating VLDL (Griffin and Perry, 1985). During lay, the flow of hepatically synthesized VLDL is largely partitioned for uptake by ovarian follicles instead of peripheral tissues (Bacon *et al*., 1978; Bacon *et al*., 1994). Thus, VLDLy in laying hens is different than VLDL in males and nonlaying females (Chen *et al*., 1999). Laying hens metabolize VLDL faster than non-laying hens (Bacon *et al*., 1978) and the VLDL particles between these hens is significantly different (Griffin and Perry, 1985). Low levels of LPL may be responsible for decreases in VLDL degradation before they reach the yolk. Only those apoB with diameters of 25-44 nm were observed distal to the granulosa basal lamina, which suggest that only a select size subclass of apoB can access oocytic receptors (Walzem *et al*., 1994, 1999). This indicates that alterations in the classical metabolism of apoB, where LPL converts VLDL into IDL and LDL lipoproteins, has significantly evolved to permit only small energy-laden VLDLy to be deposited in egg yolk. In ovarian follicles, yolk deposition is achieved through receptor-mediated endocytosis in follicles during the stage of rapid development (Perry and Gilbert, 1979; Nimpf *et al*., 1988). The distribution of VLDLy diameters has been related to EP (Walzem *et al*., 1994; Walzem, 1996), leading to the hypothesis that the livers of egg laying hens assemble unique VLDLy particles specifically targeted for yolk deposition. Yolk targeting among VLDL particles, including VLDLy, was hypothesized to depend upon the physical properties of lipoprotein particles (Walzem, 1996; Walzem *et al*., 1999), including particle diameter.

The hens' fundamental capability for and persistence in VLDLy assembly appears to be influenced by genetics and environmental factors (Walzem *et al*., 1994, 1995, 1999).

Egg yolk and plasma cholesterol levels in hens are not well correlated (Hargis, 1988). Furthermore, the rate of uptake of VLDL from plasma is not well correlated with its rate of deposition into yolk (Bacon *et al.*, 1978). However, Griffin (1992) suggested that this relationship is dependent on blood VLDL concentration. Griffin (1992) also suggested that oocyte receptors are always saturated, because concentrations of yolk precursors in the plasma of laying hens are normally at least ten-fold greater than those in non-laying hens. *In vitro* studies clearly demonstrate that receptor affinity on the oocyte plasma membrane is high, with half maximal binding of VTG at about 100 μg/ml (Stifani *et al.*, 1987) and for VLDL at about 50 μg apoprotein/ml (Griffin and Perry, 1985).

In general, it is known that yolk comprises approximately 31% of a freshly laid hen egg, and 33% of the yolk is lipid, 16% protein, and the remainder is water and solutes (Shenstone, 1968). Also, triacylglycerols comprise 65-70% of YL by weight and phospholipids comprise another 25-27%, while cholesterol contained in the egg yolk represents only ~3% of YL, and is largely unesterified (Naber, 1983; Kuksis, 1992; Johnson, 1986, 2000). Yolk deposition is continuous, and yolk follicles increase in size from 7 to 35 mm in diameter and in weight from 0.2 to 17 g in the 7-10 d period preceding ovulation (Etches, 1996).

Dietary Control Over Lipid Absorption, Metabolism, and Follicular Deposition in Egg Laying Chickens

Changes in dietary fatty acid profiles can change the fatty acid profile of fat in the yolks of eggs (Cruickshank, 1934; Sell *et al*., 1968; Sim *et al*., 1973; Hargis and Van Elswyk, 1993). A dietary regime may influence the cholesterol content of egg yolk. Harris and Wilcox (1963) indicated that added cholesterol in the diet markedly increases the cholesterol concentration in the egg yolk by 25%. An increase in excretion of cholesterol into the egg is one avenue available to the hen for maintaining a normal cholesterol level when cholesterol intake is increased. Moreover, endogenous biosynthesis of cholesterol from acetate is reduced and neutral sterol output in feces is enhanced by cholesterol feeding (Sim *et al*., 1980).

Vargas *et al.* (1986) investigated the effects of feeding an oxygenated derivative of cholesterol, 7-ketocholesterol, a known inhibitor of HMG-CoA reductase. The suppressive effects of 7-ketocholesterol on HMG-Co-A reductase activity in hen liver and cholesterol deposition in the egg were investigated, and as a result, Vargas *et al.* (1986) found that although 7-ketocholesterol effectively inhibited the activity of HMG-CoA reductase by 43%, it did not significantly alter yolk cholesterol levels. Under normal dietary conditions, the laying hen is capable of synthesizing cholesterol that exceeds levels essential for yolk deposition, and that inhibition of HMG-CoA reductase activity by more than 43% is required to alter egg cholesterol deposition (Vargas *et al.,* 1986). In rats and humans, inhibitors of HMG-CoA reductase lower plasma triglycerides primarily by decreasing hepatic secretion of VLDL (Kasim *et al*., 1993), which is believed to result

from interference in VLDL assembly. Since one molecule of apoB is required for proper assembly and secretion of VLDL and apoB may be regulated by various lipid components of the lipoproteins, it is possible that HMG-CoA reductase inhibitors decrease the secretion of apoB. Data suggest that obesity in both men and women leads to reductions in hepatic and LPL that cause a decease in the relative amount of circulatory HDL (Cominacini *et al*., 1993). High cholesterol diets sharply skew the lipoprotein profiles in birds (St. Clair, 1983) and the cholesterol content of all the lipoprotein particles (Mahley *et al.*, 1975, 1977; Riley *et al.*, 1979; Kris-Etherton and Cooper, 1980; Hermier and Dillon, 1992). Xu *et al*. (1990) suggest that changes in metabolism involving VLDL particles also alter the HDL response. In mammalian females, low-fat diets result in higher triglyceride and VLDL levels, which Cobb *et al*. (1993) believe may be a result of hormonal effects. However, females have lower baseline LDL, triglyceride, and VLDL levels, and higher HDL levels, and are less responsive to dietary pressures than males (Grundy *et al*., 1986; Grundy and Vega, 1986; Mensink and Katan, 1987; Havel, 1990).

The quantity and type of dietary fatty acids have a direct effect on sterol metabolism in the bird (Hargis, 1988). The ability of the hen to absorb dietary cholesterol is directly dependent upon the nature of the fat (March and Bily, 1959); that is, highly unsaturated oils have a synergistic effect on cholesterol absorption and result in increased egg yolk cholesterol (Bartov *et al*., 1971). Saturated fats lower the cholesterol content of the blood in birds (Weiss *et al*., 1976). Weiss *et al*. (1976) also noted that the rate of total lipid synthesis by the liver was diminished by feeding saturated fat, but rate of cholesterogenesis was not changed. Cholesterol synthesis in the liver is stimulated by

feeding polyunsaturated fats, which further increases excretion of cholesterol deposited into the egg. Hirata *et al*. (1986) found that egg yolk cholesterol content did not differ among hens fed diets containing soybean oil, coconut oil, lard, or beef tallow, although fatty acid composition of egg yolk was markedly influenced by the type of lipid in the diet.

In 1934, Cruickshank conducted an investigation of dietary influences on YL and concluded that diets have little if any effect on the amount of fat present, but that fatty acid composition of yolk fat is altered by diet. Furthermore, highly unsaturated fatty acids affect the proportion of fatty acids present while high levels of saturated fat in the diet have less influence. Hargis and Van Elswyk (1993) believe that Cruickshank's conclusions were correct and suggest that the fatty acid composition of body fat in animals and their diets generally reflect one another (Hargis, 1988). However, Di Giorgio *et al*. (1962) suggested that tissue fatty acid composition, especially adipose tissue, never completely duplicated that of the diet. Hargis *et al*. (1991) modified YL content with the addition of dietary menhaden oil; that is, diets containing fish oils increased the relative concentrations of fatty acids found in fish oils in the yolk. Couch and Saloma (1973) changed fatty acid distribution in egg yolks through changes in dietary fat. Cherian and Sim (1993) demonstrated that hens fed diets enriched with –9, –6, or n-3 fatty acids will modify yolk compositions. Feigehbaun and Fisher (1959) also suggested that high levels of various types of dietary oils influence the deposition of polyunsaturated fatty acids in body fat; whereas, the diet has no effect on either the saturated or unsaturated fractions in YL. Sim *et al*. (1973) conducted a study to investigate the effects of animal tallow,

soybean, sunflower and rapeseed oil on various tissues and found that changes in fatty acid composition of body tissues were proportional to those in the diet. Naber (1979) reported that polyunsaturated compared to saturated fatty acid levels in yolk are not as responsive to diet.

Diseased States of the Ovary and Oviduct and Their Subsequent Effects on Reproductive Function in Egg Laying Chickens

The small intestine, liver and ovary, along with the oviduct, which consists of an infundibulum, magnum (Wyburn *et al*., 1970), isthmus (Hoffer, 1971; Solomon, 1975), uterus (Breen and Debruyn, 1969; Nevalainen, 1969; Wyburn *et al*., 1973), and vagina are extremely important to internal egg formation and the production process. For the most part, the ovum remains in the infundibulum 18 minutes, magnum 180 minutes, isthmus 90 minutes, and uterus approximately 20 hours before being transferred to the vagina for oviposition and expulsion (Sturkie, 1976; Johnson, 2000). This 24-plus-hr ovulation-oviposition cycle is characteristic of the chicken (Fraps, 1955).

Infections of the ovary with bacteria of the genus *Salmonella* comprise one of the most important reservoirs of salmonellae that can be transmitted through the food chain to humans. This paratyphoid bacteria comprises more than 2,000 serotypes (Barrow, 2000). *Salmonella enteritidis* colonizes the tissues of the chicken ovary and oviduct, presumably contaminating eggs and thereby contributing to human outbreaks of salmonellosis (Keller *et al*., 1995). Keller *et al*., (1995) suggests that prior to egg deposition, forming eggs are subject to descending infections from colonized ovarian tissue, ascending infections from colonized vaginal and cloacal tissue, and lateral

infections from colonized upper oviduct tissues. Thiagarajan *et al*., (1996) demonstrated that *Salmonella enteritidis* interacts with granulosa cells, preferably of the preovulatory follicles, in a specific manner and can invade and multiply in these cells in the ovary at different stages of development. Associated diseases are Pullorum disease, which is caused by *S. pullorum*, and fowl typhoid, which is caused by *S. gallinarum*. Salmonellae can infect the ovary and result in lesions and salpingitis, leading to decreases in EP and quality.

In the female chicken embryo, two Mullerian ducts start to develop into oviducts. The left duct develops into a functional oviduct, while the right duct regresses. If this regression is not complete, partial development will result in a cystic right oviduct. Cystic right oviducts are common incidental findings in postmortem examinations of chickens. They vary in size from small elongated cysts to large fluid filled sacs up to 10 cm or more in diameter. Small cysts are of little consequence, but large cysts compress the abdominal viscera. The large sacs can result in a bird with a pendulous abdomen and should be differentiated from ascites.

The term false layer has been used to describe a bird that has the characteristics of a bird in production, visiting the nest regularly but not laying eggs (Hutt *et al.*, 1956). This bird has a normal appearing ovary and oviduct, but the infundibulum fails to engulf the ovum after it has been ovulated. At necropsy, these birds show excessive amounts of orange-colored fat and have liquid yolk or coagulated yolk in the body cavity. This defect may result as a sequel to infectious bronchitis at an early age (Broadfoot *et al.*, 1954, 1956). Other associated problems are soft-shelled eggs or fully formed eggs found in the

peritoneal cavity, indicating that the yolk progressed normally through the oviduct to a certain point and then reverse peristalsis discharged the egg into the body cavity. A bird with a large accumulation of eggs in the peritoneal cavity may assume a penguin-like posture. Occasionally, an oviduct may be occluded by masses of yolk, coagulated albumen, shell membranes, and in some instances, fully formed eggs.

Egg-bound is used to describe a condition in which an egg is lodged in the cloaca but cannot be laid. It may result from inflammation of the oviduct, partial paralysis of the muscles of the oviduct, or production of an egg so large that it is physically impossible for it to be laid. Young pullets laying unusually large eggs are more prone to the problem. Poor egg quality and depressed EP are common problems that cause great economic loss to the poultry industry. They can be due to a multitude of factors involving nutrition, management, environment, and disease. Reviews related to the topic have been written by Hanson, (1968), Overfield (1970), Wolford and Tanaka (1970), and Peckham *et al.* (1984).

Structure and Pathology of MG

Mycoplasmas are members of the taxonomic class *Mollicutes,* and are among the smallest and simplest known pleiomorphic microorganisms (Panangala *et al*., 1992; Wise *et al*., 1992) capable of self-replication (Razin, 1981). Colonies are 1 mm in diameter and have a characteristic "fried-egg" appearance when viewed through a microscope (Kleven, 1998). The majority of these are aerobes, but they can be facultative anaerobes that form filaments resembling fungi. Filaments from fragmentation have the ability to sieve through filters small enough to retain most other bacteria because they are flexible

and range in size from 0.1 - 0.25 • m. The genome size of a mycoplasma is 5 x 10^8 Daltons (ranging from about 600 to 1300 kbp), which is one-third that of *Escherichia coli,* and is bound only by a tri-layered unit membrane (Clyde *et al*., 1983). This small genome size confers very limited metabolic capabilities. Mycoplasmas are small, delicate, morphologically irregular bacteria that lack a cell wall (Kleven, 1998), which makes them susceptible to cleaning and disinfecting agents. These extracellular parasites use membrane-membrane interactions for host cell attachment in the epithelium of the respiratory and urogenital tracts for nutritional purposes and to mediate disease. Pathogenesis of mycoplasma disease is a process influenced by the genetic background, environmental factors, and the presence of other infectious agents. However, there are a number of attributes possibly affecting disease pathogenicity, including the ability to attach, to cause cell injury, to vary phenotype at high frequency, and to modulate and resist the host immune response.

Mycoplasma gallisepticum (MG) infects nearly the entire flock, and tends to be more severe in young birds and during cold weather (Yoder, 1975; Yoder and Hopkins, 1985; Ley and Yoder, 1997). This simple microorganism cannot survive outside a host environment for a long period of time. Specifically, MG will live in manure for one to three days, on muslin cloth for three days, and in egg yolk for six weeks at 70 degrees F (Zander, 1984). The survival time for MG outside the environment of the host depends upon ambient temperature, the material on which it resides, and the type of growth medium (Chandiramani *et al.*, 1966; Simon *et al.*, 1989). Simon *et al.*, (1989) found that several poultry mycoplasmas have differing survival times on selected environmental

media. Viability of MG is dependent on the strain and the temperature at which MG-contaminated materials are held (Chandiramani *et al*., 1966). Mycoplasmas exist in over 125 different species and the National Poultry Improvement Plan only recognizes *M. gallisepticum, M. synoviae, M. iowae,* and *M. meleagridis* (NPIP, 1995). Over 20 serotypes of these species have been discovered. Transmission of MG can occur through the hatching egg and it is the major route of infection for the next generation (Kleven, 1981; Glisson and Kleven, 1984). A chick infected with only a few organisms may contaminate all the rest of the chicks in a flock. Although MG is fragile, its fragility is deceptive, because this organism can move short distances through the air. This may cause the infection of birds within a pen, but probably has little role in the transfer of disease from house to house. Clothing, feed bags, feed, egg filler flats, poultry equipment, trucks, and personnel represent the main means by which MG is transferred.

Infections of MG occur mainly in commercial avian species, however, game fowl may also serve as natural hosts. When there are no secondary infections to mycoplasmas, infection is often subclinical or mild (Kerr and Olson, 1967). Manifestations of MG usually occur in the respiratory system and lesions become extensive when complicated by other bacteria. Singularly, MG is not lethal, in fact, it may not always increase bird morbidity. However, an outbreak is quickly followed by a main secondary infection, which can then be harmful. *Coliform* organisms are particularly noteworthy as secondary infectious agents. Visible identification of MG is often confused by symptoms produced by secondary invaders. Respiratory disease in poultry has a complex etiology in which various species of mycoplasmas are only a single factor. There is a marked interaction

between mycoplasma, respiratory viruses, and bacteria, especially *E. coli* (Saif *et al*., 1970; Jordan, 1972; Springer *et al*., 1974; Stipkovits, 1979; Rhoades, 1981; Gross, 1990). Chronic respiratory infections associated with MG become more severe and adversely affect producer profit if the disease is complicated with Newcastle's Disease or Infectious Bronchitis exposure (Mohammed *et al*., 1987; Patterson, 1994). Clinical infection is often associated with a concurrent virus or bacterial infection, such as Newcastle's Disease, Infectious Bronchitis, Infectious Bursal Disease, *E. coli*, *Pasteurella*, and *Hemophilus* species (Kleven, 1998).

Environmental factors such as dust and ammonia, may also exacerbate lesion incidence and severity (Jordan, 1972; Springer *et al.*, 1974; Jordan, 1985). Intensive rearing or stress, crowding, cold weather, live virus vaccination, or natural virus infection may further intensify an MG infection. Zander (1984) listed the following factors as being associated with an increased risk of flock disease: increased human contact, multiple-age flocks, inter-flock movement of machinery, presence of wild birds, absence of wild bird-proofing, increased amounts of ammonia and dust through lack of ventilation, absence of frequent stirring and replacement of manure to reduce moisture content, insufficient carcass disposal and building cleaning, and absence of litter removal before flock replacement. Dormant MG remains in many birds but when those birds are exposed to a stressor, the organism is likely to spread throughout the flock.

An intense interest in examining the mechanism(s) of attachment of mycoplasmas to host cells has arisen in order to understand the initial steps of disease pathogenesis and perhaps to devise treatments based on inhibition of adherence. An excellent review of

mycoplasma adherence was described by Razin (1985). Electron microscopy enabled the identification of an apparent attachment organelle or tip structure (Uppal and Chu, 1977; Tajima *et al*., 1979; Razin *et al*., 1980; Kirchhoff *et al*., 1984; Levisohn, 1984), which allows mycoplasma to bind to sialoglycoproteins (Glasgow and Hill, 1980; Bredt *et al*., 1981; Kahane *et al*., 1984). Based on the possession of an actin-like material, MG is very motile (Clyde *et al*., 1983). Motility of mycoplasmas may contribute to their adherence to various structures *in vivo* (Razin, 1984). Attachment to the epithelial cell surface and underlying epithelial cells occurs after MG penetrates the mucus layer of the respiratory and urogenital tracts. Adherence is an important process, but it is unlikely to singularly produce the wide variety of effects seen in mycoplasma disease. Although many virulence factors have been suggested for various mycoplasmas, there is no clear case of cause and effect between these factors and the pathogenicity of mycoplasmas.

Localization of MG mainly occurs in the air sacs of the avian respiratory tract. Air sacs which may become involved, are cloudy in appearance, and are filled with mucus. In the latter stages of the disease, this mucus develops a yellow color and a cheesy consistency. Similar exudates may encircle the heart and heart sac. The epithelium of the air passages is most susceptible to infection. Effects of MG do occur in other organs, but lesions predominate in the respiratory tract. There is hypertrophy and hyperplasia of respiratory epithelium, including cells of the mucous glands in MG-infected birds (Trampel and Fletcher, 1981; Nunoya *et al.*, 1987, 1995). An infiltration of lymphoid cells leading to follicle formation in the lamina proper and heterophilic exudation in the airways are common. Lymphoid follicles also appear in the walls of the

air sacs. If complicated by *E. coli*, extensive pneumonia and air sacculitis are common, along with fibrinopurulent pericarditis and perihepatitis (Gross, 1990). Implications indicate that MG is an arthritogenic agent in poultry and can occasionally produce a mild arthritis; but, it usually is a result of septicemia following respiratory infection (Lamas da Silva and Adler, 1969).

Clinical disease with mycoplasmas alone occurs most often when young birds become infected with more virulent strains (Jordan, 1972). In young chicks there is a rattling, sneezing, and sniffling, which are indicative of respiratory difficulty. If complicated by other similar respiratory diseases, these symptoms are accentuated, however, the symptoms in adult birds could go unnoticed. Occasionally birds will appear depressed and inactive, and diarrhea may result during the intestinal phase of the disease. Subsequent depressions in EP may also result in hens, with symptoms varying in either endemic or epidemic proportions. The mechanisms involved in the production of cell injury are not well understood in most cases, but it is clear that several mycoplasmas have the capability to directly cause cell injury. In general, mycoplasma strains appear to be highly variable in their phenotypes, and many strains within the same species differ in their ability to cause disease after experimental infection. In addition, virulence of mycoplasmas appears to be related to the ability of the organisms to evade nonspecific defense mechanisms (Howard and Taylor, 1979; Davidson *et al.*, 1988), and this feature rapidly declines through passage in artificial media. The development of immunity to mycoplasmas can protect animals, as vaccination for mycoplasma diseases in animals is commonly practiced (Barile, 1985).

Colonization of MG in Various Tissues

It has been documented that MG can be cultured from tracheal, air sac, lung, and sinus exudates (Kleven and Yoder, 1989), and the choanal cleft/palatine fissure (Branton *et al.*, 1984; Brown *et al*., 1995), as well as the brain (Chin *et al*., 1991). A detailed histological examination of MG-infected chicken air sacs was provided by Trampel and Fletcher (1981). In that report, significant increases in total volume and numbers of epithelial cells, heterophils, mononuclear cells, fibrin, blood vessels, and connective tissue components were found in the air sacs from chickens having been inoculated with MG 21 d earlier. *Mycoplasma gallisepticum* can be transmitted from a hen to her eggs (Glisson *et al*., 1984). Also, MG has been cultured from the preovarian region (Fabricant and Levine, 1963), oviduct (Yoder and Hofstad, 1964; Carlson and Howell, 1967; Domermuth *et al*., 1967; Hitchner *et al*., 1980), liver, spleen, uterus, and vagina (Sahu and Olson, 1976) and cloaca of chickens (Amin and Jordan, 1979; MacOwan *et al*., 1983).

Current Diagnosis and Treatment of MG Infections in Chickens

Production of broilers, turkeys and egg layers free of MG, has been accomplished in the past few years, which economically justify the time and cost involved. Eradication has been the philosophy of the poultry industry toward MG for some time now and has worked well with primary breeding flocks, but it is not feasible in multi-age layer complexes from an economical standpoint (Yoder, 1978). The American poultry industry has been successful in reducing the number of mycoplasma related diseases, but high percentages of MG-infected layers still exist.

Isolation, identification, and serological tests, such as serum plate agglutination (SPA), hemagglutination inhibition (HI), and ELISA are used to diagnose MG infections. Because the SPA test is quick, relatively inexpensive, and sensitive it has been widely used as an initial screening test for flock monitoring and serodiagnosis. However, nonspecific reactors occur in some flocks infected with *Mycoplasma synoviae*, or those recently vaccinated with oil-emulsion vaccines and/or vaccines of tissue-culture origin against various agents (Bradbury and Jordan, 1972; Cullen and Timms, 1972; Roberts, 1970; Yoder, 1975; Glisson *et al*., 1984; Yoder and Hopkins, 1985; Yoder, 1989). The HI test has been commonly used to confirm reactors detected by SPA or, more recently, ELISA. The HI test, however, is time consuming, the reagents are not commercially available, and the test is not very sensitive (Ryan, 1973; Hitchner *et al*., 1980; Kleven *et al*., 1988; Dingfelder *et al*., 1991). The ELISA has been used by several researchers to devise a test that would be more sensitive and more specific for MG antibodies than either the SPA or HI test (Mallinson *et al*., 1985; Talkington *et al*., 1985; Higgins and Whithear, 1986; Avakian *et al*., 1988; Czifra *et al*., 1993). Commercial ELISA test kits are being used increasingly as an initial screening test for flock monitoring and serodiagnosis. Efforts continue to improve MG ELISA sensitivity and specificity by identifying and purifying specific immunodominant MG proteins for use as ELISA antigen (Opitz and Cyr, 1986; Avakian *et al*., 1991). As well, ELISAs have been used to detect MG antibodies in respiratory tract washings (Yagihashi and Tajima, 1986; Avakian and Ley, 1993) and egg yolk samples (Mohammed *et al*., 1986). Studies comparing egg yolk and serum for the detection of MG antibodies by ELISA or HI found comparable

results, indicating that egg yolk samples could be used instead of serum samples for flock screening (Yoder and Hopkins, 1985; Mohammed *et al*., 1986; Brown *et al*., 1991).

Serum of birds inoculated with MG may cross react with other species (Olson *et al.*, 1964) making field diagnosis difficult, therefore, cultivation has been facilitated and mycoplasmas role in the etiology of chronic respiratory disease in chickens has been confirmed by using improved medium for primary isolation (Adler, 1953; Olson *et al.*, 1954; Ibid, 1956; Adler *et al.*, 1958).

Vaccination with live-attenuated or killed-bacterin vaccines prevents the clinical signs of an MG infection. These vaccines are used on farms and facilities known to be infected with MG. Many vaccine strains are relatively low in virulence, poorly transmissible, and susceptible to commonly used antibiotics. Presently, vaccines have not resulted in eradication of MG, but have provided protection against moderate drops in EP in at least three studies, where the unvaccinated challenged control hens exhibited reduced EP (Yoder *et al*., 1984). Vaccination of young layers which have the potential to contract MG may increase their production rates. Application of immunizing agents against MG was reviewed by Adler (1976). Evidence suggests that recovery from natural, and sometimes experimental MG infection does provide some degree of protection against subsequent MG infection. During recent years, the use of live cultures of F-strain MG (FMG) has been evaluated as a means of counteracting reductions in EP in multiple-age egg-layer complexes caused by infections with MG (Carpenter *et al*., 1981; Lin and Kleven, 1982; Hildebrand *et al*., 1983). This live FMG vaccine is susceptible to antibiotics and disinfectants, does not readily spread, and causes only a

mild reaction. Continuous immunization with the live FMG vaccine in replacement pullet flocks on multiple age commercial layer sites may produce chicks resistant to infection from pathogenic strains or may actually displace the original field strain of MG (Levisohn and Kleven, 1981; Kleven *et al.,* 1990). A disadvantage of FMG vaccination, is that it will cause drops in EP in hens that are in lay. Vaccines should be developed against specific MG species which specifically induce effective host responses, and induce early steps in disease pathogenesis. These concepts include the limitations of current diagnostic techniques and their interactions with environmental factors and other infectious agents, which will affect interpretation of studies on the efficacy of any vaccinated form of MG.

Medication can only be considered a temporary solution and is usually quite expensive. Mycoplasmal bacteria was first discovered when such atypical infections responded to tetracycline, indicating that the agent was non-viral. Tetracycline inhibits protein synthesis in bacteria by blocking the A site on the ribosome, inhibiting binding of the aminoacyl-tRNA's. Tylosin is an antibiotic specific for the treatment of birds infected with MG. Gallimycin, aureomycin, erthromycin, spectinomycin, doxycline, streptomycin, and broad spectrum tetracyclines have been found to be an effective antibiotic against MG in growing and adults birds (Barnes *et al.*, 1960, 1961; Yoder *et al.*, 1961). These medication treatments may not stop MG infection or prevent the disease from spreading, but will prevent severe drops in EP. Tylosin is expensive and may lead to resistant organisms. Crawley and Fahey (1955) suggested repeated injections of an antibiotic into hens to prevent the transmission of MG through eggs. Antibiotic treatment

should be weighed against other control measures to ensure increased EP would be cost effective. Antibiotic medication is also used to reduce MG induced EP losses and MG transmission within a flock.

Eradication is built around complete curtailment of embryo transmission. Thus, infected dams must be removed as sources of infection. One or two infected birds in a pen will infect all others in a very short time. Primary breeders of broiler and egg-laying stock have practiced this method of eliminating MG from their flocks. Most multiplier flocks are also now negative to MG, but it is still a common disease in table-egg flocks because of the multiple-age flock practices in common use. On farms that practice all-in all-out housing of pullets and layers, it is practical to establish and maintain an MG-free flock. A national survey in 1983 revealed that 85 percent of farms that practiced all-in all-out management were MG-free (Johnson, 1983). In contrast, 75 percent of multi-age layer farms in the same study were MG positive. Since it is often impractical to totally depopulate and disinfect a large multi-age farm, the best solution in this case may be to establish an MG vaccination program (Bermudez and Kalbac, 1988). In situations where eradication appears unobtainable, controlling the production loses appears to be the only viable alternative (Mohammed *et al*., 1987; Patterson, 1994). The best alternative for the producer is total elimination of the infection by depopulation.

The development of licensed live MG vaccines was viewed as a possible advantageous option. These types of vaccines should provide a more uniform, standardized product to industry, make live MG vaccine equally available among interested states and producers, and decrease the spread of wild MG to neighboring

operations free of MG. Recent field and research reports suggest possible future productivity and control advantages to various combined uses of live and killed MG vaccines. Careless or widespread use of live MG vaccines should not occur under the approval and supervision of official state veterinarians or regulatory agencies. The use of FMG has been shown to be workable in several locations. Until a comprehensive national MG eradication program becomes a reality, the use of a standardized live vaccine appears to be a reasonable alternative. Several states have special permits to allow numerous layer-replacement flocks to be inoculated experimentally with live MG organisms. Disease-control officials and research scientists responsible for such studies report that mild transient respiratory signs in the exposed young chickens are followed by less severe reductions in peaks and rates of EP as the flocks mature (Gentry, 1978; Van Der Heide, 1977).

Effects of MG on Layer Performance

Egg producers experience lost revenues from poor feed conversion and increased medication costs, in addition to drops in EP and poor egg quality (Mohammed *et al*., 1987; Patterson, 1994). Infected birds have a drastic decrease in EP, therefore, MG is of substantial economic importance to egg facilities. Laying flocks positive for MG have been shown to produce as many as 16 fewer eggs per hen per year than MG-negative flocks (Carpenter *et al*., 1981). In essence, layers free from MG infection will lay 16 more eggs per hen than MG infected layers. When the performance of FMG vaccinated and MG infected flocks are compared, the FMG vaccinated flocks will produce 7.0 more eggs per hen per year (Carpenter *et al*., 1981). When uninfected flocks are compared to

vaccinated flocks, the advantage decreases to 8.7 eggs per hen. Past data indicates that FMG-vaccinated hens lay fewer large eggs than mycoplasma-clean hens. Approximately 43% of eggs laid in a 45 wk laying cycle are usually graded as large, reportedly decreasing by 8% in FMG vaccinated hens (Branton *et al*., 1999).

