

The Effect of Social Stress and Vitamin C on Immunity and Response to  
Vaccination with Hemorrhagic Enteritis Virus in Turkeys

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## (ABSTRACT)

Hemorrhagic Enteritis (HE) vaccine is perhaps the most commonly used vaccine in the turkey industry. Although it provides protection against clinical disease, the vaccine is still thought to produce transient immunosuppression. In the field, HE still remains a significant concern for turkey producers.

Research conducted over the years has shown that management stressors such as movement of turkeys from brooding to finishing environments and the timing of these stressors may influence the short-term response to vaccination. Strategic stress application may be of benefit in the optimization of protective responses and the development of vaccination protocols without detrimental effects on performance. Ascorbic acid may also have important implications on social stress and may play a role in immunity and response to HE vaccination in turkeys.

Trials were conducted to examine the interrelationship among social stress, nutrition (vitamin C), immunity and their influence on response to hemorrhagic enteritis virus (HEV) vaccination.

Stress is unavoidable, however if it is managed properly, it can be beneficial. In this dissertation, it was first demonstrated that stress in the form of social disruption can have negative physiological and immunological effects on turkey poults and that these effects can be alleviated with the addition of 300mg/kg vitamin C to the diet. Secondly, it was also demonstrated that when stress is applied on the day of vaccination, response to HEV vaccination can be improved. Thirdly, vitamin C supplementation at 300mg/kg can improve responses to HEV vaccination. However, it was concluded that vitamin C supplementation during periods of simultaneous stress application and vaccination does not provide benefit to response to vaccination.

**"The person with a fixed goal, a clear picture of her desire, or an ideal always before her, causes it, through repetition, to be buried deeply in her subconscious mind and is thus enabled, thanks to its generative and sustaining power, to realize her goal in a minimum of time and with a minimum of physical effort. Just pursue the thought unceasingly. Step by step you will achieve realization, for all your faculties and powers become directed to that end."**

**~ adapted from Claude M. Bristol**

To Mom, Dad, Fiona, and Anaiah

You have been God's gracious gift to accompany me on a most enduring yet fulfilling journey-

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“Stress certainly is one of the most grandly imprecise terms in the lexicon of science. However like sin which also means different things to different people, it is probably here to stay, because it is a short, emotionally charged word for something that otherwise takes many words to say” Ganong (1963)

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# Chapter 1

## Literature Review

### 1.1 Scope of the problem

Hemorrhagic Enteritis (HE) is an acute viral disease of economic importance that affects turkeys 4-weeks-of-age or older. It is caused by hemorrhagic enteritis virus (HEV) and is characterized by bloody droppings, depression and death. Clinical signs usually persist in affected flocks for seven to 10 days (Pierson and Fitzgerald, 2003). Due to the immunosuppressive nature of HE, secondary bacterial infections may extend the course of the disease for an additional two to three weeks (Pierson and Fitzgerald, 2003). The mortality rate varies between < 1 and 60% (Rautenschlein, 2004). HE is usually vaccinated against around three to four weeks-of-age via the drinking water using an avirulent vaccine strain of HEV (Domermuth et al., 1977). Although it provides protection, the vaccine is still thought to cause transient immunosuppression.

Turkeys in most commercial environments can experience a variety of environmental, pathological, or nutritional stressors. One stressor that poultry are subjected to is social interaction competition (within species and with humans). Stress has generally been regarded as something “bad”, however some researchers feel that a certain amount of stress (‘optimum stress’) appears to be necessary for optimal performance (Gross and Siegel, 1973, 1981).

Disease-associated stress and reduced flock performance are a common cause of economic loss to the poultry industry. HEV vaccines are probably the most commonly

used vaccines in the turkey industry today. However, there are rare occasions, where flocks with “classical” HE lesions and increased mortality are seen (Pierson and Fitzgerald, 2003). HE still remains a significant concern for turkey producers. Observations in the field indicate that HE may be involved in multi-factorial disease and may be related to vaccination failures, interference with other vaccines, such as Newcastle disease virus (NDV) and *Bordetella avium* (BA); and stress (Sharma, 1999; Rautenschlein, 2004).

Ascorbic acid has been shown to have anti-stress properties and to improve immune responses. It is a water soluble antioxidant present in cytosolic and extracellular fluids that can directly scavenge superoxide hydroxyl radicals and singlet oxygen species (Clarkson and Thompson, 2000). Ascorbic acid is thought to exert its effects on the immune system through the enhancement of neutrophil production and through protection of the cell against superoxide radical damage (Bendich et al., 1986).

Current strategies to control enteric diseases of turkeys involve proper husbandry and management practices to reduce stress and help maintain a competent immune system. Ascorbic acid may also have important implications on social stress and may play a role in immunity and response to HE vaccination in turkeys.

## 1.2 Hemorrhagic Enteritis Virus

A thorough review of literature regarding HEV has been recently authored by Pierson and Fitzgerald, (2003) in the 10<sup>th</sup> edition of Diseases of Poultry.

Although, HE was first recognized in 1937 by Pomeroy and Fenstermacher in Minnesota, and later in 1957 by Gale and Wyne in Ohio, the pathogenesis of the disease

is still not well understood. This disease commonly referred to as “bankruptcy gut”, has reached epidemic proportions in Texas in the early 1960’s and in Virginia in the mid 1960’s (Gross and Moore, 1967). HE has been found to occur in both range and confinement turkeys and has a strong tendency to infect successive flocks on the same premises. Gross and Moore (1967) first transmitted HE with filtered and unfiltered intestinal contents of turkeys that had died from the disease. HE along with Marble Spleen Disease virus (MSDV) and avian adenosplénomegaly virus (AASV) have only been classified as to source (turkeys, chickens, pheasants), however it is common for isolates to be referred to as virulent or avirulent based on the degree of pathology they produce. (Pierson and Fitzgerald, 2003).

### 1.2.1 Characteristics of HE

HE is a disease that is caused by a lymphotropic, lymphocytopathic adenovirus, HEV. The main targets for HEV are B cells and macrophages (Suresh and Sharma, 1995, 1996, Rautenschlein et al., 1998). HEV is transmitted horizontally by the cloacal or oral route (Gross and Moore, 1967; Itakura et al., 1974) and affects turkeys four weeks-of-age or older (Pierson and Fitzgerald, 2003). Poults younger than two to four weeks-of-age are considered resistant to clinical HE (Fadly and Nazerian, 1989). This resistance is thought to correlate with the presence or absence of maternal antibodies against HEV which have been detectable for up to five weeks-of-age, and the need for target maturation (Fadly and Nazerian, 1989; Sharma, 1991).

With virulent HEV infections, clinical signs progress over a 24-hour period and usually persist in affected flocks for seven to 10 days. Affected poults usually die within

a 24 hour period or otherwise recover completely. The most prominent pathologic signs are found in the spleen and gastrointestinal tract. Dead poult appear pale due to blood loss and small intestines are usually distended, dark red to black in appearance and are filled with reddish-brown bloody contents (Pierson and Fitzgerald, 2003). The intestinal mucosa is congested and sometimes covered with a fibronecrotic membrane. Spleens of infected birds are enlarged and mottled in appearance, however, those of dead poult are smaller and less mottled probably due to blood loss. Splenic lesions present at death include hyperplasia of white pulp, necrosis and apoptosis of lymphoid cells, proliferation of enlarged reticuloendothelial (RE) cells and the presence of some intranuclear inclusion bodies (Rautenschlein et al., 2000a; Pierson and Fitzgerald, 2003). Due to the immunosuppressive nature of HE, secondary bacterial infections can extend the course of the disease for an additional two to three weeks (Pierson and Fitzgerald, 2003).

Commercially raised turkeys acquire the virus infection from the environment. The mortality caused by HE is normally low, but in some cases can reach as high as 60%. Economic losses are primarily attributed to the fact that infected turkeys perform poorly due to immunosuppression. Mortality is associated with secondary bacterial infections.

### 1.2.2 Reclassification of HEV

The *Aviadenovirus* genus contains viruses isolated from avian species (turkeys, chickens, goose, pheasant, duck, etc.). Among the 21 avian adenoviruses, there are 12 fowl, three turkey, three goose, one pheasant, and two duck serotypes (Pierson and Fitzgerald, 2003).

Initial deoxyribonucleic acid (DNA) sequence and molecular hybridization studies suggested that HEV was different from other aviadenoviruses affecting birds (Jucker et al., 1996). Frog adenovirus 1 (FAV-1), a Siadenovirus, appears to be phylogenetically similar to HEV. The phylogenetic similarity takes into account that FAV-1 and HEV have sialidase homologue genes present at the left side of their genomes (Davison et al., 2000). Therefore, in 2001, Davison and Harrach suggested moving HEV from the genus *Aviadenovirus* to a new genus *Siadenovirus*.

### 1.2.3. Chemical Composition and Ultrastructure

HEV is a non-enveloped, linear, double-stranded DNA virus. It has an icosohedral capsid with a diameter of 70-90 nm. Thin section tissue preparations that have been examined by electron microscopy indicate that HEV has a total capsomere count of 252, of which 240 are non-vertex capsomeres (hexons), occurring in empty and dense forms, and are arranged intranuclearly in loosely packed aggregates or crystalline arrays (Carlson et al., 1974; Fujiwara et al., 1975; Itakura and Carlson, 1975; Tolin and Domermuth, 1975). The remaining 12 are vertex capsomeres (penton bases). One penton fiber is present at each vertex distinguishing HEV from the family *Aviadenoviridae*, which possess two fibers at each vertex (Mc Ferran, 1997, van den Hurk, 1992).

A complete sequence (Genebank Accession number AF074946) and maps of the HEV genome have been published (Pitcovski et al., 1998; Davison et al., 2000). HEV has a full-length genome of 26,263 base pairs (bp), shorter than the DNA of any other

adenovirus described thus far (Pitcovski et al., 1998; Davison et al., 2000; Pierson and Fitzgerald, 2003). The G + C content of the genome is 34.93% (Pitcovski et al., 1998; Pierson and Fitzgerald, 2003). The genome is composed of six late genes: a 96-kD polypeptide believed to be a monomer of the outer capsid or hexon, 51/52-kD and 29-kD polypeptide believed to be the vertex penton base and fiber proteins, a 57-kD homologue of human adenovirus group 2 IIIa protein and two core nucleoproteins of 12.5-kD and 9.5-kD each (Nazerian et al., 1991; van den Hurk, 1992; Pierson and Fitzgerald, 2003).

#### 1.2.4 HEV Entry and Replication

HEV replication was first shown to take place in the RE cells (Carlson et al., 1974, Fujiwarwa et al., 1975, Itakura and Carlson, 1975, Tolin and Domermuth, 1975, Wyand et al., 1972). These were later, more clearly identified as non-adherent mononuclear cells, adherent mononuclear macrophages, and immunoglobulin (Ig) M bearing B cells, thus leading to the conclusion that these are the primary targets for HEV (Suresh and Sharma, 1995, 1996, van den Hurk, 1990).

At a cellular level, HEV replication is facilitated by the attachment by the virus penton base and fiber proteins to the target cells. Receptor mediated endocytosis. Virus uncoating occurs in the cytoplasm and is delivered to the cell nucleus. HEV replication is divided into early and late phases, the latter defined as beginning with the onset of DNA replication. During the early phase, DNA transcription takes place in the nucleus by cellular RNA polymerase II. During late stage, the viral genome is transcribed by cellular RNA polymerase III. Cellular RNA polymerase III encodes products which facilitate translation of late messenger RNAs (mRNAs). Primary transcripts are then

capped and polyadenylated. Translation of structural proteins can occur during the early transcription phase replication, or intermediate transcription phase of replication, or late transcription phase of replication. Translations of non-structural proteins occur in the early stage of replication, or late stage of replication. Structural proteins, or non-structural proteins are modified by post-translational processes, including proteolytic cleavage, or phosphorylation, or glycosylation

(<http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/00.001.htm>).

Genomic replication likewise occurs in the nucleus by a strand-displacement. Replication does not involve a reverse transcription step. Rather it involves virus encoded DNA-dependent DNA polymerase and the formation of an intermediate panhandle with base pairing occurring at the inverted terminal repeats (Davison and Harrach, 2001). Virions mature after proteolysis of some structural proteins by the virus-coded protease. Virions may provide helper functions to dependent virus during replication. Virions are then assembled and released upon degradation of the cell (Davison and Harrach, 2001; Pierson and Fitzgerald, 2003).

### 1.2.5 Effect of HEV on Immune Cells

Researchers have examined the effects of HEV infection on immune cells. B cells have been shown repeatedly to be the main target for HEV. Early studies demonstrated that chemical bursectomy protected turkeys against HEV-induced lesions and mortality (Fadly and Nazerian, 1982). Nagaraja et al., (1982a) revealed that antibody response against sheep red blood cells was lower in HEV-infected turkeys than in virus-

free turkeys. Flow cytometric analysis has also shown that HEV caused a reduction in the relative proportion of IgM<sup>+</sup> B lymphocytes in the spleen of infected turkeys on days 2, 3, and 9 post-infection (PI) (Suresh and Sharma, 1995, 1996). Lymphocyte depletion was also detected in the thymus and bursa of Fabricius (Saif, 1998).

Immunohistochemical studies have demonstrated that B cells in HEV-infected turkeys had lost or reduced expression of cell determinants as a possible result of apoptosis (Ossa et al., 1982; Rautenschlein et al., 1998; Rautenschlein and Sharma, 2000). Selective B cell deficiency induced by cyclophosphamide severely impaired HEV replication in the spleen of infected turkeys (Suresh and Sharma, 1995).

HEV has been shown to infect macrophages and reduce their functional capabilities (Rautenschlein et al., 1998). HEV infected macrophages have also been shown to undergo necrosis and apoptosis (Rautenschlein, 1998). Rautenschlein et al., (1998) observed that at the peak of infection, splenic macrophages from HEV-exposed birds did not produce nitric oxide (NO) after *ex vivo* stimulation with lipopolysaccharides (LPS) (Rautenschlein and Sharma, 1999). Some studies have shown that NO in avian and mammalian species destroy infected cells and prevent the spread of infectious agents (Pertile et al., 1995; Reiss and Komatsu, 1998). When cell culture supernatants from HEV-infected birds were tested for their NO inducing capabilities, there was an elevation in nitric oxide inducing factor (NOIF) three days PI. The NOIF activity of infected spleen cell supernatants was neutralized by a polyclonal-anti-chicken interferon-gamma (INF- $\gamma$ ) antiserum. These data suggested that INF- $\gamma$  is one of the NOIFs induced in spleens from HEV-infected birds (Rautenschlein et al., 2000a).

The effects of HEV infection on T-lymphocyte subpopulations and functions have also been studied. Flow cytometric analysis revealed that there was an increase in the relative percentage of CD4<sup>+</sup> cells in the spleen between four and six days post HEV infection (Suresh and Sharma, 1995). However, there were no differences observed in CD4<sup>+</sup> peripheral blood lymphocytes (PBLs) in HEV-infected poult (Pierson, 1993). Elevated levels of CD8<sup>+</sup> spleen cells were observed in infected turkey only on day 16 post-inoculation (Suresh and Sharma, 1995). Elevated CD8<sup>+</sup> levels were also observed in PBLs of infected poult on days eight and 10 PI (Pierson, 1993). The increase in the proportion of CD8<sup>+</sup> cells is thought to be associated with viral clearance and possibly a suppressor response. Nagaraja et al. (1982) demonstrated that cultures of whole blood from turkeys infected with virulent HEV responded poorly to the T cell mitogens, concanavalin A (ConA) and phytohemagglutinin (PHA). These mitogen responses were lower in infected birds than in virus-free birds for up to seven weeks. In another study, HEV infected birds showed a transient inhibition of the mitogenic response of whole blood to PHA (Nagaraja et al., 1985). Other studies have indicated that turkeys infected with HEV had a higher number of duodenal and mucosal mast cells than did non-infected controls and that an increase in vascular permeability was detected in HEV-inoculated birds with intestinal lesions (Opengart et al., 1992). It was speculated that T cells may release interleukin (IL) -3, a mast cell growth factor, and other cytokines that could lead to intestinal hemorrhaging (Razin et al., 1984).

HEV infection has also been reported to have an effect on cytokine production. HEV-infected splenocytes have been shown to produce elevated levels of IL-6-like factor

when cultured *in vitro* two to three days post HEV infection. HEV positive splenocytes have also been reported to release tumor necrosis factor alpha (TNF- $\alpha$ )-like factor after stimulation with ConA three days PI. Previous studies have shown that TNF- $\alpha$  may play a role in pathogenesis of HEV and may also induce apoptosis *in vivo* (Rautenschlein et al., 2000a). The massive release of TNF- $\alpha$  and IL-6 has been suggested to induce extensive hemorrhaging in the intestine of HEV-infected turkeys and that INF- $\gamma$ , IL-6, and TNF- $\alpha$  may contribute significantly to immunosuppression induced by HEV (Rautenschlein and Sharma, 2000).

### 1.2.6 HEV Pathogenesis

HEV pathogenesis is described as follows: Following oral inoculation, HEV is detected in the intestine, bursa of Fabricius, and cecal tonsils as early as one day PI (Suresh and Sharma, 1996; Rautenschlein and Sharma, 2000). HEV may replicate in these organs before entering systemic circulation and reaching the spleen to infect target cells. The virus reaches peak levels of replication in the spleen on day four PI coinciding with occurrences of hemorrhagic lesions in the intestine (Fasina and Fabricant, 1982; Suresh and Sharma, 1996; Rautenschlein and Sharma, 2000). Once HEV replicates to high levels, viremia occurs. Viral replication in B cells and macrophages induces necrosis and apoptosis of infected and possibly by-stander cells. These cytopathic effects may lead to the depletion and destruction of splenic IgM<sup>+</sup> cells (Suresh and Sharma, 1995; Rautenschlein, et al., 1998). Replication is associated with proliferation of the white pulp surrounding the ellipsoids on day three PI (Saunders et al., 1993). T cells are attracted to the spleen and increases in CD4<sup>+</sup> cells have been observed four to six days PI

possibly contributing to hyperplasia of the red pulp (Suresh and Sharma, 1995; Rautenschlein and Sharma, 2000). Plasma cells appear in the red pulp around six and seven days PI (Saunders et al., 1993). By day 10 PI, splenic architecture has returned to normal (Rautenschlein and Sharma, 2000).

There are several studies that examine the presence of viral antigen in different tissues. HEV antigen has been detected in low levels in the intestinal tract from as early as one to three and nine to 15 days PI, whereas high levels were found four to seven days PI. The bursa also contained detectable levels of viral antigen from two to seven days PI. The plasma and cellular fraction of blood also contained viral antigen at one and three days PI, respectively. In the spleen, viral antigen was detected two days PI in a few reticular cells and reached a peak at six days PI. Viremia followed splenomegaly, resulting in inflammation of the lamina propria of the small intestine. By five to seven days PI, the lamina propria had several lymphoreticular cells with intranuclear inclusions. Intestinal hemorrhaging and congestion was seen at this point (Hussain et al., 1993). From these studies, it was concluded that HEV first replicates in the lymphoid cells of the intestinal tract and bursa, and then in the spleen with viral distribution throughout the body. The highest levels of antigen are seen at mainly four to seven days PI in the lymphoid cells of various organs, and the occurrence of intestinal congestion and hemorrhaging occurs between five and seven days PI.

During HEV infection, the hemorrhages are more prominent in the duodenum than in other parts of the intestine (Domermuth and Gross, 1991). HEV infected cells are always detected in the lamina propria of the intestine and not in mucosal epithelial cells,

suggesting that degeneration and sloughing of intestinal cells is not due to the effects of the virus but to an immune system-mediated occurrence (Opengart et al., 1992; Hussain et al., 1993; Rautenschlein and Neuman, 2004; Suresh and Sharma, 1996). The appearance of intestinal lesions after infiltration of the lamina propria of the small intestine with lymphoreticular or lymphoid cells supports speculation that immune cells may be involved in pathogenesis of HE (Hussain et al., 1993; Rautenschlein and Sharma, 2000).

The pathogenesis of HEV infections has been compared between chickens and turkeys. Infected turkeys showed lesions indicative of HEV, including mottling and enlargement of the spleen, as well as hemorrhagic enteritis three to four days post inoculation. However, in infected chickens only splenomegaly was observed. The number of HEV infected spleen cells were higher in turkeys infected 24 days after hatching than in turkeys infected 13 days after hatching. This suggests that there is an age dependent difference in the susceptibility of turkey spleens to HEV (Rautenschlein et al., 1998). In chickens infected 13 days after hatching, very few HEV-positive cells found in the spleen and in the thymus; and none were observed in the bursa of Fabricius. The number of HEV-positive cells in chickens infected 13 days after hatching, were significantly lower than turkeys infected at the same age (Rautenschlein et al., 1998). Chickens infected 24 days after hatching had fewer HEV-positive cells, however they were present in the spleen, thymus and the bursa of Fabricius.

The exact mechanism of HEV immunosuppression is unclear. Several studies have been conducted looking at the immunosuppressive effects of HEV. Rautenschlein

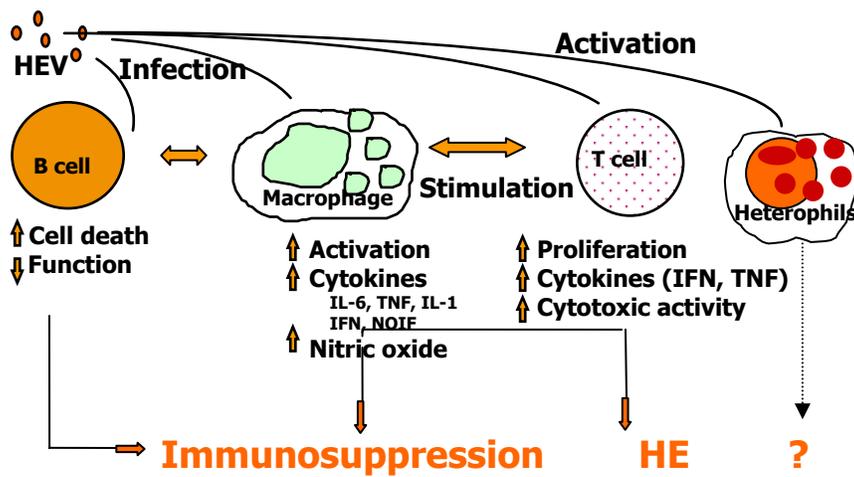
and Sharma (2000) have suggested a model that addresses HEV immunopathogenesis and its mechanism of immunosuppression. Immunosuppression is mainly due to the cell death of B cells, but also macrophage type cells. Following infection, T cells are activated, attracted to the spleen, and release various cytokines, such as interferon  $\gamma$  (Rautenschlein et al., 2000a). This replication in the spleen is associated with hyperplasia of the white pulp and mononuclear phagocytic cells leading to an increase of CD4<sup>+</sup> cells. Interferon- $\gamma$  then stimulates macrophages to release IL-6, TNF- $\alpha$ , and nitric oxide, which has modulating effects on T cell functions including stimulation of T cells to produce type II IFN and TNF- $\alpha$  (Rautenschlein et al., 2000a). It has been shown that splenocytes from HEV infected turkeys secreted type I NOIF and proinflammatory cytokines like IL-6 TNF- $\alpha$  at the peak of HEV infection (Rautenschlein et al., 2000a). The massive release of these proinflammatory cytokines may lead to systemic shock associated with HE and death and that the release of interferons may protect turkeys from the disease. It is also speculated that TNF- $\alpha$  may induce intestinal hemorrhages in turkeys exposed to HEV. Evidence for this speculation is supported by the observation that virus-induced HE was prevented by thalidomide, a potent TNF- $\alpha$  down regulatory drug (Sampaio et al., 1991). Rautenschlein et al., (2000b) also reported that HEV infection, HEV replication and virus-induced lesions were lower in S-28828 treated versus untreated turkeys given one day before or on the same day as inoculation. S-28828 is an imidazoquinolone with antiviral and antitumor activities in mammals (Sidky et al., 1992; Miller et al., 1995). It is hypothesized that inhibition of viral replication is likely to be responsible for the reduced pathologic lesions, B cell destruction and apoptosis. Other researchers have

shown that mucosal mast cells, and the vasoactive mediators contained within mucosal mast cells (histamine), may be important in the early manifestation of HEV infection (Opengart et al., 1992). These mediators provide a possible mechanism through which biochemical (decreased serum lipid and albumin concentrations) and physiologic (increased vascular permeability) changes characteristic of HEV infection can occur (Opengart et al., 1992).

Retrospective studies have been conducted to evaluate temporal relationships between seroconversion to HEV and the appearance of adenoviral inclusions in the cells of the spleen and renal epithelium. The presence of kidney inclusions was also associated with age. The probability of detecting renal inclusions increased with age up to 10 weeks-of-age and then declined at 14 weeks-of-age (Meteyer et al., 1992). Basophilic intranuclear inclusion bodies were observed in renal tubular epithelium in turkeys from six Iowa commercial flocks and three California flocks (Trampel et al., 1990). Another case reported by Trampel et al. (1992) described commercial turkeys from four Iowa, two Illinois, and three California flocks. While some of which had previously been immunized with live HEV vaccine and others had not, yet all experienced basophilic intranuclear inclusion bodies in renal tubular epithelium. These inclusions consisted of densely packed virus particles and were identified under transmission electron microscopy (TEM) as adenovirus based upon their icosohedral morphology and average particle size.

HEV infection has been shown to promote diseases caused by bacteria that are normally innocuous. A flock that is immunosuppressed due to HEV infection may

experience an increased incidence of secondary opportunistic infections, such as colibacillosis (Larsen et al., 1985; Sponenberg et al., 1985; van den Hurk et al., 1994; Pierson et al., 1996). In addition, synovitis, osteomyelitis, and green liver associated with *Escherichia coli* (*E. coli*) infection were observed in HEV-infected turkeys (Droual et al., 1996). Colibacillosis has been experimentally reported in turkeys following sequential exposure to NDV, or BA, HEV and *E. coli* (Pierson et al., 1996). Furthermore, this study demonstrated there is a positive correlation between the number of infectious agents encountered during primary exposure and the incidence of colibacillosis. Increased mortality has also been reported in turkeys that were challenged with either HEV and *E. coli* versus birds challenged either pathogen alone or negative controls (Newberry et al., 1993; van den Hurk et al., 1994). This effect was more pronounced when *E. coli* was administered intratracheally rather than by intravenous or intra air sac routes (van den Hurk et al., 1994).



**Figure 1: HEV-Immunopathogenesis:** A model HEV = hemorrhagic enteritis virus; IL = interleukin; TNF = tumor necrosis factor; IFN = interferon; NOIF = nitric oxide inducing factor; HE = hemorrhagic enteritis (Rautenschlein and Sharma, 2000; Rautenschlein, 2004).

### 1.2.7 HEV Vaccination

HEV vaccine is perhaps the most commonly used vaccine in the turkey industry. The vaccines available to the commercial poultry producers are highly effective in preventing HEV outbreaks, however they are still thought to be immunosuppressive. Turkeys are vaccinated between four and six weeks-of-age (Pierson and Fitzgerald, 2003). There are two main forms of live vaccine that are currently used. The first is a crude homogenate prepared from spleens of four to six week-old turkeys inoculated with HEV avirulent I (a.k.a. MSDV, or Domermuth strain) or HEV avirulent II (turkey origin) (Domermuth et al., 1977) via the drinking water. MSDV is a pheasant adenovirus that is non-pathogenic for turkeys, however, it is protective against HEV. Vaccination with MSDV provides full (100%) protection against mortality and morbidity caused by HEV in SPF turkeys (Sharma, 1994, Fadly et al., 1985). The other vaccine is produced *in vitro* using a lymphoblastoid cell line, MDTC-RP19 cells (Fadly et al., 1985; Pierson and Fitzgerald, 2003). A third vaccine, which is produced by propagation of avirulent virus in peripheral blood leukocytes has also been described and used in Canada (van den Hurk, 1990; Pierson and Fitzgerald, 2003).

There have also been efforts to study the effects of combined vaccination of turkeys against HEV and NDV. Dual vaccination with NDV and HEV enhances white mottling of the spleens and the apoptosis rate in turkey spleen cells (Rautenschlein and Sharma, 1999). Moreover, turkeys had fewer HEV-infected spleen cells post-vaccination than turkeys vaccinated with HEV alone. In another study, turkeys were vaccinated with MDSV tissue cultures and two MDSV spleen homogenates and were compared to HEV

vaccines. Both types of vaccine induced HEV antibodies, however, turkeys that received spleen homogenates or culture homogenates had an earlier development of antibodies versus those that were administered as tissue culture vaccines (Sharma, 1994).

*In ovo* vaccination at 24 days-of-embryonation has also been shown to induce protective immunity (Ahmad and Sharma, 1993). Other researchers reported that experimental vaccination with turkey rhinotracheitis virus four days prior to HEV vaccination suppressed HEV antibody production and compromised protection against HEV challenge (Chary et al., 2002).

Purified hexon subunit has been tested experimentally against HEV-challenge. A virus neutralizing response was induced by immunization with the native hexon, but not with the denatured protein. Turkeys that were immunized with native hexon were protected against virus induced disease and virus replication, however birds immunized with denatured hexon proteins were not protected (van den Hurk and Leittl-van den Hurk 1993). Cardona et al., (1999) examined a genetically engineered recombinant fowl poxvirus expressing the native hexon of HEV, which was shown to induce an anti-HEV humoral response in turkeys. When compared with a commercial HEV vaccine (vxHEV- a tissue culture-attenuated strain of HEV) both were shown to protect turkeys from virulent HEV challenge.

Virulent HEV and avirulent HEV have both been successfully propagated in turkey leukocyte cell cultures. In contrast to the successful infection of HEV in turkey leukocytes, the infection of chicken leukocytes with HEV or splenomegaly virus of

chickens was poor as measured by the serological response and the absence of clinical disease and HEV antigen in spleens (van den Hurk, 1990).

### HEV Vaccines

Vaccine type	Source	Propagated in	Commercially used
Non-replicating			
Full antigen inactivated	HEV/MSDV	Turkey spleen	Yes
Subunit	HEV	Turkey spleen	No
Live			
Attenuated/avirulent	HEV	MDTC RP19 cells, (PBLs), or turkey spleen	Yes
	MSDV	MDTC RP19 cells, or turkey spleen	Yes
Recombinant-FPV	HEV	Chicken fibroblasts	No

HEV = hemorrhagic enteritis virus; MSDV = marble spleen disease virus; PBL = peripheral blood leukocytes; FPV = fowl pox virus, used as vector.

**Table 1. HEV vaccines** (Rautenschlein, 2004)

## Turkey Vaccination Program

Age	Disease	Activity
1 day	TC	Turkey Bordetellosis vaccine. Live-culture <i>Bordetella avium</i> vaccine administered intranasally at the hatchery for rhinotracheitis (turkey coryza).
14 days	TC	Live-culture vaccine for rhinotracheitis administered in drinking water.
21 days	Newcastle Disease (ND)	Live-virus vaccine via drinking water.
28 days	HE	Hemorrhagic Enteritis vaccine. Live-culture vaccine (adenovirus II) of either spleen emulsion or tissue-culture origin. Administered in drinking water for hemorrhagic enteritis.
6 weeks	FC	Vaccine for fowl cholera can be either, via drinking water; or inactivated via subcutaneous injection.
8 weeks	ND	Live virus via drinking water
10 weeks	FC	Second vaccination in FC series administered as either live-culture or inactivated vaccine. This is the last vaccination in FC series for hens
12 weeks	ND	Live virus via drinking water.
14 weeks	FC	Live-culture vaccine should be used. This is the last vaccination in FC series for toms.

**Figure 2. Turkey Vaccination Program.** Adapted from Poultry Health Handbook, Fourth Edition (1994) pg. 34.

### 1.2.8 Detection of HEV

There are several methods to detect HEV. Traditionally HEV antigen is detected using agar gel immunodiffusion (AGID) or various enzyme linked immunosorbant assay (ELISA) techniques (Domermuth et al., 1973, van den Hurk, 1986, Nazerian et al., 1990; Pierson et al., 1998). Immunochemistry and immunofluorescence have also been used as a method to isolate HEV, although they are not routinely used (Fascina and Fabricant, 1982; Fitzgerald et al., 1992; Pierson et al., 1998; Rautenschlein et al., 1998). An indirect immunoperoxidase (IP) technique involving avidin-biotin peroxidase complex using a monoclonal antibody has also been developed. HEV was detected as early as 48 hours post infection up to 11 days when the experiment was terminated in frozen and formalin-fixed spleens of infected turkeys (Hussain and Nagaraja, 1993). The most recent method has been through HEV DNA by *in situ* hybridization and polymerase chain reaction (PCR) (Suresh and Sharma, 1996). Recently, a hexon gene based PCR has been developed for specific amplification of DNA sequences (Hess et al., 1999). PCR is the most useful for rapid diagnosis and epidemiological investigations of HEV infection in turkeys. MDTC-RP19 has also been used to isolate HEV (Nazerian and Fadly, 1982, 1987).

Epidemiological surveys and follow-ups of HEV infection are important factors in disease control programs. In some countries or areas where laboratory facilities are not available, blood samples and genetic material must be collected, stored and shipped to an appropriately equipped laboratory. Depending on time and distance, this can be a limiting factor. There have been methods developed to transport swab tubes, infective

viruses, transport tubes, and special monolayers (Skinner et al., 1997). Pitcovski et al. (1999) have developed a method to store viruses on filter paper without the need for prior treatment or special environmental conditions of the genetic material. Pitcovski et al., (1998) showed that HEV was able to be stored for periods of 5-30 days in this manner. HEV DNA was extracted from filter paper loaded with purified virus or crude tissue. PCR reactions were found to be of similar intensity to those of control viruses that were kept in a tube at -20°C.

### 1.2.9 Conclusion

Due to the immunosuppressive nature of HE, secondary bacterial infections are a possibility. In commercial turkey production practices, proper management and vaccination for other agents that may be exacerbated by exposure to HEV, such as BA and NDV, must also be considered into the equation for maintaining a healthy flock.

## 1.3 General Features of the Avian Immune System

A thorough review of literature regarding the avian immune system is authored by Sharma, (2003) in the 10<sup>th</sup> edition of Diseases of Poultry.

The immune system plays a big role in defending birds against invading pathogens. The primary lymphoid organs of the bird include the bursa of Fabricius and the thymus (Sharma, 1999, 2003). The bursa of Fabricius is a sac-like organ located in the region of the hind gut where B cell differentiation takes place (Sharma, 1997, 1999, 2003). The bursa of Fabricius may also serve as a secondary lymphoid organ.

Development of B lymphocytes in the bursa of Fabricius distinguishes the avian immune system from other immune systems (Glick, 1967). The thymus consists of six to seven lobes located on either side of the trachea with some extending into the anterior thoracic cavity. The thymus is the primary lymphoid organ for T cell differentiation (Sharma, 2003). There is a higher percentage of immigrant B cells in the avian thymus compared to that of other animals (Sharma 1997). Organized lymph nodes are absent in most avian species, but do have lymphoid nodules along the course of the lymphatics (Sharma, 2003).

Once cells are functional, they leave the primary lymphoid organs and migrate to the secondary lymphoid organs (spleen, bursa of Fabricius, bone marrow, Harderian gland, conjunctival associated lymphoid tissue (CALT), gut associated lymphoid tissue (GALT), and the bronchial associated lymphoid (BALT), which are the principal sites of antigen induced immune responses (Sharma, 1999, 2003). The Harderian gland, which is a distinct structure located over the eye is full of plasma cells secreting IgM and IgA (Sharma, 1997). There is also the presence of two appendix like structures (ceca) that branch from the intestines and cecal tonsils located at the ileo-cecocolic junctions (Sharma, 2003).

The mucosa associated lymphoid tissue (MALT) is the first line of defense against pathogens that enter the body via the mucosal lining of the gastrointestinal and genitourinary tract. One component of the MALT is the GALT (Sharma, 1999). The GALT in chickens is responsible for inducing immune responses against viral, parasitic, and bacterial antigens that are introduced into the body through the intestinal tract (Naqi

et al., 1984; Lillehoj and Trout, 1996; Mast and Goddeeris, 1999; Muir et al., 2000). The avian GALT consists of the cecal tonsils, bursa of Fabricius, Peyer's patches, Meckel's diverticulum, intraepithelial lymphocytes (IEL) and cells that are scattered throughout the lamina propria (Lillehoj and Trout, 1996). In addition, a number of visceral organs have diffusely scattered lymphoid cells through the body that tend to proliferate during an immune reaction (Fletcher and Barnes, 1998; Sharma, 1999, 2003).

Morphologically, avian non-lymphoid hematopoietic cells (monocytes, thrombocytes, granulocytes, and erythrocytes) are different than mammalian cells. All of these cells, including erythrocytes and thrombocytes, are nucleated making separation procedures designed for mammalian lymphocytes difficult (Sharma, 2003).

The development of the immune system of the bird begins early during embryogenesis. The thymus develops from an epithelial outgrowth of the pharyngeal pouches and the bursa of Fabricius from an outgrowth of the cloacal epithelium starting at around day five of incubation (Sharma, 2003). Bursal precursor cells can be detected in the embryo around day seven of embryonation (Sharma, 1999). IgM, IgG, and IgA can be detected by days 10, 14, and 16 of embryonation, respectively (Sharma, 1999). Lymphocytes with surface IgG develop on day 21 around the time of hatching, whereas IgA cells first appear in the intestine by three to seven days after hatching (Tizard, 1996). T cell precursor cells enter the thymus in three waves at 6.5, 12, and 18 days (Cotley et al., 1989; Sharma, 1999). Each wave lasts about two days and cells of each wave are capable of differentiating into  $T\alpha\beta$  or  $T\gamma\delta$  cells (Tizard, 1996, Sharma, 1999). Stem cells arise in the yolk sac between five and seven days of incubation. These cells differentiate

within the bursa of Fabricius and follicles develop within the bursa of Fabricius by day 14 of embryonation (Tizard, 1996). T cells with CD3 molecules appear in the embryo at nine days and those with T cell receptors (TCR) at 12 days of embryonation (Sharma, 1999).

Defense against pathogens can be divided into non-specific and specific immune mechanisms. Physical barriers such as the skin, mucosa of the airways, digestive and genital tract prevent pathogens from entering the body (Meyer et al., 1991). Non-specific defense mechanisms exist to inhibit the entrance of pathogens include enzymes, low pH, physical integrity of the epithelial lining, mucosa etc. (Sharma, 2003). For pathogens that evade these physical barriers other non-specific mechanisms such as phagocytic cells, heterophils, macrophages (Qureshi et al., 2000, Sharma, 2003) complement (Koppenheffer, 1998) and natural killer cells (Sharma and Schat, 1991) are employed. The non-specific immune mechanism is unlike the specific immune mechanism in that it does not depend on prior exposure to a particular pathogen for functioning.

“Specific” immune mechanisms (acquired immunity) are characterized by specificity, heterogeneity, and memory and are divided into cellular and humoral components.

B cells represent the humoral component of acquired immunity. B cells respond by producing antibodies against an antigen approximately five days post exposure. This lag time is due to the programming of B-cells against the antigen, followed by clonal expansion (Tizard, 1996; Sharma, 2003). When the bird is re-exposed to the antigen, it produces an anamnestic response where the bird responds quicker due to the memory of

the B cells. The activated B cells produce antibodies that attach to the antigen and may block its attachment to a specific receptor (Tizard, 1996; Sharma, 2003).

