INVASION OF AVIAN REPRODUCTIVE TISSUES BY *SALMONELLA* TYPHIMURIUM AND *SALMONELLA* ENTERIDITIS

A Thesis

by

ZOE R. HOWARD

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2003

Major Subject: Food Science and Technology

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Approved as to style and content by:

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ABSTRACT

Invasion of Avian Reproductive Tissues by *Salmonella* Typhimurium and *Salmonella* Enteritidis. (August 2003)

Zoe R. Howard, B.S., Texas A&M University

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In recent decades salmonellosis has been on the rise as a food related illness worldwide. Causing over 24% of all non-typhoidal Salmonellosis cases, SE is the most frequently isolated serovar of *Salmonella.* Increased isolation of SE from eggs has paralleled an increase in the number of transovarian infections associated with laying hens in the poultry industry. This route of infection is a fairly new line of study when compared to the more traditional path where SE originates from fecal contamination through the shell.

Salmonella Typhimurium (ST) is another concern for the egg industry. ST has caused 23.5% of all non-typhoidal salmonellosis cases. Understanding these two egg pathogens requires an in depth look at the mechanisms by which an egg may support infection and bacterial growth. Eggs were inoculated with both SE and ST onto the vitelline membrane and incubated for 24 hours. It was hoped that by gathering samples from the interior of the egg membrane, the albumen of the egg, and the membrane itself, some clarification as to when *Salmonella* is allowed to grow within the egg could be gathered. Albumen and membrane were found to be more hospitable environments to bacterial growth with increased storage times.

In order to better understand the movement of bacteria into pre-ovulatory tissues, samples were gathered from mature laying hens. Follicular tissues were separated into divisions based on maturity, and bacteria were added to an in vitro cell culture broth containing the follicles. The point of this experiment was to determine if either species of *Salmonella* preferentially moved into follicles of different maturity when inoculated in vitro.

A third experiment looked into the role of developmental stages of the vitelline membrane in exclusion of bacteria from the nutrient rich yolk. Tissues were gathered in the method described above. The follicular sack was removed from half of these samples and left intact for the other half. Another treatment group included was the yolks of eggs which had been laid by the same flock of birds. Results showed that follicles with intact follicular sacks were more susceptible to bacterial colonization than other treatment groups.

DEDICATION

My Pop:
Ryan Madden Howard
December 23 1910- December 23, 2001

"All that is gold does not glitter,
Not all those who wander are lost;
The old that is strong does not wither,
Deep roots are not reached by the frost.
From ashes a fire shall be woken,
A light from the shadows will spring;
Renewed shall be the blade that was broken,
The crownless again shall be king."
-The Fellowship of the Ring

My Friend:
Erin Marie Jackson
September 20, 1979- October 14, 2001

"I learned that there burns within us all a flame
of independence that must not be allowed to go out.
That as long as it exists within us
we cannot be destroyed."
-The Power of One

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INTRODUCTION

The Centers for Disease Control (CDC) shows a recorded incidence of more than 40,000 cases of non-typhoidal Salmonellosis each year. This does not take into account; however, the overwhelming number of cases which go unreported and untreated. The true number of Salmonellosis cases is probably more in the region of 1.4 million annually (Rabsch et al., 2001). Causing 582 deaths in the year 2001, Salmonellosis is the most lethal food borne bacteria found in the United Sates food supply (Rabsch et al, 2001). Eggs have been shown to be a significant vector for the transmission of *Salmonella* Enteritidis (SE). In fact 80% of the outbreaks of this pathogen can be attributed to eggs or egg products (Gast and Beard, 1992).

Salmonella Enteritidis accounts for over 24% of all Salmonellosis cases (Rabsch et al, 2001). Since the 1970's infections of this pathogen have been on the rise both domestically and in other countries, especially the United Kingdom. Grade A shell eggs have been the most common vehicle for the transmission of *Salmonella* Enteritidis (Schoeni et al, 1995). Shell eggs or products containing eggs were found to be the vehicles for 77% of the 35 SE outbreaks which occurred in 1985 (St Louis et al, 1988). SE was the causative agent of 43% of all *Salmonella* outbreaks involving eggs in which the particular serovar was known (St Louis et al, 1988). The most common food origin for these outbreaks has been products which contain raw or undercooked eggs including

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This thesis follows the format of Poultry Science.

hollandaise sauce, salad dressing, or homemade mayonnaise (St Louis et al, 1988).

Salmonella Typhimurium (ST) is another pathogen of concern in egg products. It is responsible for approximately 23% of all non-typhoidal cases of Salmonellosis worldwide (Rabsch et al, 2001). St. Louis et al (1988), found that 26% of *Salmonella* outbreaks involving egg products were caused by ST. This prevalence of disease incidence makes it necessity to understand the mechanisms by which S*almonella* invades the egg.

Growing concerns for the safety of U.S. shell eggs have led the Food and Drug Administration (FDA) to propose the requirement that shell eggs sold to consumers or used by retail food establishments be stored at no more than 45□F (US FDA, 1998). A 1991 study by Lock suggested that *Salmonellae* introduced onto the air cell of an egg could survive for up to 17 days at refrigeration temperatures (Lock and Board, 1992). *Salmonellae* may be able to survive refrigeration temperature high relative humidity (Rizk et al., 1966). This combination of increased susceptibility at advanced storage times and the ability of pathogens to survive refrigeration makes understanding the time line of an egg's *Salmonella* growth permissiveness crucial.

To fully understand the importance that commercial shell eggs play as a transmission vehicle for *Salmonella* Typhimurium and *Salmonella* Enteritidis an in depth knowledge of the egg's physiology, natural defenses of the egg, and infection routes of *Salmonella* are in order. The burgeoning of *Salmonella* Enteritidis as an egg pathogen demands special consideration. This pathogen seems to be closely associated

with transovarian infection of the laying hen. Therefore, a review of ovarian systems in which such an infection could occur is also necessary.

CHAPTER II

BIBLIOGRAPHIC REVIEW

EGGS AND EGG PRODUCTS

On average, Americans consumed 240 eggs per person in the year 1997. Eggs are used as an inexpensive food source in the form of shell eggs, liquid, frozen, and dried products (Ricke et al., 2001). Currently eggs are produced largely on commercial farms housing upwards of 30,000 laying hens. Commercial facilities may reach populations of over one million birds in some instances. This is a fairly new practice. Prior to the 1940's eggs were generally produced on small family owned farms thus fostering the idea of a wholesome, safe food source. Practices of the present are much more mechanistic with feeding, watering, and egg collection all accomplished through the use of automated systems. Mainstream practices of egg grading, proper cooling and storage and genetic selection of birds have led to an egg with an increase in quality (Ricke et al., 2001).

Not only do eggs and egg products provide a reliable source of nutrition, they also serve a variety of functions in other products. The emulsifying properties of lecithin and cholesterol with in the egg yolk make eggs necessary components of mayonnaise and other food systems requiring an emulsifier (Baker and Bruce, 1994). Albumen, or egg white, is noted for its ability to form heat stable foams used in cakes, meringues, and other baked products. Other products involving egg products include noodles, candy

and ice creams (Ricke et al., 2001). Eggs used in products with other primary ingredients are referred to as hidden eggs.

Recent interest in convenience foods has led to increased egg consumption (Messens et al., 2002). Dehydrated egg yolk is also sold in retail facilities to those seeking added foaming or low fat alternatives in recipes. The popularity of salad bars also increases use of eggs in the current market. These products help assure that a wide variety of consumers are exposed to egg products (Ricke et al., 2001). Due to wide spread use of eggs as a food source, the safety of this product is important.

PHYSICAL DEFENSES

Eggs function primarily as a means of reproduction to avian species. The egg must be able to protect a developing embryo for the period of 21 days in which a chick will mature until hatching (Haines, 1939). Therefore, eggs as a biological system, necessitate certain defenses, both chemical and physical, to limit the interference of microbial contaminants on a chicks development.

The cuticle is the first of the physical defenses that the egg has to exclude bacteria, yeasts, and molds from the nutrient rich yolk. Also called the bloom, it is a protein layer which is secreted over the shell of the egg immediately prior to oviposition (Mayes and Takeballie, 1983). This 0.01 mm thick protein coating serves to clog fairly large pores in the hard calcium based shell (Simkiss, 1968). By closing these pores bacteria are less able to move from the exterior of the egg to the interior. Unfortunately

the cuticle, a relatively effective barrier, has approximately a 96 hour period of effectiveness (Mayes and Takeballie, 1983). After that amount of time the barrier is largely removed due to abrasion (Fromm, 1963). Humidity, temperature of the egg, ambient air temperature surrounding the egg directly after oviposition, and washing of the egg can all have effects on the amount of time the cuticle stays in place to prevent the penetration of bacteria such as *Salmonella* to the interior of the egg.

Next in the line of physical defenses is the shell itself. Shells of chicken's eggs have between 7,000 and 17,000 pores most of which span from the outside to the inside of the shell (Mayes and Takeballie, 1983). Pores are wider at the top and taper down toward the bottom end along a spiraling path. More pores can be found at the larger end of the egg than in the small end (Walden et al., 1956). This is the reason that air cells of eggs are generally found at the broad end of the shell. Some malformed pores are much larger in diameter and can allow organisms easier access to the interior of the eggs (North, 1978).

Thickness of the shell can also play a role in the admittance of bacteria to the interior of the egg (Taylor and Martin, 1929). As shell thickness increases so does the length of the pore. Pores are not straight and instead wind through the thickness of the shell matter. Longer pores are likely to be more spiraled and therefore provide a more difficult route for bacteria to navigate due to limited motility of bacteria (Mayes and Takeballie, 1983).

Moisture on the surface of the egg can aide the process of bacterial penetration (Humphrey, 1999). According to Humphrey bacteria are better able to access the

interior of the egg if they are introduced onto the shell surface before the cuticle has sufficiently dried. Rapid cooling of the egg, usually from wash water that is too cold, causes the yolk and albumen to contract while the shell remains fixed. This produces a negative pressure thus pulling bacterial cells from the exterior of the egg through the winding pores of the shell (Lock, 1991). Conversely excessive warmth of wash water can cause interior egg contents to swell through the pores of the shell, creating a fluid conduit through which bacteria can penetrate the shell.

Directly under the shell lie two shell membranes, the outer and inner membranes. These are the last physical line of defense before contaminants are introduced into the albumen environment. The two shell membranes are attached at all points except where the respiration of gases in and out of the cell forms an air cell, usually at the blunt end of the egg shell (Mayes and Takeballie, 1983).

The outer membrane is attached directly to the shell of the egg and is composed of three layers. These layers are fibrous in nature providing a complex structure which is very difficult for bacteria and other microorganisms to penetrate (Moran and Hale, 1936). The inner shell membrane is closest to the center of the egg's interior. It is thought to be made up of two layers (Mayes and Takeballie, 1983). It is supposed that the shell membranes act as a sort of filter to potential contaminants through the intricate system of pores and fibers they present. The shell membranes acting jointly are thought to be a better defense against bacterial invasion than the egg's shell (Garibaldi, 1958). Penetration of the shell membrane is in all probability due to proteolytic activity

possessed by invading bacteria (Hartung and Stadelman, 1963). Figure II-1 is a graphical representation of the components of an egg's physical defenses.

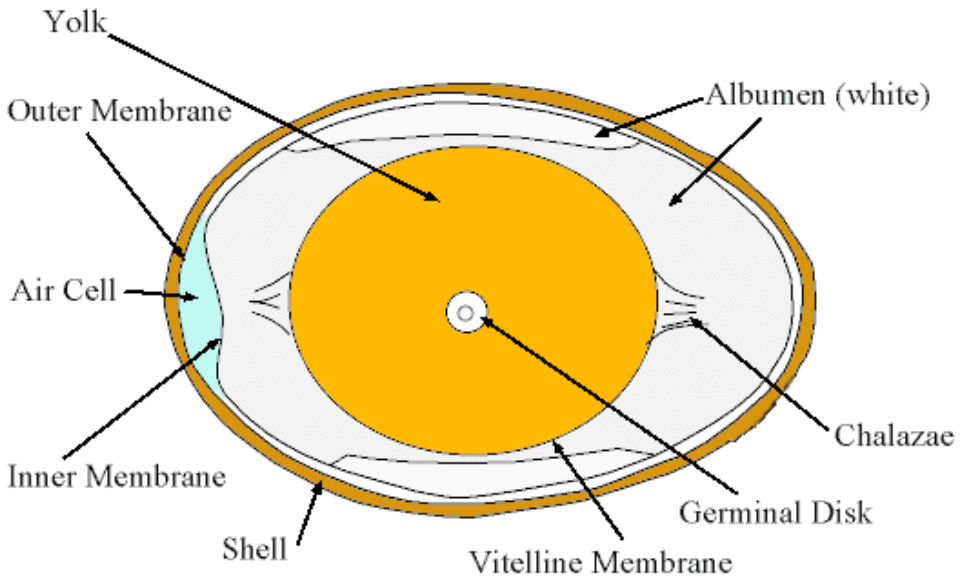

Figure II-1: Internal structures of the hen's egg. Reproduced with permission from the Purdue Animal Science web Page. http://ag.ansc.purdue.edu/poultry/

CHEMICAL DEFENSES

After managing to penetrate the physical barriers presented by the cuticle, shell, and shell membranes, microorganisms invading the egg face a number of chemical barriers in the harsh environment of the albumen (O'Leary and Busta, 1974). One such chemical defense is the enzyme lysozyme (Cuguennec et al., 2000) Lysozyme is present in biological fluids including milk, urine and blood. It serves to lyse gram positive bacteria (Cuguennec et al., 2000).

Lysozyme is a globular protein which is heavily saturated with lysine and arginine. Lysozyme acts by cleaving the (1,4) linkage between N-acetylneuramine and N–acetylglucosamine in the cell wall of gram positive bacteria (Board, 1969). Although most pathogens associated with food borne disease outbreaks are gram negative, gram positive bacteria such as *Pseudomonas* play an important role in the spoilage of eggs (Board, 1964). Gram positive bacteria do contribute to the overall bacterial load of the egg, though they are of no major significance to food safety. The natural microflora of the egg shell seems to be gram positive bacteria. This contamination is probably due to contamination by dirt or feces at the time of lay (Board, 1964).

