

EVALUATION OF THE POSSIBLE IMMUNOLOGICAL FUNCTION OF THE CHICKEN
CROP (INGLUVIES)

by

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(Under the Direction of Mark Jackwood)

ABSTRACT

The crop (ingluvies) of the chicken (*Gallus gallus*) is an anatomical diverticulum of the esophagus and is regarded as a food storage organ. Additionally, the crop may function as a component of the extensive mucosal immune system of the gastrointestinal tract. Following experimental *per os* (oral) challenge with *Salmonella enterica* serovar Enteritidis (SE), a SE-lipopolysaccharide (LPS)-specific IgA humoral immune response was detected within the crop and a recall response to SE antigen was generated after SE re-infection. Increased lymphoid tissue was observed within the crop lamina propria of SE-infected chickens compared to uninfected controls. B-lymphocytes and plasma cells were identified within isolated lymphoid follicles (ILF), thus cellular populations exist that have the capacity to generate a humoral response locally within the crop. Commercial egg-layer hen strains and specific pathogen free (SPF) White Leghorn (WL) chickens presented similar findings of an increased SE-LPS-specific IgA response and lymphoid tissue presence within the crop organ post-SE infection. Bacteriological assessment of crop lavage samples revealed viable SE harbored within the crop and that SE persisted within the organ for extended time. The crop may be a potential reservoir for spread of SE in flock and/or a potential source of SE contamination to poultry carcasses

during processing. The chicken crop deserves more attention as part of the integrated mucosal immune system and as an organ of importance in SE pathogenesis.

INDEX WORDS: Chicken, Crop, Ingluvies, *Salmonella* Enteritidis (SE), Mucosal immune system, Gut-associated-lymphoid tissue (GALT), Isolated lymphoid follicles (ILF), Immunohistochemistry (IHC), Enzyme-linked immunosorbent assay (ELISA), Immunoglobulin-A (IgA), Humoral response

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

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2007

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DEDICATION

This document is dedicated to Dorothy Christos and Blondena Parker for their roles in teaching me the importance of education and showing me the awe of nature.

“In every walk with nature one receives far more than he seeks.”

~John Muir

“When one tugs at a single thing in nature,
he finds it attached to the rest of the world”.

~John Muir

“A society grows great when old men plant trees
whose shade they know they shall never sit in.”

~Unknown

“And the day came when the risk to remain tight in a bud
was more painful than the risk it took to blossom.”

~Anais Nin

ACKNOWLEDGEMENTS

I would like to express acknowledgement of those who have helped me with this document and my academic career. Thank you to Dr. Mark Jackwood and my PhD committee members. I would especially like to thank Dr. Pete Holt for generously allowing me to become a member of his research laboratory at USDA and for facilitating completion of my PhD graduate degree. I would like to acknowledge the continuous support and love that I have received from my family and friends throughout my academic career.

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CHAPTER 1

INTRODUCTION

Salmonella Enteritidis (SE) remains a significant food safety problem in the United States due primarily to the ability of SE to infect laying hens and subsequently enter eggs (via transovarian transmission) meant for human consumption (12, 29). It has also been reported that ceca and crops from broiler chickens harbored *Salmonella* at the processing plant and therefore these organs may act as a source for carcass contamination (6-9, 14, 15, 19, 22, 28, 32). As a result, information is being sought to define in more detail the host-pathogen interaction between chickens and SE. Acquiring more knowledge about SE pathogenesis will help determine key intervention points where SE may be attacked and eliminated.

Substantial research has been undertaken to delineate the immune response of chickens against *Salmonella* and many aspects of this immunity have been defined. However, information regarding local immunity in the alimentary tract against *Salmonella* Enteritidis (SE) remains a key point that needs to be addressed. As salmonellae, including SE, invade and initiate infection in the gastrointestinal tract, an understanding of the development of immunity in this region is of paramount importance. The understanding of local gastrointestinal tract mucosal immunity against SE would potentially be of benefit in formulating more effective SE monitoring and vaccine regimens.

The chicken crop, deemed an organ of food storage, is anatomically described as a ventral diverticulum of the esophagus, with no immunological significance having been attributed to it (10, 33). Recent discoveries in the laboratory of Dr. Peter Holt (USDA, ARS, Egg Safety and Quality Research Unit, Athens, GA) indicated that strong SE-antigen-specific IgA

antibody response can be found in crops (ingluvies) of specific pathogen free (SPF) White Leghorn (WL) chickens infected with SE (16, 30, 31). Along with SE-antigen-specific IgA antibody, presence of lymphoid aggregates within the crop lamina propria were observed following SE infection (17). Researchers have demonstrated that IgA antibodies can inhibit mucosal infection by bacteria in the intestinal tract (1, 2, 18, 24, 25). The immune response in the chicken crop recently documented by Seo, *et al.* (30) and Holt, *et al.* (17) needs to be more clearly defined in order to determine the possible role for moderating enteric disease in the avian host. The presence of an immune response against SE in the crop organ may be an indicator that the anatomical site may serve a role in mucosal immunity or immune competence and is not merely a food storage organ.

The immune system of the chicken (*Gallus gallus domesticus*) has been described as consisting of primary and secondary lymphoid tissues. Thymus and bursa of Fabricius are primary or central lymphoid tissue. Secondary or peripheral lymphoid tissue includes the spleen, Harderian gland of the eye, esophageal-proventricular tonsil, ileal Peyer's patches and cecal tonsils (3, 4, 11, 23, 26, 27). Sporadically dispersed areas of lymphoid populations in the upper and lower alimentary tract (gut-associated lymphoid tissue, GALT) and respiratory tract (bronchial-associated lymphoid tissue, BALT) are also defined as secondary lymphoid tissue (5, 13, 20, 21, 34). The lymphoid aggregates and/or isolated lymphoid follicles (ILF) within the chicken crop (ingluvies) may be comparable to known GALT or BALT sites.

The role of crop immunity in the over-all protection of the host against enteric SE infection remains minimally understood. Information about the local immune response in the crop induced by SE could potentially be useful to develop more efficient surveillance protocols and intervention strategies against SE in poultry. The main research focus of the present study

was to further evaluate the potential immunological significance of the chicken crop. Temporal kinetics of development of SE-specific IgA immune response was determined pre- and post-SE challenge. Crop tissue sections were examined for presence or absence of lymphoid tissue at various times post-SE infection. These immune parameters were compared between specific pathogen free (SPF) White Leghorn (WL) chickens and commercial strains of egg-layer hens following SE infection. The evolution of humoral response within the crop was evaluated for SPF White Leghorn chickens after primary SE-challenge and then secondary SE re-challenge, and long-term immune response was assessed. Cellular composition of the lymphoid aggregates in the crop lamina propria was investigated and characterized post-SE infection.

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CHAPTER 2

LITERATURE REVIEW

Salmonella enterica subspecies *enterica* serovar Enteritidis (SE)

Bacterial strains of the genus *Salmonella* belong to the family Enterobacteriaceae. The genus *Salmonella* is currently comprised of two species, *Salmonella enterica* and *Salmonella bongori* (formerly subspecies V). The type species *Salmonella enterica* should be divided into six subspecies (subsp): I, *Salmonella enterica* subsp *enterica*; II, *Salmonella enterica* subsp *salamae*; IIIa, *Salmonella enterica* subsp *arizonae*; IIIb, *Salmonella enterica* subsp *diarizonae*; IV, *Salmonella enterica* subsp *houtenae*; and VI, *Salmonella enterica* subsp *indica* (19, 150, 151).

The facultative intracellular pathogen *Salmonella enterica* is a rod-shaped, non-sporeforming, generally motile with peritrichous flagella, gram-negative bacterium capable of causing disease in a wide range of host species (19, 150). The majority of the approximately 2500 *Salmonella* serotypes belong to *Salmonella enterica* subspecies I (*Salmonella. enterica* subsp *enterica*). Within *Salmonella enterica* subsp *enterica*, the most common somatic O-antigen serogroups are A, B, C1, C2, D and E. Strains in these serogroups cause approximately 99% of *Salmonella* infections in humans and warm-blooded (homoiotherm) animals (19). Serotypes in *Salmonella enterica* subspecies II, IIIa, IIIb, IV, VI and *Salmonella bongori* serotypes are usually isolated from cold-blooded (poikilotherm) animals and the environment but rarely from humans (19, 34, 102).

Some environmental sources of *Salmonella enterica* include water (86-88, 130), soil

(137), insects (86), and animal feces (44, 45, 90). Most types of *Salmonella* live in the intestinal tracts of animals and birds, and transmission to humans is by contaminated foods of animal origin. *Salmonella* may be spread in a factory or kitchen facility when surfaces and food preparation items come in contact with contaminated raw meats (139, 165), poultry (76, 107), and/or seafood (103, 146). Salmonellosis (gastroenteritis) may be caused in humans when *Salmonella* contaminated water (47, 130), fish (3, 146), vegetables/fruits/nuts (55, 126, 153, 155), milk (4, 56, 101), meat (3, 91, 160), and/or poultry eggs (4, 37, 73, 110) are ingested.

Salmonella enterica subspecies *enterica* serovar Enteritidis [*Salmonella* Enteritidis (SE)] is a broad host range serotype with widespread occurrence in animals, especially swine and poultry (139, 154). *Salmonella* Enteritidis (SE) rarely causes clinical disease in chickens, with the exception of very young chicks. SE colonizes the intestinal tract of poultry and SE may be shed in feces (7-9, 140). Horizontal transmission of SE by fecal-oral route to chickens in flock may occur and at time of slaughter SE may contaminate poultry meat (67, 69, 116, 162). *Salmonella* Enteritidis (SE) may also colonize the reproductive tract of hens, leading to contamination of eggs via transovarian route (13, 48, 50, 85, 147). Research by Timoney *et al.* showed that inoculation of approximately 10^6 *Salmonella* Enteritidis organisms into the crop of adult hens was followed by bacteremia and infection of many body sites, including peritoneum, ovules, and oviduct (149). SE was present in the yolk or albumen of eggs from about 10% of the hens shortly after infection and again 10 days later, which provided evidence for egg or transovarian transmission of the SE infection (131, 149).

Role of SE in Food-Safety

Salmonella Enteritidis (SE) is responsible for food-borne enteritis in man, and often human salmonellosis cases are associated with consumption of contaminated poultry meat or

eggs (132, 144). A person infected with the *Salmonella* Enteritidis (SE) bacterium usually has fever, abdominal cramps, and diarrhea beginning 12 to 72 hours after consuming a contaminated food or beverage (38, 54). The illness usually lasts 4 to 7 days, and most persons recover without antibiotic treatment. However, diarrhea can be severe, and the person may be ill enough to require hospitalization. The elderly, infants, and those with impaired immune systems may have a more severe illness (38, 54). In these patients, the infection may spread from the intestines to the blood stream, and then to other body sites and can cause death unless the person is treated promptly with antibiotics.

Salmonellosis is an important public health problem in the United States and several European countries (33). *Salmonella enterica* serovar Enteritidis (SE) accounts for an increasing proportion of the serotypes reported to the Centers for Disease Control and Prevention's (CDC) national *Salmonella* Surveillance System (28). During 1976-1994, the proportion of reported *Salmonella* isolates that were SE increased from 5% to 26%. During 1985-1995, state and territorial health departments reported 582 SE outbreaks, which accounted for 24,058 cases of illness, 2290 hospitalizations, and 70 deaths (25, 26). During the 1980s, illness related to contaminated eggs occurred most frequently in the northeastern United States, but illness caused by SE has increased in other parts of the country as well (22, 23, 110). *Salmonella* Enteritidis (SE) outbreaks continue to occur in the United States (29). Foods other than eggs have also caused outbreaks of SE disease in humans. Consumers should be aware of the disease and learn how to minimize the chances of becoming ill (6, 111).

The CDC estimates that 75% of SE outbreaks are associated with the consumption of raw or inadequately cooked grade A whole shell eggs (27, 28). SE can be inside perfectly normal-appearing eggs, and if the eggs are eaten raw or undercooked, the bacterium can cause illness

(27, 73). Stringent procedures for cleaning and inspecting eggs were implemented in the 1970s and have made salmonellosis caused by external fecal contamination of egg shells (on-egg contamination) extremely rare (158). Unlike egg-associated salmonellosis of past decades, the current epidemic is due to intact and disinfected grade A eggs (24, 58, 72, 99, 110, 148). The present situation with *Salmonella* Enteritidis (SE) in chickens is complicated by the presence of the organism inside the egg, in the yolk (51). *Salmonella* Enteritidis (SE) appears to infect the ovaries of layer hens (147). Information strongly suggests that egg-associated salmonellosis may be a result of vertical transmission of SE with deposition of SE in the yolk (in-egg contamination) prior to egg shell deposition (48). Hens appear healthy yet their reproductive tracts are infected with SE and subsequently SE may contaminate eggs before shell formation (50, 124).

Crop as Potential Reservoir for SE

Salmonella bacteria present in intestinal contents of poultry have been considered to be the major source of meat contamination during slaughter and processing (134). To reduce the amount of contents in the intestines, feed is removed from broiler chickens for 8 to 10 hours before they are transported to the processing plant. Feed withdrawal from broilers before commercial processing has been shown to result in an increased incidence of *Salmonella* within the crop (41, 92, 134). After feed removal, broilers may often begin to consume contaminated litter on the rearing house floors, and this may result in a 5-fold increase in the number of broilers with *Salmonella* in their crop contents (59). The rise in *Salmonella* within the crop is probably due to coprophagy, however, this is not definitive. The incidence of *Salmonella* in the crop has been reported to significantly increase following an 8 hour feed withdrawal in both commercial and experimental settings (74, 134). This has implicated the crop as a source of

Salmonella contamination at processing (20, 35, 36, 59).

Market age broilers were frequently contaminated with *Salmonella* at a processing plant. It was reported that the crop was 3.5 times more likely to be contaminated with *Salmonella* than the ceca, and the crop was found to rupture 85 times more frequently than ceca during commercial processing at one plant (59). Thus, the *Salmonella* contaminated crop contents become a greater source of contamination within the processing plant than do contaminated intestinal contents (21, 35, 36, 59, 134). Results show that the crop serves as a reservoir of *Salmonella* that is carried into the processing plant and represents a critical control point to reduce contamination of poultry meat products (35, 36). Furthermore, the practice of feed withdrawal to induce molting for the purpose of stimulating multiple egg-laying cycles in hens has been associated with enhanced recovery of *Salmonella* Enteritidis (SE) from crops, an increased SE organ invasion in layer-hens, and an increased risk of horizontal transfer of SE in flocks (68, 112).

Mucosal Immunity: IgA Antibody & Gut-Associated Lymphoid Tissue (GALT)

Humoral immunity can be divided into two main components: systemic and local. Systemic antibody responses involve those found in the serum. Primarily an IgG response, systemic immunity generally lasts for extended periods (31). Local immunity resides in three primary mucosal lined regions: the respiratory tract, the genitourinary system, and the gastrointestinal tract (14, 15, 17, 83). The predominant immunoglobulin of local mucosal immunity is IgA (17, 118, 138, 163), although IgM and IgG responses have been observed (32, 42, 53, 61, 119, 127). The bulk of the body's IgA antibodies are produced by plasma cells in mucosal lamina propria (LP). In the gastrointestinal tract, mucosal secretions and bile contain IgA that exists in monomeric and multimeric forms (16).

Production of IgA is especially prominent in the intestinal tract because of its length, enormous surface area, and exposure to antigens of microbes and food (65). It has been appreciated that IgA antibodies in the intestinal secretions provide a first line of immunological defense against microbial pathogens by helping to prevent microbes from adhering to and penetrating the mucosal epithelium (43, 109, 117). Correlations have been made clinically and experimentally between the content of specific IgA antibodies in the luminal secretions and resistance to challenge by a given pathogen (108, 128). It has been possible to show experimentally with a number of mucosal pathogens, including enteric pathogens, that IgA antibody acting as the immunologically specific agent can mediate effective resistance to microbial challenge (77). Such considerations have provided much of the rationale for attempts to develop oral vaccines capable of stimulating effective mucosal IgA responses against infectious agents (5, 62, 89, 114). *Salmonella* Enteritidis (SE) exposure at mucosal surfaces can induce a detectable humoral response specific against SE antigens (52, 70, 71, 143)

Mucosal epithelium is not totally impermeable to penetration by macromolecules. Intact antigens or their fragments can to a small extent gain access to the lamina propria, especially of the intestine where luminal contents are filled with constituents of food and with microflora (152, 163). This constant exposure is supplemented by periodic bouts of infection, which can result in the presence of intact microbes and their antigens in the lamina propria (1, 159). Accordingly, the IgA antibodies that are continuously secreted from local plasma cells can be expected to have ready opportunities to bind diverse antigens that happen to be in the microenvironment (95). Evidence suggests that IgA antibody *in vivo* can also transport antigen from lamina propria across intestinal epithelium (81, 82, 96). Thus, IgA antibodies appear to be capable of providing an excretory immune system that can rid the body of foreign substances and

minimize exposure to a potentially harmful burden of local or systemic immune complexes (94, 96, 136).

The gut epithelium is one of the few epithelia that expresses secretory component (SC) necessary for transport of IgA from lamina propria through epithelium to mucosal lumen (2, 84). Most of this IgA is destined for local secretion because of the existence of a polymeric immunoglobulin receptor (pIgR)-mediated transport mechanism through the epithelium (81, 138, 161). Along the gut, the epithelium and organized GALT in lamina propria can express cell determinants involved in induction of region-specific immunity against pathogens, and conversely may serve as region-specific sites for invasion and infection by pathogens (46, 60, 64, 125, 129, 145). GALT is believed to serve a function in the secretory IgA-linked immune system of antigen-recognition and antibody-production (93, 135, 159). IgA antibodies can potentially combine with antigens in three anatomical compartments in relation to mucosal epithelium: in the luminal secretions; within the epithelial cells during transcytosis; and in the lamina propria beneath the epithelium (94, 105).

The gut-associated lymphoid tissue (GALT) comprises cells and tissues that participate in the immune system response along the alimentary tract from oropharynx to cloaca (2, 66, 78, 80, 97). The organized lymphoid tissues (i.e., Peyer's patches and cecal tonsils), the isolated lymphoid follicles (ILF) and the single cells in lamina propria and epithelium are all included (57, 118, 133, 164). Lymphoid tissue can be located in the roof of the pharynx, around the junction with nasal cavity and Eustachian openings. Organized lymphoid tissue is located in lamina propria of proventriculus on top of the glandular units (104, 115, 123). The Meckel's vitelline diverticulum, situated at junction of duodenum and jejunum, is a remnant of the yolk stalk and contains lymphoid tissue (120, 122). Peyer's patches (Pp) occur regularly in the ileum

(10, 157) and cecal tonsils (CT) are located at the proximal ends of ceca (84, 113). In the avian cloaca, the anatomical structure known as bursa of Fabricius contains aggregations of lymphoid follicles (100, 121, 142). Morphological differences along the gut in architecture and/or proportion of organized lymphoid tissue may perhaps be reflective of antigen load and/or antigenic stimulus at the various alimentary tract sites (40, 79, 98, 106).

Once a bird becomes infected with *Salmonella*, changes in the systemic immune system and the local immune system of the alimentary tract ensue. Following SE infection, Sasai *et al.* (141) observed that splenic CD4⁺ T-lymphocytes and thymic CD8⁺ T-lymphocytes increased in chickens, and Berndt *et al.* (12) found elevated CD8⁺, T-cell Receptor (TCR)1⁺ ($\gamma\delta$) T-lymphocytes in the peripheral blood. The numbers of IgM⁺ and IgA⁺ B-lymphocytes in the spleen were elevated resulting in the rapid development of a humoral immune response in the serum (141). By one week post-infection, a detectable serum antibody response was observed in chickens challenged with SE (30, 49) or ST (63). Serum anti-*Salmonella* IgM responses appeared first and were followed by IgG and IgA (63). The IgM and IgA levels in serum gradually declined while IgG levels remained for extended periods (30, 63). Serum antibody responses persisted for approximately 10 weeks (49, 63, 75). Re-infection with ST resulted in a rapid enhanced serum antibody response to the challenge organism (63). Serum antibodies will, in many cases, be detectable after individuals are no longer culture positive for the organism (52, 156).

Changes in the local immune system of the alimentary tract were observed in chickens infected with *Salmonella*, although these effects are not nearly as well defined as that of the systemic immune system. Sasai *et al.* (141) and Berndt *et al.* (11, 12) found that in chickens challenged with *Salmonella*, CD3⁺ and CD8⁺ cells in the cecal tonsils decreased the first day

post-challenge, while at 4 days post-challenge CD4⁺ T-lymphocytes increased in cecal tonsils. Lymphocytes expressing CD4⁺, TCR2⁺ ($\alpha\beta$) were observed to increase in the lamina propria of cecum, and IgG⁺ and IgM⁺ B-lymphocytes increased while IgA⁺ cells remained constant (11, 12, 141). Antibody responses in the alimentary tract or in bile were delayed and not observed until 2 weeks post-challenge in chickens receiving *Salmonella* Typhimurium (63). Contrary to serum IgG immunity, mucosal IgA responses were more short term (18, 39, 63). Re-infection with ST resulted in a substantial boost in the immune response indicating that an anamnestic response is possible in mucosal tissues (63).

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CHAPTER 3**CROP IMMUNE RESPONSE POST-SALMONELLA ENTERITIDIS CHALLENGE IN
EIGHT COMMERCIAL EGG-LAYER STRAINS AND SPECIFIC PATHOGEN FREE
WHITE LEGHORN CHICKENS ¹**

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ABSTRACT

The crop immune response against *Salmonella* Enteritidis (SE)-challenge in eight commercial egg-layer strains (5 white-egg layer & 3 brown-egg layer) and specific pathogen free (SPF) White Leghorn (WL) hens was investigated. Pre- and post-SE-challenge mucosal immune responses within the crops were evaluated. Commercial layers and SPF WL hens were orally challenged with 10^8 CFU/ml *Salmonella* Enteritidis PT13a and SE nalR PT13, respectively. Crop lavage samples were collected at weekly intervals from day 0 (pre-challenge) to day 25-27 post-infection (pi) and bacteriological examination was performed to monitor progression of SE infection. Crop lavage samples were analyzed for SE-lipopolysaccharide (LPS)-specific IgA by enzyme-linked immunosorbent assay (ELISA). H&E stained slides of crop sections from day 34 pi and uninfected controls were assessed for lymphoid tissue via light microscopy. Lymphoid areas were graded based on morphology, size and cellularity using a score 0 to 5 scale. The score 0 to 5 (low to high) numerical values represented progressive increases in size and cellular density of lymphoid tissue. Bacterial culture results showed the highest percentage of SE positive crop lavage samples from all hen groups at day 5-6 pi and day 11-12 pi. A progressive decline in percentage of SE positive crop lavage samples did occur as time post-infection lengthened, however at day 25-27 pi SE persisted in crop lavage samples from SPF WL hens and three commercial white-egg layer strains. A marked increase in SE-LPS-specific IgA was measured in crop lavage samples between day 0 and day 11-12 pi for all hen groups. Crop SE-LPS-specific IgA response remained elevated above day 0 baseline for the duration of the experiment. Well-defined score 3 to 5 lymphoid tissue aggregates were observed in crop tissue sections harvested at day 34 pi. Comparison of crop sections determined a 1.2-4.0 times increase in ratio of lymphoid tissue in day 34 pi SE-challenged hens versus uninfected control hens.

Key words:

Salmonella Enteritidis,

Chicken,

Crop,

Ingluvies,

Mucosal immune system,

Gut-associated lymphoid tissue,

IgA,

Enzyme-linked immunosorbent assay

Abbreviations:

ARS = Agricultural Research Service;

BSL-2 = biosafety level-2;

BG+NB = brilliant green agar with novobiocin;

BG+NB+NA = brilliant green agar with novobiocin and nalidixic-acid;

CFU = colony-forming units;

CO₂ = carbon dioxide;

ELISA = enzyme-linked immunosorbent assay;

GALT = gut-associated lymphoid tissue;

H&E = hematoxylin and eosin;

IgA = immunoglobulin A;

ILF = isolated lymphoid follicle;

LPS = lipopolysaccharide;

nalR = nalidixic-acid resistant;

NCSU = North Carolina State University;

OD = optical density;

PT13 = phage type 13;

PT13a = phage type 13a;

PBS = phosphate-buffered saline;

pi = post-infection / post-inoculation;

RV = Rappaport-Vassiliadis;

SE = *Salmonella* Enteritidis;

ST = *Salmonella* Typhimurium;

SEPRL = Southeast Poultry Research Laboratory;

SPF = specific pathogen free;

TSB = tryptic soy broth;

USDA= United States Department of Agriculture;

WL = White Leghorn;

XLT4 = xylose lysine tergitol-4 agar

INTRODUCTION

The chicken crop, a ventral diverticulum of the esophagus, is regarded as a food storage organ in the domestic chicken *Gallus gallus domesticus*. Along with food storage, the crop may act as a depot for commensal and pathogenic organisms (2, 5, 25, 30, 32). *Salmonella enterica* subspecies *enterica* serovar Typhimurium (ST) and serovar Enteritidis (SE) are two pathogens that have been cultured from crop contents of broiler-type and layer-type chickens (4, 6, 12, 30). Conditions such as stress, feed-deprivation or immunosuppression may alter the crop environment promoting growth of ST and SE within the crop (7, 8, 13, 14, 27). ST or SE harbored within the crop may have the potential to spread to other parts of the body, cause subclinical infection and subsequently lead to establishment of carrier birds in poultry flocks.