Birds produce three classes of Igs: IgM, IgA, and an IgG-like class, (Tizard, 1996; Sharma, 1999, 2003). Although avian and mammalian IgG have similar biological functions, avian IgG is larger than its mammalian counterpart and lacks a genetically encoded hinge (Sharma, 1999, 2003). Because of the differences between avian and mammalian IgG, avian IgG is often referred to as IgY (Leslie and Clem 1969; Warr et al., 1995; Magor et al., 1998, Sharma, 1999, 2003). Molecular data suggest that IgY may be the ancestral precursor of mammalian IgG and IgE (Parvari et al. 1988). As in mammals, production of IgM precedes IgG; however, IgG and IgA producing cells are derived from IgM-positive precursors. IgG is detectable five days following exposure, peaks around 3 to 3-1/2 weeks and then slowly decreases (Sharma, 2003). IgG is the main Ig in the bird that is usually measured by serological tests. As in mammals, circulating IgA is polymeric, whereas, IgA exists as a dimer in mucosal secretions. IgM appears after four to five days post exposure and then disappears by 10-12 days. IgA is considered critical for local immunity in the respiratory and digestive tracts (Sharma, 2003). It appears five days following antigen exposure and is primarily found in the gut, eyes, and respiratory tract.

T cells are the principal effector cells of specific cellular immunity. The most prominent feature of T-lymphocytes is the presence of the T cell receptor (TCR) complex on the cell surface that functions as an antigen-binding receptor (Sharma, 2003). The avian TCR is a multichain complex and consists of the TCR chains that recognize the

antigen and CD3 complex that is important for signal transduction (Sharma, 1999). The antigen binding sites are formed by two glycoprotein chains: TCR $\alpha\beta$  and TCR $\gamma\delta$ . In the adult chicken  $\gamma\delta$  T cells constitute about 20-50% of circulating lymphocytes (Sowder et al., 1988; Chen et al., 1994; Sharma, 1999).

The TCR portion of the TCR-CD3 complex binds with the antigen and the CD3 portion is important for signal transduction. The constant region of the TCR molecule is encoded by a single nonpolymorphic gene (Sharma, 1999). The variable region is encoded by rearrangement of single V, D, and J segments resulting from multiple polymorphic genes (Sharma, 1999, 2003).

In addition to TCR and CD3, T lymphocytes also express CD4 and CD8 surface markers which are indicative of their function. The functions of CD4<sup>+</sup> and CD8<sup>+</sup> cells are Major Histocompatibility Complex (MHC) restricted. CD4<sup>+</sup> T cells are characterized as T helper cells that assist during humoral and cellular immune responses (Tizard, 1996; Sharma, 1999). Upon antigen stimulation, CD4<sup>+</sup> T helper cells are stimulated to proliferate to a greater extent than do other lymphocytes. They recognize processed exogenous antigens in conjunction with MHC II (Tizard, 1996). CD8<sup>+</sup> cells act as cytotoxic/suppressor cells that recognize endogenous antigens in conjunction with MHC I (Maccubbin and Schierman, 1986; Tizard, 1996; Sharma, 2003).

Antigen presenting cells such as macrophages, dendritic cells, and B cells must share the same MHC before T cells can recognize them. The avian MHC originally described as a blood group locus (B locus) is smaller and is organized differently than mammalian MHC (Kaufman and Wallny, 1996). The B locus consists of at least three

loci: BF (encode Class I antigens), BL (encodes Class II antigens), and BG (encodes Class IV antigens). Class III genes have not yet been described in birds (Tizard, 1996). BF molecules are located on several nucleated cells. BL expression is restricted to macrophages, dendritic cells, monocytes, B cells, and activated T cells (Pink et al., 1977). BG molecules are found on lymphocytes and their precursors in the bursa and thymus (Tizard, 1996).

Mitogenic responses of peripheral T lymphocytes were shown to be poorly developed at hatch. However, they appear to develop rapidly after hatch and reach adult levels by two weeks of age (Suresh et al., 1993). Age has not been shown to be a factor in the relative proportions of CD4<sup>+</sup> and CD8<sup>+</sup> cells in the periphery or spleen of the turkey (Suresh et al., 1993; Sharma, 2003). The average percentage of CD4<sup>+</sup> T cells in the peripheral blood was 29.8; 45.0 in the thymus, and 26.3 in the spleen. The average percentage of CD8<sup>+</sup> T cells in the peripheral blood was 13.6; 53.8 in the thymus and 15.5 in the spleen (Suresh et al., 1993; Sharma, 2003).

#### 1.4 Stress

Miriam Webster defines stress as “a physical, chemical or emotional factor in disease causation”. The effects of short-term stressors adversely affect economically important parameters, such as growth rate, immune responses, fertility, hatchability, livability and feed conversion efficiency in commercial poultry (Siegel and Gross, 2000). Individual animals do not respond to a stressor in the same manner. Whether a stressor is harmful is dependent upon the body's ability or inability to respond to a threatening situation in an attempt to maintain a state of homeostasis (Siegel and Gross, 2000). The

severity of stress is determined by the bird's previous experience with the same stressor (Baines, 1996).

The effort of the body to maintain a stable environment when challenged with various stressors was first reported by Claude Bernard (1878). The physiological stress response is primarily characterized by an activation of both the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenocortical (HPA) axis. This can manifest itself as a "fight or flight response" (Cannon, 1929) resulting in increased concentrations of catecholamines (e.g. epinephrine, norepinephrine) and glucocorticoids (corticosterone) (Besedovsky et al., 1983) with the reallocation of resources to benefit survival. The "fight or flight response", also known as the sympathoadrenal response (SA), enables the animal to act immediately to a stressor. Blood flow is redirected away from non-essential organs (reproductive and gastrointestinal) toward the heart, brain and striated muscles so that the animal may respond by fighting or fleeing the threat (Cannon, 1929). The "fight or flight" response represents a short acting stress response. A non-specific response to stress includes the release of adrenal hormones (corticoids).

Selye (1950) outlined four stages of the stress response: 1) the "alarm reaction" characterized by an immediate activation of the SA in which neurons in the hypothalamus stimulate the release of epinephrine from the adrenal medulla, increasing heart rate, glucose availability, blood pressure and volume; 2) a "resistance phase" characterized by HPA activation; 3) a stage of adrenal hypertrophy, gastrointestinal ulceration, thymic and lymphoid atrophy; and 4) an "exhaustion phase" that ultimately results in death.

Recently, new hormones mediating the stress response, have been discovered, such as stresscopin and stresscopin related-peptide, which are members of the corticotropin releasing hormone (CRH)/urocortin family (Hsu and Hsueh, 2001).

Hans Selye (1936, 1950, 1963, 1976) discovered that unrelated stimuli could invoke a state of stress in an animal, activating the HPA. In this response, a stressor causes the release of CRH from the hypothalamus, which acts on the anterior pituitary to synthesize and release adrenocorticotrophic hormone (ACTH), which is then released into peripheral circulation to cause the release of glucocorticoids from the cortex of the adrenal glands.

Corticosterone is the primary glucocorticoid in the bird, which ensures that energy needed during stress is produced through gluconeogenesis to ensure immediate survival (Baines, 1996). The blood carries glucocorticoids (cortisol and corticosterone) to all cells of the body where they enter the nucleus. Steroids such as corticosterone act by binding to specific receptors in the cytoplasm which are then capable of inducing the production of tyrosine aminotransferase in the nucleus (Gross and Siegel, 1973). There they regulate the transcription of amino acids into messenger RNA (mRNA), which migrates to the mitochondria where they are translated into proteins (Siegel, 1980; Siegel and Gross, 1997). Glucocorticoids act to increase the availability of glucose by breaking down or mobilizing protein, glycogen, and fat from body reserves for energy production (Baines, 1996). The effects of corticosteroids on lymphoid tissues depend on the ability of the steroids to pass through the cell membranes, bind to specific receptors in cell cytoplasm and then bind to the DNA (Thompson and Lippman, 1974; Siegel and Gross, 2000). The

HPA is a much slower response to stressors than the SA response because the HPA response is maintained for a long duration, which may have deleterious effects on the body. It has been hypothesized that stress-induced increases in glucocorticoid levels are not protection against the source of stress itself, but against the normal defense reactions that are activated by stress and turn off mechanisms that would threaten homeostasis (Richardson, 1985).

Activation of the SA can impair cellular immunity while having no effect on humoral immunity; the HPA does the opposite. For example, immune cells express receptors for neurotransmitters. The release of norepinephrine, substance P, and vasoactive intestinal peptide causes the SA to act upon a component of the immune system in an isolated manner, whereas the HPA releases glucocorticoids directly into circulation (Lay and Wilson, 2002).

#### 1.4.1 Stress in Poultry

Domestic fowl have been used as a model to study the development of several physiological systems including the immune system. The advantage to using young domestic fowl is that they are precocial and do not require maternal interaction during incubation or post-hatch. Stress has largely been regarded as something “bad”. This is not always the case. When stress levels are too high, viral diseases and other diseases that stimulate a lymphoid response are more common (Gross and Siegel, 1980, 1981; Gross and Siegel, 1997). Such environments enhance sensitization of cell-mediated immunity, while effectiveness of cell-mediated immunity is reduced, resulting in an

increased incidence of tumors and coccidiosis (Gross, 1972, 1976, 1985). With increasing levels of stress and release of corticosterone, there is an increase in levels of superoxide radicals in polymorphs, which enhances their ability to destroy bacteria (Som et al., 1983). In contrast when stress levels are too low, resistance to opportunistic bacteria, some internal and external parasites, and some toxins are more severe (Hall et al., 1979; Gross and Siegel, 1981; Larsen et al., 1985; Brown et al., 1986). However, at an 'optimum stress level' the incidence of essentially all diseases is reduced (Gross and Siegel, 1981; Siegel and Gross, 2000).

There are several ways in which stress is measured. In the past, stress was mainly assessed by descriptive behavioral studies using indicators presumed to be related to stress (e.g. stereotypic behaviors). More recent physiological indicators of stress include endocrine changes of the HPA by measuring corticosterone, ACTH, and catecholamines. Plasma corticosterone levels do not necessarily correlate well with an animal's stress response because the rate of corticosterone absorption and utilization varies among individuals (Gross and Siegel, 1997). As corticosterone is absorbed from the plasma, the number of heterophils increases while the number of lymphocytes decreases, resulting in very little change in the total number of cells (Gross and Siegel, 1983, 1997). Therefore avian research has relied on changes in heterophil to lymphocyte (H:L) ratios, in addition to plasma corticosterone, as an indicator of stress (Gross and Siegel, 1983).

Chickens and turkeys are reared in social situations and information on their behavior is important in evaluating their production performance. They rarely adapt to social strife or a disease in progress. Individuals fail to recognize flock mates when

separated over two weeks (Guhl, 1953) making it important to keep the composition of the flock stable. The role of social organization, social stratification and the relationships between birds, social aspects and their production performance are important when they are maintained in groups (Siegel and Gross, 1973). The social hierarchy provides a flexible system because it allows priorities associated with peck-rights to be applied to a range of competitive situations. However, when the birds are prevented from establishing hierarchies this may lead to stress (Siegel and Gross, 2000).

Corticosterone has been demonstrated to be the principal free corticosteroid found in turkey serum/plasma (Brown, 1961). Wentworth and Hussein (1985) observed increases in serum corticosterone in turkey embryos increased from 0.69 to 1.26 ng/ml in the embryos between 17 and 18 days of development and a second increase to 4 ng/ml during pipping. A decrease in serum corticosterone was noted during hatching and for the first six hours after hatching. Concentrations of corticosterone in serum fluctuated as post hatch corticosterone secretion became established between one and 15 days-of-age (Wentworth and Hussein, 1985).

Much has been written on stress in poultry, however there are very few studies that observe the effect in turkeys. Brown et al., (1973) reported that cold stress increased plasma corticosterone within three hours in four week-old turkeys. El Halawani et al., (1973), Simensen et al., (1978), and McCorkle et al., (1982) observed increases in corticosterone levels in turkeys subjected to high temperature stress, *Pastuerella multocida*, and *Alcaligenes faecalis*, respectively. Treatment of turkey embryos two days prior to hatching with exogenous doses of corticosterone (540 ng/embryo) suggested

a trend toward shorter mean incubation time and significantly increased hatchability (Wentworth and Hussein, 1985). Constant light treatments have been shown to reduce feed intake and cornea heights, heavier and larger eye size (anterior to posterior diameter and horizontal width) in turkey poults (Davis and Siopes, 1985; Davis et al., 1986). Cold water and temperature have also been reported to increase plasma corticosterone levels in poults (Davis and Siopes, 1986, 1987, 1989). Dietary protein restriction in young turkeys was reported to depress growth and adrenal weight, and increase plasma corticosterone levels (Carsia and McIlroy, 1998). Huff et al., (2001a,b) reported that brief and gentle handling of turkey poults for the first 10 days of life increased body weight and influenced later susceptibility to opportunistic infections. Later, Huff et al., (2003) demonstrated that poults receiving environmental enrichment (presence of caretakers and objects) for the first 14 days after hatch had increased body weights, disease incidence disease and mortality and lower numbers of peripheral blood leukocytes when challenged with *E. coli*.

Research on social stress has mainly been performed in chickens. It has been reported that there is less strife and higher productivity in organized flocks than those kept in the process of becoming organized (Guhl and Allee, 1944; Siegel and Gross, 1973). Siegel and Gross (1965) reported that social stress seemed to increase the resistance of domestic fowl to *E. coli* infection, but not to infection with *Mycoplasma gallisepticum*. Gross (1976) also demonstrated that increased levels of social stress resulted in increased resistance to *Eimeria necatrix* infections. Siegel and Latimer (1975) discovered that moving individuals into strange flocks induces antagonistic behavior in

domestic birds, which increased stress levels. NDV infection was shown to be more invasive and attained higher titers in stressed birds (Mohamed and Hanson, 1980). Gross (1985) later demonstrated that primary cellular resistance to *Eimeria tenella* oocysts were higher in birds housed in environments of high social stress than those in low social stress environments. Social stress, such as disruption of social order on the fourth day following vaccine administration enhanced resistance to lesion formation in chickens challenged with *Eimeria tenella* oocytes compared to unvaccinated controls (Pierson, et al., 1997).

#### 1.4.2 Stress and Immune Responses

Previous research in mammals has found that stress reproducibly alters various lymphocyte functions: responsiveness to mitogens, cytokine production and cytotoxic activation (Cunnick et al., 1992). The neuroendocrine systems, predominantly the HPA axis have been shown to modulate immune system responses, and immune system-derived cytokines can modulate neuroendocrine system activities. There are several cytokines that are implicated in responses to stress. These cytokines include, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, TNF, and IFN- $\gamma$ . These cytokines have been shown to alter behavior and mood, as administration causes fatigue, decreased appetite and a lack of interest for normal activities, characteristic of sick individuals (Tizard, 1996).

TNF- $\alpha$  and IL-6 are mainly produced by monocytes. TNF-  $\alpha$  is a potent acute ACTH-releasing compound *in vivo* (Sharp et al. 1989). IL-6, which shares many of the same biological activities with TNF- $\alpha$ , also activates the HPA axis (Naitoh et al.1988) in

a dose related manner when administered intravenously. Pituitary IL-6 production is inhibited by glucocorticoids, indicating that IL-6 regulation is similar in both the neuroendocrine and immune systems (Sarlis et al., 1993).

Several deleterious effects of HPA related substances on immune function have been well documented. Besedovsky et al. (1986) discovered that there was a relationship between the immune system and the HPA axis. Both systems contained a negative feedback loop to control the immune system, such that when an animal encountered a pathogen and macrophages are activated, they release IL-1. IL-1 then caused the release of CRH from the hypothalamus to cause an increase in glucocorticoids, which down regulated macrophages to keep the immune function in check. Since then, it has become evident that not only IL-1, but also IL-6 and TNF- $\alpha$  stimulate the HPA activation in response to threats of homeostasis (Turnbull and Rivier, 1995).

## 1.5 Ascorbic Acid

A thorough review of ascorbic acid in poultry is authored by Pardue and Thaxton (1986).

Nutrition plays a significant role in the development and function of the immune system and protection against disease. Not only does the immune system benefit directly from proper nutrition, but indirectly proper nutrition will also prepare the bird for periods of stress, reducing the adverse effects of stress and enhancing recovery from stressful periods. Essential nutrients such as vitamins may affect humoral and cell-mediated immune responses in addition to several non-specific humoral factors such as lysosomes

and hormones, which regulate the immune response. The immune system of the bird can be influenced by nutrition in several ways, including anatomical development of lymphoid tissues, mucous production synthesis of immunologically active substances, cellular proliferation, cellular activation and movement, intracellular killing of pathogens, and modulation and regulation of the immune process (Butcher and Miles, 2002). One such essential chemical is ascorbic acid (vitamin C).

Ascorbic acid is a water soluble antioxidant present in cytosolic and extracellular fluid. It is present in high concentrations in leukocytes and is utilized at a higher rate during infection and phagocytosis (Thomas and Holt, 1978). Ascorbic acid can directly scavenge superoxide radicals and singlet oxygen species (Clarkson and Thompson, 2000). The quantity of superoxide radicals present in phagocytes is increased by an optimum level of ascorbic acid. Further increases in ascorbic acid result in increased reduction of the superoxide radical (Scarpa et al., 1983; Som et al., 1983; Gross et al., 1988). Ascorbic acid has also been shown to have a sparing effect on vitamin E by acting as a redox system reducing tocopheroxyl radicals back to their reduced state tocopherol (Wilson, 1983).

To increase phagocytosis of polymorphonuclear cells and macrophages, vitamin C reduces  $\text{NADP}^+$  to NADPH. NADPH acts as an electron donor in the reduction of oxygen and as a result the respiratory burst is stimulated. Free radicals generated from the respiratory burst subsequently destroy microorganisms in phagosomes (Muggli, 1993). However in contrast to stimulating the production of intracellular free radicals, vitamin C reduces the extracellular free radicals to water. Therefore modulating the

excess production of free radicals to prevent damaging of other cells and blocking the chemotactic inhibition from hydrochlorous acid. Moreover, vitamin C accelerates chemotaxis by reducing histamine production from mast cells, which was found to suppress the movement of lymphocytes (Muggli, 1993; Cunningham-Rundles et al., 1993).

Vitamin C has also been shown to stimulate the activities of lymphocytes by stimulating the chemotaxis and phagocytosis (Cunningham-Rundles et al., 1993). It is suggested that vitamin C may interact with lymphokines such as IL-2 for lymphoproliferative stimulation, since IL-2 functions are known to stimulate lymphocyte proliferation and induce INF- $\gamma$  (Cunningham-Rundles et al., 1993).

In the adrenal gland, vitamin C functions metabolically to help control the production of corticosterone. A controlled rate of corticosterone release from the adrenal cortex is preferred in coping with stress. During stress, the increase for vitamin C is increased since there is a rise in the rate of synthesis at the kidney or the rapid decreasing of storage amount is detected. For adrenal cortical depletion of corticosterone to occur would result in death of the animal. Vitamin C plays a central role in the continued synthesis of corticosterone. The proposed mechanism for this effect is through inhibition of the 21-hydroxylase enzymes in the steroid biosynthetic pathway in the adrenal cortex (Butcher and Miles, 2002). The level of histamine released from mast cells and damage tissue was also found to be reduced after vitamin C administration. Hence, the vitamin C may also be involved in reducing stress by modulating excessive inflammatory substances (Johnston et al., 1992). Furthermore, vitamin C may also be helpful in

maintaining homeostasis by enhancing cAMP and biosynthesis of epinephrine and norepinephrine which are important in regulating metabolism.

Researchers have demonstrated that fowl can be maintained on a diet which did not contain antiscorbutic properties (Shorten and Ray, 1921; Emmett and Peacock, 1922; Plimmer and Rosedale, 1923). Roy and Guha (1958) showed that under normal conditions, birds actively synthesize adequate amounts of vitamin C in normal kidneys, therefore supplementation is not necessary when the animal is managed properly. Nestor et al. (1972) reported that blood ascorbic acid concentration in female turkeys older than six months was 16.7 µg/ml. Dorr and Balloun (1976) reported that plasma ascorbic acid concentration in male turkeys less than one month to be 9.6 µg/ml. Some of the highest concentrations of vitamin C in animals can be found in the testicles, ovaries and adrenal glands (Pardue and Thaxton, 1986; Butcher and Miles, 2002).

Several stressors have been shown to alter ascorbic acid synthesis and / or utilization in the fowl. These stressors include various environmental, pathological and nutritional stressors (Pardue and Thaxton, 1986). In 1979, Kechik and Sykes reported that plasma vitamin C may be reduced during periods of stress. The release of corticosterone creates an increased demand for vitamin C for the controlled production of adrenal hormones necessary to provide a continued energy source (Baines, 1996).

The addition of ascorbic acid (from here referred to only as vitamin C) in the diet has been reported to influence various physiological parameters especially in stressed birds. In one study, it was reported that the addition of vitamin C at 330 ppm prevented a depression in blood vitamin C levels during heat stress in turkey hen breeders (Nestor et

al., 1972). Vitamin C supplementation decreased levels of plasma corticosterone in chickens subjected to high temperatures (Abdel-Wahab et al., 1975). In another study, Pardue and Thaxton (1984) demonstrated that vitamin C supplemented at 1000 ppm in the feed ameliorated the immunosuppression induced by exogenous cortisol. From that report they postulated that the amelioration of steroid mediated immunosuppression by vitamin C was due in part to protection of immunobiological tissues from the cytotoxic effects of adrenal tissues. In 1988, Gross reported that feeding a series of rations containing a broad range of concentrations of vitamin C resulted in a decrease in severity of chickens' response to heat stress and a decrease in their H:L ratios. McKee and Harrison (1995) also reported that vitamin C increased feed intake and lowered H:L ratios when subjected to multiple concurrent stressors. However, Sell et al., (1997) documented that poults that were toe-clipped, injected and debeaked immediately after hatching showed no beneficial effect on performance when supplemented with 300 mg/kg in the feed. Recent studies have also reported that vitamin C decreased heat shock protein 70 and plasma corticosterone response in chickens subjected to cyclic heat stress (Mahmoud et al., 2003, 2004).

Vitamin C is hypothesized to be important in optimum functioning of the immune system through neutrophil production and by protection against free radical damage (Bendich et al., 1986). In the first line of defense against pathogens, phagocytosis by neutrophils involves increased consumption of both dehydroascorbate and ascorbate (Rund., 1989). Macrophages contain 40 times more vitamin C than the levels present in plasma. The higher level of vitamin C is associated with the activation of membrane

transport mechanisms to engulf and kill invading organisms, yet protect the macrophage from self destruction (Baines, 1996).

Vitamin C has been demonstrated to improve immunoresponsiveness and increase disease resistance in poultry by optimizing the immune system (Pardue et al., 1985; Rund, 1989). Antibody response against *B. abortus* was lower in neonates and higher in older chickens supplemented with 1% vitamin C in the feed (McCorkle et al., 1982). Vitamin C supplemented at 1000ppm in the feed significantly increased primary agglutins to sheep red blood cells and *B. abortus* in cockerels after immunization (Pardue and Thaxton, 1984). Vitamin C supplemented chicks, vaccinated with NDV, had significantly higher antibody titers than un-supplemented controls and showed a better performance, as well as no clinical signs of the disease after viral challenge (Edrise et al. 1986). The addition of 150 ppm or 300 ppm of vitamin C to the diet ameliorated the adrenocorticotropin-mediated suppression of cutaneous basophil hypersensitivity to PHA in young chicks in a dose-dependent manner (Murray et al., 1987a). The delayed type hypersensitivity response to sensitization of chicks to human gamma globulin was enhanced by dietary vitamin C (Murray et al., 1987b). Vitamin C treatment had no effect on the plasma corticosterone levels of donors or on the spleen weight of the recipients in a graft-versus-host reaction model, using blood from vitamin C supplemented broilers injected into the chorioallantoic vein of chicken embryos (Murray et al., 1988). Gross (1988) demonstrated that increased doses of vitamin C resulted in increased susceptibility to *E. coli* infections. In another study, it was reported that the administration of 330 ppm of dietary vitamin C prior to *E. coli* challenge reduced the incidence of pericarditis in six

week-old Leghorn chickens (Gross et al., 1988). Some researchers reported that the effects of supplemental vitamin C on growth and immuno-responsiveness were related to the quality of husbandry, length of supplemental feeding, age, exogenous-endogenous balance for vitamin C and the relationship with corticosterone (van Niekerk et al., 1989). Addition of vitamin C in the diet also increased resistance to a combined NDV and *Mycoplamsa gallisepticum* infection and secondary *E. coli* infection as well as to a primary *E. coli* infection in chickens (Gross, 1992). Broiler chicks supplemented with 500 ppm vitamin C in the diet and vaccinated with NDV and *Pasteurella anatipestifer* showed a better antibody response to both the viral and the bacterial antigen than the un-supplemented controls (Franchini et al., 1994). Wu et al. (2000) reported that chickens infected with infectious bursal disease (IBD) and fed a diet supplemented with 1000 ppm vitamin C had higher numbers of CD8<sup>+</sup> T cells in the spleen and higher IgM<sup>+</sup> cells in the bursa of Fabricius. In a similar study, researchers reported that vitamin C supplementation resulted in higher ELISA titers to IBD vaccination and higher body weights than their un-supplemented counterparts (Amakye-Anim et al., 2000).

Roche recommends that in order to achieve adequate plasma levels, vitamin C should be administered at least 24 or 48 hours prior to the onset of stress and should be continued throughout the stress period, since plasma vitamin C levels decline significantly 24 hours after the discontinuation (Pardue et al., 1984).

Roche Vitamins Recommendations of dietary ascorbic acid supplementation for turkeys:

	(mg/kg of feed)
Turkey prestarter (0-3 weeks)	100-200
Turkey starter (3-6 weeks)	100-200
Turkey grower (7-12 weeks)	100-200
Turkey finisher I (13-16 weeks)	100-200
Turkey finisher II (17 weeks-market)	100-200
Turkey breeder	100-200

These supplementation guidelines are recommended in stress conditions and to enhance reproductive performance in breeders.

## 1.6 Statement of hypothesis

Based on published work and progress to date in the area on the effect of social stress and vitamin C on immunity and response to vaccination with HEV, it is hypothesized that:

1. Disruption of social order and the time in which this stressor is introduced has an immunological and physiological effect on turkeys and their response to vaccination with HEV.
2. Vitamin C may be used to counteract the effects of social stress and/or HEV vaccination.
3. Supplemental vitamin C would improve immunological and physiological responses of turkeys to concurrent application of stress and HEV vaccination.

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## Chapter 2

### The Effect of Social Stress on the Response of Turkey Poults to Vaccination with Hemorrhagic Enteritis Virus

#### 2.1 Abstract

This study investigated the effects of social stress on response to vaccination with hemorrhagic enteritis virus (HEV). Three experiments were conducted. Each used one-day-old poults. All birds were handled daily from one day-of-age to acclimate them to handling and sampling. In Experiment 1, 32 four-week-old poults were divided into four “Stress” and four “No Stress” groups of four birds each. After two weeks of social adaptation, birds in the “Stress” group were randomly re-distributed to disrupt social order and create stress for 24 hours. After 24 hours, birds were reunited with their original pen-mates. Blood samples were taken on 0, 1, 2, 4, 6, 8, and 14 days post stress (PS). Plasma corticosterone, heterophil to lymphocyte (H:L) ratios, peripheral CD4<sup>+</sup> and CD8<sup>+</sup> percentages, plasma tumor necrosis factor alpha (TNF- $\alpha$ ), and interleukin-6 (IL-6) were measured. The ratio of CD4<sup>+</sup> to CD8<sup>+</sup> (CD4:CD8) cells were calculated from CD4<sup>+</sup> and CD8<sup>+</sup> levels. Plasma corticosterone and H:L ratios were increased 1 day after the initiation of stress. CD4<sup>+</sup> and CD8<sup>+</sup> cell percentages decreased in the “Stress” groups on day 1 PS, which resulted in increased CD4:CD8 ratios on day 1 PS.

In Experiment 2, 32 six-week-old turkeys were orally inoculated with 0.5ml of 100 turkey infectious doses 50 (100 TID<sub>50</sub>) of an avirulent vaccine strain of a HEV vaccine. Poults were bled on days 0, 1, 2, 4, 6, 8, 14, and 21 post-vaccination (PV). In

addition to previously measured parameters, plasma collected on days 0, 8, 14, and 21 PV were assayed for HEV antibody titers. On day 5 PV, four birds from each group were euthanized and spleens removed and analyzed for presence of viral antigen by agar gel immunodiffusion (AGID), viral deoxyribonucleic acid (DNA) by nested polymerase chain reaction (PCR), and histopathological analysis. On day 21, the entire protocol was repeated for the remainder of the birds. Plasma corticosterone and H:L ratios were elevated on day 6 PV in the HEV positive (HEV+) group. CD8<sup>+</sup> cells were increased on day 8 PV in the HEV+ group, which resulted in decreased CD4:CD8 ratios in the HEV+ group on day 8 PV. Plasma TNF- $\alpha$  levels expressed as percent cytotoxicity were increased on days 6 and 8 PV. Plasma IL-6 levels were increased on day 8 PV in the HEV+ group. All of the HEV+ birds were found to be seropositive on days 8, 14, and 21. HEV negative (HEV-) controls were seronegative for HEV. Spleens from HEV+ birds contained intranuclear inclusion bodies in the ellipsoid cells and lymphocytes upon histopathologic examination on day 5 PV. Controls had no inclusions. On day 21 PV, spleens from HEV+ birds still had mild expansion of the ellipsoids, whereas controls were normal. HEV antigen and DNA were positive in 4 out of 4 spleens taken from the HEV+ group on day 5, but negative on day 21 PV by AGID and PCR.

In Experiment 3, 96 four-week-old turkey poults were randomly assigned to five “treatment” groups and one “No Stress” control group of sixteen birds each. Treatment was based on the time that social stress was applied in relation to vaccination with HEV (24 and 48 hours prior to vaccination, day of vaccination, and 24 and 48 hours PV). Blood and tissue samples were taken as described in Experiment 2. Plasma

corticosterone was decreased in the 24 and 48 hour group on day 6 PV. H:L ratios were only significantly different in the 0 hour group. The percentage of CD4<sup>+</sup> cells were increased on day 0 PV in all “treatment” groups except the 0 hour group. CD4<sup>+</sup> cells were increased on day 6 PV. CD4<sup>+</sup> cells were decreased by days 14 and 21 in the -24, -48, 48, and 0 hour groups. Although all groups had an increase in CD8<sup>+</sup> on day 8 PV, levels were increased in the -24 and -48 hour groups on day 21 PV and decreased in the 24 and 48 hour groups on day 14 PV. CD4:CD8 ratios were decreased in the -24, -48, 24, and 48 hour groups on day 21 PV. CD4:CD8 ratios were increased on days 0 and 2 in the 24 and 48 hour groups. Plasma IL-6 levels were decreased on day 8 in the -48 hour group and increased on day 14 in the 24 hour group and in the 48 hour group on days 14 and 21 PV. There were no statistical differences observed in regard to HEV antibody titers, however, application of stress on the day of vaccine administration (0 hour group) had the highest titers.

In summary, social reorganization stress altered physiological and immunological effects in turkeys. These studies demonstrate that vaccination with HEV can produce transient immunosuppression and other physiological effects in turkeys. Social disruption stress also altered responses to vaccination with HEV and the alteration was dependent on the timing of social stress application. Given these observations, it is likely that the effectiveness of vaccination for HEV can be improved if birds are subjected to a form of stress on the day of vaccine administration.

*Keywords:* social stress, turkeys, hemorrhagic enteritis virus, HEV

## 2.2 Introduction

Stress is a term used to describe a state characterized by a broad range of physiological and behavioral changes resulting from one or more stressors that may be external or internal in origin (Selye, 1950). The physiological stress response is primarily characterized by an activation of both the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenocortical (HPA) axis. This result can manifest itself as a “fight or flight response” (Cannon, 1929) resulting in increased concentrations of catecholamines (e.g. epinephrine, norepinephrine) and glucocorticoids (corticosterone) (Besedovsky et al., 1983) with the reallocation of resources as a benefit to increase survival. Researchers have documented that plasma corticosterone levels do not necessarily correlate well with an animal’s response because the rate of corticosterone absorption and utilization varies among individuals (Gross and Siegel, 1997). As corticosterone is absorbed from the plasma, the numbers of heterophils increase, while the numbers of lymphocytes decrease, resulting in very little change in total number of cells (Gross and Siegel, 1983, 1997). Therefore, avian research has relied on changes in heterophil to lymphocyte (H:L) ratios in addition to plasma corticosterone as an indicator of stress (Gross and Siegel, 1983).

Stress is generally regarded as something “bad”. However, this is not always the case. Higher levels of stress enhance defense against bacteria and parasites. Although there is enhanced resistance to bacterial infection, further increases will reduce defenses, implying an optimum (Gross, 1984; Gross and Siegel, 2000). It was also demonstrated that in higher stress environments susceptibility to viral infections and tumors increased

in chickens (Gross and Colmano, 1969; Mohamed and Hanson, 1980; Thompson et al., 1980). In contrast low stress environments increase susceptibility to opportunistic bacteria such as coliforms, fecal streptococci and staphylococci (Larsen et al., 1985; Siegel et al., 1987, Gross and Siegel, 2000). However, at an 'optimal stress level' birds are not highly susceptible to bacteria, parasites, viruses or tumors, and the incidence of essentially all diseases is reduced (Siegel and Gross, 1997, 2000).

Turkeys in modern environments can experience a variety of stressors such in the form of environmental, pathological, or nutritional. Environmental stressors include social interaction (within species and with humans), extreme or unusual climates, sounds or lights at high levels or of unusual nature, changes in husbandry procedures, lack of food or water, and predation (Gross and Siegel, 1997).

Chickens and turkeys are reared in social situations and rarely adapt to social strife or a disease in progress (Guhl, 1953). The role of social organization, social stratification, and the relationships between birds and their production performance are important when they are maintained in groups (Siegel and Gross, 1973). It has been reported that there is less strife and higher productivity in organized flocks than those kept in a constant state of reorganization (Guhl and Allee, 1944). Therefore, social strife can be reduced by keeping poultry in the same flock as long as possible.

There are very few studies that observe the adrenal corticosterone responses of turkeys. Brown et al., (1971) reported that cold stress increased plasma corticosterone within three hours in four-week-old turkeys. El Halawani et al., (1973), Simensen et al., (1978), and McCorkle et al., (1982) observed increases in plasma corticosterone in

turkeys subjected to temperature stress, *Pasteurella multocida*, and *Alcaligenes faecalis*, infections. Davis and Siopes (1987) reported that turkey hens subjected to chilling with cold water had increased plasma corticosterone levels. Carsia and McIlroy (1997) reported that dietary protein restriction in young turkeys depressed growth, adrenal weight, plasma aldosterone and T<sub>3</sub> levels and increased plasma corticosterone levels. Huff et al., (2001a,b) reported that brief and gentle handling of turkey poults for the first 10 days of life increased body weight and influenced later susceptibility to opportunistic infections. Huff et al., (2003) also reported that environmental enrichment (presence of caretakers and objects) increased body weight and resulted in decreased susceptibility to respiratory infections in dexamethasone and *Escherichia coli* (*E.coli*) challenged turkeys.

Most of the research on social stress has been performed in chickens. Siegel and Gross (1965) reported that social stress seemed to increase the resistance of the domestic fowl to *E.coli* infection, but not to infection by *Mycoplasma gallisepticum*. Gross and Colmano (1967) reported that resistance to *E. coli* decreased with increasing lapse of time between exposure to stress and challenge. Gross (1976) also reported that increasing levels of social stress result in increasing levels of resistance to *Eimeria necatrix*. Mohamed and Hanson (1980) showed that Newcastle disease virus (La Sota strain) infection was more invasive and attained higher titers in stressed birds. Later, Gross (1985) demonstrated that primary cellular resistance and the sensitization phase of cellular-mediated immunity to *Eimeria tenella* oocysts are higher in birds that are housed in environments of high social stress than those housed in low social stress environments.

HEV is lymphotropic, lymphocytopathic Siadenovirus that affects young turkeys four weeks-of-age or older (Davison and Harrach, 2001; Pierson and Fitzgerald, 2003). Poults younger than three to four weeks-of-age are thought to be protected against the disease due to the presence of maternal antibodies and the need for target cell maturation (Fadly and Nazerian, 1989). Hemorrhagic enteritis (HE) is generally characterized by a sudden onset of depression, distended, congested small intestine, bloody droppings, immunosuppression and death (Domermuth and Gross, 1984; Pierson and Fitzgerald, 2003). The primary mechanism of protection against HEV is cell-mediated immunity. The principal method for the control of HEV is vaccination (Domermuth et al., 1977). In the field, it is common practice to vaccinate all turkeys against HEV with a live avirulent vaccine between three and six weeks-of-age. Because of the extensive use of vaccines, the disease is only occasionally seen in the “classic form” (Pierson and Fitzgerald, 2003).

Very few studies have been conducted examining the effect of social stress or the response to HEV vaccination on physiological and immunological parameters in turkeys. Therefore the purposes of the following studies were three-fold: first, to determine the effects of social stress on immunological and physiological parameters; second, to examine the effects of HEV vaccination on the same parameters and; third, to determine whether vaccine efficacy, could be enhanced by the strategic application of a stressful stimulus.

## 2.3 Materials and Methods

### *Animals*

Day-old poults obtained from British United Turkeys of America (BUTA) (Lewisburg, WV) were used. All birds were handled daily from one-day-of-age to acclimate them to handling and sampling and the environment in order to eliminate these factors as possible contributors to stress. Birds were placed on a 12 hour light-dark cycle and given a medicated basal diet (Appendix 1) and water *ad libitum*.

### *Housing*

At four weeks-of-age, birds were placed into 48" x 54" pens with mesh raised floor (Figure 1).

### *Experimental Protocols*

***Experiment 1.*** Thirty –two four-week-old, poults were randomly distributed into four “Stress” and four “No Stress” groups of four birds each per group. After two weeks of environmental and social acclimation, those in the “Stress” groups were randomly re-assorted among pens to disrupt social disorder such that, no bird was in the same pens with any of its original mates. They remained in these unstable social settings for 24 hrs and were then reunited with their original group mates. The control groups (No Stress) were not subjected to any social disruption. Blood samples were taken on 0, 1, 2, 4, 6, 8, and 14 days post-stress (PS).

**Experiment 2.** Thirty-two, six-week-old poultts were randomly distributed into one “treatment” group and one “control” group of 16 birds each. After two weeks of adaptation, poultts in the “treatment” group were orally inoculated with 100 Turkey Infectious Doses<sub>50</sub> (TID<sub>50</sub>) of an avirulent vaccine strain of HEV (Virginia Avirulent 1 strain) (Domermuth et al., 1977). Birds in the “control” groups were not inoculated. Blood samples were taken on 0, 1, 2, 4, 6, 8, 14, and 21 days post-vaccination (PV).

**Experiment 3.** Ninety-six , four-week-old turkey poultts were randomly assigned to five “treatment” groups and one “control” group of sixteen birds each. Treatment was based on the time that social stress was applied in relation to vaccination with HEV (48 or 24 hours prior to vaccination (-48 or -24), day of vaccination (0 hours), 24 (+24) or 48 (+48) hours PV). They were placed in pens with unfamiliar pen mates, as previously described in Experiment 1. Experiment 3 was performed in three blocks due to space limitation. Each block represented two time periods and 32 birds. Blood samples were taken as described in Experiment 2 PV.

### ***Sample Analysis***

Five milliliters of blood were removed from the jugular vein of each bird using a 23-gauge needle and a five ml heparinized syringe at 0, 1, 2, 4, 6, 8, 14, and 21 days PV.

***Plasma corticosterone assay.*** Plasma was collected and measured for corticosterone using a Coat-a-Count Rat Corticosterone In-Vitro Diagnostic Test Kit (Diagnostic Products Corporation, Los Angeles, CA). Turkey plasma samples stripped with activated charcoal were used as standards for this assay. The standards were 0.78, 1.56, 3.125, 6.25, 12.5, and 25 ng/ml.

***H:L ratios.*** H:L ratios were analyzed by making microscope slides of whole blood smears and staining them with Modified Wright's Stain (1:10 vol/vol). The ratio of heterophils to lymphocytes was determined based on a 100-cell differential count.