A more important player in the chemical defense of the egg is the protein Conalbumin. This egg white protein functions by chelating the iron of the egg albumen thus making it unavailable to bacteria for growth (Garibaldi, 1959). The conalbumin protein molecule contains two binding sites which sequester iron. These sites are

located at the C-terminus and N-Terminus. Conalbumin accounts for approximately 10% of the total solids content in egg white (Alderton et al., 1946).

Garibaldi (1960) reported that when there is enough iron to saturate all the conalbumin of the egg albumen, the protein becomes completely unable to retard bacterial growth. *Salmonella* is known to have a high demand for iron in its reproduction and metabolic pathways. Different bacterial classifications are more sensitive to the action of conalbumin than others. Micrococcus, Bacillus, and gram negative species of bacteria are particularly retarded by conalbumin, probably due to a higher need for iron (Feeny and Nagy, 1952). Through the bicarbonate buffer system, the pH of the albumen is increased from 7.6-7.9 to an alkaline state of about 9.5 (Mayes and Takeballie, 1983). Through the loss of carbon dioxide, the pH of egg white becomes more inhospitable to the growth of pathogenic bacteria such as *Salmonella* over time (Romanoff and Romanoff, 1949).

However, because of the role of Conalbumin, egg albumen has a relatively small amount of free iron available. The nutrient rich yolk on the other hand contains ample amounts of free iron (Kilic et al., 2002). Bacteria are able to colonize the albumen of shell eggs, but not to multiply rapidly. A 2001 study by Gast and Holt showed that several serovars of *Salmonella* were unable to grow in the harsh conditions provided by egg albumen. Heavy growth of bacteria in the egg does not; therefore, seem apparent until the bacteria are able to reach the contents of the yolk (Sharp and Whitaker, 1927).

Growth of bacteria in the albumen of eggs does occur despite the chemical defenses found there. Several theories have been proposed as to the mechanisms by

which bacteria with high demands for iron can survive in an environment which excludes this particular nutrient. *Salmonella* have the ability to produce siderophores which chelate iron and provide it to the parent cell thus competing with Conalbumin in the egg white (Cudjoe et al., 1994). This siderophore is a high affinity molecule which can not only sequester iron, but can also remove it from the Conalbumin complex (Chart, 1993). *Salmonella* also produces outer membrane proteins which receive the ferric-siderophore complex (Chart, 1993). In *Salmonella,* the siderophore enterobactin serves as an iron transport compound (Garibaldi, 1971).

Other factors can affect the growth of bacteria in the albumen. Ovomucioid is a proteinase inhibitor which denies bacteria the ability to utilize the proteins of the albumen (Baron et al., 1977). Egg whites also contain the compound avidin which binds biotin, a riboflavin compound, and certain thiamin binding chemicals (Baron et al., 1977).

THE VITELLINE MEMBRANE

After penetrating both the physical barriers of the shell and the chemical barriers of the albumen, bacteria are faced with only one more defense before migrating into the yolk. The vitelline or yolk membrane surrounds the yolk and is responsible for compartmentalization of the egg (Burley and Vadehra, 1989). The membrane has two fibrous layers surrounding a thin inner membrane. Should this membrane fail, yolk and albumen contents are allowed to mix. This causes an influx of iron and other compounds

into the harsh albumen environment where defenses of the egg are weakened if not destroyed (Burley and Vadehra, 1989). The vitelline membrane's function in controlling invasion of pathogens into the egg is highly debated. It is possible that the membrane serves to physically restrain bacterial penetration.

The yolk membrane serves a dual purpose. In the egg as a food source it is responsible for the segregation of yolk and albumen which is expected. In the egg as a reproductive cell, the vitelline membrane plays an important role in embryogenesis. There are several enzymes associated with the yolk membrane which are theorized to allow the penetration of materials involved in fertilization, yet exclude the transfer of nutrient molecules and bacterial cells (Debruyne and Stock, 1978). These enzymes include lysozyme, pyruvate kinase, and alkaline phosphodiesterase. Ribonuclease can also be removed from the outer layer of the membrane (DeBoeck and Stock, 1986).

EGG YOLK

At the core of the egg, is the nutrient rich yolk. The yolk is composed of 47.5% water, 33 % lipid and 17.4% protein. Lipids account for more than 60% of the dry weight of the yolk (Shenstone, 1968). Triglycerols, phospholipids, and cholesterol make up the lipid concentration. These are typically used by the developing embryo for cell membranes (Burley and Vadehra, 1989). The yolk itself, with no defense mechanisms like those in the albumen is an excellent media for bacterial growth. Studies by Gast and

Holt (2001) have shown that numerous serovars of *Salmonella* Enteritidis can grow rapidly in egg yolk.

ROUTES OF INFECTION

Humphrey (1994) indicated that shell contamination of the egg is more common than contamination of the egg's contents. This demonstrates a possible infection route for bacteria. Eggs at oviposition are almost totally sterile internally. Mayes (1983) suggested that well over 90% of all eggs are free of microorganisms at the time of lay. Bacteria originating from organic material on the external shell are able to penetrate the physical defenses posed by the cuticle, shell, and shell membranes to colonize the interior of the egg. A wide range of *Salmonella* serovars can be recovered from the shell of an egg including *S*. Enteritidis, *S*. Infantis, and *S*. Typhimurium. Though most *Salmonella* species die rapidly on shell surfaces, increased relative humidity can lengthen survival time (Lancaster, 1953). While the natural microflora of the egg shell is primarily composed of gram positive bacteria, usually spoilers, the interior contents of the egg would seem to select for gram positive bacteria which could be pathogenic (Board, 1977).

Bacteria present on the shell of an egg can also come in contact with egg contents during breaking. Bacteria from the outside environment are thereby inoculated into the interior of the egg. This route of infection could be extremely risky when egg contents are pooled (Humphrey, 1999). In this commodity, eggs are broken and component parts

of yolk and albumen are mixed. The normally harsh environment of the albumen is compromised and both spoilage and pathogenic bacteria can grow exponentially.

Another potential stimulant to bacterial penetration into shell eggs is the incidence of cracked shells. Hara-Kudo et al., (2001) showed a significant increase in the penetration of *Salmonella* into eggs which had been purposefully cracked.

Although most bacteria commonly indicated in food borne disease outbreak have the ability to penetrate into the egg from external sources, it would seem that some have an alternative route (Mayes and Takeballie, 1983). In the case of several *Salmonella* serovars, in particular SE, it seems that transovarian infection of the laying hen may be at fault (Humphrey, 1999). Lister (1988) demonstrated that SE is could be recovered from the reproductive tissues of infected birds. Transovarian infection of the laying hen occurs when a bacteria infects the reproductive tract of the bird. Eggs laid subsequent to infection are potentially laced with bacteria.

SE has begun an emergence as a primary egg pathogen since the 1970's (Schoeni et al., 1995). One possible explanation for this increased disease incidence is that transovarian infection of the laying hen is a more prominent route of infection for this pathogen. The increase in occurrence of SE seems to parallel an increased reporting of Salmonellosis cases since the early 1980's (Humphrey, 1980).

Evidence suggests that transovarian infection of the hen's ovary with SE may be similar to the same route of infection found with *Salmonella* Pullorum (SP) (Benson and Keller, 1999). Vertical transmission of SE has been documented as with SP (Benson and Keller, 1999). SP is known to produce a systemic infection in chickens, but is non

pathogenic to humans. SE seems to be the only serovar of *Salmonella* which causes disease in humans and is also able to colonize the ovary of the laying hen (Barrow, 1999)

Despite increased attention paid to the subject, transovarian infection of the laying hen seems to be somewhat rare. In commercial flocks less than 0.03% of eggs laid are contaminated with SE (Gast and Holt, 2001). In flocks that are known to be SE positive 1 in 12,000 eggs laid is contaminated. Only 1 egg in 253,000 eggs is found to be SE positive overall. The biggest potential problem with transovarian infection is presented by pooled egg products. Though a very low number of total eggs produced are laced with a very small dose of the bacteria, pooled egg components lend the perfect environment for rapid bacterial growth. Gast and Holt (2001) showed that several serovars of SE could increase by two to three logs in as little as 6 hours when grown in liquid egg yolk media. In the case of pooled egg products, one contaminated egg has the ability to spread its bacterial load to the entire liquid contents of the egg pool (Morris 1990).

FOLLICULAR DEVELOPMENT

Eggs contaminated through transovarian infection may pose a significant hazard to both industry and consumers. The possibility of egg contamination through transovarian infection necessitates an understanding of the laying hen's reproductive tract. By the time of hatch the laying hen's reproductive tract contains more than

480,000 oocytes which have the potential to become developed ova (Johnson, 2000). In a young bird the ovarian tissue is composed of immature, small white follicles. These can be visually observed upon dissection of the reproductive tissue. Approximately 2,000 of these visible white follicles are present (Johnson, 2000). Not all of these follicles will become viable within the lifetime of the hen. For several reasons even fairly developed follicles can cease to mature and be reabsorbed by the body of the hen. These are referred to as atretic follicles (Johnson, 2000). However, once a follicle has entered the large F follicle stage of maturation, it is unlikely that atresia will occur without a severe outside stress (Gilbert et al., 1981).

In a mature laying hen the ovary is composed of follicles of different size and maturity at any given time. One follicle is ovulated every 24-40 hours (Etches, 1990). The follicular hierarchy of the mature laying hen is composed of 7-10 follicles of 10-35 mm, 15-20 follicles of 1-10mm, and several thousand follicles of less than 1mm (Williams and Sharp, 1978).

The largest follicle of the hierarchy is referred to as the F1 follicle. This follicle is the most mature of the system, and will be released into the reproductive tract when its maturation is complete. The F1 follicle has the appearance of a fully formed egg yolk that is still encapsulated in a follicular sack. Visible capillaries line the surface of the follicle, for the purpose of transport and deposition of yolk material (Johnson, 2000). The F2, F3, F4, and F5 follicles are progressively smaller in diameter than the F1 follicle and are further down the hierarchy of maturity. The F follicles can collectively be referred to as large yellow follicles.

Also present are small yellow follicles in which yolk material has begun to deposit. These immature follicles will move toward the size and volume of the F follicles as more yolk material is carried via the blood stream from the liver into the developing follicle. Immature follicles which do not yet possess large volumes of yolk material contain granulosa cells (Etches, 1990). In the more mature follicles, these granulosa cells become one distinct layer of cells which release a variety of hormones during the course of ovulation (Etches et al., 1981). Still less mature are small white follicles which have not yet begun filling with yolk material, but are still visible without the use of microscopy (Johnson, 2000).

FOLLICLE GROWTH

Maturation and growth of the laying hens' follicles are divided into three separate phases. First is a slow period of growth which can last a range of time from a few months up to several years. This slow growth phase is followed by a more rapid period of a few months in which yolk protein is brought into the developing follicle. The last phase of follicle growth is characterized by rapid growth over a period of 6-11 days. During these last days before the follicle is released into the reproductive tract a vast amount of yolk material is imported into the follicle (Johnson, 2000). The length of time at each stage of development varies from follicle to follicle (Johnson, 2000). Continued lay from the time of sexual maturity is possible because of this.

Yolk components are synthesized in the liver and transported via the vascular system to the ova. Precursors for yolk contents, vitellogenin (VTG) and very low density lipoprotein (VLDL) are moved through the granulosa cells by blood capillaries (Johnson, 2000). VTG and VLDL are deposited inside the oocyte and contribute to the observed increase in follicle weight and volume.

HORMONE REGULATION OF THE REPRODUCTIVE SYSTEM

Luteinizing hormone (LH) is a particularly important hormone in the regulation of ovulation in the laying hen. A surge in LH is observed approximately 6 hours prior to ovulation of the mature F1 follicle (Wilson and Sharp, 1973). The purpose of LH in ovulation is to induce ovulation of the largest F follicle in the hierarchy (Etches, 1990).

LH also promotes the production of steroids important to the ovulatory cycle of the hen (Etches, 1990). Robinson et al. (1988), showed that androstenedione and estradiol produced in the small yellow follicles, and adrostenedione secreted by the theca layer are stimulated by LH. Luteinizing hormone also serves to stimulate the production of progesterone from granulosa cells within the follicle. Androgens are released from the theca layer of the follicle until approximately 12-36 hours prior to ovulation, and are stimulated by LH prior to its major surge (Etches et al., 1983). Although all large F follicles produce steroids and hormones used throughout the ovulatory cycle, the F1 follicle is far more responsive to the action of LH (Robinson and Etches, 1986). This

accounts for the fact that typically only the largest F follicle is released during a particular ovulation.

Follicle stimulating hormone (FSH) also plays a major role in the hen's ovulatory cycle, but its actions are less well defined than those of LH (Hammond et al., 1981). Like LH, FSH stimulates the production and release of progesterone from small yellow follicles. Etches (1990) explains that FSH has been difficult to study due to a lack of techniques which would allow for the purification of the hormone. However, it is known that FSH is bound to all follicles most frequently 12-16 hours prior to ovulation (Etches et al., 1981.)

Progesterone is released from the large F1 follicle of the follicular hierarchy. Its release appears to be stimulated by relatively low levels of LH (Etches et al., 1983) LH is secreted at varying levels throughout the ovulatory cycle. LH stimulates progesterone release which in turn stimulates secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus gland. GnRH is known to induce the large surge of LH which precedes ovulation by 4-6 hours (Wilson and Sharp, 1973). The subsequent surge in LH accelerates the production of progesterone. Both LH and progesterone have the ability to degrade the follicular sack surrounding the F1 follicle and allow it to drop into the infundibulum, the opening to the reproductive tract (Etches, 1990).

Prostaglandin (PG) is a hormone responsible for uterine contraction within the reproductive tract (Saito, 1987). This contraction of the uterus can stimulate premature oviposition. This means that the immature egg is expelled from the reproductive tract prior to being completely formed. PG is also widely associated with normal oviposition

of the egg. PG is secreted from the largest F follicle in the hierarchy and its levels in the plasma of the hens are seen to increase near time of oviposition (Simada and Saito, 1989). Arginine vasotocin (AVT) also plays a role in the regulation of oviposition. The introduction of exogenous AVT has been shown to stimulate uterine contraction and thusly, premature oviposition (Saito et al., 1987).