Branton *et al*., (1997) separated 80 commercial pullets into two treatments with four replicates of 10 chickens in each treatment. Forty pullets received no inoculation and were designated as controls, while 40 others were inoculated with FMG at 10 wk of age. Significant differences were observed for pimpling and blood meat spot incidence, and intra-follicular hemorrhage. Also, 8.25% more large eggs were observed in FMG clean hens. The results suggested that most of the reproductive tract functions similarly in FMG-vaccinated and clean hens (Branton *et al*., 1997).

In a study of 132 Pennsylvania flocks, FMG vaccinated layers produced seven more eggs per hen housed than unvaccinated MG infected layers over a 45 week laying period (Carpenter *et al*., 1981). In a similar study in California of 366 flocks, FMG vaccinated flocks produced six more eggs per hen housed than MG infected flocks (Khan *et al*., 1986). These two studies clearly demonstrate that an FMG vaccination program will prevent drops in EP due to a field strain MG infection. Glisson and Kleven (1984) tested the production of control, FMG vaccinated and MG bacterin vaccinated pullets all of which were challenged with field strain MG at 28 weeks of age. After 34 weeks of EP, controls, and FMG, and MG bacterin vaccinated layers produced 179, 192 and 193 eggs, respectively. A field study by Hildebrand *et al.*, (1983) compared FMG versus MG bacterin vaccination programs in 250,000 layers. In that study, MG bacterin vaccinated

birds produced significantly more eggs per hen housed in 64 weeks than FMG vaccinated birds. Kahn, *et al.* (1986) failed to demonstrate a protective effect with MG bacterin vaccination in layers. The efficacy of MG-bacterin and FMG in 14 commercial layer flocks was compared (Bermudez and Kalbac, 1988). In that study, a total of 1.1 million laying hens, all of the same brown egg breed, on two similar complexes, were studied. Both complexes housed FMG and MG-bacterin vaccinated birds and had a history of endemic MG infection. The five MG-bacterin vaccinated flocks and nine FMG vaccinated flocks produced an average of 275 and 264.7 eggs per hen-housed through 74 wk of age. These results were compared to the breed standard for EP of 276 eggs per hen-housed through 74 wk of age. The comparison suggested that the MG-bacterin was more efficacious in preventing drops in EP.

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CHAPTER III

EFFECTS OF F-STRAIN *MYCOPLASMA GALLISEPTICUM* INOCULATION AT TWELVE WEEKS OF AGE ON PERFORMANCE AND EGG CHARACTERISTICS OF COMMERCIAL EGG LAYING HENS

Abstract

The effects of F-strain *Mycoplasma gallisepticum* (FMG) inoculation during the pullet period on the subsequent performance and egg characteristics of commercial Single Combed White Leghorn hens were evaluated. In two trials, BW, feed consumption, egg production (EP), egg weight, egg size class, relative eggshell water vapor conductance, and relative eggshell, yolk and albumen weights were determined throughout a complete laying cycle (~ 60 wk of age). Feed and fecal compositional analyses were also performed. In each trial, pullets at 12 wk of age were randomly assigned to negative pressure biological isolation units. Birds in one-half of the total units were inoculated with FMG and the other half were sham-inoculated with sterile media. In both trials, onset of lay was delayed approximately one week in layers inoculated with FMG. Control birds, that had not been previously inoculated with FMG laid their first egg at 18 wk of age, while birds that had been previously inoculated with FMG laid their first egg at 19 wk of age. In Trial 1, FMG-inoculated hens laid significantly fewer total eggs, which became apparent at each wk after Week 42. In Trial 2, a numerical decrease in total EP occurred in FMG-inoculated chickens. In Trial 2, the percentage of undersized

eggs laid by FMG-inoculated birds was lower at 19 wk of age but was significantly higher at 20 and 21 wk when compared to controls. A significant increase in percentage eggshell occurred at 24 and 34 wk of age in FMG-inoculated birds in Trial 2. In Trial 1, fecal stearic acid was significantly lower in FMG-treated birds at 26 and 54 wk of age, which may be indicative of an FMG-related metabolic digestive disorder. Mortality was not significantly different between the treatments in either trial. These data demonstrate that when birds are housed in isolation facilities and inoculated with FMG at 12 wk of age, onset of lay and undersized EP are delayed, and total EP is decreased. It may be advisable to inoculate pullets with FMG at an age prior to 12 wk in order to avoid EPloss. (*Key words:* albumen, egg, *Mycoplasma gallisepticum,* shell, yolk)

Introduction

Mycoplasma gallisepticum (MG) is a pathogenic organism that can infect (Kreig and Holt, 1984) and cause problems primarily within the respiratory tract of laying hens (Ley and Yoder, 1978; Branton *et al*., 1984). Chronic respiratory disease, airsacculitis, air sac infection, and pleuropneumonia are commonly used synonymous names associated with MG infection (Yoder, 1978). It has been speculated that infection can also spread through the blood from the hen's respiratory tract to the oviduct, causing reduced egg production (EP) and poor egg quality (Yoder and Hofstad, 1964; Domermuth *et al*., 1967; Patterson, 1994). Feed consumption, BW, and EP have been reported to be reduced in MG-infected birds by Mohammed *et al*., (1987). A reduction in feed consumption of layer hens may alter the essential dietary components necessary to sustain adequate egg formation and EP.

The table egg industry experiences financial losses primarily attributable to decreased EP from naturally infected layers (Carpenter *et al*., 1981; Mohammed *et al*., 1987; Stadelman, 1988). Other losses experienced by egg producers include poor feed conversion and increased medication costs (Patterson, 1994). Although most commercial poultry flocks in the U. S. are raised free of MG via strategic biosecurity and monitoring programs, they are still at risk of infection. In spite of intensive eradication programs, the frequency of outbreaks in layer facilities has increased over recent years and susceptible birds can be readily infected by this organism (Kerr and Olson, 1964; Kleven, 1981; Branton *et al*., 1984; Yoder, 1991). Once a bird is infected with MG, it is generally considered chronically infected for life (Brown *et al*., 1995).

During the past several years, vaccination of commercial layers with live MG vaccine produced from F-strain (FMG) of low to moderate virulence have become available to protect flocks against natural MG infections (Branton *et al.*, 1997). The vaccine strain displaces natural field strain infections and has a low rate of bird-to-bird transmission (Levisohn and Kleven, 1981; Kleven *et al.*, 1990). Once vaccinated, the birds remain permanent carriers of FMG (Brown *et al*., 1995). Live vaccines are effective in minimizing EP losses if administered to commercial layers before exposure to more virulent field strains of MG (Luginbuhl *et al*., 1976). Inoculations with FMG between 8 and 18 wk of age allows a pullet to receive a mild infection and recover before coming into EP (Yoder *et al*., 1984). Layers vaccinated with FMG will produce more eggs than unvaccinated hens naturally infected with MG, while MG clean flocks have been reported

to lay more eggs than either FMG vaccinated or field strain MG infected hens (Carpenter *et al.*, 1981; Mohammed *et al.*, 1987).

There is a scarcity of information characterizing reproductive function and egg characteristics in commercial layers inoculated between 8 and 18 wk of age with FMG. Therefore, the present study was designed to determine EP, feed utilization, and a comprehensive profile of various egg quality parameters in commercial layers inoculated with FMG at 12 wk of age.

Materials and Methods

Pullet Housing and Management

In each of two trials, one thousand 1-d-old pullets of a single genetic strain were obtained from a commercial source that was monitored and certified free for MG and *M. synoviae* (MS) (National Poultry Improvement Plan and Auxiliary Provisions, 1995). Chickens were vaccinated at 10 d of age for infectious bursal disease via the drinking water. At 12 d and again at 4 wk of age, chickens were also vaccinated for Newcastle Disease and infectious bronchitis by the same route. At 5 wk of age, ten randomly selected pullets were bled from the left *cutanea ulnea* wing vein and tested for antibodies to MG and MS using both the serum plate agglutination (SPA) and the hemagglutination-inhibition (HI) tests (Yoder, 1975). At the same time, swabs were collected from the choanal cleft (Branton *et al.,* 1984) and placed into tubes containing Frey's broth medium (Frey *et al.*, 1968) supplemented with an additional 0.15 mg thallium acetate and 10^6 IU penicillin-G/mL. Tubes were incubated at 37 C for 30 d or until a phenol red indicator reaction occurred in the media. A sample from those that changed color was then

inoculated onto Frey's-based (Papageorgiou medium) agar and incubated at 37 C. Colonies with morphology suggestive of *Mycoplasma* species were examined by an agar plate fluorescent antibody (FA) method (Baas and Jasper, 1972) that used direct labeling of colonies stained with anti-FMG polyclonal antibodies produced in rabbits and labeled with fluorescein isothiocyanate (Kleven, 1981).

Up until the pullets were 12 wk of age, they were placed on clean dry litter in a 5.5 x 6.1 m section of a conventional house resulting in an initial flock density of 0.034 m^2/bird. A daily artificial lighting schedule followed a 13 h light (L):11 h dark (D) cycle. One 75-Watt incandescent light bulb was used to illuminate each 8.4 m^2 of floor space, providing a calculated intensity at bird level of 35.5 lux. Feed and water were provided for *ad libitum* consumption in each trial. Ingredient percentages and dietary analyses of the basal starter and grower diets used in both trials are provided in Table 3.1. All diets were formulated to meet or exceed National Research Council (1994) specifications. No medication was administered during the interval of either trial.

At 12 wk of age, 11 pullets were randomly selected and placed in each of 8 (Trial 1; total of 88 pullets) or 16 (Trial 2; total of 176 pullets) negative pressure fiberglass biological isolation units (1.16 m^2). The units were housed in a previously described poultry disease isolation facility (Branton and Simmons, 1992). Hen numbers were reduced to 10 per unit at point-of-lay (18 wk of age) so that bird density was 0.116 m^2/bird for the duration of each trial. In each trial, half of the total number of isolation units contained FMG-free control birds, whereas, the other half contained FMG-inoculated birds. There were four replicate units per treatment in Trial 1 and eight

replicate units per treatment in Trial 2. Beginning at 18 wk of age, the artificial lighting schedule was increased 15 min/day until a 16 h 15 min L:7 h 45 min D cycle was achieved. Chickens were maintained on that schedule through the remainder of the experiments. Ingredient percentages and dietary analyses of the basal developer, pre-lay, and layer diets used in both trials are also provided in Table 3.1. In both trials at 26 and 54 wk of age, quadruplicate feed samples per lot of mixed feed were analyzed for moisture, ash, CP, crude fat, and crude fiber. All determined analyses were performed according to the methods of the Association of Official Analytical Chemists (1980) and averaged for each of the two trials at each time period. Available protein and lysine percentages in the layer diet were adjusted according to the percentage of feed consumed per bird every 28 days until trial termination (54 wk in Trial 1 and 60 wk in Trial 2).

FMG Inoculation

In each trial, pullets treated with FMG were inoculated via eye drop in the right eye at 12 wk of age with 0.04 mL of a 24-hr broth culture of high-passage FMG (99^{th} passage above the unknown passage level) provided by Dr. S. H. Kleven (University of Georgia, Athens, GA). Inoculum titers were 5.0×10^6 and 1.0×10^5 cfu/mL in Trials 1 and 2, respectively. Similarly, pullets designated as controls were sham-inoculated via eye drop in the right eye at 12 wk of age with 0.04 mL of sterile Frey's broth medium.

Mycoplasma Detection

In each trial at 20 wk, and again at 54 wk in Trial 1 and 58 wk of age in Trial 2, one randomly selected hen from each of four FMG-free control and FMG-treated

isolation units was bled and swabbed. Each of these samples were tested for the presence of *Mycoplasma* species as previously described for pullets.

Data Collection

Individual BW of all hens in each unit were recorded at 12, 16, 20, 22, 24, 28, 30, 32, 36, 40, 44, 48, 52, and 54 wk of age in both trials. In Trial 2, BW was recorded as in Trial 1, but additionally at 34, 46, and 58 wk of age. Commensurate with the production of the first egg (18 wk of age) in control hens in both trials, eggs from control and treatment groups were collected daily until trial termination at 54 wk (Trial 1) and 60 wk (Trial 2). Egg production data for FMG-clean and FMG-inoculated hens in each trial was expressed as percentage hen-day production. Ten eggs per pen were weighed at 22, 24, 28, 30, 32, 36, 40, 44, 48, and 52 wk of age in both trials. In addition to egg weight (EW) being determined for the same weeks in Trial 2 as in Trial 1, in Trial 2 eggs were also weighed at 34, 46, and 58 wk of age. Egg size frequency distribution was determined by converting EW in grams to ounces and categorizing these as undersized, peewee, small, medium, large, extra-large, or jumbo sizes in accordance with the Agricultural Marketing Service of the United States Department of Agriculture (1996). At those same weeks listed above for EW, eggs were subsequently broken out to determine percentage yolk (PYW), albumen (PAW), and eggshell (PSW) weights and were expressed as percentages of EW. Eggshell moisture was removed according to the procedure of Brake *et al.* (1984). As specified by Peebles *et al.* (1998), at least ten eggs per replicate pen were gathered for measurement of relative eggshell water vapor conductance (RG). These eggs were collected separately but at the same time as those

used for the other egg quality determinations. Measurement of RG was as described by Peebles *et al.* (1994).

In both trials at 26 and 54 wk of age, one fecal sample from each of two replicate units belonging to each treatment, were randomly selected and analyzed for moisture, ash, CP, crude fat, crude fiber, and fatty acid content. Also, quadruplicate feed samples per lot of mixed feed were analyzed for fatty acid content. Determined fatty acid analyses of the layer diets are provided in Table 3.2. Between 42 and 58 wk in Trial 2, two randomly selected units (4 total) in both the control and treatment groups were used in a pilot study for the determination of feed consumption (g/bird/day) and feed conversion (g feed intake/g eggs produced). Total feed consumed and numbers of eggs produced per unit were recorded each week to derive feed consumption and conversion.

Statistical Analysis

A completely randomized experimental design was utilized. Body weight, EP, EW, egg size, PSW, PYW, PAW, RG, feed consumption and conversion, and fecal contents were subjected to a repeated measures analysis where the same experimental units were observed over an extended time period. Individual sample data within each replicate unit were averaged prior to analysis. Least-squares means were compared in the event of significant global effects (Steel and Torrie, 1980; Petersen, 1985; Freund and Wilson, 1997). All data were analyzed using the MIXED Procedure of SAS®, Version 8 (1996). Statements of significance were based on P • 0.05 unless otherwise stated.

Results

In both trials, all initial mycoplasmal cultures as well as SPA and HI test results obtained from 5-wk-old pullets were negative for MG and MS. Control serum samples obtained at 20 wk of age in each trial and also at 54 wk (Trial 1) and 58 wk (Trial 2) were SPA and HI negative for MG, while the same tests were positive for MG in the FMG-inoculated hens. Hens were considered FMG-free when they exhibited no detectable HI titers. All FMG-inoculated hens had HI titers • 1:80. Similarly, FA culture results for swabs obtained at 20 wk of age in each trial and also at 54 wk (Trial 1) and 58 wk (Trial 2) were negative for *Mycoplasma* species growth for 4 out of 4 FMG-free hens tested, while growth was evident for 4 out of 4 FMG-inoculated hens tested. Mortality was not significantly different between FMG-free and FMG-inoculated hens in either trial.

There was a significant (P • 0.0001) main effect due to layer hen age for BW in Trials 1 and 2 (Table 3.3). In general, birds in each trial grew as expected over the experimental periods, averaging 1520 g at 54 wk in Trial 1 and 1565 g at 58 wk in Trial 2. Layer hen age main effects were also observed in Trial 2 for EP (P • 0.0001), feed consumption (P • 0.03) and feed conversion (P • 0.0001). In Trial 2, at 22 (pre-peak), 32 (peak), and 60 wk of age (study termination), EP was 61.6±0.5, 80.4±0.5, and 68.2±0.5 percent, respectively. In Trial 2, at 42 and 58 wk, feed consumption (g/bird/day) was 99.5±0.5 and 118.8±0.5, respectively, and feed conversion (g feed intake/g eggs produced) was 2.35±0.5 and 2.63±0.5, respectively. In both trials, significant (P • 0.0001) hen age main effects were observed for EW. In Trial 1, at 22 (pre-peak), 32 (peak), and 54 wk of age (study termination), EW was 44.8±0.5, 54.1±0.5, and 58.8±0.5

g, respectively. In Trial 2, EW at 22 (pre-peak), 32 (peak), and 60 (study termination) wk was 45.3±0.5, 53.7±0.5, and 59.1±0.5 g, respectively. In Trial 1, a significant (P • 0.02) main effect due to layer hen age was observed for the percentage of undersized eggs that were laid. Those in the peewee, small, medium, large, and extra large egg size categories were significantly (P • 0.0001) affected by hen age in both trials. Furthermore, in both trials, significant (P • 0.0001) hen age main effects were observed for PAW, PYW, and RG, and in Trial 1, an age main effect (P • 0.0001) was observed for PSW. In Trial 1, significant main effects due to layer hen age were observed for fecal ash (P • 0.02), moisture (P • 0.03), fat (P • 0.03), fiber (P • 0.04), myristic acid (P • 0.04), and palmitoleic acid (P • 0.05) contents.

Initiation of lay was delayed eight days in Trial 1 and four days in Trial 2, for FMG-inoculated hens in comparison to uninoculated controls. In Trial 1, there was a significant (P • 0.05) reduction in total number of eggs laid per hen due to FMG-inoculation (Table 3.4). Although not significant (P = 0.1), total egg mass per hen was also reduced more than 1,000 g in Trial 1 due to FMG-inoculation. However, in Trial 2 number of eggs and egg mass per hen were not affected by FMG-inoculation (Table 3.4). In Trial 1, a significant (P • 0.003) age by FMG treatment interaction was observed for EP (Figure 3.1). A decline in EP of inoculated hens began at Week 44 so that EP became significantly less than that of uninoculated controls. This same comparative pattern of EP continued each week until the study was terminated at 54 wk of age. In Trial 2, there were significant age by FMG treatment interactions for percentage undersized EP (P • 0.0001) and PSW (P • 0.007). Percentages of undersized eggs laid by FMG-inoculated

birds were lower at 19 wk, but were higher at 20 and 21 wk when compared to controls (Table 3.5). Over the entire production period, a total of 31 undersized eggs were laid by the FMG-infected birds, compared to 18 that were laid by the FMG-free birds. Percentage eggshell weight at 24 and 34 wk of age was significantly higher in FMG-inoculated birds when compared to controls (Table 3.6). In Trial 1, there was a significant (P • 0.03) age by FMG treatment interaction for fecal stearic acid. The percentage of stearic acid in the feces of FMG-inoculated birds was significantly lower at 26 and 54 wk of age compared to controls. At 26 wk and 54 wk of age, fecal stearic acid was 7.8 and 4.2% in control hens and 5.7 and 3.3% in FMG-inoculated hens, respectively (SEM=0.59).

Discussion

As described by Zander (1984), MG is already established on many multi-age farms, and transmission from mature hens to replacement pullets ensures its existence. Unfortunately, since it is often impractical to totally depopulate and disinfect a large multi-age farm, the best solution in this case may be to establish an MG vaccination program (Bermudez and Kalbac, 1988). The development of immunity to mycoplasmas can protect animals, as vaccination for mycoplasma diseases in animals is commonly practiced (Barile, 1985), where the disease is endemic. These results from the current study indicate that there was no cross contamination between FMG-inoculated and FMG-clean birds. Zero cross contamination between FMG-inoculated and FMG-clean birds is indicative of a strategic biosecurity and sanitation program (Patterson, 1994). Mortality was negligible when FMG-inoculated birds were housed in temperature regulated

isolation units. Also, Branton and Deaton (1985), reported that although mortality may be negligible in adult flocks infected with FMG, there still can be a reduction in the number of birds in production. At the beginning and end of both trials in this study, SPA tests from swabs and sera and HI sera tests, along with the FA tests verified systemic infections in FMG-inoculated birds. Conversely, sham-inoculated birds remained FMG-free throughout each trial. Age-related changes in EW and EP in the current study were similar to those in an earlier study of layer hens (Branton *et al*., 1997). Those authors found that inoculation with FMG at 10 wk of age did not affect EW or EP. Other authors have reported that EP, BW, and feed efficiency were reduced in flocks naturally infected with MG (Yoder, 1978, 1991; Mohammed *et al.*, 1987). Variables such as PAW, PSW, PYW, and RG, along with feed consumption and conversion, and fecal analysis had not been previously explored until the present trials. All egg and eggshell quality parameters selected for this study were chosen to reflect the developmental process of eggs in chickens. Feed and fecal analysis provided additional dietary information and were similar in each trial.

In the existing MG literature, a delay in EP in MG-vaccinated or infected hens as compared to MG-free hens has not been reported. However, in both trials of this study, all birds inoculated with FMG at 12 wk of age laid their first egg approximately one wk after FMG-free controls.

Reduced EP and financial losses have been attributed to MG-infection in layers (Mohammed *et al*., 1987; Stadelman, 1988). In Trial 1, FMG-inoculated birds had a significantly lower total EP, which was mainly due to significantly lower hen-day EP

after 42 wk. Each control hen laid approximately 19 more eggs during the trial period than any FMG-inoculated hens. This has also been shown in laying flocks positive for field strain MG where they produce as many as 16 fewer eggs per hen per year than MG-negative flocks (Carpenter *et al*., 1981). When the performance of FMG vaccinated and MG infected flocks are compared, FMG-vaccinated flocks will produce 7.0 more eggs per hen per year (Carpenter *et al*., 1981). In that same study, when uninfected flocks were compared to FMG-vaccinated flocks, the advantage decreased to 8.7 eggs per hen. However, Branton *et al*. (1997) reported that there was no difference in EP over a 45 week laying period when FMG-free and FMG-inoculated hens were compared. Branton *et al.*, (2000) also reported that there was no difference in EP when control and ts-11 vaccine strain MG inoculated hens were compared. Glisson and Kleven, (1984) reported that all hens vaccinated with low virulence MG at 16 or 20 wk of age were protected against EP drops seen in unvaccinated hens challenged with virulent MG. Other reports have described variable levels of protection against decreases in EP of hens vaccinated with low-virulence live MG (Truscott *et al*., 1974; Fabricant, 1977).

Past data indicates that FMG-vaccinated hens lay fewer large eggs than Mycoplasma-clean hens (Branton *et al*., 1999). Specifically, 43% of eggs laid in a 45 wk laying cycle are usually sized as large and this phenomena reportedly decreases by approximately 8% in FMG vaccinated hens. Also, Branton *et al.* (2002) found a significant increase in jumbo-sized eggs laid by 6/85 strain MG vaccinated hens as compared to control hens. The lower percentage of undersized eggs laid by FMG-inoculated hens at 19 wk and the higher percentage of similarly sized eggs laid at both 20

and 21 wk in the present study, is indicative of a delay in EP in FMG-infected birds. As previously stated, initiation of lay in control hens began earlier than FMG-inoculated hens. Undersized eggs were being laid by control hens until the beginning of lay occurred in FMG-inoculated hens. Concurrently, when treated birds began to lay undersized eggs, control hens shifted to a larger size category.

A lack of information on shell characteristics in MG infected birds exists in the literature; however, in the present study relative PSW was significantly increased at 24 and 34 wk of age in FMG-inoculated birds. This may indicate that infected hens deposited relatively more calcium carbonate on eggs during peak-production than those not infected with FMG. Conductance through eggshell pores provides a mechanism of chemical exchange with the environment (Tullett, 1984; Paganelli *et al*., 1990). Although, RG may be insignificant for infertile eggs produced from FMG-infected laying hens, it may be an important aspect in FMG-infected breeder and broiler breeder operations. This information may provide vital insights as to the effects of MG colonization in the oviduct.

Fatty acids are essential in the growth and performance of laying hens (Austic and Scott, 1997). These data indicate that FMG-inoculated birds have a significantly lower percentage of fecal stearic acid at 26 and 54 wk of age as compared to controls. Fecal stearic acid decreased at 26 wk of age and may be associated with the delayed onset of lay, while decreases at 54 wk of age may also be associated with lowered EP observed in the FMG-inoculated hens. Since nutritional balance is a key factor in layer hen production, a detailed feed investigation is planned in a subsequent trial. After reviewing

the aforementioned variables, other possible FMG-mechanistic considerations include blood, reproductive, and digestive tissue characteristics.

Mycoplasma gallisepticum varies from being a subtle, sub-clinical, disease to an overt severe clinical disease and variation of MG phenotypes and surface antigens may account for these different virulent patterns (Howard and Taylor, 1979). In addition, virulence of mycoplasmas appears to be related to the ability of the organisms to evade nonspecific defense mechanisms and this feature rapidly declines through passage in artificial media (Davidson *et al.*, 1988). Since, environmental factors, contact with host cells, and other infectious agents influence the severity of mycoplasma infections, these isolation experiments are necessary to provide an understanding of the pathogenic processes associated with MG. These experiments may lead to new approaches for the treatment and control of MG.

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TABLE 3.1 Ingredient percentages and calculated and determined analyses of pullet and layer diets

	Starter	Grower	Developer	Prelay	Layer[1]					
Age (week)	0-6	6-12	12-18	18-20	20	28	32	36	40	44-60
Ingredients:	(%)									
Corn, 8.6%	64.51	73.64	72.22	61.35	58.11	64.93	68.39	71.33	63.38	70.47
Soybean meal, 48%	30.97	22.09	17.17	19.13	27.74	23.16	20.35	17.44	24.49	18.29
Wheat middlings	0.00	0.00	6.39	11.67	0.00	0.00	0.00	0.00	0.00	0.00
Vitamin premix[2,3]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
DL-methionine[4]	0.15	0.11	0.10	0.13	0.22	0.16	0.12	0.08	0.17	0.09
Dicalcium phosphate[5]	2.08	1.99	1.92	1.68	2.00	1.81	1.81	1.81	1.81	1.81
Limestone[6]	1.06	0.95	0.98	4.82	9.25	8.66	8.06	8.06	8.71	8.06
Sodium chloride[7]	0.48	0.47	0.47	0.47	0.53	0.53	0.53	0.53	0.53	0.53
Poultry fat	0.50	0.50	0.50	0.50	1.90	0.50	0.50	0.50	0.65	0.50
Dietary analyses:										
CP, calculated	20.50	17.00	15.50	16.34	18.09	16.40	15.31	14.12	16.95	14.47
CP, determined	ND[8]	ND	ND	ND	18.70	ND	ND	ND	ND	14.55
Crude fiber, calculated	2.29	2.24	2.55	2.76	2.17	2.21	2.21	2.20	2.21	2.20
Crude fiber, determined	ND	ND	ND	ND	3.75	ND	ND	ND	ND	2.70
Crude fat, calculated	3.22	3.52	3.68	3.48	4.27	3.12	3.23	3.32	3.22	3.30
Crude fat, determined	ND	ND	ND	ND	4.00	ND	ND	ND	ND	2.85
Ash, determined	ND	ND	ND	ND	13.25	ND	ND	ND	ND	17.50
Moisture, determined	ND	ND	ND	ND	11.35	ND	ND	ND	ND	11.30
ME, calculated kcal/kg	3,000	3,101	3,051	2,819	2,819	2,828	2,879	2,910	2,819	2,901

TABLE 3.1 Continued.

Available phosphorus, calculated	0.43	0.42	0.42	0.38	0.37	0.33	0.34	0.34	0.34	0.34
Calcium, calculated	0.88	0.82	0.82	2.25	4.00	3.73	3.50	3.50	3.75	3.50
Lysine, calculated	1.10	0.85	0.73	0.80	0.97	0.85	0.77	0.69	0.88	0.71
Methionine, calculated	0.50	0.42	0.38	0.41	0.52	0.44	0.40	0.35	0.47	0.36
Methionine + cystine, calculated	0.81	0.68	0.61	0.65	0.80	0.70	0.63	0.56	0.73	0.58
Potassium, calculated	0.81	0.66	0.55	0.56	0.72	0.65	0.61	0.56	0.67	0.57
Sodium, calculated	0.20	0.20	0.20	0.20	0.21	0.21	0.21	0.21	0.21	0.21
Tryptophan, calculated	0.28	0.23	0.20	0.22	0.25	0.22	0.20	0.19	0.23	0.19
Xanthophyll, calculated	6.45	7.36	7.22	6.14	5.81	6.49	6.84	7.13	6.34	7.05

[1]Available protein and lysine percentages in the layer diet were adjusted as needed according to the percentage of feed consumed per bird every 28 days until trial termination.

[2]Vitamin premix provided per kilogram of diet: vitamin A, 7,710 IU; cholecalciferol, 2,202 IU; vitamin E, 10 IU; menadione, 0.88 mg; vitamin B_{12}, 0.01 mg; choline, 380 mg; riboflavin, 5 mg; niacin, 33 mg; pantothenic acid, 9 mg; thiamine, 1 mg; folic acid, 0.6 mg; biotin, 0.06 mg; pyridoxine, 0.9 mg; ethoxyquin, 0.03 g.

[3]Trace minerals provided in vitamin premix: manganese, 2.2%; zinc, 2.0%; iron, 1.1%; copper, 1,400 ppm; iodine, 200 ppm; and selenium, 40 ppm.

[4]Manufactured by Degussa Corp., Ridgeland Park, NJ 07600-2100.

[5]Manufactured by IMC-Agrico Feed Ingredients, Bannockburn, IL 60015.

[6]Manufactured by Franklin Industrial Minerals, Nashville, TN 37203.

[7]Manufactured by Cargill Incorporated, Minneapolis, MN 55440.

[8]Not determined.

TABLE 3.2 Determined fatty acid analyses of layer diets at 26 and 54 weeks of age in Trials 1 and 2

		Trial			
		1	2	1	2
Age (week)		26		54	
		----------(%)----------			
Determined Fatty Acid Analyses:					
Myristic	(c14:0)	0.0	0.0	0.9	0.6
Palmitic	(c16:0)	17.8	17.9	17.0	15.4
Palmitoleic	(c16:1)	3.8	3.9	1.8	1.7
Stearic	(c18:0)	4.3	3.3	3.8	4.2
Oleic	(c18:1)	33.1	31.6	28.2	32.0
Linoleic	(c18:2)	38.3	42.3	45.3	43.4
Linolenic	(c18:3)	1.1	1.0	1.3	1.3

TABLE 3.3 Body weight (g) of laying hens at 12, 16, 20, 22, 24, 28, 30, 32, 34, 36, 40, 44, 46, 48, 52, 54, and 58 weeks of age in Trials 1 and 2

Age (week)	Trial 1[1]	Trial 2[1]
12	924.13[j,2]	936.16[j,2]
16	1160.41[i]	1107.36[i]
20	1321.11[h]	1310.95[h]
22	1364.91[g]	ND
24	1375.26[g]	1344.92[g]
28	1409.90[f]	1365.03[f]
30	1408.39[f]	ND
32	1426.25[e]	ND
34	ND[3]	1425.73[e]
36	1459.11[d]	ND
40	1468.57[d]	1468.84[d]
44	1484.05[c]	ND
46	ND	1510.81[c]
48	1538.54[a]	ND
52	1521.55[b]	1526.47[b]
54	1519.87[b]	ND
58	ND	1565.17[a]

[a-k]Means within trial among week of age with no common superscript differ significantly (P• 0.05).