The chicken crop may be a potential site along the upper gastrointestinal tract where a mucosal immune response can be produced against *Salmonella* (14, 18, 28, 29). Seo *et al.* (28, 29) revealed an IgA humoral immune response within the crop against *Salmonella* Enteritidis (SE) when specific pathogen free (SPF) White Leghorn (WL) hens were experimentally challenged with SE via oral gavage. Enzyme-linked immunosorbent assay (ELISA) detected elevated levels of SE-specific IgA in crop lavage samples collected at week 1 post-infection (pi) through week 4 pi (14, 17, 18, 28, 29). Microscopic examination by Holt *et al.* (18) of hematoxylin and eosin (H&E) stained crop tissues from post-SE-challenged SPF WL hens showed lymphoid aggregations/lymphoid follicles present in the crop lamina propria. Thus, the crop organ of the chicken may perhaps function as a component of the extensive mucosal immune system.

The ability of ST and SE to reside within and colonize the crop makes this organ an anatomical location worthy of further investigation concerning *Salmonella* pathogenesis. The

antigen-specific IgA humoral immune response detected in the chicken crop after SE infection could be of relevance as a diagnostic indicator of enteric disease status and/or useful in identifying prospective oral immunostimulants of the alimentary tract for poultry mucosal vaccines. The crop may also be an organ of importance in the study of gut-associated lymphoid tissue and mucosal immunity. More knowledge about the behavior of ST and SE in relation to the chicken crop and the mucosal immune system is needed so that more effective control measures against infection in poultry can be designed and applied.

Specific pathogen free (SPF) White Leghorn (WL) pullets and SPF WL layer hens have been the primary animal models used to study the mucosal immune response within the chicken crop against SE infection (18, 28, 29). However, there is a degree of uncertainty regarding the applicability of results obtained from crop-SE experiments using SPF White Leghorn chickens to the effects and outcomes produced by SE infection in commercial egg-layer hen strains. Information is lacking about commercial egg-layer strains of chickens in regards to the presence of an IgA humoral response and existence of lymphoid tissue in the crop organ. We sought to determine if SE-antigen-specific IgA response and lymphoid tissue would be observed in the crop organ of SPF WL hens as well as commercial egg-layer strains of chickens post-SE challenge, and to make sure that our previous crop-SE findings were not merely occurrences restricted solely to our line of SPF WL chickens.

Research was undertaken in an effort to determine if data from *Salmonella* Enteritidis (SE) infected SPF WL hens would be applicable to commercial strains of layer hens in the poultry industry. The purpose of the study was to compare the crop post-SE-challenge among eight commercial strains of layer hens and specific pathogen free (SPF) White Leghorn chickens. The experiment had three objectives: first was to monitor SE prevalence within the crop at

various time points post-SE inoculation; second was to investigate the evolution of a local humoral SE-specific IgA immune response in the crop; and third was to assess the crop for lymphoid tissue presence in SE-challenged hens versus uninfected controls.

MATERIALS & METHODS

Animals.

Specific pathogen free (SPF) White Leghorn (WL) hens used in trial 1 of the study were obtained from flocks maintained by Southeast Poultry Research Laboratory (SEPRL), USDA-ARS, Athens, Georgia. Hens at approximately seventy weeks of age were housed within individual layer cages located within a climate controlled biosafety level-2 (BSL-2) building. Birds were allowed access to antibiotic-free feed and water *ad libitum*.

Layer pullets from eight commercial layer strains (five white-egg layer and three brown-egg layer) used in trial 2 were received from the 36th North Carolina Layer Performance and Management Test conducted by the Cooperative Extension Service at North Carolina State University (NCSU) and North Carolina Department of Agriculture and Consumer Services (1). The flock was maintained at the Piedmont Research Station, Salisbury, NC. The eight commercial strains consisted of: Lohmann LSL Lite; Dekalb White; Hy-Line W-36; Hy-Line W-98; Bovans White; Bovans Goldline; Bovans Brown and Hy-Line Brown. At appropriate time points during the rearing period, day of hatch through sixteen weeks of age, the Piedmont Research Station personnel routinely vaccinated all pullets against viral diseases: Marek's (herpesvirus), Newcastle (paramyxovirus), Fowl Pox (poxvirus), and Avian Encephalomyelitis (picornavirus). The layer pullets at seventeen weeks of age were transported from the Piedmont Research Station to the J. Phil Campbell, USDA-ARS facility in Watkinsville, Georgia. Birds were grouped by strain and housed within floor pen containment units. Separate containment

modules were used for each of the eight strains. Antibiotic-free feed and water was provided *ad libitum*. At twenty-four weeks of age, birds were moved to a BSL-2 building at SEPRL, USDA-ARS, Athens, Georgia. Birds were arranged according to same strain and placed in individual layer cages (one bird per cage). Free access to antibiotic-free feed and water was provided.

Bacterial strains.

Salmonella serovar Enteritidis (SE) phage type (PT) 13 and SE PT13a were selected for experimental challenge of layer hens in trial 1 and trial 2, respectively, due to the ability of *Salmonella* serovar Enteritidis (SE) to colonize the reproductive organs of mature laying hens and the identification of phage types 13 and 13a in egg-associated foodborne salmonellosis outbreaks among humans (3, 9, 10, 15, 24). Frozen stocks maintained at -70°C of a nalidixic acid resistant (nalR) *Salmonella* serovar Enteritidis phage type 13 (strain SE89-8312) and *Salmonella* serovar Enteritidis phage type 13a (strain 6) were thawed and cultured on nutrient agar at 37°C for 18 hours. Isolated colonies were selected and cultivated in 10 ml tryptic soy broth (TSB) at 37°C for 18 hours. Broth cultures were then diluted in sterile physiological saline to prepare 10⁸ colony-forming units (CFU) /ml inoculum for experimental infection of hens.

Experimental design.

Salmonella-free status of trial 1 and trial 2 chickens was determined before commencement of the experiment. Feces and crop lavage samples were selectively enriched in Rappaport-Vassiliadis (RV) broth for 24 hours at 37°C-42°C then plated onto brilliant green agar containing 20 µg/ml novobiocin (BG+NB). *Salmonella* was not detectable.

For trial 1, twenty SPF White Leghorn hens were separated into two groups, experimental and control. Twelve hens comprised the experimental group and each hen was infected *per os* with 1

ml of 10^8 CFU/ml SE naIR PT13. Eight additional SPF WL hens remained uninfected to serve as the control group.

For trial 2, a total of sixty-three hens were randomly selected from the eight groups of commercial layer strains to serve as experimental and control birds. Five groups were of white-egg layer type hens: Lohmann LSL Lite; Dekalb White; Hy-Line W-36; Hy-Line W-98 and Bovans White. Three groups were brown-egg layer type hens: Bovans Goldline, Bovans Brown and Hy-Line Brown. Forty commercial layer strain birds (five hens from each of the eight strains) were experimentally infected *per os* with 1 ml of 10^8 CFU/ml of SE PT13a. Twenty-three birds remained uninfected as controls (one to five birds represented each of the eight strains). In an effort to prevent any perceived bias to a particular commercial strain, we randomly assigned W1-W5 to the five white-egg layer strains and Br6-Br8 to the three brown-egg layer strains. The W1-W5 and Br6-Br8 designations will be used from this point forward to refer to hens from the eight commercial strains in trial 2.

Crop lavage samples were obtained from trial 1 and trial 2 hens for bacteriological assessment of SE and for ELISA analysis of humoral immune response. Feces were collected from trial 2 hens for bacterial culture to monitor SE fecal shedding. Crop lavage samples were collected from trial 1 hens on: day 0 pre-challenge, day 5 post-infection (pi), day 12 pi, day 19 pi and day 27 pi. Trial 2 hens had crop lavage samples and feces collected on: day 0 pre-challenge, day 6 pi, day 11 pi, day 18 pi and day 25 pi. The entire crop organ was harvested at day 34 pi from trial 1 and trial 2 hens for histopathological evaluation of crop tissue. Studies conducted were approved by and in accordance with guidelines of the SEPRL Institutional Animal Care and Use Committee (IACUC).

Crop lavage sample collection.

Crop lavages were performed according to the protocol designed by Holt *et al.* (18). The crop lavage devices were fashioned using 10 ml syringes and attached tubing 7-10 inches (17.8-25.4 cm) in length with the distal 1.5 inches (3.8 cm) fenestrated. The lavage devices were aseptically filled with 5 ml of room temperature, sterile glycine flush solution, pH 7.8-8.0 (18, 20). Birds were held in an upright, vertical position with neck extended to facilitate insertion of the lavage device tubing into the esophagus. Once the fenestrated portion of tubing was positioned within the crop, the 5 ml glycine solution was slowly infused. Immediately a fluid sample was aspirated back into the syringe then the crop lavage sample (aspirate) was dispensed from syringe to a sterile collection container. The lavage procedure was repeated per individual bird using a new lavage device and separate sample collection container.

Bacteriological examination.

Appropriate techniques and media for isolation of salmonellae were utilized for bacteriological examination of samples collected post-SE infection (31). Trial 1 bacterial culture for SE naIR PT13 was performed using crop lavage samples collected at weekly time points: day 0 pre-challenge, day 5 post-infection (pi), day 12 pi, day 19 pi and day 27 pi. A 100 µl portion from each neat crop lavage sample was directly spread plated onto brilliant green agar (BG+NB+NA) containing novobiocin (NB, 20 µg/ml) and nalidixic-acid (NA, 20 µg/ml). A 1.0 ml aliquot from each crop lavage sample was added to 9 ml Rappaport-Vassiliadis (RV) broth for selective enrichment. The BG+NB+NA direct spread plates and RV sample tubes were incubated at 37°C overnight. Plates were then assessed for SE colony growth. In instances where direct plating resulted in no growth, the RV enriched samples were streak plated on BG+NB+NA agar and streak plates were incubated 24 hours at 37°C then assessed for typical SE colony growth. Crop lavage samples were recorded as positive if SE was recoverable either by direct plating or after

selective enrichment. Negative crop lavage samples were those that had no SE growth detectable.

Trial 2 crop lavage samples and feces were cultured for SE PT13a at weekly time points: day 0 pre-challenge, day 6 post-infection (pi), day 11 pi, day 18 pi, and day 25 pi. A 1.0 ml aliquot from each neat crop lavage sample and a 1.0 gram weight of feces were added to 9 ml Rappaport-Vassiliadis (RV) broth. The RV sample tubes were incubated at 37°-42°C overnight. After 24 hours selective enrichment, samples from RV were streak plated onto two media, brilliant green agar (BG+NB) containing novobiocin (NB, 20 ug/ml) and xylose lysine tergitol-4 (XLT4) agar (Beckton Dickinson-Difco, Franklin Lakes, NJ, USA). The BG+NB and XLT4 plates were incubated at 37°C overnight then examined for bacterial growth and colony morphology indicative of SE. Questionable colonies were further differentiated based on biochemical tests using triple sugar iron (TSI) agar and modified-lysine iron (MLI) agar slant tubes, and agglutination reaction with antiserum against *Salmonella* serogroup D₁ somatic antigen (Beckton Dickinson-Difco). Positive samples were those that had SE definitively identified from culture on BG+NB and/or XLT4.

Crop SE-specific IgA ELISA.

The crop lavage samples obtained at weekly time points from trial 1 and trial 2 hens were analyzed by enzyme-linked immunosorbent assay (ELISA) for SE-lipopolysaccharide (LPS)-specific IgA as previously described by Holt *et al.* (16-18). A 1.5-2.0 ml portion from each neat crop lavage sample was centrifuged at 10,000 rpm for 5 minutes to remove feed particulate debris and excess mucous. The supernatant fraction was collected and stored at -20°C. For assay, the frozen crop supernatant samples were thawed, diluted 1:1 with phosphate buffered saline (PBS) containing 0.05% Tween20 (Sigma-Aldrich, St. Louis, MO, USA) then 2-fold serial

dilutions were prepared. Sample dilutions 1:2 to 1:128 were added to flat-bottomed 96-well polystyrene ELISA plates (Corning-Costar Inc., Wilkes Barre, PA, USA) to which SE-LPS antigen (Sigma-Aldrich) at concentration 10 µg/ml had been adsorbed. Additionally, previously defined crop lavage samples with strong and weak SE-LPS-specific IgA antibody responses were run on each assay plate as positive and negative controls, respectively. Plates containing samples and controls were incubated 60 minutes at room-temperature then washed three times with PBS containing 0.05% Tween20. The next two sequential steps performed were the application of primary antibody with 60 minutes incubation time followed by three washes, then addition of an enzyme conjugated secondary antibody with protocol repeated for incubation time and washes. The primary antibody for detection of SE-LPS-specific IgA in crop lavage samples was a monoclonal mouse anti-chicken IgA (Southern Biotech, Birmingham, AL, USA) diluted 1:1000 in PBS containing 0.05% Tween20 and 1% bovine serum albumin (BSA). Goat anti-mouse IgG with alkaline phosphatase conjugate (Calbiochem, La Jolla, CA, USA) diluted 1:1000 served as the enzyme conjugated secondary antibody. Diethanolamine buffer with concentration 1 mg/ml p-nitrophenyl-phosphate (Sigma-Aldrich) was applied to plate wells as substrate solution and the chromogenic reaction proceeded for 15 to 30 minutes at room-temperature in the dark. Colorimetric analysis with Multiskan Ascent (Thermo Fisher Scientific, Milford, MA, USA) microplate photometer at 405 nm wavelength yielded absorbance/optical density (OD) values.

Crop Histology & Lymphoid Tissue Scoring.

On the final day of the experiment for trial 1 and trial 2, the SE-challenged hens at day 34 post-infection and negative control hens were euthanatized via carbon dioxide (CO₂) gas inhalation. Immediately postmortem the entire crop with 1.0 inch (2.54 cm) of attached esophagus was excised. Tissues were fixed intact in 10% neutral buffered formalin for 24 hours and the crops

were then sectioned. Transverse cuts were made at the mid-body region of the crop. The plane of the cut passed through the entirety of the hollow-bodied crop organ to produce ring-shaped circular sections. Crop tissue sections were routinely processed, paraffin embedded, microtome cut, and affixed to glass slides. Tissue slides were stained with hematoxylin and eosin (H&E) then examined via light microscopy. The H&E crop tissues were evaluated full-circle for the presence of inflammatory changes and lymphoid aggregations in the epithelium and lamina propria. All apparent sites of lymphocytic-lymphoid cell infiltrates and/or isolated lymphoid follicles (ILF) were graded as score 0 to 5, and the number of lymphoid areas observed for each score category was recorded. An ocular grid reticle (Fisher Scientific, Suwanee, GA, USA) was used to make the score 0 to 5 determinations and perform counts at 200X magnification. Criteria used to establish the score 0 to 5 grade scale were morphological character, size and cellular density of a lymphoid site. The score 0 to 5 numerical increase represented lymphoid tissue that progressively increased in structural detail, size and cellularity (**Table 3.1**).

Statistical Analyses.

Statistical analyses were performed using GraphPad InStat (GraphPad Software, Inc., San Diego, CA, USA). Analysis of variance (ANOVA) with Tukey's multiple comparison test procedure were conducted on bacterial culture results and enzyme-linked immunosorbent assay (ELISA) data for trial 1 and trial 2 hen strains to determine statistically significant differences ($p < 0.05$) among the post-SE infection time points. Analysis of variance (ANOVA) with Dunnett's multiple comparison test procedures were conducted on bacterial culture results and enzyme-linked immunosorbent assay (ELISA) data to determine statistically significant differences ($p < 0.05$) between pre-challenge day 0 control and post-SE-challenge time points for trial 1 and trial 2 hen strains. Unpaired t-tests were performed on crop lymphoid tissue score data to assess

whether or not the uninfected control group and day 34 pi SE-challenged group differed significantly ($p < 0.05$) for each of the hen strains. The humoral response within the crop of SPF WL hens and commercial layer hen strains (W1-W5, Br6-Br8) was compared for statistically significant differences ($p < 0.05$) by ANOVA and Tukey's multiple comparison post-test procedure. Significant differences ($p < 0.05$) in the mean number of crop isolated lymphoid follicles (ILFs) observed at day 34 post-SE infection between SPF WL and commercial layer hen strains were determined by ANOVA with Tukey's multiple comparison post-test procedure.

RESULTS

Bacterial Culture.

Table 3.2 represents the temporal kinetics of SE prevalence in weekly samples cultured from SPF White Leghorn hens and each of the eight commercial egg-layer strains. The prevalence of *Salmonella* Enteritidis (SE) from crop lavage samples among trial 1 SPF White Leghorn (WL) hens was 0% at day 0 pre-challenge then 100% day 5 pi, 42% day 12 pi, 50% day 19 pi and 25% day 27 pi. For the trial 2 commercial layer hen strains (W1-W5, Br6-Br8), no SE was recoverable from crop lavage samples or feces cultured at day 0 pre-challenge. The percentage of SE positive samples among the five white-egg layer (W1-W5) strains at day 6 pi ranged from 60% to 100% (mean 84%) crop and 80% to 100% (mean 92%) feces. Among the three brown-egg layer (Br6-Br8) strains at day 6 pi, SE positive samples ranged from 20% to 80% (mean 47%) crop and were 100% from feces. The percentage of SE positive samples at day 11 pi among the five white-egg layer (W1-W5) strains ranged from 40% to 100% (mean 84%) crop and 20% to 60% (mean 40%) feces. At day 11 pi the three brown-egg layer (Br6-Br8) strains ranged from 20% to 80% (mean 47%) crop and 60% to 80% (mean 73%) feces SE culture positive. Detection of SE prevalence at day 18 pi ranged from 20% to 80% (mean 48%) crop

and 20% to 60% (mean 32%) feces among W1-W5 strains. At day 18 pi, SE prevalence among Br6-Br8 strains was 0% to 40% (mean 20%) crop and 40% to 100% (mean 73%) feces. The SE culture positive samples at day 25 pi among the W1-W5 strains ranged from 0% to 20% (mean 12%) crop and 0% to 20% (mean 8%) feces. The Br6-Br8 strains had 0% SE recovered from crop lavage samples at day 25 pi, and fecal shedding of SE was 0%, 80% and 40% (mean 40%) among Br6, Br7 and Br8 strains, respectively. Comparison of trial 1 (SPF WL) and trial 2 (W1-W5, Br6-Br8) culture results from crop lavage samples and feces revealed that the average percentages of SE positive samples were highest at the earlier time points following SE challenge (**Table 3.2**). As time lengthened post-SE challenge, then a progressive decline in percentage of SE positive samples occurred. However, SE infection persisted within the crop through days 25-27 pi for SPF WL, W1, W2 and W3 hen strains. Shedding of SE in feces continued through day 25 pi for hen strains W1, W2, Br7 and Br8.

Crop SE-LPS-specific IgA.

Enzyme-linked immunosorbent assays (ELISA) detected an increased SE-lipopolysaccharide (LPS)-specific IgA response in crop lavage supernatant samples from trial 1 and trial 2 post-SE-challenged hens. Crop SE-LPS-specific IgA responses over time for SPF WL hens in trial 1 and each of eight commercial strains W1-W5, Br6-Br8 in trial 2 are depicted in **Figure 3.1**. The humoral immune response detected post-SE-challenge within the crop of trial 1 and trial 2 hens was of the same relative pattern. An IgA humoral immune response against SE-LPS appeared within the crop following SE oral challenge and SE-LPS IgA remained elevated for duration of the experiment. Prior to challenge at day 0, the SE-LPS-specific IgA levels within the crop were minimal. A slight increase in SE-LPS-specific IgA response was observed between day 0 and days 5-6 pi. By 11-12 days pi, the SE-LPS-specific IgA response in the crop increased

dramatically. Statistically significant differences ($p < 0.05$) from day 0 were observed for crop SE-LPS-specific IgA at 11-12 days pi (**Figure 3.1**). The SE-LPS-specific IgA response remained elevated greater than day 0 baseline at days 18-19 pi and days 25-27 pi. The humoral response within the crop post-SE infection was similar for the SPF WL hens and the eight commercial egg-layer hen strains (**Figure 3.1**). There were no statistically significant differences ($p > 0.05$) between the egg-layer hen strains (SPF WL hens versus commercial hen strains) at the post-SE infection time points, as determined by statistical analysis using ANOVA and Tukey's multiple comparison post-test.

Crop Histology & Lymphoid Tissue Scores.

Illustrations of the various degrees of lymphoid tissue and/or lymphocytic infiltration observed in the crop, and the criteria used for establishment of the score 0 to 5 grade classifications, are presented in **Table 3.1**. The ascending 0 to 5 numerical score scale corresponded to lymphoid tissue that progressively increased in size, structural detail, and cellular density. A score of 3 to 5 denoted a well-demarcated, organized lymphoid-lymphocytic aggregation and/or individual lymphoid follicle of moderate to heightened cellularity and of increased size diameter. We felt that the score 3 to 5 lymphoid tissue sites within the crop potentially reflected areas of enhanced antigenic stimulation and induction of an immune system inflammatory response (11, 19, 22, 23). Thus, crop lymphoid tissue sites in the score 3 to 5 range were those selected to make comparisons among the day 34 pi SE-challenged hens and uninfected control hens. **Table 3.3** shows the trial 1 and trial 2 day 34 post-SE-challenged groups' and control groups': semi-quantitative count distribution for crop lymphoid areas in the score categories 3, 4 and 5; total number count of crop lymphoid areas within score 3-5 range; calculated mean (average) number

of the score 3-5 range crop lymphoid tissue areas; and ratio of SE day 34 pi to control crop lymphoid tissue.

The H&E crop sections from uninfected control groups in trial 1 and trial 2 contained lymphoid tissue areas that were judged as score 3, 4 and 5. The total number of score 3-5 range lymphoid areas in the control groups ranked from a low count of five to a high of fifteen. Examination of H&E crop sections from day 34 post-SE-challenged hen groups found an increased number of score 3 to 5 lymphoid tissue areas in the SE-challenged compared to uninfected controls. Crop sections from day 34 pi SE-challenged groups had total number counts ranked at eighteen low to thirty-six high for lymphoid areas within score range 3-5. **Figure 3.2** compares the mean number of score 3-5 range crop lymphoid tissue areas assessed for the day 34 pi SE-challenged group versus uninfected control group from each hen strain. Averages of crop lymphoid tissue among the day 34 pi SE-challenged groups were greater than the uninfected control groups for trial 1 SPF WL hens and each of the eight commercial strains in trial 2. The SPF WL trial 1 hens and trial 2 commercial W1-W5, Br6-Br8 hen strains had 1.2 to 4.0-fold increased ratio of day 34 pi SE-challenged crop lymphoid tissue compared to uninfected controls (**Table 3.3**). An over-all increase of score 3-5 range isolated lymphoid follicles (ILFs)/lymphoid tissue areas in crop sections from day 34 pi SE-challenged groups as compared to control groups was seen for trial 1 SPF WL hens and the trial 2 hen strains W1-W5, Br6-Br8. ANOVA and Tukey's post-test determined no statistically significant differences ($p>0.05$) in the mean number of crop lymphoid tissue areas assessed at day 34 post-SE infection between SPF WL hens and the eight commercial egg-layer hen strains.

DISCUSSION

Experimental *Salmonella* Enteritidis (SE) challenge did induce a similar or comparable immune response in the crop post-SE infection among the SPF White Leghorn (SPF WL) hens and the eight commercial egg-layer strains (W1-W5, Br6-Br8). Results were similar in regards to kinetics of SE prevalence (**Table 3.2**), humoral SE-LPS IgA response (**Figure 3.1**), and lymphoid cellular infiltrate presence (**Figure 3.2**) within the crop. Trends observed for the SPF WL and commercial layer-hen strains were: higher percentages of SE culture positive crop lavage and fecal samples detected at early time points post-challenge with gradual decline in percentage of SE detected over time post-infection (**Table 3.2**); initial crop lavage samples with minimal to low SE-LPS-specific IgA then elevated IgA antibody responses specific against SE-LPS antigen at 11-12 days post-SE infection (pi) to 25-27 days pi (**Figure 3.1**); and increased lymphoid tissue evaluated in crop sections from SE-challenged hens versus uninfected controls (**Table 3.3** and **Figure 3.2**). Data obtained from this study and other SE-crop immunity studies conducted by Seo *et al.*(28, 29) and Holt *et al.* (18) that involved the use of specific pathogen free (SPF) White Leghorn (WL) chickens as the animal model do appear applicable to commercial egg-layer strains of chickens.

All of the SE-challenged hen groups (SPF WL, W1-W5, Br6-Br8) showed elevated IgA against SE-LPS antigen in the crop lavage samples that were collected at days 5-6 post-infection (pi) through days 25-27 pi (**Figure 3.1**). The increased SE-LPS-specific IgA response detected in crop lavage samples at days 5-6 pi and days 11-12 pi correlated with bacteriological examination time points in which the highest percentages of SE culture positive crop lavage samples were observed (**Table 3.2**). The concomitant findings of viable SE, SE-LPS-specific IgA, and lymphoid tissue within the crop organ seem to support the ability of the crop to actively

produce a humoral immune response when exposed to the pathogen. However, it is not conclusive that the generation of SE-specific IgA occurred locally within the crop, therefore future studies are planned to try to identify and recover SE-antigen specific B-lymphocytes from the crop of SE-infected chickens.