RESEARCH OBJECTIVES

The purpose of this research was to characterize the time line in which ST and SE may colonize the ova of domestic laying hens. The route of fecal contamination of the shell egg may be enhanced by the effect of storage time on the defense mechanisms found in eggs. To discover if this is true a study was performed which utilized egg components stored for lengthening storage time. Samples from the albumen, vitelline membrane, and yolk of eggs held from 0-8 weeks were diluted and plated to determine whether or not the amount of introduced ST or SE recovered increased at higher storage times. Yolk fluid samples were removed from intact yolks in order to ascertain whether or not bacteria introduced outside of the vitelline membrane were able to penetrate to the nutrient rich yolk contents.

Transovarian infection of the laying hen is also responsible for *Salmonella* infection of eggs. This route of infection occurs when bacteria are introduced to the reproductive tract of the hen. Since the follicular hierarchy of the reproductive tract is always maturing, there is a potential for long term prevalence of bacteria in eggs through

the transovarian infection route. Therefore, a study designed to examine the ability of *Salmonella* to invade ovarian tissues of differing maturity is in order.

CHAPTER III

EFFECT OF STORAGE TIME ON THE GROWTH OF *SALMONELLA* TYPHIMURIUM IN EGG COMPONENTS

SYNOPSIS

Salmonella enterica subspecies *enterica* serovar Typhimurium (ST) is responsible for about 23% of all non-typhodial cases of Salmonellosis worldwide. An increasing trend in disease incidence makes understanding mechanisms and specific timelines for bacterial invasion of the egg a necessity. Approximately 150 eggs were collected over a 24 hour period from a flock of single comb white leghorn hens. Eggs were held at refrigeration temperatures until time points of one-week intervals from zero to eight weeks and then removed for analysis. Each week, ten eggs were cracked, separated into yolk and albumen components, and inoculated with 10^8 CFU/mL of novobiocin and nalidixic acid (NO/NA) resistant *Salmonella* Typhimurium onto the vitelline membrane of the egg. Yolks were then covered with albumen. Eggs were incubated for twenty-four hours at 25ºC. After incubation eggs were again separated into albumen, yolk, and vitelline membrane samples. Samples were diluted ten fold and enumerated on brilliant green agar supplemented with NO/NA. Membrane counts increased from about $10^5$to 10^7 CFU/mL. Albumen samples also increased by eight weeks, but showed peaks of growth at two and five weeks of storage. After inoculation and incubation, albumen counts at zero weeks of storage were observed at 2.3 x 10^5. By

eight weeks, counts in albumen had reached 1.1 x 10^9. Yolk samples showed no clear increase over time.

INTRODUCTION

The Centers for Disease Control and Prevention (CDC) show that there are over 40,000 reported cases of Salmonellosis each year; however, this does not take into account the vast number of cases that are unreported and unconfirmed each year (Schoeni et al., 1994). The CDC estimates that the true number of non-typhoidal Salmonellosis cases is around 1.4 million annually. Causing 582 deaths in the year 2001, *Salmonella* is the most lethal variety of food borne illness in the United States (Rabsch et al.,2001). *Salmonella* Typhimurium is a pathogen of concern in eggs and egg products. Jones et al. (1995), found that *Salmonella* Typhimurium was the third most frequently isolated serotype of *Salmonella* in an egg production facility. It is responsible for around 23% of all non-typhoidal cases of Salmonellosis worldwide (Rabsch et al., 2001).

Growing concern in the safety of U.S. shell eggs have led the FDA to propose the requirement that shell eggs sold to consumers or used by retail food establishments be stored at no more than 45°F and that they carry a safe handling label (US FDA, 1998). It is possible that over time the eggs natural defenses begin to break down, even at refrigeration temperature. These breakdowns could allow for the growth of significantly hazardous pathogens such as *Salmonella.*

After penetrating both the physical barriers of the shell and the chemical barriers of the albumen, bacteria are faced with one more defense before migrating into the yolk (Burley and Vadehra, 1989). The vitelline or yolk membrane surrounds the yolk and is responsible for compartmentalization of the egg (Burley and Vadehra, 1989). The membrane has two fibrous layers surrounding a thin inner membrane. Should this membrane fail, yolk and albumen contents are allowed to mix. This causes an influx of iron and other compounds into the harsh albumen environments where defenses of the egg are weakened if not destroyed (Burley and Vadehra, 1989). It has been previously demonstrated that numerous serovars of *Salmonella* can grow rapidly in egg yolk (Gast and Holt, 2001).

The study described here undertakes to discover if, with greater storage times, the growth of introduced *Salmonella* Typhimurium (ST) increases in egg components. The most basic question of the study is does the amount of ST which crosses the vitelline membrane, thus infecting the yolk, increase over time. On possible way of analyzing this hypothesis is by testing at one week intervals of storage if there is an increase in the amount of ST isolated from aseptically obtained yolk material of infected eggs.

MATERIALS AND METHODS

Egg Collection

One day's egg production was collected from a flock of single comb white leghorn hens. Hens used in this study were approximately 60 weeks of age. Eggs were stored at refrigeration temperatures of 4ºC for a period of zero to eight weeks during experiment 1. At storage times from 0-8 weeks, ten eggs were removed from storage and sampled using the following method. Egg collection for experiment 2 differed in that eggs were picked up from a commercial laying source once a week over the course of eight weeks and stored in a refrigerated environment at 4ºC. Relative humidity of the cooler was 97% as measured by a digital read wet bulb thermometer. Eggs of 0, 1, 2, 3, 4, 5, 6, 7, and 8 weeks of storage were then sampled on one day.

Bacterial Preparation

Salmonella Typhimurium resistant to novobiocin and nalidixic acid (Ziprin et al., 1990) were grown overnight in Difco™ Tryptic Soy Broth. After incubation, cells were centrifuged for 10 minutes at 5000 rpm. Supernatant was discarded and cells were resuspended in 6 mL of sterile phosphate buffered saline. The centrifuge and resuspension steps were then repeated. Repeated plating of bacteria grown in this manner throughout the study indicated a consistent level of bacterial inoculation of

approximately 10^8 CFU/mL of ST. Each time eggs were inoculated, the bacterial suspension was plated to assure consistent CFU/mL and purity of the culture.

Sampling Method

At one week time intervals between zero and eight weeks of storage eggs were removed from the cooler and sampled. Eggs were dipped in 95% ethanol to remove surface contamination of the shell (Garibaldi, 1960). Eggs were then aseptically cracked and separated into yolk and albumen components in sterile vessels. A 0.01 mL aliquot of ST prepared as stated previously was then placed directly on the exposed vitelline membrane of the eggs to be sampled. After holding the inoculated yolk for five minutes, albumen was added back to the sterile vessel (Gast and Holt, 2001b). Inoculated eggs were incubated at 25ºC for 24 hours. Ten eggs were inoculated at each time point. In addition one egg was sampled immediately following inoculation in order to determine initial amounts of bacteria that survived transfer into egg components. To determine that all eggs were initially negative for ST, one egg was sampled prior to inoculation with bacteria.

After 24 hours of incubation, eggs were again separated into component parts of yolk and albumen. A sample of albumen was then drawn up in a sterile 3 mL syringe. The purpose of this study was to determine at which time point bacteria were able to penetrate the yolk membrane, therefore causing positive sampling from the yolk. It was then necessary to assure that ST recovered from yolk samples was not in fact due to

contamination of bacteria external to the yolk membrane. To this end, a sample method described by Gast and Holt (2001b) was used. A flame heated spatula was applied to the vitelline membrane. This created a sterile opening through which a 16-gauge needle could pass (Gast and Holt, 2001b). Membrane samples were taken by gathering the membrane with flamed rat tooth forceps followed by stomaching with 5 mL of sterile phosphate buffered samples.

All samples were diluted ten fold in sterile phosphate buffered saline and enumerated on plates of Brilliant Green Agar with novobiocin and nalidixic acid at 25_g/mL (Ziprin et al., 1990). Plates were inoculated with 0.01 mL of each dilution and then stored for 48 hours at 37ºC.

Statistical Analysis

Each trial consisted on samples taken from ten eggs per week. Population counts from multiple plates of the same dilution series were averaged to give total colony counts from each individual egg. Data was analyzed using SPSS statistical software. The analysis of variance test for significance was used, with significance set at the 0.05 level.

RESULTS AND DISCUSSION

Albumen *Salmonella* Counts Over Time

A graphical representation of albumen counts for experiment 1 can be seen in Figure III-1. Significant differences were found between counts from weeks 0, 1, 6, and 7 and those taken from week eight samples. At week zero counts from albumen samples were at an average *Salmonella* level of 5.18×10^5 CFU/mL. At week eight counts in albumen had risen to the level of 10^9. Unanticipated spikes at weeks 2 and 5 were seen in albumen counts for experiment 1.

In experiment 2 a general increase was seen in albumen counts over the course of time. Weeks 0-7 were significantly lower than those at week eight ($p<0.05$). One possible reason that results of the second trial seemed to follow a more specific trend is the time frame in which samples were taken. Experiment 2 eggs were inoculated and sampled in one day, unlike trial 1 which took samples on a week to week basis for two months. Experiment 2's method allowed all eggs to be inoculated from one bacterial culture and may have therefore had more consistent numbers of cells from egg to egg (Figure III-2).

This apparent increase in the ability of ST to grow under albumen conditions suggests that the natural bacteriostatic properties of that environment may degrade over time. Schoeni et al (1995) demonstrated that *Salmonella* Typhimurium, *Salmonella* Heidelberg, and *Salmonella* Enteritidis could grow in egg albumen when inoculated in

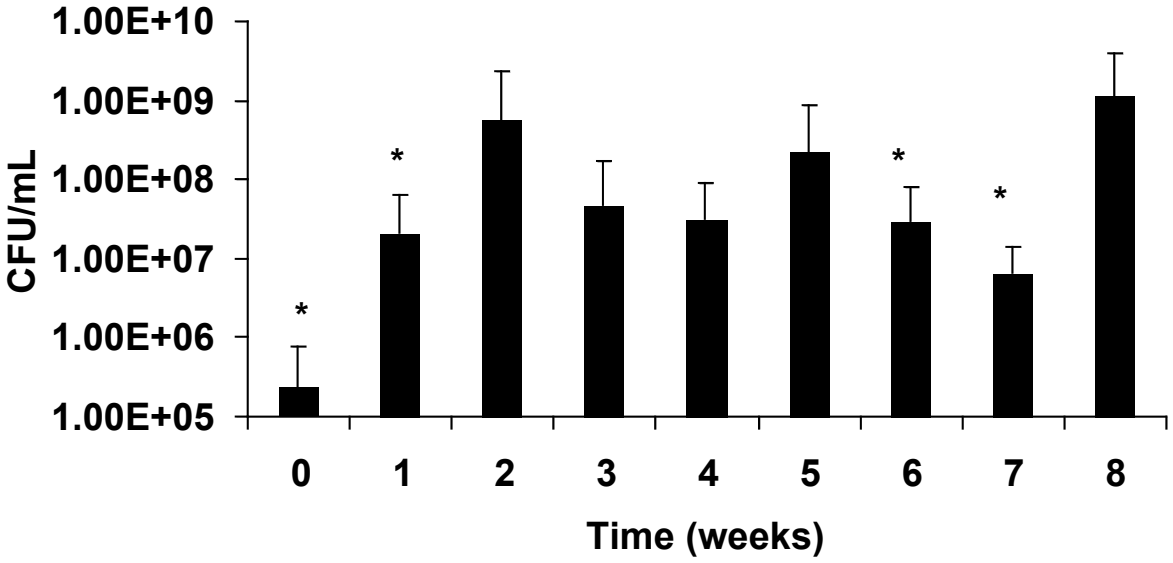

Figure III-1: Albumen counts over time (TRIAL 1). Bars represent average and standard error values for colony forming units (CFU) in eggs collected over a period of eight weeks. Samples were processed and viable *Salmonella* counts were determined as described in Methods (n=10 per week, $p<0.05$) * indicate that CFU/mL of ST for this week were significantly lower than counts at eight weeks.

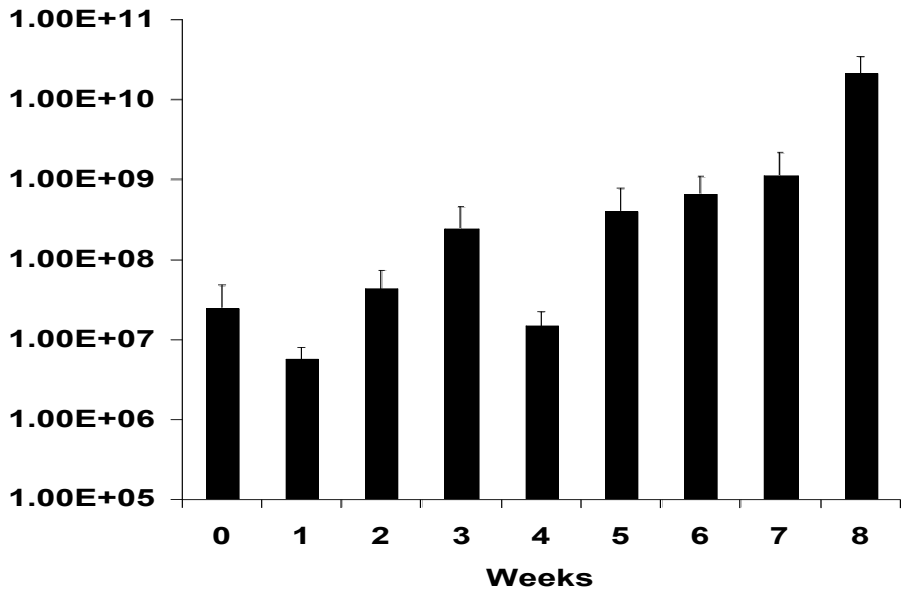

Figure III-2: Albumen counts over time (TRIAL 2). Bars represent average and standard error values for colony forming units (CFU) in eggs collected over a period of eight weeks. Samples were processed and viable *Salmonella* counts were determined as described in Methods (n=10 per week, $p<0.05$).

relatively small doses and incubated over several days. The higher level of inoculation used in this study would explain the more rapid growth of ST in albumen.