***Flow cytometric analysis.*** Peripheral blood lymphocytes (PBLs) were removed using a modified slow spin method (Hudson and Hay, 1980): blood was centrifuged twice at 40 x g for 10 minutes with no brake at room temperature. The buffy coat containing peripheral blood leukocytes (PBLs) was collected on the second spin by gently swirling the buffy coat layer off the erythrocyte layer and then washed three times using Hanks Balanced Salt Solution (HBSS) by centrifuging twice at 120 x g for 10 minutes at 7°C with the brake on. Supernatant was removed and cells were resuspended in 5mls incomplete RPMI 1640 (Media Tech) media and enumerated on a CASY1 cell counter. Viability and purity of the cells were determined using the Trypan Blue Exclusion Assay. Once cells were enumerated, they were adjusted to a concentration of  $2 \times 10^6$  cells/ml. One hundred microliters of PBLs were placed into 12 x 75 mm tissue culture tubes and incubated with 100µl of either 5µg/100µl mouse anti-chicken CD4 (R-

PE) or 1µg/100µl mouse anti-chicken CD8 (FITC) (Southern Biotechnology Associates, Inc.). Cells were then washed with phosphate buffered solution (PBS) to remove any unbound antibodies and identified by flow cytometric analysis.

***Plasma TNF-α.*** . Plasma TNF-α was chosen as a measurable cytokine because of its involvement in the pathogenicity of HEV infection. Currently, there are no known commercial TNF-α enzyme-linked immunosorbent assay (ELISA) kits available for poultry, therefore plasma TNF-α was measured using a modified assay based on the ability of TNF-α to induce spontaneous lysis of murine L929 fibroblasts. (Gehad et al., 2002). Briefly, L929 murine fibroblast cells (ATCC, Rockville, MD), were plated at a concentration of  $2 \times 10^6$  cells/ml in 100µl RPMI 1640 with 5% FBS and 1% L-glutamine using 96-well plates. Cells were incubated overnight at 37°C in 5% CO<sub>2</sub> to allow the cells to adhere to the plate. The next day, cell supernatants were removed by gently but quickly decanting the plates without disturbing the cells. One hundred microliters of diluted plasma (1:2) samples were added to each well. Control wells received 100µl of RPMI with 5% FBS instead of diluted plasma plus 100µl of (2 µg/ml in RPMI 1640, 5% FBS) actinomycin D. Three wells that did not contain cells served as negative controls. Another 100 µl of actinomycin D was added to all wells. The cells were then incubated for 18-20 h at 37 °C in 5% CO<sub>2</sub>. Following this incubation with the samples/standards, 20 µL (10% vol) of AlamarBlue, which incorporates an oxidation-reduction (REDOX) indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth, was added to each well and the plate was

returned to the incubator for an additional 24 hours. Fluorescence was monitored at 530 nm excitation wavelength and 590 nm emission wavelength. TNF- $\alpha$  activity in plasma was expressed as percent cytotoxicity against L929 using the following equation: Percent cytotoxicity =  $(A-B) / A \cdot 100$ , where  $A$  is the fluorescence of control wells, and  $B$ , the fluorescence of sample wells. TNF- $\alpha$  results are reported as percent cytotoxicity.

***Plasma IL-6.*** Plasma IL-6 was chosen as a measurable cytokine because of its involvement in the pathogenicity of HEV infection. Currently, there are also no known commercial IL-6 ELISA kits available for poultry, therefore plasma bioactivity of IL-6 was determined by measuring the proliferation of the B9 cell line, a modified IL-6 dependent murine hybridoma (courtesy of Dr. N.C. Rath) bioassay (Helle *et al.*, 1988; Rath *et al.*, 1995; Xie *et al.*, 2000). Plasma, diluted with RPMI medium (1:10; vol/vol), was assayed using proliferation of B9 cells as a measure of IL-6 activity. One hundred microliters of diluted plasma were added to cell cultures at a concentration of  $1 \times 10^4$  cells/100 $\mu$ L per well in a 96-well plate and incubated for 72 hours. Cell proliferation was assayed using the AlamarBlue Assay, as previously described (Ahmed *et al.*, 1994). Following 72 hours of incubation with the samples/standards, 200 $\mu$ L (10% vol) of alamarBlue was added to each well and the plates were returned to the incubator for an additional 24 hours. Fluorescence was monitored at 530 nm excitation wavelength and 590 nm emission wavelength. The concentrations of IL-6 in plasma samples were calculated from a standard curve obtained using recombinant human IL-6. Standards were prepared in dilutions of human recombinant IL-6 in 10% RPMI (serially dilute

standards 1:2 to yield a final concentration of 300 pg/ml to 4.6875 pg/ml IL-6). Results were reported as IL-6 pg/ml of IL-6.

***HEV antibody titer.*** Plasma collected on days 0, 8, 14, and 21 of Experiment 2 and 3 were assayed for the presence of antibodies against HEV using ELISA (Synbiotics Corporation, San Diego, CA). The ProFlok<sup>®</sup> HE-T ELISA kit is a rapid serologic test for the detection of HE antibody in turkey serum samples. The assay is designed to measure HE antibody bound to HE antigen coated plates. Plates are read on an ELISA plate reader at 405nm-410nm.

***Histopathologic analysis.*** Four birds each from the infected and the control group in Experiment 2 and four birds from each block in Experiment 3 were euthanized via cervical dislocation on days 5 and 21 PV. One-half of each spleen was placed in 10% neutral-buffered formalin for histopathologic analysis, the other half was used to determine the presence of HEV antigen and viral DNA.

***Agar Gel Immunodiffusion (AGID).*** The presence of HEV antigen in the spleen was determined by AGID Domermuth et al., 1972, 1973; (Pierson et al., 1998) (Experiments 2 and 3). Immunodiffusion gels were prepared using 32 g sodium chloride, 0.8 g sodium azide, and 32 g agarose added to 400 ml water. The mixture was heated to just boiling with constant agitation. The liquefied gel was then divided into immunodiffusion plates (ICN Pharmaceuticals, Costa Mesa, Ca) and allowed to cool and solidify.

Wells were cut after the gel was cooled using a prefabricated immunodiffusion cutter (ICN Pharmaceuticals, Costa Mesa, Ca). Unknown splenic samples and known positive control antigen were placed in alternating wells and both placed in opposition to wells containing positive control antiserum. Plates were incubated in a humidified chamber at room temperature and read at 24-48 hours. Bands of precipitation showing lines of identity with known positive antigen were indicative of HEV infection.

***PCR analysis.*** Viral DNA (Experiments 2 and 3) was extracted from spleens and confirmed using a modified version of nested PCR (Cardona, personal comm.). Specifically, splenic samples were homogenized 50:50 v/v in sterile PBS. Next, 200-300µl of each sample was placed in microcentrifuge tubes. Then, 200µl of InstaGene™ Matrix (Bio-Rad Laboratories, Hercules, Ca) was added, and samples were vortexed for 10 seconds. Samples were placed in 56°C water bath for 10 minutes, vortexed a second time, placed in 100°C heat block for eight minutes and vortexed for an additional 10 seconds. Splenic samples were spun down at 15 K rpm for at least 5 minutes and supernatant collected to obtain the DNA extract. The appropriate mastermix containing a platinum ready-mix (Introgen, Carlsband, Ca) and each primer was calculated and mixed thoroughly. For the first round of PCR, primers HEV 14521 and HEV 14122 were used. The second round (nested) of PCR used primers nHEVF and nHEVR (Description of reaction and HEV primers are described in Appendix 1). Twenty-four (24) µl of mastermix was dispensed into each PCR tube and 1 µl of each DNA extract was added to

the appropriate tube. The tubes were then placed in a thermocycler and run. The HEV PCR program was as follows:

Phase 1: 95°C: 3 minutes initial denaturation

Phase 2: 95°C: 15 seconds

(repeat 35 x) 58°C: 15 seconds

72°C: 30 seconds

Phase 3: 72°C: 10 minutes final extension

PCR samples were then removed and stored at 4°C. One tenth (1/10<sup>th</sup>) final PCR reaction was electrophoresed on an agarose gel containing ethidium bromide, and visualized with ultraviolet light.

At the end of each experiment, all birds were euthanized by cervical dislocation.

***Statistical Analysis.*** Data were analyzed using the SAS statistical software with a repeated measures analysis of variance (RMANOVA) and a Tukey-Kramer post-hoc analysis for Experiment 3 with a p value of < 0.05. Numbers are numerically statistical unless otherwise indicated. Statistical analysis assistance was provided by Daniel Ward (statistical analyst at VA-MD Regional College of Veterinary Medicine).

## **Results**

### ***Experiment 1***

Plasma corticosterone concentrations were increased on day 1 in the “Stress” group compared to the control group (74.89 ng/ml vs. 29.51 ng/ml, respectively) and

remained elevated up to 2 days (71.88 ng/ml vs. 34.36 ng/ml, respectively) after the initiation of stress (Figure 2). Corticosterone levels returned to pre-stress levels by day 4 PS. There were no significant differences found between the groups thereafter. H:L ratios were significantly higher in the “Stress” group on day 1 PS compared to the control (0.38 vs. 0.80) and returned to pre-stress levels by day 2 (Figure 3). There were no statistical differences observed in H:L ratios after day 2 between the groups. The percentages of peripheral blood T-lymphocyte subpopulations are shown in Figures 4, 5, and 6. There was a significant decrease in the percentage of CD4<sup>+</sup> cells on day 1 PS in the “Stress” group when compared to the control group (10.00% vs. 12.44%, Figure 4). CD8<sup>+</sup> cells were also significantly decreased in the “Stress” group 1 day after the initiation of the stress (3.48% vs. 6.07%) (Figure 5). CD4:CD8 ratios were elevated in the “Stress” group (Figure 6) on day 1 PS (2.90 vs. 2.05, respectively). There was no difference in regard to plasma IL-6 or TNF- $\alpha$ , however levels were numerically higher in the “Stress” group compared to the “No Stress” group on day 1 PS (Figure 7 and 8).

## ***Experiment 2***

In Experiment 2, there was an increase in plasma corticosterone on day 6 PV in the HEV+ birds compared to the HEV- controls (20.27 ng/ml vs. 10.44 ng/ml, respectively) (Figure 9). H:L ratios were also increased in the HEV+ group on day 6 PV compared to the controls (1.16 vs. 0.49) (Figure 10). Flow cytometric analysis did not show any significant differences in regard to the percentage of CD4<sup>+</sup> cells (Figure 11). However, there was an increase in the percentage of CD8<sup>+</sup> cells (22.15% vs. 10.24%) on day 8 PV in HEV+ birds compared to the controls (Figure 12). CD4:CD8 ratios in the

HEV + group were decreased compared to the HEV- group on day 8 PV (0.25 vs. 0.53, respectively, Figure 13). Plasma TNF- $\alpha$  levels expressed as percent cytotoxicity was increased in HEV+ birds on day 6 (38.12% vs. 25.78%) and day 8 (42.19% vs. 27.34%) PV compared to the uninfected controls (Figure 14). Plasma IL-6 was also increased in HEV+ birds compared to the control (51.47 pg/ml vs. 38.21 pg/ml) on day 8 PV (Figure 15). All 12 of the HEV+ birds were found to be seropositive on days 8, 14, and 21. The negative controls were seronegative for HEV (Figure 16). HEV antigen was positive on AGID in four out of four spleens taken from the infected group at day 5, but not at day 21 PV. HEV+ spleens had intranuclear inclusion bodies in the ellipsoid cells and lymphocytes diagnostic of HEV (Figure 17) upon histopathologic examination on day 5. The negative control group had no inclusions. On day 21 post-inoculation, HEV+ spleens had mild expansion of the ellipsoids that was consistent with antigenic stimulus. Viral DNA was also confirmed in 4 out of 4 spleens taken from the HEV+ group using nested PCR at day 5, but not at day 21 (Figure 18). Viral antigen was not present in any of the spleens taken from the HEV- group at day 5 or day 21 post-inoculation. Viral DNA was also absent in the spleens using nested PCR at day 5 and 21 PV.

### ***Experiment 3***

A timeline outlining occurrences during stress and HEV vaccination are described in Figure 19. Treatment times and their respective responses to vaccination with HEV are shown in Figures 20 through 24 (Figure 20., -48, hours; Figure 21., -24 hours; Figure 22., +24 hours; Figure 23., +48 hours; Figure 24., 0 hours). Plasma corticosterone was decreased in the +24 hour group on day 6 (9.53 ng/ml) and in the +48 hour group (11.51

ng/ml) compared to the No Stress group (26.78 ng/ml) (Figures 22A, 23A). H:L ratios were only significantly different in the 0 hour group on day 4 PV compared to the No Stress group (1.71 vs. 0.64, respectively) (Figure 24B).

CD4<sup>+</sup> cell percentages were increased in the -24 hour group on day 0 (7.02% vs. 3.27%) and decreased on days 14 (2.99% vs. 5.46%) and 21 (3.76% vs. 7.09%) PV compared to the No Stress group (Figure 21C). The percentage of CD4<sup>+</sup> cells were also increased in the -48 hour group on day 0 (5.88% vs. 3.27%) and decreased on days 14 (3.11% vs. 5.46%) and 21 (3.33% vs. 7.09%) PV compared to the controls (Figure 20C). In the +24 hour group, CD4<sup>+</sup> cells were increased on days 0 (7.16% vs. 3.27%), 2 (7.22% vs. 3.07%), and 4 (12.52% vs. 5.66%) and decreased on days 6 (5.75% vs. 6.82%) and 21 (4.29% vs. 7.09%) PV compared to the No Stress group (Figure 22C). In the 48 hour group, CD4<sup>+</sup> cell percentages were increased on days 0 (7.07% vs. 3.27%), 2 (6.46% vs. 3.07%), 4 (13.40% vs. 5.66%) and decreased on days 14 (3.36% vs. 5.46%) and 21 (4.33% vs. 7.09%) PV (Figure 23C). CD4<sup>+</sup> cells were increased on day 6 (14.31% vs. 6.82) and decreased on day 14 (3.05% vs. 5.46%) in the 0 hour group (Figure 24).

The percentage of CD8<sup>+</sup> cells were increased in the -48 and -24 hour groups on day 21 (11.52% and 11.31% vs. 5.61%; Figures 20, 21D) and decreased in the +24 hour and +48 group on day 14 (4.35% and 3.73% vs. 8.63%; Figures 22, 23D) compared to the No Stress group.

CD4:CD8 ratios were decreased in the -24 hour group on days 14 (0.31 vs. 0.63) and 21 (0.33 vs. 1.27) PV (Figure 21E). Ratios were also decreased on day 21 (0.29 vs. 1.27) in the -48 hour group (Figure 20E). CD4:CD8 ratios were increased on days 0

(1.15 vs. 0.44) and 2 (1.33 vs. 0.44) and decreased on day 21 (0.47 vs. 1.27) in the +24 hour group (Figure 22E) compared to the control. Ratios were also increased on days 0 (1.32 vs. 0.44), 1 (1.50 vs. 0.67), and 2 (1.20 vs. 0.44) and decreased on day 21 (0.60 vs. 1.27) in the +48 hour group compared to the controls.

There were no differences in regard to plasma TNF- $\alpha$  levels (Figures 20-24F). Plasma IL-6 levels were decreased on day 8 in the -48 hour group (18.62 pg/ml vs. 42.87 pg/ml; Figure 20G). IL-6 levels were increased on day 14 in the +24 hour group (41.98 pg/ml vs. 17.20 pg/ml; Figure 22G) and increased in the +48 hour group on days 14 (37.99 pg/ml vs. 17.20 pg/ml) and 21 (44.48 pg/ml vs. 24.71 pg/ml; Figure 23G).

There were no differences observed in regard to HEV antibody titers (Figures 20H-24H). Although there were no statistical differences noted between experimental groups, application of stress on the day of vaccine administration produced the highest titers numerically compared to the No Stress group. Viral DNA was detected by nested PCR at 21 days post-vaccination. The number of positive results is shown in Table 1. All samples were positive on AGID on day five and negative on day 21 PV.

## **2.5 Discussion**

Turkeys are reared in social settings. They fail to recognize flock mates when separated over two weeks making it important to keep the composition of the flock stable (Guhl, 1953). Once hierarchy is established, there is resistance to change.

Several studies have shown that social competition may initiate stress responses in poultry as measured by increases in plasma corticosterone and H:L ratios (Siegel and Latimer, 1975; Seigel and Gross, 1973). Physiological and immunological changes

occurred within one day of the initiation of the stressor and returned to pre-stress levels by day 2 PS. Plasma corticosterone increased one day after the initiation of stress, however levels did not return to pre-stress levels until day 4 PS. Other researchers have also reported increases in plasma corticosterone to social stress in chickens (Gross, 1984; Hester et al., 1996; Littin and Cockrem, 2001).

H:L ratios have been widely accepted in avian research as a measure of the animal's perception of its environment over plasma corticosterone (Gross and Siegel, 1983). In the first experiment, social randomization increased H:L ratios one day after the initiation of stress and returned to pre-stress levels by day 2. This finding is consistent with other researchers who have reported that social stress resulted in increased H:L ratios (Gross and Siegel, 1983, 1985; Gross, 1984).

Social stress altered the distribution of peripheral T lymphocyte subpopulations. Social stress caused a decrease in CD4<sup>+</sup> and CD8<sup>+</sup> cells on day 1 PS. Trout and Mashaly (1994) reported decreases in CD4<sup>+</sup> and CD8<sup>+</sup> cells in the blood of heat stressed birds. It is speculated that the decrease in circulating lymphocytes in poultry appears to be primarily the result of decreased T cell numbers (Glick, 1958; Siegel, 1971; Trout and Mashaly, 1994). Glucocorticoids have been reported to cause a redistribution of B and T lymphocytes from the circulation to secondary lymphoid tissues in mice (Fauci, 1975). Social stress has also resulted in marked decreases in blood CD4<sup>+</sup> and CD8<sup>+</sup> lymphocyte distribution in laboratory rats (Stefanski and Engler, 1999; Stefanski, 2000; Engler et al., 2004).

There are very few reports on cytokine response in poultry. Most of the research has been performed in mammals. Social stress did not reveal any significant changes in regard to plasma IL-6 or TNF- $\alpha$  in the current experiment, however levels were numerically higher in the stressed group. This suggests that the elevation in these proinflammatory cytokines was due in part to the acute response to the psychological stress of social disruption (Merlot et al., 2003). One study by Franchini et al., (2004) reported that *in vivo* effects of acute stress induced by corticosterone 21-acetate stimulated cytokine immunoreactivity to anti-IL-6 and TNF- $\alpha$  antibodies in epithelial cells and interdigitating cells in chickens. Other researchers have demonstrated that social disruption elevated plasma IL-6 levels in mice and rats (Stark et al., 2002; Merlot et al., 2003; Nukina et al., 1998). Nukina et al., (1998) was the first to demonstrate an increase in plasma TNF- $\alpha$  levels after restraint stress in mice. They hypothesized that TNF- $\alpha$  increases have not been seen before because of the invariable increase in plasma IL-6 levels over TNF- $\alpha$  levels. The mechanisms of induction of plasma IL-6 and TNF- $\alpha$  after psychological stress still remains to be clarified. However, the increase in plasma IL-6 seems to be a result from sympathetic activation and may play a protective role against acute and chronic inflammation (Takaki et al., 1996; Nunkina et al., 1998; Gornikiewicz et al., 2000; Mohamed-Ali et al., 2001; Merlot et al., 2003)

In Experiment 2, the effect of vaccination with an avirulent strain of HEV on immunological and physiological parameters was studied. During HE infection, maximum viral titer in the spleen occurs around the same time, 4-5 days PV. HEV+ birds showed a significant increase in plasma corticosterone and H:L ratios on day 6 PV

compared to the HEV- birds during this time. The increase in plasma corticosterone and H:L ratios occurred at the peak of viral infection. These results are similar to those observed by other researchers who reported increases in plasma corticosterone and H:L ratios in HEV + birds (Opengart et al., 1992). Some studies have also reported increases in plasma corticosterone and H:L ratios in other infectious diseases in poultry including infectious bursal disease (al-Afaleq, 1998; Amakye-Anim et al., 2000), ascites syndrome (Luger et al., 2003) and rous sarcoma virus (Janes et al., 1994). However, others have reported that vaccination with Marek's or Newcastle disease virus did not result in consistent increases in plasma corticosterone (Freeman and Manning, 1984).

Previous studies have demonstrated an elevated number of splenic CD4<sup>+</sup> cells during the acute phase (4-6 days) of HEV infection (Suresh and Sharma, 1995), however there was no evidence of differences in peripheral CD4<sup>+</sup> cells in the current experiment using PBLs. This observation is in agreement with Pierson (1993) who reported that there was no change in peripheral CD4<sup>+</sup> cells. The increase in splenic CD4<sup>+</sup> cells of HEV-infected poultz possibly contributes to the hyperplasia of the white pulp (Suresh and Sharma, 1995; Rautenschlein and Sharma, 2000). The differences in percentages of CD4<sup>+</sup> cells observed in the spleen and in PBLs may be attributed due to the fact that HEV replication occurs mainly in the spleen and may explain why the differences may only be observed there. The percentage of CD8<sup>+</sup> cells were increased at day 8 PV in HEV-vaccinated poultz. The increases in the proportion of CD8<sup>+</sup> cells have been suggested to be associated with viral clearance (Rautenschlein and Sharma, 2000). CD8<sup>+</sup> cells have also been demonstrated to have suppressor activity, as shown by the complement-

mediated elimination of CD8 reactive cells *in vitro* in response to T-cell growth factor (Carney et al., 1981). This suppressor response probably represents a subpopulation of CD8<sup>+</sup>-lymphocytes involved in cell-mediated, antiviral immune responses, which are distinct from cytolytic T lymphocytes. Although there were no changes in the percentage of CD4<sup>+</sup> PBLs, CD4:CD8 ratios were decreased on day 8 PV.

Researchers have demonstrated that spleen cells from HEV+ turkeys produced TNF-like factor after *ex vivo* stimulation with concanavalin A at 3 days post infection (Rautenschlein et al., 2000a). Current experiments demonstrated that plasma TNF- $\alpha$  levels were increased on day 6 and 8 PV in HEV+ birds. It is speculated that HEV-induced release of TNF- $\alpha$ , may lead to systemic shock, intestinal hemorrhaging and death. Supporting this speculation, experiments conducted demonstrated that intestinal hemorrhage was abrogated by thalidomide a potent TNF regulatory drug (Rautenschlein and Sharma, 2000). Experiment 2 provides further evidence that TNF- $\alpha$  may play a role in HEV pathogenesis. In this study, plasma IL-6 was released on day 8 PV. *Ex vivo* cultured spleen cells from HEV-infected birds secreted IL-6 like factor on days two and three post infection (Rautenschlein et al., 2000a). In murine adenovirus type 5 infections it was also demonstrated that pro-inflammatory cytokines TNF, IL-1 and IL-6 play an important role in pneumonia (Ginsberg et al., 1991).

A suggested HEV immunopathogenesis includes immunosuppression caused by a depletion of B cells either as a result of direct cytopathic effects or virus induced apoptosis (Suresh and Sharma, 1996; Rautenschlein and Sharma, 2000). Another possible mechanism for immunopathogenesis of HEV could be a suppressor response caused by

the elevation in CD8<sup>+</sup> cells on day 8. The increase in CD8<sup>+</sup> cells is hypothesized to be a limiting factor on viral replication by down-regulating the immune system and thus limit target cell proliferation (Pierson, 1993; Pierson and Fitzgerald, 2003). An HEV suppressor response is speculated to be a result of a period of immunosuppression (8-10 days PV). This period of immunosuppression is thought to create a “window of opportunity” that would enable secondary bacterial pathogens such as *Escherichia coli* to occur. This “window of opportunity” may be the case with regard to HEV vaccination reactions that would manifest themselves as an increase in mortality due to colibacillosis, observed 10-14 days PV (Pierson, 1993; Pierson, et al., 1996). It is speculated that the increase in CD8<sup>+</sup> seen in this experiment at 8 days post-vaccination may constitute a suppressor response. This would be the most opportune time for environmental *E. coli* to become a challenge 2-5 days after this response (e.g. 10-14 days post-vaccination). A similar mechanism has been proposed to explain immunosuppression during the acute phase of infection with herpes viruses such as Epstein-Barr and herpes zoster (Glaser et al., 1987; Schmader et al., 1990).

The last experiment examined the effects of the strategic application of stress in the form of social disruption on response to vaccination with HEV. Plasma corticosterone was significantly decreased when birds were stressed after vaccination (24 and 48 hour groups). Birds stressed prior to vaccination had elevated plasma corticosterone levels. Other researchers observed elevated plasma corticosterone in socially stressed chickens infected with Newcastle disease virus (Mohamed and Hanson, 1980) and *E. coli* infections (Gross and Colmano 1967). In other reports, social

disruption and infection produced significant elevations in serum cortiosterone in acute Theiler's virus infection in mice (Campbell et al., 2001; Welsh et al., 2004).

Social stress application times did not have a consistent effect on plasma or H:L ratios. H:L ratios were higher in all the stress groups on day 4 PV, where the No Stress control had higher H:L ratios on day 6 PV. H:L ratios were only significant in the 0 hour stress group on day 4 PV. Although there were not significant changes in these parameters, the stress-induced effects previously observed (Experiment 2) were abolished by HE infection.

In the first experiment, social stress altered T lymphocyte subpopulations. In the second experiment, there was no difference in numbers of peripheral CD4<sup>+</sup> cells. However in the last experiment the application of stress resulted in an increased percentage of CD4<sup>+</sup> cells. The increase in CD4<sup>+</sup> cells is theorized to be due to the increased activity of the immune system to the simultaneous application of stress and HEV vaccination. It appears that HEV vaccination abolished the effects of stress suggesting that HEV infection may induce stronger immunosuppressive effects in turkeys than stress.

Stress-induced alterations in TNF- $\alpha$  and IL-6 have been investigated in several infectious animal models. In studies of the effect of restraint stress on influenza and infection, TNF- $\alpha$  was not detected (Konstantinos and Sheridan, 2001). There were no differences detected in to plasma TNF- $\alpha$  levels, which appear to be similar to findings by Welsh et al., (2004) who reported that TNF- $\alpha$  production appeared slightly elevated after Thieler's virus infection. However, restraint stress in *Listeria monocytogenes* infection

of mice resulted in decreased levels of TNF- $\alpha$  (Zhang et al., 1998). These contrasting results are probably due to a combination of factors, such as the different experimental systems and timing of stressors. Plasma IL-6 levels were decreased when birds were stressed prior to vaccination, but increased when birds were vaccinated and then subjected to stress at a later time. Other researchers have reported that IL-6 was elevated in lung tissue in mice subjected to restraint stress and influenza infection.

Previous experiments have also demonstrated that the time in which stress is applied can have a beneficial or detrimental effect in response to disease. Pierson et al., (1997) reported that vaccinated chickens were more resistant to cecal lesion formation and protection against *Eimeria tenella* when social disruption was applied on the fourth day following vaccination. It has been reported that in high-stress environments susceptibility to viral infections is increased. Gross and Colmano (1967) reported that a high degree of social interaction decreased resistance to Newcastle disease virus infection and that a low degree of social interaction had an opposite effect. Mohamed and Hanson (1980) also reported that social stress appeared to make chickens more susceptible to Newcastle disease virus infection as indicated by higher antibody titers and invasion of more tissues. In this experiment, social stress applied prior to vaccination resulted in lower HEV antibody titers. Whereas, when social stress was applied on the day of vaccination, HEV antibody titers were numerically higher compared to the controls. These findings are similar to results where social disruption applied concurrently with Theiler's virus infection in mice resulted in less severe inflammation (Johnson et al., 2004). In contrast, Johnson et al., (2004) reported that social disruption applied prior to

Theiler's virus infection resulted in more severe disease course with increased inflammation.

In summary, the stress associated with social reorganization can alter physiological and immunological effects in turkeys. These studies have also demonstrated that vaccination with HEV can produce transient immunosuppression and other physiological effects in turkeys. Social disruption altered responses to vaccination with HEV and the alteration was dependent on the timing of social stress application. However, it appeared that HEV vaccination overshadowed the initial effects of social disruption. Given these observations, it is likely that the effectiveness of vaccination for HEV may be improved if birds are subjected to a form of stress on the day of vaccine administration.

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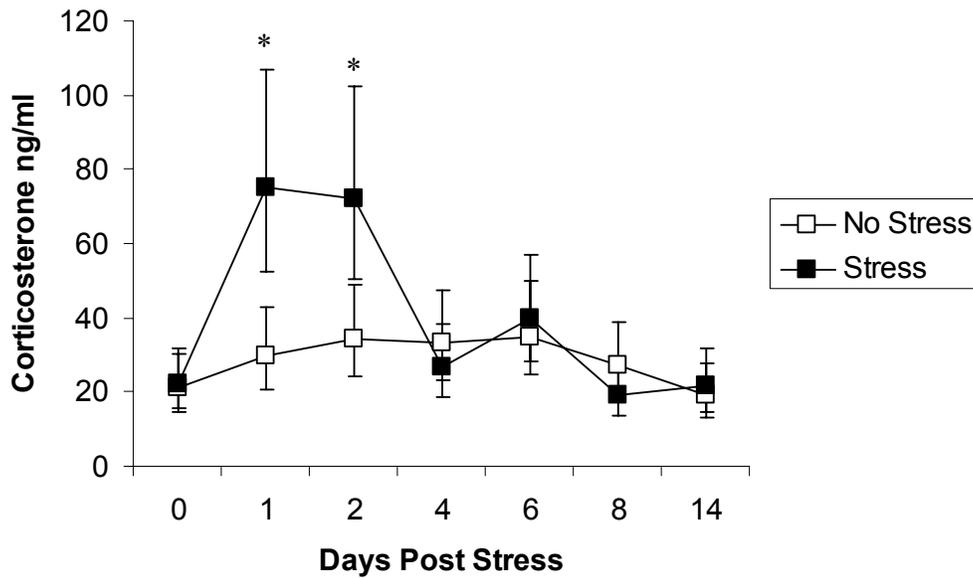
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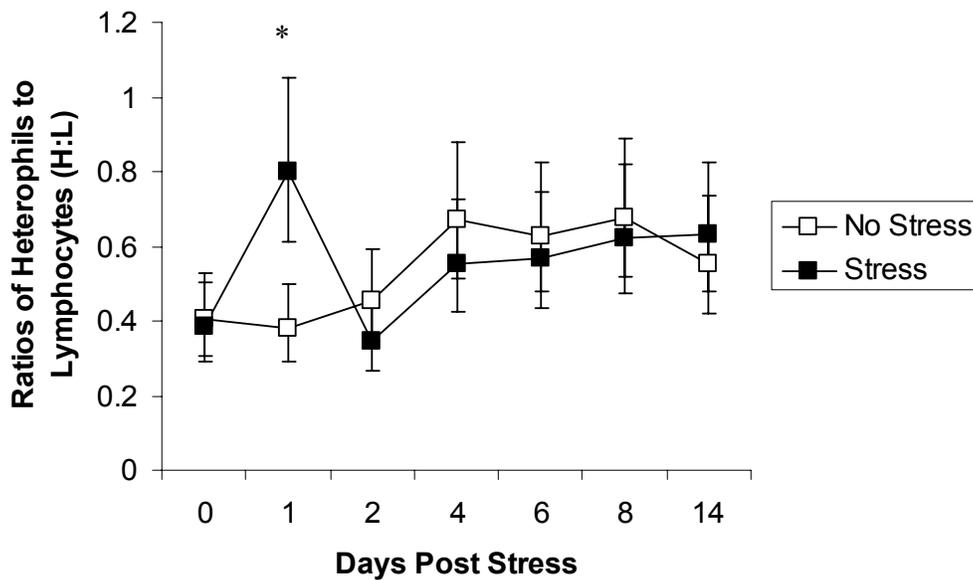
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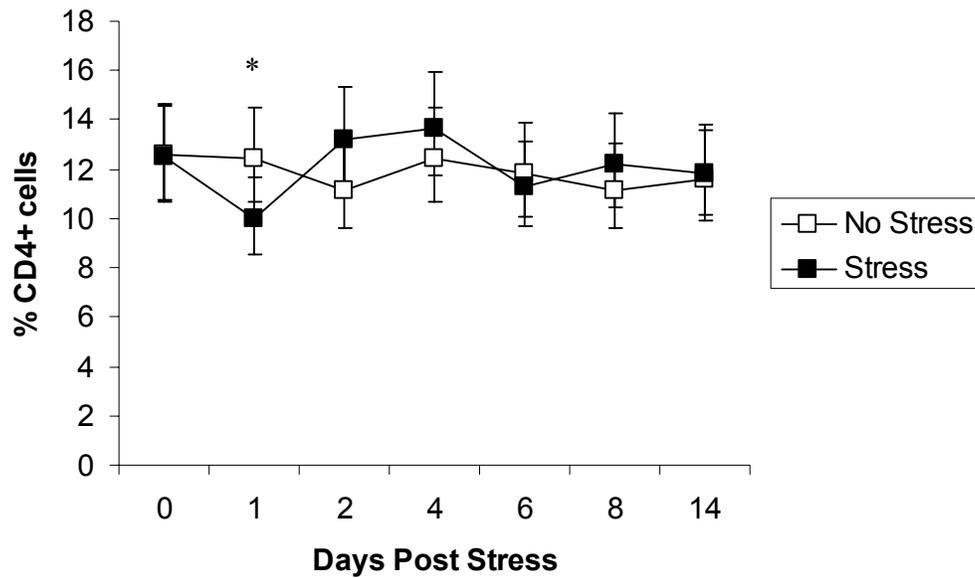
**FIGURE 1. Social Randomization Setup.** Setup for social disruption experiments. There are two rows of 3' x 5' pens equipped with automatic waterers. Birds were housed 4 to a pen in a total of 8 pens (4 on each side)



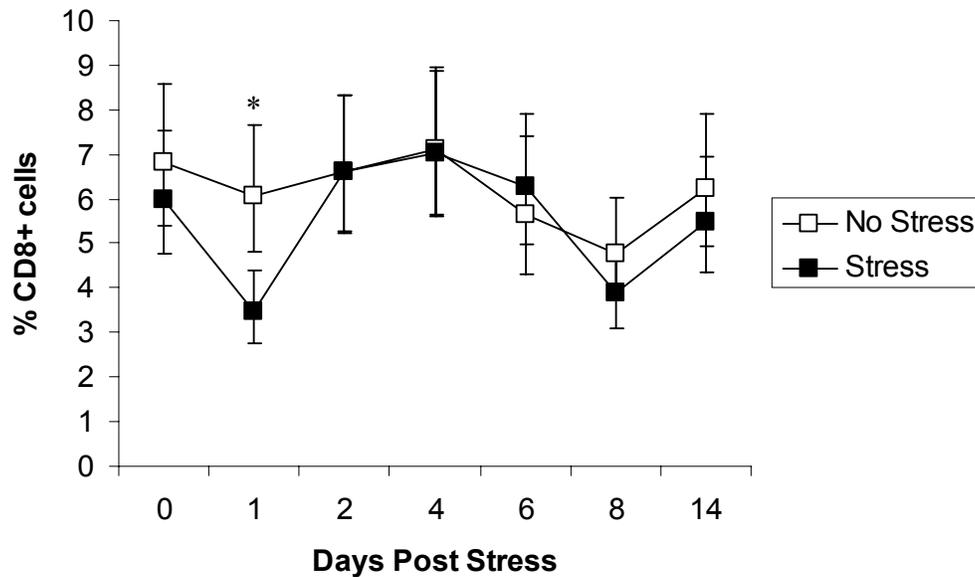
**Figure 2. Effect of Stress on plasma corticosterone.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. Asterisks indicate a significant difference ( $P < 0.05$ ). Asterisks indicate a significant difference ( $P < 0.05$ ) from the respective No Stress control group at the specified time period.



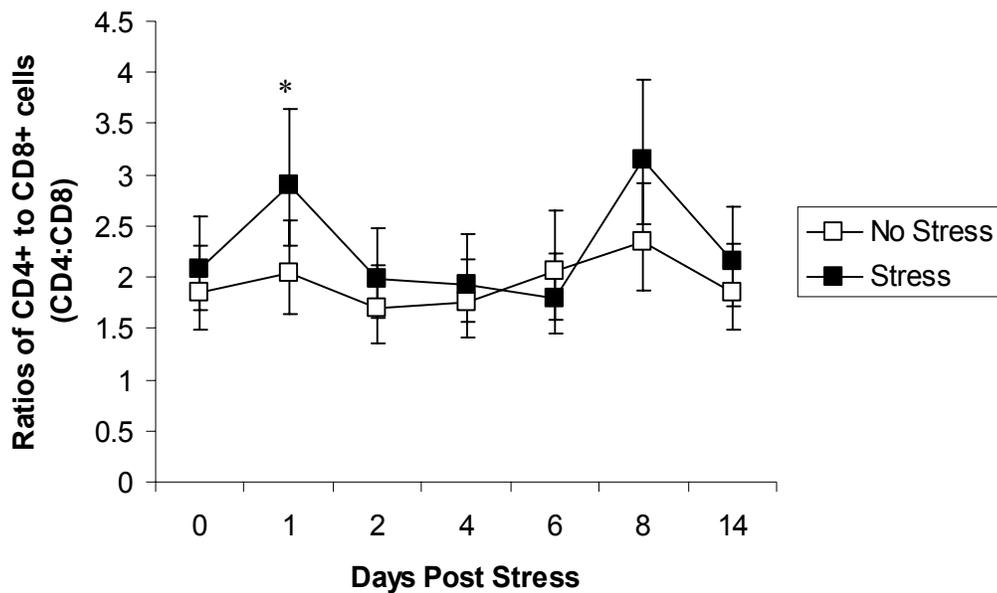
**Figure 3. Effect of Stress on the Heterophil to Lymphocyte Ratios (H:L).** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a significant difference ( $P < 0.05$ ).



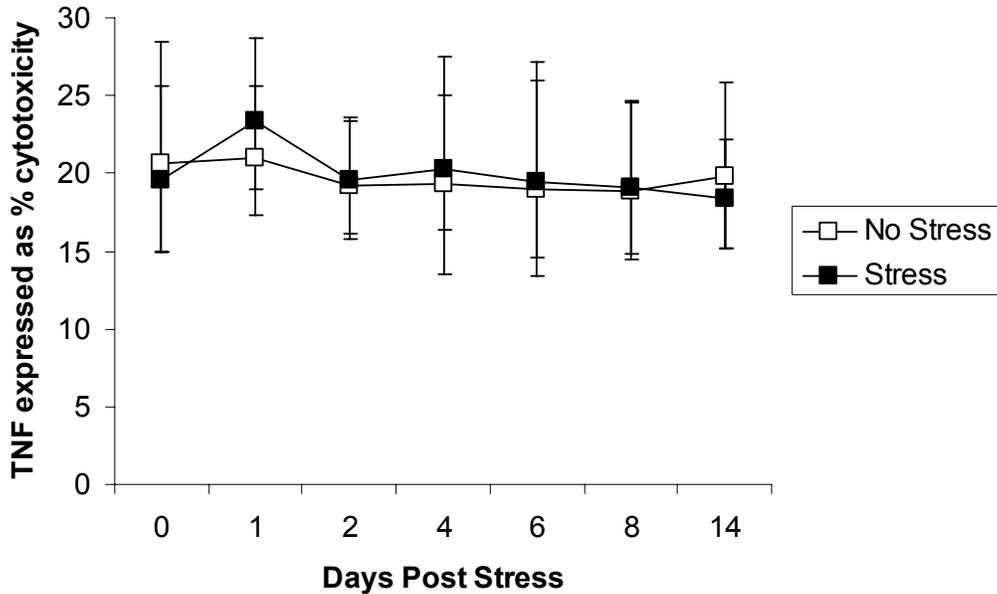
**Figure 4. Effect of Stress on peripheral CD4+ cell populations.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a significant difference ( $P < 0.05$ ).