The prevalence of *Salmonella* Enteritidis within the crop did decline over time post-SE inoculation, which may have been a result of an enhanced humoral immune response. However, the humoral IgA response found within the crop may not have been capable of providing complete protection against SE colonization nor have the ability to fully combat SE infection of the alimentary tract, as complete clearance of SE from the crop and feces of all the experimentally infected hens did not occur. At days 25-27 pi, *Salmonella* Enteritidis survived and persisted within the crop (25% SPF WL; 20% W1; 20% W2; 20% W3) and feces (20% W1; 20% W2; 80% Br7; 40% Br8) of a few of the experimentally challenged hens despite the moderate to heightened SE-specific IgA response detected within the crop (**Table 3.2** and **Figure 3.1**). A slightly more similar outcome in regards to SE prevalence within the crop of SPF WL and commercial white-egg layer strains, as compared to SPF WL and brown-egg layer hen strains, might perhaps be linked to genetics. The SPF WL hens and commercial white-egg layer hen strains used for this study could have perhaps had a close genetic relatedness. A shared ancestral linkage between the SPF WL and commercial white-egg layer strains to that of White Leghorn layer might have perhaps been a factor that influenced mucosal immune response and persistence/clearance of the SE pathogen. A genetic divergence of the brown-egg layer strains away from White Leghorn to perhaps incorporation of broiler stock lineage might have contributed to variation of SE persistence in the crop between the SPF WL and commercial

brown-egg layer strains. Genetic variation of poultry breeds can influence immunocompetence and resistance to infection (21, 26).

In the present study, specific pathogen free (SPF) White Leghorn (WL) chickens and commercial egg-layer hens exhibited a comparable SE-LPS-specific IgA response (**Figure 3.1**) and presence of lymphoid tissue (**Figure 3.2**) within the crop, thus we feel confident to advance with mucosal immunology experiments using SPF WL chickens as the animal model.

Additional SE infection studies over an extended time period will be performed to assess long-term temporal dynamics of the humoral immune response against SE within the crop. Future research will be conducted to characterize the cellular nature of lymphoid aggregations observed in the crop post-SE infection. Flow cytometry and/or immunohistochemical (IHC) staining methods will be employed in an effort to identify the cellular repertoire of the crop, and determine if cellular populations (i.e., B-lymphocytes and plasma cells) exist that are capable of generating the mucosal humoral immune response locally within the crop organ.

ACKNOWLEDGEMENTS

The authors would like to thank Mrs. Joyce Jacks for her technical help with the animal experiments. We thank the North Carolina Department of Agriculture and Consumer Services, Piedmont Research Station, Poultry Unit for providing the commercial strains of chickens.

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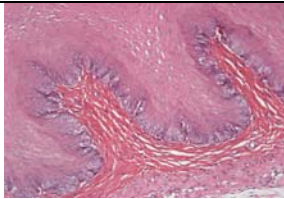
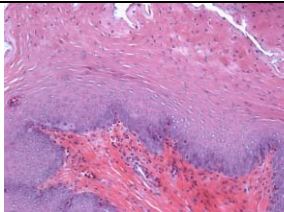
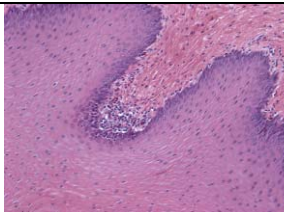
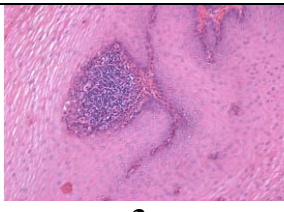
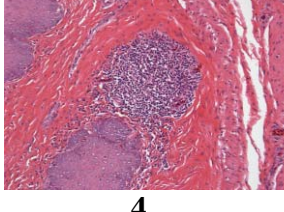
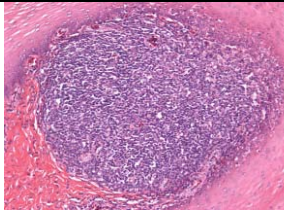
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Table 3.1. Interpretive illustrations for the score 0 to 5 crop lymphoid tissue grade scale

Score 0 to 5 Classification of Lymphoid Tissue Areas in Chicken Crop			
<u>SCORE</u>	^{a b} Size Criteria:	^a Morphologic/Cellular Criteria:	Photomicrographs:
0	0 to < 1 grid square	Absence of inflammatory cell infiltrate. (Lymphocytic-lymphoid cells within normal limits for resident population in epithelium and lamina propria of gastrointestinal tract.)	 0
1	1 x 1 grid square; ~50 µm diameter	Locally diffuse area of lymphocytic-lymphoid cell infiltration; low cellular density.	 1
2	2 x 2 grid squares; >50 to 100 µm diameter	Focal coalescence of lymphocytic-lymphoid cells; low to slightly mild-moderate cellular density.	 2
3	3 x 3 grid squares; >100 to 150 µm diameter	Defined focal aggregation of lymphocytic-lymphoid cells; moderate to heightened cellularity.	 3
4	4 x 4 grid squares; >150 to 200 µm diameter	Well-demarcated, circular-ellipsoidal focal area of lymphocytic-lymphoid cells; hypercellular; organized aggregation.	 4
5	5 x 5 grid squares or larger; >200 to 250+µm diameter	Sharply defined, focal area of lymphocytic-lymphoid cells; extreme hypercellularity; organized aggregation; locally expansive, may extend from lamina propria into basal epithelium.	 5

^a Light microscopy examinations, morphometric analyses and score 0 to 5 grading of lymphoid tissue sites were conducted at 200X magnification with aid of ocular grid reticle (1 grid square=50 µm length x 50 µm width).

^b Size of lymphoid area was established based on measurements of the diameter at widest point across the lymphoid structure and/or the number of grid squares (length x width) that defined entire area of involvement.

Table 3.2. Recovery of *Salmonella* Enteritidis (SE) from crop lavage samples and feces of specific pathogen free White Leghorn (SPF WL) hens and eight commercial layer strains of chickens (white-egg layer, W1-W5 and brown-egg layer, Br6-Br8).

Hen Strain:	<i>Salmonella</i> Enteritidis positive/total ^A									
	day 0		5-6 days		11-12 days		18-19 days		25-27 days	
	pre-infection		post-infection		post-infection		post-infection		post-infection	
	crop	feces	crop	feces	crop	feces	crop	feces	crop	feces
SPF WL	0/12	0/12	12/12	no data	5/12	no data	6/12	no data	3/12	no data
W1	0/5	0/5	4/5	4/5	2/5	1/5	3/5	3/5	1/5	1/5
W2	0/5	0/5	4/5	5/5	4/5	3/5	1/5	1/5	1/5	1/5
W3	0/5	0/5	3/5	5/5	5/5	2/5	2/5	1/5	1/5	0/5
W4	0/5	0/5	5/5	5/5	5/5	3/5	4/5	1/5	0/5	0/5
W5	0/5	0/5	5/5	4/5	5/5	1/5	2/5	2/5	0/5	0/5
Br6	0/5	0/5	2/5	5/5	2/5	3/5	1/5	2/5	0/5	0/5
Br7	0/5	0/5	4/5	5/5	4/5	4/5	2/5	4/5	0/5	4/5
Br8	0/5	0/5	1/5	5/5	1/5	4/5	0/5	5/5	0/5	2/5
Total	0/52 a	0/52 a	40/52 b	38/40 b	33/52 b	21/40 b	21/52 b	19/40 b	6/52 a	8/40 b

^A Totals in bottom row with different lowercase letters are significantly different ($P < 0.05$) from day 0 pre-infection.

Table 3.3. Numerical data for lymphoid tissue areas graded as score 3-5 that were observed in H&E stained crop sections from *Salmonella* Enteritidis challenged (SE day 34 pi) group and uninfected (control) group per each of the different hen strains.

Strain	Group	Crop Lymphoid Tissue Counts				Lymphoid Tissue Ratios	
		Score 3	Score 4	Score 5	=total #	=mean #	(SE day 34 pi /Control)
SPF WL	SE day 34 pi (n=12)	16	11	9	36	3.0	3.3
	Control (n=8)	1	5	1	7	0.9	
W1	SE day 34 pi (n=5)	11	6	14	31	6.2	1.2
	Control (n=3)	4	4	7	15	5.0	
W2	SE day 34 pi (n=5)	10	8	5	23	4.6	1.3
	Control (n=4)	6	2	6	14	3.5	
W3	SE day 34 pi (n=5)	5	7	6	18	3.6	1.4
	Control (n=2)	2	1	2	5	2.5	
W4	SE day 34 pi (n=5)	15	8	11	34	6.8	4.0
	Control (n=3)	2	1	2	5	1.7	
W5	SE day 34 pi (n=5)	8	7	7	22	4.4	1.7
	Control (n=5)	2	3	8	13	2.6	
Br6	SE day 34 pi (n=5)	12	3	15	30	6.0	1.5
	Control (n=2)	2	2	4	8	4.0	
Br7	SE day 34 pi (n=5)	9	6	8	23	4.6	2.3
	Control (n=1)	1	1	0	7	2.0	
Br8	SE day 34 pi (n=5)	9	6	11	26	5.2	1.6
	Control (n=3)	5	2	3	10	3.3	

n = number of crops sampled.

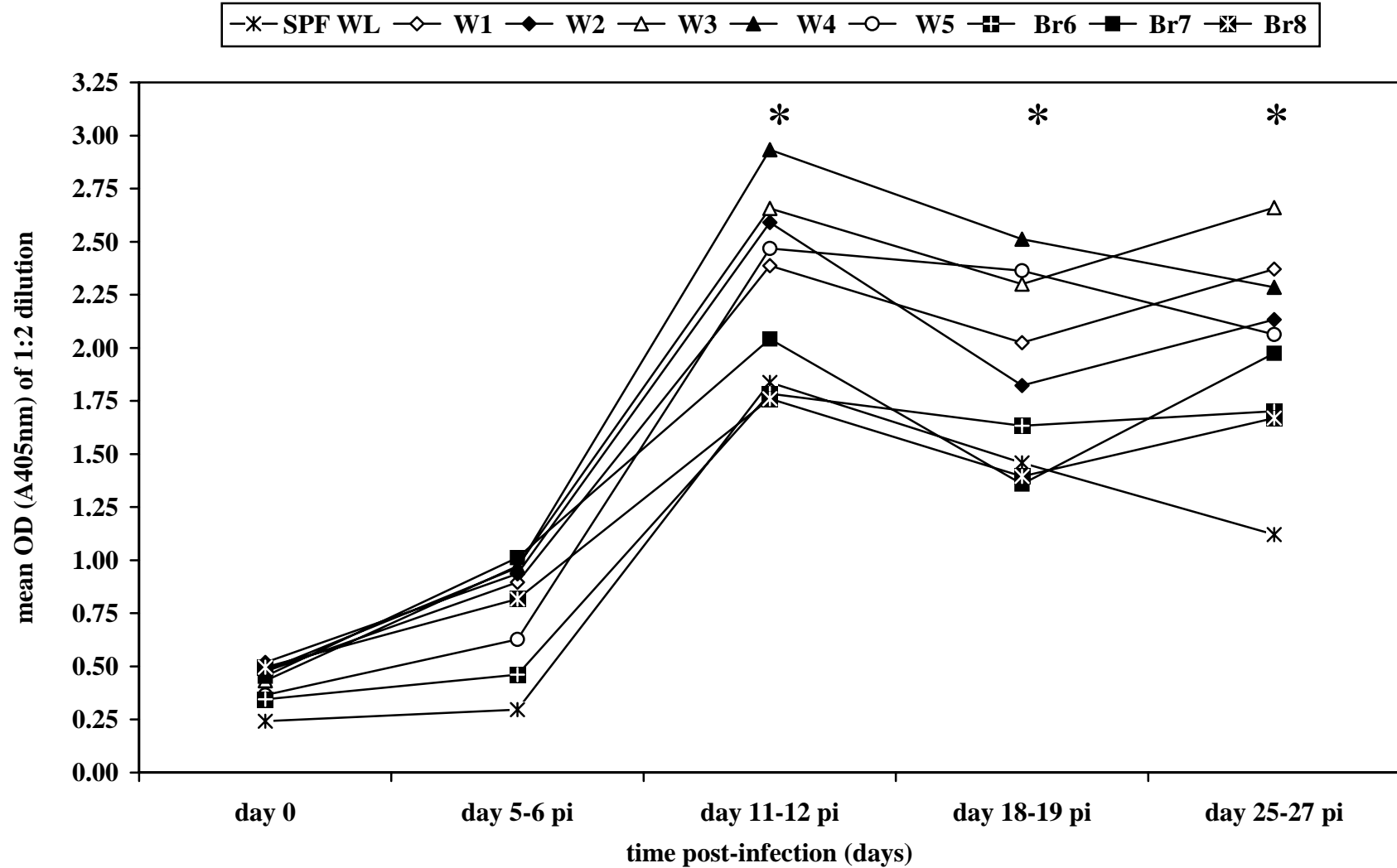


Figure 3.1. Enzyme-linked immunosorbent assay (ELISA) analyses of SE-LPS-specific IgA in crop lavage samples from specific pathogen free White Leghorn (SPF WL) hens and the eight commercial strains (W1-W5, Br6-Br8). Mean optical density (OD) values are representative of SE-LPS-specific IgA detected in 1:2 dilution crop lavage supernatant samples at pre-challenge (day 0) and post-*Salmonella* Enteritidis (SE) infection. * Data significantly different ($P < 0.05$) from pre-challenge day 0.

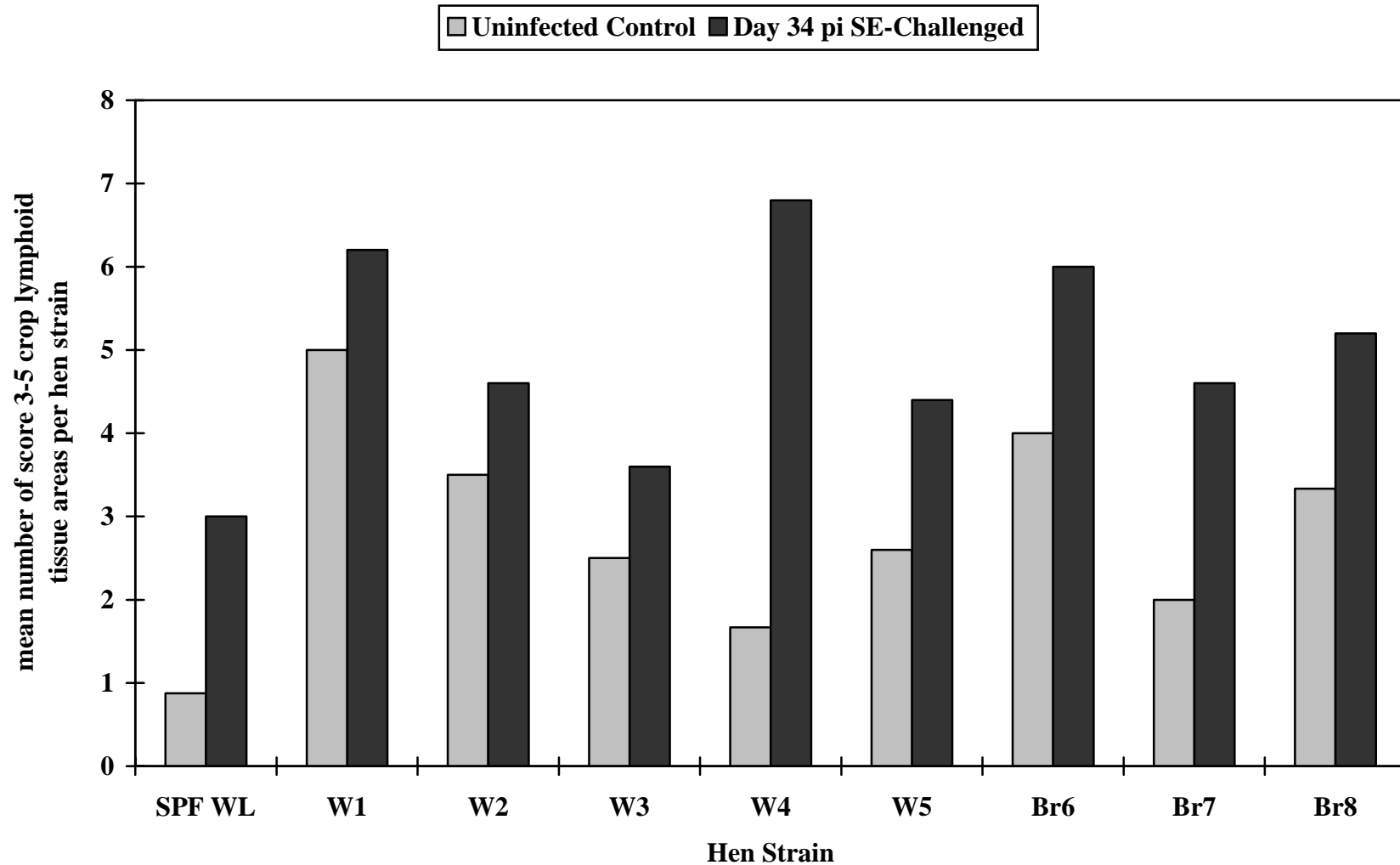


Figure 3.2. Average number of score 3-5 range lymphoid tissue areas counted in hematoxylin-and-eosin (H&E) stained crop sections prepared from uninfected control group and day 34 post-infection (pi) *Salmonella* Enteritidis (SE) challenged group for each of the hen strains. SPF WL=specific pathogen free White Leghorn; W1-W5=commercial strains, white-egg layer; Br6-Br8=commercial strains, brown-egg layer.

CHAPTER 4**PRIMARY AND SECONDARY IMMUNE RESPONSE IN THE CROP OF SPECIFIC
PATHOGEN FREE WHITE LEGHORN CHICKENS AFTER INFECTION
WITH *SALMONELLA* ENTERITIDIS ¹**

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ABSTRACT

Primary and secondary crop immune responses against *Salmonella* Enteritidis (SE) in specific pathogen free (SPF) White Leghorn (WL) chickens were investigated. Birds were challenged *per os* with 10^8 CFU/ml *Salmonella* Enteritidis (SE nalR PT13) at 13 weeks of age for primary SE-infection and then at 24 weeks of age for secondary SE-infection. Crop lavage samples were collected at bi-weekly intervals from day 0 (pre-infection) through 11 weeks post-primary infection (pi1) to evaluate primary immune response, then from 1 week post-secondary infection (pi2) through 9 weeks pi2 to investigate anamnestic secondary immune response. Crop lavage samples were analyzed for SE-lipopolysaccharide (LPS)-specific IgA by ELISA. Bacteriological examination was performed on feces and/or cecal contents at bi-weekly intervals to monitor progression of SE infection. H&E stained slides of crop sections prepared from 1 week pi1 to 11 weeks pi1 and from 1 week pi2 to 9 weeks pi2, along with uninfected controls, were assessed for lymphoid tissue via light microscopy. Lymphoid areas were graded using the score 0 (minimal) to 5 (sizable) scale. Results revealed 100% SE positive fecal and cecum samples cultured at 1 week pi1 and a progressive decline in percentage SE positive samples over time with samples negative by 7 weeks pi1 to 11 weeks pi1. Following secondary infection, SE was detectable in feces and cecum samples at 1 week pi2 through 9 weeks pi2. Crop SE-LPS-specific IgA markedly increased between day 0 to 3 weeks pi1, and remained elevated above baseline through 11 weeks pi1. IgA humoral immune response after secondary infection was increased at 1 week pi2, however, the values at 3 weeks pi2 through 9 weeks pi2 were lower than those of the post-primary infection (pi1) time points. Score 3 to 5 isolated lymphoid follicles were observed in crop tissue sections, with approximately 2 times increase in ratio of lymphoid tissue in post-SE-infected versus uninfected controls.

Key words:

Salmonella Enteritidis,
Chicken,
Crop,
Mucosal immune system,
Primary response,
Secondary response,
IgA,
Gut-associated lymphoid tissue

Abbreviations:

ARS = Agricultural Research Service;
BSL-2 = biosafety level-2;
BG = brilliant green agar
CFU = colony-forming units;
CO₂ = carbon dioxide;
ELISA = enzyme-linked immunosorbent assay;
GALT = gut-associated lymphoid tissue;
H&E = hematoxylin and eosin;
iBALT = inducible bronchial-associated lymphoid tissue;
IgA = immunoglobulin A;
ILF = isolated lymphoid follicle;
LPS = lipopolysaccharide;

nalR = nalidixic-acid resistant;

NA = nalidixic-acid;

NB = novobiocin;

OD = optical density;

PT13 = phage type 13;

PBS = phosphate-buffered saline;

pi = post-infection;

pi1= post-primary infection;

pi2= post-secondary infection;

PMN = polymorphonuclear cells;

RV = Rappaport-Vassiliadis;

SE = *Salmonella* Enteritidis;

SEPRL = Southeast Poultry Research Laboratory;

SPF = specific pathogen free;

TSB = tryptic soy broth;

USDA= United States Department of Agriculture;

WL = White Leghorn;

INTRODUCTION

The chicken crop, ingluvies, appears to have the functional capacity to produce a mucosal immune response against *Salmonella* (12, 15, 32, 33). Seo *et al.* revealed a humoral immune response within the crop against *Salmonella* Enteritidis (SE) when specific pathogen free (SPF) White Leghorn (WL) hens were experimentally challenged with SE via oral gavage (32, 33). Enzyme-linked immunosorbent assay (ELISA) detected elevated levels of SE-specific IgA in crop lavage samples collected at 1 week post-infection (pi) through 4 weeks pi (12, 14, 15, 32, 33). Microscopic examination by Holt *et al.* of hematoxylin and eosin (H&E) stained crop tissues from post-SE-challenged hens showed lymphoid aggregations/lymphoid follicles in the crop lamina propria (15). Thus, the crop may be an organ of importance in the study of gut-associated lymphoid tissue and mucosal immunity.

More knowledge about the behavior of SE in relation to the chicken crop and the mucosal immune system is needed so that more effective control measures against SE infection in poultry can be established. Mucosal immunity in the chicken is still poorly understood, and a better comprehension of long-term mucosal immune response is necessary to enable development of methodologies for generating protective immunity against SE. Previous studies have evaluated the crop IgA humoral response against SE-lipopolysaccharide (LPS) and/or SE-flagella antigens to the time point of 5 weeks pi when detectable SE fecal shedding tends to decline or abate (32, 33). In the present study, research was undertaken to analyze long-term humoral immune response in the chicken crop after primary and secondary *Salmonella* Enteritidis (SE) infection. SE-LPS-specific IgA in the crop was monitored through 11 weeks post-primary infection. A secondary *per os* SE-infection was administered to investigate if an anamnestic immune response would be elicited in the crop. Objectives were: to monitor kinetics of SE fecal shedding and SE

persistence in cecum; to compare humoral SE-specific IgA response in the crop after primary and secondary SE exposure; and to assess crop sections for isolated lymphoid follicles (ILF) in primary and secondary SE-infected birds versus age-matched uninfected control birds.

MATERIALS & METHODS

Animals.

The experiment was conducted with 140 specific pathogen free (SPF) White Leghorn (WL) chickens, 13 weeks of age, originating from a flock maintained by Southeast Poultry Research Laboratory (SEPRL), USDA-ARS, Athens, Georgia. Birds were housed within individual layer cages located within a climate controlled biosafety level-2 (BSL-2) building. Birds were allowed access to antibiotic-free feed and water *ad libitum*.

Bacterial strains.

Salmonella serovar Enteritidis (SE) phage type (PT) 13 was selected for experimental challenge of birds. A frozen stock maintained at -70°C of nalidixic-acid resistant (nalR) *Salmonella* serovar Enteritidis phage type 13 (strain SE89-8312) was thawed and cultured on nutrient agar at 37°C for 18 hours. Isolated colonies were selected and cultivated in 10 ml tryptic soy broth (TSB) at 37°C for 18 hours. Broth culture was then diluted in sterile physiological saline to prepare 10⁸ colony-forming units (CFU) /ml inoculum for experimental infection of birds.

Experimental design.

Studies conducted were approved by and in accordance with guidelines of the SEPRL Institutional Animal Care and Use Committee (IACUC). *Salmonella*-free status of chickens was determined before commencement of the experiment. From all 140 birds, a 1.0 gram weight of feces was collected and selectively enriched in Rappaport-Vassiliadis (RV) broth for 24 hours at

37°C-42°C then plated onto brilliant green (BG) agar containing 20 µg/ml novobiocin (NB).

Salmonella was not detectable in fecal cultures.

At 13 weeks of age, the 140 chickens were separated into two groups, experimental SE infection and control. Ninety-six chickens (eight for trial 1 and eighty-eight for trial 2) represented the experimental SE infection group. Forty-four chickens served as the age-matched uninfected control group. On day 0 pre-infection, eight SPF White Leghorn chickens for both trial 1 and trial 2 were crop lavaged to establish baseline crop IgA by enzyme-linked immunosorbent assay (ELISA). Eight chickens for trial 2 were euthanized via carbon dioxide (CO₂) gas inhalation to collect the crop organ for histopathological evaluation and assessment of lymphoid tissue presence at day 0 pre-infection.