The antimicrobial properties of conalbumin in the egg white have been well documented and reviewed (Mayes 1983). Bacteria such as ST require extraneous iron to survive, and conalbumin makes this nutrient unavailable to the bacterial cell (Garibaldi, 1959). One possible explanation for the increase in counts in albumen over time is that as the pH of the albumen rises through the bicarbonate buffer system, conalbumin is denatured and becomes less effective at iron binding (Mayes 1983). This pH effect could also play a direct role in the suppression of ST. Another possibility is that as the vitelline membrane degrades with time, iron from the nutrient rich yolk is allowed to profuse into the albumen thus overpowering the amounts of conalbumin contained there.

Membrane *Salmonella* Counts Over Time

Membrane counts at week zero averaged 3.4×10^5 in trial 1. At week 8 counts for this sample set were recorded at 1.3×10^7. Weeks 0-7 were found to be significantly lower in *Salmonella* Typhimurium counts than those at week 8 (Figure III-3). Though not statistically significant, experiment 2 appeared to support the results of experiment 1 for membrane counts (Figure III-4). Membrane counts were observed to be somewhat lower than those in the albumen. It should be remembered though, that membrane samples were subjected to an initial dilution with 5 mL of sterile phosphate buffered saline which other samples were not.

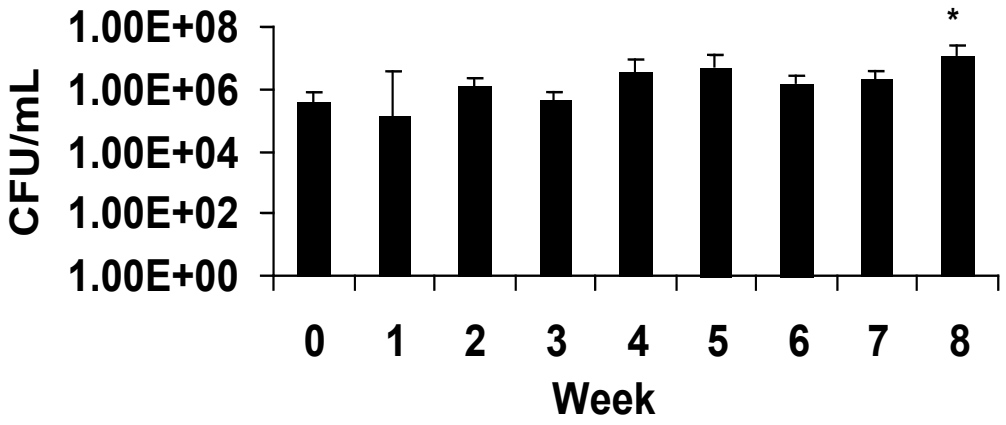

Figure III-3: Membrane counts over time (TRIAL 1). Bars represent average and standard error values for colony forming units (CFU) in eggs collected over a period of eight weeks. Samples were processed and viable *Salmonella* counts were determined as described in Methods (n=10 per week, $p<0.05$) * indicates time point was significantly higher in ST counts than all other time points.

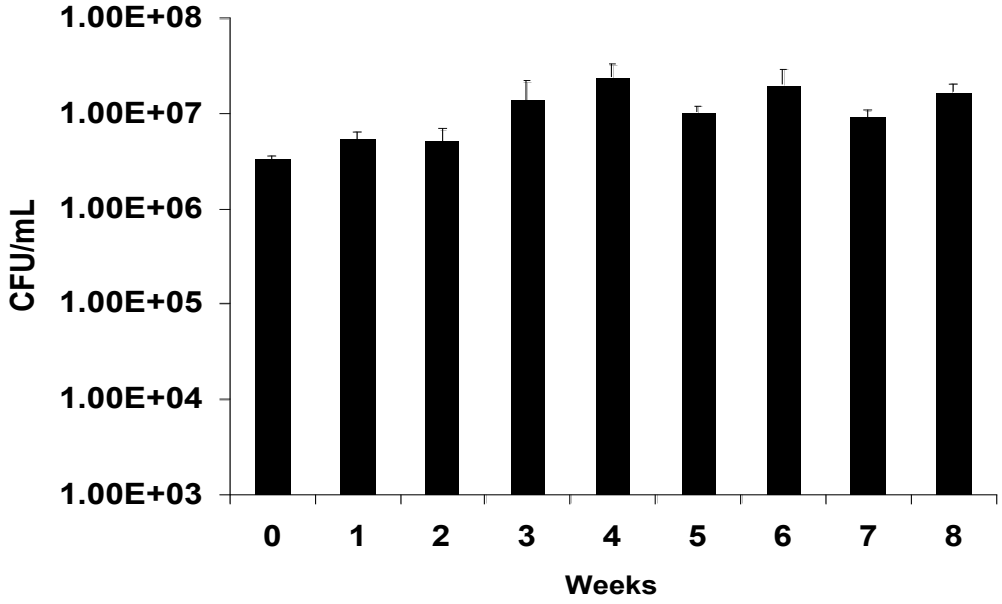

Figure III-4: Membrane counts over time (TRIAL 2). Bars represent average and standard error values for colony forming units (CFU) in eggs collected over a period of eight weeks. Samples were processed and viable *Salmonella* counts were determined as described in Methods (n=10 per week, $p<0.05$)

Yolk *Salmonella* Counts Over Time

Care was taken to assure that membrane integrity was not disrupted in the process of separating yolks from albumen. Therefore, bacterial counts taken from the yolk samples were most likely to indicate that *Salmonella* Typhimurium had successfully crossed from the exterior of the membrane to the nutrient rich yolk.

Previous studies have indicated that bacteria introduced directly into yolk contents are able to grow very rapidly. Gast and Holt (2001b) indicated that *Salmonella* Enteritidis could not be isolated from aseptically obtained yolk material when inoculated onto the vitelline membrane after 6 and 24 hour incubation at 25ºC. Conversely when inoculated directly into yolk contents, isolation was possible after just 6 hours. *Salmonella* Enteritidis grew by almost 4 $\log_{10}$ in 48 hours in yolk material (Gast and Holt 2001b).

Significantly higher peaks in yolk counts were seen at weeks four and six. Graphical representation of yolk counts in experiment 1 is provided in Figure III-5. Alternative graphical representation of yolk data can be found in the appendix. However, results seem some what misleading. Significant spikes were caused solely by one egg sampled. For instance a peak at week four suggests that average counts from yolk sampled were around the 10^5 level. Nine eggs sampled at this time point showed

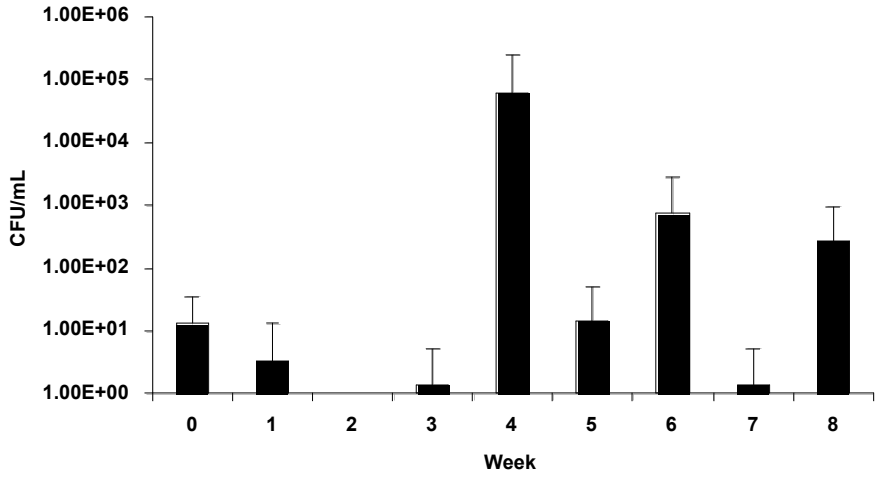

Figure III-5: Yolk counts over time (TRIAL 1). Yolk samples were taken from intact egg yolks. Bars represent average and standard error values for colony forming units (CFU) in eggs collected over a period of eight weeks. Samples were processed and viable *Salmonella* counts were determined as described in Methods (n=10 per week, $p<0.05$)

virtually no colonies on BGA, while one egg was counted at around 10^6 colony forming units. This would seem to suggest that there was no time point at which *Salmonella* Typhimurium was able to penetrate the yolk membrane. It is more likely that these eggs were somehow of less quality than other eggs sampled. This would have allowed easier access by bacteria to the nutrient yolk. The trend seen in this study seems to parallel the natural incidence of *Salmonella* in eggs. Naturally contaminated eggs are a rarity, but when they occur, growth of bacteria is rapid. Also possible is that through the process of separating yolks from albumen, the vitelline membrane was mechanically damaged, thus allowing easy penetration of bacteria. Experiment 2 supported results found in trial 1 (figure III-6).

CONCLUSIONS

This study was not able to pinpoint an exact time point at which bacteria were allowed to fully penetrate the yolk membrane of the egg. It is possible that ST instead relies on weakened defenses of the albumen to fully infect the egg. Since counts at the membrane site were seen to increase over time it could be inferred that this barrier weakens with storage, but not to a degree that allows movement of bacteria into the yolk. If the membrane were to become permeable to iron and other nutrients found within the yolk, samples immediately surrounding it would be expected to increase much in the manner demonstrated by this study.

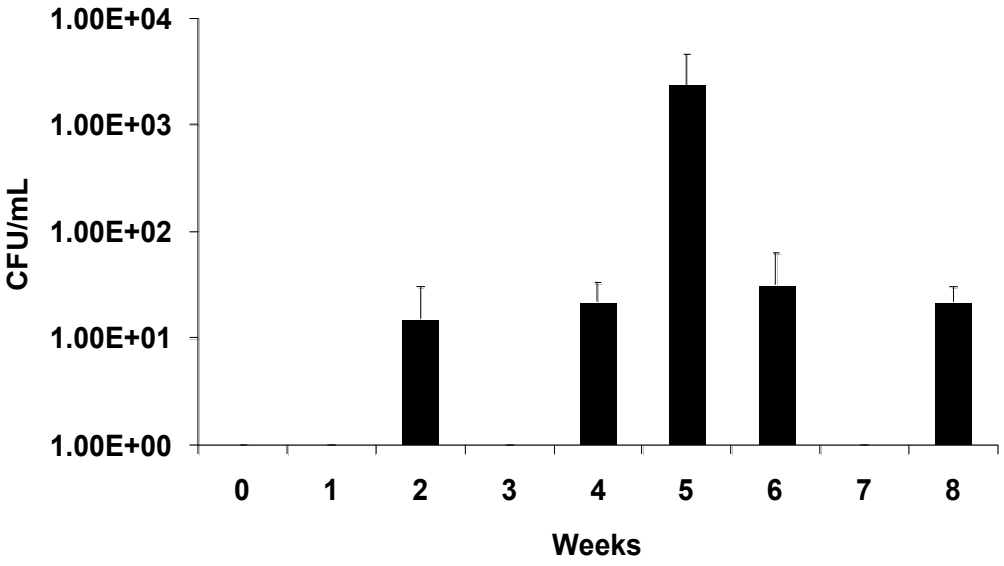

Figure III-6: Yolk counts over time (TRIAL 2). Yolk samples were taken from intact egg yolks. Bars represent average and standard error values for colony forming units (CFU) in eggs collected over a period of eight weeks. Samples were processed and viable *Salmonella* counts were determined as described in Methods (n=10 per week, $p<0.05$)

CHAPTER IV

EFFECT OF STORAGE TIME ON THE GROWTH OF *SALMONELLA* ENTERITIDIS IN EGG COMPONENTS

SYNOPSIS

Salmonella enterica serovars *enterica* subspecies Enteritidis (SE) accounts for over 24% of all food borne Salmonellosis cases. Since the 1970's, infections of this pathogen have been on the rise domestically and in other countries, especially the United Kingdom. Grade A shell eggs are the most common vehicle for the transmission of SE. Eggs were collected from a commercial laying facility at one-week intervals for eight weeks, and stored at refrigeration temperature. After storage, eggs were dipped in ethanol, cracked aseptically and separated into yolk and albumen samples. *Salmonella* Enteritidis resistant to novobiocin and nalidixic acid were inoculated on to the surface of the yolk membrane at a concentration of approximately 10^6 CFU/mL. Yolks were then covered with albumen and incubated for 24 hours at 25ºC. After incubation, eggs were separated into component parts. Samples were removed from yolk, albumen and yolk membrane and diluted 10-fold in sterile phosphate buffered saline. CFU/ml of SE was then enumerated on Difco's Brillant Green Agar supplemented with novobiocin and nalidixic acid. Over the course of storage time, albumen counts were significantly higher by seven weeks than those of fresh eggs. *Salmonella* counts in the albumen of fresh eggs were enumerated at 10^5 CFU/mL. After seven weeks of storage counts were observed at approximately 10^9 CFU/mL.

INTRODUCTION

Salmonella Enteritidis accounts for over 24% of all salmonellosis cases (Rabsch et al., 2001). Since the 1970's infections of this pathogen have been on the rise domestically and in other countries, including the United Kingdom (Rabsch, 2001). Grade A shell eggs have been the most common vehicle for the transmission of *Salmonella* Enteritidis (Schoeni et al., 1995; St. Louis et al., 1988). This increasing trend in disease incidence requires understanding the mechanisms by which S*almonella* invades the egg.

Several previous studies suggest that as length of storage time increases, the natural defenses of the egg to pathogenic bacteria decreases substantially. A study by Lock (1992) suggested that *salmonellae* introduced onto the air cell of an egg could survive for up to 17 days at refrigeration temperatures. A study by Rizk et al. (1996), indicates that at high relative humidity *Salmonellae* may be able to survive refrigeration temperature. This combination of increased susceptibility at advanced storage times with the ability of pathogens to survive refrigeration makes understanding the time line of an egg's *Salmonella* growth permissiveness critical.