[1]Based on pooled estimate of variance SEM = 14.5 in Trial 1 and 14.07 in Trial 2.

[2]n = 80 in Trial 1 and n = 160 in Trial 2.

[3]Not determined.

TABLE 3.4 Mean eggs per hen and egg mass per hen from F-strain *Mycoplasma gallisepticum* (FMG)-free and FMG-inoculated hens throughout lay in Trials 1 and 2

	Trial 1[1]		Trial 2[2]	
Treatment	Eggs/hen	Egg mass/hen (g)	Eggs/hen	Egg mass/hen (g)
FMG-free	197[a]	10,800	192	10,565
FMG-inoculated	178[b]	9,775	190	10,500

[a,b]Means within trial and parameter among treatment groups with no common superscript differ significantly (P• 0.05).

[1]n = 80, and based on pooled estimate of variance SEM = 5.27 for eggs/hen and SEM = 490 for egg mass/hen.

[2]n = 160, and based on pooled estimate of variance SEM = 12.05 for eggs/hen and SEM = 1,060 for egg mass/hen.

TABLE 3.5 Percentage undersized eggs in F-strain *Mycoplasma gallisepticum* (FMG)-free and FMG-inoculated hens at 19, 20, and 21 weeks of age in Trial 2[1]

	Undersized Eggs	
Age (week)	FMG-free	FMG-inoculated
	----------(%)----------	
19	6.25[a,2]	0.00[b]
20	2.80[b]	13.64[a]
21	0.78[b]	4.10[a]

[a,b]Means within week of age among treatment groups with no common superscript differ significantly (P• 0.05).

[1]Based on pooled estimate of variance SEM = 1.16.

[2]n = 80.

TABLE 3.6 Shell weight as a percentage of fresh egg weight in F-strain *Mycoplasma gallisepticum* (FMG)-free and FMG-inoculated hens at 22, 24, 28, 34, 40, 46, 52, and 58 weeks of age in Trial 2[1]

Age (week)	FMG-free	FMG-inoculated
	----------(%)----------	
22	8.92[2]	9.02
24	8.45[b]	9.21[a]
28	8.96	9.15
34	8.56[b]	8.97[a]
40	8.74	8.95
46	8.71	8.51
52	8.59	8.37
58	8.57	8.70

[a,b]Means within age among treatment group with no common superscript differ significantly (P• 0.05).
[1]Based on pooled estimate of variance SEM = 0.13.
[2]n = 40.

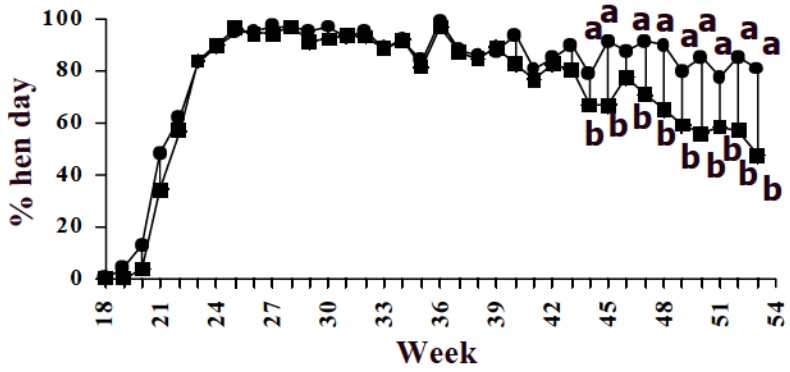

FIGURE 3.1 Weekly percent hen day egg production at 18-54 weeks of age for F-strain *Mycoplasma gallisepticum* (FMG)-free (•) versus FMG-inoculated (•) layer hens in Trial 1. Symbols within a week having different letters are significantly different (P • 0.05). Based on pooled estimate of variance SEM = 4.72.

CHAPTER IV

EFFECTS OF F-STRAIN *MYCOPLASMA GALLISEPTICUM* INOCULATION AT TWELVE WEEKS OF AGE ON DIGESTIVE AND REPRODUCTIVE ORGAN CHARACTERISTICS OF COMMERCIAL EGG LAYING HENS

Abstract

Because experimental inoculation with the F-strain of *Mycoplasma gallisepticum* (FMG) during the pullet period is known to affect reproductive performance in commercial layers, two trials were conducted to determine if changes in digestive and reproductive organ characteristics also occur in commercial laying hens infected with FMG at 12 wk of age. In Trial 1, liver weight, liver lipid and moisture contents, ovary weight, ovarian follicular hierarchy, and the weights, lengths, and histologies of the infundibulum, magnum, isthmus, uterus, and vagina were determined. In Trial 2, Fatty Liver Hemorrhagic Syndrome (FLHS) incidence, and the weights, lengths, and histologies of the duodenum, jejunum, and ileum were determined in addition to the parameters examined in Trial 1. In both trials, fewer ripe (diameter • 12 mm) ovarian follicles existed in FMG-inoculated hens. In Trial 1, FMG-inoculation resulted in a significant reduction in ovarian follicle size. Also, magnum/oviduct (cm/cm) length was significantly reduced in treated birds. In Trial 2, 25% of the FMG-inoculated birds had zero ripe follicles at 20 wk of age compared to 0% in control hens. Approximately 50% of FMG-inoculated hens at 44 wk of age had an average of seven ripe ovarian follicles

compared to 0% in control hens. Also, in Trial 2, isthmus/BW and isthmus/oviduct (g/g) weight was significantly decreased at 46 wk of age, and vagina/BW and vagina/oviduct (g/g) weight was decreased at both 20 and 36 wk of age due to FMG treatment. In Trial 2, FMG treatment resulted in a 50% increase in the number of FLHS birds. Furthermore, treatment caused a decrease at 20 wk of age and an increase at 44 wk of age in liver moisture content. Altered liver, ovarian, and reproductive organ characteristics were associated with FMG infection in commercial layers. More specifically, FMG-inoculation at 12 wk resulted in a higher incidence of FLHS, ovarian follicular regression, and decreased isthmal and vaginal proportions of the reproductive tract. These data clearly demonstrate that alterations in performance and egg characteristics of layers inoculated with FMG at 12 wk of age are related to mutual functional disturbances in the liver, ovary, and oviduct without concomitant intestinal changes.

(*Key words:* layer, liver, *Mycoplasma gallisepticum,* reproductive tract, small intestine)

Introduction

Mycoplasma gallisepticum (MG) is an infectious gram negative bacterium (Razin and Freundt, 1984), which infects nearly the entire flock, and tends to be more severe in young birds and during cold weather (Ley and Yoder, 1997). Layer hens infected with MG develop conjunctivitis and other air sac distresses (Soeripto *et al.*, 1989; Nunoya *et al.*, 1995). A detailed histological examination of MG-infected chicken air sacs was provided by Trampel and Fletcher (1981). In that report, significant increases in total volume and numbers of epithelial cells, heterophils, mononuclear cells, fibrin, blood vessels, and connective tissue components were found in the air sacs from chickens

having been inoculated with MG 21 d earlier. Also, it has been documented that MG can be cultured from tracheal, air sac, lung, and sinus exudates (Kleven and Yoder, 1989), and the choanal cleft/palatine fissure (Branton *et al.*, 1984), as well as the brain (Chin *et al*., 1991). Along with affecting the respiratory apparatus, MG also affects the reproductive performance of commercial layers (Mohammed *et al*., 1987; Burnham *et al.*, 2001, 2002). Early inoculation or vaccination with F-strain MG (FMG) may help reduce losses in the performance of birds as a result of infection by field strains of MG (Luginbuhl *et al*., 1976; Yoder *et al*., 1984).

Mycoplasma gallisepticum can be vertically transmitted from a hen to her eggs (Glisson *et al*., 1984). Also, MG has been cultured from the preovarian region (Fabricant and Levine, 1963), oviduct (Carlson and Howell, 1967; Domermuth *et al*., 1967; Hitchner *et al*., 1980), liver, spleen, uterus, and vagina (Sahu and Olson, 1976) and cloaca of chickens (Amin and Jordan, 1979; MacOwan *et al*., 1983). This information warrants investigation into the possible involvement of digestive and reproductive organ changes with those of performance in MG-infected hens. No literature is available concerning these organ system characteristics in MG-infected layers. Hepatic lipidosis, referred to as Fatty Liver Syndrome, often precedes Fatty Liver Hemorrhagic Syndrome (FLHS), which has been associated with heat stress in environmental birds (Couch 1956; Riddell, 1997). However, Branton *et al*., (2001) also reported that FLHS incidence was delayed in hens inoculated at 10 wk of age with *Mycoplasma gallinarum*. Because *Mycoplasma gallinarum* is non-pathogenic in avian species (Kleven, 1991), the affects of FMG, a pathogenic strain, on FLHS incidence was also investigated in this study.

This study was designed to characterize possible physiological changes, including digestive and reproductive organ characteristics, throughout a complete egg laying cycle in commercial layers vaccinated with FMG. The organs examined included the liver, small intestine, ovary, and oviduct. Furthermore, the relative lengths and weights of the individual segments that comprise the small intestine and oviduct were examined.

Materials and Methods

Pullet Housing and Management

In each of two trials, one thousand 1-d-old pullets of a single genetic strain were obtained from a commercial source that was monitored and certified free for MG and *M. synoviae* (MS) (National Poultry Improvement Plan and Auxiliary Provisions, 1995). Chickens were vaccinated at 10 d of age for infectious bursal disease via the drinking water. At 12 d and again at 4 wk of age, chickens were also vaccinated for Newcastle Disease and infectious bronchitis by the same route. At 5 wk of age, ten randomly selected pullets were bled from the left *cutanea ulnea* wing vein and tested for antibodies to MG and MS using both the serum plate agglutination (SPA) and the hemagglutination-inhibition (HI) tests (Yoder, 1975). At the same time, swabs were collected from the choanal cleft (Branton *et al.,* 1984) and placed into tubes containing Frey's broth medium (Frey *et al.*, 1968) supplemented with an additional 0.15 mg thallium acetate and 10^6 IU penicillin-G/mL. Tubes were incubated at 37 C for 30 d or until a phenol red indicator reaction occurred in the media. A sample from those that changed color was then inoculated onto Frey's-based (Papageorgiou medium) agar and incubated at 37 C. Colonies with morphology suggestive of *Mycoplasma* species were examined by an agar

plate fluorescent antibody (FA) method (Baas and Jasper, 1972) that used direct labeling of colonies stained with anti-FMG polyclonal antibodies produced in rabbits and labeled with fluorescein isothiocyanate (Kleven, 1981).

Up until the pullets were 12 wk of age, they were placed on clean dry litter in a 5.5 x 6.1 m section of a conventional house resulting in an initial flock density of 0.034 m^2/bird. A daily artificial lighting schedule followed a 13 h light (L):11 h dark (D) cycle. One 75-Watt incandescent light bulb was used to illuminate each 8.4 m^2 of floor space, providing a calculated intensity at bird level of 35.5 lux. Feed and water were provided for *ad libitum* consumption in each trial. Ingredient percentages and dietary analyses of the basal starter and grower diets used in both trials are provided in Table 4.1. All diets were formulated to meet or exceed National Research Council (1994) specifications. No medication was administered during the interval of either trial.

At 12 wk of age, 11 pullets were randomly selected and placed in each of 8 (Trial 1; total of 88 pullets) or 16 (Trial 2; total of 176 pullets) negative pressure fiberglass biological isolation units (1.16 m^2). The units were housed in a previously described poultry disease isolation facility (Branton and Simmons, 1992). Hen numbers were reduced to 10 per unit at point-of-lay (18 wk of age) so that bird density was 0.116 m^2/bird for the duration of each trial. In each trial, half of the total number of isolation units contained FMG-free control birds, whereas, the other half contained FMG-inoculated birds. There were four replicate units per treatment in Trial 1 and eight replicate units per treatment in Trial 2. Beginning at 18 wk of age, the artificial lighting schedule was increased 15 min/day until a 16 h 15 min L:7 h 45 min D cycle was

achieved. Chickens were maintained on that schedule through the remainder of the experiments. Ingredient percentages and dietary analyses of the basal developer, pre-lay, and layer diets used in both trials are also provided in Table 4.1. In both trials at 26 and 54 wk of age, quadruplicate feed samples per lot of mixed feed were analyzed for moisture, ash, CP, crude fat, and crude fiber. All determined analyses were performed according to the methods of the Association of Official Analytical Chemists (1980) and averaged for each of the two trials at each time period. Available protein and lysine percentages in the layer diet were adjusted according to the percentage of feed consumed per bird every 28 days until trial termination (54 wk in Trial 1 and 60 wk in Trial 2).

FMG Inoculation

In each trial, pullets treated with FMG were inoculated via eye drop in the right eye at 12 wk of age with 0.04 mL of a 24-hr broth culture of high-passage FMG (99^{th} passage above the unknown passage level) provided by Dr. S. H. Kleven (University of Georgia, Athens, GA). Inoculum titers were 5.0×10^6 and 1.0×10^5 cfu/mL in Trials 1 and 2, respectively. Similarly, pullets designated as controls were sham-inoculated via eye drop in the right eye at 12 wk of age with 0.04 mL of sterile Frey's broth medium.

Mycoplasma Detection

In each trial at 20 wk, and again at 54 wk in Trial 1 and 58 wk of age in Trial 2, one randomly selected hen from each of four FMG-free control and FMG-treated isolation units was bled and swabbed. Each of these samples were tested for the presence of *Mycoplasma* species as previously described for pullets.

Data Collection

Trial 1 was terminated at 54 wk and Trial 2 at 60 wk of bird age. At those times, five birds from each of four control and four treated units in Trial 1 and four birds from each of six control and six treated units in Trial 2 were randomly selected and euthanized by cervical dislocation and their organs removed. Similarly, in Trial 2, two randomly selected birds from each of two control and two treated units were euthanized and their organs removed at 20, 36, 44, 46, and 48 wk of age. In both trials, organ analyses included liver weight, lipid, and moisture content, ovary weight and follicular hierarchy, and the weights, lengths, and histologies of the oviduct, infundibulum, magnum, isthmus, uterus, and vagina (Figure 7.6). In Trial 2, incidence of FLHS and the weights, lengths, and histologies of the duodenum, jejunum, and ileum were also examined in addition to the above mentioned parameters (Figure 7.5). Intestinal and oviductal segment weights were calculated as percentages of total body and organ weight; and segment lengths were calculated as percentages of total organ length.

Ripe Follicle Quantitation

In both trials, the entire ovary was removed and the number of ripe (diameter • 12 mm) yellow ovarian follicles was recorded for each bird. A caliper was used to measure follicle diameter. Based on the number of ripe ovarian follicles present in each bird, a categorical number from zero to seven was assigned. A number of zero indicated that a bird had no ripe follicles present. A maximum of seven ripe follicles were recorded. Average number of ripe follicles and the percentage of birds having zero, one, two, three, four, five, six, or seven ripe follicles in each replicate unit were analyzed.

Liver Moisture and Lipid Analysis

For analysis of liver moisture content, fresh liver samples (2 g) were dried according to the procedure of Peebles *et al*. (1999) in a commercial oven (Model EL20, General Electric Co., Chicago Heights, IL 60411). Liver moisture content was calculated as the difference between the wet and dry weights of the sample and was expressed as a percentage of wet sample weight. For analysis of liver lipid content, lipid was extracted from fresh liver samples (3 g) according to the procedure previously described by Bligh and Dryer (1959), and as modified by Latour *et al.* (1998). Liver lipid content was expressed as a percentage of total fresh liver sample weight.

Histopathologic Examination

Upon termination of each trial, one tissue sample from the ovary, infundibulum, magnum, isthmus, uterus, and vagina was harvested from one hen in each of four units in both control and treatment groups. Tissue samples were placed in 10% buffered neutral formalin, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin. Each tissue sample was observed and scored for the presence or absence of lymphoid and heterophil infiltrates as described by Branton *et al*., (2000). Treatment assignments were unknown to the evaluator.

Statistical Analysis

A completely randomized experimental design was utilized. All data were subjected to a one-way analysis in Trial 1 and a repeated measures analysis in Trial 2. Individual sample data within each replicate unit were averaged prior to analysis. Least-squares means were compared in the event of significant global effects (Steel and Torrie,

1980; Petersen, 1985; Freund and Wilson, 1997). All data were analyzed using the MIXED Procedure of SAS®, Version 8 (1996). Statements of significance were based on P • 0.05 unless otherwise stated.

Results

In both trials, all initial mycoplasmal cultures as well as SPA and HI test results obtained from 5-wk-old pullets were negative for MG and MS. Control serum samples obtained at 20 wk of age in each trial and also at 54 wk (Trial 1) and 58 wk (Trial 2) were SPA and HI negative for MG, while the same tests were positive for MG in the FMG-inoculated hens. Hens were considered FMG-free when they exhibited no detectable HI titers. All FMG-inoculated hens had HI titers • 1:80. Similarly, FA culture results for swabs obtained at 20 wk of age in each trial and also at 54 wk (Trial 1) and 58 wk (Trial 2) were negative for *Mycoplasma* species growth for 4 out of 4 FMG-free hens tested, while growth was evident for 4 out of 4 FMG-inoculated hens tested. At necropsy, all digestive and reproductive tracts appeared normal through gross observation. Interestingly, one FMG-inoculated hen possessed functional left and right reproductive tracts. No significant differences were observed through histopathologic lesion scoring between treatments for any of the tissues sampled.

When expressed as percentages of BW, significant main effects due to layer age were observed for liver (P • 0.0001), ovary (P • 0.0001), oviduct (P • 0.004), infundibulum (P • 0.009), magnum (P • 0.03), uterus (P • 0.0001), small intestine (P • 0.0001), duodenum (P • 0.0001), jejunum (P • 0.0001), and ileum (P • 0.004) weights in Trial 2. In that same trial, liver lipid content changed significantly (P • 0.005) with hen

age. Furthermore, infundibulum weight (P • 0.05) as a percentage of oviduct weight; absolute lengths of the small intestine (P • 0.006) and oviduct (P • 0.0001); and infundibulum (P • 0.0002), magnum (P • 0.003), isthmus (P • 0.0001), uterus (P • 0.0001), and vagina (P • 0.0001) lengths as percentages of total oviduct length, were significantly affected by layer age. In general, oviduct and small intestine weights and lengths from birds in Trial 2 increased normally over the entire experimental period. Oviduct and small intestine weights reached approximately 52.06 and 18.74 g, respectively, and their subsequent lengths reached approximately 69.32 and 111.50 cm, respectively, at 60 wk of age in Trial 2. Also, in that same trial, percentage liver lipid content was 8.96% at 20 wk and 10.22% at 60 wk of age.

In Trials 1 and 2, there was a significant (P • 0.05) main effect due to FMG-inoculation on numbers of ripe ovarian follicles. In both trials, the average number of ripe follicles in FMG-inoculated birds was significantly lower compared to those in controls. In Trial 1, FMG-free hens averaged 5.33 ripe follicles, while FMG-inoculated hens averaged 5.00 ripe follicles (SEM=0.124). Likewise, in Trial 2, FMG-free hens averaged 5.33 ripe follicles, while FMG-inoculated hens averaged 4.63 ripe follicles (SEM=0.207). In Trial 1, there was a significant main effect due to FMG-inoculation for the percentage of birds having five (P • 0.009) or six (P • 0.03) ripe ovarian follicles (Table 4.2). The percentage of FMG-inoculated birds having five follicles was significantly higher, while the percentage of FMG-inoculated birds having six follicles was significantly lower in comparison to those in sham-inoculated control birds. Inoculation with FMG, therefore, resulted in an overall reduction in ovarian follicle size

by shifting the numbers of ripe follicles from 6 to 5 in a significant number of birds. There was a significant (P • 0.02) main effect due to FMG-inoculation on magnum length as a percentage of oviduct length in Trial 1. Magnum length as a percentage of oviduct length in FMG-inoculated birds was significantly decreased in comparison to that in controls. Magnum length as a percentage of oviduct length was 45.11 in control hens and 43.17 in FMG-inoculated hens (SEM=0.387).

In Trial 2, there was a significant (P • 0.0009) main effect due to FMG-inoculation for incidence of FLHS. The percentage of FLHS in FMG-inoculated birds was significantly higher (48.77%) compared to that in control birds. There was a 7.43% incidence of FLHS in control hens and a 56.20% level of FLHS incidence in FMG-inoculated hens (SEM=9.707).

In Trial 2, there were significant age by FMG treatment interactions for percentages of birds having zero (P • 0.05) or seven (P • 0.03) ripe ovarian follicles (Table 4.3). At 20 wk, a greater percentage of FMG-inoculated birds had zero ripe follicles than controls, and at 44 wk of age a greater percentage of FMG-free birds had seven ripe follicles compared to those in controls. In Trial 2, there was a significant (P • 0.04) age by FMG treatment interaction for percentage liver moisture (Table 4.4). Inoculation with FMG resulted in a decrease in liver moisture content at 20 wk followed by an increase at 44 wk of age. In Trial 2, there were significant age by FMG treatment interactions for isthmus weight as a percentage of BW (P • 0.04) and as a percentage of oviduct weight (P • 0.006) (Table 4.5), and for vagina weight as a percentage of BW (P • 0.01) and as a percentage of oviduct weight (P • 0.05) (Table 4.6). Isthmus/BW and

isthmus/oviduct (g/g) weights were significantly decreased at 46 wk of age, and vagina/BW and vagina/oviduct (g/g) weights were decreased at both 20 and 36 wk of age due to FMG.

Discussion

Many investigators have reported the impacts of both field and vaccine strain infections of MG in birds (Van der Heide, 1977; Gentry, 1978; Carpenter *et al*., 1981; Lin and Kleven, 1982; Hildebrand *et al*., 1983; Glisson *et al*., 1984; Yoder *et al*., 1984; Branton and Deaton, 1985; Khan *et al*., 1986; Mohammed *et al*., 1987; Stadelman, 1988; Kleven *et al.,* 1990; Patterson, 1994; Burnham *et al*., 2001, 2002). In each of these reports, whether the investigator used field or vaccine strains of MG, layer hen egg production (EP) was altered or reduced. *Mycoplasma gallisepticum* may colonize various regions of the female reproductive tract and disrupt egg formation. Earlier reports indicated that MG may be cultured from the ovary (Fabricant and Levine, 1963), oviduct (Carlson and Howell, 1967; Domermuth *et al*., 1967; Hitchner *et al*., 1980), liver, uterus, and vagina (Sahu and Olson, 1976) and cloaca of chickens (Amin and Jordan, 1979; MacOwan *et al*., 1983). Later, Glisson *et al*., (1984) also reported that hens may transmit MG to their eggs via this same reproductive pathway. These reports indicate that MG may have the unique ability to colonize and impair certain reproductive processes in commercial birds. Furthermore, altered liver, ovarian, and other reproductive organ characteristics have been associated with FMG infection in commercial layers (Burnham *et al*., 2001). At the beginning and end of both trials in this study, SPA tests from swabs and sera and HI sera tests, along with the FA tests verified systemic infections in FMG-

inoculated birds. Conversely, sham-inoculated birds remained FMG-free throughout each trial. These data also showed that FMG-inoculation at 12 wk resulted in a higher incidence of FLHS, ovarian follicular regression, and decreased isthmal and vaginal proportions of the reproductive tract.

The ovaries of egg producing birds usually contain four to six large yolk filled follicles (2-4 cm in diameter), accompanied by a greater number of smaller (2-10 mm in diameter) follicles and numerous tiny white follicles (Sturkie and Mueller, 1976; Johnson, 2000). Delayed onset of lay and decreased weekly EP after 42 wk and overall average weekly EP in treated birds (Burnham *et al*., 2002) may be associated with a delay in ovarian follicular development during pre-peak EP. As indicated in each of the current trials, fewer ripe (diameter • 12 mm) follicles existed in FMG-inoculated hens than in controls. Infection with FMG slowed follicular development early in lay, as demonstrated by the fact that 25% of the FMG-inoculated birds had zero ripe follicles at 20 wk, whereas all FMG-free birds contained some ripe follicles. Because 50% of FMG-inoculated birds at 44 wk had an average of seven ripe ovarian follicles compared to 0% in controls, ovulation was also retarded after peak production. Ovarian regression may be related to retarded production (liver), transport (blood), and/or uptake (ovary) of yolk particles. Atrophic ovarian follicles may be a consequence of FMG colonization in specific ovarian cell groups, as reported by Fabricant and Levine (1963). It has been proposed by Williams and Sharp, (1978) and Palmer and Bahr, (1992), that decreases in EP with hen age is in part caused by both an increase in the incidence of atresia and a reduction in the number of follicles that reach the final phase of rapid growth.

Consequently, fewer follicles receive a greater proportion of yolk, resulting in larger sized eggs. The influence of FMG on ovarian tissue function may, therefore, imitate those of the aging process.

To develop an egg in a 25 h period, each reproductive tract segment has a task to complete within a certain time frame (Wyburn *et al*., 1970; Sturkie and Mueller, 1976; Johnson, 2000). However, any alteration in this process could result in reduced rates in EP. Furthermore, timing of EP has been shown to be affected by oviductal colonization by MG (Domermuth *et al*., 1967; Carlson and Howell, 1967; Hitchner *et al*., 1980). Reductions in relative magnum length due to FMG may also interfere with albumen deposition. Furthermore, as isthmal and vaginal functions control shell deposition and then oviposition, respectively, decreases in relative isthmus weight at 46 wk of age, and vagina weight at both 20 and 36 wk of age may have delayed EP in FMG-treated birds.

Branton *et al*., (2001) reported that FLHS incidence was delayed in hens inoculated at 10 wk of age with non-pathogenic *Mycoplasma gallinarum*. However, in the current study, FLHS incidence was increased by approximately 50% in FMG-inoculated birds. Walzem *et al*., (1999) reported that the liver is primarily responsible for the production of yolk very low density lipoprotein (VLDLy), which is a major yolk precursor. They also reported that inefficient or aging hens appear to lose the ability to correctly assemble VLDLy, which results in decreased EP. *Mycoplasma* species may be cultured from the avian liver (Sahu and Olson, 1976), therefore, the livers of birds infected with MG may react similarly to that of inefficient or aging hens. A decrease in liver moisture at 20 wk and an increase at 44 wk of age in treated birds, also suggest a

decrease in lipid catabolism pre-lay and an increase in lipid catabolism in the liver of infected birds after peak production. Intestinal characteristics were not influenced by FMG inoculation, and this may be due to the fact that the average temperature of the avian intestinal tract is above 37 C, which is the optimal growth temperature of MG (Razin and Freundt, 1984; Kleven, 1997). Also, mycoplasmas lack a cell wall and are very fragile (Yoder, 1975; Kleven, 1997; Ose *et al*., 1979; Timms *et al*., 1989), and a highly acidic intestinal environment may not be adequate for reproduction and growth. In fact mycoplasmas tend to colonize in more basic (pH = 7.8) environments, such as the upper respiratory and lower reproductive tracts (Hall, 1962; Vardaman, 1967; Kleven and Yoder, 1989). Reductions in feed efficiency in MG infected birds have been reported (Domermuth *et al*., 1967; Rodriquez and Kleven, 1980; Patterson, 1994), however, this may in part be due to initial colonization of MG in the upper respiratory tract.

An understanding of the pathogenic and physiological processes associated with MG infections may lead to new approaches to the treatment and control of MG. These data demonstrate that alterations in performance and egg characteristics of layers inoculated with FMG at 12 wk of age are related to mutual functional disturbances in the liver, ovary, and oviduct without concomitant intestinal changes.

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TABLE 4.1 Ingredient percentages and calculated and determined analyses of pullet and layer diets

	Starter		Grower	Developer	Prelay	Layer[1]					
Age (week)	0-6		6-12	12-18	18-20	20	28	32	36	40	44-60
Ingredients:	(%)										
Corn, 8.6%	64.51		73.64	72.22	61.35	58.11	64.93	68.39	71.33	63.38	70.47
Soybean meal, 48%	30.97		22.09	17.17	19.13	27.74	23.16	20.35	17.44	24.49	18.29
Wheat middlings	0.00		0.00	6.39	11.67	0.00	0.00	0.00	0.00	0.00	0.00
Vitamin premix[2,3]	0.25		0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
DL-methionine[4]	0.15		0.11	0.10	0.13	0.22	0.16	0.12	0.08	0.17	0.09
Dicalcium phosphate[5]	2.08		1.99	1.92	1.68	2.00	1.81	1.81	1.81	1.81	1.81
Limestone[6]	1.06		0.95	0.98	4.82	9.25	8.66	8.06	8.06	8.71	8.06
Sodium chloride[7]	0.48		0.47	0.47	0.47	0.53	0.53	0.53	0.53	0.53	0.53
Poultry fat	0.50		0.50	0.50	0.50	1.90	0.50	0.50	0.50	0.65	0.50
Dietary analyses:											
CP, calculated	20.50		17.00	15.50	16.34	18.09	16.40	15.31	14.12	16.95	14.47
CP, determined	ND[8]		ND	ND	ND	18.70	ND	ND	ND	ND	14.55
Crude fiber, calculated	2.29		2.24	2.55	2.76	2.17	2.21	2.21	2.20	2.21	2.20
Crude fiber, determined	ND		ND	ND	ND	3.75	ND	ND	ND	ND	2.70
Crude fat, calculated	3.22		3.52	3.68	3.48	4.27	3.12	3.23	3.32	3.22	3.30
Crude fat, determined	ND		ND	ND	ND	4.00	ND	ND	ND	ND	2.85
Ash, determined	ND		ND	ND	ND	13.25	ND	ND	ND	ND	17.50
Moisture, determined	ND		ND	ND	ND	11.35	ND	ND	ND	ND	11.30
ME, calculated kcal/kg	3,000	3,101		3,051	2,819	2,819	2,828	2,879	2,910	2,819	2,901

TABLE 4.1 Continued.