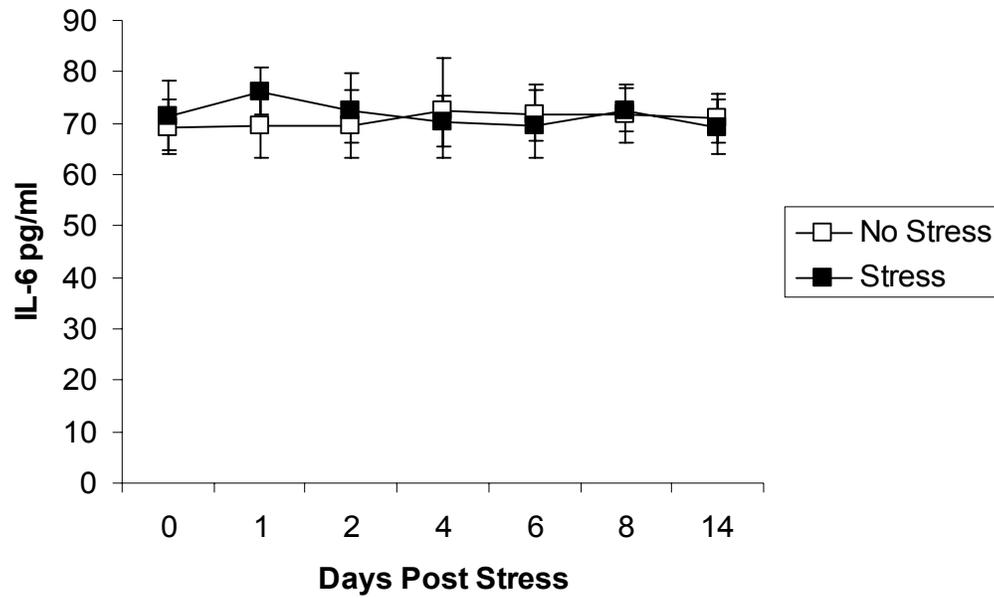
**Figure 5. Effect of Stress on peripheral CD8+ cell populations.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a significant difference ( $P < 0.05$ ).



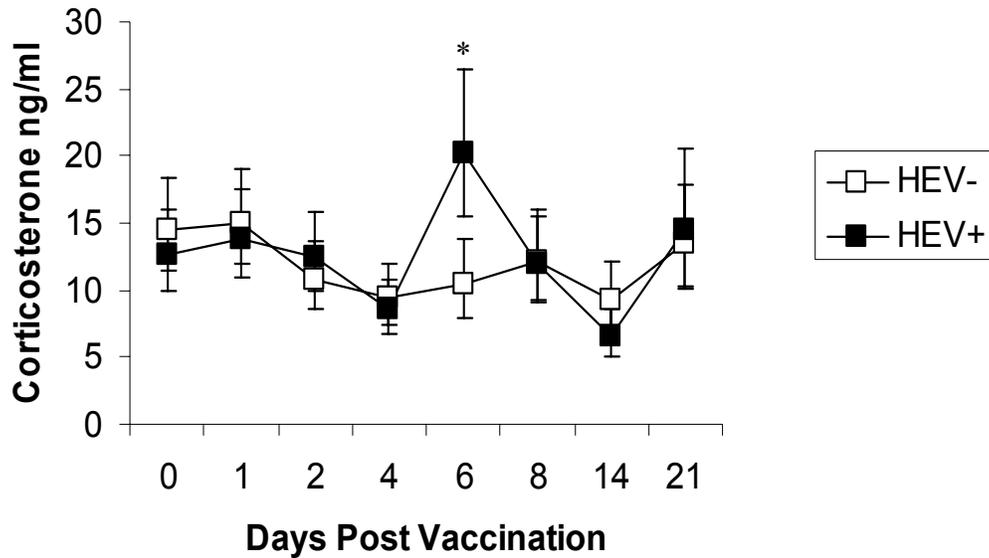
**Figure 6. Effect of Stress of CD4+ to CD8+ cell populations.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a significant difference ( $P < 0.05$ ).



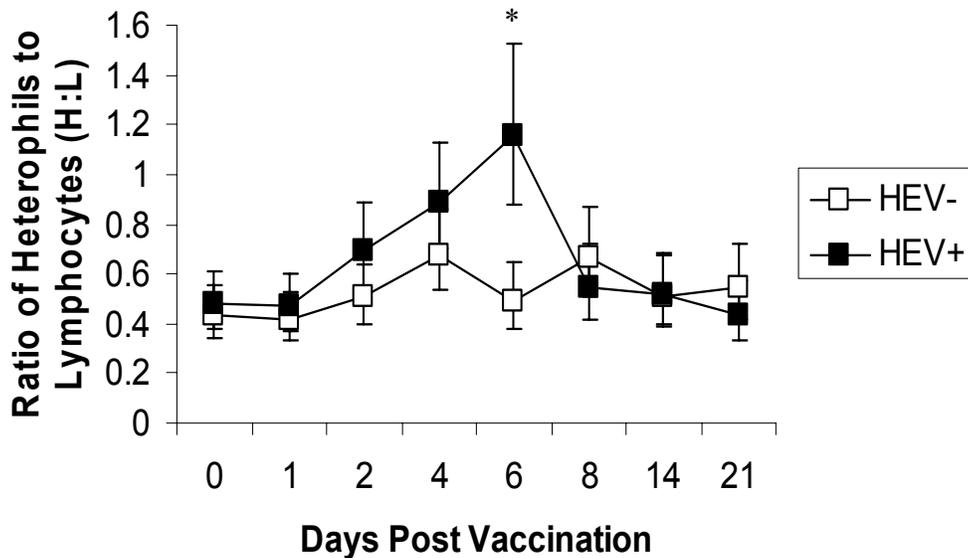
**Figure 7. Effect of Stress on plasma TNF.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits.



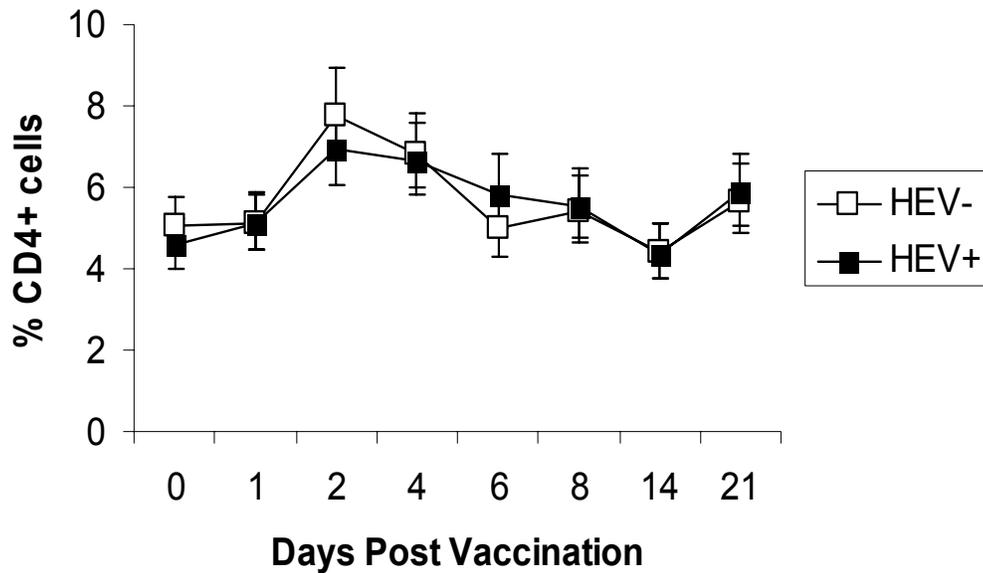
**Figure 8. Effect of Stress on plasma IL-6 levels.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits.



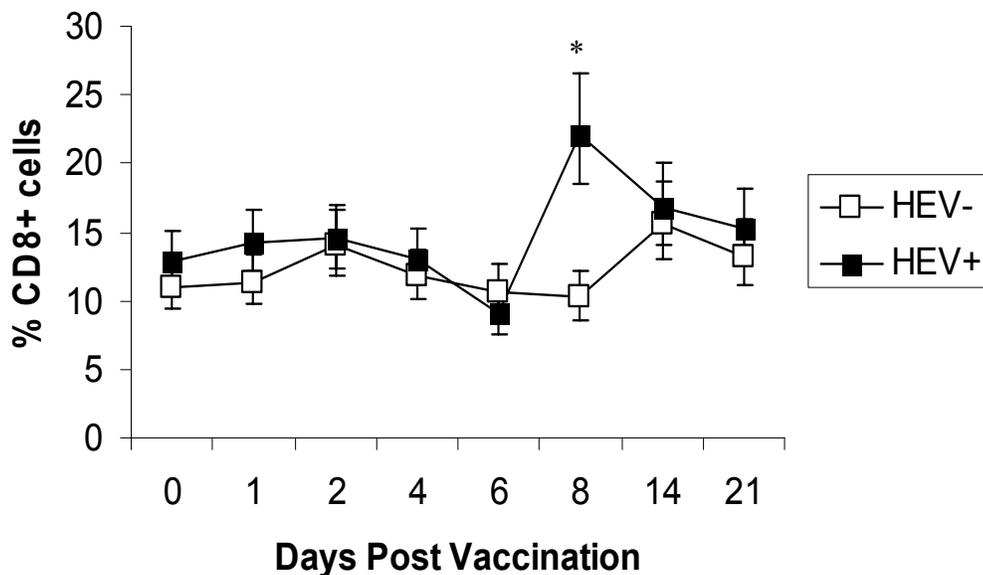
**Figure 9. Effect of HEV on corticosterone levels.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a significant difference ( $P < 0.05$ ) from the respective HEV- control group at the specified time period.



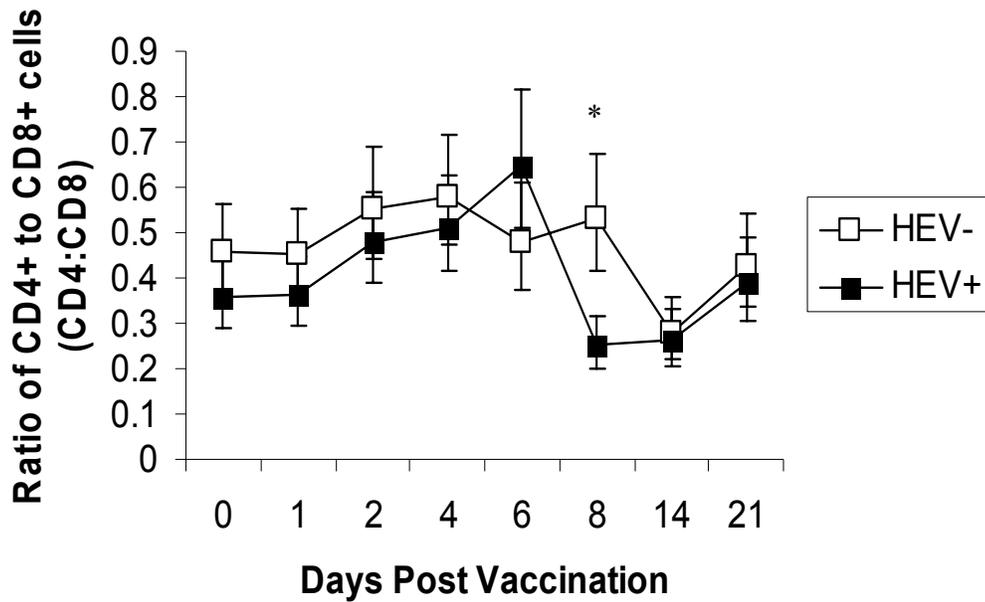
**Figure 10. Effect of HEV on Heterophil to Lymphocyte ratios (H:L).** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a significant difference ( $P < 0.05$ ).



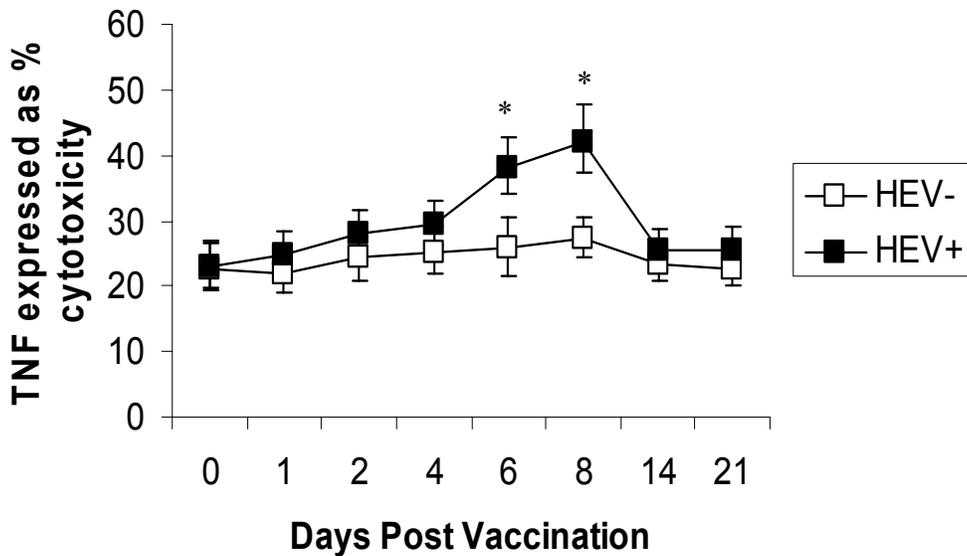
**Figure 11. Effect of HEV on peripheral CD4+ cell populations.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits.



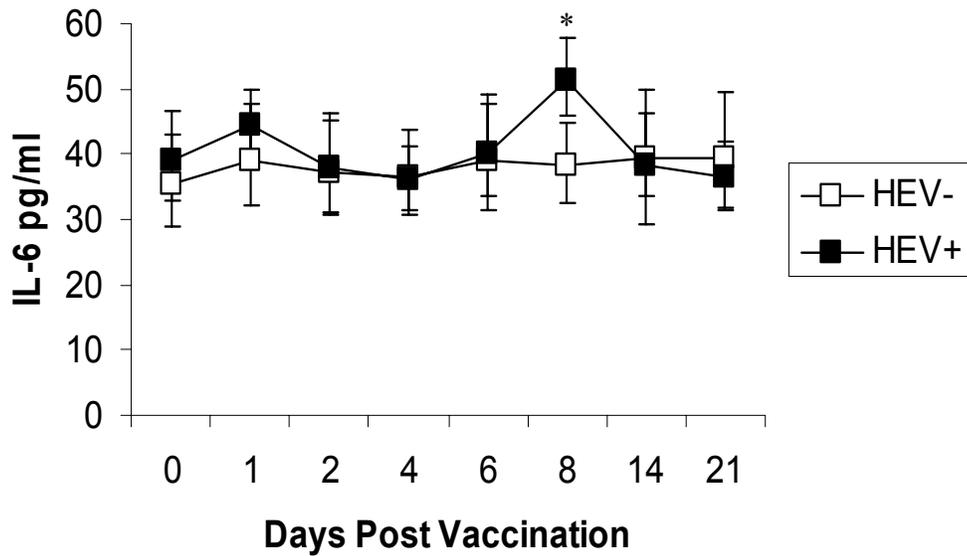
**Figure 12. Effect of HEV on peripheral CD8+ cell populations.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a significant difference ( $P < 0.05$ ).



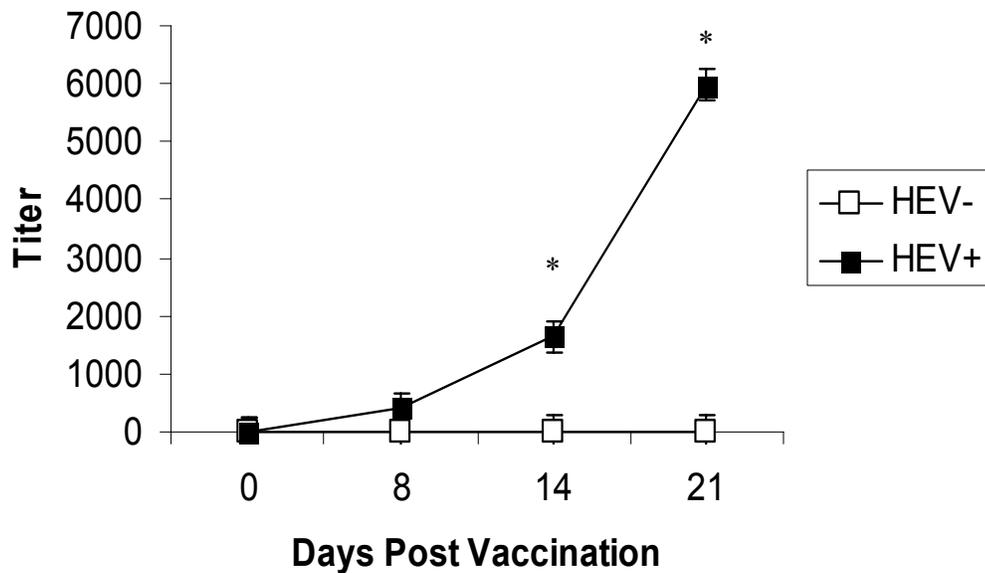
**Figure 13. Effect of HEV on ratios of CD4<sup>+</sup> to CD8<sup>+</sup> cell populations.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a significant difference ( $P < 0.05$ ).



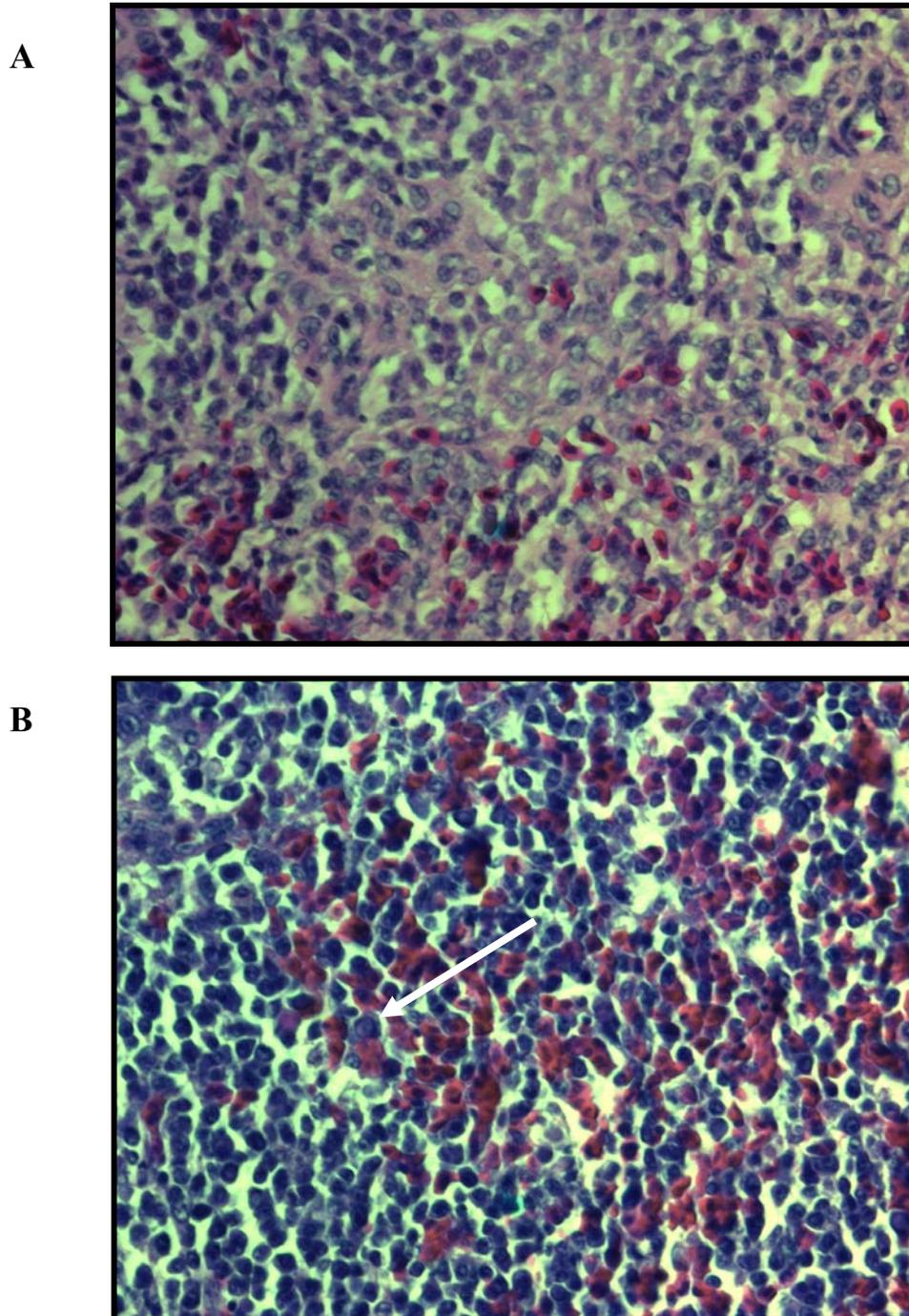
**Figure 14. Effect of HEV on plasma TNF.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. Asterisks indicate a significant difference ( $P < 0.05$ ).



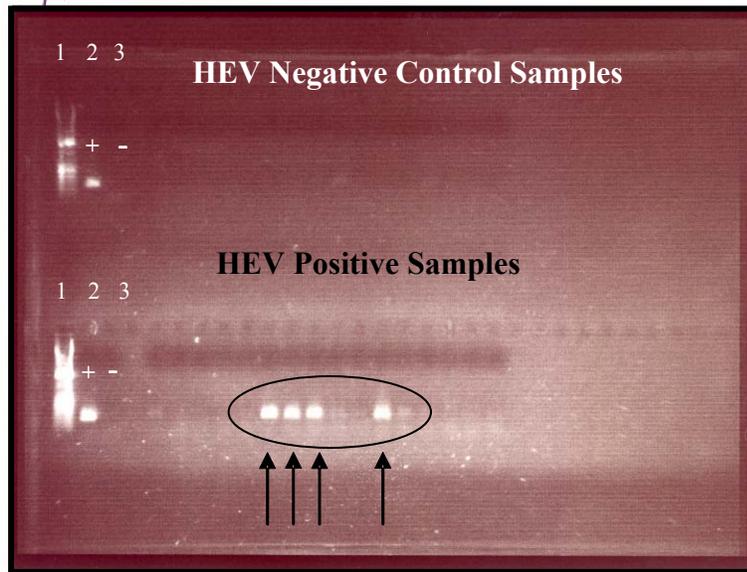
**Figure 15. Effect of HEV on plasma IL-6 levels.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a significant difference ( $P < 0.05$ ).



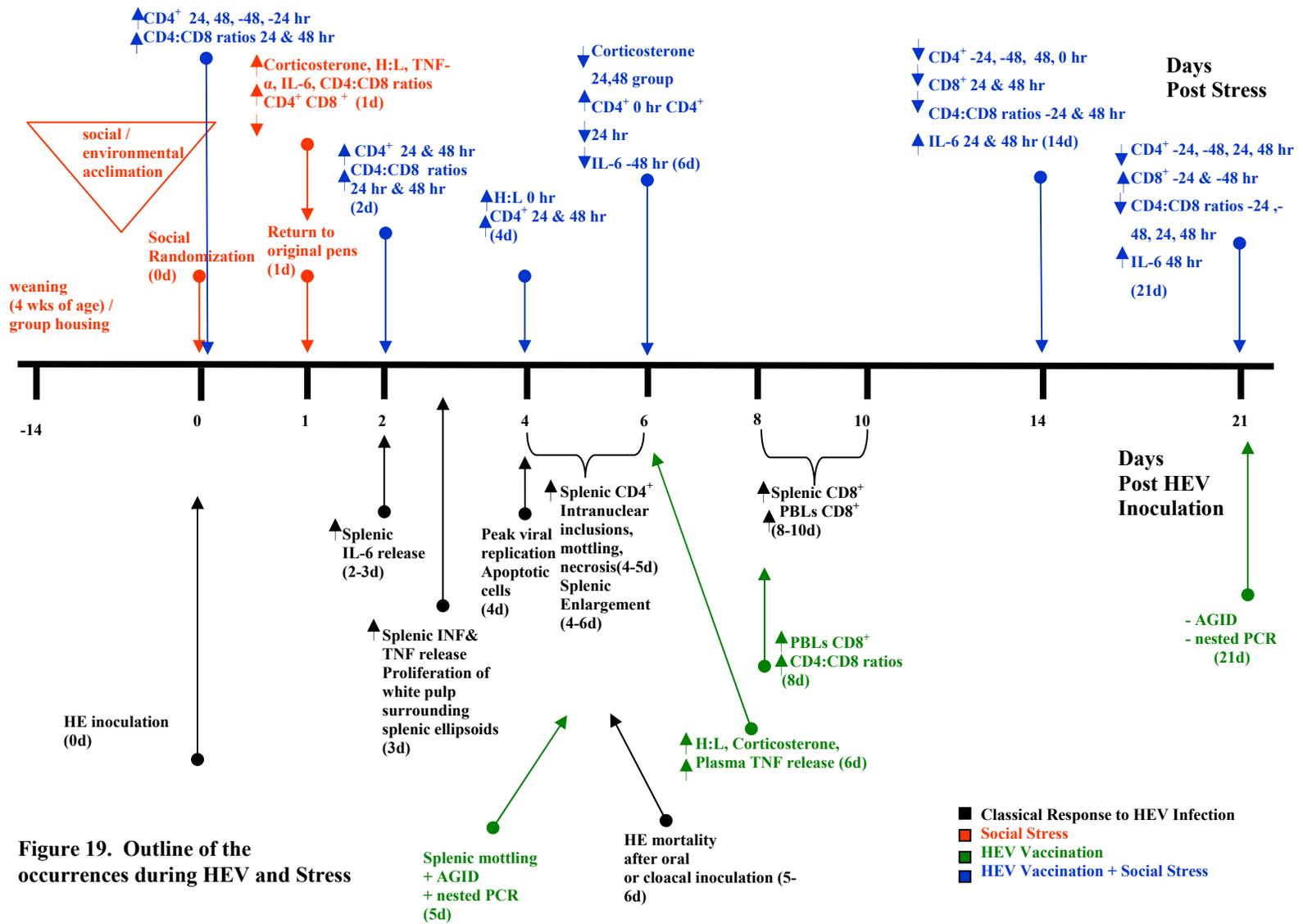
**Figure 16. Effect of HEV on HEV antibody titers.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. Asterisks indicate a significant difference ( $P < 0.05$ ).



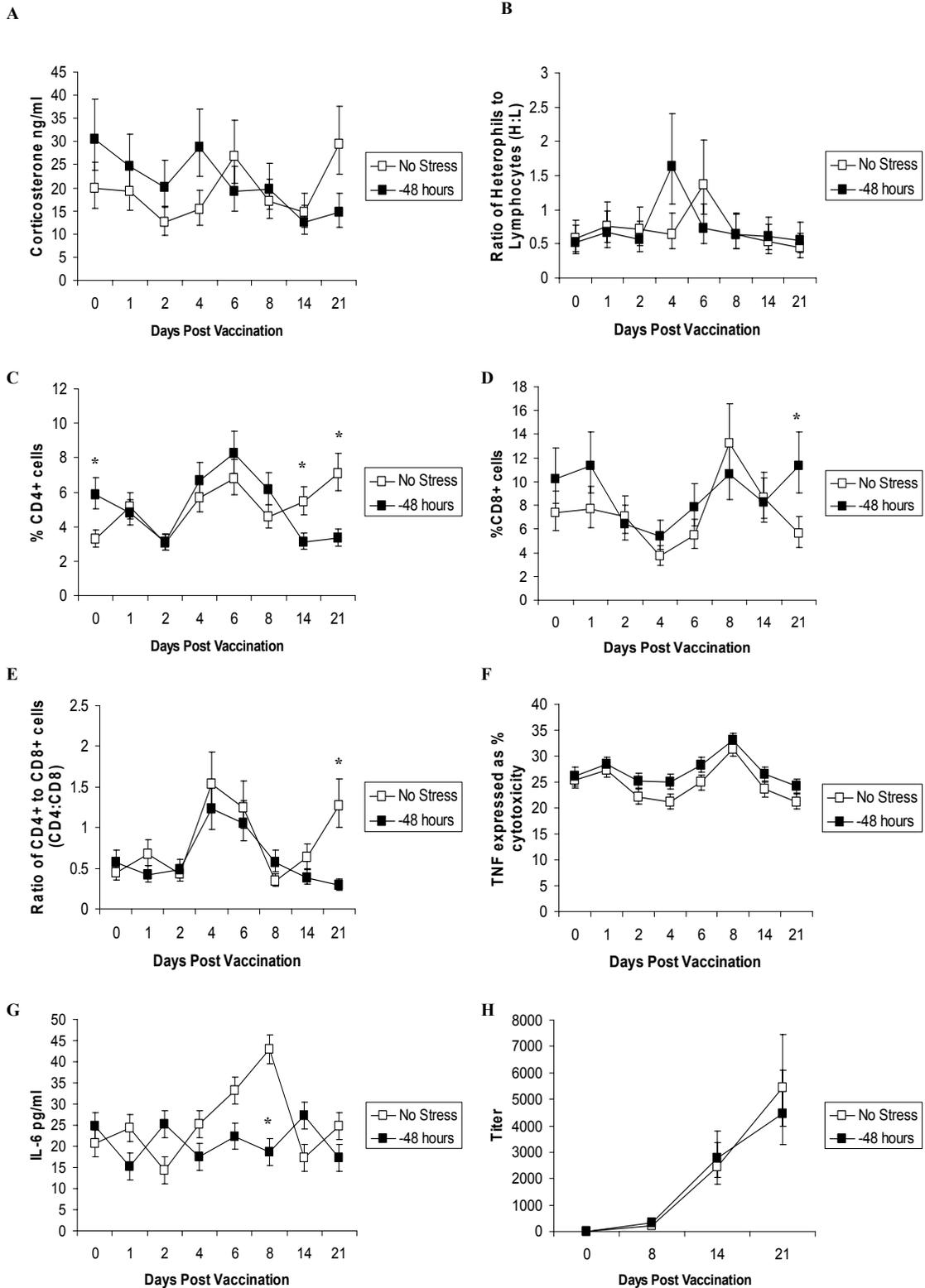
**Figure 17. The effect of vaccination with HEV on splenic histopathology day 5 PV.** **A.** HE negative spleen on day 5 PV (40x). There are no inclusions present. **B.** HEV positive spleen on day 5 (40x). Arrow indicates an intranuclear inclusion body.



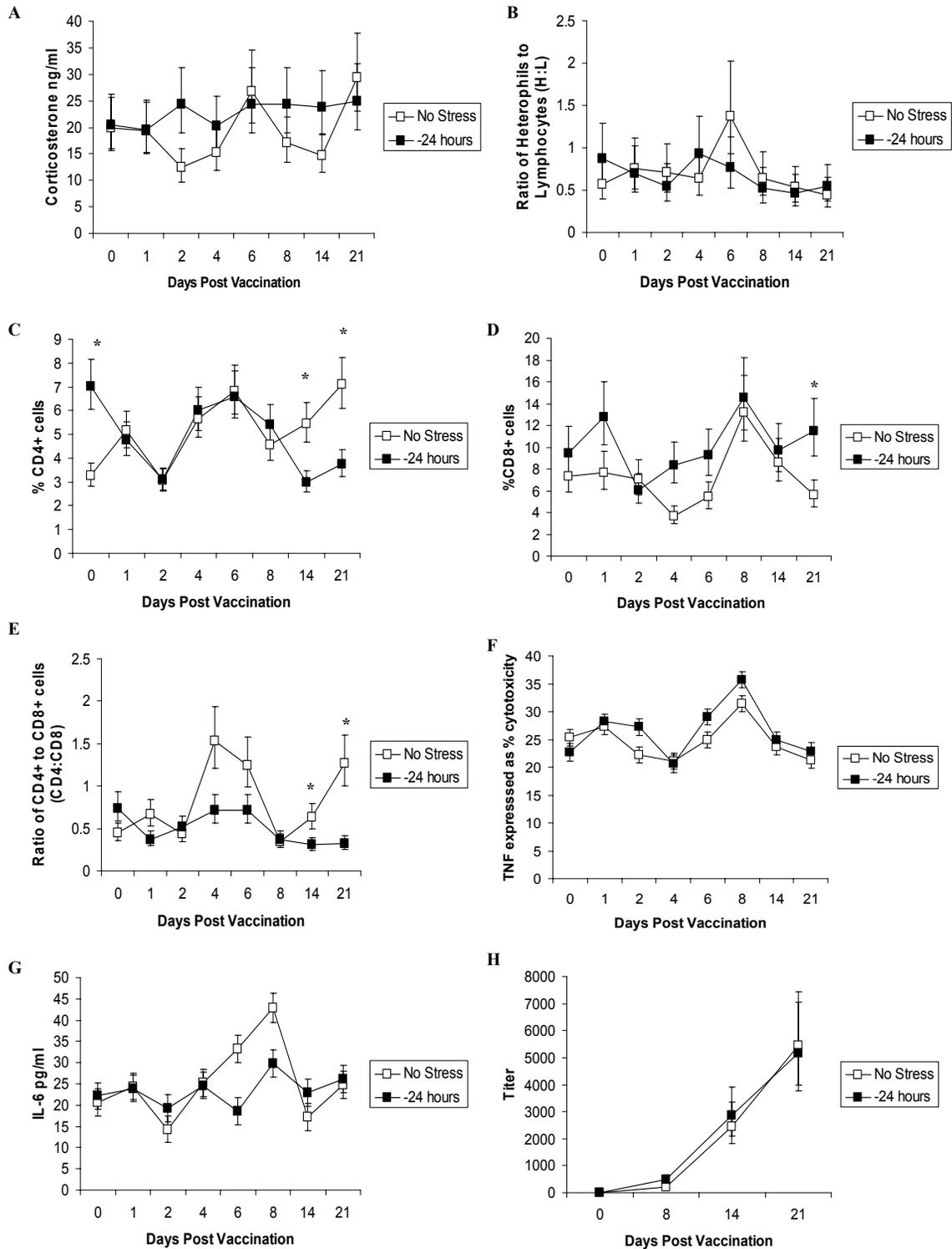
**Figure 18. Nested PCR performed on DNA isolated from spleens of HEV.** Negative (top lane) with HEV positive birds (bottom lane). Lane 1=1kb ladder, Lane 2=Positive control, Lane 3=Negative control. Bands indicate positive results on day 5 PV.



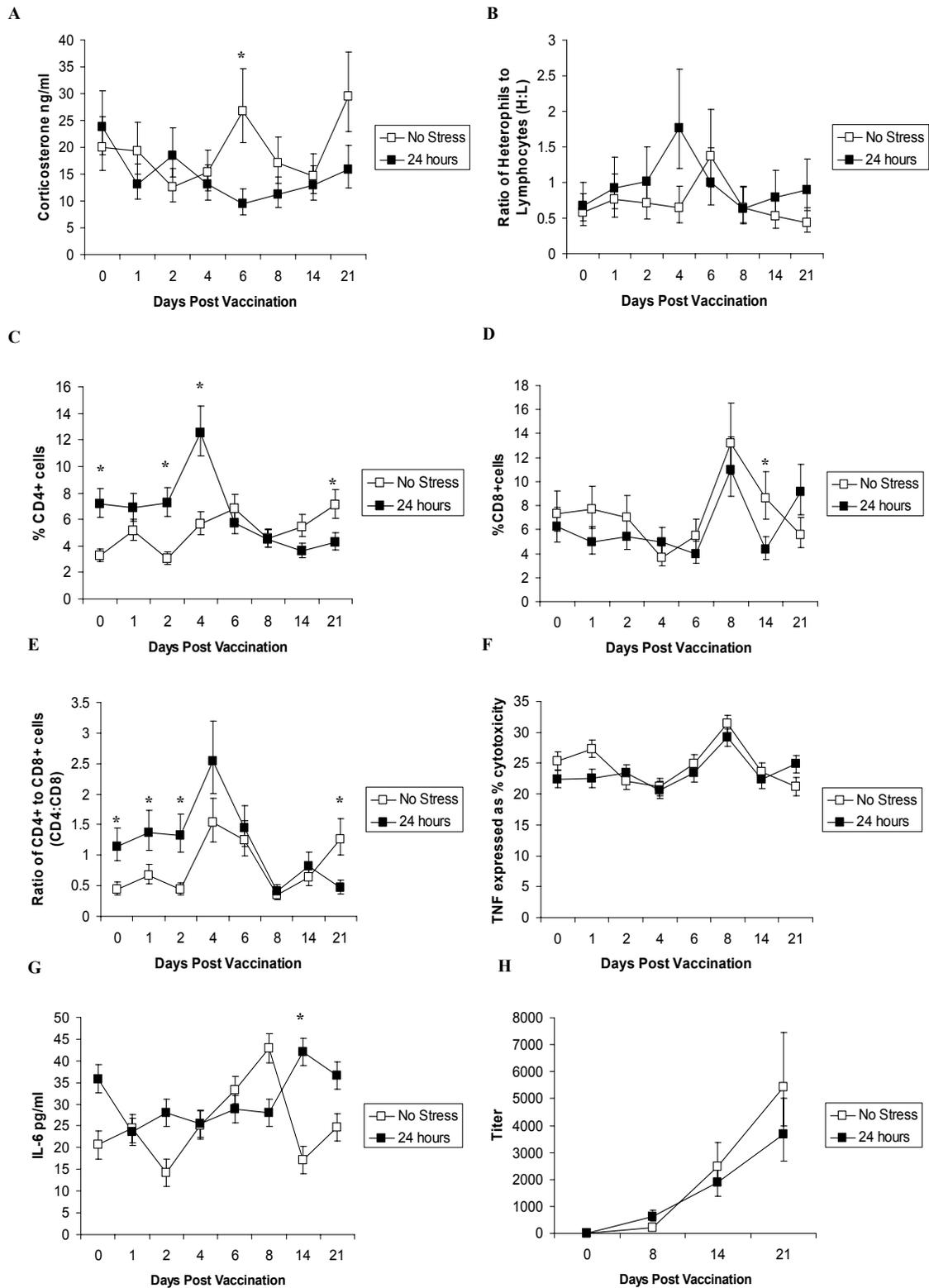
**Figure 19. Outline of the occurrences during HEV and Stress**



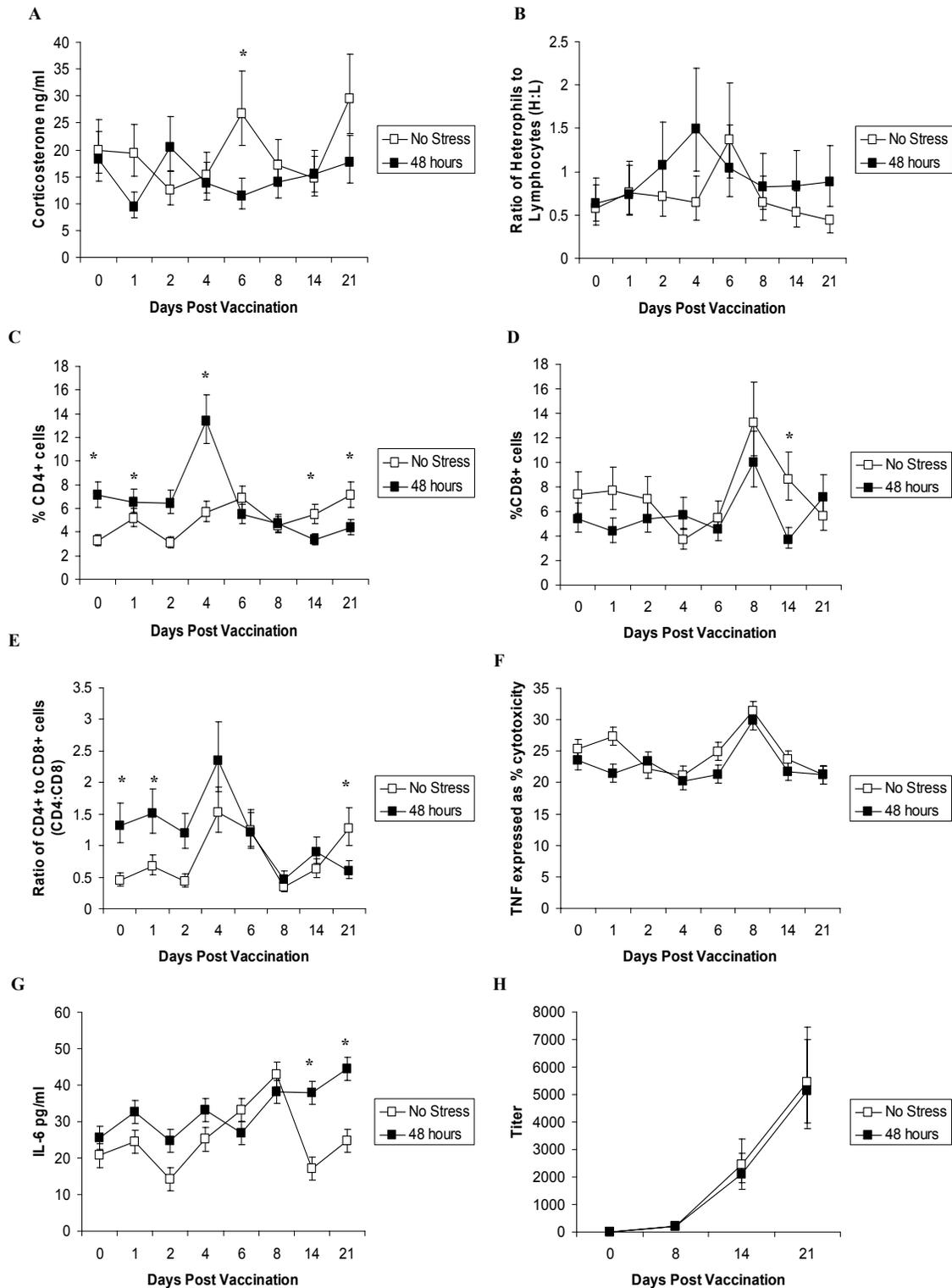
**Figure 20. The Effect of Stress at -48 hours on HEV vaccination.** Values were calculated from 16 individual birds per group and expressed as geometric means (A,C-E, H) with 95% confidence limits or means (B, F, G)  $\pm$ SEM. Asterisks indicate a significant difference ( $P < 0.05$ ).