A key to bacterial penetration of the egg is the role of degradation of the vitelline membrane over time. The vitelline membrane, commonly called the yolk membrane is a multilayer fibrous sac which surrounds the nutrient rich yolk (Humphrey, 1999). The break down of this crucial membrane could either allow bacteria such as *Salmonella* Enteritidis to gain access to yolk material, or allow nutrient compounds such as iron to

leak out into the albumen. Either mechanism would permit the growth of bacteria in an otherwise well protected system. However, which theory is correct is still a much debated topic (Burley and Vadehra, 1989).

Albumen or egg white is a harsh environment for the growth of bacteria. Several chemical defenses are found in this viscous liquid that inhibits infection by pathogens. The egg white protein conalbumen functions by chelating iron in the albumen thus making it unavailable to bacteria for growth. (Garibaldi, 1960) *Salmonella* is known to have a high demand for iron in its reproduction and metabolic pathways.

Through the bicarbonate buffer system, the pH of the albumen is increased from 7.6-7.9 to an alkaline range of about 9.5 (Mayes and Takeballie, 1983). Through the loss of carbon dioxide, the pH of egg white becomes more inhospitable to the growth of pathogenic bacteria such as *Salmonella* over time. Other factors can affect the growth of bacteria in the albumen. Ovomucioid is a proteinase inhibitor which denies bacteria the ability to utilize the proteins of the albumen (Baron et al., 1977). Egg whites also contain avidin which binds biotin, a riboflavin compound, and certain thiamin binding chemicals (Baron et al., 1977). Bacteria are able to colonize the albumen of shell eggs, but not to multiply rapidly. Gast and Holt (2001), showed that several serovars of *Salmonella* were unable to grown in the harsh conditions provided by egg albumen.

A previous study at explored the ability of *Salmonella* Typhimurium to grow in stored eggs (Howard et al., 2003). *Salmonella* Enteritidis, is the primary concern of research involving pathogenic colonization of eggs. This study undertakes to determine a time line of growth permissiveness in eggs held at refrigeration temperature. Another

concern is any evidence which may suggest differences in the way these two *Salmonella* species invade the egg.

MATERIALS AND METHODS

Two separate trials were run using this method and bacteria. Both trials used the same set of saved eggs. For each trial 10 eggs were inoculated and later sampled for each time point from 0-8 weeks of storage.

Egg Collection

Over the course of eight weeks, eggs were collected from a commercial flock of 60 week old single comb white leghorn hens at one week intervals. Eggs were stored at refrigeration temperatures of 4ºC under a relative humidity of 97% for a period of zero to eight weeks. After refrigerated storage, eggs were separated into time points of 0, 1,2,3,4,5,6,7, and 8 week old eggs and inoculated in the following manner.

Bacterial Preparation

A strain of *Salmonella* Enteritidis isolated from poultry at the University of Iowa, Ames Iowa, resistant to novobiocin and nalidixic acid were grown overnight in Difco™ Tryptic Soy Broth. After incubation, cells were centrifuged for 10 minutes at 8000 rpm.

Supernatant was discarded and cells were resuspended in 6 mL of sterile phosphate buffered saline. The centrifuge and resuspension steps were subsequently repeated. The final concentration of cells was 10^6 CFU/mL.

Sampling Method

Eggs were dipped in 95% ethanol in order to remove surface contamination of the shell (Garibaldi, 1960). Eggs were then aseptically cracked and separated into yolk and albumen components in sterile vessels. A 0.01 mL aliquot of *Salmonella* Enteritidis prepared as stated previously was then placed directly on the exposed vitelline membrane of the eggs to be sampled. After holding the inoculated yolk for five minutes to allow bacterial attachment, albumen was added back to the sterile vessel. Ten eggs were inoculated at each time point. Inoculated eggs were incubated at 25ºC for 24 hours. In addition one egg was sacrificed following the 5 minute holding period in order to determine initial population methods of bacteria that survived transfer into egg components. To determine that eggs used were initially free of *Salmonella*, one egg was sampled prior to inoculation with bacteria.

After 24 hours of incubation eggs were again separated into component parts of yolk and albumen. A 1 mL sample of albumen was then drawn using a 3 mL syringe. The following sample method previously described by Gast and Holt (2001b) was used to gather yolk samples. A flame heated spatula was applied to the vitelline membrane. This created a sterile opening through which a 16-gauge needle could pass (Gast and

Holt, 2001b). Membrane samples were taken by gathering the membrane with flamed rat tooth forceps followed by stomaching with 5 mL of sterile phosphate buffered samples.

All samples were then diluted ten fold in sterile phosphate buffered saline at neutral pH and plated on Brilliant Green Agar (BGA) with novobiocin and nalidixic acid. Plates were inoculated with 0.01 mL of each dilution and then stored for 48 hours at 37ºC. Colonies of typical *Salmonella* morphology were enumerated on BGA.

Statistical Analysis

Each trial consisted on samples taken from ten eggs per week. Population counts from multiple plates of the same dilution series were averaged to give total colony counts from each individual egg. Data was analyzed using SPSS statistical software. The analysis of variance test for significance was used, with significance set at the 0.05 level.

RESULTS AND DISCUSSION

Albumen counts in Trial 1 were increased significantly by seven weeks of storage. *Salmonella* Enteritidis counts at weeks 0, 1, 4, 5, and 6 were significantly lower than those at week seven. It is noteworthy that using the same amount of bacterial cells, fresh eggs and those of short storage times were more resistant to infection than were

older eggs. This would seem to reinforce the idea that over time, the defenses of the albumen decline. For unexplained reasons, counts obtained from eggs stored eight weeks were numerically lower than those at week 7 (Figure IV-1). This unexpected trend was not seen in trial 2.

A general increase in *Salmonella* Enteritidis counts in albumen over time was seen. All earlier time points in trial 2 were found to be significantly lower than counts from eight week old eggs. ($p<0.05$) Gast and Holt (2000) showed that fresh albumen is inhospitable to the growth of *Salmonella* Enteritidis. Fresh egg albumen in this trial yielded an average count of 7.3×10^4 CFU/mL. Albumen counts from eggs stored eight weeks were seen at an average of 5.7×10^6 (Figure IV-2).

Vitelline membrane counts from both trials showed that there was a trend toward increase in counts over the course of eight weeks. However, there were no significant differences in membrane counts over time (Figure IV-3 trial 1 Figure IV-4 trial 2). Although membrane samples were subjected to an initial dilution that other samples were not, counts were comparable and occasionally exceeded those of albumen. The lack of significance in this sample set could be due to the initial dilution of vitelline membrane sample with 5 mL of sterile phosphate buffered saline. Also, it was not possible to remove all yolk material from the membrane prior to sampling; this may have led to a variation in sample volume that could have affected variability in the counts taken.

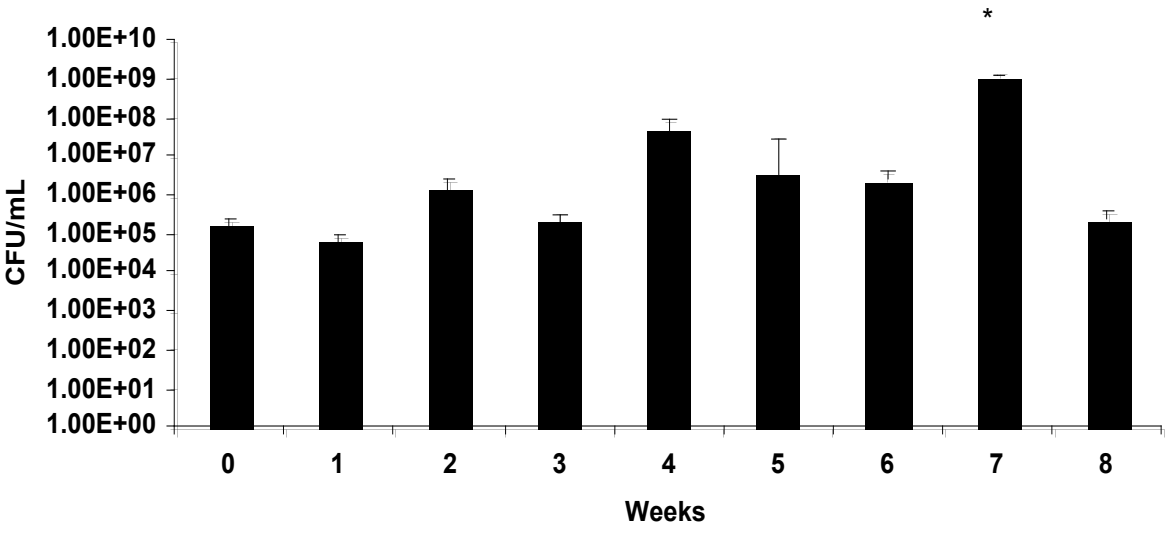

Figure IV-1: Albumen counts over time (TRIAL 1). Bars represent average and standard error values for colony forming units (CFU) in eggs collected over a period of eight weeks. Samples were processed and viable *Salmonella* counts were determined as described in Methods (n=10 per week, $p<0.05$) * indicates that week 7 ST counts were significantly higher than those at all other time points.

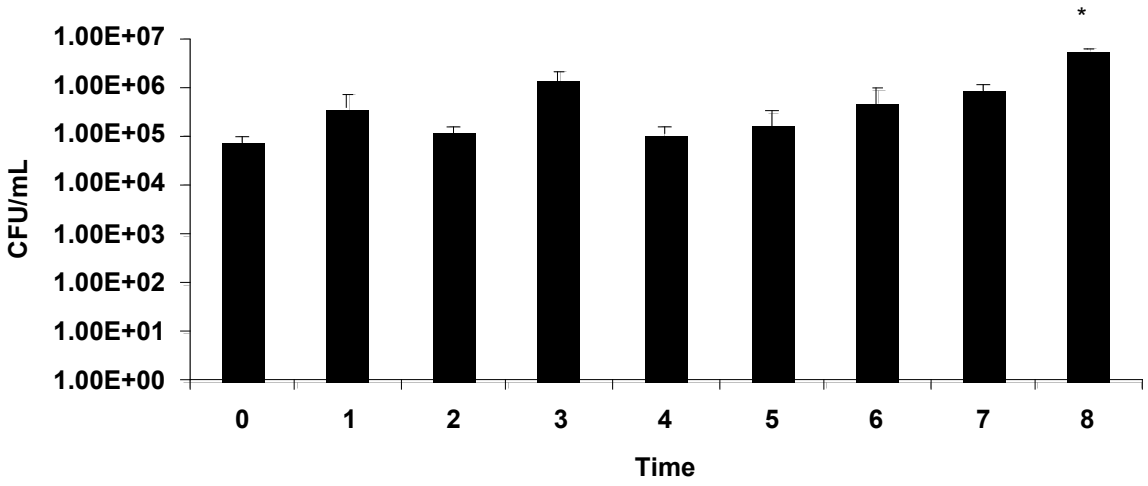

Figure IV-2: Albumen counts over time (TRIAL 2). Bars represent average and standard error values for colony forming units (CFU) in eggs collected over a period of eight weeks. Samples were processed and viable *Salmonella* counts were determined as described in Methods (n=10 per week, $p<0.05$). * indicates that counts in week 8 were significantly higher than those at all other time points.

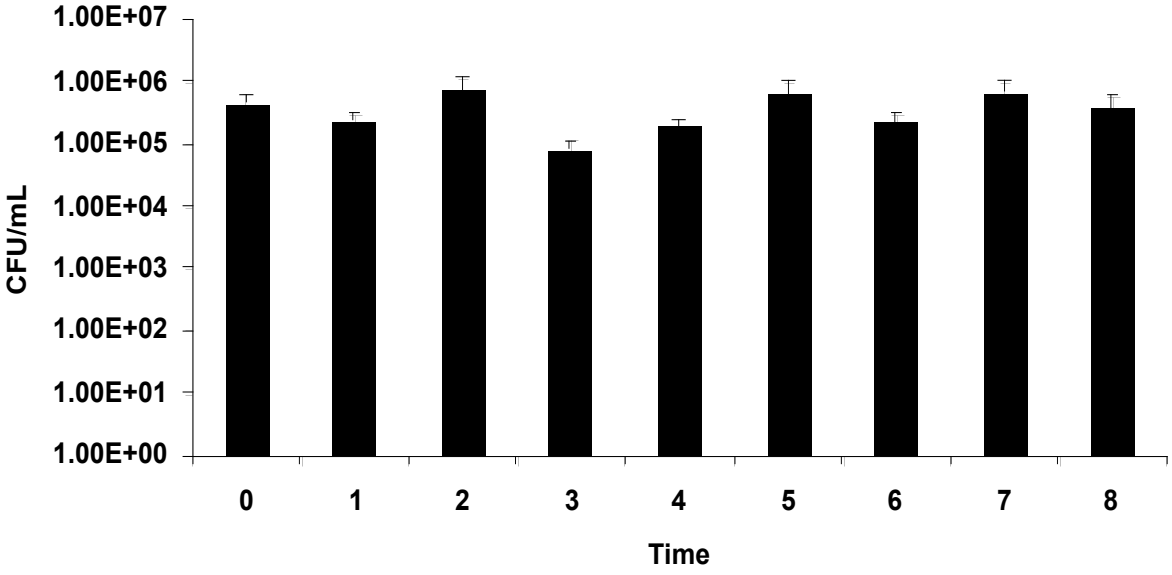

Figure IV-3: Membrane counts over time (TRIAL 1). Bars represent average and standard error values for colony forming units (CFU) in eggs collected over a period of eight weeks. Samples were processed and viable *Salmonella* counts were determined as described in Methods (n=10 per week, $p<0.05$)