Available phosphorus, calculated	0.43	0.42	0.42	0.38	0.37	0.33	0.34	0.34	0.34	0.34
Calcium, calculated	0.88	0.82	0.82	2.25	4.00	3.73	3.50	3.50	3.75	3.50
Lysine, calculated	1.10	0.85	0.73	0.80	0.97	0.85	0.77	0.69	0.88	0.71
Methionine, calculated	0.50	0.42	0.38	0.41	0.52	0.44	0.40	0.35	0.47	0.36
Methionine + cystine, calculated	0.81	0.68	0.61	0.65	0.80	0.70	0.63	0.56	0.73	0.58
Potassium, calculated	0.81	0.66	0.55	0.56	0.72	0.65	0.61	0.56	0.67	0.57
Sodium, calculated	0.20	0.20	0.20	0.20	0.21	0.21	0.21	0.21	0.21	0.21
Tryptophan, calculated	0.28	0.23	0.20	0.22	0.25	0.22	0.20	0.19	0.23	0.19
Xanthophyll, calculated	6.45	7.36	7.22	6.14	5.81	6.49	6.84	7.13	6.34	7.05

[1]Available protein and lysine percentages in the layer diet were adjusted as needed according to the percentage of feed consumed per bird every 28 days until trial termination.

[2]Vitamin premix provided per kilogram of diet: vitamin A, 7,710 IU; cholecalciferol, 2,202 IU; vitamin E, 10 IU; menadione, 0.88 mg; vitamin B_{12}, 0.01 mg; choline, 380 mg; riboflavin, 5 mg; niacin, 33 mg; pantothenic acid, 9 mg; thiamine, 1 mg; folic acid, 0.6 mg; biotin, 0.06 mg; pyridoxine, 0.9 mg; ethoxyquin, 0.03 g.

[3]Trace minerals provided in vitamin premix: manganese, 2.2%; zinc, 2.0%; iron, 1.1%; copper, 1,400 ppm; iodine, 200 ppm; and selenium, 40 ppm.

[4]Manufactured by Degussa Corp., Ridgeland Park, NJ 07600-2100.

[5]Manufactured by IMC-Agrico Feed Ingredients, Bannockburn, IL 60015.

[6]Manufactured by Franklin Industrial Minerals, Nashville, TN 37203.

[7]Manufactured by Cargill Incorporated, Minneapolis, MN 55440.

[8]Not determined.

TABLE 4.2 Percentage of F-strain *Mycoplasma gallisepticum* (FMG)-free and FMG-inoculated Single Combed White Leghorn laying hens having zero, one, two, three, four, five, six, or seven ripe (diameter • 12 mm) ovarian follicles at 54 wk in Trial 1 and at 60 wk of age in Trial 2

	Trial 1[3]		Trial 2[4]	
	FMG-free	FMG-inoculated	FMG-free	FMG-inoculated
	(%)			
Number of Follicles:				
Zero	0.00	0.00	0.00	4.17
One	0.00	0.00	4.17	4.17
Two	0.00	0.00	0.00	0.00
Three	0.00	0.00	5.11	9.66
Four	6.67	5.00	1.55	21.03
Five	53.33[b,1]	90.00[a]	54.17	31.25
Six	40.00[a,2]	5.00[b]	25.76	16.67
Seven	0.00	0.00	9.03	12.50

[a,b]Means within trial and number of follicles among treatment groups with no common superscript differ significantly (P• 0.05).
[1]Based on pooled estimate of variance SEM = 6.22.
[2]Based on pooled estimate of variance SEM = 7.99.
[3]n = 40.
[4]n = 88.

TABLE 4.3 Percentage of F-strain *Mycoplasma gallisepticum* (FMG)-free and FMG-inoculated Single Combed White Leghorn laying hens having either zero or seven ripe (diameter • 12 mm) ovarian follicles at 20, 36, 44, 46, 48, and 60 wk of age in Trial 2

	Zero		Seven	
	FMG-free[1]	FMG-inoculated[1]	FMG-free[3]	FMG-inoculated[3]
Age (week)	----------(%)----------			
20	0.0[b]	25.0[a]	0.0	0.0
36	0.0	0.0	0.0	0.0
44	0.0	0.0	0.0[b]	50.0[a]
46	0.0	0.0	50.0	25.0
48	0.0	0.0	0.0	0.0
60	0.0[2]	0.0[2]	4.2[4]	0.0[4]

[a,b]Means within week of age and follicle number (0 or 7) category among treatment groups with no common superscript differ significantly (P• 0.05).

[1]n = 4, and based on pooled estimate of variance SEM = 5.02 for Weeks 20, 36, 44, 46, and 48.

[2]n = 24, and based on pooled estimate of variance SEM = 2.11 at Week 60.

[3]n = 4, and based on pooled estimate of variance SEM = 10.96 for Weeks 20, 36, 44, 46, and 48.

[4]n = 24, and based on pooled estimate of variance SEM = 4.48 at Week 60.

TABLE 4.4 Percentage liver moisture in F-strain *Mycoplasma gallisepticum* (FMG)-free and FMG-inoculated Single Combed White Leghorn laying hens at 20, 36, 44, 46, 48, and 60 wk of age in Trial 2

Age (week)	FMG-free[1]	FMG-inoculated[1]
	----------(%)----------	
20	29.3[b]	34.8[a]
36	26.5	26.1
44	32.2[a]	26.3[b]
46	31.7	33.6
48	27.0	28.6
60	26.8[2]	27.2[2]

[a,b]Means within week of age among treatment groups with no common superscript differ significantly (P• 0.05).

[1]n = 4 duplicate samples, and based on pooled estimate of variance SEM = 1.58 for each treatment at Weeks 20, 36, 44, 46, and 48.

[2]n = 24 duplicate samples, and based on pooled estimate of variance SEM = 0.65 for each treatment at Week 60.

TABLE 4.5 Isthmus weight as a percentage of BW and as a percentage of oviduct weight in F-strain *Mycoplasma gallisepticum* (FMG)-free and FMG-inoculated Single Combed White Leghorn laying hens at 20, 36, 44, 46, 48, and 60 wk of age in Trial 2

Age (week)	FMG-free[1]	FMG-inoculated[1]	FMG-free[3]	FMG-inoculated[3]
	--------(% of BW)--------		--------(% of oviduct weight)-------	
20	0.37	0.26	11.01	9.78
36	0.38	0.35	8.82	8.73
44	0.43	0.50	12.76	13.43
46	0.55[a]	0.40[b]	15.03[a]	10.58[b]
48	0.40	0.37	10.98	11.06
60	0.41[2]	0.40[2]	10.98[4]	10.89[4]

[a,b]Means within parameter and week of age among treatment groups with no common superscript differ significantly (P• 0.05).

[1]n = 4, and based on pooled estimate of variance SEM = 0.042 for Weeks 20, 36, 44, 46, and 48.

[2]n = 24, and based on pooled estimate of variance SEM = 0.017 at Week 60.

[3]n = 4, and based on pooled estimate of variance SEM = 0.765 for Weeks 20, 36, 44, 46, and 48.

[4]n = 24, and based on pooled estimate of variance SEM = 0.312 at Week 60.

TABLE 4.6 Vagina weight as a percentage of BW and as a percentage of oviduct weight in F-strain *Mycoplasma gallisepticum* (FMG)-free and FMG-inoculated Single Combed White Leghorn laying hens at 20, 36, 44, 46, 48, and 60 wk of age in Trial 2

Age (week)	FMG-free[1]	FMG-inoculated[1]	FMG-free[3]	FMG-inoculated[3]
	--------(% of BW)--------		--------(% of oviduct weight)-------	
20	0.32[a]	0.17[b]	9.75[a]	7.00[b]
36	0.26[a]	0.17[b]	5.81[a]	4.29[b]
44	0.17	0.17	4.93	4.38
46	0.20	0.19	5.43	5.21
48	0.19	0.13	5.04	4.01
60	0.21[2]	0.23[2]	5.63[4]	6.16[4]

[a,b]Means within parameter and week of age among treatment groups with no common superscript differ significantly (P• 0.05).

[1]n = 4, and based on pooled estimate of variance SEM = 0.029 for Weeks 20, 36, 44, 46, and 48.

[2]n = 24, and based on pooled estimate of variance SEM = 0.012 at Week 60.

[3]n = 4, and based on pooled estimate of variance SEM = 0.765 for Weeks 20, 36, 44, 46, and 48.

[4]n = 24, and based on pooled estimate of variance SEM = 0.312 at Week 60.

CHAPTER V

EFFECTS OF F-STRAIN *MYCOPLASMA GALLISEPTICUM* INOCULATION AT TWELVE WEEKS OF AGE ON THE BLOOD CHARACTERISTICS OF COMMERCIAL EGG LAYING HENS

Abstract

In two trials, the effects of F-strain *Mycoplasma gallisepticum* (FMG) on the blood characteristics of commercial Single Combed White Leghorn laying hens was investigated throughout lay. Variables measured in both trials were whole blood hematocrit (HCT), plasma protein (PP), and serum cholesterol, triglycerides (ST), and calcium. In addition, in Trial 1, serum vitellogenin was determined, and in Trial 2, serum lipoprotein profiles were investigated. Very low density lipoproteins were categorized into particle diameter (nm) ranges (10^{th}, 50^{th}, and 90^{th} population percentiles). Also, mean diameter (nm) of the total population of very low density lipoprotein particles were determined. Percentiles belonging to the 10^{th}, 50^{th}, and 90^{th} population percentiles had small, medium, and large relative diameters, respectively. Percentages of total serum cholesterol recovered in very low density lipoprotein, low density lipoprotein, and high density lipoprotein particle classes were determined. In both trials, HCT at 20 wk of age was significantly increased in birds inoculated with FMG. In Trial 1, ST and PP were significantly increased by FMG at 22 wk of age, while ST and PP were significantly decreased in FMG-inoculated birds at Weeks 54 and 52, respectively. Eight wk post-

challenge, FMG-inoculated birds exhibited an increased HCT, which may have been as a result of a compensatory polycythemic response. Between 8 and 10 wk post-challenge, ST and PP increased, which suggests that the initial weeks of egg production are stressful to the bird, particularly when combined with the establishment of an FMG-infection. Increases in these independent blood parameters may indeed be useful initial indicators of a bird's compensatory response to an FMG-challenge. Post-peak decreases in both ST (54 wk) and PP (52 wk) in FMG-infected birds suggest a more chronic effect of FMG on lipid and protein synthesis in the liver. Although overall levels of PP and ST were altered by FMG in this study, these data demonstrate that FMG-inoculation at 12 wk of age does not affect egg production through alterations in specific organized carriers of fats and lipids (vitellogenin and lipoproteins) between the liver and ovary. However, because these birds were housed in biological isolation units, these results do not preclude the possibility that these yolk precursors may be affected in FMG-infected birds that are housed in stressful facilities or environments. These data suggest that alterations in egg production in response to FMG-infection in commercial layers, as noted in a previous report, may be indirectly controlled by changes in blood lipid and protein concentrations. (*Key words:* blood, hematology, layer, lipid, *Mycoplasma gallisepticum*)

Introduction

Knowledge of the normal blood characteristics of avian species is essential for the evaluation of their health status. Epidemiological status is known to influence the composition of blood (Campbell, 1995). Other reports have described differential leukocyte counts in birds after being exposed to *Mycoplasma* species (Gross, 1961; Kerr

and Olson, 1967,1970; Branton *et al*., 1997), but further characterization of the blood from birds infected with *Mycoplasma gallisepticum* (MG) is lacking in the literature.

Estrogen release and the onset of egg production (EP) in laying hens drastically increases liver metabolism (Lorentz *et al*., 1938; Hillyard *et al*., 1956) and its production of neutral lipids (Heald and Badman, 1963), triacylglycerols and phospholipids (Dashti *et al*., 1983), vitellogenin (VTG) (Bergink *et al*., 1974; Deeley *et al*., 1975, 1977; Christmann *et al*., 1977), and lipoproteins (Kudzma *et al*., 1975; Khan *et al*., 1990; Castillo *et al*., 1992). Eventually, these components are destined for yolk lipid (YL) deposition (Lusky *et al*., 1974; Burley and Vadehra, 1989; Nimph and Schneider, 1991; Schneider, 1996; Walzem *et al*., 1994, 1999), but colonization of the liver by F-strain MG (FMG) (Sahu and Olson, 1976) may disrupt YL synthesis and subsequently reduce EP. Vitellogenin, likewise, may also potentially be affected by an MG-infection. Layer hens with elevated levels of very low density lipoprotein (VLDL) particles with diameters between 25 and 44 nm commonly are successful egg producers. Those that are deficient in VLDL particles within that particular size exhibit reproductive dysfunction (Walzem, 1996). Fatty livers are also known to impair VLDL particle assembly (Walzem *et al*., 1993). Burnham *et al*., (2002b) reported that laying hens reared in isolation units and inoculated with FMG at 12 wk of age had a 50% higher incidence of fatty liver hemorrhagic syndrome.

The objective of the current study was to determine possible changes in blood characteristics associated with changes in performance of FMG inoculated hens. Blood characteristics included, whole blood hematocrit (HCT), plasma protein (PP), and serum

cholesterol (SCH), triglycerides (ST), and calcium (SCA). In addition, in Trial 1, VTG was determined, and in Trial 2, serum lipoprotein profiles were investigated. Determinations of the effects of FMG on blood components in association with various production characteristics, as noted in an earlier related study, may provide vital information as to the physiological mechanisms behind the previously observed alterations in the performance of FMG-infected hens.

Materials and Methods

Pullet Housing and Management

In each of two trials, one thousand 1-d-old pullets of a single genetic strain were obtained from a commercial source that was monitored and certified free of MG and *M. synoviae* (MS) (National Poultry Improvement Plan and Auxiliary Provisions, 1995). Chickens were vaccinated at 10 d of age for infectious bursal disease via the drinking water. At 12 d and again at 4 wk of age, chickens were also vaccinated for Newcastle Disease and infectious bronchitis by the same route. At 5 wk of age, ten randomly selected pullets were bled from the left *cutanea ulnea* wing vein and tested for antibodies to MG and MS using both serum plate agglutination (SPA) and hemagglutination-inhibition (HI) tests (Yoder, 1975). At the same time, swabs were collected from the choanal cleft (Branton *et al.,* 1984) and placed into tubes containing Frey's broth medium (Frey *et al.*, 1968) supplemented with an additional 0.15 mg thallium acetate and 10^6 IU penicillin-G/mL. Tubes were incubated at 37 C for 30 d or until a phenol red indicator reaction occurred in the media. A sample from those that changed color was then inoculated onto Frey's-based (Papageorgiou medium) agar and incubated at 37 C.

Colonies with morphology suggestive of *Mycoplasma* species were examined by an agar plate fluorescent antibody (FA) method (Baas and Jasper, 1972) that used direct labeling of colonies stained with anti-FMG polyclonal antibodies produced in rabbits and labeled with fluorescein isothiocyanate (Kleven, 1981).

Up until the time that pullets were 12 wk of age, they were maintained on clean dry litter in a 5.5 x 6.1 m section of a conventional house. Flock density at placement was 0.034 m^2/bird. A daily artificial lighting schedule followed a 13 h light (L):11 h dark (D) cycle. One 75-watt incandescent light bulb was used to illuminate each 8.4 m^2 of floor space, providing a calculated intensity at bird level of 35.5 lux. Feed and water were provided for *ad libitum* consumption in each trial. Ingredient percentages and dietary analyses of the basal starter and grower diets used in both trials are provided in Table 5.1. All diets were formulated to meet or exceed National Research Council (1994) specifications. No medication was administered during the interval of either trial.

At 12 wk of age, 11 pullets were randomly selected and placed in each of 8 (Trial 1; total of 88 pullets) or 16 (Trial 2; total of 176 pullets) negative pressure fiberglass biological isolation units (1.16 m^2). The units were housed in a previously described poultry disease isolation facility (Branton and Simmons, 1992). In each trial, half of the total number of isolation units contained FMG-free control birds, whereas, the other half contained FMG-inoculated birds. There were four replicate units per treatment in Trial 1 and eight replicate units per treatment in Trial 2. Beginning at 18 wk of age, the artificial lighting schedule was increased 15 min/day until a 16 h 15 min L:7 h 45 min D cycle was achieved. Chickens were maintained on that schedule through the remainder of the

experiments. Ingredient percentages and dietary analyses of the basal developer, pre-lay, and layer diets used in both trials are also provided in Table 5.1. Hen numbers were reduced to 10 per unit at point-of-lay (18 wk of age) so that bird density was 0.116 m^2/bird for the duration of each trial. In both trials at 26 and 54 wk of age, quadruplicate feed samples per lot of mixed feed were analyzed for moisture, ash, CP, crude fat, and crude fiber. All determined analyses were performed according to the methods of the Association of Official Analytical Chemists (1980) and averaged for each of the two trials at each time period. Beginning at Week 20, available protein and lysine percentages in the layer diet were adjusted according to the percentage of feed consumed per bird every 28 days until trial termination (54 wk in Trial 1 and 60 wk in Trial 2).

FMG Inoculation

In each trial, pullets treated with FMG were inoculated via eye drop in the right eye at 12 wk of age with 0.04 mL of a 24-hr broth culture of high-passage FMG (99^{th} passage above the unknown passage level) provided by Dr. S. H. Kleven (University of Georgia, Athens, GA). Inoculum titers were 5.0×10^6 and 1.0×10^5 cfu/mL in Trials 1 and 2, respectively. Similarly, pullets designated as controls were sham-inoculated via eye drop in the right eye at 12 wk of age with 0.04 mL of sterile Frey's broth medium.

Mycoplasma detection

In each trial at 20 wk, and again at 54 wk in Trial 1 and 58 wk of age in Trial 2, one randomly selected hen from each of four FMG-free control and FMG-treated isolation units was bled and swabbed. Each of these samples were tested for the presence of *Mycoplasma* species as previously described for pullets.

Data Collection

In both trials, layer hens were bled following an overnight fast. Blood was harvested at 16, 20, 22, 24, 28, 30, 32, 36, 40, 44, 48, 52, and 54 wk of age in Trial 1 and at 16, 20, 24, 28, 34, 40, 46, 52, and 58 wk of age in Trial 2 from four hens per isolation unit. Hematocrit, expressed as percentage blood packed cell volume, was determined through use of capillary tubes that were centrifuged in a micro HCT centrifuge and were then read with a micro-capillary reader. Serum cholesterol and ST expressed in mg/dl and PP expressed in g/dl were determined by placing 10 ul of serum or plasma for each test on test slides which were analyzed on a Kodak Ektachem DT-60 analyzer (Eastman Kodak Co., Rochester, NY 14619) according to procedures of Elliott (1984) and as described by Latour *et al*., (1996). Similarly, SCA concentrations expressed in mg/dl were determined by placing 10 ul of serum on a test slide which was inserted in a Kodak Ektachem DTSC module analyzer according to procedures of Tietz (1986). Specific slides were required for each type of assay. Control analyses was required to assure that each sample was in the appropriate range category for accurate analysis.

Vitellogenin Analysis

In Trial 1, at 20, 22, 24, 28, 30, 32, 36, 40, 44, 48, 52, and 54 wk of age blood was centrifuged and serum was collected and analyzed for the distribution of VTG. High speed subnatant fluid from the blood of estrogenized roosters known to be highly concentrated with VTG was used as a comparison in this study. Estrogenized rooster serum and the serum from FMG-free and FMG-inoculated hens were diluted 1:20 with sample buffer. Dilutions of serum (10 • l) were analyzed in vertical slab gels using the

Mini-Protean III Power Pac 300 (Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules CA 94547) discontinuous gel electrophoresis (SDS-PAGE) system (Laemmli, 1970). Separating gels (7.5%) and stacking gels (5%) were prepared from a stock solution in which the ratio of acrylamide to bisacrylamide was 30:0.8 by weight. The final composition of the sample buffer was 10 mM Tris, pH 6.8, 1 mM EDTA, 1% (wt/vol) SDS, 10% (wt/vol) glycerol, and 0.001% (wt/vol) bromphenol blue. Separating gel slabs (14 cm long and 0.15 cm thick) were formed between glass plates and electrophoresis was conducted following the description of Studier (1973). Electrophoresis was carried out at 50 V until the leading edge entered the separating gel (• 1 hr) and then at 100 V until the leading edge almost reached the bottom of the gel (• 7 hr). After electrophoresis, the slabs were fixed in 12% (wt/vol) trichloroacetic acid for 20 minutes, stained in 0.2% (wt/vol) Coomassie brilliant blue R250 dissolved in methanol-water (1:1), and destained in 10% (vol/vol) acetic acid (Wallace and Selman, 1985; Wallace and Begovac, 1985).

The molecular weight of VTG in the serum samples was estimated by comparing their electrophoretic mobilities in polyacrylamide gels with the mobilities of proteins of known molecular weight, and additionally compared with the VTG induced serum. Image analysis and quantitation of serum VTG were assessed with a Flour-S Multi-Imager with Quantity One software (BIO-RAD). Broad spectrum molecular mass markers were used to estimate the molecular weight of VTG. Marker proteins and their respective molecular weights were ribonuclease A 14,000; deoxyribonuclease 31,000; ovalbumin 43,000; catalase 58,000; bovine serum albumin 66,000; phosphorylase b

94,000; ß-galactosidase 116,200; myosin heavy chain 200,000; and human apolipoprotein B 250,000.

Lipoprotein Analysis

In Trial 2, at 16, 20, 24, 28, 34, 40, 46, 52, and 58 wk of age serum was collected and analyzed for lipoprotein particle size and cholesterol concentration distributions. A complete serum lipoprotein distribution profile was determined as previously described by Walzem *et al.*, (1994). Very low density lipoprotein particles were categorized according to diameter (nm) (10^{th}, 50^{th}, and 90^{th} population percentiles). In addition, mean diameter (MA; nm) of the total VLDL population was determined as described by Chen *et al*., (1999). Each of the percentiles represents a specific particle diameter (nm) range within a bell-shaped curve. Small VLDL particles are represented in the 10^{th}, medium in the 50^{th}, and large in the 90^{th} population percentiles. In some populations, the total population mean and mean of 50^{th} percentile may be equal. Percentages of total SCH recovered in VLDL, low density lipoprotein (LDL), and high density lipoprotein (HDL) particle classes were determined.

Lipoproteins were isolated from 0.5 mL aliquots of serum, at a background density of 1.02 g/mL (Hermier *et al*., 1985, 1989) by flotation ultracentrifugation (148,600 x *g* at 4• C for 20 h) in a Beckman 50.4 rotor (Beckman, Palo Alto, CA 94304) as described by Walzem *et al.*, (1994), and with similarities to Fless (1991). Diameters and size distributions of serum VLDL particles were determined optically by dynamic light scattering using a Microtrak® Series 9200 Ultrafine Particle Analyzer (Leeds and Northrup, North Wales, PA) (Mack *et al*., 1994; Kraayenhof *et al*., 1993). Centrifugally

isolated VLDL were suspended as a 1:30 dilution in a NaCl solution (d 1.006 g/mL) and placed into the sample well. These dilutions were run in parallel with serum samples to verify salt densities at each step of the density fractionation. Densities of all salt solutions were measured with a digital solution density meter (Mettler/Parr, DMA 46, Graz, Austria). System software, and 3 mWatt, ? = 780 nm laser beam were activated. Light scattering from lipoprotein particles was recorded for 4 min, adapted to the audio range, and deconvoluted by system software. Using this method, light from the laser diode is scattered from each particle and its frequency subsequently Doppler-shifted by Brownian motion of the particle. The Doppler effect is proportional to particle velocity. Velocity distribution is a known function of particle size, fluid temperature, and fluid viscosity. Both temperature and viscosity of the suspending fluid are known, thus with compensation, the velocity distribution becomes a unique function of particle diameter distribution. The results of this primary data collection can be expressed in different ways in order to adequately describe different aspects of the same VLDL particle population with respect to their colloidal properties (Walzem, 1996). These descriptions are collectively termed density functions and include particle number, particle area, and particle volume distributions. Particle number distribution describes the frequency distribution of VLDL particles of the specified diameter. Particle volume distribution describes the distribution of total VLDL volume among particles of different diameters. The two distributions provide complementary information. Volume distributions can be converted to weight distributions if all particles are known to have the same specific gravity. This assumption was not made in the present study, as VLDL could only be

described within a possible range of 0.90 to 1.02 g/mL. In highly productive Single Comb White Leghorn hens, particle number distribution is nearly superimposable upon particle volume distribution. Particles (25-44 nm) of yolk VLDL (VLDLy) (Walzem, 1996) are spherical in shape, and the diameter of a sphere is a function of particle radius (diameter = 2r). Particle volume distribution is rather sensitive to the presence of small numbers of larger diameter particles because the volume of a sphere increases as a cubic function of particle radius (volume = 4/3 pr^3). A third distribution, particle area distribution, is calculated using information from both particle number and particle volume distribution. Particle area distributions are often calculated first because they are less sensitive to the presence of a few large particles. Particle area, number, and volume distributions are collectively termed density functions of lipoprotein particles, including VLDLy.

Although considered a primary method (Freud and Trainer, 1990), validity of size measurements were routinely assessed using monosized latex beads (Bangs Laboratory, Carmel, IN). Polydispersity of particle populations was calculated as the width (nm) of the measured particle size distribution. Concentration of particles within the sample well is automatically calculated by summing all of the scattered light within the measurement region and dividing by the scattering efficiency of the differently sized particles. This value, corrected for dilution of sample, was designated as Concentration Index and its arbitrary units are proportional to particle concentration on a volume basis.

Recovery of cholesterol was determined as a relative percentage of total cholesterol recovered in VLDL, LDL, and HDL particles in serum as described by

German *et al*., (1996). Lipoproteins were prepared by density gradient ultracentrifugation and size-exclusion high performance liquid chromatography (HPLC). In the density gradient ultracentrifugation preparation, lipoproteins were isolated from 2 mL of serum using sequential density gradient ultracentrifugation essentially according to the methods described by Lindgren (1975) and Orr *et al*. (1991). Solutions of varying density were prepared by mixing either NaCl or NaBr with a basal salt (d=1.0063) solution containing 0.01% EDTA and 50 kU/L each of streptomycin and penicillin. The VLDL were isolated at d<1.019, LDL at 1.019<d<1.063 and HDL at 1.063<d<1.200 by aspiration with a narrow-bore pipet. Densities of all salt solutions were measured with a digital density meter. Cholesterol in isolated lipoproteins were measured with commercial reagents (Sigma, St. Louis, MO). Parallel analyses were performed using size-exclusion HPLC that separated the lipoproteins on the basis of size (Kieft *et al*., 1991). Typically, 15-20 • L of serum was injected by an autosampler (WISP 710B, Waters Associates, Milford, MA) onto a Superose 6HR FPLC column (Pharmacia LKB Biotechnology, Piscataway, NJ). The lipoproteins were eluted with a buffer containing 0.15 M NaCl, pH 7.0, and 0.02% sodium azide at a flow rate of 1.0 mL/min (Waters model 510 solvent delivery pump). Lipoprotein cholesterol was determined using a post-column reactor consisting of a mixing coil (1615-50, Bodman, Aston, PA) in a temperature-controlled water jacket (Aura Industrials, Staten, NY), and a Hewlett-Packard (Palo Alto, CA) HPLC pump (model 79851A) was used to deliver cholesterol reagent (Boehringer-Mannheim Diagnostics, Indianapolis, IN) at a rate of 0.2 mL/min. The absorbance at 505 nm was compared with that of cholesterol standards (Sigma) to quantitate cholesterol.

Statistical Analysis

A completely randomized experimental design was utilized. All parameters were subjected to a repeated measures analysis were subjected to a repeated measures analysis where the same experimental units were observed over an extended time period. Individual sample data within each replicate unit were averaged prior to analysis. Least-squares means were compared in the event of significant global effects (Steel and Torrie, 1980; Petersen, 1985; Freund and Wilson, 1997). All data were analyzed using the MIXED Procedure of SAS®, Version 8 (1996). Statements of significance were based on P • 0.05 unless otherwise stated.

Results

In both trials, all initial mycoplasmal cultures as well as SPA and HI test results obtained from 5-wk-old pullets were negative for MG and MS. Control serum samples obtained at 20 wk of age in each trial and also at 54 wk (Trial 1) and 58 wk (Trial 2) were SPA and HI negative for MG, while the same tests were positive for MG in the FMG-inoculated hens. Hens were considered FMG-free when they exhibited no detectable HI titers. All FMG-inoculated hens had HI titers • 1:80. Similarly, FA culture results for swabs obtained at 20 wk of age in each trial and also at 54 wk (Trial 1) and 58 wk (Trial 2) were negative for *Mycoplasma* species growth for 4 out of 4 FMG-free hens tested, while growth was evident for 4 out of 4 FMG-inoculated hens tested.

In Trials 1 and 2, there were significant (P • 0.05) age by FMG treatment interactions for HCT (Table 5.2). In comparison to FMG-free birds, inoculation with FMG resulted in an increase in the percentage of packed blood cells at 20 wk in both

trials. In Trial 1, there were significant (P • 0.05) age by FMG treatment interactions for both ST (Table 5.3) and PP (Table 5.4). Results from Trial 1 indicated a significant increase in ST and PP at 22 wk of age, followed by a significant decrease in ST at 54 wk and a significant decrease in PP at 52 wk in FMG-inoculated birds. Distribution of VLDL particle diameters among the 10^{th}, 50^{th}, and 90^{th} population percentiles, MA, and percentage of SCH recovered from VLDL, LDL, and HDL particles in Trial 2 were not significantly affected by inoculation with FMG at 12 wk. Furthermore, serum VTG in Trial 1 was not significantly effected by the 12 wk FMG inoculation. Gel electrophoresis image analysis of VTG in Trial 1 are provided in Figure 5.1. Trace quantitation resulted in VTG levels of 3.324, 3.053, and 3.040 ODu x mm in sera from control hens, FMG-inoculated hens, and estrogenized roosters, respectively.