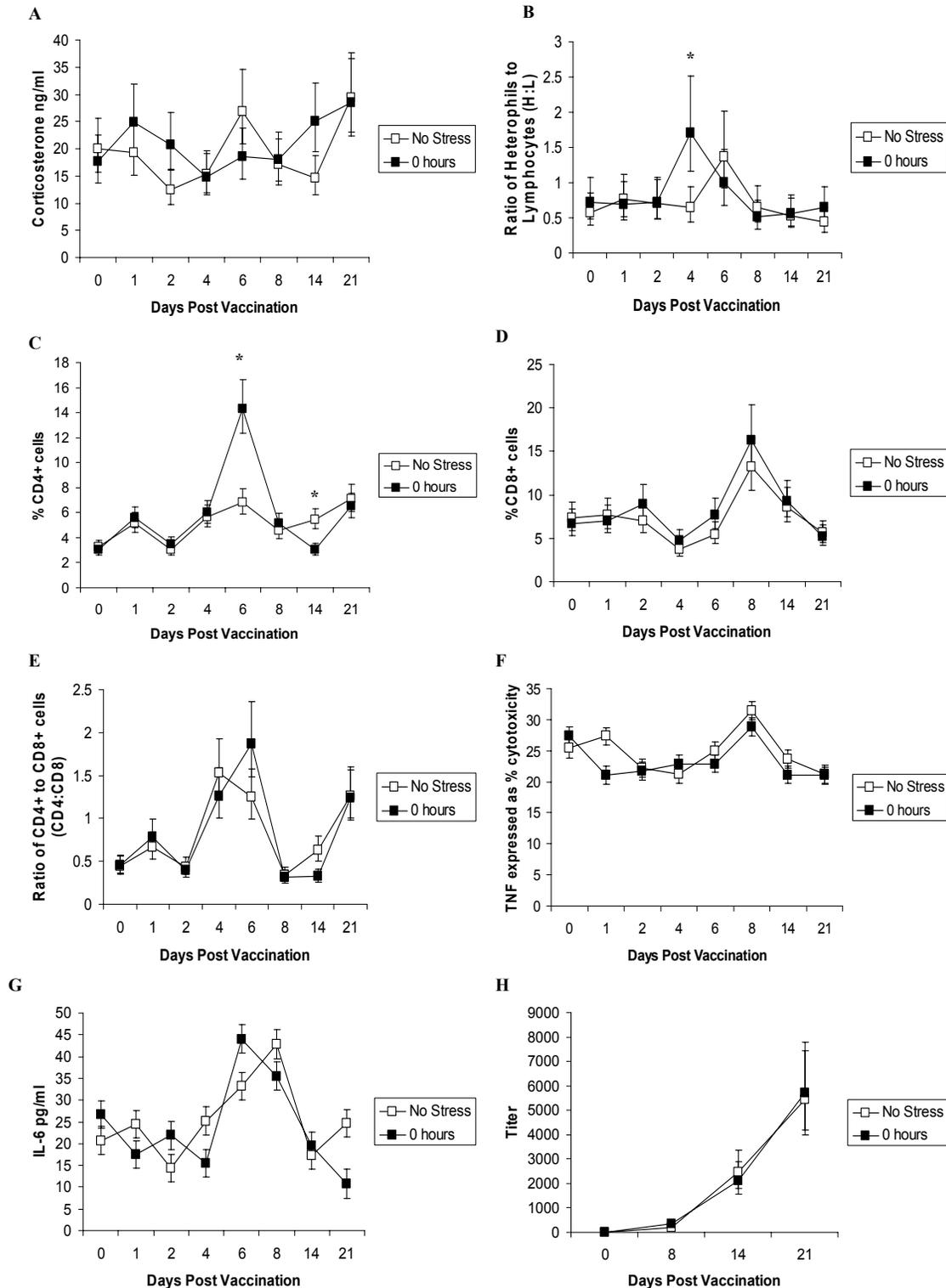
**Figure 21. The Effect of Stress at -24 hours on HEV vaccination.** Values were calculated from 16 individual birds per group and expressed as geometric means (A,C-E, H) with 95% confidence limits or means (B, F, G)  $\pm$ SEM. Asterisks indicate a significant difference ( $P < 0.05$ ).



**Figure 22. The Effect of Stress at +24 hours on HEV vaccination.** Values were calculated from 16 individual birds per group and expressed as geometric means (A-E, H) with 95% confidence limits or means (F, G)  $\pm$ SEM. Asterisks indicate a significant difference ( $P < 0.05$ ).



**Figure 23. The Effect of Stress at +48 hours on HEV vaccination.** Values were calculated from 16 individual birds per group and expressed as geometric means (A, C-E, H) with 95% confidence limits or means (B, F, G)  $\pm$ SEM. Asterisks indicate a significant difference ( $P < 0.05$ ).



**Figure 24. The Effect of Stress at 0 hours on HEV vaccination.** Values were calculated from 16 individual birds per group and expressed as geometric means (A-E, H) with 95% confidence limits or means (F, G)  $\pm$ SEM. Asterisks indicate a significant difference ( $P < 0.05$ ).

<b>Treatment</b>	<b>No. Positive Birds/ Total Birds</b>
-24 hours	11/16
-48 hours	13/16
24 hours	10/16
48 hours	12/16
0 hours	13/16
No Stress	16/16

**Table 1. The effect of Stress on HEV DNA on day 21 post-vaccination**

## Chapter 2 Summary Table

	Corticosterone	H:L	CD4 <sup>+</sup>	CD8 <sup>+</sup>	CD4:CD8	TNF- $\alpha$	IL-6	HEV Titer	Histopathology	AGID	PCR
Exp 1	↑	↑	↓	↓	↑	↑	↑				
Exp 2	↑	↑	--	↑	↓	↑	↑	↑	↑ Lymphoid Proliferation	+	--
Exp 3		↑	↑, ↓					↑	+, -	+	↓

↑ = Increase

↓ = Decrease

-- = No Change

+ = Positive

- = Negative

Table 2. Summary.

## Chapter 3

### The Effect of Vitamin C Supplementation on Social Stress in Turkeys

#### 3.1 Abstract

These studies were conducted to investigate the effects of supplementing diets of turkeys with vitamin C on specific immunological and physiological parameters while being subjected to a social stressor. Two experiments were conducted. In Experiment 1, 16 four-week-old poults were divided into three “treatment” and one “control” group of four birds each. After two weeks of environmental and social adaptation, birds were given feed supplemented with 0 (control), 75, 150, or 300 mg/kg vitamin C. Plasma corticosterone, heterophil to lymphocyte (H:L) ratios, and plasma vitamin C were measured on days 0, 1, and 2 post-supplementation. Birds supplemented with 300 mg/kg vitamin C had lower H:L ratios and higher plasma vitamin C values compared to the other “treatment” groups.

In Experiment 2, 32 four-week-old poults were divided into four “treatment” groups and four “control” groups. At six weeks-of- age and one day before stress application (Day-1), “treatment” birds were placed on feed containing 300 mg/kg vitamin C. On day 0, birds were randomly redistributed to disrupt social order for 24 hours. After 24 hours, birds were reunited with their original pen mates. Blood samples were taken on 0, 1, 2, 4, 6, 8, 14, and 21 days post-stress. Plasma corticosterone, H:L ratios, CD4<sup>+</sup> and CD8<sup>+</sup> T cell percentages, tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-6

(IL-6), plasma vitamin C, splenic vitamin C were measured. Histopathological analysis was also performed on the spleens on day 21 PV. H:L ratios decreased, CD4<sup>+</sup> and CD8<sup>+</sup> T cell percentages increased, CD4:CD8 ratios decreased, and plasma and splenic vitamin C levels increased with vitamin C supplementation. There were no differences in regard to plasma corticosterone, IL-6 or TNF- $\alpha$ .

These experiments demonstrated that vitamin C supplementation at 300mg/kg was able to alleviate stress-induced affects and elevate plasma vitamin C in turkeys. These physiological and immunological findings suggest that vitamin C may be an anti-stress agent and may be an essential nutrient if added to poultry diets when poults are subjected to a social stressor.

*Keywords:* vitamin C, social stress, turkeys

### **3.2 Introduction**

Ascorbic acid is a water soluble antioxidant present in cytosolic and extracellular fluids that can directly scavenge superoxide hydroxyl radicals and singlet oxygen species (Clarkson and Thompson, 2000). Ascorbic acid is thought to exert its effects on the immune system through the enhancement of neutrophil production and through protection of the cell against superoxide radical damage (Bendich et al., 1986).

Under normal conditions, birds actively synthesize adequate amounts of ascorbic acid (Roy and Guha, 1958), therefore supplementation is not necessary when the animal is managed properly. However in stressful circumstances (environmental, pathological, and nutritional stressors) supplementation may be beneficial to provide non-specific resistance against infections and overcome stress, therefore improving productivity (Pardue and Thaxton, 1985).

Phagocytosis by neutrophils involves increased consumption of both ascorbate and dehydroascorbate (Rund, 1989). The quantity of superoxide radicals present in phagocytes is increased by an optimum level of ascorbic acid. Further increases in the level of ascorbic acid result in a reduction of the superoxide radical (Scarpa et al., 1983; Som et al., 1983). Viral infections have been shown to cause a depletion of leukocyte ascorbate, which resulted in varying degrees of non-specific immunosuppression and a reduction in serum ascorbic acid concentration (Thomas and Holt, 1978; Bendich et al., 1986).

The effect of ascorbic acid (from here on referred to as vitamin C) on environmental stressors in birds have been well documented. In one study it was

reported that the addition of vitamin C at 330 ppm prevented a depression in blood vitamin C levels during heat stress in turkey breeder hens (Nestor et al., 1972). In another study, Pardue and Thaxton, (1984) reported that 1000 ppm vitamin C ameliorated immunosuppression associated with exogenous cortisol. They postulated that the amelioration of steroid mediated immunosuppression by vitamin C was due in part to protection of immunobiological tissues from the cytotoxic effects of adrenal tissues. In 1988, Gross reported that feeding a series of rations containing a broad range of concentrations of vitamin C resulted in a decrease in severity of chickens' response to heat stress and a decrease in H:L ratios. McKee and Harrison (1995) also reported that vitamin C increased feed intake and lowered H:L ratios when subjected to multiple concurrent stressors. Recent studies have also reported that vitamin C decreased heat shock protein 70 and plasma corticosterone response in chickens subjected to cyclic heat stress (Mahmoud et al., 2003, 2004).

We considered the potential advantage of dietary supplementation of vitamin C to improve immunoresponsiveness under stressful conditions. Therefore, the objectives of these studies were to determine if the addition of supplemental vitamin C to the diet would have a beneficial effect on social stress and response to HEV vaccination in turkeys.

### **3.3 Materials and Methods**

#### ***Animals***

Day-old turkey poults were used. Birds were obtained at one day-of-age from British United Turkeys of America (BUTA, Lewisburg, WV). All birds were handled daily to acclimate them to handling and sampling and the environment in order to eliminate these factors as possible contributors to stress. Birds were placed on a 12 hour light-dark cycle and given a medicated basal diet (Appendix 1) and water *ad libitum*.

#### ***Housing***

At four weeks-of-age, birds were placed into 48" x 54" pens with mesh raised floor (Figure 1).

#### ***Diet***

A standard turkey ration (Appendix 1) was supplemented with a chemically modified form of vitamin C (Rovimix<sup>®</sup> STAY-C 35<sup>®</sup>) with molar equivalence to L-ascorbic acid to retard oxidation.. Analysis of vitamin C (Appendix 3) was performed by Craft Technologies, Inc. (Wilson, NC) to validate the biological activity of the concentration of vitamin C used.

## ***Experimental Protocols***

***Experiment 1.*** Four-week-old birds were divided into three “Supplemented” groups and one “Un-Supplemented” (0 mg) group of four birds each for a total of 16 birds. After two weeks of social and environmental acclimation, birds were given a feed supplemented with 0 (control), 75, 150, or 300 mg/kg vitamin C *ad libitum*. Birds were bled on 0, 1, and 2 days post-vitamin C supplementation (PVC). Birds were maintained on the vitamin C supplemented diet throughout the experiment.

***Experiment 2.*** Four-week-old poults were randomly divided into four “Un-Supplemented” and four “Supplemented” groups of four birds each per group for a total of 32 birds. The “Supplemented” group received feed supplemented with vitamin C and the “Un-supplemented” remained on the standard turkey ration. At 6 weeks-of-age (one day before social re-assortment Day -1), “Supplemented” birds were placed on 300 mg/kg vitamin C and maintained on the diet throughout the experiment. On day 0, birds were re-assorted among groups to disrupt social disorder as previously described (Chapter 2). Birds were bled at 0, 1, 2, 4, 6, 8, 14, and 21 days post-stress (PS).

## ***Sample Analysis***

Five milliliters of blood were removed from the jugular vein of each bird using a 23-gauge needle and a 5ml heparinized syringe.

***Plasma Corticosterone, IL-6, and TNF- $\alpha$  assays.*** Plasma was collected and assayed for corticosterone using a Coat-a-Count Rat Corticosterone In-Vitro Diagnostic Test kit and then frozen for subsequent vitamin C, IL-6, and TNF- $\alpha$  assays. Plasma IL-6 and TNF- $\alpha$  were analyzed (Experiment 2) as previously described (Chapter 2).

***H:L ratios.*** (H:L) ratios were analyzed by making microscope slide whole blood smears and stained with Modified Wright's Stain (1:10 vol/vol). H:L ratios were determined based on a 100-cell differential count as previously described (Chapter 2).

***Flow cytometric analysis.*** Peripheral blood lymphocytes (PBLs) were removed using a modified slow spin method as previously described (Chapter 2). PBLs were analyzed for percentage of CD4<sup>+</sup> and CD8<sup>+</sup> T cells flow by cytometric analysis (Experiment 2). CD4:CD8 ratios were calculated from CD4<sup>+</sup> and CD8<sup>+</sup> percentages.

***Plasma Vitamin C.*** Plasma was also collected and assayed for vitamin C using a modified spectrophotometric assay (Liu et al., 1982). Samples were analyzed indirectly by measuring the absorbance of a complex of ferrous ion, 2,2' dipyridyl (2,2'bipyridine), and sodium acetate. The product is formed by reducing the ferric iron complex under acidic conditions. Ascorbic acid was specifically quantified by the pretreatment of one pair of replicate samples with ascorbate oxidase (ao) to oxidize the

ascorbic acid. The samples were then reacted with the ferric iron complex and the difference measured at an absorbance of 525nm.

Specifically, 50  $\mu$ l of plasma was added to two microcentrifuge tubes. One tube contained 25  $\mu$ L of ao (ao+) and the other tube contained 25 $\mu$ L of distilled water (ao-). The samples were then incubated at 37°C for 15 minutes. After 15 minutes of incubation, 750 $\mu$ L of a plasma ascorbic acid reagent (625 $\mu$ L Na acetate: 75 $\mu$ L dipyridyl: 50 $\mu$ L ferric chloride for a total volume of 750 $\mu$ L per sample tested) was added. Two hundred microliters of sample was added in triplicate to 96-well culture plates and the absorbance read. Ascorbic acid standards were prepared at 0, 6.25, 12.5, 25, 50, and 100  $\mu$ g/mL using L-Ascorbic Acid. Plasma was reanalyzed in Experiments 1 to determine the loss of plasma vitamin C activity with multiple freeze thaws.

***Splenic Vitamin C.*** On day 21 PS, splenic samples, previously frozen (Experiment 2) were homogenized with phosphate buffered saline (PBS) 1:1 (vol/vol) and measured for vitamin C using the above assay. Spleen samples were also placed in 10% neutral buffered formalin for histopathological analysis (Experiment 2). At the end of each experiment, birds were euthanized by cervical dislocation.

***Statistical Analysis*** Data were analyzed using the SAS statistical software with a repeated measures analysis of variance (RMANOVA) and a Tukey-Kramer post-hoc analysis for Experiment 1 with a p value of < 0.05. Numbers are numerically statistical

unless otherwise indicated. Statistical analysis assistance was provided by Daniel Ward (statistical analyst at VA-MD Regional College of Veterinary Medicine).

### **3.4 Results**

#### ***Experiment 1***

There were no statistical differences observed in regard to plasma corticosterone between the supplemented groups and “Un-Supplemented” (0 mg) control group, however higher doses of vitamin C resulted in numerically lower corticosterone values (Figure 1). Birds supplemented with 300 mg/kg vitamin C had significantly decreased H:L ratios on day 1 (0.34 vs. 0.54) and day 2 (0.31 vs. 0.53) PVC compared to the Un-Supplemented (0 mg) control group (Figure 2). Supplementation with 150 mg/kg increased plasma vitamin C levels on days 1 (17.39 µg/ml vs. 8.85 µg/ml) and 2 PVC (14.08 µg/ml vs. 8.35 µg/ml) compared to the control (0 mg) group. Vitamin C supplementation with 300mg/kg vitamin C also resulted in increased plasma vitamin C levels on days 1 (20.53 µg/ml vs. 8.85 µg/ml) and 2 PVC (20.26 µg/ml vs 8.35 µg/ml) in the 0mg group (Figure 3). Vitamin C levels returned to pre-treatment levels by day 2 PVC in all treatment groups except the 300 mg/kg group. When treatment groups were compared to each other, a Tukey-Kramer post-hoc analysis revealed that poult supplemented with 150 mg/kg vitamin C were increased compared to the 75 mg group on days 1 (17.39 µg/ml vs. 9.16µ gml) and 2 (14.98 µg/ml vs. 7.46 µg/ml) PVC. Plasma vitamin C levels were also increased in the 300mg/kg group compared to the 75mg groups on days 1 (20.53 µg/ml vs. 9.16 µg/ml) and 2 (20.26 µg/ml vs. 7.46 µg/ml) PVC.

Plasma vitamin C was also increased in the 300 mg compared to the 150 mg group (20.26  $\mu\text{g/ml}$  vs. 14.08  $\mu\text{g/ml}$ ) on day 2 PVC. After an additional freeze-thaw, there was an average of 33.89% loss of ascorbate in the samples (data not shown). All plasma samples were adjusted for the loss of ascorbate. The average plasma vitamin C value for unsupplemented turkeys in this experiment was approximately 9.26  $\mu\text{g/ml}$ .

## ***Experiment 2***

In Experiment 2, there were no significant changes in regard to plasma corticosterone levels between the “Supplemented” and the “Un-Supplemented” group, however values were numerically lower in the treatment group (Figure 5). H:L ratios were significantly lower in the Supplemented group on day 0 (0.55 vs. 0.70) and day 2 (0.54 vs. 0.71) PS compared the Un-Supplemented control group. H:L ratios were also numerically lower on day 1 PS (0.63 vs 0.75) in the “Supplemented” group compared to the “Un-Supplemented” (Figure 6).

Vitamin C supplementation increased  $\text{CD4}^+$  cell percentages on day 1 PS compared to the “Un-Supplemented” group (14.14% vs. 12.12%, respectively) (Figure 7). The percent of  $\text{CD8}^+$  cells also increased on day 1 PS in the “Supplemented” group compared to the “Un-Supplemented” group (9.75% vs. 6.72%, respectively) (Figure 8).  $\text{CD4}:\text{CD8}$  ratios were decreased in the Supplemented group on day 1 PS (1.45% vs. 1.81%) (Figure 9). All T- cell percentages returned to pre-stress levels by day 2 PS. There were no statistical differences in regard to plasma IL-6 or TNF-. However, both IL-6 and TNF- $\alpha$  were numerically lower in the supplemented group on day 1 PS (Figures 10 and 11).

Plasma vitamin C was statistically higher in the “Supplemented” group compared to the “Un-Supplemented” group. However, plasma vitamin C in the “Supplemented” group decreased (16.96 $\mu$ g/ml) and vitamin C increased in the “Un-Supplemented group” (11.33 $\mu$ g/ml) on day 1 PS (Figure 12). All plasma values returned to pre-stress levels by day 2 PS. After an additional freeze-thaw, there was an average of 33.59% loss of ascorbate in the samples (data not shown). All plasma samples were adjusted for the loss of ascorbate. Vitamin C supplementation increased splenic vitamin C on day 21 PS (48.38 $\mu$ g/ml vs. 14.53 $\mu$ g/ml) (Figure 13). There were no differences observed between the groups upon histopathological evaluation on day 21.

### **3.5 Discussion**

The efficacy of supplementing birds with vitamin C under stressful conditions depends upon its ability to elevate plasma vitamin C (Pardue et al., 1984). Therefore, in the first experiment, we determined whether 0, 75, 150, and 300 mg/kg of vitamin C in the diet would elevate plasma vitamin C and influence specific physiological parameters. Dorr and Balloun (1976) reported that the average plasma vitamin C level for turkeys was 9.6  $\mu$ g/ml under normal conditions. In the current experiment under normal conditions, the average plasma vitamin C was 10  $\mu$ g/ml. There were no significant changes in plasma corticosterone between the groups. Vitamin C supplemented at 300 mg/kg was able to elevate plasma vitamin C and reduce H:L ratios. Gross and Siegel (1983) reported that H:L ratios are a better indicator of stress than plasma corticosterone as it reflects the stress status more accurately. Plasma vitamin C increases are consistent with findings by

Nestor et al., (1972) who reported that 330 ppm vitamin C elevated blood vitamin C in turkeys. Pardue et al., (1984) also reported that vitamin C supplementation at 250 ppm increased plasma vitamin C concentrations in broilers. However, Sell et al., (1997) found that 300 mg/kg vitamin C was unable to increase plasma vitamin C in turkey breeder toms. The inability of vitamin C to elevate plasma levels in the experiment by Sell et al., (1997) may be due to the fact that turkeys were provided dietary vitamin C only between 20 and 40 days-of-age. These birds were also on a feed restriction program to prevent obesity, which may not have allowed for sustained circulating levels of vitamin C. The present experiments, poults were provided vitamin C continuously from 41 days-of-age to 63 days-of age.

Bobrowicz et al., (2001) reported an estimation of vitamin C (ascorbic acid) in that freezing in a regular freezer at -25 degrees C causes an approximately 14% loss of ascorbate in the sample. In the first and second experiment, vitamin C levels were adjusted for the 33.89% loss of ascorbate in the plasma samples. Therefore, it is suggested that samples be analyzed as soon as possible to prevent loss of ascorbate and flawed data.

In the second experiment, the effect of supplementing vitamin C at 300 mg/kg on stress was examined. Kolb (1984) strongly suggested that supplemental vitamin C should be provided in poultry and livestock diets as a stress alleviator. Hill and Garren (1958) agreed and stated that vitamin C was an essential nutrient in chickens experiencing stress. Vitamin C supplementation at 300 mg/kg was able to decrease plasma corticosterone in stressed birds. This observation is consistent with others who

reported that supplemental vitamin C alleviated heat stress-induced elevations of plasma corticosterone (Pardue and Thaxton, 1982, 1986; Kutlu and Forbes, 1993; Mahmoud et al., 2004). These responses are supported by research suggesting that vitamin C suppresses adrenocortical steroidogenesis and depresses plasma corticosterone levels, therefore limiting some of the deleterious responses associated with stress and delays the depletion of steroid hormone precursors (Pardue et al., 1985; Gross, 1992; Kutlu and Forbes, 1993). The action of vitamin C on adrenal steroidogenesis is associated with its ability to inhibit adrenal steroid hydroxylating enzymes (Kitabchi, 1967). Supplementation also decreased H:L ratios which is consistent with previous research demonstrating that vitamin C alleviates stress-induced increases in H:L ratios in chickens (Gross, 1988; Pardue and Williams, 1990).

Supplementation with 300 mg/kg resulted in a significant increase in plasma vitamin C throughout the experiment when compared to the “Un-Supplemented” birds. These observations are consistent with findings by Pardue et al., (1984). However, “Supplemented” birds did not show an increase in the plasma vitamin C levels. Plasma vitamin C might have a saturation point defined by the amount of intake of vitamin C (Pardue et al., 1984), which in turn can affect the renal excretion of vitamin C. Other researchers observed similar results in chickens subjected to cyclic heat stress and supplemented with dietary vitamin C (Mahmoud et al., 2004). It has been documented that vitamin C requirements increase as the number and intensity of various stressors in the environment increase and psychological stress (Siegel, 1971; Nockels et al., 1973; Pardue and Thaxton, 1985). Therefore the increase in plasma vitamin C in the “Un-

Supplemented” group may be due to the increased body requirement for vitamin C during stress. Either way, “Supplemented” birds maintained higher levels of plasma vitamin C, which appeared to be enough to supply the increasing tissue demand for vitamin C.

In Experiment 2, splenic vitamin C was found to be 37% higher in the supplemented group on day 21 compared to plasma levels on day 21. Other tissues, including the spleen have been documented to contain ascorbic acid concentrations several times greater than those in plasma. The sizeable difference between plasma and tissue ascorbic acid suggests active transport from the blood to the tissue (Pardue and Thaxton, 1985).

Also in the second experiment and previous experiments, there was an increase in plasma IL-6 and TNF- $\alpha$  levels associated with stress. Exercise resembles trauma (stress) and has similar effects on cytokine production (Pedersen and Hoffman-Goetz, 2000). Yamashita et al., (1999) reported that rats that underwent an exercise regime had increased plasma IL-6 and TNF- $\alpha$  levels. Supplemental vitamin C in the diets reduced the levels of these pro-inflammatory cytokines. These observations are consistent with other researchers who demonstrated that after antioxidant supplementation TNF- $\alpha$  levels and IL-6 levels were reduced (Pedersen and Hoffman-Goetz, 2000; Vassilakopoulos et al., 2003).

There is limited research examining the effects of antioxidants (vitamin C) on lymphocyte subset populations in chickens. However, those experiments that have been demonstrated the effects of vitamin C on lymphocyte populations in association with

disease or vaccination (Wu et al., 2000). Therefore the effects of supplemental vitamin C on lymphocyte subpopulations will be discussed in Chapter 4.

Although research examining the effects of dietary vitamin C supplementation is still controversial, these experiments demonstrated that vitamin C supplementation at 300 mg/kg alleviated stress-induced affects in turkeys. These physiological and immunological findings suggest that vitamin C may be an anti-stress agent and may be an essential nutrient in poultry when poult are subjected to stressful conditions.

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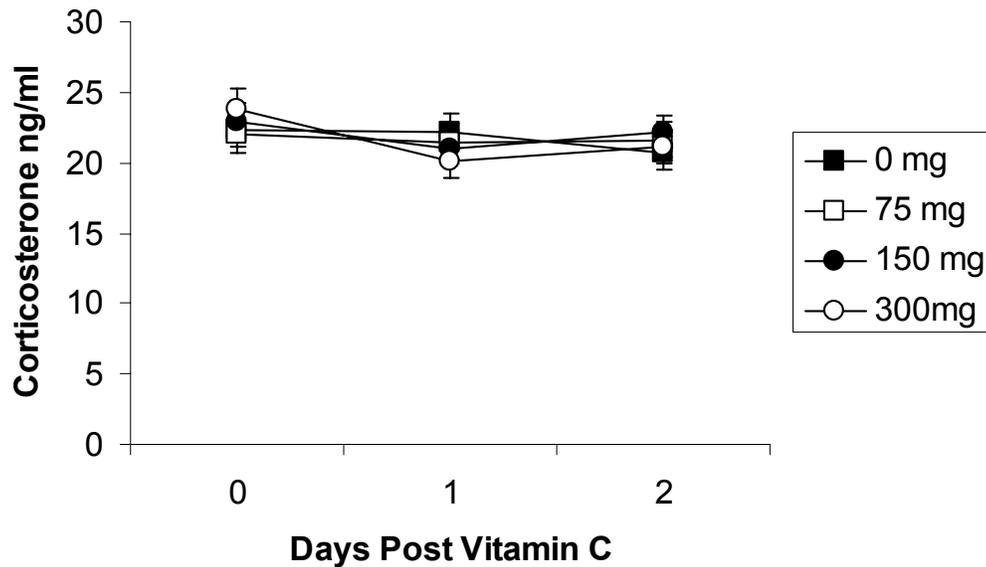
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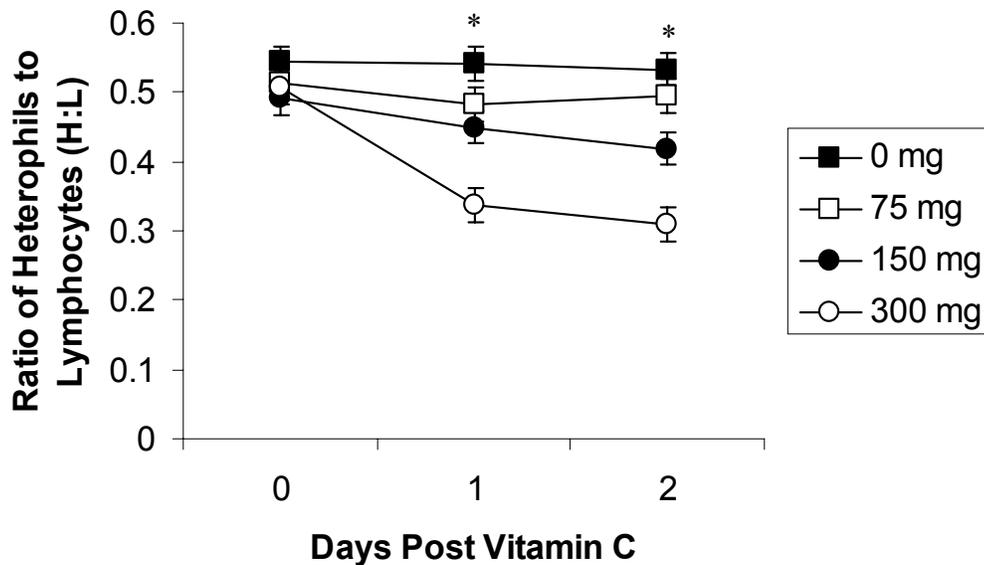
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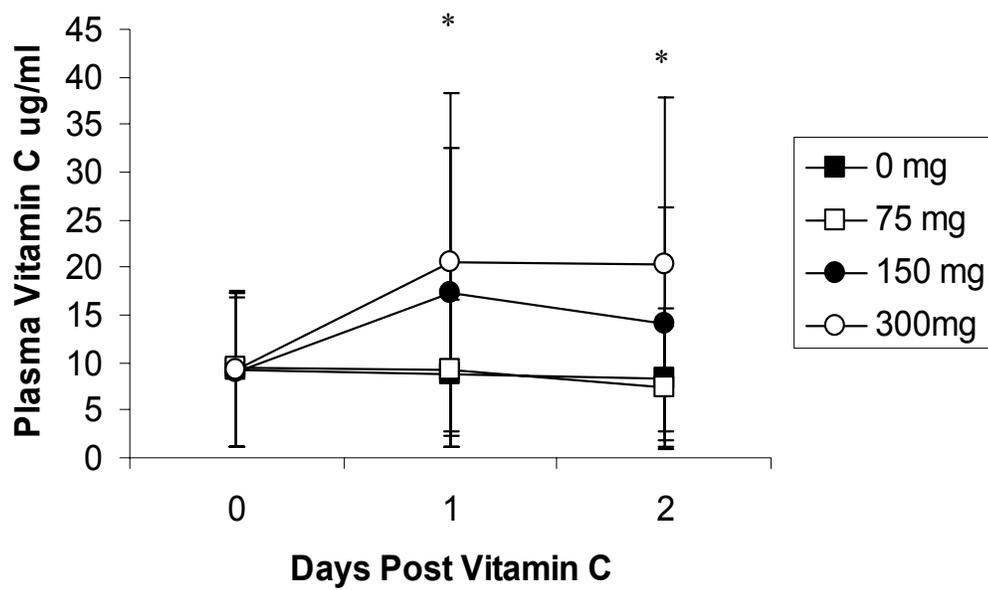
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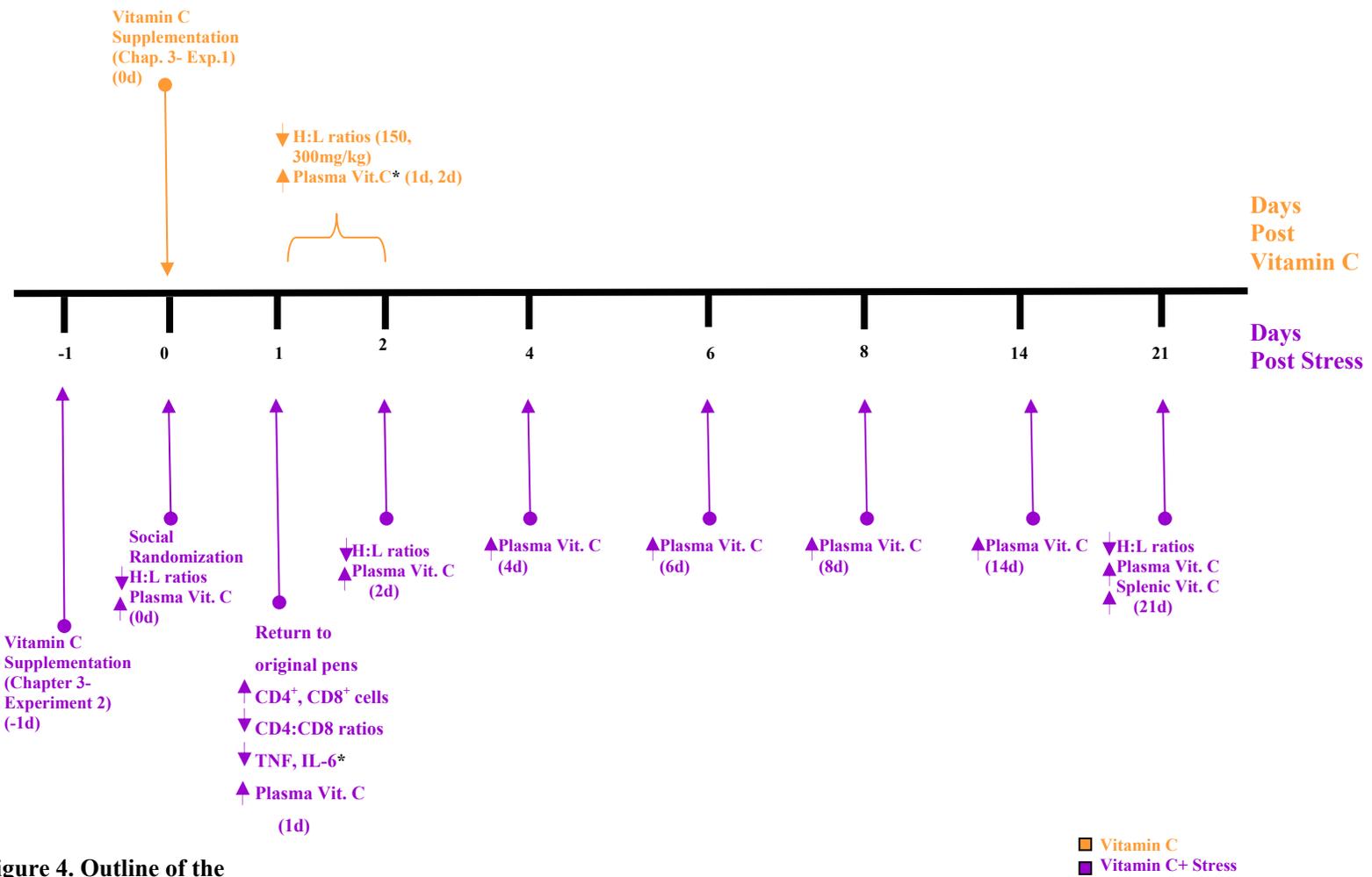
**Figure 1. Effect of Supplemental Vitamin C on plasma corticosterone.** Values were calculated from 4 individual birds per group and expressed as geometric means with 95% confidence limits.



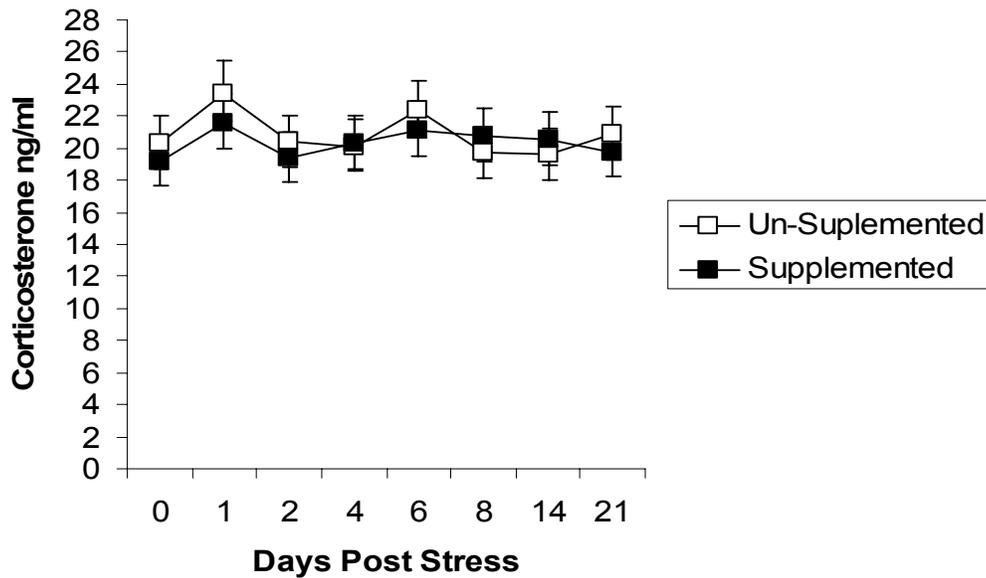
**Figure 2 Effect of Supplemental Vitamin C on Heterophil to Lymphocyte ratios (H:L).** Values were calculated from 4 individual birds per group and expressed as geometric means with 95% confidence limits. Asterisks indicate a significant difference ( $P < 0.05$ ).