Chickens of the experimental SE-infection group for trial 1 and trial 2 were challenged *per os* with 1 ml of 10⁸ CFU/ml SE nalR PT13 at 13 weeks of age for primary-infection then the group was re-challenged at 24 weeks of age for secondary-infection. For trial 1, in an effort to monitor persistence of SE infection and crop humoral immune response over time, eight SE-infected birds were repeatedly sampled at bi-weekly time points following primary infection and post-secondary SE re-infection. Feces and crop lavage samples in trial 1 were successively collected from the same eight SE-infected birds for the duration of the experiment. For trial 2, crop lavage samples, cecal contents and crop tissues were collected from eight SE-infected birds, along with four uninfected age-matched control birds, at bi-weekly intervals: 1 week post-primary infection (pi1), 3 weeks pi1, 5 weeks pi1, 7 weeks pi1, 9 weeks pi1 and 11 weeks pi1. At 24 weeks of age the SE-infection group was re-challenged with SE, then eight SE-infected birds along with four uninfected control birds continued to be sampled at bi-weekly intervals: 1 week post-secondary infection (pi2), 3 weeks pi2, 5 weeks pi2, 7 weeks pi2 and 9 weeks pi2.

Crop lavage sample collection.

Crop lavages in trial 1 and trial 2 were performed according to the protocol designed by Holt *et al.* (15). The crop lavage devices were fashioned using 10 ml syringes and attached tubing 7-10 inches (17.8-25.4 cm) in length with the distal 1.5 inches (3.8 cm) fenestrated. The lavage devices were aseptically filled with 5 ml of room temperature, sterile glycine flush solution, pH 7.8-8.0 (15, 17). Birds were held in an upright, vertical position with neck extended to facilitate insertion of the lavage device tubing into the esophagus. Once the fenestrated portion of tubing was positioned within the crop, the 5 ml glycine solution was slowly infused. Immediately a fluid sample from the crop was aspirated back into the syringe and then the crop lavage sample (aspirate) was dispensed from syringe to a sterile collection container. The lavage procedure was repeated per individual bird using a new lavage device and separate sample collection container. A 1.5-2.0 ml portion from each neat crop lavage sample was centrifuged at 10,000 rpm for 5 minutes to remove feed particulate debris and excess mucous. The supernatant fraction was collected and stored at -20°C .

Bacteriological examination.

Appropriate techniques and media for isolation of salmonellae were utilized for bacteriological examination of feces and cecum samples collected post-SE infection (4, 6, 35, 38, 39). Bacterial culture for SE naIR PT13 was performed on feces from trial 1 and cecal contents from trial 2. Feces and cecum samples were collected at bi-weekly time points: 1 week post-primary infection (pi1), 3 weeks pi1, 5 weeks pi1, 7 weeks pi1, 9 weeks pi1 and 11 weeks pi1. Following secondary SE re-infection, bacterial culture continued to be conducted on samples harvested biweekly at: 1 week post-secondary infection (pi2), 3 weeks pi2, 5 weeks pi2, 7 weeks pi2 and 9 weeks pi2. In trial 1, feces were obtained by placement of sterile catch trays under each bird. A

1.0 gram portion of each fecal sample was added to 9 ml Rappaport-Vassiliadis (RV) broth to prepare 1:10 dilution, then 100 μ l volumes were directly spread plated onto brilliant green (BG) agar containing novobiocin (NB, 20 μ g/ml) and nalidixic-acid (NA, 20 μ g/ml). For trial 2, one cecum per bird was aseptically harvested postmortem. Cecum samples of 1.0 gram weight were placed into sterile whirl-pack bags (Fisher Scientific, Suwanee, GA, USA), RV broth added at 9x volume/tissue weight and ceca were macerated in a stomacher for 30 seconds to evacuate cecal contents. A 100 μ l portion from each macerated cecum sample was directly spread plated onto brilliant green (BG) agar containing novobiocin (NB, 20 μ g/ml) and nalidixic-acid (NA, 20 μ g/ml). The BG+NB+NA direct spread plates and RV samples were incubated at 37°C overnight. Plates were then assessed for SE colony growth. In instances where direct plating resulted in no growth, the RV-enriched samples were streak plated on BG+NB+NA agar and streak plates were incubated 24 hours at 37°C then assessed for typical SE colony growth. Questionable colonies were further differentiated based on biochemical tests using triple sugar iron (TSI) agar and modified-lysine iron (MLI) agar slant tubes, and agglutination reaction with antiserum against *Salmonella* serogroup D₁ somatic antigen (Beckton Dickinson-Difco, Bedford, MA, USA). Feces and cecum samples were recorded as positive if SE was recoverable either by direct plating or after selective RV-enrichment. SE enumerations were performed and reported as colony-forming units (CFU)/ml. Samples with no growth on direct plating but positive in RV-enrichment were assigned the arbitrary count of 50 CFU/ml (half of the theoretical detection limit). Negative samples, those with no SE growth detectable either by direct plating or in RV-enrichment, were given a count of 0. SE concentrations in transformed metric (logarithmic CFU/ml) were used to calculate the mean \log_{10} CFU/ml at each biweekly interval.

Crop SE-specific IgA ELISA.

The trial 1 and trial 2 crop lavage samples obtained at bi-weekly time points following primary and secondary SE-infection were analyzed by enzyme-linked immunosorbent assay (ELISA) for SE-lipopolysaccharide (LPS)-specific IgA as previously described by Holt *et al.* (13-15). For assay, the frozen crop supernatant samples were thawed, diluted 1:1 with phosphate buffered saline (PBS) containing 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA), and then 2-fold serial dilutions were prepared. Sample dilutions 1:2 to 1:128 were added to flat-bottomed 96-well polystyrene ELISA plates (Corning-Costar Inc., Wilkes Barre, PA, USA) to which SE-LPS antigen (Sigma-Aldrich) at concentration of 10 µg/ml had been adsorbed. Additionally, previously defined crop lavage samples with strong and weak SE-LPS-specific IgA antibody responses were run on each assay plate as positive and negative controls, respectively. Plates containing samples and controls were incubated 60 minutes at room-temperature then washed three times with PBS containing 0.05% Tween 20. The next two sequential steps performed were the application of primary antibody with 60 minutes incubation time followed by three washes, then addition of an enzyme-conjugated secondary antibody with protocol repeated for incubation time and washes. The primary antibody for detection of SE-LPS-specific IgA in crop lavage samples was a monoclonal mouse anti-chicken IgA (Southern Biotech, Birmingham, AL, USA) diluted 1:1000 in PBS containing 0.05% Tween 20 and 1% bovine serum albumin (BSA). Goat anti-mouse IgG with alkaline phosphatase conjugate (Calbiochem, La Jolla, CA, USA) diluted 1:1000 served as the enzyme conjugated secondary antibody. Diethanolamine buffer with concentration 1 mg/ml p-nitrophenyl-phosphate (Sigma-Aldrich) was applied to plate wells as substrate solution and the chromogenic reaction proceeded for 15 to 30 minutes at room-temperature in the dark. Colorimetric analysis with Multiskan Ascent (Thermo Fisher Scientific,

Milford, MA, USA) microplate photometer at 405 nm wavelength yielded absorbance/optical density (OD) values.

Crop Histology & Lymphoid Tissue Scoring.

In trial 2, eight of the SE-infected birds, along with four negative control birds, were euthanatized at bi-weekly time points via carbon dioxide (CO₂) gas inhalation in accordance with guidelines established by USDA, Institutional Animal Care and Use Committee (IACUC). Immediately postmortem the entire crop with 1.0 inch (2.54 cm) of attached esophagus was excised. Tissues were fixed intact in 10% neutral buffered formalin for 24 hours and the crops were then sectioned. Transverse cuts were made at the mid-body region of the crop. The plane of the cut passed through the entirety of the hollow-bodied crop organ to produce ring-shaped circular sections. Crop tissue sections were routinely processed, paraffin embedded, microtome cut, and affixed to glass slides. Tissue slides were stained with hematoxylin and eosin (H&E) then examined via light microscopy. The H&E crop tissues were evaluated full-circle for the presence of inflammatory changes and lymphoid aggregations in the epithelium and lamina propria. An ocular grid reticle (Fisher Scientific) was used to grade lymphoid areas as score 0 to 5 and perform counts at 200X magnification. The score 0 (minimal) to score 5 (sizable) numerical grade scale established by Vaughn, *et al.* was used to score apparent sites of lymphocytic-lymphoid cell infiltrates and/or isolated lymphoid follicles (ILF) in crop tissue sections (36). Scores of 3 to 5 denoted well-demarcated, organized lymphoid-lymphocytic aggregations and/or individual lymphoid follicles of moderate to heightened cellularity and of increased size diameter. The score 3 to 5 lymphoid tissue sites within the crop potentially reflected areas of enhanced antigenic stimulation and induction of an immune system inflammatory response, thus crop lymphoid tissue sites in the score 3 to 5 range were those

selected to make comparisons between post-primary SE-infected and post-secondary SE infected birds, and among SE-infected and uninfected age-matched control birds (9, 22, 23).

Statistical Analyses.

Statistical analyses were performed using GraphPad InStat (GraphPad Software, Inc., San Diego, CA, USA). Levels of *Salmonella* Enteritidis/ml detected in feces and cecum samples were log₁₀ transformed and means (mean log₁₀ CFU/ml) were calculated. Analysis of variance (ANOVA) with Tukey's multiple comparison test and Dunnett's multiple comparison test procedures were conducted to determine statistically significant differences ($p < 0.05$) between post-primary infection and post-secondary infection time points and between day 0/uninfected controls and post-SE-infection time points, respectively, for bacterial culture results and enzyme-linked immunosorbent assay (ELISA) data. Unpaired t-tests were performed on crop lymphoid tissue score data to assess whether or not age-matched uninfected controls and SE-infected bird groups differed significantly.

RESULTS

Bacterial Culture.

Figure 4.1 and **Figure 4.2** represent the temporal kinetics of SE prevalence (SE/ml enumerations and percentage SE culture positive bird samples) in bi-weekly trial 1 fecal samples and trial 2 cecal contents cultured from SPF White Leghorn birds following primary and secondary SE-infection. The SE levels detected from feces and ceca were significantly increased ($p < 0.001$) at 1 week pi1 as compared to the SE levels at subsequent post-SE infection time points. At 1 week post-primary infection (pi1), levels of SE were 5.141 mean log₁₀ CFU/ml from feces (**Figure 4.1**) and 5.008 mean log₁₀ CFU/ml from cecal contents (**Figure 4.2**). SE was recovered from

100% of fecal samples (**Figure 4.1**) and 100% of cecal contents (**Figure 4.2**) cultured from trial 1 and trial 2, respectively, at 1 week pi1.

The prevalence of *Salmonella* Enteritidis (SE) in feces collected from the trial 1 eight birds repeatedly sampled at post-primary SE-infection intervals was: 100% at 1 week pi1; 38% at 3 weeks pi1; and then SE was not detectable by culture at 5 weeks pi1, 7 weeks pi1, 9 weeks pi1 and 11 weeks pi1 (**Figure 4.1**). Recovery of SE from fecal culture after secondary SE re-infection was: 50% at 1 week post-secondary infection (pi2); 63% at 3 weeks pi2; 50% at 5 weeks pi2; 57% at 7 weeks pi2; and 25% at 9 weeks pi2 (**Figure 4.1**). SE culture positive feces were detected less frequently during post-primary infection (at two of six bi-weekly time points) compared to the post-secondary infection (at five of five bi-weekly time points).

Trial 2 cecum samples cultured after primary SE-infection had a *Salmonella* Enteritidis (SE) prevalence of: 100% at 1 week pi1; 63% at 3 weeks pi1; 13% at 5 weeks pi1; and then 0% at 7 weeks pi1, 9 weeks pi1 and 11 weeks pi1 (**Figure 4.2**). After secondary SE-infection the SE prevalence in cecum samples cultured was: 25% at 1 week post-secondary infection (pi2); 0% at 3 weeks pi2; 13% at 5 weeks pi2; 25% at 7 weeks pi2; and 38% at 9 weeks pi2 (**Figure 4.2**). Cecum samples from the uninfected age-matched control birds remained negative for SE throughout the experiment.

Crop SE-LPS-specific IgA.

Enzyme-linked immunosorbent assays (ELISA) demonstrated an increased SE-LPS-specific IgA response in crop lavage supernatant samples from the post-primary and post-secondary SE-infected birds. Crop SE-LPS-specific IgA responses over time are depicted in **Figure 4.3** for the trial 1 eight birds sampled repeatedly and in **Figure 4.4** for trial 2 different bird groups sampled at bi-weekly time points. Prior to infection at day 0, the SE-LPS-specific IgA levels were

minimal. SE-LPS-specific IgA was elevated greater than day 0 baseline after experimental SE challenge at all bi-weekly intervals (1 week pi1 to 11 weeks pi1 and 1 week pi2 to 9 weeks pi2). For the sampling time points nearest to SE challenge, a more exaggerated SE-LPS-specific IgA humoral response was noted, however, as time lengthened a progressive wane of SE-LPS-specific IgA in the crop occurred. A similar relative pattern was observed between post-primary infection (pi1) and post-secondary infection (pi2). The humoral immune response detected in the crop following SE infection increased at 1 week post-infection then over time the antibody response progressively declined.

A markedly increased SE-LPS-specific IgA response was observed between day 0 pre-infection to 3 weeks pi1. Following a secondary SE re-infection, crop SE-LPS IgA response at 1 week post-secondary infection (pi2) increased, however, values at the subsequent post-secondary infection (pi2) time intervals were lower than the SE-LPS IgA values of the post-primary infection (pi1) time points (**Figures 4.3 & 4.4**). The slightly diminished humoral response detected within the crop post-secondary infection (pi2) appeared to coincide with a reduced SE bacterial load detected in the feces (**Figures 4.1**) and cecal contents (**Figures 4.2**) following secondary re-infection.

Crop Histology & Lymphoid Tissue Scores.

The ascending 0 to 5 numerical score scale corresponded to lymphoid tissue that progressively increased in size, structural detail, and cellular density (36). The calculated mean (average) of score 3 to 5 range lymphoid tissue in crop sections from age-matched birds at bi-weekly time points from the trial 2 SE-infected chickens versus uninfected controls are shown in **Figure 4.5**. The H&E crop sections from uninfected control birds contained some lymphoid tissue areas that were judged as score 3, 4 and 5. However, examination of H&E crop sections from age-

matched SE-infected birds found an increased number of score 3 to 5 lymphoid tissue areas in the SE-infected compared to uninfected control birds of the same age (**Figure 4.5**). The increase of lymphoid tissue in crop sections from SE-infected as compared to controls was determined by comparison of the mean number of score 3-5 range isolated lymphoid follicles/lymphoid tissue areas. Crop sections from post-primary infection (pi1) compared to controls had 1.78-fold increase ratio of lymphoid tissue, and post-secondary infection (pi2) compared to controls had 1.99-fold increase ratio of crop lymphoid tissue. Overall, crop sections from all SE-infected birds as compared to all uninfected control birds had 1.87-fold increase of isolated lymphoid follicles in score 3-5 range.

Comparisons of the mean score 3 to 5 range crop lymphoid tissue areas in crop sections from post-primary infection (pi1) and post-secondary re-infection (pi2) chickens are given in **Figure 4.5**. No statistically significant differences ($p > 0.05$) in crop lymphoid tissue from primary SE infection (pi1) versus secondary SE re-infection (pi2) time points were determined. A greater incidence of heterophilic infiltrations within crop epithelium and lamina propria following the secondary SE-infection was noted with microscopy evaluation. Moderate to severe, focally extensive, heterophil polymorphonuclear cell (PMN) inflammation with concomitant epithelial necrosis was observed in 8 of 40 post-secondary SE-infection (pi2) crop sections as compared to 1 of 48 post-primary SE-infection (pi1) crop sections.

DISCUSSION

A humoral immune response within the crop of SPF White Leghorn (SPF WL) chickens was detected after primary infection and secondary re-infection with *Salmonella* Enteritidis (**Figures 4.3 & 4.4**). The increased SE-LPS-specific IgA response and presence of lymphoid tissue/isolated lymphoid follicles (ILF) within crop samples from SE-infected birds would

appear to demonstrate that a local mucosal immune response can be elicited in the chicken crop. The SE-infected bird groups showed elevated IgA against SE-LPS antigen in crop lavage samples that were collected at all bi-weekly intervals post-infection (pi). Increased SE-LPS-specific IgA responses in crop lavage samples correlated with time points when SE positive feces and cecum samples were detected by bacteriological culture (**Figures 4.1 & 4.2**). Higher SE-LPS IgA values, along with higher percentages of SE culture positive feces and cecum samples, were detected at similar time points following SE challenge. Crop lavage samples had elevated IgA antibody responses specific against SE-LPS antigen at 1 week pi following both the primary SE-infection and secondary SE re-infection (**Figures 4.3 & 4.4**). It appeared that ingestion of the SE pathogen resulted in selective induction of SE-LPS-specific IgA humoral response within the crop. Resurgence of SE-LPS-specific IgA antibody response at 1 week pi2 (**Figures 4.3 & 4.4**) after the second experimental bacterial re-exposure implied that recall response associated with SE antigen was induced. However, overall the humoral SE-LPS IgA response detected within the crop after primary SE-infection was slightly greater than the SE-LPS IgA response of the secondary SE re-infection (**Figures 4.3 & 4.4**). Perhaps reduced SE bacterial levels and/or diminished SE-antigen presence along the alimentary tract resulted in a slight reduction in crop IgA response.

The humoral IgA response within the crop may not be capable of providing complete protection against SE-infection of the gastrointestinal tract. After chickens were re-infected with *Salmonella* Enteritidis, the pathogen persisted within the ceca (**Figure 4.2**) and SE fecal shedding (**Figure 4.1**) occurred despite a moderate to heightened SE-specific IgA response within the crop following initial infection (**Figures 4.3 & 4.4**). Post-primary infection (pi1), SE was recovered from feces at 1 week pi1 through 3 weeks pi1, and then SE was not detected at 5

weeks pi1, 7 weeks pi1, 9 weeks pi1 and 11 weeks pi1. Post-secondary infection (pi2), viable SE was actively shed in feces at 1 week pi2 and SE fecal shedding persisted through 9 weeks pi2 (**Figure 4.1**). During primary infection (pi1), SE was recovered from cecal contents at 1 week pi1 through 5 weeks pi1 and then SE was not detected at 7 weeks pi1, 9 weeks pi1 and 11 weeks pi1. Following secondary infection (pi2), SE positive cecum samples occurred at 1 week pi2, but dropped to zero at 3 weeks pi2, and then SE reoccurred in cecal contents at 5 weeks pi2 and SE persisted through 9 weeks pi2 (**Figure 4.2**).

Mucosal lymphoid tissue of the alimentary tract, known as gut-associated lymphoid tissue (GALT), is equipped with a cellular repertoire necessary to sample luminal antigens and to produce humoral (i.e., secretory-IgA) and/or cell-mediated response against microorganisms at mucosal surfaces (16, 25). Exposure of the gut to enteroinvasive pathogenic bacteria may promote expansion of existent secondary gut-associated lymphoid tissues (GALT), and the genesis of tertiary lymphoid tissue (7). An increase of lymphoid tissue was evaluated in crop sections from SE-infected birds versus uninfected age-matched controls (**Figure 4.5**). The observation of a greater proportion of lymphoid tissue in the crop sections from SE-infected versus the uninfected age-matched control birds would appear to be related to the introduction of *Salmonella* Enteritidis and the antigenic stimulus evoked by the enteroinvasive pathogen, as opposed to an age-associated change (**Figure 4.5**). The lymphoid tissue identified in the chicken crop might perhaps be comparable to isolated lymphoid follicles of murine small intestine and/or inducible bronchial-associated lymphoid tissue (iBALT) that have been described by Hamada and Moyron-Quiroz, respectively (9, 26). The crop, situated within the upper gastrointestinal tract, would appear to be a prospective location for gut-associated lymphoid tissue and/or for tertiary lymphoid tissue to arise in response to bacterial pathogens harbored within the crop or to

agents that traverse the crop on descent to the lower alimentary tract. Well-defined lymphoid follicles were apparent in crop sections after primary SE-infection (pi1) and secondary SE re-infection (pi2), yet there were no statistically significant differences ($p>0.05$) in the mean number of crop lymphoid tissue sites/isolated lymphoid follicles (**Figure 4.5**).

It should be noted that within post-secondary SE-infection (pi2) crop sections, an increased incidence of heterophilic influx and epithelial damage was observed. Heterophil infiltration and concomitant epithelial necrosis was seen in 8 of 40 crop sections post-secondary SE-infection as compared to 1 of 48 post-primary SE-infection crop sections. Research by Parkos suggests that a functional consequence of polymorphonuclear (PMN) cell migration across intestinal epithelium is disruption of important barrier function, and that the magnitude of barrier dysfunction is related to PMN cell number migrating across the epithelium (28). Factors such as increased polymorphonuclear (PMN) cells, reactive enzymatic components, and/or cytokines/chemokines (i.e., interferon-gamma, tumor necrosis factor-alpha, interleukin-8, MIC-A/MIC-B) may contribute to damage of gastrointestinal tract epithelium and reduced structural integrity of the epithelial layer (1-3, 8, 20, 30). Break down of the GI mucosal barrier may result in a compromised first line of defense against enteric pathogens (18). In the present study, it is unknown if the heterophilic influx and epithelial necrosis within the crop were associated with secondary SE exposure, or if inflammatory changes were perhaps attributable to altered normal intestinal flora with subsequent proliferation of *Pseudomonas aeruginosa* capable of causing disease and sepsis, or due to unidentified agents (12, 24, 27). Oral re-infection with SE might have perhaps lead to the local inflammatory response and mucosal injury within the crop (5, 34), however, no definitive explanation can be offered in this study.

Within the crop, the precise roles of lymphoid cells (i.e., lymphocytes), myeloid cells (i.e., macrophages, heterophils), and epithelium remain to be determined, and the function of these cells may perhaps differ depending on primary exposure or secondary re-exposure to SE (10, 11, 19, 21, 29, 31, 37). Additional experiments are warranted to investigate variations in the avian mucosal immune response against SE. A more detailed understanding of how SE influences the host immune system would be important in determining if a protective immune response might be generated within the crop of poultry. Acquiring more knowledge about SE pathogenesis in the chicken might ultimately help to establish mucosal vaccine strategies that would perhaps yield long-term protective immunity against SE.

The notion that the chicken crop may have the ability to actively produce a humoral immune response locally appears to be supported by findings of a SE-LPS-specific IgA response and lymphoid tissue development within the crop after oral SE exposure. However, further studies need to be conducted to characterize cellular populations of the crop isolated lymphoid follicles (ILFs)/lymphoid tissue. General histochemical and immunohistochemical (IHC) staining techniques will be employed in an attempt to identify cellular components (i.e., B-lymphocytes and plasma cells) that have the capacity to generate a humoral response locally within the crop following SE infection.

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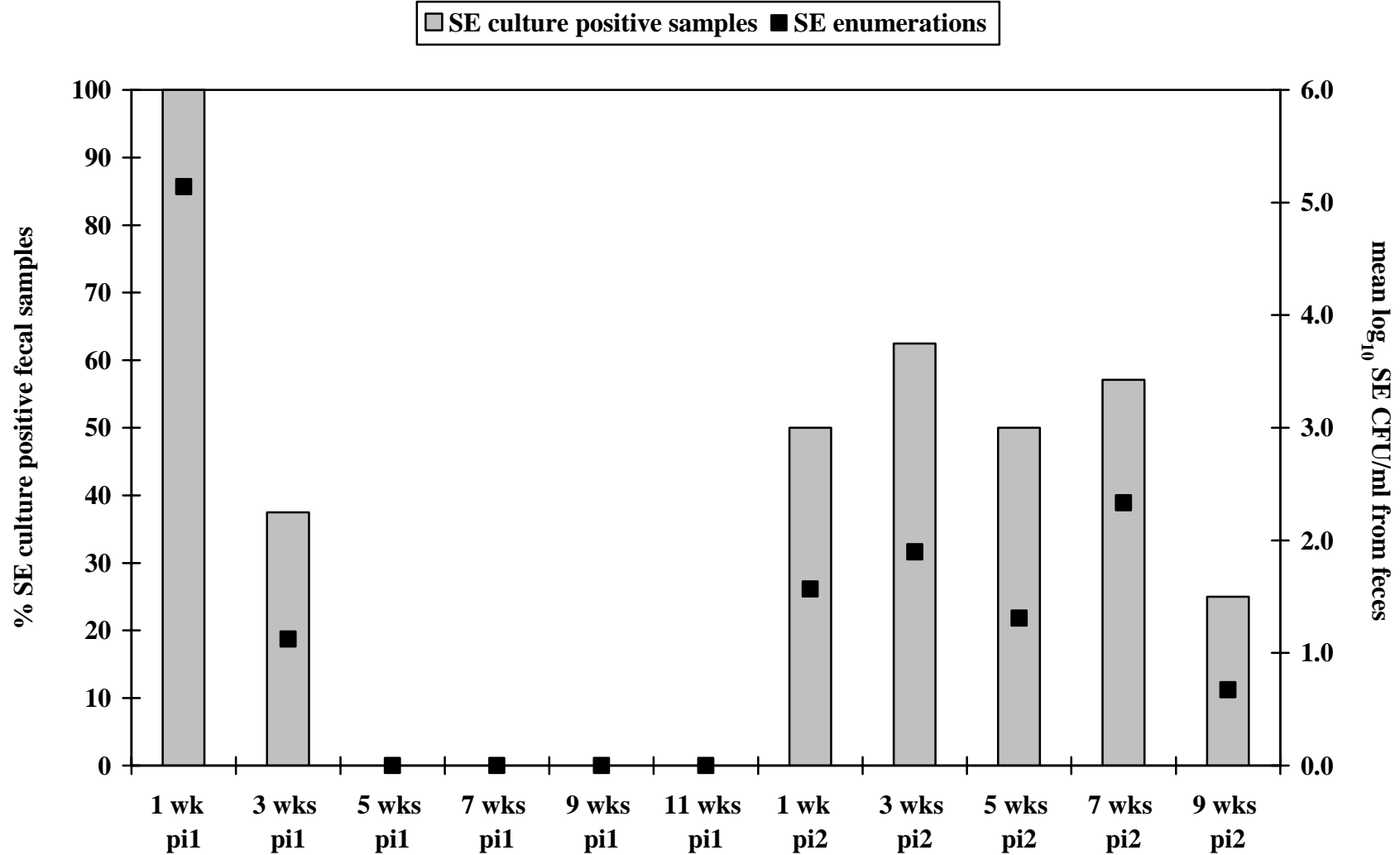


Figure 4.1. Trial 1: Comparison of percentage *Salmonella* Enteritidis (SE) culture positive fecal samples and SE enumerations (mean log₁₀ SE/ml) at bi-weekly intervals from specific pathogen free (SPF) White Leghorn (WL) chickens post-primary SE infection (pi1) and post-secondary SE re-infection (pi2). Same birds (n=8) were repeatedly sampled at each time point for the duration of experiment.