In both trials, significant (P • 0.0001) main effects due to layer age were observed for SCH and SCA. In Trial 1, SCH and SCA were 115.57 and 11.20 mg/dl at 16 wk (initiation of study), 171.54 and 39 mg/dl at 28 wk, and 160.71 and 30.79 mg/dl at 54 wk (trial termination), respectively. In Trial 2, SCH and SCA were 114.41 and 10.71 mg/dl at 16 wk (initiation of study), 138.9 and 20.72 mg/dl at 28 wk, and 103.65 and 20.03 mg/dl at 58 wk (trial termination), respectively. Also in Trial 2, significant (P • 0.0001) main effects due to layer age were observed for ST and PP. At 16 wk ST and PP were 35.78 mg/dl and 4.54 g/dl, at 28 wk 2043.75 mg/dl and 5.48 g/dl, and at 58 wk 1895.31 mg/dl and 4.96 g/dl, respectively. In Trial 2, significant (P • 0.0001) main effects due to layer age were observed for the distribution of serum VLDL particle diameters among the 10^{th}, 50^{th}, and 90^{th} population percentiles, MA, and the percentage of SCH recovered from

VLDL, LDL, and HDL particles. Mean VLDL particle diameters were 31.72, 23.34, 31.21, and 42.68 nm at 16 wk, for the total population and for the 10th, 50th, and 90th population percentiles, respectively. These approximate particle diameters were found with little variation throughout the remainder of the study. Also in Trial 2, SCH recovered in each of the VLDL, LDL, and HDL particles were 65.59, 15.94, and 18.47% at 16 wk (initiation of study) and 45.97, 27.03, and 15.35% at 58 wk (trial termination), respectively.

Discussion

When there are no secondary infections to mycoplasmas, infection is often subclinical or mild (Kerr and Olson, 1967). Manifestations of MG usually occur in the respiratory system and lesions become extensive when complicated by other bacteria. There is a marked interaction between mycoplasma, respiratory viruses, and bacteria (Saif *et al*., 1970; Jordan, 1972; Springer *et al*., 1974; Stipkovits, 1979; Rhoades, 1981; Gross, 1990; Patterson, 1994; Kleven, 1998). Environmental factors such as dust and ammonia, along with intensive rearing or stress, crowding, cold weather, live virus vaccination, or natural virus infection may also be important in lesion incidence and severity (Jordan, 1972; Springer *et al.*, 1974; Jordan, 1985). At the beginning and end of both trials, SPA tests from swabs and sera, and HI sera tests along with the FA tests, verified systemic infections in FMG-inoculated birds. Conversely, sham-inoculated birds remained FMG-free throughout each trial.

Hematocrit is a percentage of the blood occupied by cells, primarily red blood cells. Normally, about 97 percent of the oxygen transported from the lungs to the tissues

is carried in chemical combination with hemoglobin in the red blood cells (Guyton and Hall, 1996). By eight wk after challenge (20 wk) FMG-inoculated birds exhibited an increase in HCT. Birds at this age may, therefore, be exhibiting a compensatory polycythemic response to any insult imposed on their respiratory system by the FMG. Nevertheless, HCT levels returned to those of controls after this time, indicating that the birds are apparently adjusting through other physiological means to the FMG. Serum triglycerides are normally elevated in hens during lay. When a stressor such as FMG is introduced, ST may become elevated to compensate for the infection. An elevation in ST is known to be a common response to the presence of infectious disease agents (Guyton and Hall, 1996). During pre-peak EP (22 wk) the concentrations of ST and PP in FMG-inoculated birds were elevated, but during post-peak lay ST (54 wk) and PP (52 wk) levels were depressed. It is known that the establishment of systemic FMG infections in birds takes approximately six weeks (Truscott *et al*., 1974; Mallinson and Rosenstein, 1976; Yoder, 1986; McMartin *et al*., 1987; Soeripto *et al*., 1989; Nunoya *et al*., 1995). Increases in HCT, ST, and PP between 20 and 22 wk of age or between 8 and 10 wk post-inoculation, suggest that the initial weeks of EP are stressful to the bird, particularly when combined with the establishment of a full systemic FMG infection. Increases in these independent blood parameters may indeed be useful initial indicators of a bird's compensatory response to an FMG challenge. Post-peak decreases in both ST (54 wk) and PP (52 wk) in FMG-infected birds suggest a more chronic inhibition on lipid and protein synthesis in the liver by FMG.

Burnham *et al*., (2002a) have reported that initiation of lay was delayed and that weekly EP after 42 wk and overall average weekly EP were reduced in layer hens inoculated with FMG at 12 wk of age. Other reports have indicated a reduction in ripe ovarian follicles, ovarian follicle size, and magnal, isthmal, and vaginal proportions, along with increased incidences of fatty liver hemorrhagic syndrome in birds having been previously inoculated with FMG at 12 wk of age (Burnham *et al*., 2002b). Decreases in ST and PP may lead to ovarian follicular regression, atrophic reproductive tissues, and higher incidences of fatty livers during the egg laying cycle. Burnham *et al*., (2002c) have reported decreases in YL and yolk cholesterol between 22 and 28 wk of age in birds inoculated with FMG. In that same study in FMG-inoculated birds, decreases in overall yolk myristic, palmitoleic, and oleic acid percentages, along with increases in yolk linoleic, stearic and arachidonic acid percentages were reported. Decreased concentration of lipids in the blood may be directly responsible for the reductions in YL, yolk cholesterol, and yolk fatty acid deposition in FMG-inoculated hens. At this particular time, birds may need additional dietary supplementation. Additional fat may help provide the lipids necessary for the maintenance of egg yolk formation and subsequent EP in infected birds. Decreased PP may contribute to drops in EP similar to that caused by ST. Protein alterations may, likewise, affect egg white formation in the magnum, as egg white is primarily composed of the protein albumen. Although SCA is known to increase in response to accelerated estrogen release in association with increased EP, there were no differences in either SCA or SCH with FMG-inoculation.

Vitellogenin concentration and the physical characteristics of VLDL particles, were not effected by the inoculation of FMG. Although overall levels of PP and ST were altered by FMG in this study, these data demonstrate that FMG-inoculation at 12 wk of age does not affect EP through alterations in the specific quantity of VTG (carrier of yolk granules) or the characteristics of circulating VLDL particles. Because changes in the total lipid and protein levels in the blood of FMG-infected birds were evidenced without associated changes in the characteristics of the various classes of lipoprotein particles, it would be probable that the concentrations of lipids and proteins among all lipoprotein particles in circulation change similarly. Subsequent studies are needed to verify this hypothesis. Because these birds were housed in biological isolation units, these results do not preclude the possibility that these yolk precursors may be affected differently in FMG-infected birds that are housed in stressful facilities or environments.

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TABLE 5.1 Ingredient percentages and calculated and determined analyses of pullet and layer diets

	Starter	Grower	Developer	Prelay	Layer[1]					
Age (week)	0-6	6-12	12-18	18-20	20	28	32	36	40	44-60
Ingredients:	(%)									
Corn, 8.6%	64.51	73.64	72.22	61.35	58.11	64.93	68.39	71.33	63.38	70.47
Soybean meal, 48%	30.97	22.09	17.17	19.13	27.74	23.16	20.35	17.44	24.49	18.29
Wheat middlings	0.00	0.00	6.39	11.67	0.00	0.00	0.00	0.00	0.00	0.00
Vitamin premix[2,3]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
DL-methionine[4]	0.15	0.11	0.10	0.13	0.22	0.16	0.12	0.08	0.17	0.09
Dicalcium phosphate[5]	2.08	1.99	1.92	1.68	2.00	1.81	1.81	1.81	1.81	1.81
Limestone[6]	1.06	0.95	0.98	4.82	9.25	8.66	8.06	8.06	8.71	8.06
Sodium chloride[7]	0.48	0.47	0.47	0.47	0.53	0.53	0.53	0.53	0.53	0.53
Poultry fat	0.50	0.50	0.50	0.50	1.90	0.50	0.50	0.50	0.65	0.50
Dietary analyses:										
CP, calculated	20.50	17.00	15.50	16.34	18.09	16.40	15.31	14.12	16.95	14.47
CP, determined	ND[8]	ND	ND	ND	18.70	ND	ND	ND	ND	14.55
Crude fiber, calculated	2.29	2.24	2.55	2.76	2.17	2.21	2.21	2.20	2.21	2.20
Crude fiber, determined	ND	ND	ND	ND	3.75	ND	ND	ND	ND	2.70
Crude fat, calculated	3.22	3.52	3.68	3.48	4.27	3.12	3.23	3.32	3.22	3.30
Crude fat, determined	ND	ND	ND	ND	4.00	ND	ND	ND	ND	2.85
Ash, determined	ND	ND	ND	ND	13.25	ND	ND	ND	ND	17.50
Moisture, determined	ND	ND	ND	ND	11.35	ND	ND	ND	ND	11.30
ME, calculated kcal/kg	3,000	3,101	3,051	2,819	2,819	2,828	2,879	2,910	2,819	2,901

TABLE 5.1 Continued.

Available phosphorus, calculated	0.43	0.42	0.42	0.38	0.37	0.33	0.34	0.34	0.34	0.34
Calcium, calculated	0.88	0.82	0.82	2.25	4.00	3.73	3.50	3.50	3.75	3.50
Lysine, calculated	1.10	0.85	0.73	0.80	0.97	0.85	0.77	0.69	0.88	0.71
Methionine, calculated	0.50	0.42	0.38	0.41	0.52	0.44	0.40	0.35	0.47	0.36
Methionine + cystine, calculated	0.81	0.68	0.61	0.65	0.80	0.70	0.63	0.56	0.73	0.58
Potassium, calculated	0.81	0.66	0.55	0.56	0.72	0.65	0.61	0.56	0.67	0.57
Sodium, calculated	0.20	0.20	0.20	0.20	0.21	0.21	0.21	0.21	0.21	0.21
Tryptophan, calculated	0.28	0.23	0.20	0.22	0.25	0.22	0.20	0.19	0.23	0.19
Xanthophyll, calculated	6.45	7.36	7.22	6.14	5.81	6.49	6.84	7.13	6.34	7.05

[1]Available protein and lysine percentages in the layer diet were adjusted as needed according to the percentage of feed consumed per bird every 28 days until trial termination.

[2]Vitamin premix provided per kilogram of diet: vitamin A, 7,710 IU; cholecalciferol, 2,202 IU; vitamin E, 10 IU; menadione, 0.88 mg; vitamin B_{12}, 0.01 mg; choline, 380 mg; riboflavin, 5 mg; niacin, 33 mg; pantothenic acid, 9 mg; thiamine, 1 mg; folic acid, 0.6 mg; biotin, 0.06 mg; pyridoxine, 0.9 mg; ethoxyquin, 0.03 g.

[3]Trace minerals provided in vitamin premix: manganese, 2.2%; zinc, 2.0%; iron, 1.1%; copper, 1,400 ppm; iodine, 200 ppm; and selenium, 40 ppm.

[4]Manufactured by Degussa Corp., Ridgeland Park, NJ 07600-2100.

[5]Manufactured by IMC-Agrico Feed Ingredients, Bannockburn, IL 60015.

[6]Manufactured by Franklin Industrial Minerals, Nashville, TN 37203.

[7]Manufactured by Cargill Incorporated, Minneapolis, MN 55440.

[8]Not determined.

TABLE 5.2 Whole blood hematocrit in FMG-free and FMG-inoculated Single Combed White Leghorn laying hens at 16, 20, 22, 24, 28, 30, 32, 34, 36, 40, 44, 46, 48, 52, 54, and 58 wk of age in Trials 1 and 2

	Trial 1[1]		Trial 2[1]	
Age (week)	FMG-free[2]	FMG-inoculated[2]	FMG-free[2]	FMG-inoculated[2]
	----------(%)----------			
16	30.9	30.2	29.5	29.1
20	30.1[b]	31.7[a]	26.9[b]	28.1[a]
22	27.6	27.2	ND	ND
24	27.0	26.2	28.2	27.2
28	26.7	25.7	27.8	27.6
30	26.3	25.0	ND	ND
32	25.2	25.7	ND	ND
34	ND[3]	ND	25.2	25.3
36	25.3	26.6	ND	ND
40	26.9	26.5	27.9	27.2
44	26.1	25.7	ND	ND
46	ND	ND	27.2	26.1
48	25.4	25.6	ND	ND
52	24.9	24.7	27.6	28.0
54	23.9	23.7	ND	ND
58	ND	ND	28.3	29.4

[a,b]Means within trial and week among treatment group with no common superscript differ significantly (P• 0.05).

[1]Based on pooled estimate of variance SEM = 0.51 in Trial 1 and 0.48 in Trial 2.

[2]n = 16 samples for the calculation of means within treatment and week.

[3]Not determined.

TABLE 5.3 Serum triglycerides in FMG-free and FMG-inoculated Single Combed White Leghorn laying hens at 16, 20, 22, 24, 28, 30, 32, 34, 36, 40, 44, 46, 48, 52, 54, and 58 wk of age in Trials 1 and 2

	Trial 1[1]		Trial 2[1]	
Age (week)	FMG-free[2]	FMG-inoculated[2]	FMG-free[2]	FMG-inoculated[2]
	----------(mg/dl)----------			
16	167	131	34	37
20	2597	1781	3338	2925
22	2857[b]	4239[a]	ND	ND
24	3274	3518	1765	1573
28	3219	3214	2548	1540
30	3362	3029	ND	ND
32	3558	2813	ND	ND
34	ND[3]	ND	2358	2184
36	3127	2802	ND	ND
40	3803	3386	1892	984
44	3551	4089	ND	ND
46	ND	ND	2076	1843
48	3321	3364	ND	ND
52	3393	3013	1091	1360
54	4028[a]	2758[b]	ND	ND
58	ND	ND	1820	1971

[a,b]Means within trial and week of age among treatment group with no common superscript differ significantly (P• 0.05).

[1]Based on pooled estimate of variance SEM = 364.3 in Trial 1 and 508.6 in Trial 2.

[2]n = 16 samples for the calculation of means within treatment and week.

[3]Not determined.

TABLE 5.4 Plasma protein in FMG-free and FMG-inoculated Single Combed White Leghorn laying hens at 16, 20, 22, 24, 28, 30, 32, 34, 36, 40, 44, 46, 48, 52, 54, and 58 wk of age in Trials 1 and 2

	Trial 1[1]		Trial 2[1]	
Age (week)	FMG-free[2]	FMG-inoculated[2]	FMG-free[2]	FMG-inoculated[2]
	----------(g/dl)----------			
16	4.5	4.4	4.6	4.5
20	5.5	5.6	5.4	5.5
22	4.9[b]	5.9[a]	ND	ND
24	5.7	5.4	4.1	4.3
28	5.6	5.4	4.2	4.0
30	5.6	5.5	ND	ND
32	5.7	5.4	ND	ND
34	ND[3]	ND	4.1	4.5
36	5.1	4.8	ND	ND
40	6.2	5.7	4.5	4.3
44	5.6	5.8	ND	ND
46	ND	ND	4.7	4.6
48	6.1	5.7	ND	ND
52	5.8[a]	5.0[b]	4.9	4.7
54	5.6	5.0	ND	ND
58	ND	ND	5.0	4.9

[a,b]Means within trial among week of age and treatment groups with no common superscript differ significantly (P• 0.05).

[1]Based on pooled estimate of variance SEM = 0.22 in Trial 1 and 0.22 in Trial 2.

[2]n = 16 samples for the calculation of means within treatment and week.

[3]Not determined.

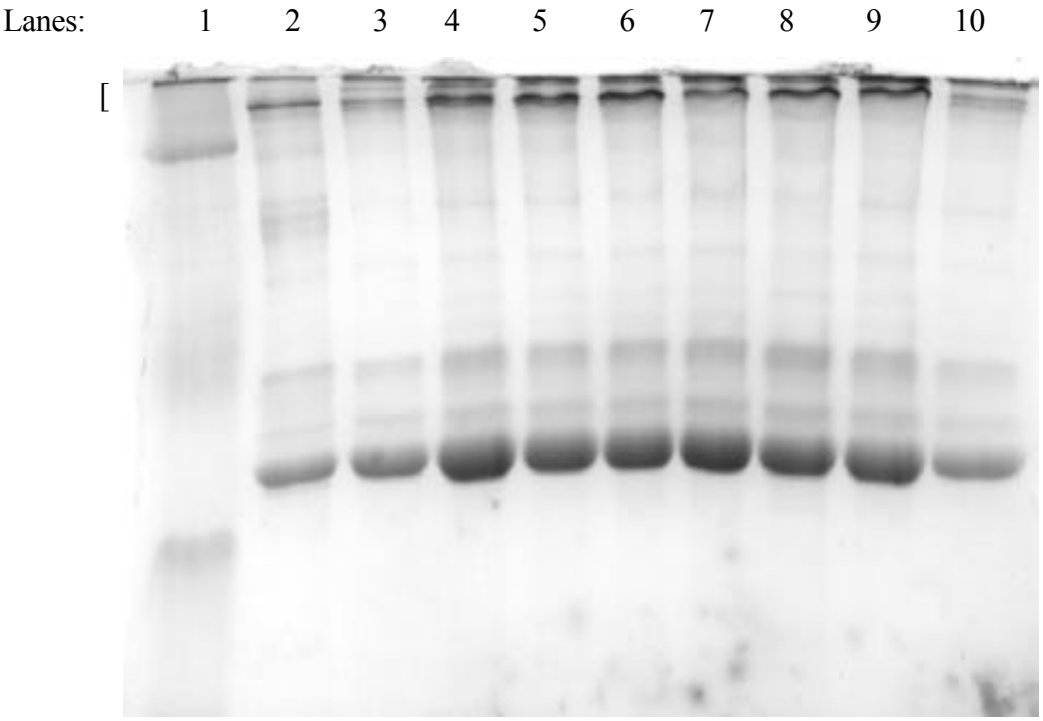

FIGURE 5.1 Sodium dodecylsulfate (SDS) gel electrophoresis, image analysis, and quantitation of serum vitellogenin (indicated by "["). Scanned with a Flour-S Multi-Imager and accessed with Quantity One software (BIO-RAD). Lane 1 = Kaleidoscope pre-stained standards, Lane 2 = Estrogenized rooster serum, Lanes 3-6 = F-strain *Mycoplasma gallisepticum* (FMG)-free layer hen serum, and Lanes 7-10 = FMG-inoculated layer hen serum.

CHAPTER VI

EFFECTS OF F-STRAIN *MYCOPLASMA GALLISEPTICUM* INOCULATION AT TWELVE WEEKS OF AGE ON EGG YOLK COMPOSITION IN COMMERCIAL EGG LAYING HENS

Abstract

In two trials, the effects of F-strain *Mycoplasma gallisepticum* (FMG) on the content of egg yolks from commercial Single Combed White Leghorn laying hens were investigated over a production cycle. Ten hens were assigned to each of eight (Trial 1) or sixteen (Trial 2) negative pressure fiberglass biological isolation units. Birds in half of the total units served as sham-inoculated controls and those in the other half were inoculated with FMG at 12 wk of age. Eggs were collected and yolks were harvested at various times during the pre-peak, peak, and post-peak periods of both trials for constituent analysis. Yolk constituents analyzed in these trials included moisture, total lipids, cholesterol, triglycerides, phospholipids, and fatty acids. In both trials, total yolk lipid at 22 wk of age was significantly decreased in birds inoculated with FMG. In Trial 1, yolk cholesterol at 28 wk was significantly decreased in FMG-inoculated birds. Yolk linoleic acid in Trial 1, and yolk stearic and arachidonic acids in Trial 2, were significantly increased in FMG- inoculated birds compared to FMG-free birds. In Trial 2, yolk myristic, palmitoleic, and oleic acid percentages were significantly decreased in FMG-inoculated birds compared to FMG-free birds. These data suggest that alterations

in egg production in commercial layers in response to an FMG infection at 12 wk of age, as noted in a previous report, are associated with changes in yolk composition.

(*Key words:* egg, layer, lipid, *Mycoplasma gallisepticum,* yolk)

Introduction

Many investigators have suggested that *Mycoplasma gallisepticum* (MG) is present in a high percentage of multi-age commercial egg producing complexes (Carpenter *et al*., 1981; Mohammed *et al*., 1986a, 1987; Kleven, 1998). Current treatments for MG may not prevent the disease from spreading, but may prevent severe drops in egg production (EP) (Barnes *et al.*, 1960; Barnes *et al.*, 1961; Yoder *et al.*, 1961; Cummings *et al*., 1986; Timms *et al*., 1989). The most commonly used live vaccine in the United States is F-strain MG (FMG) (Barbour *et al*., 2000).

Burnham *et al*., (2002a) have reported that initiation of lay was delayed and that weekly EP after 42 wk and overall average weekly EP were reduced in layer hens inoculated with FMG at 12 wk of age. In these same birds, it was also determined that on average, FMG-inoculated hens had fewer ripe ovarian follicles and decreased magnal, isthmal, and vaginal proportions of the reproductive tract at trial termination (60 wk) when compared to FMG-free hens (Burnham *et al*., 2002b). Previous research has been aimed at determining the effects of FMG on general egg characteristics of commercial egg laying chickens. However, no literature is available concerning specific possible concomitant alterations in the constituents of egg yolks from these birds.

Mycoplasma gallisepticum has been cultured from the liver (Sahu and Olson, 1976) and preovarian region (Fabricant and Levine, 1963) of chickens. Burnham *et al*.,

(2002b) have reported increased incidences of fatty liver hemorrhagic syndrome in birds having been previously inoculated with FMG at 12 wk of age. Furthermore, other studies comparing egg yolk and serum for the detection of MG antibodies by enzyme linked immunosorbent assays or hemagglutination inhibition tests, showed that egg yolk samples could be used instead of serum samples for flock screening (Brown *et al*., 1991, Mohammed *et al*., 1986b; Yoder and Hopkins, 1985). Therefore, colonization of MG in the liver and ovary has the potential of altering yolk composition.

The objectives of this study were to assess yolk characteristics in birds inoculated with FMG at 12 wk of age and describe possible relationships between these effects with those associations in previously reported work concerning the effects 12 wk inoculations of FMG on reproductive organ characteristics and the performance parameters of laying hens. The yolk constituents described in this study included yolk moisture (YM), total lipids (YL), fatty acids, cholesterol (YCH), triglycerides (YT), and phospholipids (YP).

Materials and Methods

Pullet Housing and Management

In each of two trials, one thousand 1-d-old pullets of a single genetic strain were obtained from a commercial source that was monitored and certified free of MG and *M. synoviae* (MS) (National Poultry Improvement Plan and Auxiliary Provisions, 1995). Chickens were vaccinated at 10 d of age for infectious bursal disease via the drinking water. At 12 d and again at 4 wk of age, chickens were also vaccinated for Newcastle Disease and infectious bronchitis by the same route. At 5 wk of age, ten randomly selected pullets were bled from the left *cutanea ulnea* wing vein and tested for antibodies

to MG and MS using both serum plate agglutination (SPA) and hemagglutination-inhibition (HI) tests (Yoder, 1975). At the same time, swabs were collected from the choanal cleft (Branton *et al.,* 1984) and placed into tubes containing Frey's broth medium (Frey *et al.*, 1968) supplemented with an additional 0.15 mg thallium acetate and 10^6 IU penicillin-G/mL. Tubes were incubated at 37 C for 30 d or until a phenol red indicator reaction occurred in the media. A sample from those that changed color was then inoculated onto Frey's-based (Papageorgiou medium) agar and incubated at 37 C. Colonies with morphology suggestive of *Mycoplasma* species were examined by an agar plate fluorescent antibody (FA) method (Baas and Jasper, 1972) that used direct labeling of colonies stained with anti-FMG polyclonal antibodies produced in rabbits and labeled with fluorescein isothiocyanate (Kleven, 1981).

Up until the time that pullets were 12 wk of age, they were maintained on clean dry litter in a 5.5 x 6.1 m section of a conventional house. Flock density at placement was 0.034 m^2/bird. A daily artificial lighting schedule followed a 13 h light (L):11 h dark (D) cycle. One 75-watt incandescent light bulb was used to illuminate each 8.4 m^2 of floor space, providing a calculated intensity at bird level of 35.5 lux. Feed and water were provided for *ad libitum* consumption in each trial. Ingredient percentages and dietary analyses of the basal starter and grower diets used in both trials are provided in Table 6.1. All diets were formulated to meet or exceed National Research Council (1994) specifications. No medication was administered during the interval of either trial.

At 12 wk of age, 11 pullets were randomly selected and placed in each of 8 (Trial 1; total of 88 pullets) or 16 (Trial 2; total of 176 pullets) negative pressure fiberglass

biological isolation units (1.16 m^2). The units were housed in a previously described poultry disease isolation facility (Branton and Simmons, 1992). In each trial, half of the total number of isolation units contained FMG-free control birds, whereas, the other half contained FMG-inoculated birds. There were four replicate units per treatment in Trial 1 and eight replicate units per treatment in Trial 2. Beginning at 18 wk of age, the artificial lighting schedule was increased 15 min/day until a 16 h 15 min L:7 h 45 min D cycle was achieved. Chickens were maintained on that schedule through the remainder of the experiments. Ingredient percentages and dietary analyses of the basal developer, pre-lay, and layer diets used in both trials are also provided in Table 6.1. Hen numbers were reduced to 10 per unit at point-of-lay (18 wk of age) so that bird density was 0.116 m^2/bird for the duration of each trial. In both trials at 26 and 54 wk of age, quadruplicate feed samples per lot of mixed feed were analyzed for moisture, ash, CP, crude fat, and crude fiber. All determined analyses were performed according to the methods of the Association of Official Analytical Chemists (1980) and averaged for each of the two trials at each time period. Beginning at Week 20, available protein and lysine percentages in the layer diet were adjusted according to the percentage of feed consumed per bird every 28 days until trial termination (54 wk in Trial 1 and 60 wk in Trial 2).

FMG Inoculation

In each trial, pullets treated with FMG were inoculated via eye drop in the right eye at 12 wk of age with 0.04 mL of a 24-hr broth culture of high-passage FMG (99^{th} passage above the unknown passage level) provided by Dr. S. H. Kleven (University of Georgia, Athens, GA). Inoculum titers were 5.0 x 10^6 and 1.0 x 10^5 cfu/mL in Trials 1

and 2, respectively. Similarly, pullets designated as controls were sham-inoculated via eye drop in the right eye at 12 wk of age with 0.04 mL of sterile Frey's broth medium.

Mycoplasma detection

In each trial at 20 wk, and again at 54 wk in Trial 1 and 58 wk of age in Trial 2, one randomly selected hen from each of four FMG-free control and FMG-treated isolation units was bled and swabbed. Each of these samples were tested for the presence of *Mycoplasma* species as previously described for pullets.

Data Collection

Eggs were collected and yolks were harvested several times during the pre-peak, peak, and post-peak periods of both trials for constituent analysis. More specifically, a pool of ten egg yolks per replication from control and treatment groups were harvested at 22, 24, 28, 30, 32, 36, 40, 44, 48, and 52 wk of age in Trial 1 and at 22, 24, 28, 34, 40, 46, 52, and 58 wk of age in Trial 2.

Quantitation of Yolk Moisture and Total Lipid Content

For analysis of YM content, duplicate fresh yolk samples (2 g) were dried according to the procedure of Peebles *et al*. (1999) in a commercial oven (Model EL20, General Electric Co., Chicago Heights, IL 60411). Yolk moisture contents were calculated as the difference between their wet and dry weights and were expressed as a percentage of wet yolk sample weight. For analysis of YL content, lipid was extracted from duplicate fresh yolk samples (3 g) according to the procedure previously described by Bligh and Dryer (1959), and as modified by Latour *et al.* (1998). Total YL was expressed as a percentage of fresh yolk sample weight. The YL was dissolved in 2 mL of

hexane, 200 • L of 0.83% butylated-hydroxy toluene, and refrigerated, as described by Christie (1982) for further content analyses as described below.

Methyl Esterification of Yolk Lipids

Duplicate lipid samples were methylated according to the procedure described by Morrison and Smith (1964). A Multi-Block (Lab-Line, Melrose Park, IL 60160) system was used to boil each sample in a test tube at 80±0.5 C for 30 minutes. A 200 • L aliquot of the solution was placed in a 2 mL gas chromatography (GC) vial along with 400 • L of iso-octane and sealed with a rubber lined cap for further fatty acid analyses by GC as described below.

Chromatographic Analysis of Yolk Contents

Fatty acid profiles of duplicate YL samples were determined at 24, 28, 32, 36, 40, and 44 wk of age in Trial 1 and at 24, 34, 40, 46, 52, and 58 wk of age in Trial 2 with a Hewlett Packard (Hewlett Packard, Boise, ID 83714) 5890 A, Series I GC according to the procedure by Latour *et al.* (1998). Fatty acids were identified by comparing peak retention times against polyunsaturated fatty acids and rapeseed oil. The standards were injected periodically to ensure accurate measurement by the GC. The individual fatty acids retained by the GC were expressed as a percentage of the total fatty acid content of the fresh yolk sample. Determination of YCH was performed at 24, 28, 32, 36, 40, and 44 wk of age in Trial 1 and at 22, 24, 28, 34, 40, 46, 52, and 58 wk of age in Trial 2 by direct saponification followed by a procedure that utilized capillary liquid GC (Maurice *et al.*, 1994). Fresh yolks were used to determine YCH in mg/g of total yolk.

Yolk Phospholipid and Triglyceride Analysis

For determination of YP and YT, lipids were previously extracted by the method described by Bligh and Dryer (1959) and Folch *et al*. (1957). Rapid, efficient, high recovery Sep-Pak (Sep-Pak, Waters Corporation, Milford, MA 01757) cartridges were used to separate and isolate polar (PL) and neutral/non-polar (NPL) lipids (Hamilton and Comai, 1984, 1988; Kaluzny *et al*., 1985; Figewicz *et al*., 1985; Juaneda and Rocquelin, 1985) at 24, 28, 32, 36, 40, and 44 wk of age in Trial 1. Non-polar lipids were eluted with 20 ml of chloroform to collect triglycerides, followed by 20 ml of chloroform/methanol (49:1, v/v) to discard monoacyl and diacyl glycerides. Polar lipids [primarily (98%) composed of YP (Hamilton and Comai, 1988] were then eluted with 30 ml of methanol, all with a flow rate of 25 ml/min. After evaporation using a rotary evaporator at 40• C, the respective weights of YT and YP were determined with an electronic balance, and their concentrations were quantified as percentages of total YL.

Statistical Analysis

A completely randomized experimental design was utilized. Egg YM, YL, fatty acids, YCH, YT, and YP were subjected to a repeated measures analysis where the same experimental units were observed over multiple age periods. Individual sample data within each replicate unit were averaged prior to analysis. Least-squares means were compared in the event of significant global effects (Steel and Torrie, 1980; Petersen, 1985; Freund and Wilson, 1997). All data were analyzed using the MIXED Procedure of SAS®, Version 8 (1996). Statements of significance were based on P • 0.05 unless otherwise stated.