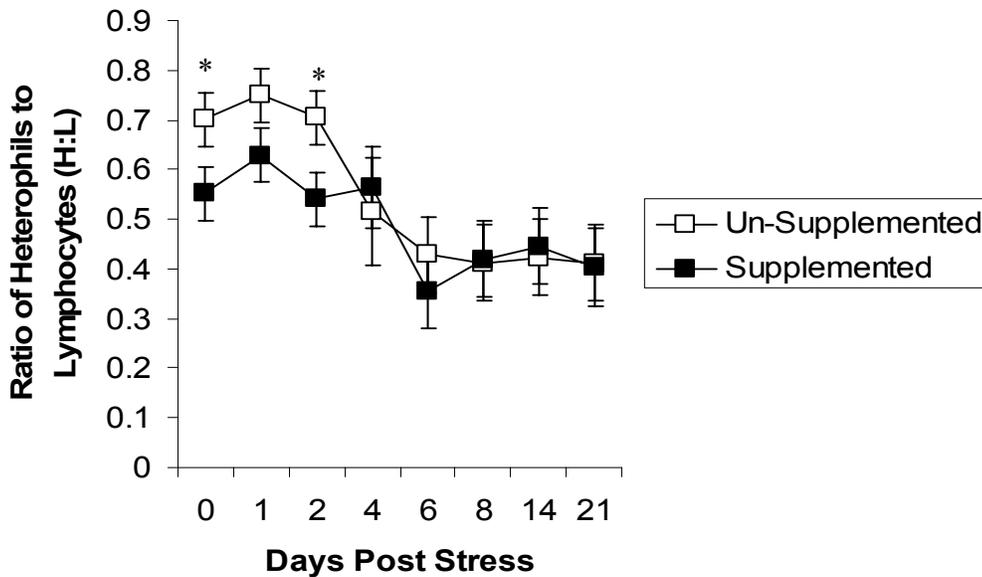
**Figure 3. Effect of Supplemental Vitamin C on plasma Vitamin C.** Values were calculated from 4 individual birds per group and expressed as geometric means with 95% confidence limits. Asterisks indicate a significant difference ( $P < 0.05$ ).



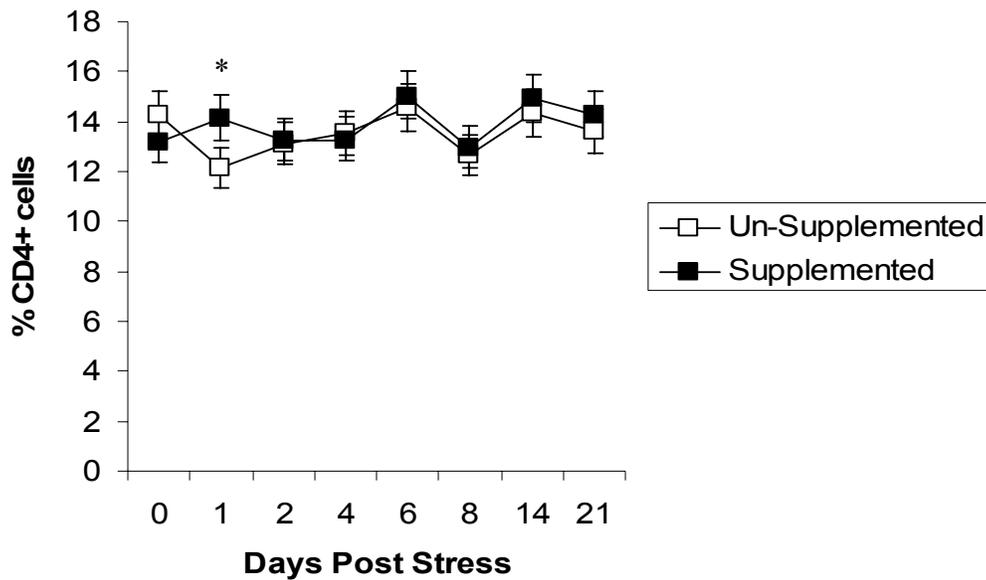
**Figure 4. Outline of the occurrences during Vitamin C Supplementation and Stress**



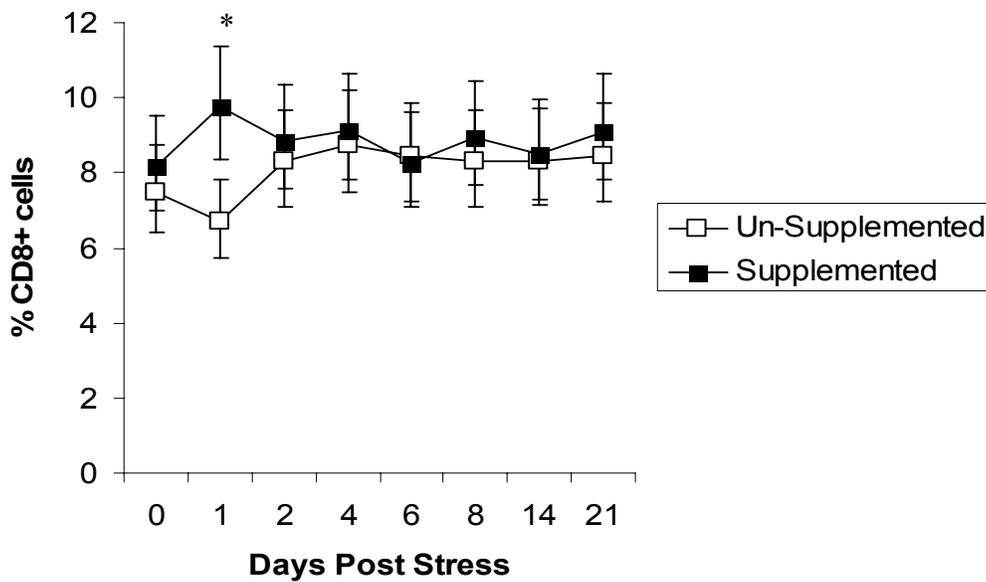
**Figure 5. Effect of Supplemental Vitamin C on plasma corticosterone in stressed poult.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits.



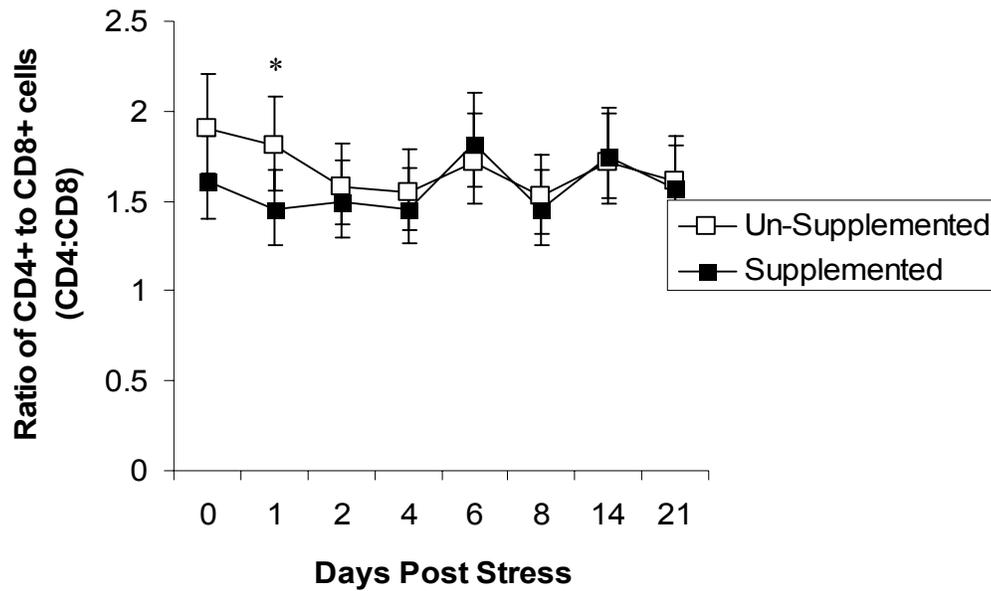
**Figure 6. Effect of Supplemental Vitamin C on H:L ratios in stressed poult.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a significant difference ( $P < 0.05$ ).



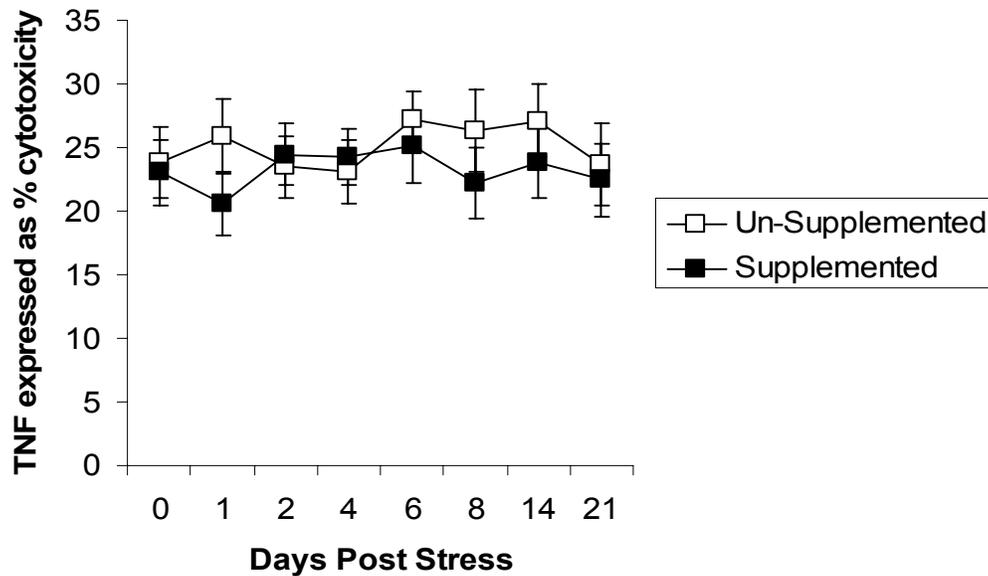
**Figure 7. Effect of Supplemental Vitamin C on CD4+ cell populations in stressed poult.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a significant difference ( $P < 0.05$ ).



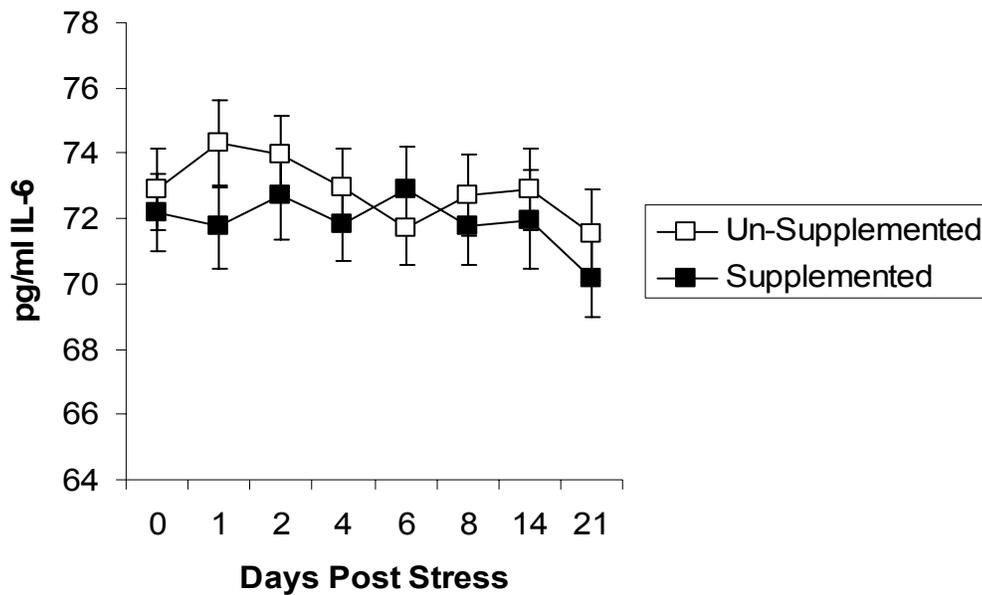
**Figure 8. Effect of Supplemental Vitamin C on CD8+ cell populations in stressed poult.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a significant difference ( $P < 0.05$ ).



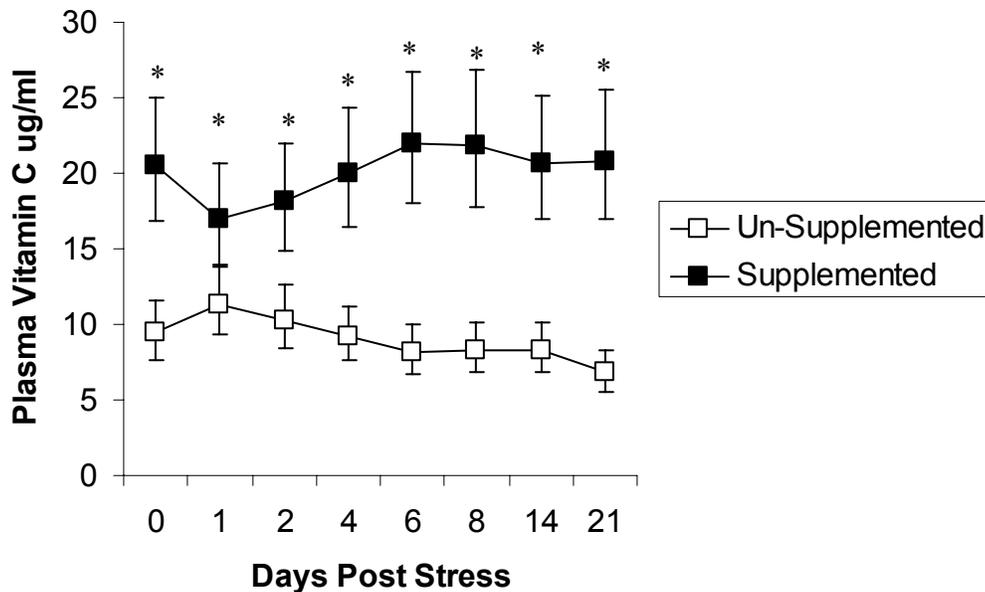
**Figure 9. Effect of Supplemental Vitamin C on CD4: CD8 ratios in stressed poult.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a significant difference ( $P < 0.05$ ).



**Figure 10. Effect of Supplemental Vitamin C on plasma TNF- $\alpha$  levels in stressed poult.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits.



**Figure 11. Effect of Supplemental Vitamin C on plasma IL-6 levels in stressed poult.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits.



**Figure 12. Effect of Supplemental Vitamin C on plasma Vitamin C levels in stressed poult.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. Asterisks indicate a significant difference ( $P < 0.05$ ).



**Figure 13. Effect of Supplemental Vitamin C on splenic Vitamin C levels in stressed poult on day 21.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a significant difference ( $P < 0.05$ ).

### Chapter 3 Summary Table

	Corticosterone	H:L	CD4 <sup>+</sup>	CD8 <sup>+</sup>	CD4:CD8	TNF- $\alpha$	IL-6	HEV Ab Titer	Plasma Vit C	Splenic Vit C
Exp 1	--	↓							↑	
Exp 2	↓	↓	↑	↑	↓	↓	↓		↑	↑

↑ = Increase

↓ = Decrease

-- = No Change

+ = Positive

- = Negative

Table 1. Summary.

## Chapter 4

### Effect of Social Stress on the Responses of Vitamin C-Treated Turkeys to Vaccination with Hemorrhagic Enteritis Virus

#### 4.1 Abstract

The purpose of these experiments was to investigate the effects of supplementing diets with vitamin C on vaccination with an avirulent strain of hemorrhagic enteritis virus (HEV) and to determine if supplementation would have a beneficial effect on the simultaneous application of social stress and vaccination. Two experiments were conducted. In Experiment 1, 32, four-week-old poult were placed into 1 “treatment” group supplemented with 300 mg/kg of Rovimix<sup>®</sup> STAY C-35<sup>®</sup> and one “Un-Supplemented” control group. Social adaptation and vitamin C supplementation (Day-1) were as described in Chapter 3. At 6 weeks-of-age (Day 0), both groups were orally inoculated with 100 turkey infectious dose 50 (TID<sub>50</sub>) of avirulent HEV. Plasma corticosterone, heterophil to lymphocyte ratios (H:L), CD4<sup>+</sup> and CD8<sup>+</sup> cell percentages, CD4:CD8 ratios, tumor necrosis factor-alpha (TNF- $\alpha$ ) levels, plasma interleukin-6 (IL-6) levels, plasma vitamin C levels, splenic vitamin C levels and histopathology were determined on 0, 1, 2, 4, 6, 8, 14 and 21 days post-vaccination (PV). HEV antibody titers were analyzed on days 0, 8, 14, and 21 PV. On days 5 and 21 PV, splenic samples were analyzed for the presence of viral antigen and deoxyribonucleic acid (DNA) using, agar gel immunodiffusion (AGID), and nested polymerase chain reaction (PCR). H:L ratios

were lower, CD8<sup>+</sup> cell percentages increased and CD4:CD8 ratios decreased in the “Supplemented” group. Vitamin C supplementation resulted in higher HEV antibody titers, and increased plasma and splenic vitamin C levels. On day 5, AGID and PCR revealed the presence of viral antigen in the spleen in both groups. Upon visualization, birds in the “Supplemented” group were larger and less mottled than those in the “Un-Supplemented” group. Spleen histopathology showed “Supplemented” birds had less lymphoid proliferation in the ellipsoids and fewer aggregates of small lymphocytes throughout the red pulp. Spleens from “Un-Supplemented” birds had moderate proliferation of the ellipsoids and obliteration of the red pulp. On day 21 PV, there were no differences observed between the groups on histopathological analysis or AGID. However, there were more positive splenic samples for viral DNA in the “Supplemented” group than the “Un-Supplemented” group.

In Experiment 2, thirty-two poultts were divided into four “Supplemented” and four “Un-Supplemented” control groups. Social adaptation, vitamin C supplementation and HEV vaccination were as described in Experiment 1 and Chapter 3. On day 0, birds were orally inoculated with HEV and re-assorted among groups to disrupt social order. They remained in these unstable settings for 24 hours and then were reunited with their original pen mates. Blood samples were taken and parameters were analyzed as were described in Experiment 1. “Supplemented” birds had higher plasma vitamin C levels, lower HEV antibody titers, and increased plasma TNF- $\alpha$  levels. H:L ratios were lower in the supplemented group on day 4, but increased ratios on day 6 PV. No histopathological

differences were detected; however it appears that there was more viral DNA present in the spleens on day 21 PV in the “Supplemented” group.

Results of the first study suggested that although supplementation alleviated some of the stress-induced immunosuppression, it may also have reactivated latent HEV virus in macrophages. When stress was added in the second experiment, vitamin C supplementation appeared to alleviate the negative effects of stress, however, viral DNA was still present in higher amounts than the “Un-Supplemented” group.

*Keywords:* Vitamin C, stress, HEV, hemorrhagic enteritis virus, social stress, turkeys

## 4.2 Introduction

The effects of vitamin C in poultry have been well documented. Vitamin C has been shown to influence immunological parameters and improve performance in poultry subjected to disease. In early studies, McCorkle et al., (1980) reported that supplementing vitamin C in the diet prior to immunization increased antibody production against *Brucella abortus*. The addition of vitamin C was reported to increase the resistance to *Escherichia coli* (*E.coli*) challenge and reduce the incidence of pericarditis in chickens (Gross et al., 1988). Gross (1992) also reported that the addition of ascorbic acid in the diet increased resistance to combined Newcastle disease virus (NDV) and *Mycoplasma gallisepticum* infection and primary and secondary *E.coli* infections in chickens. Recent studies have documented that birds supplemented with vitamin C had elevated antibody titers to infectious bursal disease (IBD) following vaccination and higher body weights than unsupplemented controls (Amakye-Anim et al., 2000). Others researchers have shown that supplemental vitamin C elevated the percentage of CD8<sup>+</sup> cells in the spleen, IgM<sup>+</sup> cells in the bursa, and increased production of IL-2 by splenocytes in birds vaccinated and challenged with IBD (Wu et al., 2000).

Most experimental work involving environmental stressors in poultry have usually involved the effect of one or two stressors. In most situations, these experiments are not representative of poultry production practices. Many of these stressors vary in duration, intensity, and time and can often occur concurrently with other stressors (Curtis, 1983; McFarlane and Curtis, 1988; Mc Kee and Harrison, 1995). Stressors that can

affect poultry include extreme environmental temperatures, disease, handling, transport, beak trimming, vaccinations, crowding and nutrition (Pardue and Thaxton, 1985).

There are very few experiments that focus on the effects of concurrent multiple stressors in poultry and its effect on disease. McFarlane et al., (1989a) reported that chicks exposed to coccidiosis, atmospheric ammonia, beak trimming, electric shock, heat stress and continuous noise had decreased weight gain, decreased feed intake and decreased feed efficiency, regardless of whether the stressor occurred singularly or concurrently. In another study, McFarlane et al., (1989b) demonstrated that heterophil, monocyte and eosinophil percentages were increased and lymphocyte and basophil percentages were decreased, using the same stressors as previously reported, regardless of the order of application.

Experimental work examining the effects of ascorbic acid on multiple stressors in poultry has been limited. Gross (1992) reported that birds that were socially stressed and supplemented with ascorbic acid, resulted in increased resistance to *E.coli*. In a later study, McKee and Harrison (1995) demonstrated that addition of ascorbic acid enhanced performance of broiler chicks exposed to multiple concurrent environmental stressors.

Hemorrhagic enteritis (HE) is an acute viral disease that affects turkeys 4 weeks-of age or older. It is characterized by bloody droppings, depression, splenomegaly, transient immunosuppression, and sometimes death. HE is caused by a siadenovirus (Davison, and Harrach 2001) and is considered to be lymphotropic and lymphocytopathic with B cells and macrophages being the target cells (Hussain et al., 1993; Saunders et al., 1993). HEV induces necrosis and apoptosis in infected and

possibly by-stander cells (Rautenschlein et al., 2000a). The ability of birds to mount an optimal immune response as well as normal macrophage functions such as phagocytosis may be impaired. The principal method for the control of HE is vaccination using a live avirulent strain in the water around four to six weeks-of-age. Although vaccination has proved to be a successful strategy to protect against disease, the vaccine itself causes transient immunosuppression and thus predisposes turkeys to various bacterial and viral diseases (Pierson et al., 1996).

Poults are subjected to multiple concurrent stressors and these stressors can have an additive detrimental effect on performance, (McFarlane and Curtis, 1989; McFarlane et al., 1989a,b). McKee and Harrison (1995) theorized that one could subtract out the detrimental effect of a single stressor in a multi-stressor situation by incorporating a specific stress alleviator, such as vitamin C. Experimental work observing the effects of supplemental vitamin C on response to HEV vaccination or the application of stress during vaccination on immunological and physiological parameters in turkeys have not been previously performed until now.

Therefore, the purpose of these experiments was to investigate the effects of supplementing diets with vitamin C on vaccination with an avirulent vaccine strain of hemorrhagic enteritis virus (HEV) and to determine if supplementation would have a beneficial effect with the simultaneous application of social stress and vaccination.

## 4.3 Materials and Methods

### *Animals*

Thirty-two, day-old turkeys were obtained from British United Turkeys of America (BUTA, Lewisburg, WV). They were handled daily to acclimate them to restraint and sampling and placed on a 12 hour light-dark cycle as described in Chapter 2.

### *Diet*

A standard turkey ration (Appendix 1) was supplemented with a chemically modified form of vitamin C (Rovimix<sup>®</sup> STAY-C 35<sup>®</sup>) as described in Chapter 3. Analysis of vitamin C (Appendix 3) was performed by Craft Technologies, Inc. (Wilson, NC) to validate the biological activity of the concentration of vitamin C added to the diet

### *Experimental Protocols*

***Experiment 1.*** Four-week-old poults were divided into 1 “Supplemented” and 1 “Un-Supplemented” control group of 16 birds each for a total of 32 birds. At six weeks-of-age, and 1 (Day -1) day before inoculation, birds in the “Supplemented” group were placed on feed containing 300 mg/kg vitamin C in the form of Rovimix<sup>®</sup> STAY C-35<sup>®</sup>. The “control” group remained on un-supplemented feed. On day 0, birds were orally inoculated with 0.5ml of 100 turkey infectious dose 50 (TID<sub>50</sub>) of an avirulent strain of HEV (Domermuth et al., 1977). Blood samples were taken on 0, 1, 2, 4, 6, 8, 14, and 21 days post vaccination (PV).

**Experiment 2.** At 4-weeks-of-age, birds were randomly divided into four “Supplemented” and four “Un-Supplemented” groups of 4 birds each group for a total of 32 birds. After two weeks of environmental and social adaptation and one day before social re-arrangement (Day-1), vitamin C was supplemented as described in Experiment 1. Birds in the “Un-Supplemented” group remained on the standard turkey ration. On day 0, birds were re-assorted among groups to disrupt social disorder and orally vaccinated with HEV as previously described. Birds remained in these unstable social settings for 24 hours and were then reunited with their original group mates. Blood samples were taken as described in Experiment 1.

### ***Sample Analysis***

Five milliliters of blood were removed from the jugular vein of each bird using a 23-gauge needle and a 5ml heparinized syringe.

***Plasma corticosterone, IL-6 and TNF- $\alpha$  assays.*** Plasma was collected and assayed for corticosterone using a Coat-a-Count Rat Corticosterone In-Vitro Diagnostic Test kit and then frozen for subsequent vitamin C, IL-6, and TNF- $\alpha$  assays. Plasma IL-6 and TNF- $\alpha$  were analyzed as previously described (Chapter 2).

***Heterophil to lymphocyte ratios (H:L).*** H:L ratios were analyzed by making microscope slide whole blood smears and stained with Modified Wright’s Stain

(1:10 vol/vol). H:L ratios were determined based on a 100-cell differential count as previously described (Chapter 2).

***Flow cytometric analysis.*** Peripheral blood lymphocytes (PBLs) were removed using a modified slow spin method as previously described (Chapter 2). PBLs were analyzed for percentage of CD4<sup>+</sup> and CD8<sup>+</sup> cells flow cytometric analysis. CD4:CD8 ratios were calculated from CD4<sup>+</sup> and CD8<sup>+</sup> percentages.

***Plasma Vitamin C.*** Plasma was also collected and assayed for vitamin C using a modified spectrophotometric assay developed by Liu et al., (1982) as described in Chapter 3.

***Splenic Vitamin C.*** On days 5 (Experiment 1) and 21 (Experiment 1 and 2) PS, splenic samples, previously frozen were homogenized with phosphate buffered saline (PBS) 1:1 (vol/vol) and measured for vitamin C as described in Chapter 3.

***Histopathology.*** Spleen samples were also placed in 10% neutral buffered formalin for histopathological analysis. On days 5 (Experiment 1) and 21 (Experiment 1 and 2) PV.

***Agar Gel Immunodiffusion (AGID).*** The presence of HEV antigen in the spleen was determined by AGID Domermuth et al., 1972, 1973; (Pierson et al., 1998) on days 5 (Experiment 1) and 21 (Experiment 1 and 2) PV as described in Chapter 2.

***PCR analysis.*** Viral DNA was extracted from spleens and confirmed using a modified version of nested PCR on days 5 (Experiment 1) and 21 (Experiment 1 and 2) PV as previously described in Chapter 2.

At the end of each experiment, birds were euthanized by cervical dislocation.

***Statistical Analysis*** Data were analyzed using the SAS statistical software with a repeated measures analysis of variance (RMANOVA) with a p value of <0.05. Numbers are numerically statistical unless otherwise indicated. Statistical analysis assistance was provided by Daniel Ward (statistical analyst at VA-MD Regional College of Veterinary Medicine).

## **4.4 Results**

### ***Experiment 1***

An outline of the major occurrences for Experiment 1 is shown in Figure 1. Plasma corticosterone was lower in the “Supplemented” group compared to the “Un-Supplemented” group on day 6 PV (23.68 ng/ml vs. 27.47 ng/ml, respectively) (Figure 2). H:L ratios were lower in the “Supplemented” group, however significance was only observed on day 0 PV (0.38 vs. 0.55, respectively) (Figure 3).

There were no differences in CD4<sup>+</sup> cell populations (Figure 4). Vitamin C supplementation increased the percentage of CD8<sup>+</sup> cells on days 1 (17.25% vs. 14.26%), 2 (23.66% vs. 17.42%), 6 (18.41% vs. 15.74%), 8 (32.61% vs. 26.48%), and 21 (17.60% vs. 14.06%) PV (Figure 5). CD4:CD8 ratios decreased in the vitamin C supplemented group on days 1 (1.02 vs. 1.24), 2 (0.77 vs. 1.07), 6 (0.87 vs. 1.05), 8 (0.59 vs. 0.72), day 21 (0.91 vs. 1.18) PV (Figure 6).

There were no significant differences in regard to plasma TNF- $\alpha$  or IL-6 between the “Supplemented” and “Un-Supplemented” groups (Figure 7, 8).

There were no statistical differences in HEV antibody titer in regard to supplementation, however titers were numerically higher in the “Supplemented” group compared to the “Un-Supplemented” controls (Figure 9).

Vitamin C supplementation at 300 mg/kg resulted in an increased plasma vitamin C on all days. (Figure 10). After an additional freeze-thaw cycle, there was an average of 34.43% loss of ascorbate in the plasma samples (data not shown). Plasma samples were adjusted for the loss. Supplementation also resulted in an increased splenic vitamin C on day 5 PV (71.98 $\mu$ g/ml vs. 27.83 $\mu$ g/ml) (Figure 11) and day 21 PV (27.83 $\mu$ g/ml vs. 12.48 $\mu$ g/ml) (Figure 12)

On day 5, histopathology analysis revealed that spleens from birds in the vitamin C group had less lymphoid proliferation in the ellipsoids and fewer nodular aggregates of small lymphocytes throughout the red pulp. Visually, splenic samples in the “Supplemented” were smaller in size in the vitamin C birds and were less mottled in appearance, resembling normal, healthy spleens. Spleens from the “Un-Supplemented”

group had moderate proliferation of the ellipsoids and marked expansion of the red pulp with large lymphocytes and plasma cells (Figure 13). Upon histopathological analysis and gross visualization, there were no differences between the vitamin C “Supplemented” group and the “Un-Supplemented” controls on day 21.

On day 5, nested PCR (Figure 14) and AGID revealed the presence of viral DNA and viral antigen in the spleen in both groups. On day 21PV, there was more viral DNA present in the “Supplemented” group compared to the “Un-Supplemented” group.

## ***Experiment 2***

A diagram depicting the events of Experiment 2 are shown in Figure 15. Plasma corticosterone (Figure 16) was reduced in the “Supplemented” compared to the “Un-Supplemented” group on day 0 PV (14.32 ng/ml vs. 22.34 ng/ml). The reduction in plasma corticosterone occurred one day after supplementation. There was no difference between the groups thereafter. H:L ratios were decreased in the “Supplemented” group on day 4 (0.58 vs. 0.81, Figure 17). Although H:L ratios were decreased in the “Supplemented” group on day 4, they were numerically higher compared to the “Un-Supplemented” group on day 6 PV. There were no differences in regard to the percentage of CD4<sup>+</sup> or CD8<sup>+</sup> among PBLs (Figure 18 and 19). CD4:CD8 ratios were increased in the “Supplemented” group on day 21 PV compared to the “Un-Supplemented” controls (0.53 vs. 0.37, respectively, Figure 20). There was also a decrease in plasma TNF- $\alpha$  on day 6, PV in the vitamin C “Supplemented” group compared to the “Un-Supplemented” controls (Figure 21). The interaction of social

stress, vitamin C, and HEV vaccination caused a decrease in plasma IL-6 levels on days 0, 2, 4, and 6, days PV (Figure 22). HEV antibody titers were lower in the vitamin C supplemented group compared to the control group however they were only significant on day 14 PV ((1485.60 vs. 23.84, Figure 23). Plasma vitamin C was significantly increased in the “Supplemented” group compared to the “Un-Supplemented” controls on all days except days 14 and 21 PV (Figure 24). In addition, “Supplemented” birds displayed an increase in plasma vitamin C on day 8 PV. Both groups showed a decrease in plasma vitamin C on days 14 and 21 PV. Birds that received vitamin C supplemented feed resulted in an 85% elevation of splenic vitamin C (29.55 $\mu$ g/ml) compared to the control unsupplemented group (4.47 $\mu$ g/ml, Figure 25). All birds were positive for viral antigen on AGID on day 5 PV and negative on day 21 PV. Upon histopathological analysis, there was no difference between the vitamin C supplemented group and the “Un-Supplemented” group on day 21 PV. Ten out of sixteen (10/16) spleens were positive for viral DNA in the vitamin C “Supplemented” group compared to the 14 out of 16 (14/16) in the “Un-Supplemented” control group. (Figure 26).

## **4.5 Discussion**

Vaccination has been shown to be a stressor that can interfere with adequate biosynthesis of vitamin C in poultry (Gross, 1988; Satterlee et al., 1989). Results of the first study demonstrated that although vitamin C supplementation lowered HEV-induced increases in plasma corticosterone observed in Chapter 2, the differences were not significant. However, Amakye-Anim et al., (2000) reported that supplemental vitamin C

did not have a consistent effect on plasma corticosterone in birds vaccinated with IBD. Vitamin C supplementation also lowered the increase in H:L ratios on day 6 observed in association with HEV vaccination (Chapter 2). These results are similar to those reported by Gross (1992) in which chickens inoculated with *Mycoplasma gallisepticum* and NDV and fed vitamin C had lower H:L ratios than untreated controls.

HEV infection has been reported to show changes in lymphocyte subpopulations. There were no differences observed in birds vaccinated with HEV (Chapter 2) nor differences observed in birds vaccinated and supplemented with vitamin C in Experiment 1. These findings are consistent with work by Wu et al., (2000) who was unable to find differences in splenic CD4<sup>+</sup> T cells in birds vaccinated with IBD and supplemented with vitamin C. Results from Chapter 2 and previous researchers have demonstrated an increase in peripheral CD8<sup>+</sup> T cells on days 8-10 PV (Pierson, 1993). “Supplemented” birds had increased peripheral CD8<sup>+</sup> T cell on days 1, 2, 6, 8, and 21 PV. Others have reported an increase in splenic CD8<sup>+</sup> T cells in birds vaccinated with IBD and supplemented with vitamin C (Wu et al., 2000). This change in lymphocyte subpopulations is hypothesized to reflect the response of the immune system to viral challenge such. enhancement of viral clearance.

In several reports, humoral immunity was shown to be partially influenced by supplemental vitamin C. In the first experiment, “Supplemented” birds showed a better HEV antibody response than their “Un-Supplemented” counterparts. This finding is similar to those of previous reports in which dietary supplementation increased the antibody response of chickens to infectious bronchitis vaccine and IBD (Tuekam et al.,

1994; Amakye-Anim et al., 2000). Franchini et al., (1994) also demonstrated that broilers supplemented with vitamin C and vaccinated with NDV and *Pasteurella anatipestifer*, showed a better antibody response to both antigens than the unsupplemented controls.

HEV infection has also been shown to cause changes in cytokine production. In Chapter 2, plasma IL-6 and TNF- $\alpha$  were elevated in HEV+ birds, however vitamin C supplementation in Experiment 1 did not result in any significant changes in cytokine levels.

On visualization, the spleens from the “Supplemented” group in Experiment 1 appeared smaller with less mottling compared to those of the “Un-Supplemented” group on day 5 PV. Upon histopathological analysis, birds in the “Supplemented” group had decreased lymphocyte proliferation and fewer numbers of lymphocytes obliterating the red pulp than the “Un-Supplemented” group. These findings are similar to those of Gross (1988) demonstrating that the addition of supplemental vitamin C one day prior to *E. coli* challenge significantly reduced pericarditis and death in chickens. Spleens in the “Un-Supplemented group were larger and mottled in appearance which is consistent with dogma that HEV reaches peak levels of replication in the spleen around 5-6 days after oral inoculation as observed previously (Chapter 2) (Domermuth et al., 1972; Pierson and Fitzgerald, 2003). It is speculated that the decrease in lymphocyte numbers may be caused by vitamin C’s ability to improve macrophage activity. Improved macrophage activity may increase phagocytosis of infected cells and possibly antigen processing,

therefore, decreasing the need for a splenic response (proliferation of infected lymphocytes).

Although vitamin C supplementation decreased the mottling effects in HEV infection, viral DNA was still present on days 5 and 21 PV. B cells have been shown repeatedly to be the main target cell population for HEV infection, however it has also been shown to infect macrophages and reduce their functional capabilities (Rautenshlein et al., 1998). Therefore, it is speculated that the increased number of splenic DNA in the “Supplemented” group may be due to reactivation of latent virus present in macrophages.

In Experiment 2, supplemental vitamin C appeared to alleviate some of the effects caused by the application of stress when combined with HEV vaccination. Throughout the experiments, it appeared that vitamin C alleviated the effects of stress, rather than improving response to vaccination with HEV.

Decreases in plasma corticosterone were observed (Experiment 2) in the “Supplemented” group on day 1 PV. These effects are different than what was observed in Chapter 2 in which HEV vaccination caused an increase in plasma corticosterone on day 6 PV and stress application caused shifts in these elevations. There is no scientific explanation to the difference in observations. H:L ratios have been documented to be a more reliable indicator of psychological stress than plasma corticosterone in poultry (Gross and Siegel, 1983). Therefore, H:L ratios were used to better estimate psychological stress than plasma corticosterone in this experiment. In this experiment, H:L ratios in the “Supplemented” group resembled effects observed in birds that were

vaccinated and unstressed in Chapter 2. From this it is speculated that vitamin C was able to decrease stress induced elevations in H:L ratios in vaccinated birds.

Although vitamin C alleviated stress-induced elevations in H:L ratios, it caused no changes in the percent of CD4<sup>+</sup> or CD8<sup>+</sup> cells.

Supplementation also in the current study indicates that the addition of supplemental vitamin C possibly alleviated the negative effects caused by stress. This effect may have a damaging effect on turkeys subjected to simultaneous social stress and HE vaccination. McKee and Harrison (1995) hypothesized that one could factor out the detrimental effect of a single stressor in a multi-stressor situation by incorporating a specific inhibitor of the single stressor, such as ascorbic acid. However, there may be circumstances where alleviating the effects of a single stressor may not be beneficial to the animal, such as during simultaneous stress application and T cell mediated vaccines.

Elevated serum corticosterone levels during stress conditions have been shown to decrease with dietary supplementation of vitamin C (Satterlee et al., 1989; Kutlu and Forbes, 1993). However supplementation in the present study was found to have no consistent effects on plasma corticosterone levels in poults. This observation is consistent with other researchers who found no consistent findings in serum corticosterone of vitamin C supplemented and vaccinated birds (Amakey-Anim et al., 2000).

Addition of ascorbic acid decreased H:L ratios on day 4 PV. The decrease in H:L ratios are in agreement with McKee and Harrison (1995) who reported that vitamin C alleviated the stress-induced increases in H:L ratios in birds who were subjected to

multiple stressors. However, H:L ratios increased in the supplemented group on day 6 PV. The increase in H:L ratios is speculated to be the virus's attempt to manipulate the lymphoproliferative effects of vitamin C on social stress during the peak of HEV infection. H:L ratios have been documented to be a more reliable indicator of psychological stress than plasma corticosterone (Gross and Siegel, 1983).