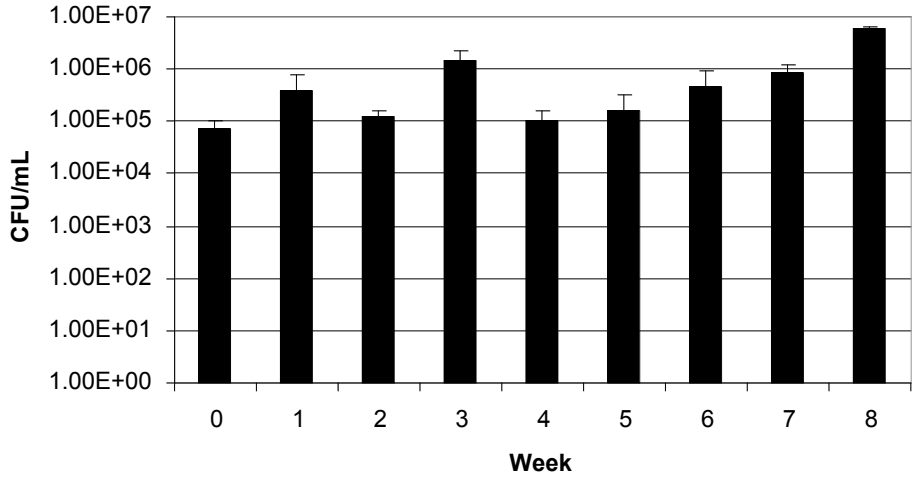

Figure IV-4: Membrane counts over time (TRIAL 2). Bars represent average and standard error values for colony forming units (CFU) in eggs collected over a period of eight weeks. Samples were processed and viable *Salmonella* counts were determined as described in Methods (n=10 per week, $p<0.05$)

Counts taken from yolk samples in both trials were somewhat erratic over time. Although there were significant peaks in colonies formed at different lengths of storage time there was no noticeable trend in contamination levels over time. Time points which yielded significant differences were usually the result of only a few eggs within that group that supported significant growth. For instance, in Trial 1 at four weeks of storage the average amount of *Salmonella* Enteritidis counted was 2.5×10^4 CFU/mL (Figure IV-4). One sample from this time point gave counts of 2.7×10^4 CFU/mL while the other nine samples contained close to undetectable levels of SE. Previous studies have illustrated that when SE is introduced directly to yolk material, rapid growth is experienced (Gast and Holt, 2001.) Therefore, if *Salmonella* Enteritidis was able to penetrate the vitelline membrane, counts from yolk samples should have been much higher.

A lack of trend among yolk samples was also seen in a previous study using *Salmonella* Typhimurium (Howard et al., 2003). Contamination of yolk samples from the trials using *Salmonella* Enteritidis did seem to be at an appreciably higher rate, even though eggs were contaminated with 2 logs less of bacteria. Yolk samples from trial one are presented in figure IV-5. Figure IV-6 is a representation of yolk samples from trial 2. Alternative graphical representation of yolk data can be found in the appendix.

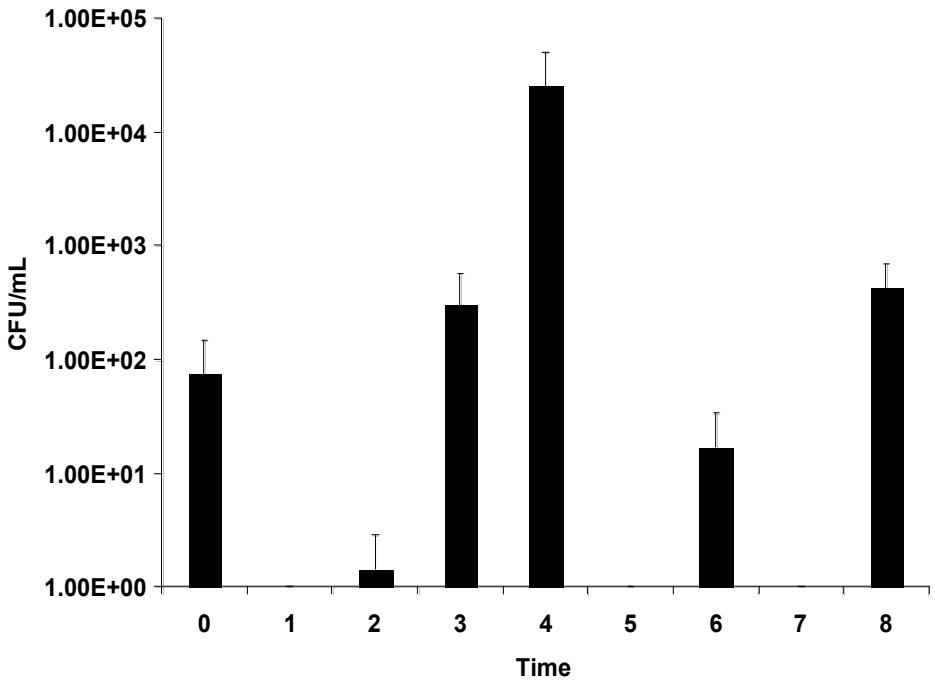

Figure IV-5: Yolk counts over time (TRIAL 1). Bars represent average and standard error values for colony forming units (CFU) in eggs collected over a period of eight weeks. Samples were processed and viable *Salmonella* counts were determined as described in Methods (n=10 per week, $p<0.05$)

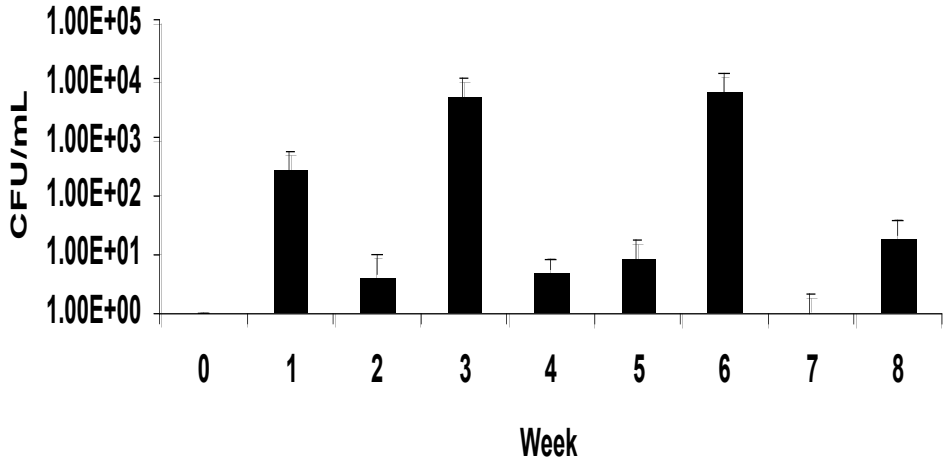

Figure IV-6: Yolk counts over time (TRIAL 2). Bars represent average and standard error values for colony forming units (CFU) in eggs collected over a period of eight weeks. Samples were processed and viable *Salmonella* counts were determined as described in Methods (n=10 per week, $p<0.05$)

CONCLUSIONS

Clearly the length of storage time plays a considerable role in the ability of *Salmonella* Enteritidis to infect contents of shell eggs. Significant increases were seen in counts obtained from albumen samples after longer storage periods. It is possible that increasing storage time decreases the physical and chemical defenses of the egg (Mayes 1983).

Also possible is that the vitelline membrane degrades over time allowing mixing of yolk and albumen components. A study by Fromm (1963) indicated that with increased storage time, the strength of the vitelline membrane is significantly decreased. Membrane samples were not seen to dramatically increase with time possibly due to variation innate to the sampling procedure. However, it would seem that bacteria are better able to grow in this environment than in the harsh albumen of the egg. Though no conclusion concerning the invasion of *Salmonella* Enteritidis into the yolk of eggs can be reached through numerical data presented here, it is evident that some difference between this pathogen and *Salmonella* Typhimurium do exist. In the future, an in depth examination of observed characteristics of SE's invasion of the vitelline membrane is in order.

CHAPTER V

EFFECTS OF OVARIAN FOLLICULAR MATURATION ON IN VITRO *SALMONELLA* INVASION

SYNOPSIS

Transovarian transmission of paratyphoid *Salmonella* is well documented and occurs at a low incidence in chickens. However, the exact mechanism of follicular invasion is not well understood. The following study investigates the ability of *Salmonella* to invade ovarian follicles at different stages of follicular maturity *in vitro*. Ovarian follicles were collected from Leghorn hens and separated into three stages of maturity: 1) large yellow follicles or F follicles (LYF), 2) small yellow follicles (SYF), and 3) small white follicles (SWF). All follicles were incubated at 37°C in RPMI 1640 medium. Initially, follicles were incubated with $1*10^6$ CFU/mL of *Salmonella* Typhimurium (ST) and *Salmonella* Enteritidis (SE) sensitive to gentamicin for 0, 0.5, 1, or 2 h. Follicles were then removed from the bacterial culture, rinsed in fresh medium, and placed in medium containing gentamicin sulfate for 5 h to kill any ST or SE which had not invaded the follicular membrane. After the 5 h incubation, follicles were rinsed in fresh medium and stomached in phosphate buffered saline. Serial dilutions were made of each follicle and viable ST and SE cells were enumerated on brilliant green agar. Two identical trials were conducted. Data suggest that ST and SE may

differentially invade ovarian follicles depending on maturity of the follicle, and that SWF may be more susceptible to ST and SE invasion than either the SYF or the LYF.

INTRODUCTION

Though most bacteria commonly indicated in food borne disease outbreak have the ability to penetrate into the avian egg through fecal contamination of the shell, it would seem that some have an alternative route (Mayes and Takeballie, 1983). In the case of several *Salmonella* serovars, in particular *Salmonella* Enteritidis, it seems that transovarian infection of the laying hen may be at fault (Humphrey, 1999). Lister (1988), demonstrated that *Salmonella* Enteritidis could be recovered from the reproductive tissues of infected birds. Transovarian infection of the laying hen occurs when a bacteria infects the reproductive tract of the animal. Eggs laid subsequent to infection are potentially laced with bacteria.

Salmonella Enteritidis has begun an emergence as a primary egg pathogen since the 1970's (Schoeni et al., 1995). One possible explanation for this increased disease incidence is that transovarian infection of the laying hen is a more prominent route of infection for this pathogen. Transovarian infection has also been largely noticed and debated in the past few decades. The increase in occurrence of *Salmonella* Enteritidis seems to parallel an increased reporting of salmonellosis cases since the early 1980's (Humphrey 1994).

According to Rabsch et al. (2001),*Salmonella* Enteritidis (SE) is responsible for 24.7% of all non-typhoidal Salmonellosis cases world wide. Another closely related

pathogen *Salmonella* Typhimurium (ST) causes 23.5% of these reported illnesses. As the incidence of SE increases over time, the question becomes apparent: Does SE preferentially invade avian reproductive tissues more easily than other serovars of *Salmonella*.

By the time of hatch the laying hen's reproductive tract contains more than 480,000 oocytes which have the potential to become developed ova. In a young bird the ovarian tissue is composed of immature, small white follicles. These can be visually observed upon dissection of the reproductive tissue. Johnson (2000) estimates that close to 2,000 of these visible follicles are present.

Maturation and growth of the laying hens' follicles are divided into three separate phases. First is a slow period of growth which can last a range of time from a few months up to several years. This slow growth phase is followed by a more rapid period of a few months in which yolk protein is brought into the developing follicle. The last phase of follicle growth is characterized by rapid growth over a period of 6-11 days. During these last days before the follicle is released into the reproductive tract a vast amount of yolk protein is imported into the follicle (Johnson, 2000). These distinct levels of follicular maturation give rise to an ovarian system characterized by diversity of follicle size. A step in understanding the processes by which pathogens invade ovarian tissue could be in understanding the role that maturation plays in the ability of bacteria to permeate into developing follicles. The following study investigates the ability of *Salmonella* to invade ovarian follicles at different stages of follicular maturity *in vitro.*

MATERIALS AND METHODS

Tissue Collection

Tissues were collected from a flock of single comb white Leghorn hens provided by the Texas A&M poultry science farm. Birds were from between 60 and 80 weeks of age and had not yet been subjected to the rigors of forced molting. Tissues of three designations were removed from the bird aseptically. Large yellow (LY) or F follicles of larger than 2cm in diameter, small yellow follicles of approximately 0.5cm, and clusters of small white follicles around 2cm in diameter were removed from the ovary of the hens. Tissues were then placed in the sterile culture media RPMI 1640.

Bacterial Preparation

A strain of *Salmonella* Typhimurium or *Salmonella* Enteritidis resistant to novobiocin and nalidixic acid were grown over night in tryptic soy broth. Cell suspensions were then centrifuged at 8,000 rpm for 10 minutes. Supernatant was discarded and the bacterial pellet was then resuspended in 6 mL of sterile phosphate buffered saline (PBS). The centrifugation step was repeated and cells were brought up in 0.5 mL PBS. This concentrated cell suspension was then used to form a solution which yielded an optical density reading which corresponded to 10^9 CFU/mL of either *Salmonella* serovars.

Assay for *Salmonella* Invasion

After preparation in the above manner, bacteria were added to RPMI cell medium containing collected ovarian tissues to a final concentration of 10^6 CFU/mL of SE or ST. Tissues were then incubated with bacteria for 2 hours at 37ºC. After incubation tissues were removed from the inoculated medium, rinsed with sterile culture fluid, and then placed in sterile RPMI which had been amended with gentamicin sulfate at a concentration of 500_g/mL tissues then incubated for an additional 5 hours at 37ºC . The concentration of gentamicin to be used was determined through repeated antibiotic sensitivity testing. This was done in order to kill off any bacteria present in the culture media which had not invaded or attached to the target tissue. After incubation with gentamicin, follicles were removed from the culture media, rinsed, separated into LY, SY, and SW designations and then stomached individually with 5 mL of sterile phosphate buffered saline. Samples were then diluted ten-fold in PBS and enumerated on Difco™ Brilliant Green Agar with novobiocin and nalidixic acid. Plates were incubated for 24 hours at 37ºC and then counted. Culture media in which cells were incubated with gentamicin was also plated to assure sufficient kill of introduced *Salmonella* cell.

Both strains of bacteria used in this experiment were tested for susceptibility to gentamicin. ST was seen to be only marginally more resistant. The concentration of antibiotic used in this study was shown to eliminate both serovars adequately within 5 hours of addition to culture media. Two trials were run in the above method using both

SE and ST. An additional independent trial was run using both bacteria in the same day to confirm earlier results.