Results

In both trials, all initial mycoplasmal cultures as well as SPA and HI test results obtained from 5-wk-old pullets were negative for MG and MS. Control serum samples obtained at 20 wk of age in each trial and also at 54 wk (Trial 1) and 58 wk (Trial 2) were SPA and HI negative for MG, while the same tests were positive for MG in the FMG-inoculated hens. Hens were considered FMG-free when they exhibited no detectable HI titers. All FMG-inoculated hens had HI titers • 1:80. Similarly, FA culture results for swabs obtained at 20 wk of age in each trial and also at 54 wk (Trial 1) and 58 wk (Trial 2) were negative for *Mycoplasma* species growth for 4 out of 4 FMG-free hens tested, while growth was evident for 4 out of 4 FMG-inoculated hens tested.

Yolk moisture in both trials and YCH content in Trial 2 changed significantly (P • 0.0001) with bird age. Yolk moisture was highest at 22 wk in both trials and lowest at 52 wk in Trial 1 and at 58 wk in Trial 2. Yolk cholesterol was highest at 22 wk and lowest at 58 wk in Trial 2. In both trials, yolk myristic (P • 0.03), palmitic (P • 0.0001), stearic (P • 0.0003), palmitoleic (P • 0.006), oleic (P • 0.01), linoleic (P • 0.0004), linolenic (P • 0.01), and arachidonic (P • 0.05) acid concentrations changed with bird age. Non-essential fatty acid concentrations, which included myristic, palmitic, and stearic acids, were lowest and essential fatty acid concentrations, which included palmitoleic, oleic, linoleic, linolenic, and arachidonic acids, were highest at 24 wk in both trials. However, at 44 wk in Trial 1 and at 58 wk in Trial 2 non-essential fatty acid concentrations were highest and essential fatty acid concentrations were lowest.

There were significant age by FMG treatment interactions for YL in Trial 1 (P • 0.02) and in Trial 2 (P • 0.0001) (Table 6.2). In both trials, when compared to controls, YL from FMG-treated hens was significantly lower at 22 wk. Also, YL from FMG-treated hens was significantly higher at 32 and 44 wk and significantly lower at 48 wk of age in Trial 1. There was a significant (P • 0.02) age by FMG treatment interaction in Trial 1 for YCH concentration (Table 6.3). Concentration of YCH in Trial 1 was significantly decreased at 28 wk of age in birds infected with FMG compared to FMG-free controls.

There were significant main effects due to FMG-inoculation for the percentage of yolk linoleic acid (P • 0.04) in Trial 1, and stearic (P • 0.03), arachidonic (P • 0.01), myristic (P • 0.03), palmitoleic (P • 0.05), and oleic (P • 0.01) acid concentrations in Trial 2 (Table 6.4). Linoleic, stearic, and arachidonic acid concentrations were significantly increased, while myristic, palmitoleic, and oleic acid concentrations were significantly decreased in FMG-inoculated birds compared to FMG-free birds.

Discussion

At the beginning and end of both trials in this study, SPA tests from swabs and sera, HI sera tests, and FA tests, verified systemic infections in FMG-inoculated birds. Conversely, sham-inoculated birds remained FMG-free throughout each trial. *Mycoplasma gallisepticum* has been cultured from the liver (Sahu and Olson, 1976) and preovarian region (Fabricant and Levine, 1963) of chickens. Furthermore, egg yolks can be used for the detection of MG infections in birds (Brown *et al*., 1991, Mohammed *et al*., 1986b; Yoder and Hopkins, 1985). Although it is known that circulating triglyceride

levels increase in response to infections by pathogens (Meyer, 1985; Funder and Sheppard, 1987; Guyton and Hall, 1996), no other information is available concerning possible alterations in yolk content after an MG challenge. A significant decrease in YL at 22 wk in FMG-infected hens suggests that FMG may inhibit YL deposition at that time. Increased percentages of YL post-peak, however, may represent a compensatory increase in YL deposition in response to the earlier depression in YL. Furthermore, decreases in YCH at 28 wk (pre-peak) due to FMG-infection support the conclusion that FMG may interfere with the deposition of YL in young layers.

Because specific lipid profiles, including those of fatty acids in the blood of layer hens inoculated with FMG at 12 wk of age has never been determined, it is not possible to ascertain whether or not changes in yolk characteristics of FMG-infected hens is due to alterations in the liver only or in both the liver and ovary. However, because enzymatic activity in follicles destined to become yolks can change drastically during reproduction (Chalana and Guraya, 1978) it is possible that changes in yolk characteristics may involve FMG colonization of the ovary. However, changes in YL deposition are likely to occur through colonization of the liver. Formation of yolk protein and lipid mainly occur in the liver (Johnson, 1986, 2000). A chicken's liver is also responsible for synthesis of fatty acids *de novo* (O'Hea and Leveille, 1969; Donaldson, 1990). Mammalian and avian livers are quite rich in lipid and the process of fatty acid synthesis in each have many common features (Poulose *et al*., 1981; Girard and Ferre, 1982; Wakil *et al*., 1983; Naggert *et al*., 1988; Gurr, 1992). The distribution of fatty acids of various chain lengths is relatively constant in YL and is related to their synthesis in the hens' liver (Watkins and

Kratzer, 1987; Watkins, 1995; Walzem, 1996; Speake and Thompson, 1999). Because MG may be cultured from the livers of infected chickens (Sahu and Olson, 1976), liver colonization by MG may play a role in the changes in YL profiles of infected birds.

In the current study, yolk fatty acid composition was altered by inoculation with FMG at 12 wk of age. Altered fatty acid profiles of yolks from MG-inoculated hens may be due more specifically to altered activities of various liver lipid enzymes. The conversion of palmitate to palmitoleate occurs when a double bond is introduced into the fatty acid chain by an oxidative reaction catalyzed by ? 9-fatty acyl-CoA desaturase (Lehninger, 1993; Cook, 1991). Palmitate can also be elongated by further additions of acetyl groups from the smooth endoplasmic reticulum and mitochondria to form Stearoyl CoA (Stearate), which can further be desaturated by ? 9-desaturase to produce oleate (Cook, 1991). These data suggest that there is a shift in the elongation process from palmitic to stearic acid instead of the ? 9-fatty acyl-CoA desaturase process to form palmitoleic acid in the livers of FMG-inoculated birds. Also, it is possible that ? 9-desaturase activity is depressed to allow for the build up of stearic acid, while oleic acid concentration is depleted. Ding and Lilburn (1996) reported that oleic acid comprised the largest proportion of total yolk fatty acids. Although linoleate, an essential fatty acid, cannot be synthesized from oleate in animals, once ingested, linolenic acid is derived from linoleic acid through appreciable ? 6-desaturation activity (Noble and Cocchi, 1990; Noble and Shand, 1985). Eicosatrienoate is elongated to form linoleate, and subsequently arachidonic acid is derived from eicosatrienoate acid through appreciable ? 5-desaturation activity (Lehninger, 1993; Cook, 1991). Increased yolk arachidonic acid concentrations

may have ultimately resulted from increased liver ? 5-desaturase activity. Evans *et al*., (1962) reported that dietary cottonseed oil supplementation increased the percentage of stearic acid and decreased the percentage of oleic acid in the yolks of layer hen eggs. Raju and Reiser (1967) showed that the cyclopropenoid fatty acids (constituents of crude cottonseed oil) inhibited a fatty acyl desaturase which normally converts stearic acid to oleic acid. Alterations in fatty acid precursors or fatty acyl desaturase activities may yield variations in the formation of subsequent fatty acids, the degree or type of MG colonization in the liver might also lead to changes in fatty acid metabolites due to conformational changes in liver fatty acid precursors and enzymes.

In the current study, inoculation with FMG at 12 wk of age did not significantly effect the concentrations of YT and YP within the egg yolk. Since YT and YP concentrations are similar in FMG-free control and FMG-treated birds, it appears that quantity of total lipid and the types of fatty acids deposited in yolk can change without concomitant changes in YT and YP due to an FMG infection.

Burnham *et al*., (2002a) have reported that initiation of lay was delayed and that weekly EP after 42 wk and overall average weekly EP were reduced in layer hens inoculated with FMG at 12 wk of age. Inoculation with FMG at 12 wk altered lipid concentrations in blood (Burnham *et al*., 2000), which may lead to decreases in YL concentration at the pre-peak period. This in turn may have resulted in a delay in onset of lay and a reduction in EP. Palmer and Bahr (1992) have suggested that increased follicular atresia and reduced follicle numbers during the final 6-11 d prior to ovulation can result in decreased EP. It has also been reported (Burnham *et al*., 2002b) that FMG-

inoculated layer hens possessed fewer ripe follicles when compared to FMG-free hens. Colonization of FMG within the ovary probably does not affect YL, including YT, YP, and fatty acid distribution, but may alter the ovulatory process. These data suggest that F-strain MG may be affecting EP, as noted in a previous report, through changes in yolk deposition, and rates of follicle formation and ovulation. It is concluded that FMG colonization in the liver of laying hens significantly affects EP through alterations in YL concentrations and also affects the metabolism and production of various fatty acids that are ultimately deposited in the yolk.

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TABLE 6.1 Ingredient percentages and calculated and determined analyses of pullet and layer diets

	Starter	Grower	Developer	Prelay	Layer[1]					
Age (week)	0-6	6-12	12-18	18-20	20	28	32	36	40	44-60
Ingredients:	(%)									
Corn, 8.6%	64.51	73.64	72.22	61.35	58.11	64.93	68.39	71.33	63.38	70.47
Soybean meal, 48%	30.97	22.09	17.17	19.13	27.74	23.16	20.35	17.44	24.49	18.29
Wheat middlings	0.00	0.00	6.39	11.67	0.00	0.00	0.00	0.00	0.00	0.00
Vitamin premix[2,3]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
DL-methionine[4]	0.15	0.11	0.10	0.13	0.22	0.16	0.12	0.08	0.17	0.09
Dicalcium phosphate[5]	2.08	1.99	1.92	1.68	2.00	1.81	1.81	1.81	1.81	1.81
Limestone[6]	1.06	0.95	0.98	4.82	9.25	8.66	8.06	8.06	8.71	8.06
Sodium chloride[7]	0.48	0.47	0.47	0.47	0.53	0.53	0.53	0.53	0.53	0.53
Poultry fat	0.50	0.50	0.50	0.50	1.90	0.50	0.50	0.50	0.65	0.50
Dietary analyses:										
CP, calculated	20.50	17.00	15.50	16.34	18.09	16.40	15.31	14.12	16.95	14.47
CP, determined	ND[8]	ND	ND	ND	18.70	ND	ND	ND	ND	14.55
Crude fiber, calculated	2.29	2.24	2.55	2.76	2.17	2.21	2.21	2.20	2.21	2.20
Crude fiber, determined	ND	ND	ND	ND	3.75	ND	ND	ND	ND	2.70
Crude fat, calculated	3.22	3.52	3.68	3.48	4.27	3.12	3.23	3.32	3.22	3.30
Crude fat, determined	ND	ND	ND	ND	4.00	ND	ND	ND	ND	2.85
Ash, determined	ND	ND	ND	ND	13.25	ND	ND	ND	ND	17.50
Moisture, determined	ND	ND	ND	ND	11.35	ND	ND	ND	ND	11.30
ME, calculated kcal/kg	3,000	3,101	3,051	2,819	2,819	2,828	2,879	2,910	2,819	2,901

TABLE 6.1 Continued.

Available phosphorus, calculated	0.43	0.42	0.42	0.38	0.37	0.33	0.34	0.34	0.34	0.34
Calcium, calculated	0.88	0.82	0.82	2.25	4.00	3.73	3.50	3.50	3.75	3.50
Lysine, calculated	1.10	0.85	0.73	0.80	0.97	0.85	0.77	0.69	0.88	0.71
Methionine, calculated	0.50	0.42	0.38	0.41	0.52	0.44	0.40	0.35	0.47	0.36
Methionine + cystine, calculated	0.81	0.68	0.61	0.65	0.80	0.70	0.63	0.56	0.73	0.58
Potassium, calculated	0.81	0.66	0.55	0.56	0.72	0.65	0.61	0.56	0.67	0.57
Sodium, calculated	0.20	0.20	0.20	0.20	0.21	0.21	0.21	0.21	0.21	0.21
Tryptophan, calculated	0.28	0.23	0.20	0.22	0.25	0.22	0.20	0.19	0.23	0.19
Xanthophyll, calculated	6.45	7.36	7.22	6.14	5.81	6.49	6.84	7.13	6.34	7.05

[1]Available protein and lysine percentages in the layer diet were adjusted as needed according to the percentage of feed consumed per bird every 28 days until trial termination.

[2]Vitamin premix provided per kilogram of diet: vitamin A, 7,710 IU; cholecalciferol, 2,202 IU; vitamin E, 10 IU; menadione, 0.88 mg; vitamin B_{12}, 0.01 mg; choline, 380 mg; riboflavin, 5 mg; niacin, 33 mg; pantothenic acid, 9 mg; thiamine, 1 mg; folic acid, 0.6 mg; biotin, 0.06 mg; pyridoxine, 0.9 mg; ethoxyquin, 0.03 g.

[3]Trace minerals provided in vitamin premix: manganese, 2.2%; zinc, 2.0%; iron, 1.1%; copper, 1,400 ppm; iodine, 200 ppm; and selenium, 40 ppm.

[4]Manufactured by Degussa Corp., Ridgeland Park, NJ 07600-2100.

[5]Manufactured by IMC-Agrico Feed Ingredients, Bannockburn, IL 60015.

[6]Manufactured by Franklin Industrial Minerals, Nashville, TN 37203.

[7]Manufactured by Cargill Incorporated, Minneapolis, MN 55440.

[8]Not determined.

TABLE 6.2 Yolk lipid in FMG-free and FMG-inoculated Single Combed White Leghorn laying hens at 22, 24, 28, 30, 32, 34, 36, 40, 44, 46, 48, 52, and 58 wk of age in Trials 1 and 2

	Trial 1[1]		Trial 2[1]	
Age (week)	FMG-free	FMG-inoculated	FMG-free	FMG-inoculated
	----------(% of total yolk)----------			
22	27.6[a,2]	26.7[b]	26.5[a]	13.1[b]
24	27.0	27.2	28.4	28.9
28	26.0	26.1	28.1	28.4
30	26.1	26.4	ND	ND
32	27.3[b]	28.5[a]	ND	ND
34	ND[3]	ND	29.2	28.7
36	28.7	28.3	ND	ND
40	29.0	28.3	30.1	29.2
44	28.4[b]	29.8[a]	ND	ND
46	ND	ND	28.2	28.5
48	30.2[a]	29.2[b]	ND	ND
52	29.4	28.7	30.4	30.4
58	ND	ND	29.2	29.4

[a,b]Means within trial among week of age and treatment group with no common superscript differ significantly (P• 0.05).

[1]Based on pooled estimate of variance SEM = 0.37 and 0.99 in Trial 1 and 2, respectively.

[2]n = 4 samples for the calculation of means within treatment and week.

[3]Not determined.

TABLE 6.3 Yolk cholesterol in FMG-free and FMG-inoculated Single Combed White Leghorn laying hens at 22, 24, 28, 32, 34, 36, 40, 44, 46, 52, and 58 wk of age in Trials 1 and 2

	Trial 1[1]		Trial 2[1]	
Age (week)	FMG-free	FMG-inoculated	FMG-free	FMG-inoculated
	----------(mg/g)----------			
22	ND[3]	ND	13.5	13.4
24	12.1[2]	12.8	13.1	12.9
28	13.5[a]	12.2[b]	13.1	13.0
32	12.2	12.8	ND	ND
34	ND	ND	12.7	12.9
36	12.6	12.4	ND	ND
40	11.4	11.7	12.3	12.0
44	11.8	12.2	ND	ND
46	ND	ND	12.0	12.1
52	ND	ND	12.0	11.9
58	ND	ND	11.9	12.1

[a,b]Means within trial among week of age and treatment groups with no common superscript differ significantly (P• 0.05).

[1]Based on pooled estimate of variance SEM = 0.54 and 0.23 in Trial 1 and 2, respectively.

[2]n = 4 samples for the calculation of means within treatment and week.

[3]Not determined.

TABLE 6.4 Myristic, palmitic, stearic, palmitoleic, oleic, linoleic, linolenic, and arachidonic acids in FMG-free and FMG-inoculated Single Combed White Leghorn laying hens in Trials 1 and 2

	Trial 1[1]		Trial 2[2]	
Fatty Acids	FMG-free[3]	FMG-inoculated[3]	FMG-free[4]	FMG-inoculated[4]
	----------(% of total fatty acids)----------			
Myristic	0.3	0.3	0.28[a]	0.25[b]
Palmitic	27.9	27.9	27.8	28.1
Stearic	12.2	12.0	11.7[b]	12.3[a]
Palmitoleic	2.3	2.3	2.2[a]	2.0[b]
Oleic	32.9	33.0	34.5[a]	32.9[b]
Linoleic	13.0[b]	13.5[a]	12.7	12.7
Linolenic	0.4	0.4	0.3	0.3
Arachidonic	4.6	4.3	4.2[b]	4.8[a]
Other	6.6	6.3	6.3	6.6

[a,b]Means within trial and fatty acid among treatment groups with no common superscript differ significantly (P• 0.05).

[1]Based on pooled estimate of variance SEM for myristic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, and arachidonic fatty acids in Trial 1 = 0.01, 0.18, 0.07, 0.16, 0.41, 0.12, 0.01, and 0.18.

[2]Based on pooled estimate of variance SEM for myristic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, and arachidonic fatty acids in Trial 2 = 0.01, 0.14, 0.06, 0.15, 0.29, 0.11, 0.02, and 0.13.

[3]n = 24.

[4]n = 32.

CHAPTER VII

INOCULATION WITH F-STRAIN *MYCOPLASMA GALLISEPTICUM* BEFORE INITIATION OF LAY AND AFTER INITIATION OF LAY AND THEIR SUBSEQUENT EFFECTS ON THE PERFORMANCE CHARACTERISTICS OF COMMERCIAL EGG LAYING HENS HOUSED IN A CAGED LAYER FACILITY

Abstract

The specific objectives of this study were to determine the performance and physiological characteristics of commercial layers through ~ 60 wk of age after being inoculated with F-strain *Mycoplasma gallisepticum* (FMG) before initiation of lay (12 wk) or after initiation of lay (22 wk) and to compare the responses of these birds in a caged layer environment with those of birds that had previously been housed in fiberglass isolation units. At 12 wk of age, sixty layer hens were randomly assigned to individual cages in each of two enclosed ends of a temperature regulated caged layer facility. Birds were either FMG (treated)- or sham (control)- inoculated. The separated ends of the facility housed either FMG or sham treated birds. At 22 wk of age, an additional sixty layer hens were randomly assigned to individual cages in each enclosed end of the same facility and were likewise either FMG- or sham-inoculated. Variables investigated included BW, feed conversion, egg production (EP), egg weight (EW), eggshell and internal egg quality, yolk constituent analyses, hematocrit, blood lipid and protein

constituent analyses, liver weight and composition, ovary weight and follicular hierarchy, segmental weights, lengths, and histologies of the intestine and oviduct, and percentage incidence of Fatty Liver Hemorrhagic Syndrome. Birds housed in negative pressure isolation units were also previously noted to experience changes in EP and egg characteristics in response to a 12 wk FMG inoculation. Furthermore, in those same birds, plasma protein and serum triglycerides increased at 22 wk and decreased at 54 wk of age by FMG-inoculation at 12 wk. Alterations in EP and feed conversion were associated with timing (12 and 22 wk of age) of inoculation with FMG in commercial layers in the current study. Layer hen inoculation with FMG at 22 wk rather that at 12 wk of age significantly improved weekly EP among pre-peak and post-peak EP periods and caused an increase in the percentage of small sized eggs at 22, 23, 24, and 25 wk. Total EP over the entire laying cycle was not affected by timing of FMG inoculation. Inoculation with FMG at 12 wk rather than 22 wk of age reduced weekly EP at these same time periods and caused an increase in the percentage of peewee sized eggs at 18 and 19 wk and a decrease at 20 wk, along with a decrease in the percentage of small sized eggs at 19, 20, 22, 23, 24, and 25 wk. However, FMG inoculation at 12 wk did not significantly affect total EP. Because weekly EW was not influenced by FMG-inoculation, increased EP at the previously mentioned time periods by the 22 wk FMG-inoculation may have been a result of both increased yolk deposition and rate of ovulation. However, unlike a previous report, in which lipoprotein profiles were not affected by FMG-inoculation in birds housed in low stress isolation units; birds that were inoculated with FMG in the caged layer facility experienced changes in lipoprotein

profiles. On the other hand, hematocrit, plasma protein, serum triglycerides, serum cholesterol, and serum calcium levels in birds housed in the caged layer facility were not influenced by FMG when inoculated at 12 or 22 wk of bird age. These data suggest that although inoculation of pullets with FMG at 12 or 22 wk of age may lead to improvements in layer performance at particular time periods when compared to birds naturally infected by field strains of MG, birds inoculated with FMG at 12 wk of age in a caged layer facility do not perform as adequately as those similarly infected birds in biological isolation units. Furthermore, in a caged layer facility, inoculation with FMG at 12 wk ultimately refines layer performance as opposed to a 22 wk FMG inoculation in these same facilities. Nevertheless, birds infected with FMG in either environment, do not perform as well as those that remain clean of any form of MG throughout production. This research also provides information necessary for the formulation of vaccination timetables and the prediction of their impacts on performance.

(*Key words:* blood, inoculation, *Mycoplasma gallisepticum,* performance, yolk)

Introduction

Mycoplasma gallisepticum (MG) has economically challenged the table egg industry and has globally affected producer profits (Mohammed *et al*., 1987; Patterson, 1994). Virulence of MG appears to be related to the ability of the organism to evade nonspecific defense mechanisms (Howard and Taylor, 1979; Davidson *et al.*, 1988;). Immunity to MG is accomplished by vaccination (Barile, 1985). Earlier reports indicated that MG may be cultured from the ovary (Fabricant and Levine, 1963), oviduct (Carlson and Howell, 1967; Domermuth *et al*., 1967; Hitchner *et al*., 1980), liver, uterus, and

vagina (Sahu and Olson, 1976) and cloaca of chickens (Amin and Jordan, 1979; MacOwan *et al*., 1983). In the United States, F-strain MG (FMG) is the most common live vaccine (Branton *et al.*, 1997, 1999; Barbour *et al*., 2000), and natural field strains may eventually be displaced by continuous FMG vaccination (Levisohn and Kleven, 1981; Kleven *et al.*, 1990). Pullets are generally vaccinated with FMG between 8 and 18 wk of age (Yoder *et al*., 1984) and remain infected for life (Brown *et al*., 1995). It has been suggested that vaccination of pullets before the laying cycle may enable hens to maintain full egg production (EP) (253 eggs per year), whereas, vaccination during lay may cause a reduction in EP from hens (Branton and May, 1999).

Burnham *et al*., (2002a) have reported that initiation of lay was delayed, and that weekly EP after 42 wk and total EP per bird over a complete cycle were reduced in layer hens previously inoculated with FMG at 12 wk of age and housed in biological isolation units (Branton and Simons, 1992). Other reports of 12 wk FMG-inoculated layers in these same environments have indicated a reduction in ripe ovarian follicles, ovarian follicle size, and magnal, isthmal, and vaginal proportions, as well as increased incidences of fatty liver hemorrhagic syndrome (Burnham *et al*., 2002b). In concurrent work with these birds, Burnham *et al*., (2002c) have reported decreases in yolk lipid (YL) and cholesterol between 22 and 28 wk of age in birds inoculated with FMG. Throughout the entire laying cycle, decreases in yolk myristic, palmitoleic, and oleic acid percentages, along with increases in yolk linoleic, stearic and arachidonic acid percentages were also reported. At eight wk post-challenge, those birds exhibited a compensatory polycythemic response. Furthermore, between 8 and 10 wk post challenge, serum triglycerides (ST)

and plasma protein (PP) increased, which suggested that the initial weeks of EP were stressful to the bird, particularly when combined with the establishment of an FMG infection (Burnham *et al*., 2000).

The first objective of this study was to verify the importance of age (12 and 22) of FMG-inoculation on a common flock housed in a caged layer facility. The second objective was to compare the effects of a 12 wk FMG-inoculation in birds housed in fiberglass isolation units, as previously mentioned, with the effects of a 12 wk FMG-inoculation in birds housed in a caged layer facility. Research data collected in caged layer facilities concerning the effects of FMG may be more appropriate than those obtained from birds housed in isolation units, as birds housed in a caged layer facility are subjected to social and environmental stressors encountered by birds in industry.

Materials and Methods

Pullet Housing and Management

One thousand 1-d-old Hy-Line W-36 Single Combed White Leghorn pullets of a single genetic strain were obtained from a commercial source that was monitored and certified free of MG and *M. synoviae* (MS) (National Poultry Improvement Plan and Auxiliary Provisions, 1995). Chickens were vaccinated at 10 d of age for infectious bursal disease via the drinking water. At 12 d and again at 4 wk of age, chickens were also vaccinated for Newcastle Disease and infectious bronchitis by the same route. At 5 wk of age, ten randomly selected pullets were bled from the left *cutanea ulnea* wing vein and tested for antibodies to MG and MS using both the serum plate agglutination (SPA) and the hemagglutination-inhibition (HI) tests (Yoder, 1975). At the same time, swabs

were collected from the choanal cleft (Branton *et al.,* 1984) and placed into tubes containing Frey's broth medium (Frey *et al.*, 1968) supplemented with an additional 0.15 mg thallium acetate and 10^6 IU penicillin-G/mL. Tubes were incubated at 37 C for 30 d or until a phenol red indicator reaction occurred in the media. A sample from those that changed color was then inoculated onto Frey's-based (Papageorgiou medium) agar and incubated at 37 C. Colonies with morphology suggestive of *Mycoplasma* species were examined by an agar plate fluorescent antibody (FA) method (Baas and Jasper, 1972) that used direct labeling of colonies stained with anti-FMG polyclonal antibodies produced in rabbits and labeled with fluorescein isothiocyanate (Kleven, 1981).

Up until the pullets were 12 wk of age, they were maintained on clean dry litter in a 5.5 x 6.1 m section of a conventional house with an initial flock density of 0.034 m^2/bird. A daily artificial lighting schedule followed a 13 h light (L):11 h dark (D) cycle. One 75-watt incandescent light bulb was used to illuminate each 8.4 m^2 of floor space, providing a calculated intensity at bird level of 35.5 lux. Feed and water were provided for *ad libitum* consumption. Ingredient percentages and dietary analyses of the basal starter and grower diets are provided in Table 7.1. All diets were formulated to meet or exceed National Research Council (1994) specifications. No medication was administered during the trial. ***Caged Layer Housing and Management***

At 12 wk of age, sixty layer hens were randomly assigned to individual cages in each of two enclosed ends of a temperature regulated caged layer facility. Birds in one end were FMG (treated)- and those on the other end were sham (control)- inoculated. At 22 wk of age, an additional sixty layer hens were randomly assigned to individual cages

in each enclosed end of the same facility and were likewise either FMG- or sham-inoculated. Each inoculation period group in each side of the house (treatment) were replicated 6 times, with ten hens in each replication. Birds in each side of the house were watered, fed, and ventilated separately. Beginning at 18 wk of age, the artificial lighting schedule was increased 15 min/d until a 16 h 15 min L:7 h 45 min D cycle was achieved. Chickens were maintained on that schedule through the remainder of the experiment. All birds were fed a common basal diet. Ingredient percentages and dietary analyses of the basal developer, pre-lay, and layer diets are also provided in Table 7.1. At 36 and 56 wk of age, quadruplicate feed samples per lot of mixed feed were analyzed for moisture, ash, CP, crude fat, and crude fiber. All determined analyses were performed according to the methods of the Association of Official Analytical Chemists (1980). Available protein and lysine percentages in the layer diet were adjusted as needed according to the percentage of feed consumed per bird every 28 days until trial termination (60 wk).

FMG Inoculation

Pullets treated with FMG were inoculated via eye drop in the right eye at either 12 or 22 wk of age with 0.04 mL of a 24-hr broth culture of high-passage FMG (99^{th} passage above the unknown passage level) provided by Dr. S. H. Kleven (University of Georgia, Athens, GA). The inoculum titer was 2.0×10^6 cfu/mL, respectively. Similarly, pullets designated as controls were sham-inoculated via eye drop in the right eye at either 12 or 22 wk of age with 0.04 mL of sterile Frey's broth medium.

Mycoplasma Detection

At 20 and 60 wk of age, one randomly selected hen from three replicate groups within 12 and 22 wk FMG-free and 12 and 22 wk FMG-inoculated treatment groups were bled and swabbed. Each of these samples were tested for the presence of *Mycoplasma* species as previously described for pullets at 5 wk of age.

Data Collection

Individual BW of all hens in each replication were recorded at 12, 20, 24, 28, 32, 34, 36, 40, 44, 48, 50, 52, 56, 58, and 60 wk of age. Bird mortalities were recorded daily. Commensurate with the production of the first egg (18 wk of age) in control hens, eggs from control and treatment groups were collected and weighed daily until trial termination at 60 wk. Egg production data for 12 and 22 wk FMG-clean and FMG-inoculated hens was expressed as percentage hen-day production. The percentage of eggs belonging to undersized, peewee (PS), small (SS), medium, large, extra-large, or jumbo size classes in accordance with the Agricultural Marketing Service of the United States Department of Agriculture (1996), was accomplished by converting individual egg weights in grams to ounces and expressing the number in each class as a percentage of the total eggs classified. ***Egg Constituent Analysis***

At 24, 34, 44, 50, and 58 wk of age ten eggs per each treatment, inoculation, and replication group were collected and yolks were harvested for constituent analysis. Eggs were subsequently broken out to determine percentage yolk, albumen, and eggshell weights and were expressed as percentages of egg weight. Eggshells were dried according to the procedure of Brake *et al.* (1984) before being weighed.

Quantitation of Yolk Moisture and Total Lipid Content

For analysis of yolk moisture (YM) content, duplicate fresh yolk samples (2 g) were dried according to the procedure of Peebles *et al*. (1999) in a commercial oven (Model EL20, General Electric Co., Chicago Heights, IL 60411). Yolk moisture contents were calculated as the difference between their wet and dry weights and were expressed as a percentage of wet yolk sample weight. For analysis of YL content, lipid was extracted from duplicate fresh yolk samples (3 g) according to the procedure previously described by Bligh and Dryer (1959), and as modified by Latour *et al.* (1998). Total YL was expressed as a percentage of fresh yolk sample weight. The YL was dissolved in 2 mL of hexane, 200 • L of 0.83% butylated-hydroxy toluene, and refrigerated, as described by Christie (1982) for further content analyses as described below.