Flow cytometric analysis revealed that there were no differences in regard to percent CD4<sup>+</sup> cells in peripheral blood between the groups, although both groups were increased on day 4 PV. This is in agreement with previous studies that reported an increase in CD4<sup>+</sup> cells when birds were subjected to social stress and vaccinated with HEV (Chapter 3). Other researchers have observed similar increases in CD4<sup>+</sup> lymphocytes in people infected with human immunodeficiency virus type 1 (HIV) and treated with vitamin C (Muller et al., 2000). There were no differences in regard to CD8<sup>+</sup> cells between the groups; however cell percentages were increased in both groups on day 4 and 8 PV. Other researchers reported higher numbers of CD8<sup>+</sup> cells in the spleens of birds supplemented with vitamin C and vaccinated with infectious bursal disease (Wu et al., 2000).

Currently, there is limited information on the effects of the interaction between vitamin C, stress and disease on the production of cytokines. Current research demonstrates that plasma IL-6 and TNF- $\alpha$  levels were decreased in vitamin C supplemented birds that were subjected to simultaneous social stress and HEV vaccination. Previous experimental work only examined the individual effects of stress, vitamin C and disease on cytokine production. Siegel (1974, 1975) reported that

supplemental ascorbic acid in mice enhanced interferon production. Others reported that circulating IL-6 and TNF- $\alpha$  are increased by psychological stress (Zhou et al., 1993; Nukina et al., 1998). Nukina et al., (1998) hypothesized that TNF- $\alpha$  observations have not been seen before because of the invariable increase in plasma IL-6 levels over TNF- $\alpha$  levels. Hartel et al., (2004) reported that supplemental vitamin C resulted in an inhibition of IL-6 and TNF- $\alpha$  producing monocytes in healthy humans. Vassilakopoulos et al., (2003) also reported that vitamin C supplementation decreased the exercise induced increase in plasma IL-6 and TNF- $\alpha$ .

There have been inconsistencies with regard to the relationship between plasma vitamin C and antibody titers in poultry. McCorkle et al., (1980) reported that antibody response to sheep red blood cells were not influenced by feeding supplemental vitamin C. Other researchers have reported that vitamin C supplementation may improve the humoral immune response to infectious bursal disease by increasing the number of IgG antibody secreting cells in the spleen (Wu et al., 2000). Amakye-Anim et al., (2000) documented similar observations showing that vitamin C supplementation increased serum antibody titers. In Chapter 3, birds supplemented with vitamin C, showed elevated HEV antibody titers, however, when social stress was introduced into the equation, there were significantly lower HEV antibody titers in poult fed the diet supplemented with vitamin C as compared to those poult fed the diet not supplemented with vitamin C at 14 days PV.

Effectiveness of vitamin C supplementation depends on the dosage of vitamin C in its ability to elevate vitamin C levels in the plasma thus limiting the possibility of

tissue depletion. In the current experiment, 300 mg/kg of vitamin C was able to significantly increase both plasma and splenic vitamin C. It appears that vitamin C supplementation alleviated the negative effects of stress associated with social disruption.

Although vaccination against HEV has been useful in control of the disease, management measures may be undertaken to help turkeys cope with vaccination and other stress and natural infections in the field. In these studies, it has been demonstrated that poultry feed supplemented with 300 mg/kg vitamin C may improve responses to HEV vaccination. However it was concluded that vitamin C supplementation during periods of simultaneous stress application and vaccination does not provide benefit to response to HEV vaccination.

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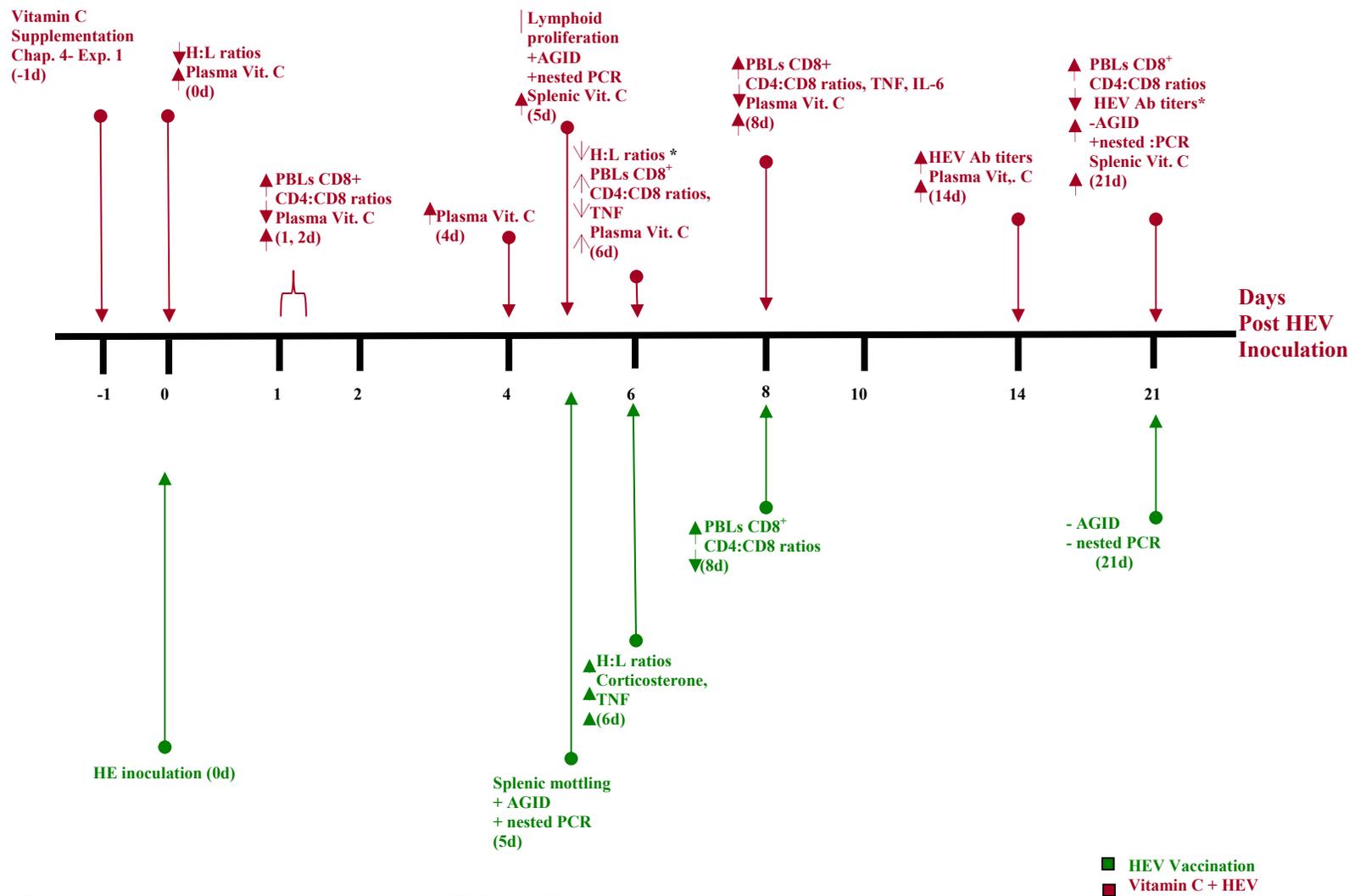
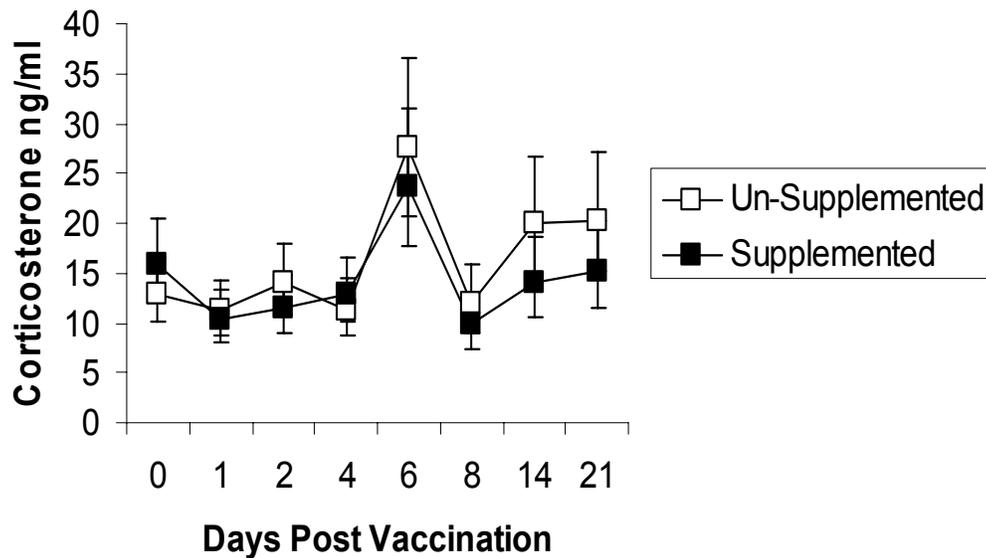
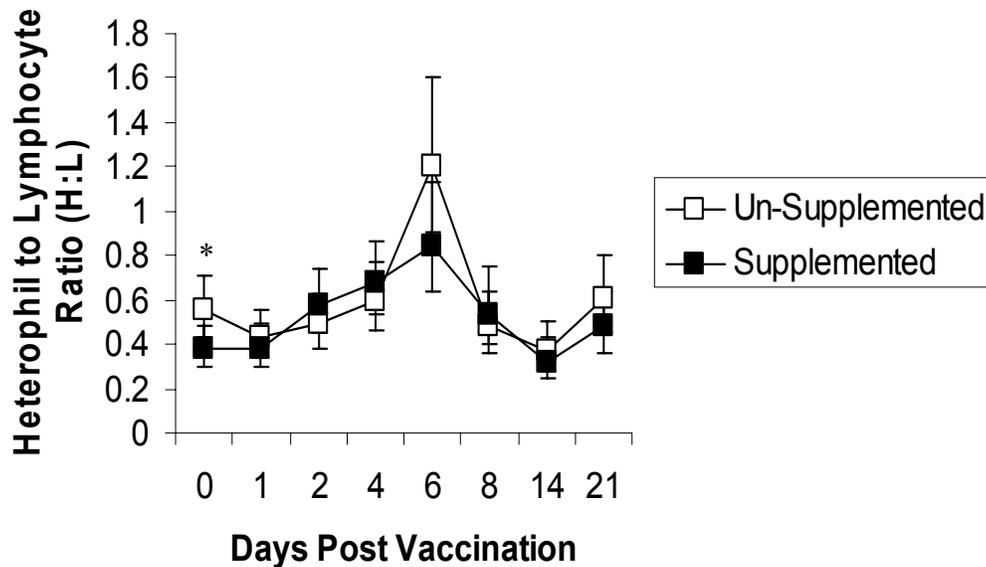


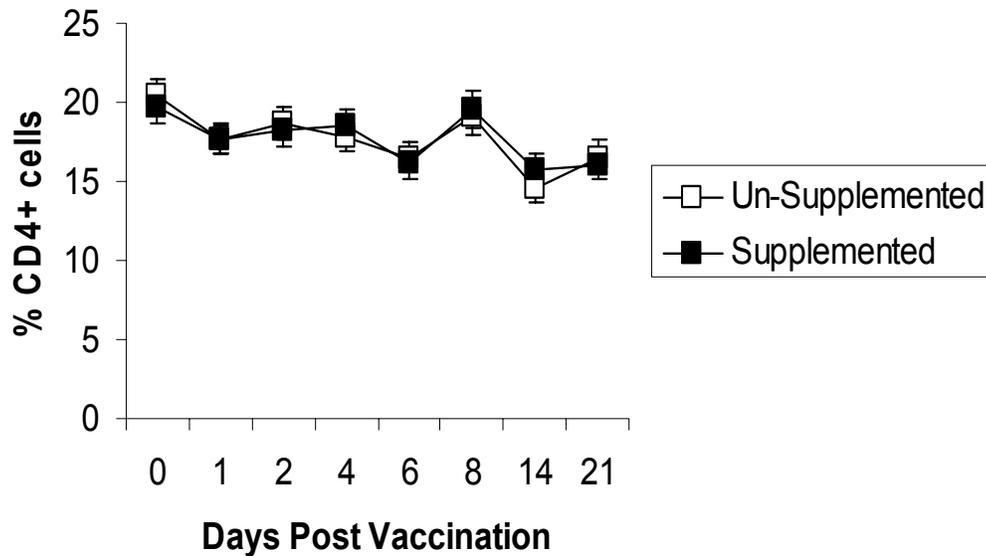
Figure 1. Outline of the occurrences during HEV and Vitamin C



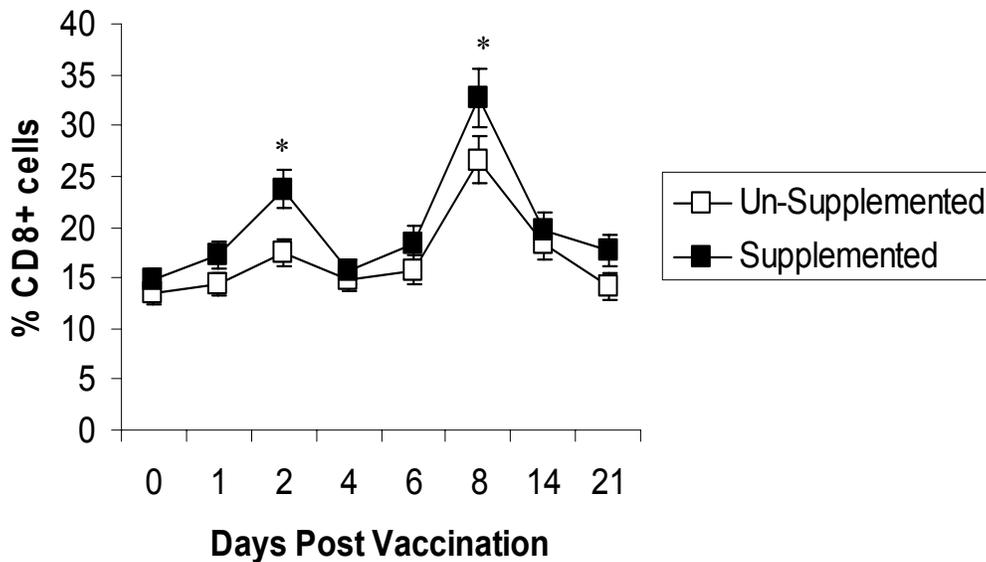
**Figure 2. Effect of supplemental Vitamin C on plasma corticosterone levels in HEV vaccinated poult.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits.



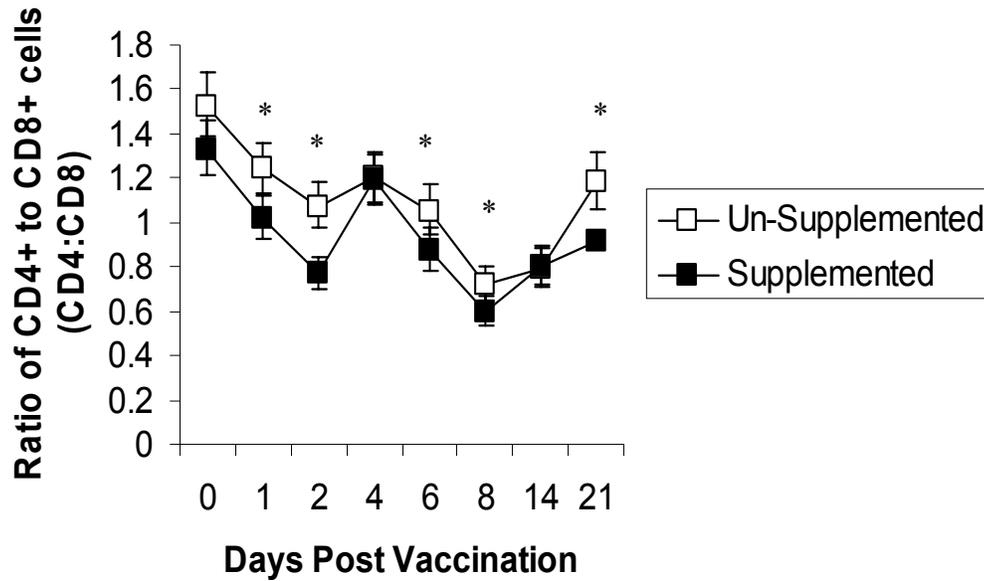
**Figure 3. Effect of supplemental Vitamin C on H:L ratios in HEV vaccinated poult.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a significant difference ( $P < 0.05$ ) at the specified time period.



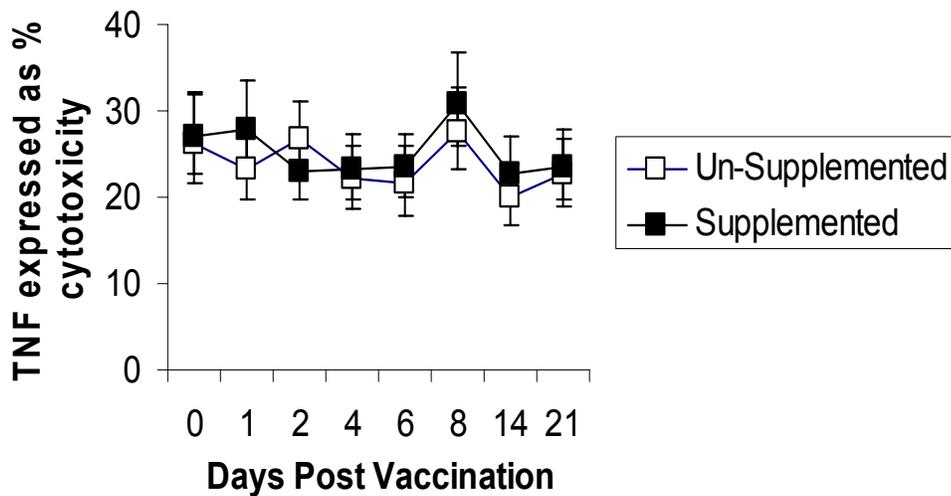
**Figure 4. Effect of supplemental Vitamin C on peripheral CD4+ cell populations in HEV vaccinated poult.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits.



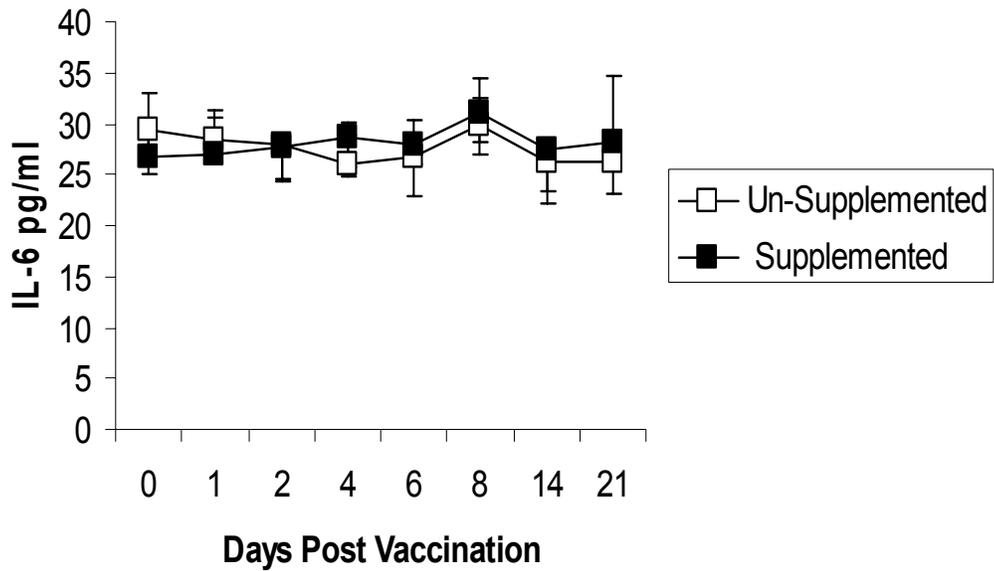
**Figure 5. Effect of supplemental Vitamin C on peripheral CD8+ cell populations in HEV vaccinated poult.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. Asterisks indicate a significant difference ( $P < 0.05$ ).



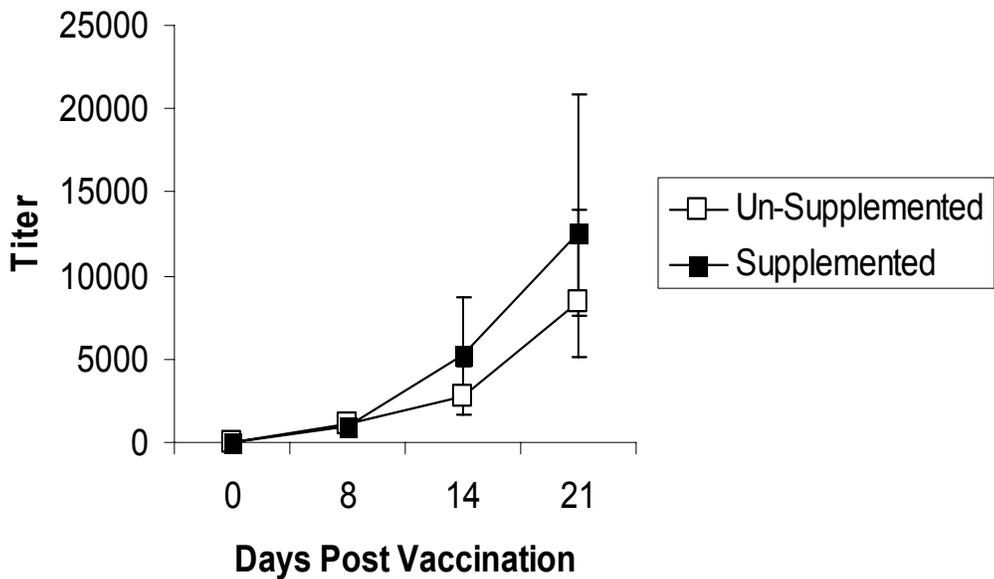
**Figure 6. Effect of supplemental Vitamin C on peripheral CD4:CD8 ratios in HEV vaccinated poult.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. Asterisks indicate a significant difference ( $P < 0.05$ ).



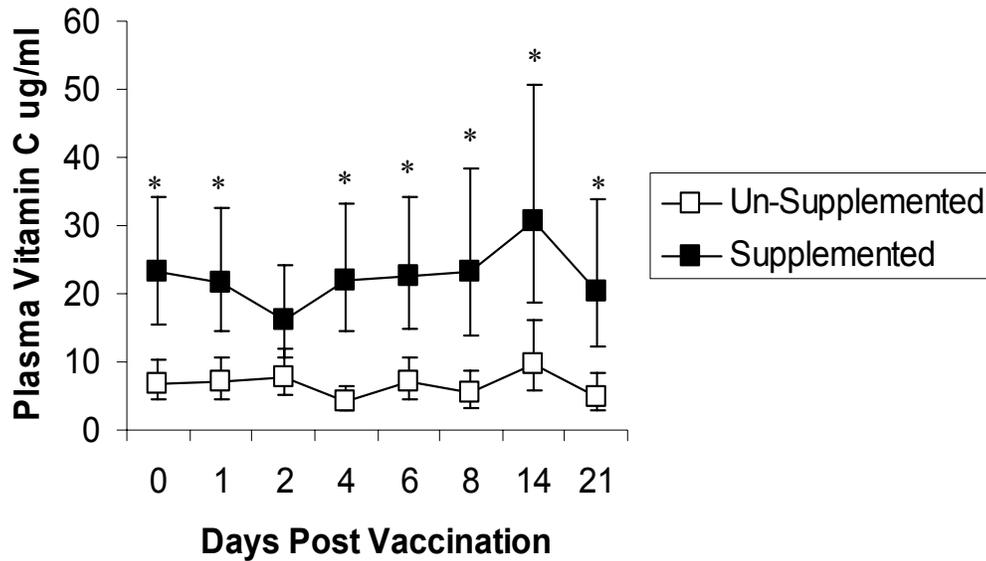
**Figure 7. Effect of supplemental Vitamin C on peripheral TNF- $\alpha$  vaccinated poult.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits.



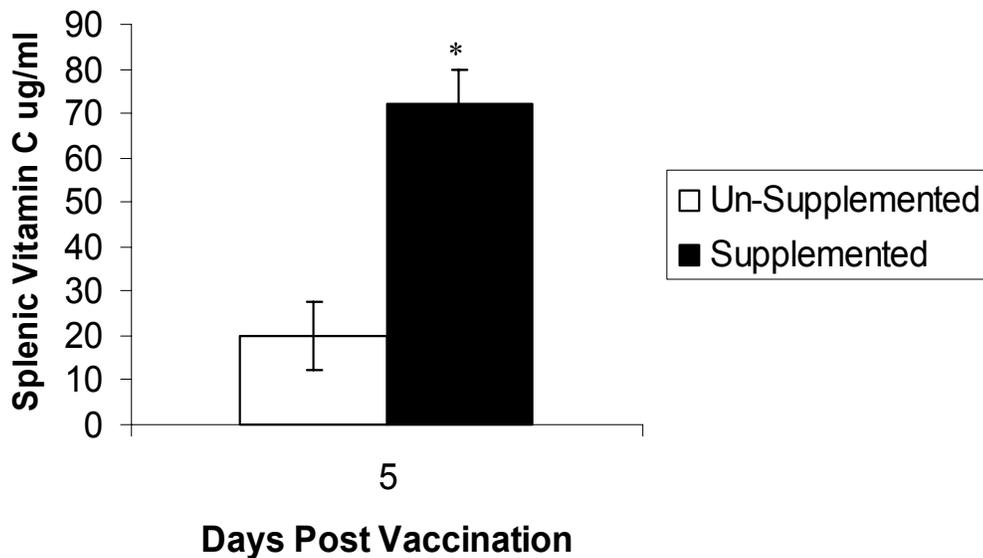
**Figure 8. Effect of supplemental Vitamin C on peripheral plasma IL-6 in HEV vaccinated poult.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits.



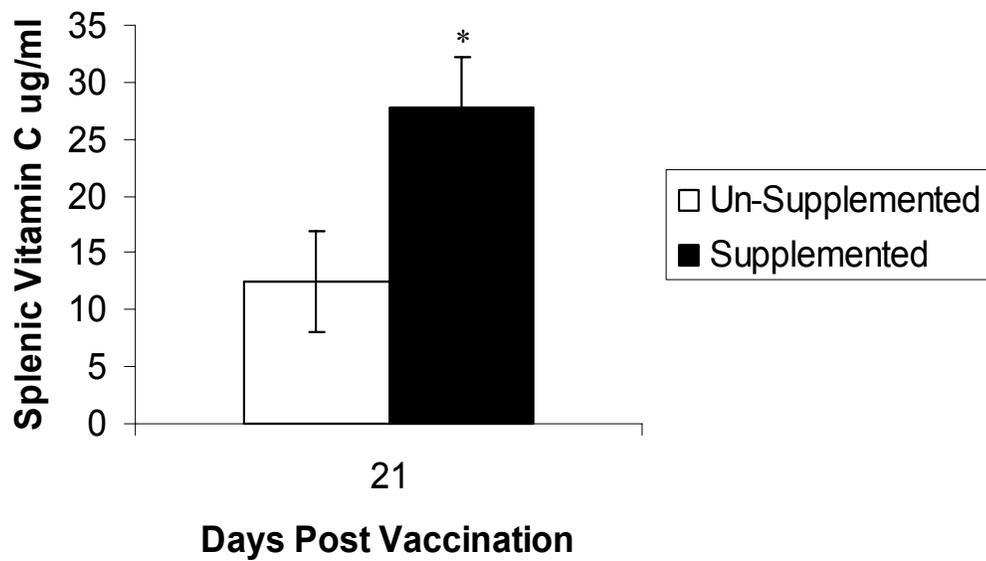
**Figure 9. Effect of supplemental Vitamin C on HEV antibody titers in HEV vaccinated poult.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits.



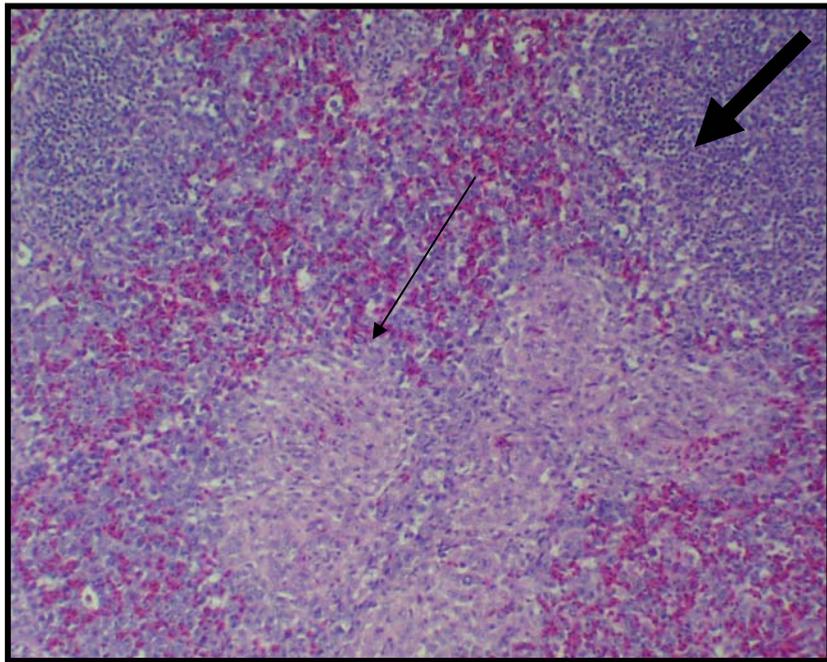
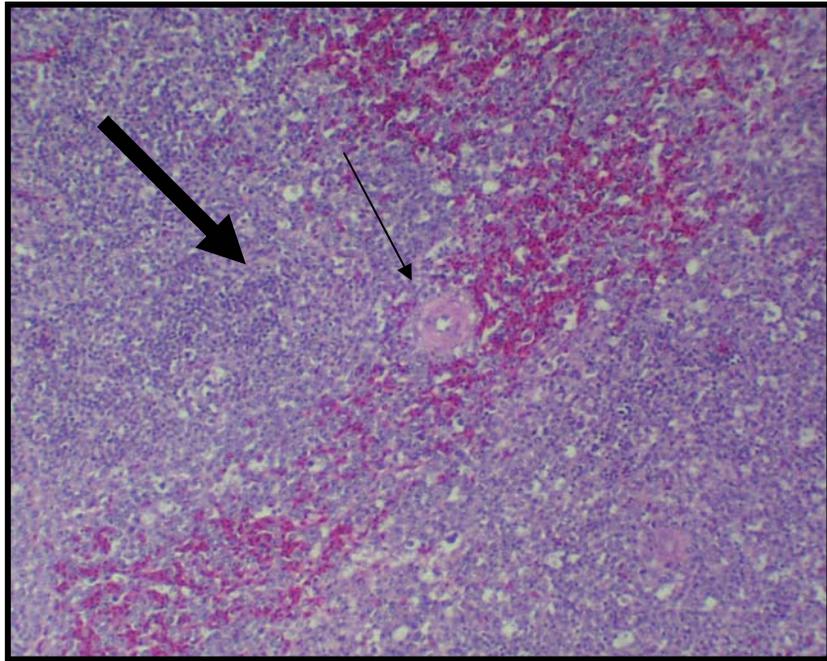
**Figure 10. Effect of supplemental Vitamin C on plasma Vitamin C levels in HEV vaccinated poult.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. Asterisks indicate a significant difference ( $P < 0.05$ ).



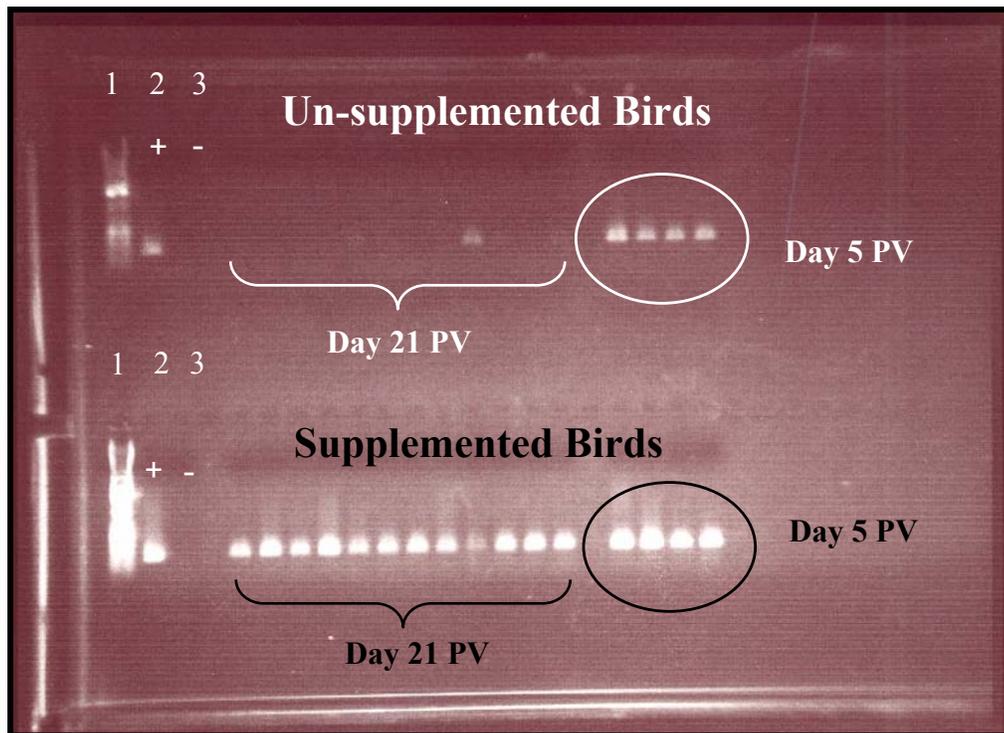
**Figure 11. Effect of supplemental Vitamin C on splenic Vitamin C levels in HEV vaccinated poult on day 5 PV.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a significant difference ( $P < 0.05$ ).



**Figure 12. Effect of supplemental Vitamin C on splenic Vitamin C levels in HEV vaccinated poult on day 21 PV.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a significant difference ( $P < 0.05$ ).



**Figure 13. The effect of Vitamin C and vaccination with HEV on splenic histopathology day 5 PV.** A. Un-supplemented spleen on day 5 PV (10x). There are several lymphocytes obliterating the red pulp. Splenic sheathed vessels are constricted. B. Supplemented spleen on day 5 PV (10x). Small arrow indicates sheathed vessels lined with macrophages. Large arrow indicates proliferation of infected lymphocytes.



**Figure 14.** Nested PCR performed on DNA isolated from spleens of “Un-Supplemented” (top lane) and “Supplemented” (bottom lane) HEV inoculated birds. Lane 1=1kb ladder, Lane 2=Positive control, Lane 3=Negative control. Bands indicate positive results on days 5 and 21 PV.