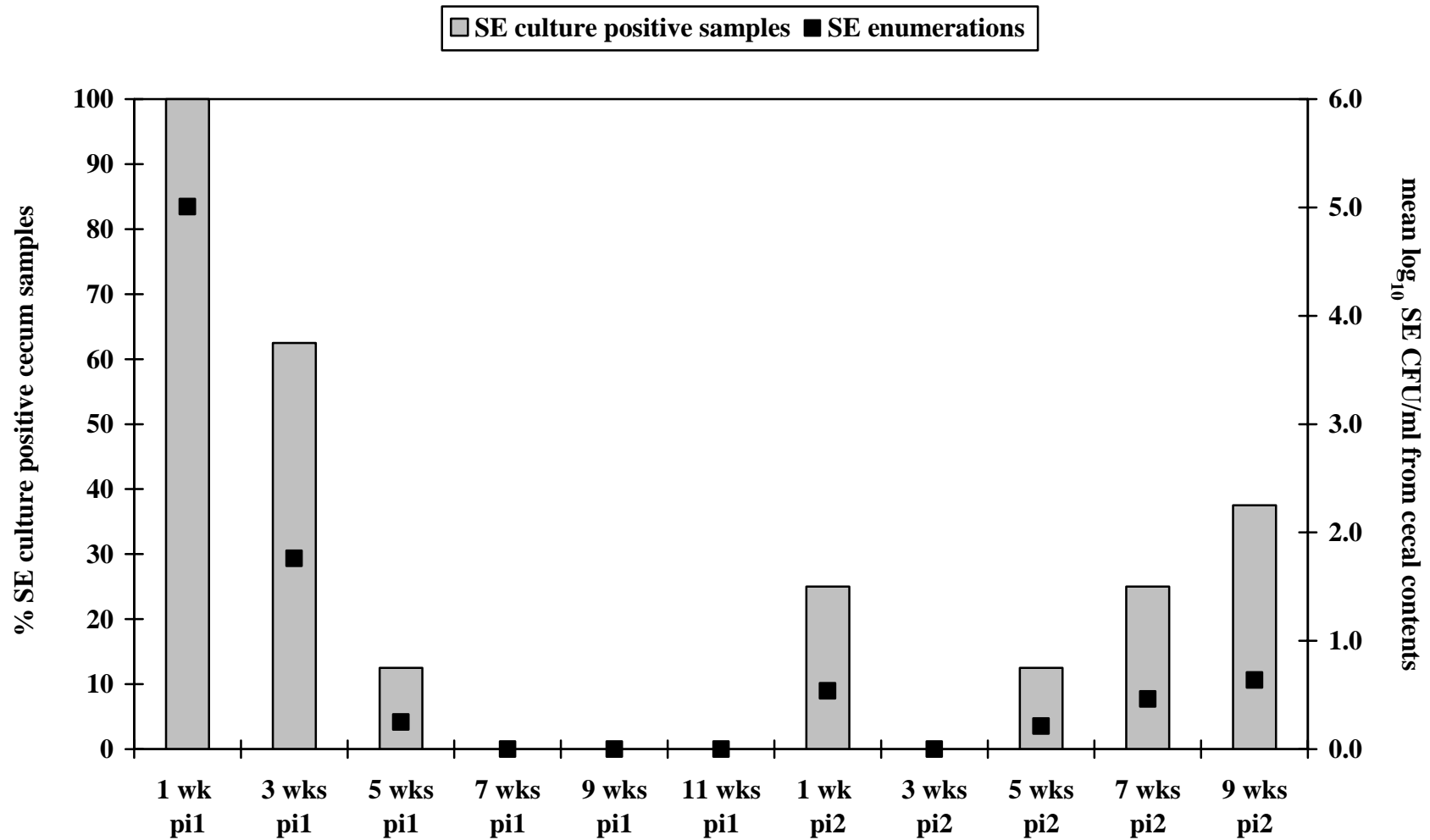


Figure 4.2. Trial 2: Comparison of percentage *Salmonella* Enteritidis (SE) culture positive cecum samples and SE enumerations (mean log₁₀ SE/ml) at bi-weekly intervals from specific pathogen free (SPF) White Leghorn (WL) chickens post-primary SE infection (pi1) and post-secondary SE re-infection (pi2). Different birds (n=8) were sampled at each time point.

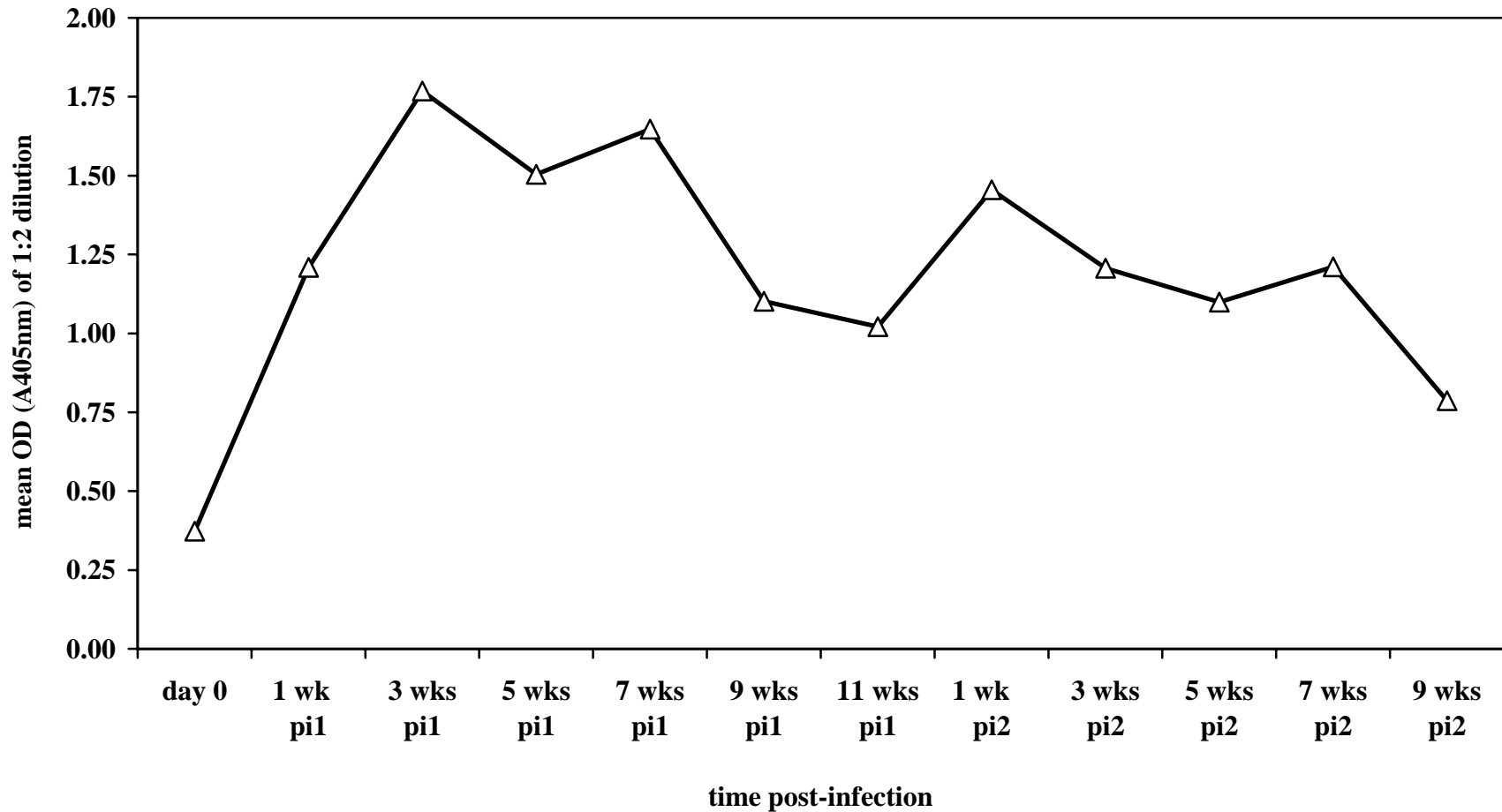


Figure 4.3. Trial 1: Enzyme-linked immunosorbent assay (ELISA) analyses of SE-LPS-specific IgA in crop lavage samples from specific pathogen free White Leghorn (SPF WL) birds following *Salmonella* Enteritidis (SE) primary-infection (pi1) and secondary SE re-infection (pi2). Mean optical density (OD) values are representative of SE-LPS-specific IgA detected in 1:2 dilution crop lavage supernatant samples at day 0 pre-infection then at bi-weekly intervals post-SE infection. Same birds (n=8) were repeatedly sampled at each time point for the duration of experiment.

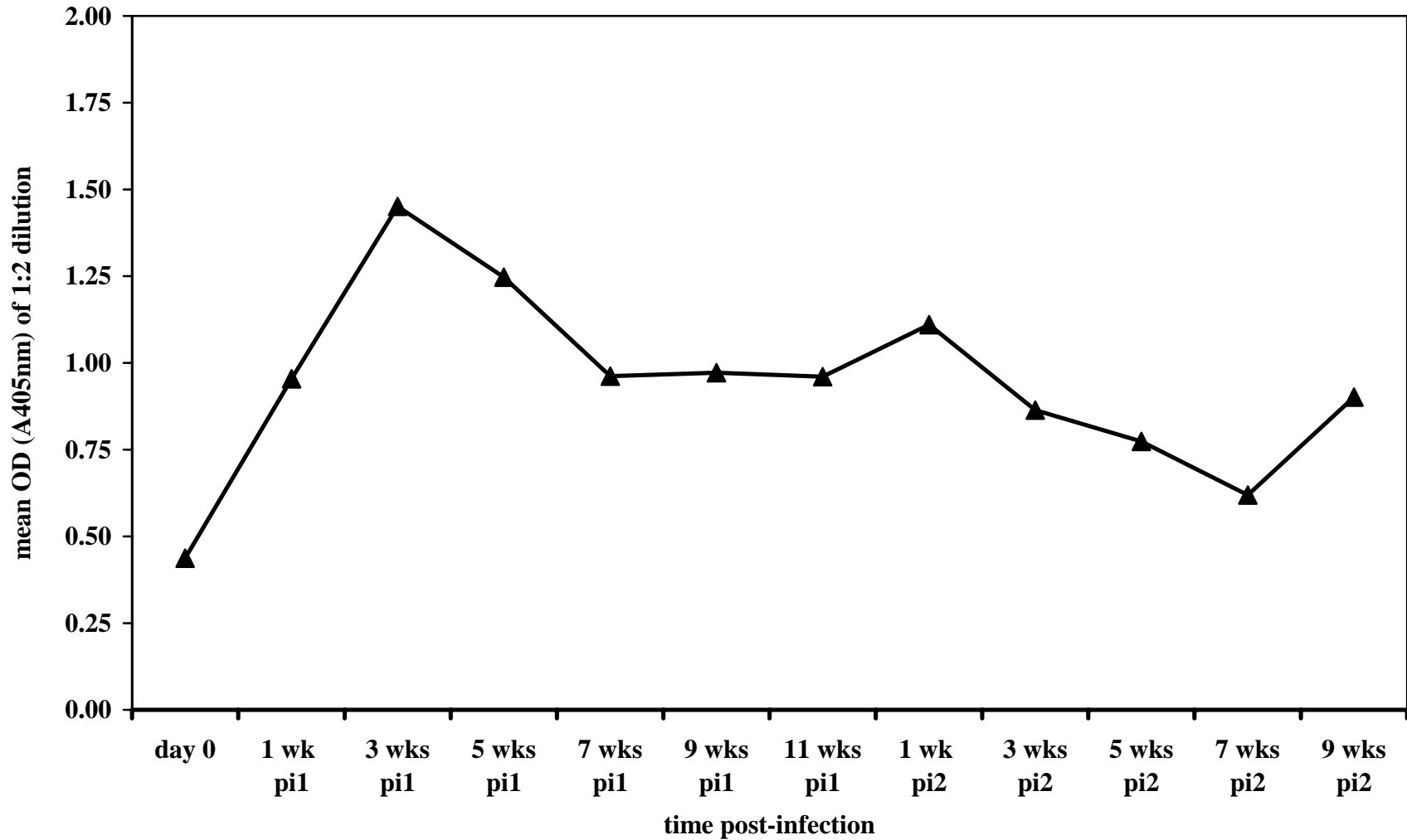


Figure 4.4. Trial 2: Enzyme-linked immunosorbent assay (ELISA) analyses of SE-LPS-specific IgA in crop lavage samples from specific pathogen free White Leghorn (SPF WL) chickens after *Salmonella* Enteritidis (SE) primary-infection (pi1) and secondary SE re-infection (pi2). Mean optical density (OD) values are representative of SE-LPS-specific IgA detected in 1:2 dilution crop lavage supernatant samples at day 0 pre-infection then at bi-weekly intervals post-SE infection. Different birds (n=8) were sampled at each time point.

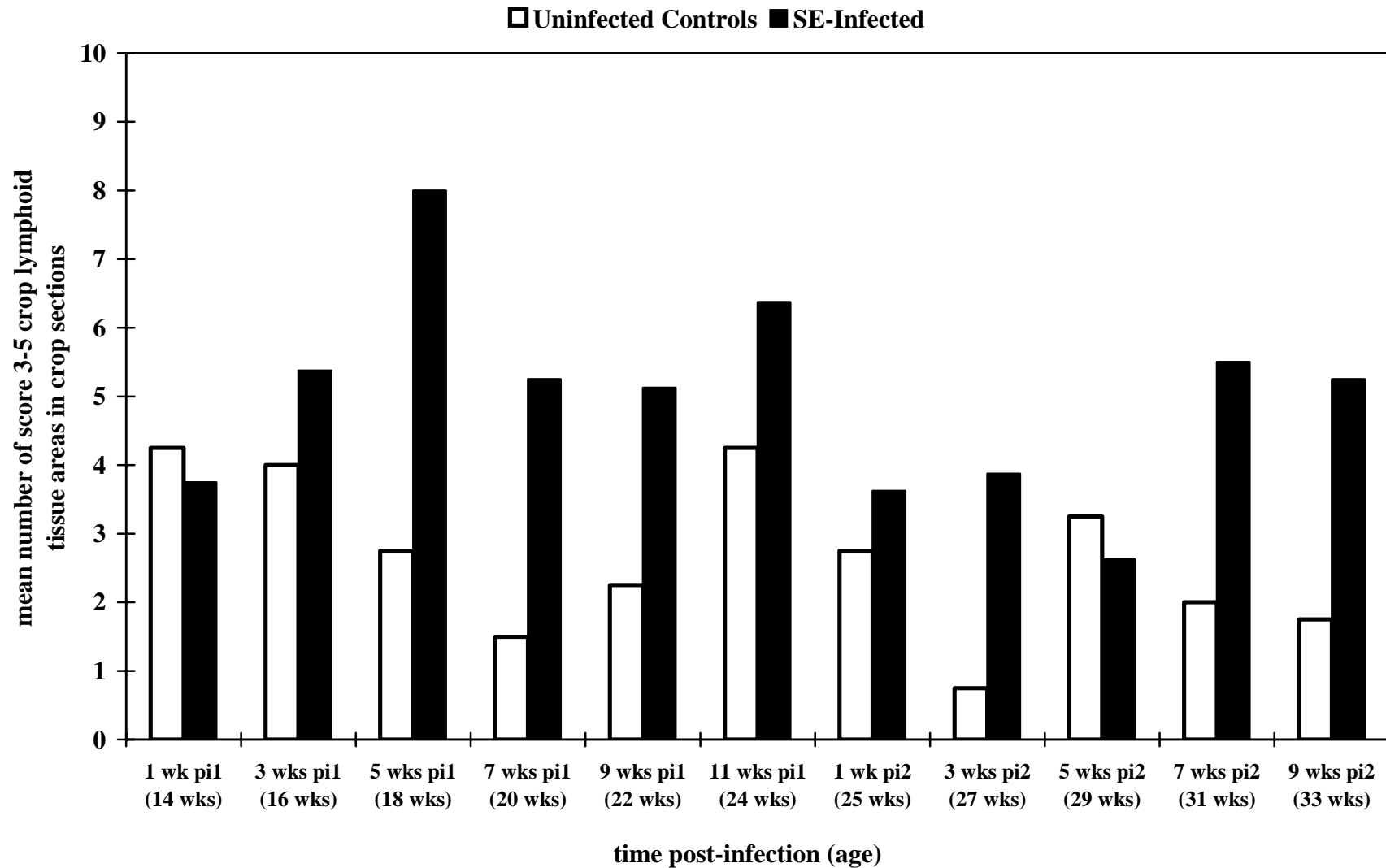


Figure 4.5. Comparison of the average number of score 3-5 lymphoid tissue areas assessed in hematoxylin-and-eosin (H&E) stained crop sections from trial 2 post-primary *Salmonella* Enteritidis (SE) infection (pi1) and post-secondary SE re-infection (pi2) chickens, and age-associated uninfected controls.

CHAPTER 5**CELLULAR ASSESSMENT OF CROP LYMPHOID TISSUE FROM
SPECIFIC PATHOGEN FREE WHITE LEGHORN CHICKENS
AFTER *SALMONELLA* ENTERITIDIS CHALLENGE¹**

¹Vaughn, L.E., P.S. Holt and R.K. Gast. To be submitted to *Avian Diseases*.

ABSTRACT

The cellular repertoire of the local mucosal immune response within the crop (ingluvies) of specific pathogen free (SPF) White Leghorn (WL) chickens against *Salmonella* Enteritidis (SE) was investigated. Three separate trials were performed using SPF WL pullets at 5-6 weeks of age. Trial 1 consisted of seventy-seven birds that were evaluated for 10 weeks post-SE infection (pi); Trial 2 was comprised of seventy-two birds that were monitored through 8 weeks pi; and Trial 3 was made up of thirty birds that were assessed for 5 weeks pi. Birds were challenged *per os* with 10^8 CFU/ml *Salmonella* Enteritidis phage type 13 (SE PT13). Crop lavage samples, crop tissues, ceca and/or internal organs were collected pre-infection then weekly post-SE infection. Crop lavages were analyzed for SE-lipopolysaccharide (LPS)-specific IgA by ELISA. General histochemical (hematoxylin & eosin, methyl green-pyronin) and immunohistochemical (IHC) staining techniques were applied to crop serial sections to identify lymphoid tissue via light microscopy and to grade lymphoid areas using the score 0 (minimal) to 5 (sizable) scale. Bacteriological examinations of cecal contents and/or liver-spleen were conducted at weekly intervals. Results from ELISA revealed markedly increased crop SE-LPS-specific IgA by 2-3 weeks pi and humoral response remained elevated above week 0 baseline for the duration of each experiment. Cecum samples were 100% SE culture positive at 1 week pi and then the percentage of SE positive samples progressively declined over time. Isolated lymphoid follicles (ILF) scored 3 to 5 were observed in H&E stained crop tissue sections, with increased proportion of ILFs in post-SE-infected crops versus uninfected. IHC staining revealed CD45 (pan-leukocyte) and Bu-1 (B-lymphocyte) positive cells within crop ILFs. Plasma cells were visible within and at periphery of ILFs. The chicken crop appears to be a lymphoid tissue site capable of generating a local humoral immune response against SE.

Key words:

Salmonella Enteritidis,
Chicken,
Crop,
Mucosal immune system,
IgA,
Gut-associated lymphoid tissue,
Isolated lymphoid follicles,
Immunohistochemistry

Abbreviations:

ARS = Agricultural Research Service;
AP = alkaline phosphatase;
BSL-2 = biosafety level-2;
BG = brilliant green agar
CFU = colony-forming units;
CO₂ = carbon dioxide;
ELISA = enzyme-linked immunosorbent assay;
GALT = gut-associated lymphoid tissue;
H&E = hematoxylin and eosin;
HRP = horseradish peroxidase;
IgA = immunoglobulin A;
IHC = immunohistochemistry;

ILF = isolated lymphoid follicle(s);

LPS = lipopolysaccharide;

nalR = nalidixic-acid resistant;

NA = nalidixic-acid;

NB = novobiocin;

OD = optical density;

PT13 = phage type 13;

PBS = phosphate-buffered saline;

pi = post-infection;

RV = Rappaport-Vassiliadis;

SE = *Salmonella* Enteritidis;

SEPRL = Southeast Poultry Research Laboratory;

SPF = specific pathogen free;

TBS = tris-buffered saline;

TSB = tryptic soy broth;

USDA= United States Department of Agriculture;

WL = White Leghorn;

INTRODUCTION

The chicken crop, ingluvies, appears to have the functional capacity to produce a mucosal immune response against *Salmonella* Enteritidis (11, 14, 29, 30, 33). A humoral immune response against *Salmonella* Enteritidis (SE) can be detected within the crop of specific pathogen free (SPF) White Leghorn (WL) hens and commercial egg-layer strains of chickens following *per os* experimental SE challenge (29, 30, 33). Enzyme-linked immunosorbent assay (ELISA) detected SE-specific IgA humoral response in crop lavage samples at 1 week post-infection (pi) with an elevated SE-LPS-specific response generated by 2-3 weeks pi (11, 13, 14, 29, 30). Microscopic examination of hematoxylin and eosin (H&E) stained crop tissues from post-SE-challenged hens revealed tentative sites of lymphoid aggregations in the crop lamina propria (14, 32, 33).

The chicken crop may be an organ of importance in the study of gut-associated lymphoid tissue (GALT) and mucosal immunity. Lymphoid tissue aggregations and/or isolated lymphoid follicles (ILFs) within SE-infected chicken crops were identified by routine histological H&E stain (11, 14, 33), however, characterization of cellular populations of the speculative lymphoid tissue of the crop has not been performed. Isolated lymphoid follicles (ILF) of the murine small intestine have been shown by Lorenz *et al.* to contain predominantly B-2 B-lymphocytes, and formation of the murine small intestine ILFs appeared to occur *de novo* in response to luminal stimuli (9, 10, 22, 23). The production of antigen-specific IgA increased in mice possessing ILFs when orally challenged with *Salmonella* Typhimurium (23). The ILFs of the chicken crop might perhaps be somewhat comparable to ILFs of the murine small intestine recently identified by Hamada *et al.* and Hitotsumatsu *et al.* (9, 10).

In the present study, research was undertaken to analyze the local mucosal immune response in the crop of specific pathogen free (SPF) chickens after *Salmonella* Enteritidis (SE) infection, and to definitively determine the presence of lymphoid tissue within the crop post-SE infection. In three separate trials, SE-LPS-specific IgA of the crop was monitored through 10 weeks post-SE infection (pi), 8 weeks pi, and 5 weeks pi, respectively. Objectives were to: monitor persistence of SE in cecum and evaluate SE dissemination to liver and spleen; compare temporal evolution of SE-specific IgA humoral response in the crop after SE exposure; and assess crop sections from SE-infected birds for presence of isolated lymphoid follicles (ILF) and identify ILF cellular populations by histochemical/immunohistochemical (IHC) staining.

MATERIALS & METHODS

Animals.

Three trials were conducted with specific pathogen free (SPF) White Leghorn (WL) chickens, approximately 5-6 weeks of age, originating from flock maintained by Southeast Poultry Research Laboratory (SEPRL), USDA-ARS, Athens, Georgia. Trial 1 consisted of seventy-seven birds, Trial 2 was comprised of seventy-two birds, and Trial 3 was made up of thirty birds. For each trial, birds were housed within a climate controlled biosafety level-2 (BSL-2) building where birds were placed within multiple brooder cages and allowed access to antibiotic-free feed and water *ad libitum*.

Bacterial strains.

Salmonella enterica serovar Enteritidis (SE) phage type 13 (PT13) was selected for experimental challenge of birds (7). Frozen stock maintained at -70°C of nalidixic-acid resistant (nalR) *Salmonella* serovar Enteritidis (SE) phage type 13 (strain SE89-8312) was thawed and cultured on nutrient agar at 37°C for 18 hours. Isolated colonies were subcultured on brilliant green (BG)

agar containing 20 µg/ml novobiocin (NB) and 20 µg/ml nalidixic-acid (NA) to ensure conserved nalidixic-acid resistance. The nalR SE PT13 culture was further cultivated in 10 ml tryptic soy broth (TSB) at 37°C for 18 hours, and then the broth culture was diluted in sterile physiological saline to prepare 10⁸ colony-forming units (CFU) /ml inoculum for experimental infection of birds.