Methyl Esterification of Yolk Lipids

Duplicate lipid samples were methylated according to the procedure described by Morrison and Smith (1964). A Multi-Block (Lab-Line, Melrose Park, IL 60160) system was used to boil each sample in a test tube at 80±0.5 C for 30 minutes. A 200 • L aliquot of the solution was placed in a 2 mL gas chromatography (GC) vial along with 400 • L of iso-octane and sealed with a rubber lined cap for further fatty acid analyses by GC as described below.

Chromatographic Analysis of Yolk Contents

Fatty acid profiles of duplicate YL samples were determined at 44 and 58 wk of age with a Hewlett Packard (Hewlett Packard, Boise, ID 83714) 5890 A, Series I GC according to the procedure described by Latour *et al.* (1998). Fatty acids were identified

by comparing peak retention times against polyunsaturated fatty acids and rapeseed oil. The standards were injected periodically to ensure accurate measurement by the GC. The individual fatty acids retained by the GC were expressed as a percentage of the total fatty acid content of the fresh yolk sample. Determination of yolk cholesterol was performed at 44 and 58 wk of age by direct saponification followed by a procedure that utilized capillary liquid GC (Maurice *et al.*, 1994). Fresh yolks were used to determine yolk cholesterol in mg/g total yolk.

Feed Utilization

At 36 and 56 wk of age, quadruplicate feed samples per lot of mixed feed belonging to each treatment, were randomly selected and analyzed for moisture, ash, CP, crude fat, crude fiber, and fatty acid content. Determined fatty acid analyses of the layer diets are provided in Table 7.2. Weekly between 20 and 60 wk, total feed consumed and numbers of eggs produced within each replicate unit in both the control and treatment groups were used for the determination of feed consumption (g/bird/day) and feed conversion (g feed intake/g eggs produced).

Hematological Evaluation

Layer hens were bled following an overnight fast. Blood was harvested at 20, 24, 34, 44, 50, and 58 wk of age. Two hens from each treatment, inoculation, and replication group were vena punctured in the left brachial wing vein, and 4 mL of blood were extracted. Hematocrit (HCT), expressed as percentage blood packed cell volume, was determined through use of capillary tubes that were centrifuged in a micro HCT centrifuge and were then read with a micro-capillary reader. Serum cholesterol and ST,

expressed in mg/dl, and PP, expressed in g/dl, were determined by placing 10 ul of serum or plasma for each test on test slides which were analyzed on a Kodak Ektachem DT-60 analyzer (Eastman Kodak Co., Rochester, NY 14619) according to procedures of Elliott (1984) and as described by Latour *et al*., (1996). Similarly, serum calcium concentrations, expressed in mg/dl, were determined by placing 10 ul of serum on a test slide which was inserted in a Kodak Ektachem DTSC module analyzer according to procedures of Tietz (1986). Specific slides were required for each type of assay. Control analyses was required to assure that each sample was in the appropriate range category for accurate analysis.

Lipoprotein Analysis

At 44 and 58 wk of age serum was collected and analyzed for lipoprotein particle size and cholesterol concentration distributions. A complete serum lipoprotein distribution profile was determined as previously described by Walzem *et al.*, (1994). Very low density lipoprotein particles were categorized according to diameter (nm) (10^{th}, 50^{th}, and 90^{th} population percentiles). In addition, mean diameter (MA; nm) of the total VLDL population was determined as described by Chen *et al*., (1999). Each of the percentiles represents a specific particle diameter (nm) range within a bell-shaped curve. Small VLDL particles are represented in the 10^{th}, medium in the 50^{th}, and large in the 90^{th} population percentiles. In some populations, the total population mean and mean of 50^{th} percentile may be equal. Percentages of total SCH recovered in VLDL, low density lipoprotein (LDL), and high density lipoprotein (HDL) particle classes were determined.

Lipoproteins were isolated from 0.5 ml aliquots of serum, at a background density of 1.02 g/ml (Hermier *et al*., 1985, 1989) by flotation ultracentrifugation (148,600 x *g* at 4• C for 20 h) in a Beckman 50.4 rotor (Beckman, Palo Alto, CA 94304) as described by Walzem *et al.*, (1994), and with similarities to Fless (1991). Diameters and size distributions of serum VLDL particles were determined optically by dynamic light scattering using a Microtrak® Series 9200 Ultrafine Particle Analyzer (Leeds and Northrup, North Wales, PA) (Mack *et al*., 1994; Kraayenhof *et al*., 1993). Centrifugally isolated VLDL were suspended as a 1:30 dilution in a NaCl solution (d 1.006 g/ml) and placed into the sample well. These dilutions were run in parallel with serum samples to verify salt densities at each step of the density fractionation. Densities of all salt solutions were measured with a digital solution density meter (Mettler/Parr, DMA 46, Graz, Austria). System software, and 3 mWatt, ? = 780 nm laser beam were activated. Light scattering from lipoprotein particles was recorded for 4 min, adapted to the audio range, and deconvoluted by system software. Using this method, light from the laser diode is scattered from each particle and its frequency subsequently Doppler-shifted by Brownian motion of the particle. The Doppler effect is proportional to particle velocity. Velocity distribution is a known function of particle size, fluid temperature, and fluid viscosity. Both temperature and viscosity of the suspending fluid are known, thus with compensation, the velocity distribution becomes a unique function of particle diameter distribution. The results of this primary data collection can be expressed in different ways in order to adequately describe different aspects of the same VLDL particle population with respect to their colloidal properties (Walzem, 1996). These descriptions

are collectively termed density functions and include particle number, particle area, and particle volume distributions. Particle number distribution describes the frequency distribution of VLDL particles of the specified diameter. Particle volume distribution describes the distribution of total VLDL volume among particles of different diameters. The two distributions provide complementary information. Volume distributions can be converted to weight distributions if all particles are known to have the same specific gravity. This assumption was not made in the present study, as VLDL could only be described within a possible range of 0.90 to 1.02 g/ml. In highly productive Single Comb White Leghorn hens, particle number distribution is nearly superimposable upon particle volume distribution. Particles (25-44 nm) of yolk VLDL (VLDLy) (Walzem, 1996) are spherical in shape, and the diameter of a sphere is a function of particle radius (diameter = 2r). Particle volume distribution is rather sensitive to the presence of small numbers of larger diameter particles because the volume of a sphere increases as a cubic function of particle radius (volume = 4/3 pr^3). A third distribution, particle area distribution, is calculated using information from both particle number and particle volume distribution. Particle area distributions are often calculated first because they are less sensitive to the presence of a few large particles. Particle area, number, and volume distributions are collectively termed density functions of lipoprotein particles, including VLDLy.

Although considered a primary method (Freud and Trainer, 1990), validity of size measurements were routinely assessed using monosized latex beads (Bangs Laboratory, Carmel, IN). Polydispersity of particle populations was calculated as the width (nm) of the measured particle size distribution. Concentration of particles within the sample well

is automatically calculated by summing all of the scattered light within the measurement region and dividing by the scattering efficiency of the differently sized particles. This value, corrected for dilution of sample, was designated as Concentration Index and its arbitrary units are proportional to particle concentration on a volume basis.

Recovery of cholesterol was determined as a relative percentage of total cholesterol recovered in VLDL, LDL, and HDL particles in serum as described by German *et al*., (1996). Lipoproteins were prepared by density gradient ultracentrifugation and size-exclusion high performance liquid chromatography (HPLC). In the density gradient ultracentrifugation preparation, lipoproteins were isolated from 2 mL of serum using sequential density gradient ultracentrifugation essentially according to the methods described by Lindgren (1975) and Orr *et al*. (1991). Solutions of varying density were prepared by mixing either NaCl or NaBr with a basal salt (d=1.0063) solution containing 0.01% EDTA and 50 kU/L each of streptomycin and penicillin. The VLDL were isolated at d<1.019, LDL at 1.019<d<1.063 and HDL at 1.063<d<1.200 by aspiration with a narrow-bore pipet. Densities of all salt solutions were measured with a digital density meter. Cholesterol in isolated lipoproteins were measured with commercial reagents (Sigma, St. Louis, MO). Parallel analyses were performed using size-exclusion HPLC that separated the lipoproteins on the basis of size (Kieft *et al*., 1991). Typically, 15-20 • L of serum was injected by an autosampler (WISP 710B, Waters Associates, Milford, MA) onto a Superose 6HR FPLC column (Pharmacia LKB Biotechnology, Piscataway, NJ). The lipoproteins were eluted with a buffer containing 0.15 M NaCl, pH 7.0, and 0.02% sodium azide at a flow rate of 1.0 mL/min (Waters model 510 solvent delivery

pump). Lipoprotein cholesterol was determined using a post-column reactor consisting of a mixing coil (1615-50, Bodman, Aston, PA) in a temperature-controlled water jacket (Aura Industrials, Staten, NY), and a Hewlett-Packard (Palo Alto, CA) HPLC pump (model 79851A) was used to deliver cholesterol reagent (Boehringer-Mannheim Diagnostics, Indianapolis, IN) at a rate of 0.2 mL/min. The absorbance at 505 nm was compared with that of cholesterol standards (Sigma) to quantitate cholesterol.

Necropsy Evaluation

This trial was terminated at 60 wk of bird age. At that time, two birds from each treatment, inoculation, and replication were randomly selected and euthanized by cervical dislocation and their organs removed. Organ analyses included liver weight, lipid, and moisture content, ovary weight and follicular hierarchy, and the weights, lengths, and histologies of the oviduct, infundibulum, magnum, isthmus, uterus, and vagina (Figure 7.6). Incidence of FLHS and the weights, lengths, and histologies of the duodenum, jejunum, and ileum were also examined in addition to the above mentioned parameters (Figure 7.5). Intestinal and oviductal segment weights were calculated as percentages of total body and organ weight; and segment lengths were calculated as percentages of total organ length.

Ripe Follicle Quantitation

The entire ovary was removed and the number of ripe (diameter • 12 mm) yellow ovarian follicles was recorded for each bird. A caliper was used to measure follicle diameter. Based on the number of ripe ovarian follicles present in each bird, a categorical number from zero to seven was assigned. A number of zero indicated that a bird had no

ripe follicles present. A maximum of seven ripe follicles were recorded. Average number of ripe follicles and the percentage of birds having zero, one, two, three, four, five, six, or seven ripe follicles in each replicate unit were calculated.

Liver Moisture and Lipid Analysis

For analysis of liver moisture content, fresh liver samples (2 g) were dried according to the procedure of Peebles *et al*. (1999) in a commercial oven (Model EL20, General Electric Co., Chicago Heights, IL 60411). Liver moisture content was calculated as the difference between the wet and dry weights of the sample and was expressed as a percentage of wet sample weight. For analysis of liver lipid content, lipid was extracted from fresh liver samples (3 g) according to the procedure previously described by Bligh and Dryer (1959), and as modified by Latour *et al.* (1998). Liver lipid content was expressed as a percentage of total fresh liver sample weight.

Histopathologic Examination

Upon trial termination, one tissue sample from the ovary, infundibulum, magnum, isthmus, uterus, and vagina was harvested from one hen in each treatment and inoculation group. Tissue samples were placed in 10% buffered neutral formalin, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin. Each tissue sample was observed and scored for the presence or absence of lymphoid and heterophil infiltrates as described by Branton *et al*., (2000). Treatments were unknown to the evaluator.

Statistical Analysis

A completely randomized experimental design was utilized. All inoculation and treatment data were subjected to a repeated measures analysis where the same experimental units were observed over an extended time period. Individual sample data within each replicate unit were averaged prior to analysis. Least-squares means were compared in the event of significant global effects (Steel and Torrie, 1980; Petersen, 1985; Freund and Wilson, 1997). All data were analyzed using the MIXED Procedure of SAS®, Version 8 (1996). Statements of significance were based on P • 0.05 unless otherwise stated.

Results

All initial mycoplasmal cultures as well as SPA and HI test results obtained from 5-wk-old pullets were negative for MG and MS. Control serum samples obtained at 20 wk of age and also at 60 wk were SPA and HI negative for MG, while the same tests were positive for MG in the 12 and 22 wk FMG-inoculated hens. Hens were considered FMG-free when they exhibited no detectable HI titers. All FMG-inoculated hens had HI titers • 1:80. Similarly, FA culture results for swabs obtained at 20 and 60 wk of age were negative for *Mycoplasma* species growth for 12 out of 12 FMG-free hens tested, while growth was evident for 12 out of 12 FMG-inoculated hens tested. There were no effects due to inoculation age or inoculation treatment on bird mortality.

A significant (P • 0.0001) age by treatment by inoculation interaction was apparent for weekly percent hen day EP (Figure 7.1); however, total EP (# of total eggs/bird) was not effected by FMG or age of inoculation. At 20, 22, 23, 46, 47, 48, 49,

50, and 58 wk of age EP decreased in flocks inoculated with FMG at 12 wk, as compared to those sham-inoculated at 12 wk (Figure 7.2). Initiation of lay was delayed ten days in treated birds inoculated with FMG at 12 wk of age when compared to 12 wk sham-inoculated controls. At 22, 56, 57, 58, and 59 wk of age EP increased in flocks inoculated with FMG at 22 wk, as compared to those sham-inoculated at 22 wk (Figure 7.3). At 22, 23, 46, 47, 57, and 58 wk of age EP increased in flocks inoculated with FMG at 22 wk, as compared to those inoculated with FMG at 12 wk (Figure 7.4). Inoculation with FMG at 22 wk significantly (P • 0.02) decreased total egg mass more than 1,000 g when compared to 22 wk sham-inoculated controls. Also, the 22 wk FMG-inoculation significantly decreased total egg mass more than 1,000 g when compared to birds inoculated with FMG at 12 wk. Total egg mass (SEM=122.94) for control birds sham-inoculated at 12 and 22 wk was 16,358 and 16,363 g, respectively and treated birds FMG-inoculated at 12 and 22 wk was 16,276 and 15,289 g, respectively.

There were significant (P • 0.0001) main effects due to layer hen age for each of the variables listed above, but due to the previous description of these effects in earlier chapters and for sake of brevity, age main effects are not described. Sham or FMG inoculation at 12 wk significantly (P • 0.0005) reduced BW at 40, 48, 50, 52, 56, 58, and 60 wk in comparison to the 22 wk inoculation period (Table 7.3). Feed conversion between 22 and 24 wk was significantly (P • 0.0001) increased in flocks inoculated with FMG at 22 wk, as compared to those inoculated with FMG at 12 wk (Table 7.4). In comparison to the 12 wk sham inoculation, the percentage of PS EP was significantly (P • 0.0001) decreased at 18 and 19 wk, but was increased at 20 wk by FMG inoculation at

12 wk (Table 7.5). Also, SS EP was significantly (P • 0.0001) decreased at 19, 20, 22, 23, 24, and 25 wk in birds inoculated with FMG at 12 wk compared to those sham-inoculated at 12 wk of age. Also, SS EP was significantly (P • 0.0001) increased at 22, 23, 24, and 25 wk in birds inoculated with FMG at 22 wk compared to those sham-inoculated at 22 wk of age (Table 7.6). In comparison to the 12 wk inoculation, sham or FMG inoculation at 22 wk significantly increased medium sized (P • 0.002) EP at 23, 35, and 37 wk of age, while large sized (P • 0.007) EP was decreased at 35 and 37 wk and increased at 56 and 58 wk of age (Table 7.7).

Inoculation with FMG significantly (P • 0.007) increased YM at 44, 50, and 58 wk of age compared to controls (Table 7.8). Sham or FMG inoculation at 12 wk significantly (P • 0.01) reduced YM at 24 and 44 wk in comparison to the 22 wk inoculation period (Table 7.8). Birds that were sham- or FMG-inoculated at 22 wk of age had significantly higher serum calcium (P • 0.02) and PP (P • 0.02) levels at 34 wk and significantly lower PP levels at 58 wk compared to those inoculated at 12 wk (Table 7.9). Average particle diameter within each percentile and MA were significantly (P • 0.02) increased in 12 wk treated birds when compared to 12 wk control birds. These same variables were also significantly (P • 0.02) decreased in 22 wk treated birds when compared to 22 wk control birds (Table 7.10). Furthermore, these variables were significantly decreased in birds inoculated with FMG at 22 wk when compared to those inoculated with FMG at 12 wk. At 58 wk of age, percentage of serum cholesterol recovered in VLDL (Table 7.11) was significantly (P • 0.03) decreased in birds inoculated with FMG at 12 wk as compared to 12 wk sham-inoculated controls; however,

serum cholesterol derived from LDL (Table 7.12) was significantly (P • 0.05) increased in birds inoculated with FMG at 12 wk as compared to 12 wk sham-inoculated controls.

Birds inoculated with FMG at 12 wk of age had significantly increased yolk palmitic acid (P • 0.03) and significantly decreased oleic (P • 0.02) acid concentrations when compared to 12 wk sham-inoculated (FMG-free) controls (Table 7.13). Relative liver weight (P • 0.03) and liver moisture (P • 0.05) and lipid (P • 0.05) contents were significantly decreased in birds inoculated with FMG at 22 wk of age when compared to sham-inoculated (FMG-free) birds (Table 7.14). Relative (% of BW) oviduct (P • 0.03), infundibulum (P • 0.04), isthmus (P • 0.02), and uterus (P • 0.05) weights were significantly increased in birds inoculated with FMG at 22 wk of age when compared to sham-inoculated (FMG-free) birds (Table 7.15). Also, FMG-inoculation significantly increased yolk palmitic acid (P • 0.03) and decreased oleic acid (P • 0.02) concentrations at 58 wk of age (Table 7.16). Sham or FMG inoculation at 22 wk rather than at 12 wk of age significantly increased overall serum cholesterol (P • 0.03) and ST (P • 0.02). Concentrations of serum cholesterol and ST in the blood of 22 wk sham- or FMG-inoculated birds was significantly higher overall when compared to 12 wk sham- or FMG-inoculated birds. Concentrations of serum cholesterol and ST in the blood of 12 wk sham- and FMG-inoculated birds were 218.7 and 4939.7 mg/dl (SEM=30.34), respectively, and in 22 wk sham- or FMG-inoculated birds were 292.2 and 7214.4 mg/dl (SEM=574.69), respectively.

Discussion

Although there were significant age and inoculation main effects, along with significant age by inoculation interactions, only significant age by treatment, treatment by inoculation, and age by treatment by inoculation interaction data will be discussed. Pullets are generally vaccinated with FMG between 8 and 18 wk of age (Yoder *et al*., 1984) and remain infected for life (Brown *et al*., 1995). At the beginning and end of this study, SPA tests from swabs and sera and HI sera tests, along with the FA tests verified systemic infections in FMG-inoculated birds. Conversely, sham-inoculated birds remained FMG-free. Current study results indicate that there was no cross contamination between FMG-inoculated and FMG-clean birds. Reports have described variable levels of protection against decreases in EP of hens vaccinated with low-virulence live MG (Truscott *et al*., 1974; Fabricant, 1977). Glisson and Kleven (1984) reported that all hens vaccinated with low virulence MG at 16 or 20 wk of age were protected against EP drops seen in unvaccinated hens challenged with virulent MG. It has been suggested that vaccination of pullets with FMG before the laying cycle may enable them to maintain full EP (253 eggs per year), whereas, vaccination during lay may reduce EP (Branton and May, 1999).

Pre- and post-peak weekly EP were increased in birds inoculated with FMG at 22 wk of age in comparison to those inoculated at 12 wk; however, total EP in birds inoculated with FMG at 12 or 22 wk in the current investigation were not significantly different. This suggests that birds inoculated at 12 wk of age compensated for these reductions during peak EP. Total egg mass was decreased by • 1000 g and feed

conversion was increased by • 0.5 g feed/g egg in 22 wk FMG-inoculated birds when compared to either 22 wk sham-inoculated or 12 wk FMG-inoculated birds. Percentages of SS eggs laid by 22 wk FMG-inoculated birds were higher at 22, 23, 24, and 25 wk when compared to 22 wk sham-inoculated controls and at each of these time periods, 12 wk FMG-inoculated birds had already shifted to a larger egg size category. Although the establishment of systemic FMG infections in young birds takes approximately six weeks (Truscott *et al*., 1974; Mallinson and Rosenstein, 1976; Yoder, 1986; McMartin *et al*., 1987; Soeripto *et al*., 1989; Nunoya *et al*., 1995), the higher percentage of SS eggs laid during pre-peak production by 22 wk FMG-inoculated hens in the current study may be due to a faster and more severe colonization rate of FMG in older birds. Also, 22 wk sham-inoculated control hens also may have already shifted to a larger egg size category by this time.

In the current study, decreases in relative liver weight and lipid content in 22 wk FMG-inoculated birds suggest an overall decrease in lipid synthesis in the liver of infected birds. Colonization of FMG in the liver may be responsible for metabolic and compositional alterations necessary for successful EP. Also, decreased production in the liver of the components necessary for YL deposition may lead to decreased lipid uptake in the ovary. Reproductive impairments in FMG-inoculated birds demonstrate the oviducts role in FMG effects on EP and the importance of inoculation timing on oviduct function. Intestinal characteristics were not influenced by FMG inoculation, which suggests that FMG does not affect lipid metabolism through changes in lipid absorption in the gut. This may be related to the fact that average temperature of the avian intestinal

tract is above the optimal temperature (37 C) for growth of MG (Razin and Freundt, 1984; Kleven, 1997). Inoculation of isolated pullets with FMG at 12 wk of age may potentially lead to greater improvements in layer performance when compared to those inoculated at later time periods or subjected to a natural field strain of MG. Inoculation with FMG at 12 wk of age may, therefore, prove to be a more suitable and cost efficient vaccination time period.

Although birds were housed in caged facilities in the current investigation, instead of isolation units (Burnham *et al*., 2002a), mortality was still not significantly different between treatment groups or inoculation periods. Branton and Deaton (1985) reported that although mortality may be negligible in adult flocks infected with FMG, there still can be a reduction in the number of birds in production. Burnham *et al*., (2002a) were the first to report delayed (• 1 week) onset of EP in FMG-inoculated as compared to FMG-free hens housed in biological isolation units. Similarly, in the current study, onset of lay was delayed ten days in birds inoculated with FMG at 12 wk of age compared to those sham-inoculated at 12 wk while housed in a caged layer facility. Reports concerning birds housed in industry caged layer facilities have also indicated that there was no difference in EP over a 45 week laying period when FMG-free and FMG-inoculated hens (Branton *et al*. 1997) or ts-11 vaccine strain MG inoculated hens and controls (Branton *et al*., 2000) were compared. However, Carpenter *et al*., (1981) also using birds housed in caged layer facilities reported that MG-negative flocks produced 16 more eggs per hen per year than laying flocks positive for field strain MG. In that same study, when uninfected flocks were compared to FMG-vaccinated flocks, the advantage decreased to

8.7 eggs per hen. Also, FMG-vaccinated flocks produced 7.0 more eggs per hen per year than field strain MG-infected flocks. Other authors have reported that EP and feed efficiency were reduced in flocks naturally infected with MG (Yoder, 1978, 1991; Mohammed *et al.*, 1987). Burnham *et al*., (2002a) have reported that weekly EP after 42 wk and total EP per bird over a complete cycle in isolation units were reduced in layer hens inoculated with FMG at 12 wk of age. Weekly EP of birds housed in the caged layer facility and those housed in isolation units were similar, but total EP in birds inoculated with FMG at 12 wk in the current investigation were not significantly different. Burnham *et al*., (2002a) also reported that percentages of undersized eggs laid by 12 wk FMG-inoculated birds were lower at 19 wk, but were higher at 20 and 21 wk when compared to controls. These same type of age affects as were observed for undersized eggs from birds housed in isolation units in the previous study, were observed for PS and SS eggs in the current study. Percentages of PS eggs laid by 12 wk FMG-inoculated birds were lower at 18 and 19 wk, but were higher at 20 wk when compared to controls. Also, percentages of SS eggs laid by 12 wk FMG-inoculated birds were lower at 19, 20, 22, 23, 24, and 25 wk when compared to 12 wk sham-inoculated controls. The lower percentages of PS and SS eggs laid during pre-peak by 12 wk FMG-inoculated hens in the present study, is indicative of a delay in EP in 12 wk FMG-infected birds. As previously stated, initiation of lay in 12 wk sham-inoculated control hens began earlier than 12 wk FMG-inoculated hens. Similar to that for undersized eggs (Burnham *et al*., 2002a), PS and SS eggs in the current study were being laid by 12 wk sham-inoculated control hens prior to their lay by 12 wk FMG-inoculated hens. Concurrently, when 12 wk treated birds began to lay PS

and SS eggs, 12 wk control hens shifted to a larger size category. Burnham *et al*., (2002c) have reported decreases in YL and cholesterol between 22 and 28 wk of age in birds inoculated with FMG. In that same study, decreases in overall yolk myristic, palmitoleic, and oleic acid percentages, along with increases in yolk linoleic, stearic and arachidonic acid percentages were reported. However, in the current study, only YM and yolk palmitic and oleic acid concentrations were affected. Increased palmitic and decreased oleic acid concentrations across age of bird in 12 wk FMG-inoculated compared to 12 wk sham-inoculated control birds, and in FMG-inoculated compared to sham-inoculated control birds at 58 wk of age may be due more specifically to altered activities of various liver lipid enzymes.

Previous reports have indicated that the characteristics of lipoproteins from birds housed in fiberglass isolation units were not significantly affected by FMG-inoculation. However, higher degrees of physiological stress experienced by hens in a caged layer facility may exacerbate the effects of FMG-inoculation and lead to changes in lipoprotein synthesis. Other reports have indicated a reduction in ripe ovarian follicles, ovarian follicle size, and magnal, isthmal, and vaginal proportions, along with increased incidences of fatty liver hemorrhagic syndrome in birds having been previously inoculated with FMG at 12 wk of age and maintained in biological isolation units (Burnham *et al*., 2002b). In the caged layer facility these same effects were less evident than those observed in the isolation units. Inoculation with FMG at 12 wk of age in birds housed in caged layer facilities, compared to isolation units, may, therefore, prove to be a more suitable and cost efficient vaccination time period and environmental condition.

Birds inoculated with FMG at 12 or 22 wk of age may perform less adequately when housed in a caged layer facility when compared to those housed in biological isolation units. Individuals in both types of facilities, nevertheless, perform better when uninfected with any form of MG than when vaccinated at 12 or 22 wk with FMG. This research provides information necessary for formulating vaccination timetables and for predicting the effects of pre-lay or lay period FMG vaccinations on performance. Research in caged layer facilities may be more appropriate than that attained from isolation units from a practical perspective. Isolation units may be more suitable for research at the cellular and molecular levels, because environmental factors influence the severity of mycoplasma infections. These experiments may lead to new approaches for the treatment and control of MG.

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TABLE 7.1 Ingredient percentages and calculated and determined analyses of pullet and layer diets

	Starter	Grower	Developer	Prelay	Layer[1]				
Age (week)	0-6	6-12	12-18	18-20	20	24	28	32-40	40-60
Ingredients:	(%)								
Corn, 8.6%	64.51	73.64	72.22	61.35	64.41	63.06	65.00	66.39	66.64
Soybean meal, 48%	30.97	22.09	17.17	19.13	23.29	24.76	20.70	17.47	16.27
Wheat middlings	0.00	0.00	6.39	11.67	0.46	0.47	2.77	4.43	5.55
Vitamin premix[2,3]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
DL-methionine[4]	0.15	0.11	0.10	0.13	0.17	0.17	0.14	0.11	0.10
Dicalcium phosphate[5]	2.08	1.99	1.92	1.68	1.81	1.81	1.78	1.70	1.63
Limestone[6]	1.06	0.95	0.98	4.82	8.58	8.45	8.33	8.62	8.53
Sodium chloride[7]	0.48	0.47	0.47	0.47	0.53	0.53	0.53	0.53	0.53
Poultry fat	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Dietary analyses:									
CP, calculated	20.50	17.00	15.50	16.34	16.50	17.10	15.64	14.44	14.05
CP, determined	ND[8]	ND	ND	ND	ND	ND	ND	14.00	14.40
Crude fiber, calculated	2.29	2.24	2.55	2.76	2.23	2.24	2.35	2.41	2.47
Crude fiber, determined	ND	ND	ND	ND	ND	ND	ND	3.10	2.40
Crude fat, calculated	3.22	3.52	3.68	3.48	3.12	3.08	3.21	3.31	3.36
Crude fat, determined	ND	ND	ND	ND	ND	ND	ND	3.20	3.10
Ash, determined	ND	ND	ND	ND	ND	ND	ND	16.30	13.50
Moisture, determined	ND	ND	ND	ND	ND	ND	ND	10.90	12.10
ME, calculated kcal/kg	3,000	3,101	3,051	2,819	2,819	2,808	2,819	2,819	2,819

TABLE 7.1 Continued.

Available phosphorus, calculated	0.43	0.42	0.42	0.38	0.34	0.34	0.34	0.33	0.31
Calcium, calculated	0.88	0.82	0.82	2.25	3.70	3.65	3.60	3.70	3.65
Lysine, calculated	1.10	0.85	0.73	0.80	0.85	0.89	0.79	0.70	0.68
Methionine, calculated	0.50	0.42	0.38	0.41	0.46	0.47	0.42	0.37	0.35
Methionine + cystine, calculated	0.81	0.68	0.61	0.65	0.71	0.73	0.65	0.58	0.56
Potassium, calculated	0.81	0.66	0.55	0.56	0.65	0.68	0.60	0.54	0.52
Sodium, calculated	0.20	0.20	0.20	0.20	0.21	0.21	0.21	0.21	0.21
Tryptophan, calculated	0.28	0.23	0.20	0.22	0.22	0.23	0.21	0.19	0.18
Xanthophyll, calculated	6.45	7.36	7.22	6.14	6.44	6.30	6.49	6.63	6.66

[1]Available protein and lysine percentages in the layer diet were adjusted as needed according to the percentage of feed consumed per bird every 28 days until trial termination.