Statistical Analysis

Each trial consisted on ten follicles per treatment group. Population counts from multiple plates of the same dilution series were averaged to give total colony counts from each individual egg. Data was analyzed using SPSS statistical software. The analysis of variance test for significance was used, with significance set at the 0.05 level.

RESULTS AND DISCUSSION

Follicles from all three designations of maturity were able to be penetrated by SE and ST, although only during trial 1 and trial 1&2 combined using ST were significant differences found. Small white follicles incubated for 2 hours were seen to be significantly higher in ST counts than were those incubated for 0.5 hours and 1 hour. It also seems that a higher level of *Salmonella* was recovered from SW follicle samples than SY or LY follicles. No direct comparison was possible, however, due to differences in surface area and morphology of between the SW follicles and more mature ones. Clusters of SW follicles are not smooth and rounded as are individual SY and LY follicles. This could have provided introduced bacteria with an environment more protected from gentamicin than that of the SY or LY follicle samples.

During trials 1&2 with each bacterium, samples were removed from culture fluid and exposed to gentamicin after 30minutes, 1 hour, and 2 hours of incubation. Invasion of SW follicles was seen to occur at a much faster rate than other follicle sizes. A graphical representation of this progression of ST's invasion is seen in figure V-1, V-2 and V-3.

After 2 hours of incubation over $8X10^3$ CFU/mL of ST was recovered from SW follicle samples. Approximately one log less was recovered from SY and LY follicles at this time point. In addition ST concentration reached 10^3 CFU/mL in SW follicles in just 0.05 hours. This suggests that SW follicles are more quickly colonized by ST than are follicles of increased maturity.

Results of trials utilizing SE instead of ST were somewhat surprising. The increase in disease incidence, and heavy association of SE with transovarian infection would seem to suggest that SE has a greater ability to invade ovarian tissues. However, results presented here are contrary to that idea. SE was shown to more quickly penetrate SW follicles just as did ST, but the rate of infection seemed to be much slower using this pathogen. During trials 1&2 no bacteria were recovered from samples of SY and LY follicles. Only samples taken after two hours of incubation allowed recovery of bacteria after gentamicin was added. In no trial performed were differences in SE's invasion of ovarian tissues statistically significant. This would seem to suggest that SE is less able to penetrate the barriers presented by the developing membranes of the egg yolk.

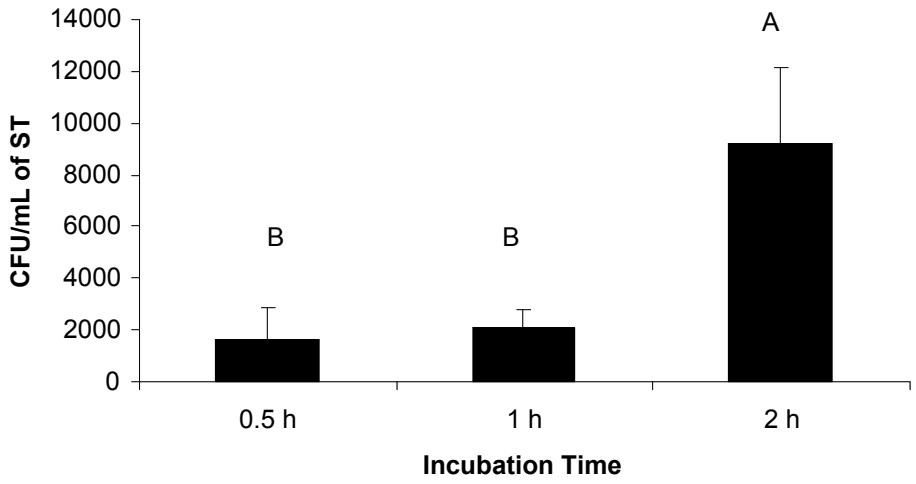

Figure V-1: *Salmonella* Typhimurium counts for SW follicles at increasing incubation times. Data from trials 1&2 combined. Bars represent average and standard error values for colony forming units (CFU) in SW follicles at varying incubation times. Samples were processed and viable *Salmonella* counts were determined as described in Methods (n=10 per follicle type, $p<0.05$)

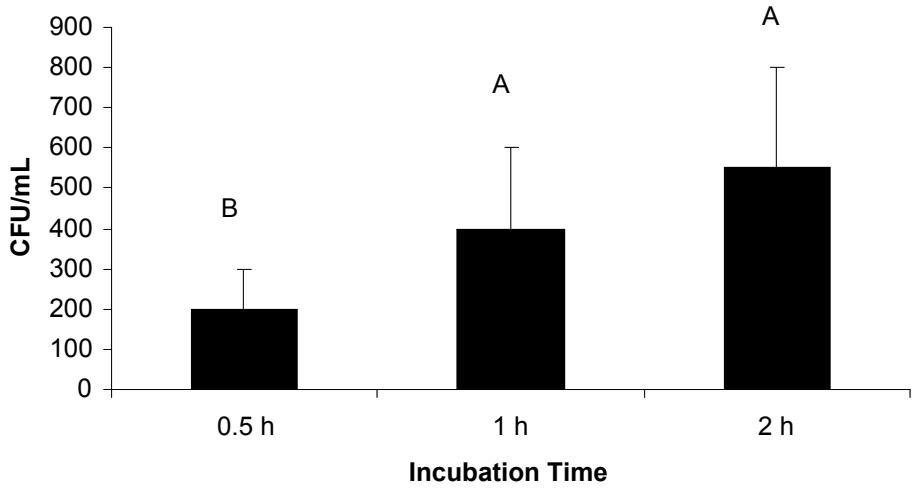

Figure V-2: *Salmonella* Typhimurium counts for LY follicles at increasing incubation times. Data from trials 1&2 combined. Bars represent average and standard error values for colony forming units (CFU) in LY follicles at varying incubation times. Samples were processed and viable *Salmonella* counts were determined as described in Methods (n=10 per follicle type, $p<0.05$)

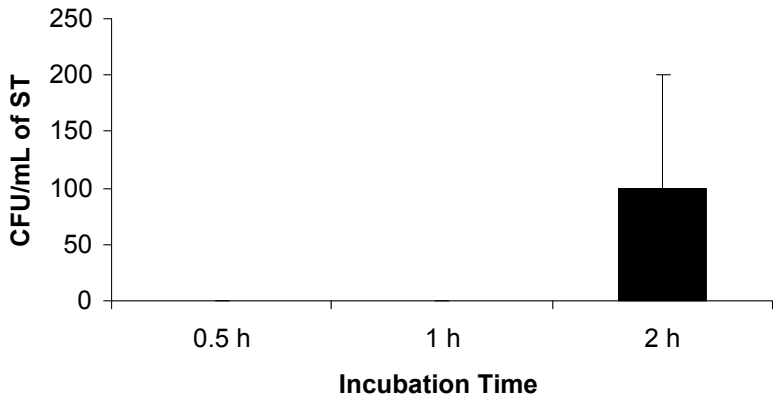

Figure V-3: *Salmonella* Typhimurium counts for SY follicles at increasing incubation times. Data from trials 1&2 combined. Bars represent average and standard error values for colony forming units (CFU) in SY follicles at varying incubation times. Samples were processed and viable *Salmonella* counts were determined as described in Methods (n=10 per follicle type, $p<0.05$)

CONCLUSIONS

Salmonella Enteritidis and *Salmonella* Typhimurium do seem to possess the ability to more quickly penetrate the immature follicles of the laying hen's reproductive tract. This could be a startling revelation if these pathogens are able to survive within the living ovary for long periods of time. Since SW follicles will eventually mature into large F follicles, early exposure to *Salmonella*s could have a profound effect on the safety of eggs produced in the hen through out the reproductive cycle. Future research should concentrate on the ability of *Salmonella* introduced orally to preferentially invade follicles of different maturity. This would more closely simulate the route of transovarian infection. In addition, methodology should be devised which would allow the direct comparison of follicles of different maturity.

CHAPTER VI

EFFECT OF VITELLINE MEMBRANE MATURATION ON THE INVASION OF *SALMONELLA* ENTERITIDIS INTO AVIAN EGG YOLKS

SYNOPSIS

The vitelline membrane is the barrier of the avian egg that is responsible for compartmentalization of egg yolk and egg albumen. Segregation of the nutrient rich yolk from the relatively bacteriostatic environment of the albumen is presumed to have a large effect on the ability of pathogenic bacteria to infect the egg. The vitelline membrane is composed of three layers. The outer membrane, or that which is closest to albumen, is laid down in the reproductive tract after ovulation. The inner membrane, that which is closest to yolk material, is fully developed just prior to the large F follicle's release from the follicular sack into the reproductive tract. A continuous layer exists between the outer and inner membranes. The role of membrane layers in controlling pathogenic invasion, especially the egg pathogen *Salmonella* Enteritidis (SE) is not well documented. Ovarian tissues were collected from a flock of single comb white leghorn hens of approximately 60 weeks in age. Large F follicles of approximately 2 cm in diameter were removed. From half of these follicles, the surrounding follicular sack was removed manually. Freshly laid eggs from the same flock of birds were collected, aseptically cracked and yolks were collected. In separate culture vessels follicles with

intact follicular sacks (FS), follicles with follicular sacks removed (NFS), and post oviposition egg yolks were introduced to a culture of SE which was resistant to novobiocin and nalidixic acid at approximately 10^6 CFU/mL. After two hours of incubation at 37ºC culture media was removed and fresh media supplemented with 500_g/mL of gentamicin was added to the vessels. Follicles were allowed to incubate for 5 hours at 37ºC in order to kill SE which had not penetrated to the exterior of the follicles. Follicles were then removed from the culture media, separated into sterile stomacher bags, and stomached for 30 seconds with 5 mL of sterile phosphate buffered saline. All samples were then diluted ten fold and enumerated on Difco™ Brilliant Green Agar supplemented with novobiocin and nalidixic acid. Plates were incubated for 24 hours at 37ºC and then counted.

INTRODUCTION

The incidence of *Salmonella* occurring in eggs has become a major concern for the poultry industry (Morris, 1990). *Salmonella* Enteritidis (SE) is of specific concern to egg producers and consumers due to the apparent increase in disease caused by this pathogen. SE accounted for 27 out of 35 outbreaks involving *Salmonella* in food products between 1985 and 1987. These outbreaks caused the illness of some 2,119 and 11 deaths (Madden, 1990). SE was still the most frequently isolated serovars of *Salmonella* in 1997 (Rabsch et al., 2001).

Salmonella Enteritidis has also become strongly associated with the transovarian infection of laying hens (Hara-Kudo et al., 2001). Hens exposed to the pathogen can be susceptible to a systematic infection which can reside within the reproductive tract for long periods of time. This provides another route of infection other than the more widely understood incidence of fecal contamination on the shell of the intact egg.

Despite these concerns the egg remains an important commodity that is usually considered safe (Morris, 1990). This apparent safety is due to innate defenses of the egg against bacterial penetration. The cuticle, shell and shell membranes provide a first line of defense against external bacteria. Within the egg, albumen presents a harsh environment to pathogens through increasing pH, viscosity, and various chemical mechanisms (Mayes and Takeballie, 1983).

The last line of defense before an invading bacterium is able to reach the yolk is the vitelline, or yolk, membrane. This membrane is composed of three separate layers, the outer, inner, and continuous layers (Burley and Vadehra, 1989). The inner layer of the vitelline membrane is approximately 3 mm thick. It is formed by the time of ovulation. Using light microscopy, yolk material can be seen attaching directly to the inside of this membrane. The outer layer of the membrane is secreted within the reproductive tract of the hen and would seem to closely associate with the albumen contents of the egg (Burley and Vadehra, 1989).

Though several studies have examined the properties of the individual membranes physical properties, little has been recorded as to the role each plays in

excluding bacteria from the yolk material of an egg. This study seeks to characterize the roles of different vitelline membrane layers in excluding *S.* Enteritidis.

MATERIALS AND METHODS

Tissue Collection

Tissues for this study were collected from a flock of single comb white leghorn hens housed at the Agricultural Research Service Southern Plains Research Center in College Station, Texas. Hens were approximately 60 weeks in age and were determined to be actively producing eggs. Large yellow follicles were removed aseptically from the ovary of the hens. Half of the follicles removed were then stripped of the surrounding follicular sacks using flamed forceps. Additionally, post oviposition eggs were gathered from the same flock of birds. These were aseptically cracked and yolks were collected intact. Treatment groups consisted of 10 each of follicles with intact follicular sack (FS), follicles with no follicular sack adhering (NFS), and egg yolks.

Bacterial Preparation

An overnight culture of a novibiocin and nalidixic acid resistant strain of SE was grown in Difco™ Tryptic Soy Broth at 37ºC. The cell suspension was then centrifuged at 8,000 rpm for 10 minutes. Supernatant was discarded and the bacterial pellet was

brought up in sterile phosphate buffered saline (PBS) at a pH of approximately 7.5. The centrifugation step was then repeated and supernatant was again discarded. Cells were brought up in 0.5 mL of PBS. This concentrated cell suspension was then used to form a solution of approximately 10^9 CFU/mL. Concentration of bacteria was determined by optical density followed by enumeration of BGA.

SE Invasion

Treatment groups of FS, NFS, and yolk were separated into three sterile culture vessels containing 300 mL of sterile Sigma™ RPMI culture media. Cell suspension was added to the culture media to a final concentration of 10^6 CFU/mL. Following inoculation follicles were incubated for 2 hours at 37ºC. Follicles were then removed from culture media and added to new sterile vessels containing RPMI amended with 500_/mL of gentamicin sulfate. This was done in order to kill any bacteria which had not successfully penetrated to the interior of the samples. Follicles were then incubated for 5 hours at 37ºC. The appropriate concentration of gentamicin to be used was determined through repeated antibiotic sensitivity testing.

After incubation with gentamicin, individual follicles were removed from the culture media and placed in sterile stomacher bags with sterile PBS. Samples were then stomached for 30 seconds. Ten fold serial dilutions were performed and all samples were then enumerated on BGA with novobiocin and nalidixic acid. Plates were incubated for 24 hours at 37ºC.