Experimental design.

Studies conducted were approved by and in accordance with guidelines of the SEPRL Institutional Animal Care and Use Committee (IACUC). *Salmonella*-free status of chickens was determined before commencement of the experiment. A 1.0 gram weight of feces was collected by placement of sterile catch trays under birds and feces were selectively enriched in Rappaport-Vassiliadis (RV) broth for 24 hours at 37°C-42°C then streak plated onto brilliant green (BG) agar containing 20 µg/ml novobiocin (NB). *Salmonella* was not detectable in fecal cultures. Three trials were conducted using SPF WL pullets at 5-6 weeks of age and birds were challenged *per os* with 10⁸ CFU/ml SE nalR PT13. The three separate trials were set-up to ensure reproducibility of research findings. Trial 1 consisted of seventy-seven birds that were evaluated from week 0 pre-infection to 10 weeks post-SE infection (pi); Trial 2 was comprised of seventy-two birds that were monitored from week 0 through 8 weeks pi; and Trial 3 was made up of thirty birds that were assessed from week 0 to 5 weeks pi. Crop lavage samples, crop tissues, ceca and/or internal organs were collected at week 0 pre-infection then collected at weekly intervals post-SE infection. During week 0 pre-infection for trials 1-3, crop lavages were performed on SPF White Leghorn chickens to establish baseline crop IgA by enzyme-linked immunosorbent assay (ELISA). The chickens were then euthanized via carbon dioxide (CO₂) gas inhalation and the crop organ harvested from each bird for histopathology assessment of

lymphoid tissue at week 0 pre-infection. After chickens were challenged *per os* with SE naIR PT13, crop lavage samples, cecal contents and crop tissues were collected weekly. Crop lavage samples were analyzed for SE-lipopolysaccharide (LPS)-specific IgA by ELISA. General histochemical and immunohistochemical (IHC) staining techniques were applied to crop serial sections to identify lymphoid tissue and to determine cellular populations of the isolated lymphoid follicles (ILFs) via light microscopy. H&E stained crop sections were selected to grade lymphoid tissue using the score 0 (minimal) to score 5 (sizable) scale (33). Bacteriological examinations of cecal contents and/or liver-spleen were conducted weekly to monitor progression of SE infection (7).

Crop lavage sample collection.

Crop lavages were performed according to the protocol designed by Holt *et al.* (14). The crop lavage devices were fashioned using 10 ml syringes and attached tubing 7-10 inches (17.8-25.4 cm) in length with the distal 1.5 inches (3.8 cm) fenestrated. The lavage devices were aseptically filled with 5 ml of room temperature, sterile glycine flush solution, pH 7.8-8.0 (14, 17). Birds were held in an upright, vertical position with neck extended to facilitate insertion of the lavage device tubing into the esophagus. Once the fenestrated portion of tubing was positioned within the crop, the 5 ml glycine solution was slowly infused. Immediately a fluid sample from the crop was aspirated back into the syringe and then the crop lavage sample (aspirate) was dispensed from syringe to a sterile collection container. The lavage procedure was repeated per individual bird using a new lavage device and separate sample collection container. A 1.5-2.0 ml portion from each neat crop lavage sample was centrifuged at 10,000 rpm for 5 minutes to remove feed particulate debris and excess mucous. The supernatant fraction was collected and stored at -20°C .

Bacteriological examination.

Appropriate techniques and media for isolation of salmonellae were utilized for bacteriological examination of cecum and liver-spleen organ samples collected post-SE infection (5, 34). Bacterial culture for SE nalR PT13 was performed on cecum samples collected at weekly intervals post-SE infection for trials 1, 2 and 3. Liver and spleen organ samples were harvested weekly post-SE infection for trial 1 and trial 3 for bacterial culture of SE nalR PT13. One cecum (mid-cecum to cecal apex) per bird was aseptically harvested immediately postmortem. Cecum samples of 1.0 gram weight were placed into sterile whirl-pack bags (Fisher Scientific, Suwanee, GA, USA) and Rappaport-Vassiliadis (RV) broth added at 9x volume/tissue weight, and then ceca were macerated in a stomacher for 30 seconds to evacuate cecal contents. A 2.0 cm x 2.0 cm portion of right liver lobe and the entire spleen were aseptically harvested and liver-spleen combined in whirl-pack bags, then RV broth 9x volume/tissue weight was added and organs were macerated for 30 seconds in a stomacher. A 100 µl portion from each macerated cecum and liver-spleen sample was directly spread-plated onto brilliant green (BG) agar containing 20 µg/ml novobiocin (NB) and 20 µg/ml nalidixic-acid (NA). The BG+NB+NA direct spread plates and RV samples were incubated at 37°C overnight. Plates were then assessed for SE colony growth. In instances where direct plating resulted in no growth, the RV-enriched samples were streaked onto BG+NB+NA agar and streak plates were incubated 24 hours at 37°C then assessed for typical SE colony growth. Questionable colonies were further differentiated based on biochemical tests using triple sugar iron (TSI) agar and modified-lysine iron (MLI) agar slant tubes, and agglutination reaction with antiserum against *Salmonella* serogroup D₁ somatic antigen (Beckton Dickinson-Difco, Bedford, MA, USA). Cecum and liver-spleen samples were recorded as positive if SE was recoverable either by direct plating or after selective RV-

enrichment. For trial 3, SE enumerations were performed from cecum samples and data reported as colony-forming units (CFU)/ml. Cecum samples with no growth on direct plating but positive in RV-enrichment were assigned the arbitrary value of 50 CFU/ml, half of the theoretical detection limit (7). Samples with no SE growth detectable either by direct plating or in RV-enrichment were deemed as negative and given a count of 0. SE concentrations in transformed metric (logarithmic CFU/ml) were used to calculate mean \log_{10} CFU/ml at each weekly interval for cecum samples from trial 3.

Crop SE-specific IgA ELISA.

The crop lavage samples obtained at weekly time points following SE-infection were analyzed by enzyme-linked immunosorbent assay (ELISA) for SE-lipopolysaccharide (LPS)-specific IgA as previously described by Holt *et al.* (12-14). For assay, the frozen crop supernatant samples were thawed, diluted 1:1 with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) then 2-fold serial dilutions were prepared. Sample dilutions 1:2 to 1:128 were added to flat-bottomed 96-well polystyrene ELISA plates (Corning-Costar Inc., Wilkes Barre, PA, USA) to which SE-LPS antigen (Sigma-Aldrich) at concentration of 10 $\mu\text{g/ml}$ had been adsorbed. Additionally, previously defined crop lavage samples with strong and weak SE-LPS-specific IgA antibody responses were run on each assay plate as positive and negative controls, respectively. Plates containing samples and controls were incubated 60 minutes at room-temperature then washed three times with PBS containing 0.05% Tween 20. The next two sequential steps performed were the application of primary antibody with 60 minutes incubation time followed by three washes, then addition of an enzyme-conjugated secondary antibody with protocol repeated for incubation time and washes. The primary antibody for detection of SE-LPS-specific IgA in crop lavage samples was a monoclonal

mouse anti-chicken IgA (Southern Biotech, Birmingham, AL, USA) diluted 1:1000 in PBS containing 0.05% Tween 20 and 1% bovine serum albumin (BSA). Goat anti-mouse IgG with alkaline phosphatase conjugate (Calbiochem, La Jolla, CA, USA) diluted 1:1000 served as the enzyme-conjugated secondary antibody. Diethanolamine buffer with concentration 1 mg/ml p-nitrophenyl-phosphate (Sigma-Aldrich) was applied to plate wells as substrate solution and the chromogenic reaction proceeded for 15 to 30 minutes at room-temperature in the dark.

Colorimetric analysis using a Multiskan Ascent (Thermo Fisher Scientific, Milford, MA, USA) microplate photometer at 405 nm wavelength yielded absorbance/optical density (OD) values.

Crop Histology & Lymphoid Tissue Scoring.

Pre-infection week 0 and at weekly time points following SE-infection, chickens were euthanized via carbon dioxide (CO₂) gas inhalation and immediately postmortem the entire crop with 1.0 inch (2.54 cm) of attached esophagus was excised. Tissues were fixed intact in 10% neutral buffered formalin for 24 hours and the crops were then sectioned through the mid-body region. The plane of the cut passed through the entirety of the hollow-bodied crop organ to produce ring-shaped circular sections. One to two tissue sections per crop were inserted into standard histology tissue cassettes (Fisher Scientific), routinely processed, paraffin embedded, microtome cut, and affixed to glass slides. Crop tissue slides were stained with hematoxylin and eosin (H&E) then examined via light microscopy. The H&E crop tissues were evaluated full-circle for the presence of inflammatory changes and lymphoid aggregations in the epithelium and lamina propria. All apparent sites of lymphocytic-lymphoid cell infiltrates and/or isolated lymphoid follicles (ILF) were graded using the score 0 minimal to score 5 sizable scale that was established by Vaughn *et al.*, and then the number of lymphoid areas observed for each score category was recorded (33). An ocular grid reticle (Fisher Scientific) was used to make the score

determinations and perform counts at 200X magnification. Scores 3 to 5 denoted the isolated lymphoid follicles (ILFs) that were well-organized, of moderate (score 3) to heightened (score 5) cellularity and of increased size. Lymphoid tissue sites within the score 3 to 5 range were those selected to make comparisons between crop lymphoid tissue from SE-infected chickens and uninfected controls (33).

Monoclonal Antibodies.

Monoclonal antibodies used in the study were: mouse anti-chicken CD45 (pan-leukocyte) unlabeled as the primary antibody (Southern Biotech, Birmingham, AL, USA) at working concentration 50 µg/ml and goat anti-mouse IgM conjugated with alkaline phosphatase (AP) enzyme as secondary antibody (Calbiochem, La Jolla, CA, USA) at 50 µg/ml; and mouse anti-chicken Bu-1 (B-lymphocyte) unlabeled as primary antibody (Southern Biotech) at concentration 50 µg/ml and goat anti-mouse IgG labeled with horseradish peroxidase (HRP) enzyme as secondary antibody (Calbiochem) at 1:50 dilution.

General Histochemical and Immunohistochemical Staining.

Optimal staining conditions were determined using slides prepared from a multiple-tissue block that contained formalin-fixed, paraffin-embedded 6mm biopsy sections of gastrocnemius muscle, thymus, bursa of Fabricius, spleen, liver, crop, proventriculus, Peyer's patch and cecal tonsil from a normal, healthy chicken. The multiple-tissue slides were included as staining controls during the staining of each group of crop tissue slides.

Replicate tissue cuts 3-5µm thickness were made from paraffin-embedded crop tissue blocks and tissues mounted on positive-charged slides (Fisher Scientific). Crop slide tissues were deparaffinized with EZ-DeWax solution (BioGenex, San Ramon, CA, USA) and washed in three changes of deionized-water. For general histochemical staining to visualize plasma cells, a

methyl green-pyronin (MGP) solution (Sigma-Aldrich, St. Louis, MO, USA) was used according to the manufacturer's instructions to identify pyroninophilic cells indicative of plasma cells (20, 21, 28). For the immunohistochemical (IHC) staining of crop tissue slides, a heat induced epitope retrieval (HIER) method was used after deparaffinization (4, 15). Slides for IHC were immersed in antigen retrieval buffer 0.01M Tris-base/0.001M EDTA disodium salt, placed inside microwave pressure cooker (BioGenex) and then microwave heated at 40-50% power for 3-5 minutes. Slides were then allowed to cool in Tris/EDTA antigen retrieval buffer for 20-30 minutes at room temperature. When appropriate, 3% hydrogen peroxide (H_2O_2) was applied to slides for 5 minutes to quench endogenous peroxidases in tissues. All slides were rinsed in deionized-water for 5 minutes. Immunohistochemical (IHC) staining to identify aggregations of leukocytes in crop tissue slides was accomplished using mouse anti-chicken CD45 (Southern Biotech) pan-leukocyte primary monoclonal antibody, alkaline phosphatase (AP) conjugated goat anti-mouse IgM (Calbiochem) as secondary enzyme-conjugated antibody and fast red substrate (BioGenex). IHC staining to identify B-lymphocytes was conducted using mouse anti-chicken Bu-1 (Southern Biotech) primary monoclonal antibody, horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Calbiochem) as secondary enzyme-labeled antibody and diaminobenzidine (DAB) substrate (BioGenex). In brief, steps of the IHC staining process involved application of appropriate primary monoclonal antibody, mouse anti-chicken CD45 or mouse anti-chicken Bu-1, and slides were incubated at 4°C overnight inside a humidified chamber. Slides were then washed for 5 minutes in tris-buffered saline (TSB) or phosphate-buffered saline (PBS) to remove unbound excess antibody from tissues. Appropriate enzyme-labeled secondary antibody, goat anti-mouse AP or goat anti-mouse HRP, was applied and slides incubated 45-60 minutes at room temperature, then slides were washed for 5 minutes in TBS or

PBS to remove any unbound secondary enzyme-conjugated antibody. After washing, bound antibody was detected in crop tissue sections by application of fast red or diaminobenzidine (DAB) chromogen-substrate solution (BioGenex) according to manufacturer's instructions and slides incubated 5-10 minutes at room temperature. Slides were washed in deionized-water to stop the enzyme-chromogen reaction, and then slides were counterstained with Mayer's hematoxylin (BioGenex). All slides were coverslipped and examined using light-microscopy.

Statistical Analyses.

Statistical analyses were performed using GraphPad InStat (GraphPad Software, Inc., San Diego, CA, USA). Levels of *Salmonella* Enteritidis/ml detected in cecum samples were \log_{10} transformed and means (mean \log_{10} CFU/ml) were calculated. For bacterial culture results and enzyme-linked immunosorbent assay (ELISA) data, analysis of variance (ANOVA) with Tukey's multiple comparison test were performed to determine statistically significant differences ($p < 0.05$) among post-SE infection time points, and ANOVA with Dunnett's multiple comparison test procedure conducted to determine statistically significant differences ($p < 0.05$) between controls (week 0 uninfected) versus post-SE-infection time points. Kruskal-Wallis nonparametric analysis of variance (ANOVA) with Dunn's multiple comparison post-test were performed on crop lymphoid tissue score data to assess whether or not statistical differences ($p < 0.05$) existed between uninfected controls and SE-infected bird groups.

RESULTS

Bacterial Culture.

Figure 5.1 represents the temporal kinetics of SE prevalence in cecal contents and liver-spleen organ samples that were cultured weekly following SE-infection for the SPF White Leghorn chickens from trial 1. Cecum samples cultured post-SE-infection (π) had a *Salmonella*

Enteritidis (SE) prevalence of: 100% at 1 week pi; 57% at 2 weeks pi; 43% at 3 weeks pi; 14% at 4 weeks pi; 29% at 5 weeks pi; 14% at 6 weeks pi; 14% at 7 weeks pi; 14% at 8 weeks pi; 57% at 9 weeks pi; and no SE recoverable in cecal contents at 10 weeks pi (**Figure 5.1**). SE dissemination to internal organs did occur. Liver-spleen culture results for SE were: 100% at 1 week pi; 29% at 2 weeks pi; 14% at 3 weeks pi; no detectable SE at 4 weeks pi to 6 weeks pi; 14% at 7 weeks pi; no SE cultured at 8 weeks pi; 14% at 9 weeks pi; and then no SE recoverable at 10 weeks pi (**Figure 5.1**).

Figure 5.2 demonstrates the temporal kinetics of SE prevalence in cecal contents cultured weekly post-SE-infection for SPF WL chickens from trial 2. After SE-infection the SE prevalence in cecum samples cultured was: 100% at 1 week pi; 86% at 2 weeks pi; 50% at 3 weeks pi; 75% at 4 weeks pi; 14% at 5 weeks pi, 25% at 6 weeks pi, 50% at 7 weeks pi and 38% at 8 weeks pi (**Figure 5.2**).

Figure 5.3 shows the temporal kinetics of SE prevalence in cecal contents (percentage SE culture positive and SE enumerations) and SE prevalence in liver-spleen organ samples cultured weekly following SE-infection from trial 3. The prevalence of *Salmonella* Enteritidis (SE) in cecal contents collected from chickens post-SE infection was: 100% at 1 week pi; 80% at 2 weeks pi; no detectable SE at 3 weeks pi and 4 weeks pi; and then 20% at 5 weeks pi (**Figure 5.3**). SE levels were highest at 1 week pi with 5.31 mean log₁₀ SE CFU/ml in cecal contents (**Figure 5.3**) and 100% of the birds having SE recoverable from cecum. The increased SE levels detected in ceca cultured at 1 week pi differed significantly ($p < 0.001$) when compared to week 0 pre-infection and to subsequent post-SE infection time points. Dissemination of SE to liver-spleen samples cultured was: 100% at 1 week pi; 100% at 2 weeks pi; 40% at 3 weeks pi; and then no detectable SE at 4 weeks pi and 5 weeks pi (**Figure 5.3**).

Crop SE-LPS-specific IgA.

Enzyme-linked immunosorbent assays (ELISA) demonstrated an increased SE-LPS-specific IgA humoral response in crop lavage supernatant samples from post-SE infection chickens in trial 1 (**Figure 5.4**), trial 2 (**Figure 5.5**) and trial 3 (**Figure 5.6**). A similar relative pattern of SE-LPS-specific IgA response within the chicken crop was observed in all three trials following SE infection. Prior to infection at week 0, the SE-LPS-specific IgA levels were considered minimal. Following oral SE challenge, the SE-LPS-specific IgA response was elevated greater than week 0 during the weekly post-SE infection intervals. A markedly increased SE-LPS-specific IgA response was detected between week 0 pre-infection and approximately 2-3 weeks pi. For the sampling time points closest to SE challenge, a more exaggerated SE-LPS-specific IgA humoral response was noted, however, as time lengthened from the initial SE challenge a progressive wane of SE-LPS-specific IgA within the crop occurred.

Crop Histology & Lymphoid Tissue Scores.

The calculated mean (average) of crop lymphoid tissue in score 3-5 range at week 0 (pre-infection controls) and at weekly time points post-SE infection for trials 1, 2 and 3 are shown in **Figures 5.7, 5.8 and 5.9**, respectively. The H&E crop sections from uninfected control chickens contained some lymphoid tissue areas that were judged as score 3, 4 and 5. However, examination of H&E crop sections from post-SE-infected chickens found an increased number of score 3 to 5 lymphoid tissue areas in the SE-infected compared to uninfected controls. **Figures 5.7-5.9** compare the means of crop lymphoid tissue assessed over time for SE-infected birds versus uninfected controls. An increase of lymphoid tissue in crop sections from SE-infected as compared to controls (week 0) was determined by comparison of the mean number of score 3-5 range isolated lymphoid follicles (ILFs)/lymphoid tissue areas. Overall, the crop sections from

SE-infected chickens as compared to uninfected controls had from 2.07 to 4.92-fold increase in isolated lymphoid follicles of score 3-5 range. At 3 weeks pi to 10 weeks pi, crop lymphoid tissue within the crops of post-SE infection birds differed significantly ($p < 0.05$) from the pre-infection controls.

General Histochemical and IHC Staining.

Isolated lymphoid follicles (ILFs) in crop lamina propria were identified via routine hematoxylin and eosin (H&E) staining (**Figure 5.10A**). Immunohistochemical (IHC) staining of the lymphoid aggregates within replicate crop tissue sections from SE-infected chickens detected a generalized positive cell staining for CD45 pan-leukocytes within the isolated lymphoid follicles (**Figure 5.10B**). The isolated lymphoid follicles (ILFs) in crop serial sections appeared to consist primarily of B-lymphocytes stained positive for Bu-1 (**Figure 5.10C**). The methyl green-pyronin (MGP) staining method revealed plasma cells scattered throughout the ILFs and at periphery of ILFs (**Figure 5.10D**).

DISCUSSION

The mucosal immune system of the avian includes secondary (peripheral) lymphoid tissues of the respiratory, urogenital and gastrointestinal tracts. Mucosal lymphoid tissue of the alimentary tract, known as gut-associated lymphoid tissue (GALT), is equipped with a cellular repertoire necessary to sample luminal antigens and to provide protective humoral (i.e., secretory-IgA) and/or cell-mediated responses against microorganisms at mucosal surfaces (16, 25). Locations where GALT has been identified in the chicken are the oropharynx, esophagus-proventriculus junction, Meckel's vitelline diverticulum, ileal Peyer's patches and cecal tonsils (1, 2, 8, 19, 26, 27). The crop, situated within the upper gastrointestinal tract, would appear to be a prospective location for gut-associated lymphoid tissue and/or for tertiary lymphoid tissue to

arise in response to bacterial pathogens harbored within the crop or to agents that traverse the crop on descent to the lower alimentary tract.

The increased SE-LPS-specific IgA and increased presence of lymphoid tissue/isolated lymphoid follicles (ILF) within crop samples from SE-infected chickens appears to demonstrate that a local mucosal immune response can be elicited in the crop. Experimental oral *Salmonella* Enteritidis (SE) infection of SPF White Leghorn chickens induced a humoral immune response that was detectable within the crop (**Figures 5.4-5.6**). IgA antibody responses specific against SE-LPS antigen were observed at 1 week post-SE-infection (pi) with a more dramatic increase in humoral response noted by 2-3 weeks pi. Increased SE-LPS-specific IgA responses in crop lavage samples correlated with time points when SE was detected by bacteriological culture from cecum samples and/or liver-spleen organs (**Figures 5.1-5.3**). Higher SE-LPS IgA values along with SE culture positive samples were detected at time points nearer to initial oral SE infection, and then a gradual decline over time of the crop SE-LPS IgA response occurred as the percentage of SE culture positive samples declined. SE bacterial load and/or SE prevalence seemed to perhaps dictate the vigorousness of the SE-LPS-specific IgA response within the crop.

The observation of a greater proportion of lymphoid tissue in the crop sections from SE-infected versus the uninfected week 0 control chickens (**Figures 5.7-5.9**) would appear to be related to the introduction of *Salmonella* Enteritidis and the antigenic stimulus evoked by the enteroinvasive pathogen. Research data presented in the current study, and by Seo and Holt (11, 14, 29, 30), support the notion that the chicken crop is a location where GALT and/or tertiary *de novo* lymphoid tissue can be found in the upper alimentary tract. The finding of a slight amount of lymphoid tissue present in the crop of uninfected control chickens could perhaps be

considered a relatively normal observation, as a variety of exogenous environmental antigens or mitogens would be encountered from time of hatch to maturation.

Exposure of the gut to enteroinvasive pathogenic bacteria may promote expansion of existent secondary gut-associated lymphoid tissues (GALT), and the genesis of tertiary lymphoid tissue (6). The lymphoid tissue identified in the chicken crop might perhaps be comparable to the isolated lymphoid follicles (ILFs) of the murine small intestine described by Hamada *et al.* (9) and Lorenz *et al.* (22, 23). Hamada *et al.* (9) identified lymphoid clusters filled with B220+ cells (B-lymphocytes) distributed throughout the antimesenteric mucosa of the mouse small intestine. The ILFs observed by Hamada *et al.* (9) along the antimesenteric wall of the murine small intestine were structurally and functionally similar to follicular units that compose Peyer's patches (Pp), however, the small intestine ILFs were smaller than B cell-enriched follicular units of Pp and the ILFs lacked the T cell-enriched interfollicular regions that are a component of Pp. Hamada *et al.* (9, 10) proposed that isolated lymphoid follicles (ILF) of the mouse small intestine are tertiary lymphoid tissue with *de novo* formation. Tertiary lymphoid tissues arise from lymphoid neogenesis, a process thought to perhaps be inducible by antigen or driven by the expression of cytokines and/or lymphoid chemokines (9, 10, 23, 24, 31). A portion of the lymphoid tissue observed in H&E stained crop sections from SE-challenged chickens may be tertiary lymphoid tissue that developed *de novo* subsequent to SE infection.

Immunohistochemical (IHC) and general histochemical staining techniques applied to the crop isolated lymphoid follicles (ILFs)/crop lymphoid tissue identified CD45, Bu-1 and pyroninophilic positive cells which were indicative of leukocytes, B-lymphocytes and plasma cells, respectively (**Figure 5.10**). The CD45 pan-leukocyte stain confirmed the presence of lymphoid tissue at sites previously identified as isolated lymphoid follicles (ILFs) by cursory

examination of H&E stained crop tissue sections. Cellular components, such as B-lymphocytes and plasma cells, have the capacity to generate an IgA humoral immune response at mucosal surfaces of the alimentary tract (3, 16, 18). The finding of crop isolated lymphoid follicles composed of B-lymphocytes and plasma cells, along with SE-LPS-specific IgA within the crop after exposure to SE, supports the ability of the crop organ to actively produce the humoral immune response locally. The chicken crop appears to be another site along the alimentary tract where inducible lymphoid tissue may be found that contributes to the integrated gut mucosal immune system.

The local humoral SE-LPS-specific IgA response within the crop may not be capable of providing complete protection against a primary SE-infection of naïve birds, as SE invasion of the alimentary tract and subsequent hematogenous dissemination to internal organs (liver and spleen) probably occurs before an adequate crop immune response has the chance to arise. SE positive liver-spleen samples were detected at 1 week pi (**Figures 5.1 and 5.3**) and a heightened IgA response in the crop appeared at 2-3 weeks pi (**Figures 5.4-5.6**). Despite moderate to heightened SE-specific IgA responses in the crop, *Salmonella* Enteritidis survived and persisted within the cecum at 9 weeks pi, 8 weeks pi, and 5 weeks pi for Trials 1, 2 and 3, respectively (**Figures 5.1-5.3**). The immune response of the crop organ might not yield full protection against the entero-invasive SE pathogen, however, the crop may have merit as an easy sampling site to measure mucosal humoral response of the upper gastrointestinal tract and thus help to determine chickens with active SE infection (14, 30).