[2]Vitamin premix provided per kilogram of diet: vitamin A, 7,710 IU; cholecalciferol, 2,202 IU; vitamin E, 10 IU; menadione, 0.88 mg; vitamin B_{12}, 0.01 mg; choline, 380 mg; riboflavin, 5 mg; niacin, 33 mg; pantothenic acid, 9 mg; thiamine, 1 mg; folic acid, 0.6 mg; biotin, 0.06 mg; pyridoxine, 0.9 mg; ethoxyquin, 0.03 g.

[3]Trace minerals provided in vitamin premix: manganese, 2.2%; zinc, 2.0%; iron, 1.1%; copper, 1,400 ppm; iodine, 200 ppm; and selenium, 40 ppm.

[4]Manufactured by Degussa Corp., Ridgeland Park, NJ 07600-2100.

[5]Manufactured by IMC-Agrico Feed Ingredients, Bannockburn, IL 60015.

[6]Manufactured by Franklin Industrial Minerals, Nashville, TN 37203.

[7]Manufactured by Cargill Incorporated, Minneapolis, MN 55440.

[8]Not determined.

TABLE 7.2 Determined fatty acid analyses of layer diets at 36 and 56 weeks of age

		Trial 3	
Age (week)		36	56
		----------(%)----------	
Determined Fatty Acid Analyses:			
Myristic	(c14:0)	0.3	0.2
Palmitic	(c16:0)	16.5	15.8
Palmitoleic	(c16:1)	2.3	1.8
Stearic	(c18:0)	4.3	4.1
Oleic	(c18:1)	32.6	29.7
Linoleic	(c18:2)	41.5	49.1
Linolenic	(c18:3)	1.3	1.9

TABLE 7.3 Body weight at 12, 20, 24, 28, 32, 34, 36, 40, 44, 48, 50, 52, 56, 58, and 60 week of age in Single Combed White Leghorn laying hens inoculated (sham and FMG) at 12 or 22 weeks of age[1]

	Inoculation (week)	
Age (week)	Twelve[2]	Twenty-two[2]
	----------(g)----------	
12	877	874
20	1467	1532
24	1550	1535
28	1571	1601
32	1607	1654
34	1635	1686
36	1662	1717
40	1714[b]	1811[a]
44	1774	1833
48	1785[b]	1865[a]
50	1797[b]	1874[a]
52	1809[b]	1891[a]
56	1831[b]	1916[a]
58	1855[b]	1934[a]
60	1876[b]	1951[a]

[a,b]Means within week of age among inoculation periods with no common superscript differ significantly (P• 0.05).
[1]Based on pooled estimate of variance SEM = 27.4.
[2]n = 60.

TABLE 7.4 Feed conversion in twelve and twenty-two week sham- and FMG-inoculated Single Combed White Leghorn laying hens between 20 and 24, 24 and 28, 28 and 32, 32 and 36, 36 and 40, 40 and 44, 44 and 48, 48 and 52, 52 and 56, and 56 and 60 weeks of age[1]

Inoculation (week)	Twelve[2]		Twenty-two[2]	
Age (week)	Sham-inoculated	FMG-inoculated	Sham-inoculated	FMG-inoculated
	----------(g feed/g eggs)----------			
20-24	2.0[a]	1.8[b]	1.7[b,3]	2.3[a,3]
24-28	1.9	1.8	1.8	1.8
28-32	1.9	1.8	1.9	1.8
32-36	1.8	1.8	1.9	1.8
36-40	1.8	1.8	1.9	1.8
40-44	1.8	1.8	1.8	1.8
44-48	1.7	1.7	1.7	1.7
48-52	1.7	1.6	1.7	1.7
52-56	1.6	1.7	1.7	1.6
56-60	1.7	1.7	1.7	1.7

[a,b]Means within week of age and inoculation period among treatment groups with no common superscript differ significantly (P• 0.05).

[1]Based on pooled estimate of variance SEM = 0.057.

[2]n = 60.

[3]Values adjusted for 22-24 wk feed conversion.

TABLE 7.5 Percentage of pee-wee sized eggs in twelve and twenty-two week sham- and FMG-inoculated Single Combed White Leghorn laying hens at 18, 19, and 20 weeks of age[1]

Inoculation (week)	Twelve[2]		Twenty-two[2]	
Age (week)	Sham-inoculated	FMG-inoculated	Sham-inoculated	FMG-inoculated
	----------(%)----------			
18	41.7[a]	0.0[b]	ND[3]	ND
19	61.9[a]	33.3[b]	ND	ND
20	38.1[b]	54.4[a]	ND	ND

[a,b]Means within week of age and inoculation period among treatment groups with no common superscript differ significantly (P• 0.05).
[1]Based on pooled estimate of variance SEM = 4.4.
[2]n = 60.
[3]Not determined.

TABLE 7.6 Percentage of small sized eggs in twelve and twenty-two week sham- and FMG-inoculated Single Combed White Leghorn laying hens at 19, 20, 21, 22, 23, 24, and 25 weeks of age[1]

Inoculation (week)	Twelve[2]		Twenty-two[2]	
Age (week)	Sham-inoculated	FMG-inoculated	Sham-inoculated	FMG-inoculated
	(%)			
19	33.3[a]	0.0[b]	ND[3]	ND
20	61.9[a]	45.6[b]	ND	ND
22	100.0[a]	85.7[b]	85.7[b]	100.0[a]
23	71.4[a]	57.1[b]	33.3[c]	57.1[b]
24	47.6[a]	19.1[b]	19.1[b]	33.3[a]
25	19.1[a]	0.0[b]	0.0[b]	14.3[a]

[a,b]Means within week of age and inoculation period among treatment groups with no common superscript differ significantly (P• 0.05).

[1]Based on pooled estimate of variance SEM = 4.4.

[2]n = 60.

[3]Not determined.

TABLE 7.7 Percentage of medium and large sized eggs from twelve and twenty-two week inoculated (sham and FMG) Single Combed White Leghorn laying hens at 23, 35, 37, 56, and 58 weeks of age

	Medium[1]		Large[1]	
	Inoculation (week)			
Age (week)	Twelve[2]	Twenty-two[2]	Twelve[2]	Twenty-two[2]
	(%)			
23	33.3[b]	54.8[a]	0.0	0.0
35	61.9[b]	92.9[a]	38.1[a]	7.1[b]
37	26.2[b]	40.5[a]	73.8[a]	59.5[b]
56	2.4	0.0	83.3[b]	100.0[a]
58	0.0	0.0	78.6[b]	92.9[a]

[a,b]Means within egg size classes and week of age among inoculation periods with no common superscript differ significantly (P• 0.05).

[1]Based on pooled estimate of variance SEM = 4.83 and 5.63 for medium and large sized eggs, respectively.

[2]n = 60.

TABLE 7.8 Yolk moisture in sham- and FMG-inoculated, and in twelve and twenty-two week inoculated (sham and FMG) Single Combed White Leghorn laying hens at 24, 34, 44, 50, and 58 weeks of age[1]

	Treatment		Inoculation (week)	
Age (week)	Sham-inoculated[2]	FMG-inoculated[2]	Twelve[2]	Twenty-Two[2]
	----------(%)----------		----------(%)----------	
24	63.8	64.4	63.7[b]	64.5[a]
34	60.5	60.9	60.5	60.9
44	58.2[b]	59.1[a]	59.1[b]	58.2[a]
50	58.5[b]	59.4[a]	59.2	58.7
58	58.2[b]	59.0[a]	58.5	58.6

[a,b]Means within week of age among treatment groups or inoculation periods with no common superscript differ significantly (P• 0.05).

[1]Based on pooled estimate of variance SEM = 0.46 and 0.5 for inoculation and treatment group means, respectively.

[2]n = 60.

TABLE 7.9 Plasma protein and calcium in twelve and twenty-two week inoculated (sham and FMG) Single Combed White Leghorn laying hens at 20, 24, 34, 44, 50, and 58 weeks of age[1]

	Plasma protein		Serum Calcium	
	Inoculation (week)			
Age (week)	Twelve[2]	Twenty-two[2]	Twelve[2]	Twenty-two[2]
	----------(g/dl)----------		----------(mg/dl)----------	
20	3.8	ND[3]	25.1	ND
24	3.0	3.2	35.5	34.8
34	5.1[b]	6.6[a]	24.5[b]	25.9[a]
44	5.1	5.0	22.0	21.8
50	4.9	5.3	26.6	25.7
58	5.0	5.2	25.2[a]	22.8[b]

[a,b]Means within parameter and week of age among inoculation periods with no common superscript differ significantly (P• 0.05).

[1]Based on pooled estimate of variance SEM = 0.33 and 1.62 for plasma protein and calcium, respectively.

[2]n = 12.

[3]Not determined.

TABLE 7.10 Very low density lipoprotein (VLDL) particle diameter distributions (10th, 50th, and 90th percentiles), and mean VLDL particle diameter (MA) in twelve and twenty-two week sham- and FMG-inoculated Single Combed White Leghorn laying hens[1]

Inoculation (week)	Twelve[2]		Twenty-two[2]	
	Sham-inoculated	FMG-inoculated	Sham-inoculated	FMG-inoculated
	----------(nm)----------			
Tenth	22.0[b]	25.1[a]	23.5[a]	20.2[b]
Fiftieth	30.4[b]	33.2[a]	32.1[a]	28.0[b]
Ninetieth	45.5[b]	46.7[a]	47.1[a]	42.8[b]
MA	32.7[b]	35.0[a]	34.2[a]	30.2[b]

[a,b]Means within inoculation periods among treatment groups with no common superscript differ significantly (P• 0.05).

[1]Based on pooled estimate of variance SEM = 0.92, 1.06, 1.2, and 1.04 for 10th, 50th, and 90th VLDL percentiles, and MA, respectively.

[2]n = 12.

TABLE 7.11 Percentage serum cholesterol recovered from very low density lipoprotein particles in twelve and twenty-two week sham- and FMG-inoculated Single Combed White Leghorn laying hens at 44 and 58 weeks of age[1]

Inoculation (week)	Twelve[2]		Twenty-two[2]	
Age (week)	Sham-inoculated	FMG-inoculated	Sham-inoculated	FMG-inoculated
	----------(%)----------			
44	58.1	58.2	65.4	61.2
58	90.7[a]	82.0[b]	81.9	87.8

[a,b]Means within week of age and inoculation period among treatment groups with no common superscript differ significantly (P• 0.05).
[1]Based on pooled estimate of variance SEM = 2.47.
[2]n = 12.

TABLE 7.12 Percentage serum cholesterol recovered from low density lipoprotein particles in twelve and twenty-two week sham- and FMG-inoculated Single Combed White Leghorn laying hens at 44 and 58 weeks of age[1]

Inoculation (week)	Twelve[2]		Twenty-two[2]	
Age (week)	Sham-inoculated	FMG-inoculated	Sham-inoculated	FMG-inoculated
	----------(%)----------			
44	28.6	27.1	24.8	30.4
58	9.3[b]	18.0[a]	18.1	12.2

[a,b]Means within week of age and inoculation periods among treatment groups with no common superscript differ significantly (P• 0.05).
[1]Based on pooled estimate of variance SEM = 3.66.
[2]n = 12.

TABLE 7.13 Yolk palmitic and oleic acids in twelve and twenty-two week sham- and FMG-inoculated Single Combed White Leghorn laying hens[1]

Inoculation (week)	Twelve[2]		Twenty-two[2]	
	Sham-inoculated	FMG-inoculated	Sham-inoculated	FMG-inoculated
	----------(% of total fatty acids)----------			
Palmitic acid	26.2[b]	27.5[a]	27.0	27.2
Oleic acid	39.0[a]	35.9[b]	37.9	37.7

[a,b]Means within inoculation period among treatment groups with no common superscript differ significantly (P• 0.05).

[1]Based on pooled estimate of variance SEM = 0.29 and 0.57 for yolk palmitic and oleic acids, respectively.

[2]n = 12.

TABLE 7.14 Relative liver weight, liver moisture, and liver lipid in twelve and twenty-two week sham- and FMG-inoculated Single Combed White Leghorn laying hens[1]

Inoculation (week)	Twelve[2]		Twenty-two[2]	
	Sham-inoculated	FMG-inoculated	Sham-inoculated	FMG-inoculated
	(%)			
Liver weight	2.1	1.7	2.9[a]	1.7[b]
Liver moisture	24.4	30.6	44.3[a]	32.1[b]
Liver lipid	7.1	8.7	27.8[a]	10.8[b]

[a,b]Means within inoculation period among treatment groups with no common superscript differ significantly (P• 0.05).

[1]Based on pooled estimate of variance SEM = 0.34, 3.6, and 4.8 for relative liver weight, moisture, and lipid, respectively.

[2]n = 12.

TABLE 7.15 Relative oviduct, infundibulum, isthmus, and uterus weights in twelve and twenty-two week sham- and FMG-inoculated Single Combed White Leghorn laying hens[1]

Inoculation (week)	Twelve[2]		Twenty-two[2]	
	Sham-inoculated	FMG-inoculated	Sham-inoculated	FMG-inoculated
	----------(%)----------			
Oviduct weight	3.8	3.4	2.1[b]	3.3[a]
Infundibulum weight	0.1	0.1	0.1[b]	0.2[a]
Isthmus weight	0.4	0.4	0.2[b]	0.3[a]
Uterus weight	1.2	1.1	0.8[b]	1.2[a]

[a,b]Means within inoculation period among treatment groups with no common superscript differ significantly (P• 0.05).

[1]Based on pooled estimate of variance SEM = 0.34, 0.02, 0.04, and 0.11 for relative oviduct, infundibulum, isthmus, and uterus weights, respectively.

[2]n = 12.

TABLE 7.16 Yolk palmitic and oleic acids in sham- and FMG-inoculated Single Combed White Leghorn laying hens at 44 and 58 weeks of age[1]

Fatty Acid	Palmitic[2]		Oleic[2]	
Age (week)	Sham-inoculated	FMG-inoculated	Sham-inoculated	FMG-inoculated
	----------(% of total fatty acids)----------			
44	27.1	27.4	38.0	37.5
58	26.1[b]	27.3[a]	38.9[a]	36.1[b]

[a,b]Means within fatty acid and week of age among treatment groups with no common superscript differ significantly (P• 0.05).

[1]Based on pooled estimate of variance SEM = 0.29 and 0.57 for yolk palmitic and oleic acids, respectively.

[2]n = 12.

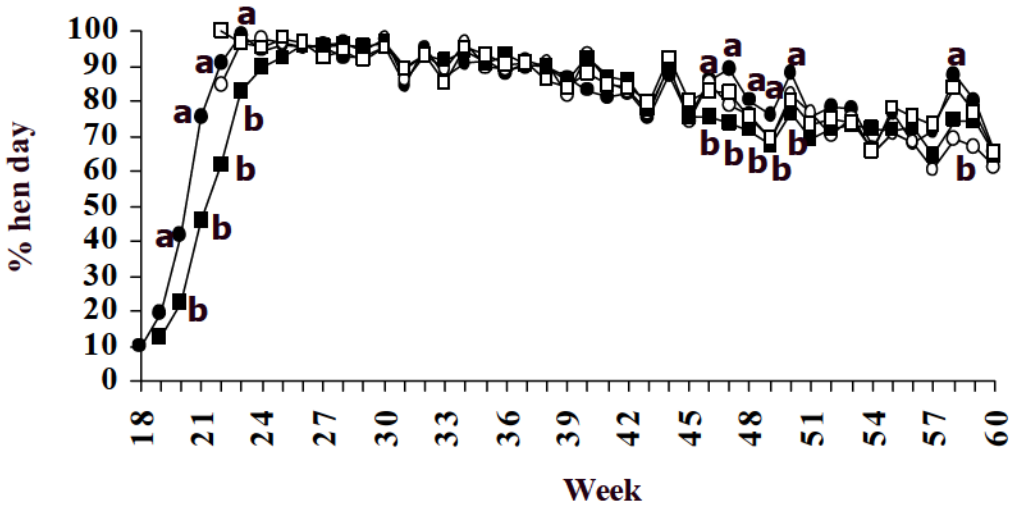

Figure 7.1 Weekly percent hen day egg production between 18 and 60 weeks of age for twelve wk sham-inoculated (•), twelve wk FMG-inoculated (•), twenty-two wk sham-inoculated (•), and twenty-two wk FMG-inoculated (•) layer hens. Symbols within a week having different letters are significantly different (P • 0.05). Based on pooled estimate of variance SEM = 3.6.

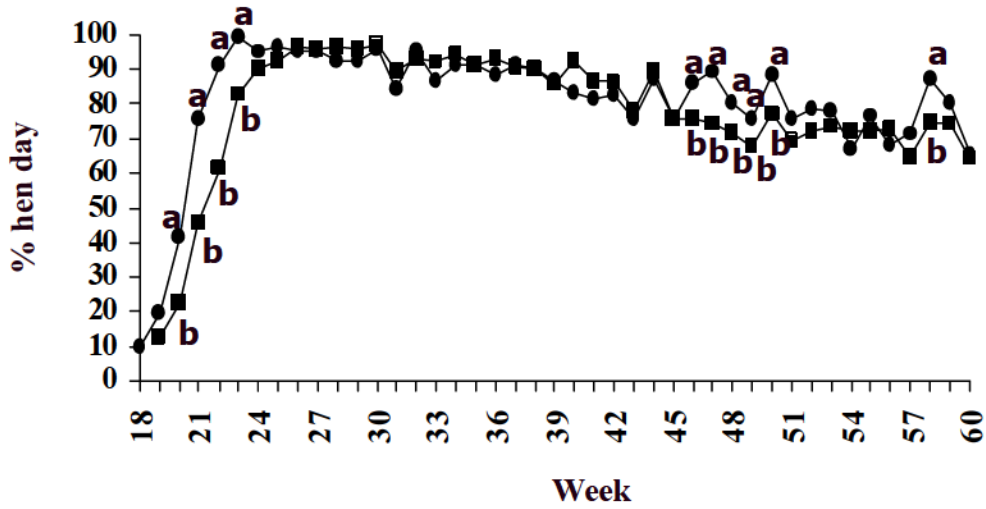

Figure 7.2 Weekly percent hen day egg production between 18 and 60 weeks of age for twelve wk sham-inoculated (•) and twelve wk FMG-inoculated (•) layer hens. Symbols within a week having different letters are significantly different (P • 0.05). Based on pooled estimate of variance SEM = 3.6.

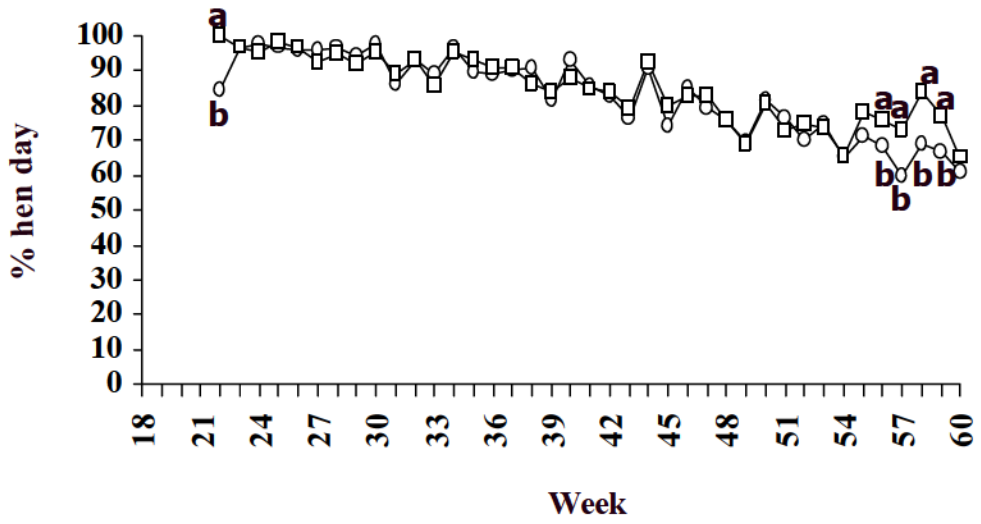

Figure 7.3 Weekly percent hen day egg production between 22 and 60 weeks of age for twenty-two wk sham-inoculated (•) and twenty-two wk FMG-inoculated (•) layer hens. Symbols within a week having different letters are significantly different (P • 0.05). Based on pooled estimate of variance SEM = 3.6.

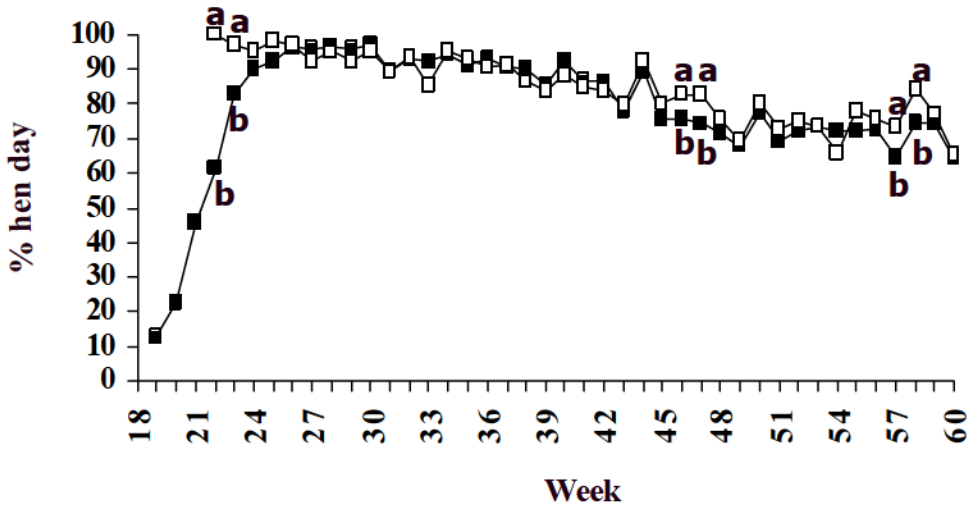

Figure 7.4 Weekly percent hen day egg production between 18 and 60 weeks of age for twelve wk FMG-inoculated (•) and twenty-two wk FMG-inoculated (•) layer hens. Symbols within a week having different letters are significantly different (P • 0.05). Based on pooled estimate of variance SEM = 3.6.

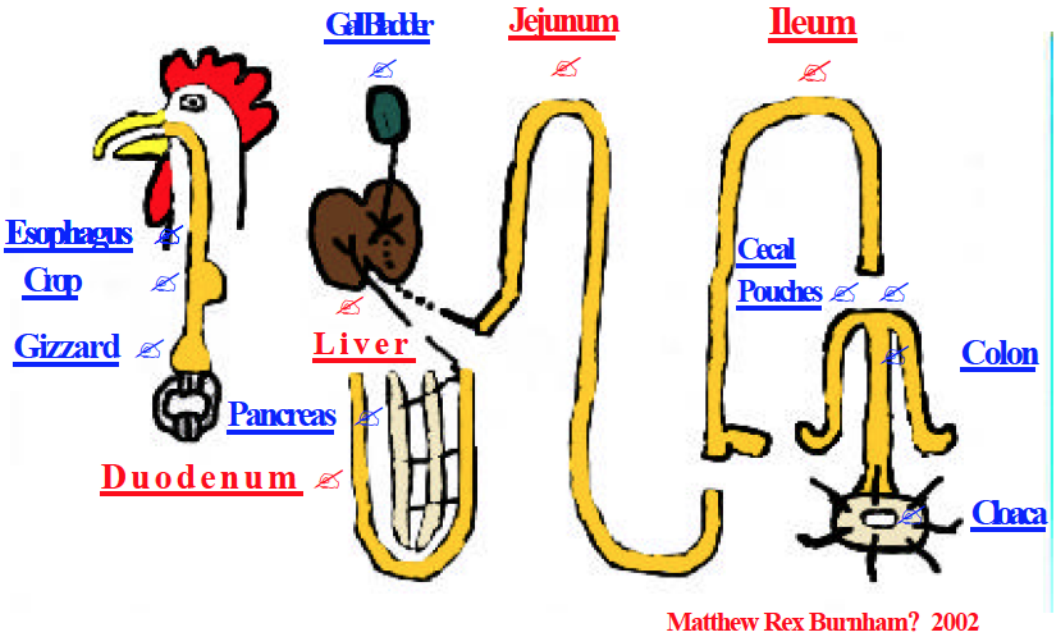

Figure 7.5 Diagram of the avian digestive tract.

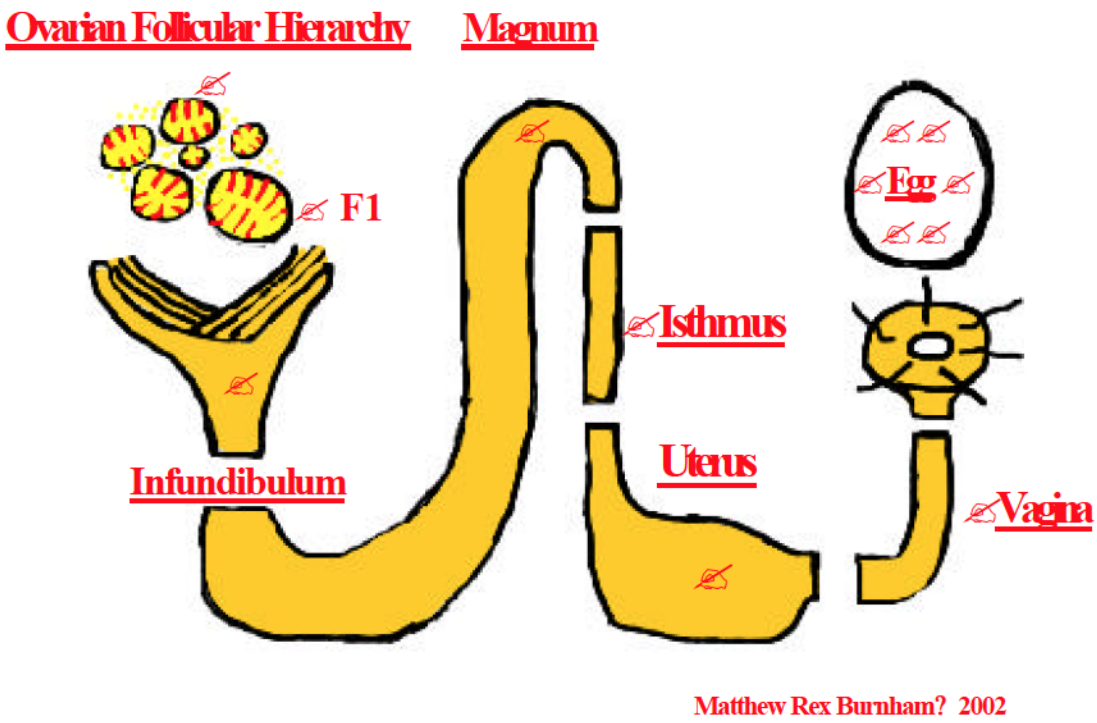

Figure 7.6 Diagram of the avian reproductive tract.

CHAPTER VIII

SUMMARY, IMPLICATIONS, AND CONCLUSIONS

The F-strain of *Mycoplasma gallisepticum* (FMG) was used in these experimental trials. These experiments were conducted in order to gain a better understanding of the mechanisms responsible for altered egg production (EP) and egg quality in commercial layers, as these alterations can cause economic loss to the U.S. layer industry. An understanding of the pathogenic and physiological processes associated with *Mycoplasma gallisepticum* (MG) infections may lead to new approaches to the treatment and control of MG. This study was designed to examine potential mechanism(s) responsible for alterations in production parameters and physiological characteristics of birds inoculated with FMG at 12 wk in fiberglass isolation units and at 12 and 22 wk in a caged layer facility. All parameters selected for examination in this study have a potential role in influencing the uptake, metabolism, and deposition of materials necessary for EP in chickens infected with FMG. It is clear that eradication of MG is the best solution in terms of maximizing EP and reducing vaccination and/or medication costs. However, it is equally important to realize that on some multi-age farms, MG eradication is not currently practical. Vaccination programs using FMG can provide significant protection against decreased EP caused by field strain MG infection.

In isolation units, 12 wk FMG-inoculation delayed onset of lay • 1 wk, and decreased EP 34 wk post-inoculation and overall EP, and resulted in a higher incidence of fatty liver hemorrhagic syndrome, ovarian follicular regression, and decreased isthmal and vaginal proportions of the reproductive tract. Ovarian regression may be related to retarded production (liver), transport (blood), and/or uptake (ovary) of yolk particles. Changes in blood (i.e. lipid) characteristics with FMG colonization of the liver may become manifest through changes in egg constituents. Liver FMG colonization significantly affects EP through alterations in yolk lipid and fatty acid concentrations. As evidenced through changes in the relative weights of various reproductive organs, colonization of these organs by FMG, in addition to the liver, may also be a cause of the effects observed on EP. Increases in hematocrit, serum triglycerides, and plasma protein between 8 and 10 wk post FMG-inoculation, suggest that the initial weeks of EP are stressful, and post-peak decreases in both serum triglycerides and plasma protein suggest a more chronic inhibition on lipid and protein synthesis in the liver. Decreased blood lipid concentration may be directly responsible for the reductions in yolk lipid, cholesterol, and fatty acid deposition in 12 wk FMG-inoculated hens.

In the caged layer facility, onset of lay was also delayed • 1 wk in birds inoculated with FMG at 12 wk and total egg mass was decreased by • 1000 g and feed conversion was increased by • 0.5 g feed/g egg in 22 wk FMG-inoculated birds. Inoculation with FMG at 12 wk of age, therefore, appears to be more desirable and cost efficient than an FMG inoculation at 22 wk of age. Lower percentages of undersized, peewee, and small sized eggs laid during pre-peak by 12 wk FMG-inoculated hens is indicative of a delay in

EP. Higher degrees of physiological stress experienced by hens in a caged layer facility may exacerbate the effects of FMG-inoculation seen in the isolation units. These data demonstrate that alterations in performance and egg characteristics of commercial layers inoculated with FMG at either 12 or 22 wk of age and housed in either isolation units or caged layer facilities are related to mutual functional disturbances in the blood, liver, ovary, and oviduct without concomitant intestinal changes. Research in caged layer facilities may be more appropriate than that attained from isolation units from a practical perspective. On the other hand, isolation units may be more suitable for research at the cellular and molecular levels, because environmental factors influence the severity of mycoplasma infections. Numerous citations have reported alterations in EP due to FMG inoculation, but previous information in the literature is controversial concerning the degree of effects on EP under different housing conditions and the timing of administration. Certainly, the effects of MG are due to type and degree of tissue colonization. This may vary under the different environmental conditions (i.e. facility, disease, etc.) imposed on the birds and may thus have an enormous impact on the performance of commercial laying hens.