Statistical Analysis

Each trial consisted on ten follicles per treatment group. Population counts from multiple plates of the same dilution series were averaged to give total colony counts from each individual egg. Data was analyzed on SPSS statistical software using ANOVA. The analysis of variance test for significance was used, with significance set at the 0.05 level.

RESULTS AND DISCUSSION

Salmonella Enteritidis preferentially invaded large yellow follicles with an intact follicular sack. The mean level of contamination for this treatment group was 144 CFU/mL in trial 1. It is possible that the vascular structures of the follicular sack that penetrate the developing membrane allow easy penetration of pathogens such as *Salmonella* Enteritidis. This could be of considerable concern in the case of transovarian infection. FS samples were found to be significantly higher than NFS samples. NFS samples showed a mean contamination level of 34 CFU/mL in trial 1. This would seem to indicate that though less susceptible to penetration than follicles not yet released into the reproductive tract, contamination is still possible. Trial 2 data, however, revealed a much lower level of contamination for NFS follicles. In both trials, FS samples were significantly higher than the NFS follicles.

The major difference between NFS samples and yolk samples is the structure of the surrounding membrane. What is called the vitelline membrane is not present until its outer membrane layer is laid down within the reproductive tract (Burley and Vadehra, 1989). By studying the infection of samples prior to and following deposition of this layer, its role in bacterial exclusion was explored. Though a numerical difference was seen between NFS samples and yolk samples in trial 1, no significance was observed. In trial 2, contamination levels between the two samples were virtually identical. Combined data for the two trials also show now significant differences (figure VI-1). This result would seem to indicate that the deposition of the outer membrane layer has little significance to the ability of bacteria to invade yolk tissue. NFS and yolk samples yielded very little contamination whatsoever, suggesting that the inner membrane itself is an effective barrier to pathogens.

CONCLUSIONS

A 1990 study by Gast and Beard revealed that the prevalent site of contamination within an egg is outside of the vitelline membrane (Gast and Beard, 1990). The present study suggests that the level of maturation attained by that membrane has a profound effect on the microbial stability of eggs. Location of infection also may have bearing on the amount of bacteria which are able to invade the nutrient rich yolk environment.

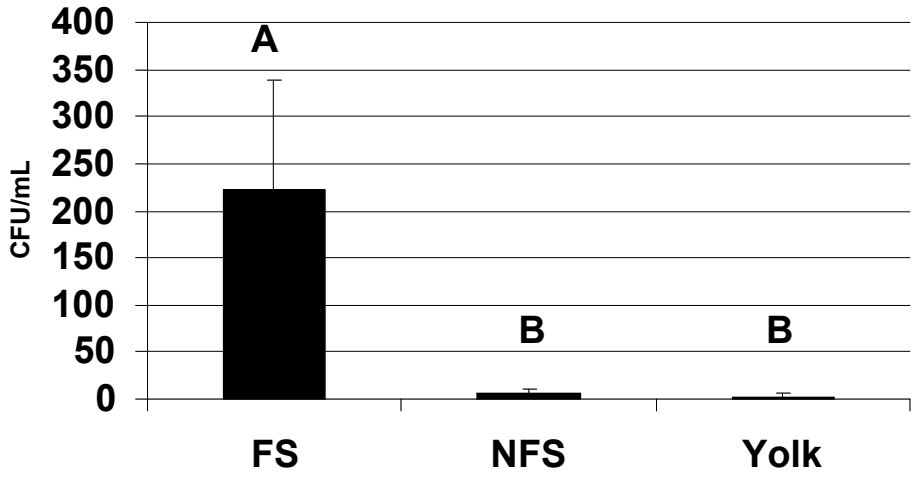

Figure VI-1: *Salmonella* Enteritidis counts for trials 1 and 2 combined. Bars represent average and standard error values for colony forming units (CFU) in SW follicles at varying incubation times. Samples were processed and viable *Salmonella* counts were determined as described in Methods (n=10 per follicle type, $p<0.05$)

CHAPTER VII

CONCLUSIONS

Salmonella Enteritidis (SE) and *Salmonella* Typhimurium (ST) are major pathogens affecting eggs and egg products. Through the use of previously described experiments it is possible to determine that over time, and egg's susceptibility to these bacteria increases. This suggests that even at refrigeration temperatures, microbial stability of an egg is not assured. Though not of statistical import, visual observations of eggs subjected to inoculation with either bacterium suggest that SE may have a mechanism for penetration of the vitelline membrane that ST does not possess. Clarification of this mechanism would alleviate questions pertaining to the increase in disease incidence of SE.

Experiments presented here suggest that avian reproductive tissues are more susceptible to bacterial invasion when in the extremely immature, small white phase. Concern should be paid to this area, as small white follicles which become infected with pathogens such as SE and ST may mature into fully developed egg yolks possible of inciting foodborne illness.

Another point of interest is the apparent importance of the inner layer of the vitelline membrane as a barrier to bacterial invasion. Data from these experiments show that follicles still incased in the follicular sack are quite prone to invasion by SE. There is no evidence to suggest, however, that the addition of the outer layer of the membrane impedes bacterial penetration.

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APPENDIX

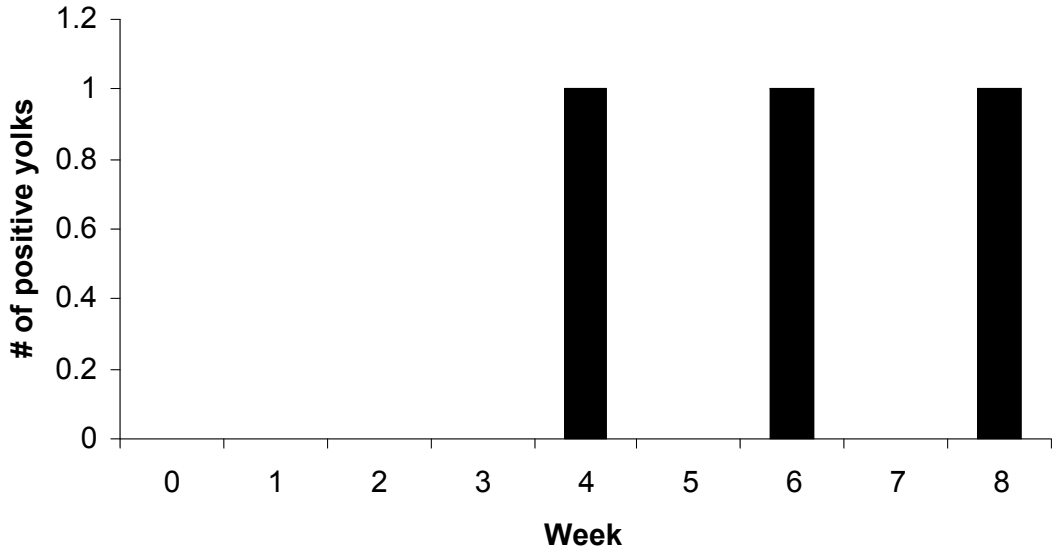

Figure A-1: Frequency of *Salmonella* Typhimurium levels over 10^3 CFU/mL in yolk contents from trial 1. Ten eggs a week for eggs stored 0-8 weeks were sampled. Bars represent the number of yolk samples whose contamination level was above 10^3 CFU/mL at each week of the trial.

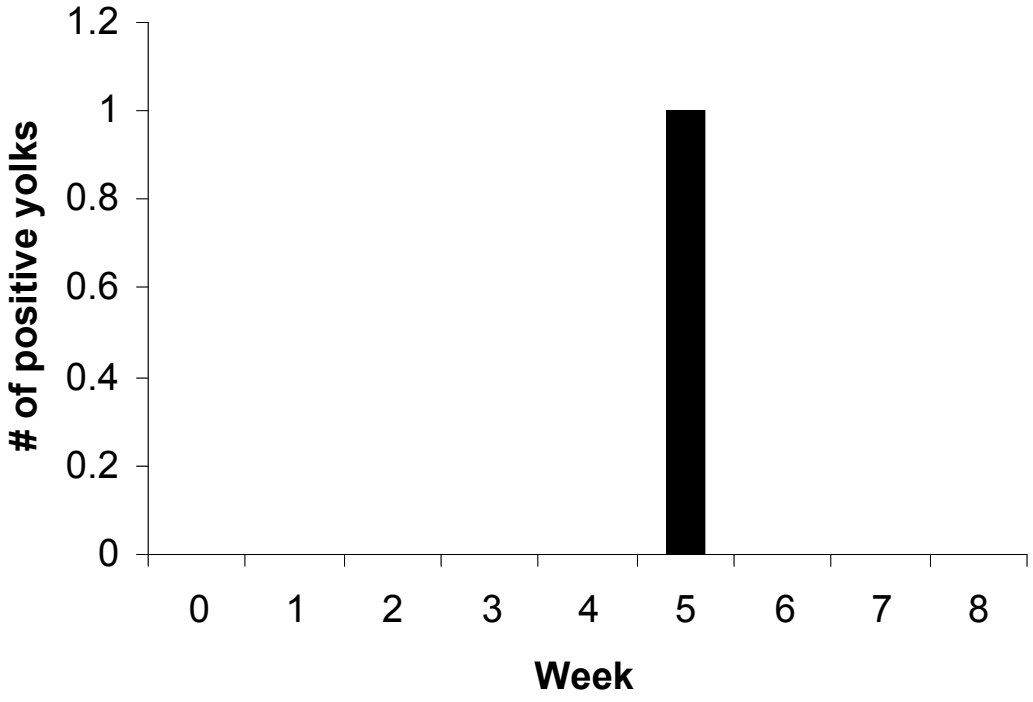

Figure A-2: Frequency of *Salmonella* Typhimurium levels over 10^3 CFU/mL in yolk contents from trial 2. Ten eggs a week for eggs stored 0-8 weeks were sampled. Bars represent the number of yolk samples whose contamination level was above 10^3 CFU/mL at each week of the trial.

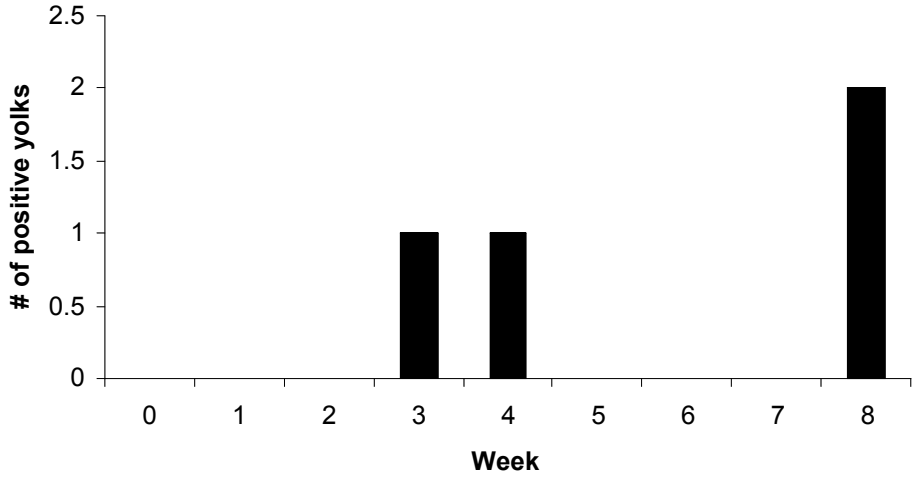

Figure A-3: Frequency of *Salmonella* Enteritidis levels over 10^3 CFU/mL in yolk contents from trial 1. Ten eggs a week for eggs stored 0-8 weeks were sampled. Bars represent the number of yolk samples whose contamination level was above 10^3 CFU/mL at each week of the trial.

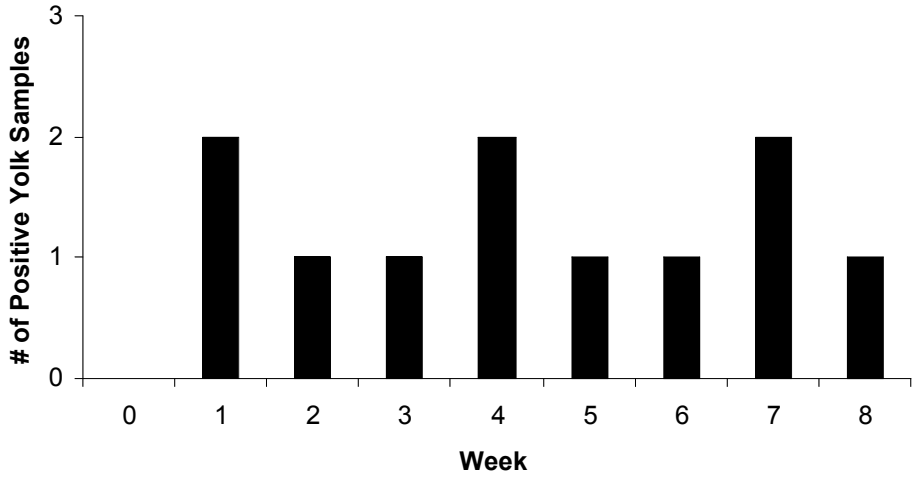

Figure A-4: Frequency of *Salmonella* Enteritidis levels over 10^3 CFU/mL in yolk contents from trial 2. Ten eggs a week for eggs stored 0-8 weeks were sampled. Bars represent the number of yolk samples whose contamination level was above 10^3 CFU/mL at each week of the trial.

V I T A

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Abstracts:

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Li X., Z.R. Howard, I.D. Zabala, K.L. Medvedev, and S.C. Ricke. Development of research paper writing skills of poultry science undergraduate students taking food microbiology. Poultry Science Association, 91st Annual Meeting, August 11-14, 2002. Newark, Delaware.

Medvedev K.L., Z.R. Howard, S.G. Birkhold, and S.C. Ricke. Consumer sensory and mechanical evaluations of quality attributes of eggs from commercial laying hens molted by alfalfa. Poultry Science Association, 91st Annual Meeting, August 11-14, 2002. Newark, Delaware.

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