Much remains to be learned about cellular and molecular events underlying formation and function of GALT isolated lymphoid follicles. Cytokine and chemokine analyses will be conducted on the chicken crop, and such data should provide researchers with more information

about SE behavior and the mucosal immune response of the avian host. A more detailed understanding of how SE influences the host immune response in the upper gastrointestinal tract (i.e., crop) may be important in determining how and where protective immunity to the SE enteric pathogen can be generated in poultry.

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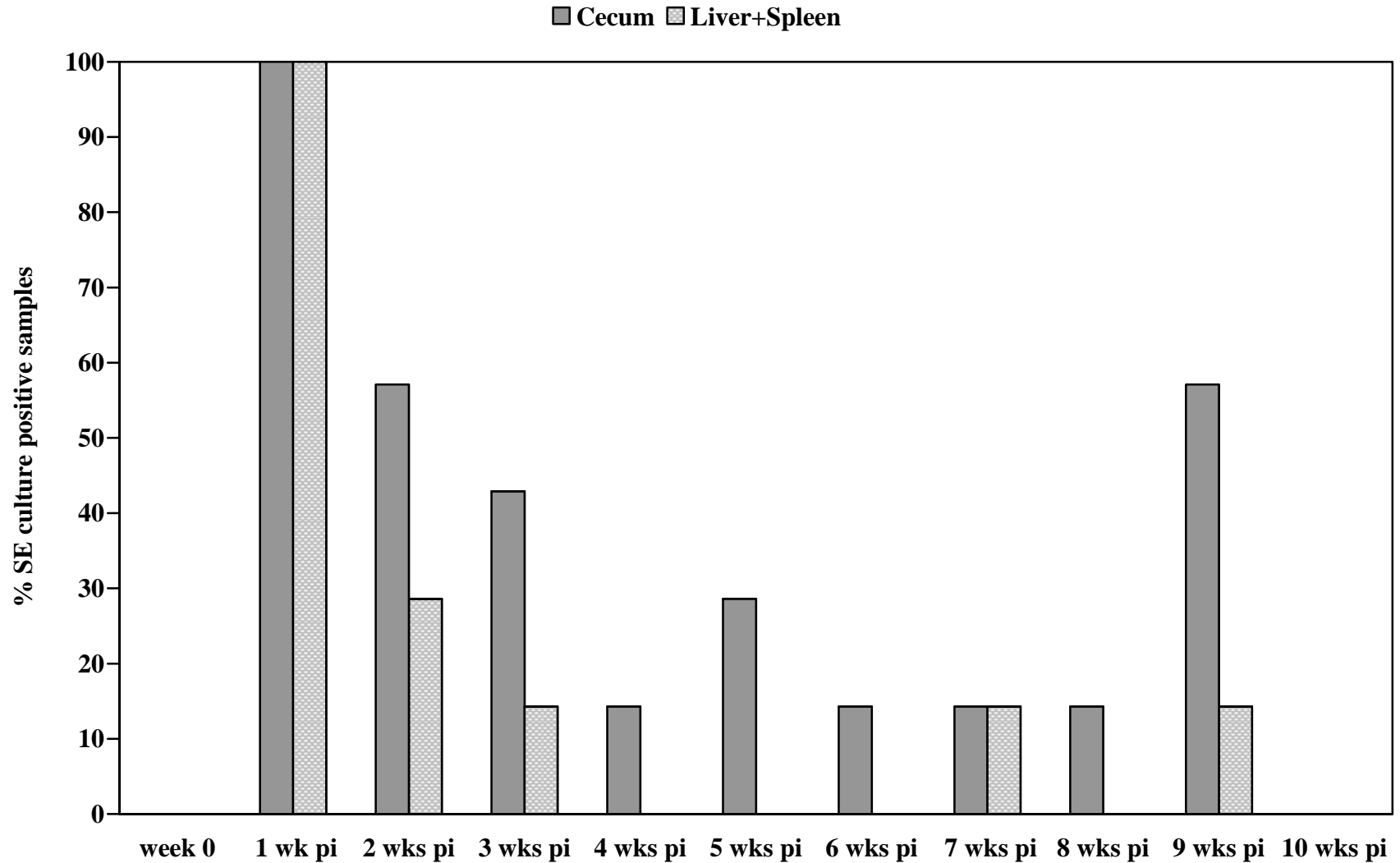


Figure 5.1. Trial 1: Comparison of percentage *Salmonella* Enteritidis (SE) culture positive cecum samples and SE culture positive liver & spleen organ samples at weekly intervals from specific pathogen free (SPF) White Leghorn (WL) chickens following SE infection (pi). Different birds (n=7) were sampled weekly.

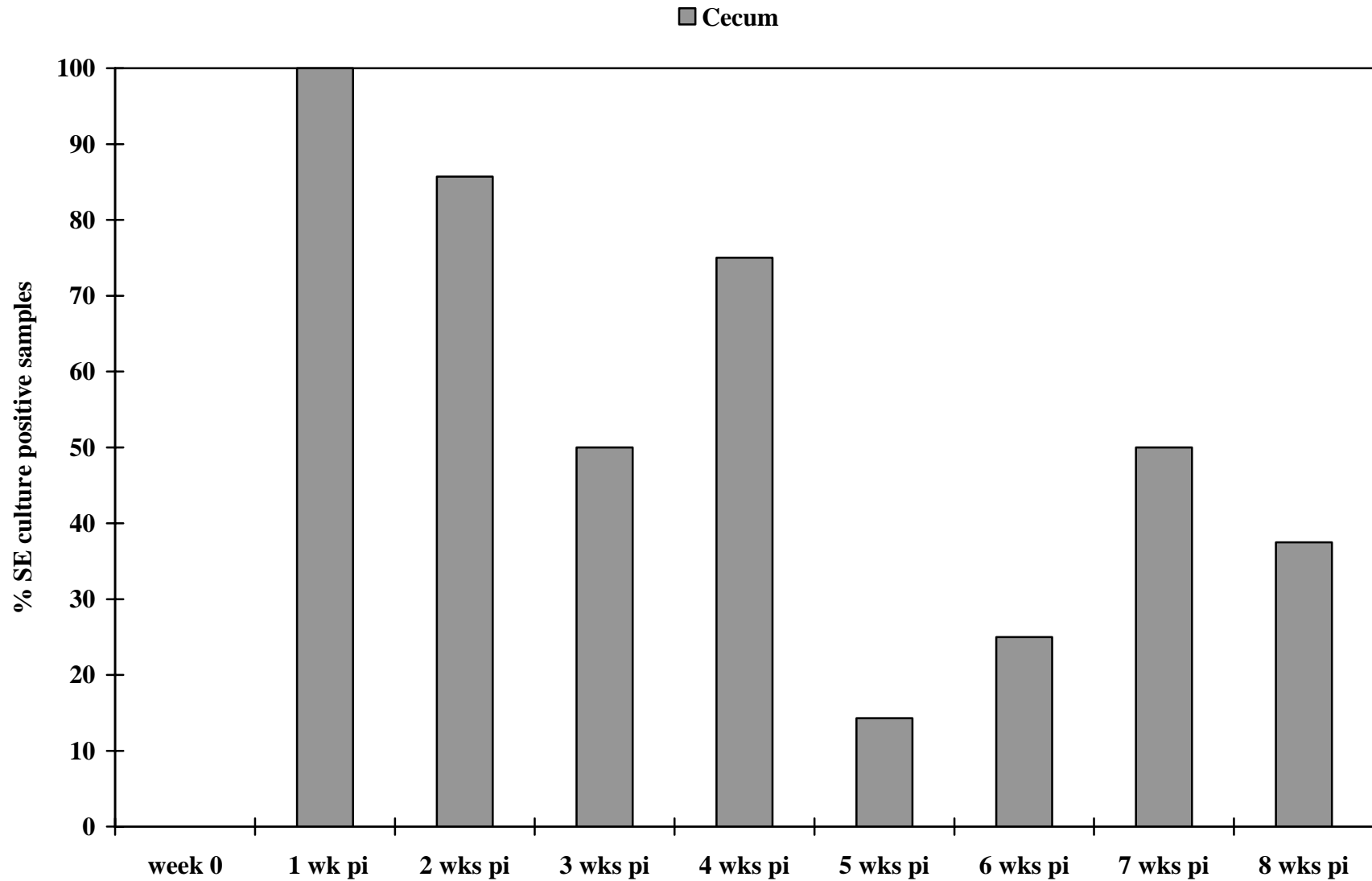


Figure 5.2. Trial 2: Comparison of percentage *Salmonella* Enteritidis (SE) culture positive cecum samples at weekly intervals from specific pathogen free (SPF) White Leghorn (WL) chickens post-SE infection (pi). Different birds (n=8) sampled weekly.

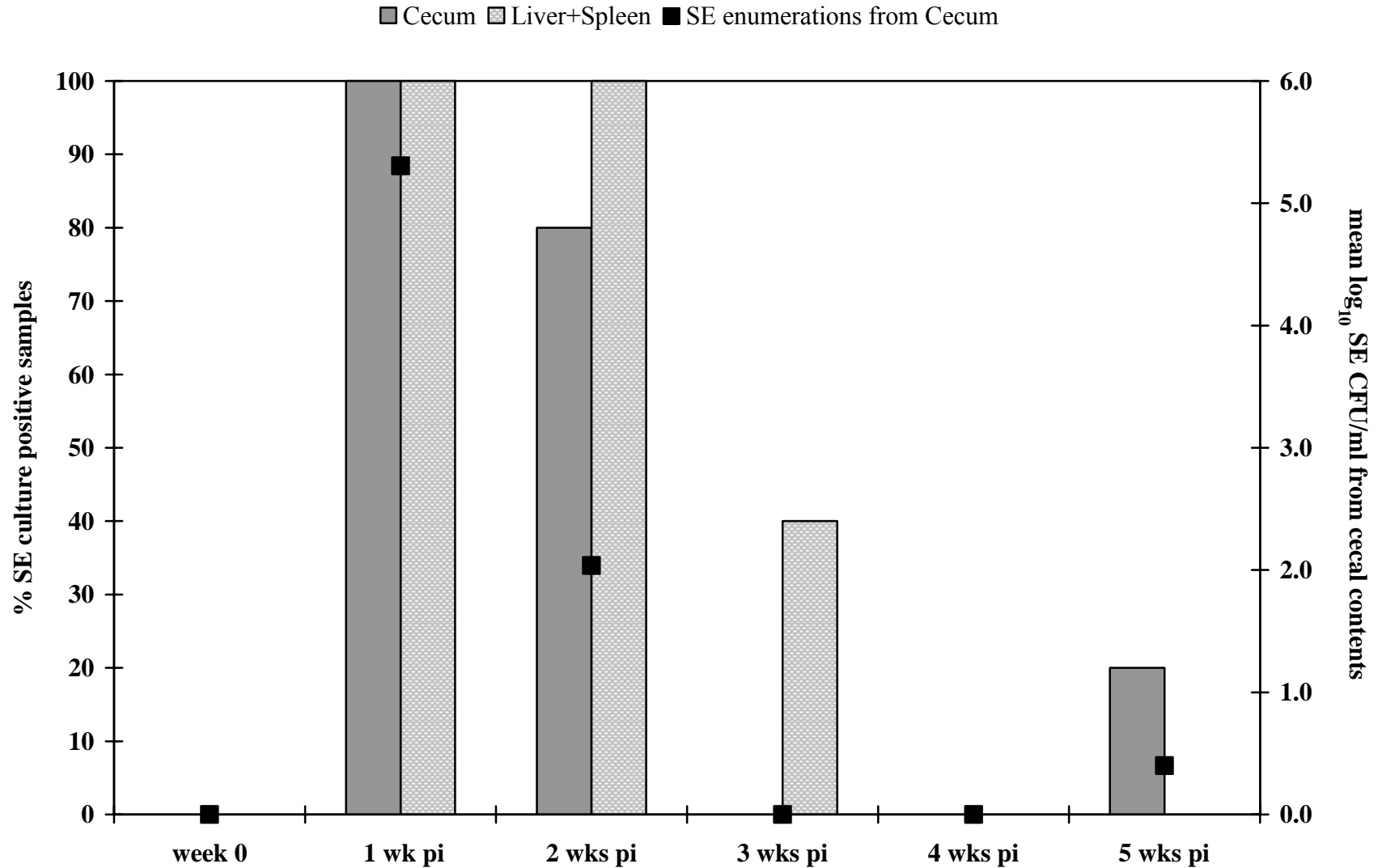


Figure 5.3. Trial 3: Comparison of percentage *Salmonella* Enteritidis (SE) culture positive cecum samples, SE enumerations (mean log₁₀ SE CFU/ml) from cecal contents, and percentage of SE culture positive liver & spleen organ samples at weekly intervals for specific pathogen free (SPF) White Leghorn (WL) chickens post-SE infection (pi). Different birds (n=5) sampled weekly.

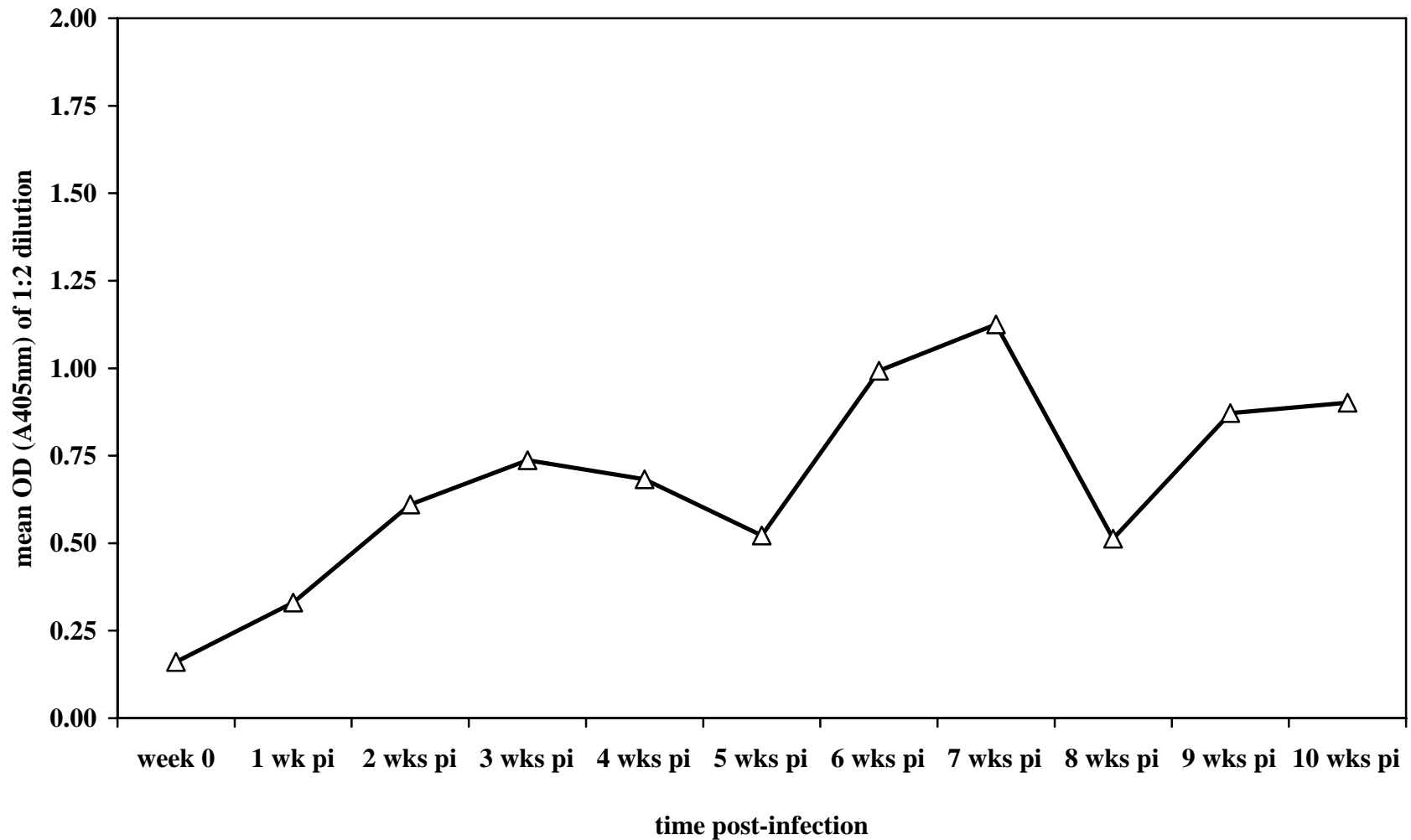


Figure 5.4. Trial 1: Enzyme-linked immunosorbent assay (ELISA) analyses of SE-LPS-specific IgA in crop lavage samples from specific pathogen free (SPF) White Leghorn (WL) chickens following *Salmonella* Enteritidis (SE) infection (pi). Mean optical density (OD) values are representative of SE-LPS-specific IgA detected in 1:2 dilution crop lavage supernatant samples at week 0 pre-infection then at weekly intervals post-SE infection. Different birds (n=8) were sampled at the weekly time points.

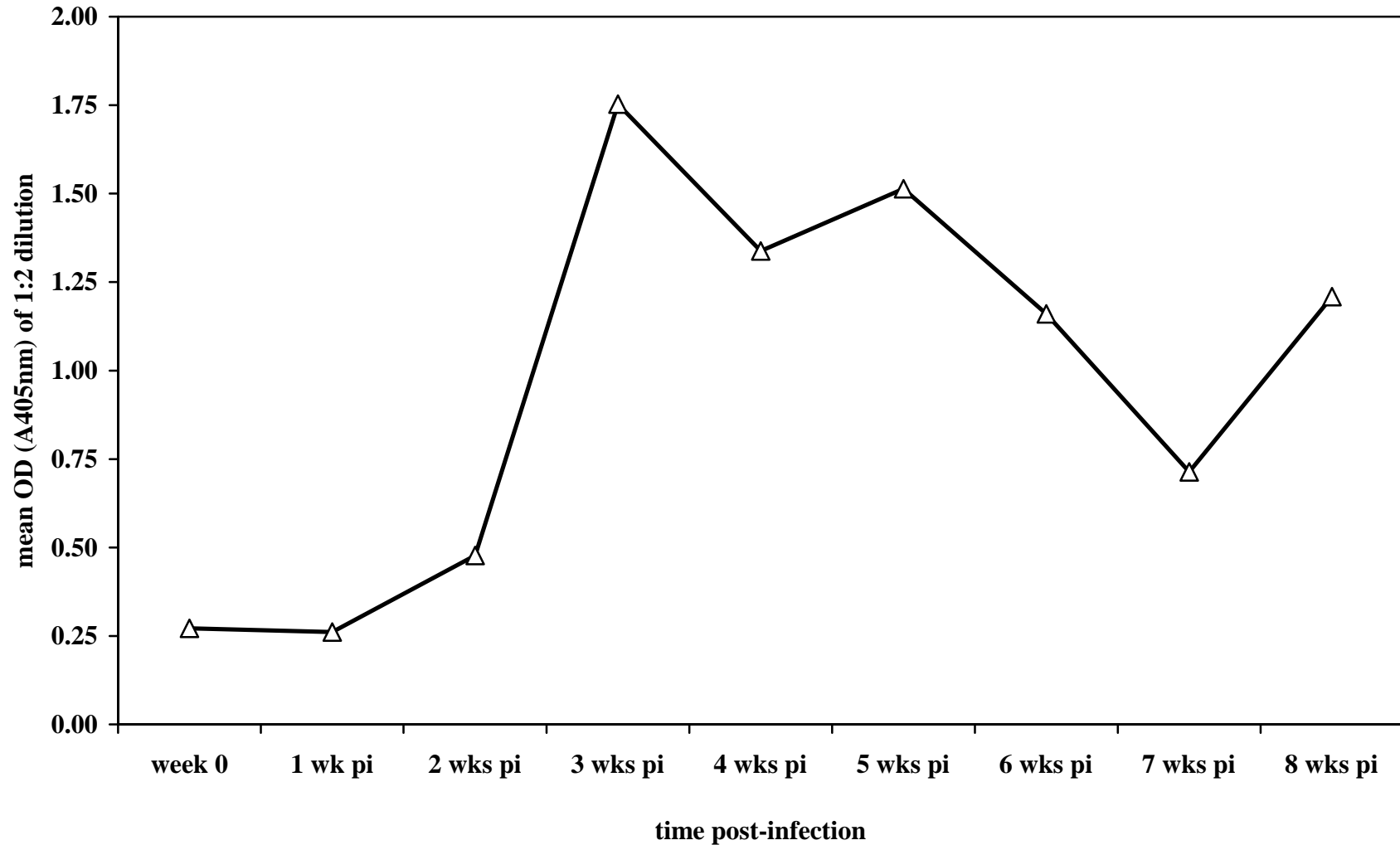


Figure 5.5. Trial 2: Enzyme-linked immunosorbent assay (ELISA) analyses of SE-LPS-specific IgA in crop lavage samples from specific pathogen free (SPF) White Leghorn (WL) chickens following *Salmonella* Enteritidis (SE) infection (pi). Mean optical density (OD) values are representative of SE-LPS-specific IgA detected in 1:2 dilution crop lavage supernatant samples at week 0 pre-infection then at weekly intervals post-SE infection. Different birds (n=7) were sampled at the weekly time points.

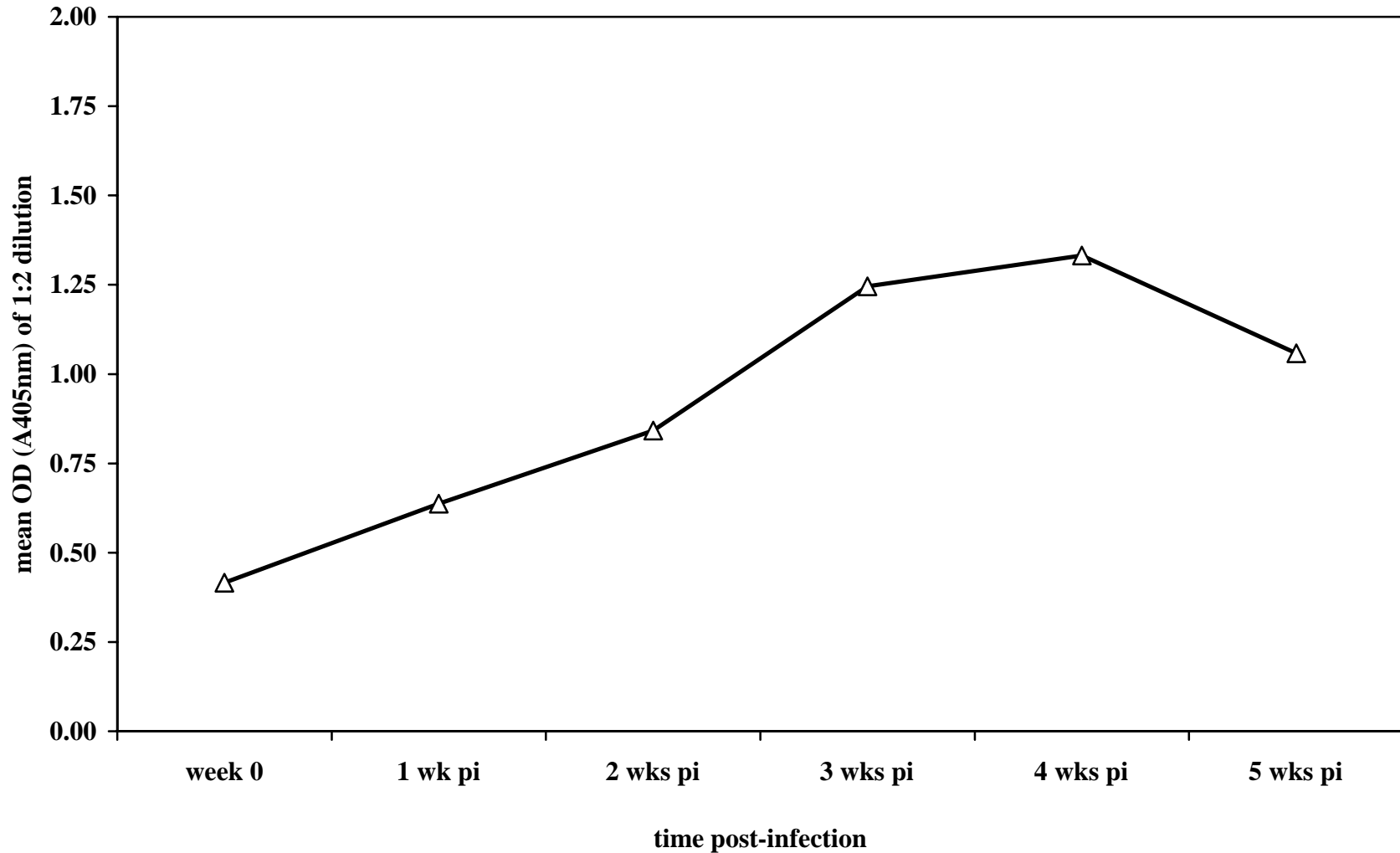


Figure 5.6. Trial 3: Enzyme-linked immunosorbent assay (ELISA) analyses of SE-LPS-specific IgA in crop lavage samples from specific pathogen free (SPF) White Leghorn (WL) chickens following *Salmonella* Enteritidis (SE) infection (pi). Mean optical density (OD) values are representative of SE-LPS-specific IgA detected in 1:2 dilution crop lavage supernatant samples at week 0 pre-infection then at weekly intervals post-SE infection. Different birds (n=5) were sampled at the weekly time points.

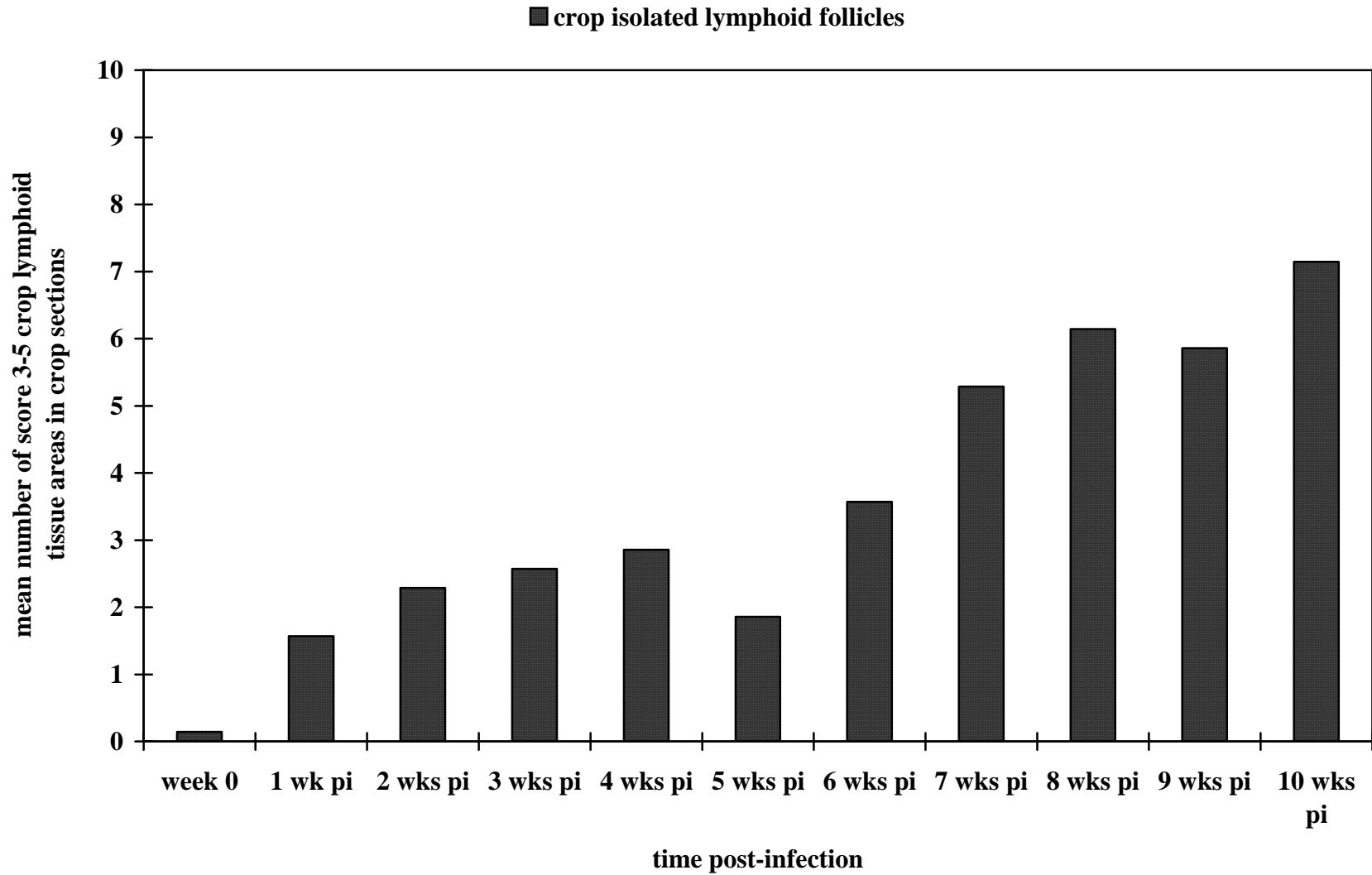


Figure 5.7. Trial 1: Comparison of the average number of score 3-5 lymphoid tissue areas assessed in hematoxylin-and-eosin (H&E) stained crop sections prepared from week 0 pre-infection controls and post-*Salmonella* Enteritidis (SE) infected chickens.

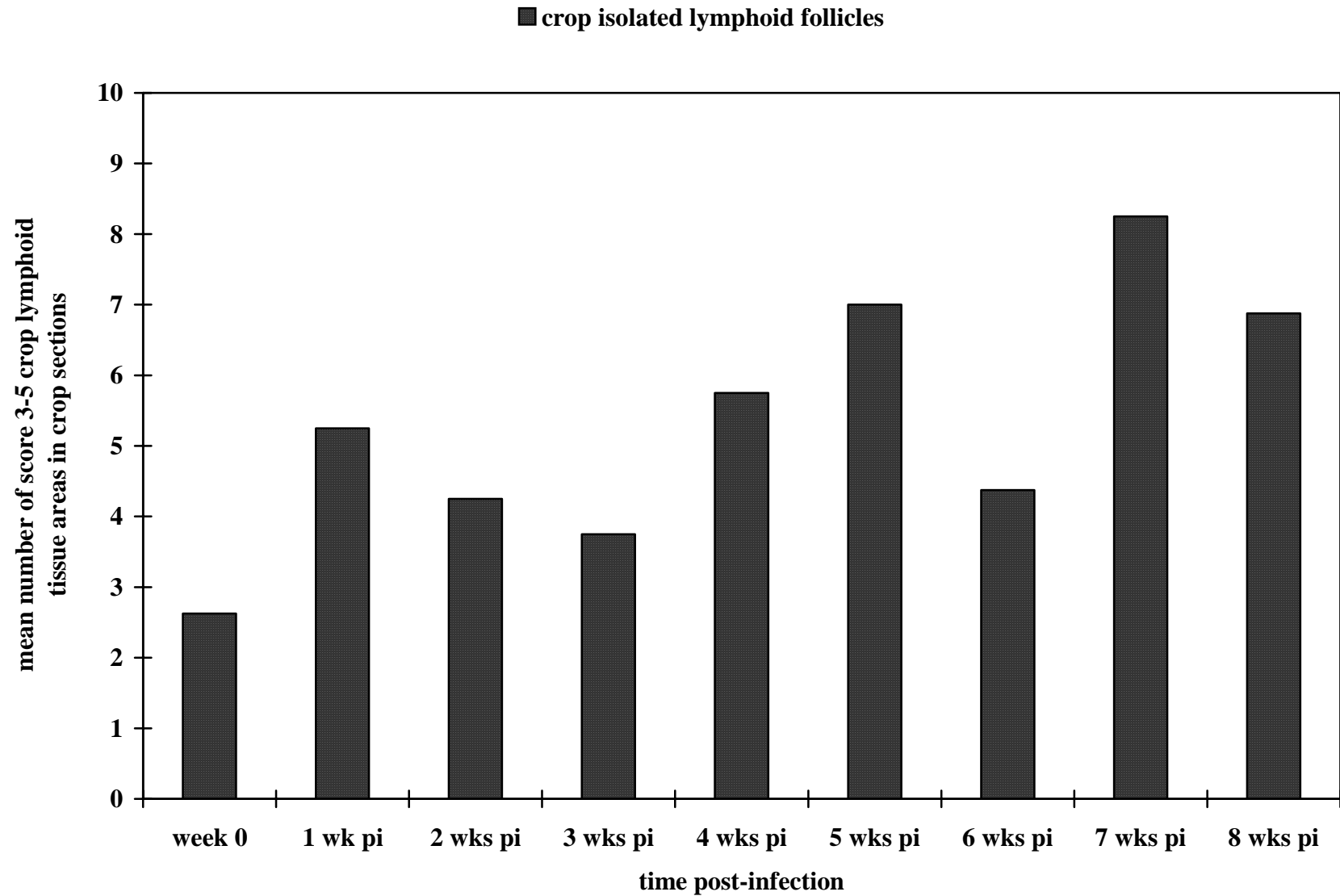


Figure 5.8. Trial 2: Comparison of the average number of score 3-5 lymphoid tissue areas observed in hematoxylin-and-eosin (H&E) stained crop sections prepared from week 0 pre-infection controls and post-*Salmonella* Enteritidis (SE) infected chickens.

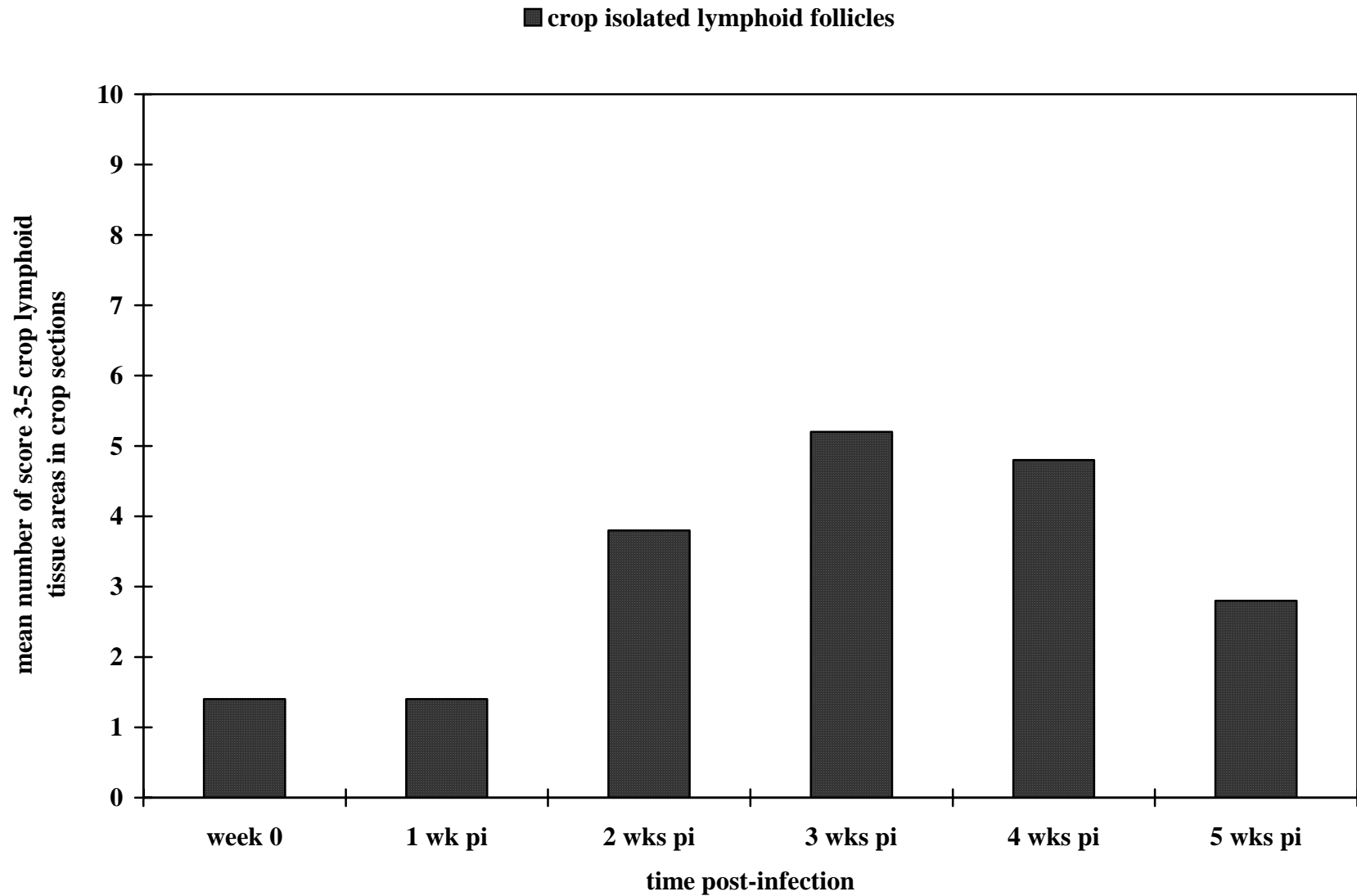


Figure 5.9. Trial 3: Comparison of the average number of score 3-5 lymphoid tissue areas observed in hematoxylin-and-eosin (H&E) stained crop sections prepared from week 0 pre-infection controls and post-*Salmonella* Enteritidis (SE) infected chickens.

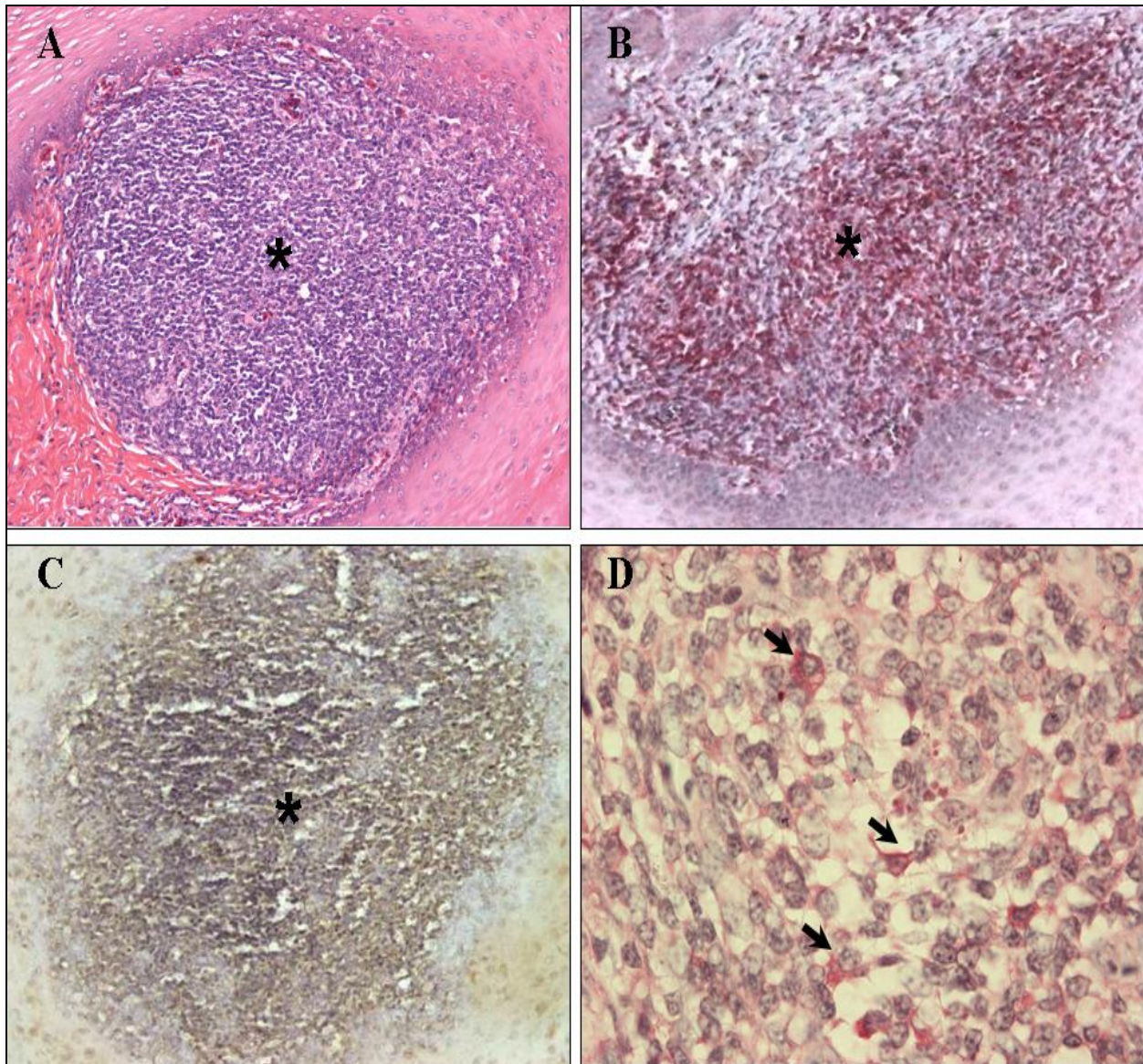


Figure 5.10. General histochemical and immunohistochemical (IHC) staining of lymphoid follicles within the chicken crop post-*Salmonella* Enteritidis (SE) infection. Areas of positive staining are indicated by asterisks * or by arrows. **(A)** Hematoxylin and eosin (H&E) staining of an isolated lymphoid follicle (ILF) within the lamina propria of crop. **(B)** IHC staining of CD45 pan-leukocyte positive cells in the follicular structure. **(C)** IHC staining of Bu-1 positive cells indicative of B-lymphocytes within the follicle. **(D)** Methyl green-pyronin (MGP) staining for pyroninophilic cells indicative of plasma cells within an isolated lymphoid follicle of crop.

CHAPTER 6

CONCLUSION

Salmonella Enteritidis (SE) is a gram-negative, enteroinvasive bacterial pathogen that infects a broad host range of animal species (23). SE infection may cause gastroenteritis in humans, a condition referred to as Salmonellosis (4). SE infection in adult poultry most often creates only a mild to subclinical disease state. Chickens infected with SE may become carriers of the pathogen and show no overt clinical signs (16, 20). In numerous human salmonellosis cases, SE contaminated poultry meat, eggs and poultry by-products have been implicated as sources of the food-borne illness.

SE tends to have a propensity to colonize the ileum and ceca of chickens, and a common sequela of intestinal SE infection and invasion is that SE organisms will disseminate to other internal organs, such as the liver, spleen, and the reproductive tract. Additionally, the chicken crop has been documented as an organ that may harbor viable SE (1-3, 5, 7, 15). SE colonization of the gastrointestinal tract, with active fecal shedding of SE, may result in on-egg contamination to the exterior egg shell (13, 14). Egg-layer hens with SE infection of the reproductive tract may produce eggs internally contaminated with SE, referred to as in-egg contamination (14, 22). Rupture of crop or lower alimentary tract during evisceration at the processing facility may lead to *Salmonella* contamination of poultry meat (1, 3, 5, 15). Thus, poultry with SE infection of the intestinal and reproductive tracts pose a threat to food-safety.

In an effort to obtain more knowledge about the behavior of SE in poultry and to try to identify strategies that may ultimately help reduce incidences of food-borne illness related to SE, my research focused on SE pathogenesis in egg-layer chickens and on the study of the mucosal

immune system of the avian gastrointestinal tract. The chicken crop (ingluvies) of the upper alimentary tract was selected to monitor SE infection and to investigate gut-associated lymphoid tissue (GALT) and mucosal immune response against SE within the crop organ.

Objectives of the research were to: (1) Investigate the mucosal immunological response as it related to IgA and lymphoid tissue development in the crop of chickens after experimental oral challenge with the enteroinvasive bacterial pathogen *Salmonella enterica* subspecies *enterica* serovar Enteritidis (SE); (2) Compare the kinetics of the SE-specific IgA antibody response and lymphoid tissue presence within the crop of specific pathogen free (SPF) White Leghorn (WL) chickens and various commercial strains of egg-layer chickens after oral *Salmonella* Enteritidis challenge; (3) Evaluate the evolution of crop immune parameters after primary SE-infection and secondary SE re-infection, and assess the immune response long-term post-SE infection for SPF WL chickens; and (4) Identify the cellular repertoire of crop lymphoid tissue areas using general histochemical and immunohistochemical (IHC) staining methods, and determine if there was a correlation between IgA response within the crop and the presence of cellular populations (i.e., B-lymphocytes and plasma cells) capable of producing the humoral response locally.

Results revealed that: (1) Post-*Salmonella* Enteritidis (SE) infection, a SE-antigen-specific IgA antibody response was detectable within the crop. Microscopy examination of H&E stained crop sections revealed lymphoid tissue aggregates in the crop lamina propria. Gut-associated lymphoid tissue (GALT) sites were observed in the form of isolated lymphoid follicles (ILFs). SE infection appeared to stimulate gut-associated lymphoid tissue (GALT) expansion and/or *de novo* GALT development within the crop organ; (2) Comparable SE-specific IgA antibody responses were found post-SE infection within the crop of SPF WL hens

and the commercial egg-layer hen strains. Crop SE-specific IgA increased by 1-2 weeks post-SE infection. The crop IgA SE-specific response was elevated at 2-3 weeks pi, a slight plateau was observed at approximately 3-5 weeks pi, and then gradually over time the humoral response waned but remained greater than the pre-infection baseline. GALT isolated lymphoid follicles (ILFs) were observed within the lamina propria of crop sections from the SE-infected egg-layer hen groups; (3) At 1 week pi following both primary SE-infection and secondary SE re-infection, a rise in IgA antibody response specific against SE-LPS antigen was detected in crop lavage samples. Primary oral ingestion of the SE pathogen resulted in selective induction of SE-LPS-specific IgA humoral response within the chicken crop. Resurgence of SE-LPS-specific IgA antibody response after a second experimental bacterial exposure implied that recall response associated with the SE antigen occurred. Long-term assessment of crop humoral response revealed an increase in SE-LPS-specific IgA by 1-2 weeks pi and the SE-LPS-specific IgA response remained greater than pre-infection baseline through 11 weeks pi. Mucosal antibody response within the crop tended to increase, plateau and then decrease with extended time following SE oral challenge; and (4) Immunohistochemical (IHC) staining of serial crop sections, identified CD45 pan-leukocyte positive cells and the presence of Bu-1 positive B-lymphocytes within the lymphoid tissue areas. Methyl green-pyronin (MGP) staining demonstrated plasma cells within and at periphery of the crop lymphoid follicles. Therefore, cellular populations do exist within the crop that may secrete a portion of the SE-specific IgA locally.

Data indicate that infection with SE via oral transmission can stimulate a mucosal IgA immune response (18, 19). The presence of viable SE, increased SE-LPS-specific IgA, and lymphoid tissue within crop of SE-challenged chickens appears to demonstrate that a local

mucosal immune response to the SE pathogen can be elicited in the chicken crop. The inclusion of crop lavage sampling for analysis of SE-specific IgA levels could assist in determining SE exposure status of a flock. The SE-infected chickens and uninfected controls in our study groups could be distinguished immunologically by comparison of the SE-LPS-specific IgA humoral response. Thus, mucosal secretions within the chicken crop may have potential as a diagnostic tool. Salivary IgA from humans has been used successfully to indicate infection status and humoral immune response to bacterial and viral agents (6, 8, 10, 21). Herath (6) analyzed human salivary samples by ELISA for IgA against *Salmonella* Typhi and the human patient groups that were *Salmonella* Typhi positive, convalescent or negative/healthy could be differentiated based on antibody level in saliva.

Crop SE-LPS-specific IgA ELISA may be useful as an additional aid to identify a flock that is positive for subclinical SE carrier hens that are not actively shedding SE in feces or shedding SE at a low level not detectable by culture. Birds that appear healthy with no overt signs of disease may actually be infected with *Salmonella* Enteritidis (SE). Carrier hens of SE are persistently infected but intermittently shed the bacterium in feces, thus SE may not be recoverable by culture at the time when fecal or environmental sample collection occurred (12). A flock might erroneously be given SE-free status when in fact undetected positive carrier hens were present.

SE infected chickens, actively or periodically shedding SE in excrement, might also have SE colonization within the crop, as SE has been readily recovered from crop lavage samples in experimental challenge studies (7). The crop may potentially serve as a reservoir source for environmental contamination and subsequent spread of SE through a flock. Culture results obtained from crop lavage samples might yield information to the poultry producer about the

behavior of SE crop colonization and/or SE persistence in varying age groups of chickens in an integrated poultry operation.

Implementing the combined bacterial testing for *Salmonella* Enteritidis (SE) from feces, cloacal swabs, the poultry environment, as well as from crop lavages may improve SE detection and provide researchers with more in-depth knowledge of SE pathogenesis. Acquiring additional knowledge about the behavior of SE in the alimentary tract of chickens and the environment might ultimately help to identify critical control points where procedures still need to be established to halt dissemination of SE within a poultry facility.

Crop lavage is an inexpensive, noninvasive, easy sampling method to perform. Birds do not have to be sacrificed to obtain crop lavage samples, thus chickens can be sampled repeatedly. The crop should perhaps be viewed as a prospective site to monitor SE infection and the mucosal immune response due to the feasibility of performing non-traumatic sample collections repeatedly over time, and because a single crop lavage sample can be used for both bacterial culture and for immunoassays. Incorporation of crop lavage to the list of standard samples harvested for surveillance of SE could perhaps assist in earlier detection of an SE-infected flock. A more complete picture of a flock's humoral immunity against SE might be provided by immunoassay analyses of crop and sera for SE-specific IgA and IgG, respectively. Crop lavage samples appear to have potential to be used as an investigational aid to detect and monitor progression of SE enteric infection, and to assess the alimentary tract mucosal immune response of flocks against SE or to *Salmonella* vaccines.

A large fraction of SE-specific IgA may be generated locally by the isolated lymphoid follicles (ILFs) within the crop organ, and a contributing portion of SE-specific IgA might be produced by regional lymphoid tissues such as the Harderian gland of the eye (17), the

oropharyngeal tonsil (11) and the organized lymphoid tissue at esophagus-proventriculus junction (9). The crop may be a site to evaluate a local mucosal immune response produced directly by the crop organ, and the crop may also function as a site where the general mucosal immune status of the upper alimentary tract could be assessed.

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