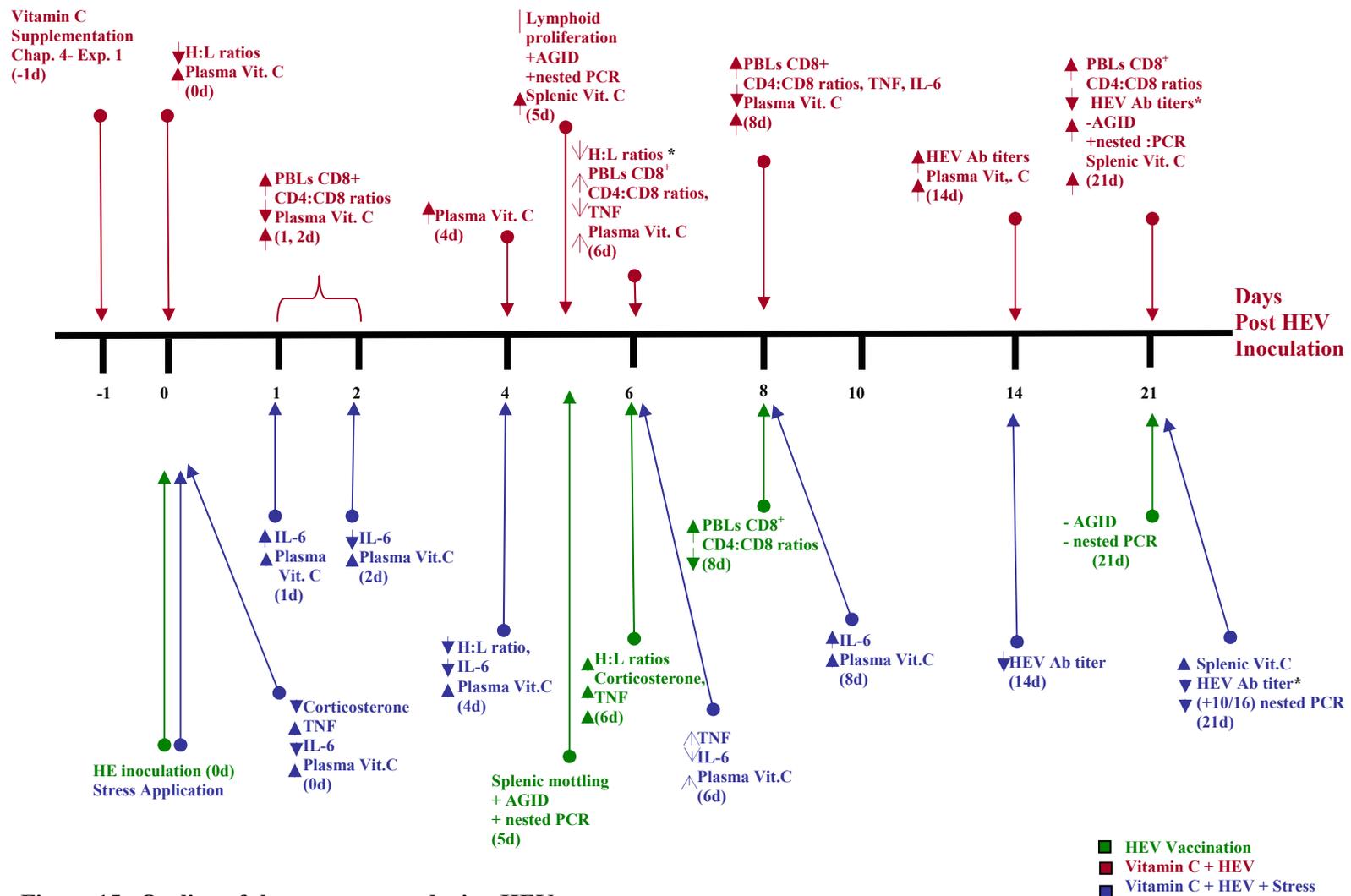
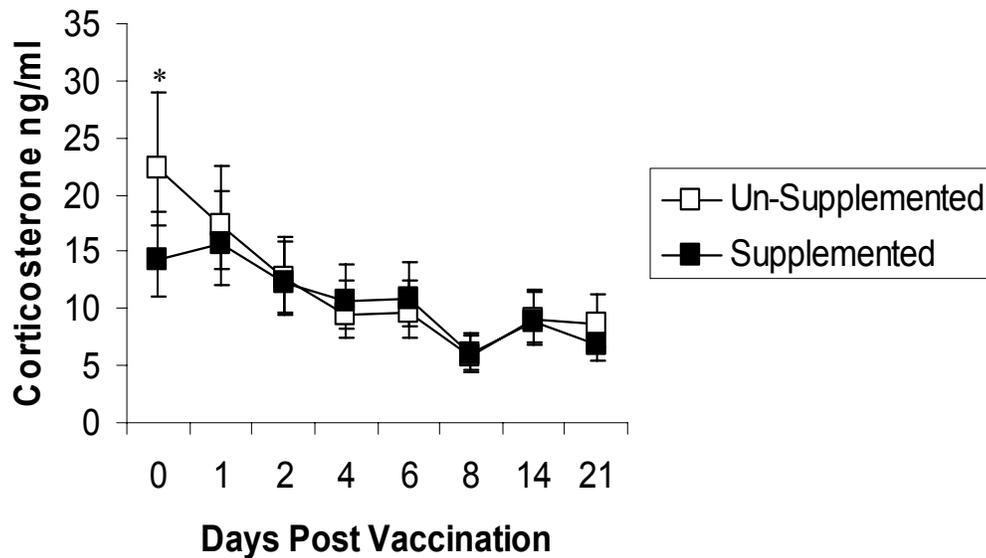
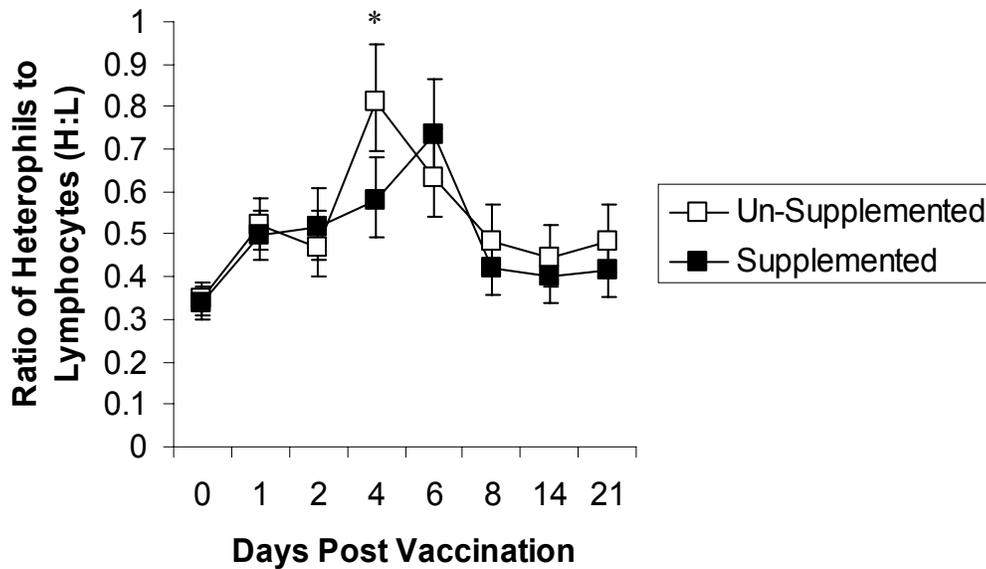


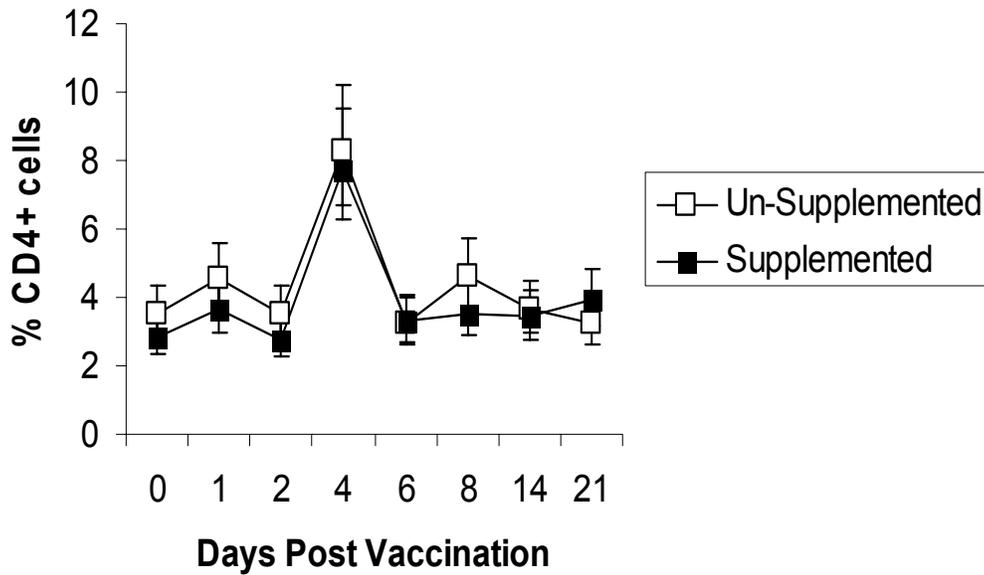
Figure 15. Outline of the occurrences during HEV and Vitamin C and Stress



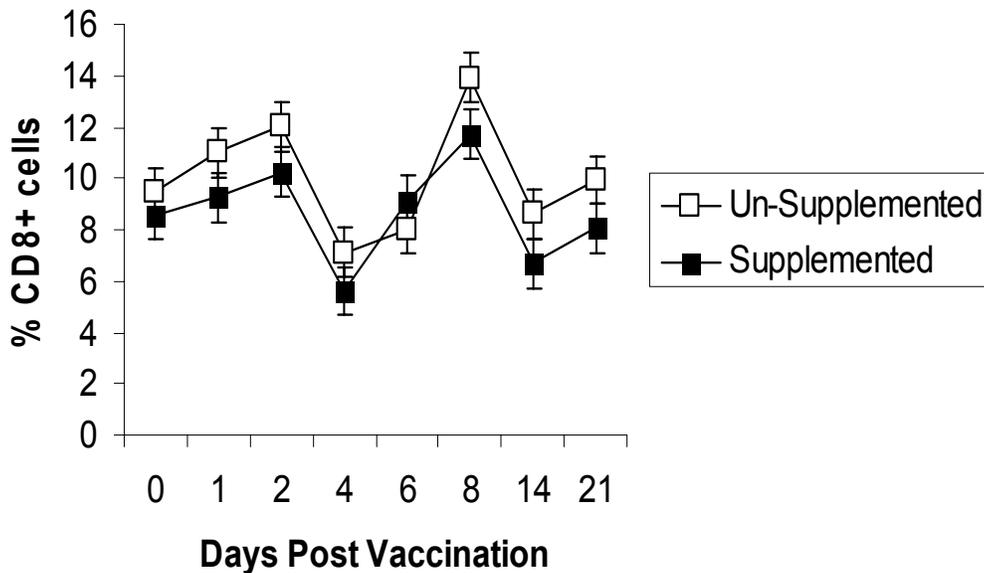
**Figure 16. Effect of Vitamin C, Stress, and HE on corticosterone levels.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a significant difference ( $P < 0.05$ ).



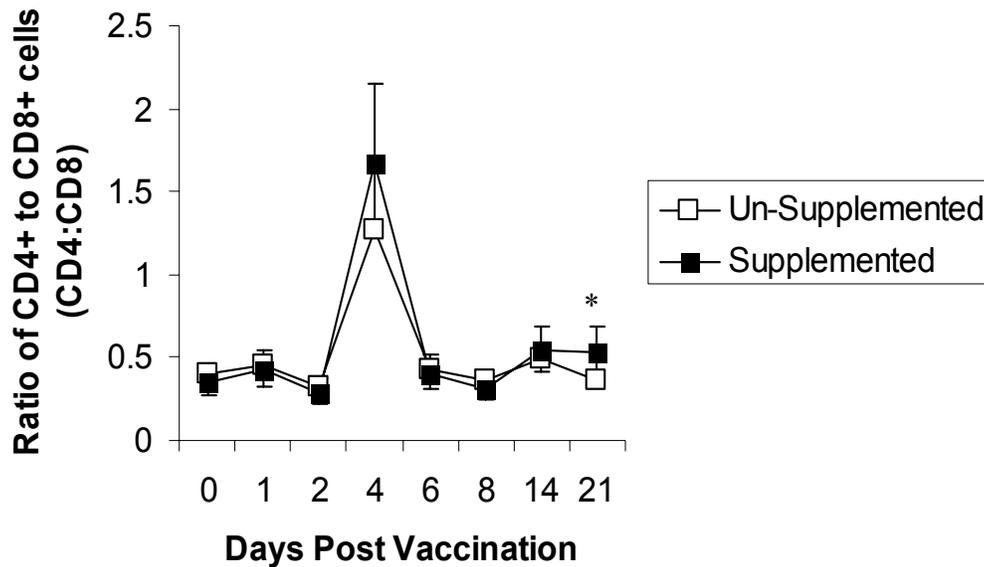
**Figure 17. Effect of Vitamin C, Stress and HE on H:L ratios.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a significant difference ( $P < 0.05$ ).



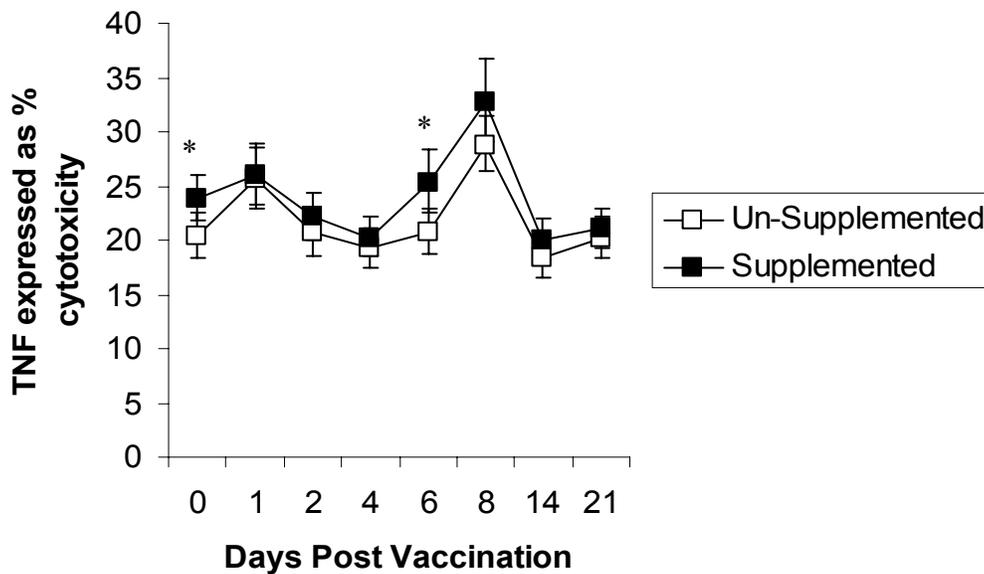
**Figure 18. Effect of Vitamin C, Stress, and HEV vaccination on peripheral CD4<sup>+</sup> cell populations.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits.



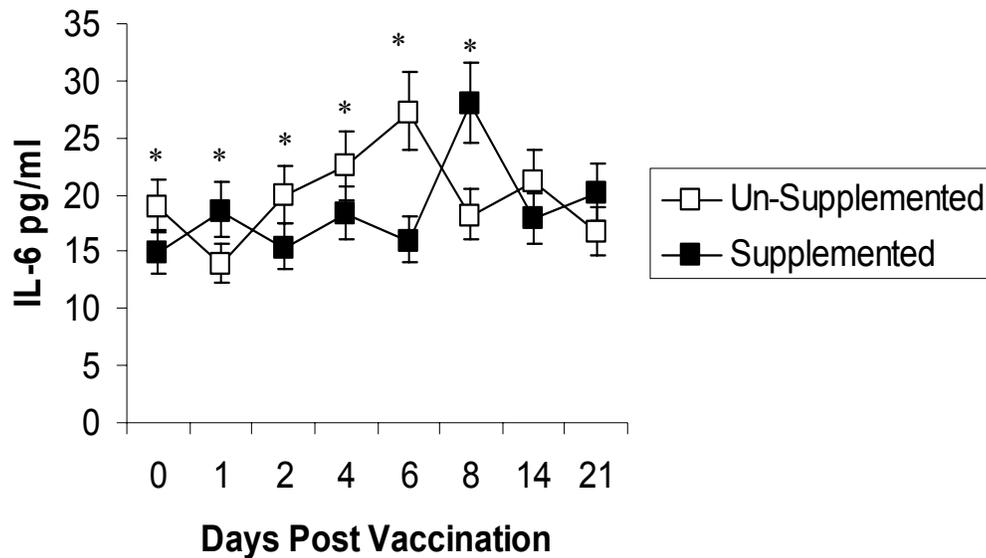
**Figure 19. Effect of Vitamin C, Stress, and HEV vaccination on peripheral CD8<sup>+</sup> cell populations.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits.



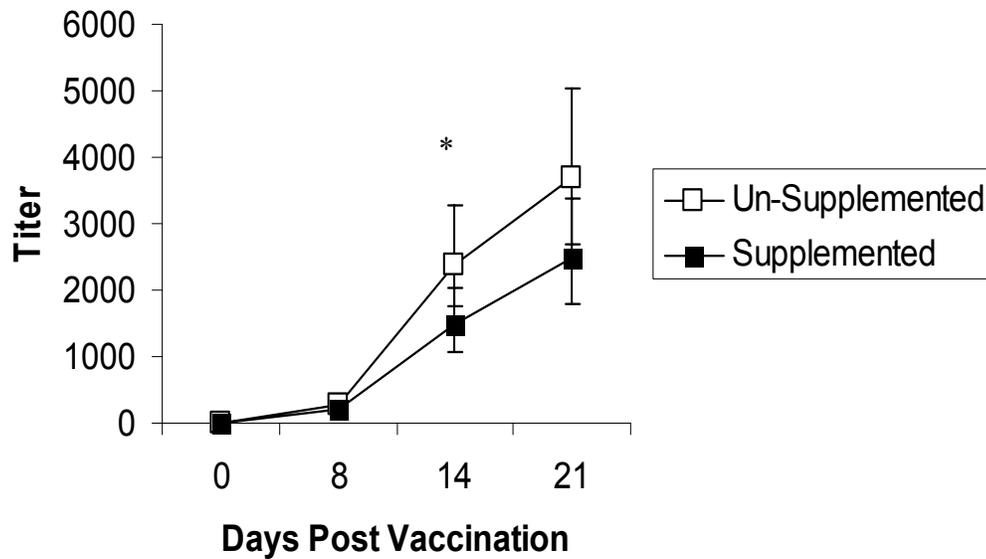
**Figure 20. Effect of Vitamin C, Stress, and HEV vaccination on CD4<sup>+</sup> to CD8<sup>+</sup> ratios.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a significant difference ( $P < 0.05$ ).



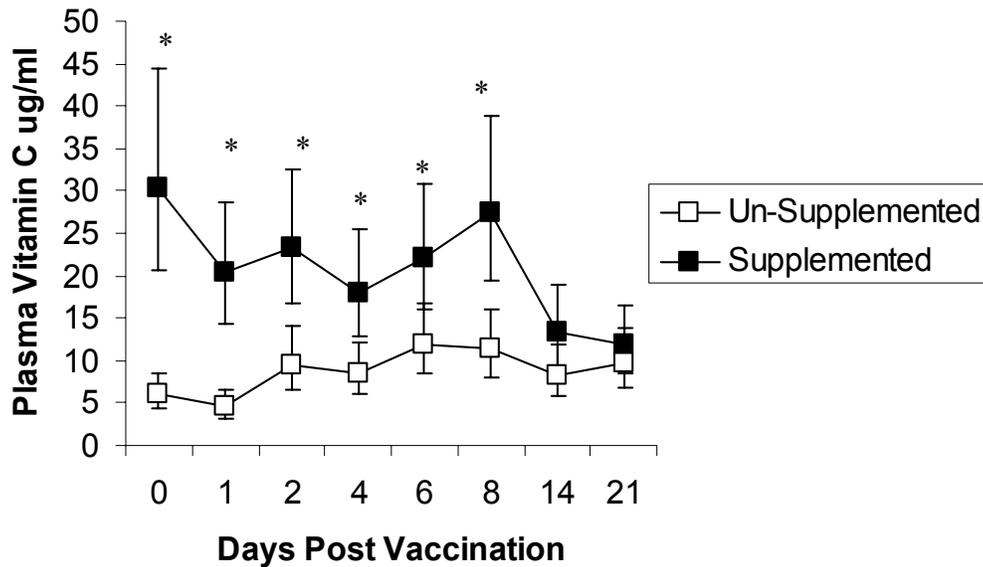
**Figure 21. Effect of Vitamin C, Stress, and HEV on plasma TNF- $\alpha$ .** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. Asterisks indicate a significant difference ( $P < 0.05$ ).



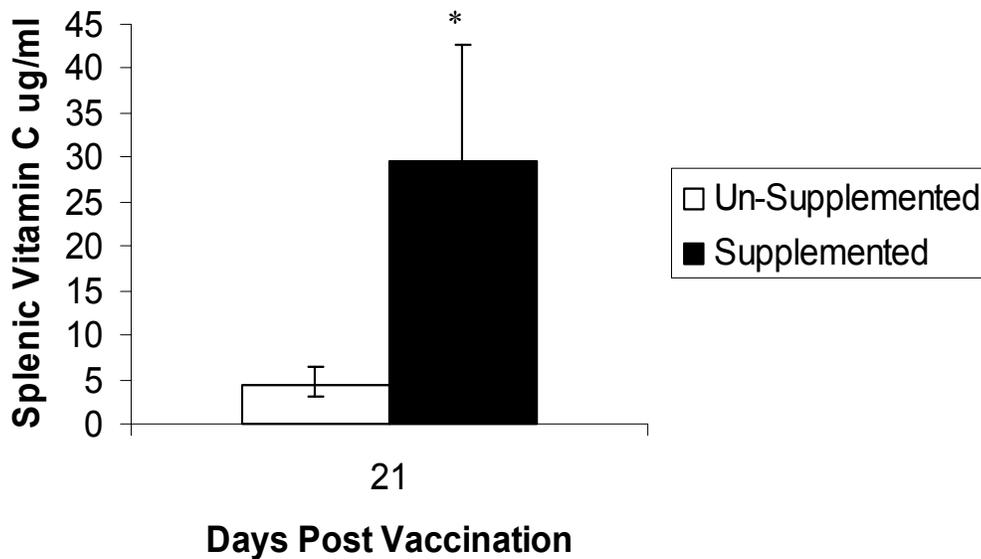
**Figure 22. Effect of Vitamin C, Stress, and HEV vaccination on plasma IL-6.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. Asterisks indicate a significant difference ( $P < 0.05$ ).



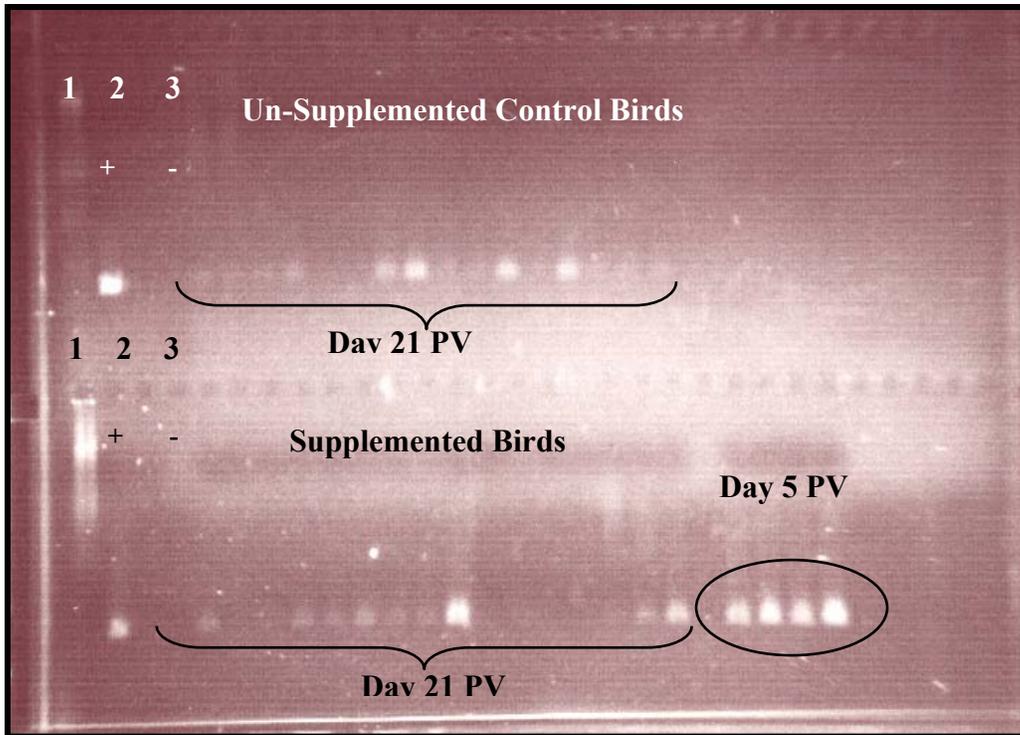
**Figure 23. Effect of Vitamin C, Stress, and HEV vaccination on HEV antibody titers.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a significant difference ( $P < 0.05$ ).



**Figure 24. Effect of Vitamin C, Stress, and HEV vaccination on Plasma Vitamin C.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. Asterisks indicate a significant difference ( $P < 0.05$ )



**Figure 25. Effect of Vitamin C, Stress, and HEV vaccination on splenic ascorbic acid.** Values were calculated from 16 individual birds per group and expressed as geometric means with 95% confidence limits. The asterisk indicates a difference ( $P < 0.05$ ).



**Figure 26.** Nested PCR performed on DNA isolated from spleens of un-supplemented (top lane) and supplemented (bottom lane) HEV inoculated and stressed birds. Lane 1=1kb ladder, Lane 2=Positive control, Lane 3=Negative control. Bands indicate positive results on days 5 and 21 PV

### Chapter 4 Summary Table

	Corticosterone	H:L	CD4 <sup>+</sup>	CD8 <sup>+</sup>	CD4:CD8	TNF- $\alpha$	IL-6	HEV Ab Titer	Plasma Vit C	Splenic Vit C	Histopath	PCR
Exp 1	--	↓	--	↑	↓	↑	↑	↑	↑	↑	↓ Lymphoid Proliferation	↑
Exp 2	↓	↓	--	--	↓	↑, ↓	↑, ↓	↓	↑	↑	--	↓

↑ = Increase

↓ = Decrease

-- = No Change

+ = Positive

- = Negative

Table 1. Summary.

## Chapter 5

### General Conclusions and Applications

The research presented in this dissertation addresses the interrelationship among stress, nutrition (vitamin C), immunity and their influence on response to HEV vaccination. Figure 1 is a Venn diagram depicting the individual as well as the independent vs. dependent effects of social stress, vitamin C and HEV vaccination. Figure 2 is a detailed expansion of the center of diagram 1 and represents the changes associated with the interactions of stress, HEV, and vitamin C. The moving of turkeys is a stressful time. Birds are moved from the hatchery to the brooding area, and then to the finishing area. When a flock is assembled, there are social encounters between birds to establish pecking order. However, when poults are moved at different stages, this forces them to re-socialize and re-establish social hierarchies which can be stressful on the bird. In this dissertation, we tried to mimic the practice of moving birds in the field with an established method of social randomization. With this stressor, we were able to demonstrate that a change in the social environment (e.g. moving birds into different pens) was able to create stress and cause immunological and physiological changes within the bird. It was found that stress in the form of social disruption caused a physiological increase in H:L ratios (increase in heterophils and a decrease in lymphocytes). Immunosuppression was also observed in stressed birds manifesting as a depression in CD4<sup>+</sup> and CD8<sup>+</sup> cells 24 hours after the application of stress. Therefore, the difference in H:L ratios is hypothesized to be a change predominantly in T cell

populations, and possibly others. Additional immunosuppressive effects observed were increases in the proinflammatory cytokines IL-6 and TNF- $\alpha$ .

In disease prevention programs, vaccination with live vaccines is a routine procedure under commercial conditions. Vaccination taxes the immune system and may require a reallocation of immunological resources. Under practical conditions stress due to vaccination is severe during the first few weeks of life. This is also the time of movement in turkeys from the hatchery to the brooding to the finishing area. During this time of moving, turkey poults are subjected to a rigorous vaccination schedule. Birds are vaccinated with several vaccines including *B. avium*, NDV, and HEV. The degree of stress caused by vaccination depends on the immunocompetence of the turkey with regards to such factors as maternal antibodies, development of the immune system and environmental stress.

One of the most commonly used vaccines in the turkey industry is avirulent live HEV. HEV is a lymphotropic, lymphocytopathic virus, and it has been demonstrated that the vaccine itself can cause transient immunosuppression. During HEV replication, there is apoptosis and necrosis of target cells in the spleen. Cytokines produced by activated T cells potentially induce apoptosis and necrosis of by-stander cells, which is accompanied by a transient period of immunosuppression. This dissertation demonstrated similar effects of immunosuppression in birds inoculated with a vaccine strain of HEV. These effects included enlarged, mottled spleens, increased H:L ratios and plasma corticosterone during the time when peak viral replication occurs in the spleen. The increase in H:L ratios on day 6 PV is speculated to be due to the destruction

of IgM bearing B cells occurring during peak replication. Immunological changes observed also include increased CD8<sup>+</sup> PBLs, on day 8 PV. The increase in the percentage of CD8<sup>+</sup> cells observed on day 8 PV coincided with the decrease in H:L ratios on day 8 PV. The increase in CD8<sup>+</sup> cells appears to be due to the immune system's attempt to clear the virus. The decrease in H:L ratios is speculated to be the result of an increase in T cells, particularly CD8<sup>+</sup> cells. Proinflammatory cytokines, IL-6 and TNF- $\alpha$  have been observed in HEV infection. Increases in TNF- $\alpha$  and IL-6 were observed on days 6 and 8 PV which appears to occur during the period of peak of replication. Therefore the increase in these proinflammatory cytokines in addition to the increase in CD8<sup>+</sup> PBLs is speculated to contribute to HEV-induced immunosuppression.

Moving turkeys around 5-6 weeks of age coincides with the timing of HEV vaccination. It has been well documented that when birds are subjected to a high stress environment, the susceptibility to viral infections are more common. HEV is a lymphotropic, lymphocytopathic virus, and in combination with environmental stressors may potentially exacerbate the immunosuppressive effects that each may cause individually. However, there is no way to completely eliminate stressors. Therefore the objective was to find the "best" time to minimize the negative effects of moving birds during vaccination and improve response to HEV vaccination.

As described in Chapter 2, birds were moved at different times in relation to HEV vaccination. It was found that when birds were vaccinated, the "normal" stress-induced increase in H:L ratios were cancelled out by HEV and increases occurred on day 4 PV, regardless of the time in which stress was applied. It seemed as though the HEV

infection overshadowed the effects of stress and caused changes in different parameters suggesting that HEV's effects on the bird are stronger than those induced by the social stressor. When stress times were compared against each other and the "No Stress" control group, it appeared that the best time to move birds in relation to HEV vaccination was on the day of vaccination. Birds that were vaccinated and moved the same day had higher HEV antibody titers and appeared to have "better" humoral immune responses than the other stress time treatments. This may have enabled the bird to clear and process virus more efficiently.

It has been demonstrated that vitamin C may be used as an anti-stress agent and may be an essential nutrient that can be added when poults are subjected to stressful conditions. Vitamin C efficacy is dependent upon its ability to elevate plasma vitamin C levels. Therefore, various doses of vitamin C supplementation were examined for their ability to elevate plasma vitamin C and decrease H:L ratios. After determining that 300 mg/kg vitamin C was able to decrease H:L ratios as well as elevate plasma vitamin C levels, feed was supplemented and the effect on birds subjected to a social stressor was examined. Vitamin C supplementation at 300 mg/kg alleviated stress-induced physiological and immunological changes. This suggests that during periods of stress, (e.g. movement, handling, temperature fluctuations, ventilation, food and water deprivation, etc.), supplementation may be beneficial in alleviating the negative effects caused by these stressors.

In addition to examining the effects of supplemental vitamin C on a social stress, the response to vaccination with HEV was also observed. HEV vaccination served as

disease prevention, and also the act of vaccination as a stressor. Although vitamin C supplementation did not produce significant changes in many of the parameters measured, supplementation appeared to improve humoral response and cause changes in the spleen. Antibody response is critical to neutralizing HEV. Supplementation improved the humoral response to HEV as observed with increased antibody production. Upon gross examination, supplementation reduced the amount of mottling normally observed in the spleen during HEV infection. In fact, the spleen appeared normal. On histopathological analysis, it appeared that vitamin C somehow limited the number of infected lymphocytes that obliterated the red pulp of the spleen, therefore creating smaller areas of proliferation. Extensive documentation has been reported on macrophages representing the first line of defense following viral infections. Macrophages are known to contain 40 times more vitamin C than the levels in plasma and the high level of vitamin C is associated with phagocytosis and antigen presenting. Therefore, it is speculated that vitamin C supplementation may have improved macrophage phagocytosis and antigen presentation of HEV. Although vitamin C is speculated to improve macrophage function and limit the proliferation of infected lymphocytes in the spleen, viral DNA was present in more splenic samples of “Supplemented” birds on day 21 PV than the “Un-Supplemented” controls. The increase in splenic samples containing viral DNA may be attributed to the improved function of splenic macrophages from vitamin C supplemented birds during phagocytosis of HEV and infected cellular debris, which may reactivate latent virus, therefore speculating that macrophages control antigenic dose. Vitamin C may have also affected antigen

processing and presentation, therefore improving the response/efficiency of antibody production in a T or B mediated process against the virus. Therefore, this may be an effective method to improve the immunosuppressiveness caused by vaccination with HEV.

As previously stated, the overall objective of the dissertation was to observe the interaction between stress, vitamin C and HEV vaccination. In the last experiment, vitamin C supplementation appeared to alleviate some of the stress-induced physiological responses, however it had no consistent effect on some immunological parameters. In addition, antibody responses were lower in the “Supplemented” group compared to the “Un-Supplemented” group.

Although stress is unavoidable, if it is managed properly, it can be beneficial. This dissertation demonstrated that stress in the form of social disruption can have negative physiological and immunological effects on turkey poults and that these effects can be alleviated with the addition of 300mg/kg vitamin C to the diet. It was also shown that stress applied on the day of vaccination can improve response to HEV vaccination. In addition it was shown that poultry feed supplemented with 300mg/kg vitamin C can improve responses to HEV vaccination. However it was concluded that vitamin C supplementation during periods of simultaneous stress application and vaccination does not provide benefit to response to HEV vaccination.

### Applications

During stressful times in poultry production, efforts should be made to reduce the amount of stress. The timing of moving birds from brooding to the finishing area may be

manipulated to improve response to vaccination. Moving birds on the day of vaccination or coordinating the time of vaccination so that it would coincide with the time in which birds are to be moved may improve responses to HEV vaccination.

Vitamin C supplementation may be used during periods of stress, such as during temperature fluctuation, ventilation, lack of food or water, loud or unfamiliar sounds or objects, or movement from the hatchery to brooding to the finishing area. Vitamin C supplementation should be provided prior to the onset of stress. Since vitamin C levels decline significantly 24 hours following discontinuation of its supplement, a continuous supplementation should be supplied during periods of stress.

Vitamin C may also be supplemented to improve immunological and pathological effects associated with disease or vaccination and possibly prevent resultant secondary infections. However, vitamin C is not suggested to be given during concurrent movement and vaccination (5-6 weeks-of-age). However, if supplementation is desired during a period of stress and vaccination, animals should be stressed and vaccinated on the same day supplement with vitamin C later.

## Further Research

As a continuation of research presented in this dissertation, the following areas of investigation should be considered:

1. *Re-examine the effects of social stress in turkeys using corticosterone binding globulin (CBG) in addition than H:L ratios and corticosterone.* Studies evaluating stress primarily focus on the adrenal cortical response evaluating plasma levels of

corticosterone, however factors ‘downstream’ of corticosterone secretion can alter organismal responses such as CBG. CBG in plasma is thought to regulate tissue availability and clearance rates of hormone and may act to regulate corticosterone action. Therefore it is hypothesized that there is a correlation between CBG and increases in plasma corticosterone and that there is a need for measuring CBG in addition to plasma corticosterone in evaluating the stress induced immune response.

2. *Investigate the impact of social stress on blood T cell migration.* Stress has been shown to result in less effective recruitment of T cell subsets into lymph nodes and spleens in immunized animals. Therefore the aim of such a study would be to investigate the impact of social stress on blood T cell migration into the spleen, bone marrow, and bursa of Fabricius. Social stress could be induced by social randomization. <sup>51</sup>Cr-labeled blood T cells isolated from synergistic donors would be intravenously injected into recipients immediately before application of stress. It is hypothesized that the accumulation of <sup>51</sup>Cr-labeled T cells in the spleen would be lower in socially stressed than in control poult. It is also hypothesized that there will be higher localization observed in the bone marrow and bursa of Fabricius of socially stressed birds

3. *Investigate the impact of HEV infection and response to vaccination on blood T cell migration.* The aim of such a study would be to investigate the impact of HEV infection and response to vaccination on blood T cell migration into the spleen, bone marrow, and bursa of Fabricius. One group of birds would be orally inoculated with

HEV, while the other would be an un-inoculated control. <sup>51</sup>Cr-labeled blood T cells isolated from synergistic donors would be intravenously injected into recipients immediately before vaccination. It is hypothesized that the accumulation of <sup>51</sup>Cr-labeled T cells in the spleen would be lower in HEV vaccinated than in control poult. It is also hypothesized that there will be higher localization observed in the bone marrow and bursa of Fabricius of HEV inoculated birds.

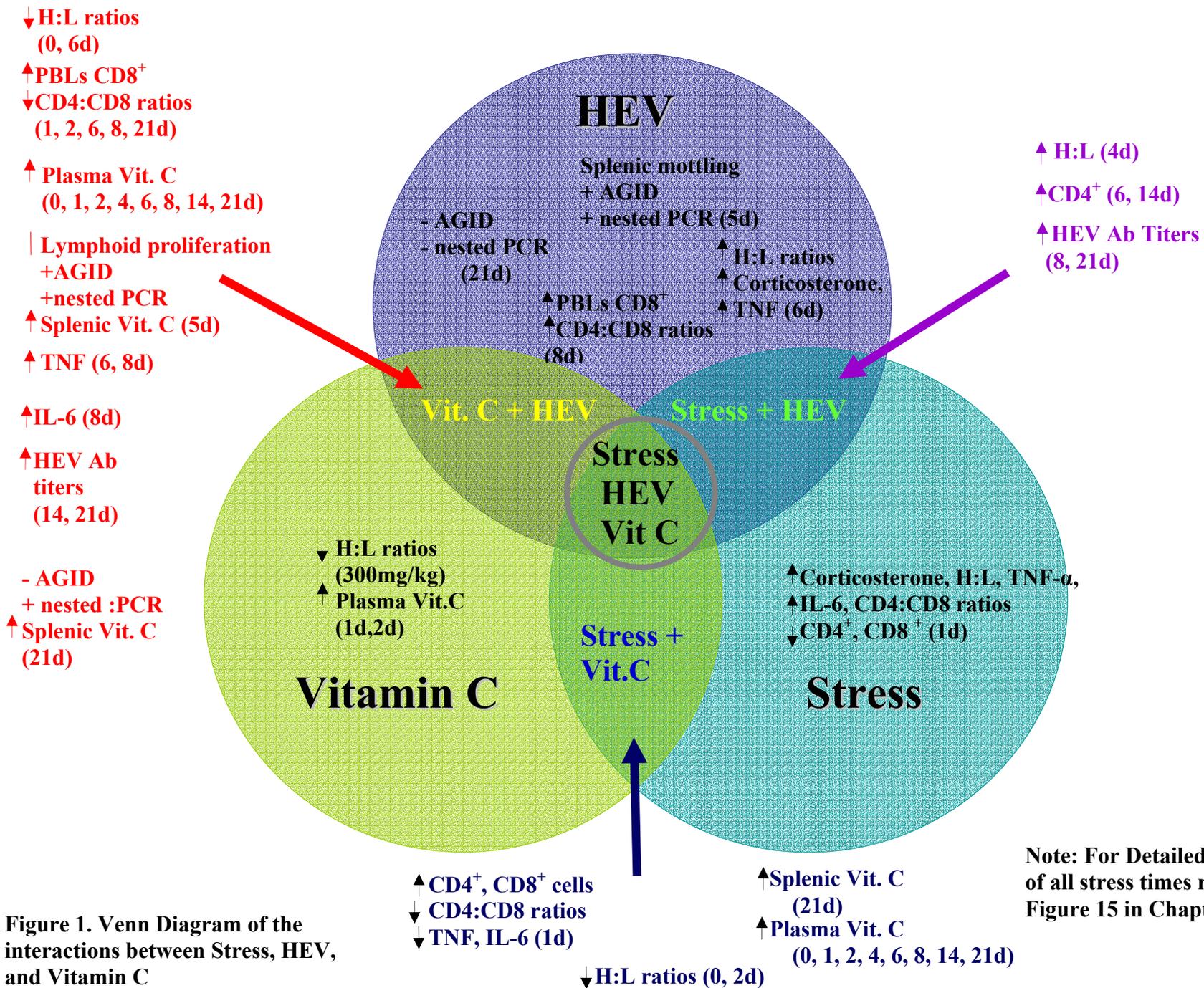
4. *Determine the effects of dietary vitamin E supplementation and the interaction of vitamins E and C on feed consumption, body weight, in vitro lymphocyte proliferation, percentage of T cell populations, H:L ratios and plasma corticosterone of turkeys during periods of social stress.* It has been shown that Vitamin E can reduce the negative effects of corticosterone induced by stress and that vitamins E and C can act synergistically.. Such a study would involve supplementing the diet of birds with either vitamin E or a combination of vitamins E and C prior to the application of stress. Prior to supplementing birds with the respective diets, trials would need to be conducted to find the optimal dose of vitamin E needed to cause a change in H:L ratios. Supplementation at levels of 0, 12, 50, 150, and 300 IU dl- $\alpha$ -tocopherol acetate would be appropriate. To examine the interaction of vitamins E and C, diets would be supplemented with two levels of vitamin C (0 or 300mg) and two levels of vitamin E (0 IU) based on the amount calculated from the first experiment and parameters measured as previously described. Feed consumption, body weight, lymphocyte proliferation, percentage of T-lymphocyte subpopulations H:L ratios, and plasma corticosterone in turkeys during social stress

should be evaluated. It is hypothesized that vitamin E supplementation may enhance feed consumption, body weight, induction of *in vitro* lymphocyte proliferation by ConA and LPS, increase CD4<sup>+</sup> and CD8<sup>+</sup> cell populations and reduce increases in H:L ratios and plasma corticosterone in turkeys during periods of social stress and that the interaction of vitamins E and C may enhance the response to stress in a synergistic fashion.

5. *Examine the effects of vitamin C on specific splenic responses to HEV vaccination.* This study would involve looking at splenic and immunological responses to HEV vaccination over the course of infection. Birds would be euthanized via cervical dislocation on a daily basis and gross observation, histopathology, splenic T lymphocyte populations (CD4<sup>+</sup> and CD8<sup>+</sup>) and production of cytokines (TNF, NOIF, IL-6) by *in vitro* cultures would be examined. It is hypothesized that vitamin C supplementation will alter the changes (mottling, CD4<sup>+</sup>, CD8<sup>+</sup>, cytokine release) associated with HEV infection

6. *Examine the effect of supplementing vitamin E and the interaction of vitamins E and C on response to HEV vaccination.* Vitamin E appears to exert a complementary effect on the immune system. Vitamin E has been reported to protect and improve the function of cells involved in the immune response. In addition, vitamin C has been demonstrated to enhance the antioxidant activity of vitamin E by reducing the tocopheroxyl radicals back to their active form of vitamin E. There appears to be a synergistic effect of vitamins E and C on the immune response. Vitamin E could be supplemented in the diet prior to HEV vaccination (as previously described in proposed

research #4). In addition, to examine the interaction of vitamins E and C, as described in proposed research #4, diets would be supplemented with two levels of vitamin C and two levels of vitamin E. Peripheral and splenic parameters would be measured as described in Chapter 3 of this dissertation in addition to parameters described in proposed research #4. It is hypothesized that vitamin E will have a positive effect on response to HEV vaccination since vitamin E and C exert similar effects on the immune system. It is also speculated that the effect of dietary supplementation of vitamins E and C would further enhance the response to HEV vaccination in a synergistic fashion.



Note: For Detailed Outline of all stress times refer to Figure 15 in Chapter 2

Figure 1. Venn Diagram of the interactions between Stress, HEV, and Vitamin C

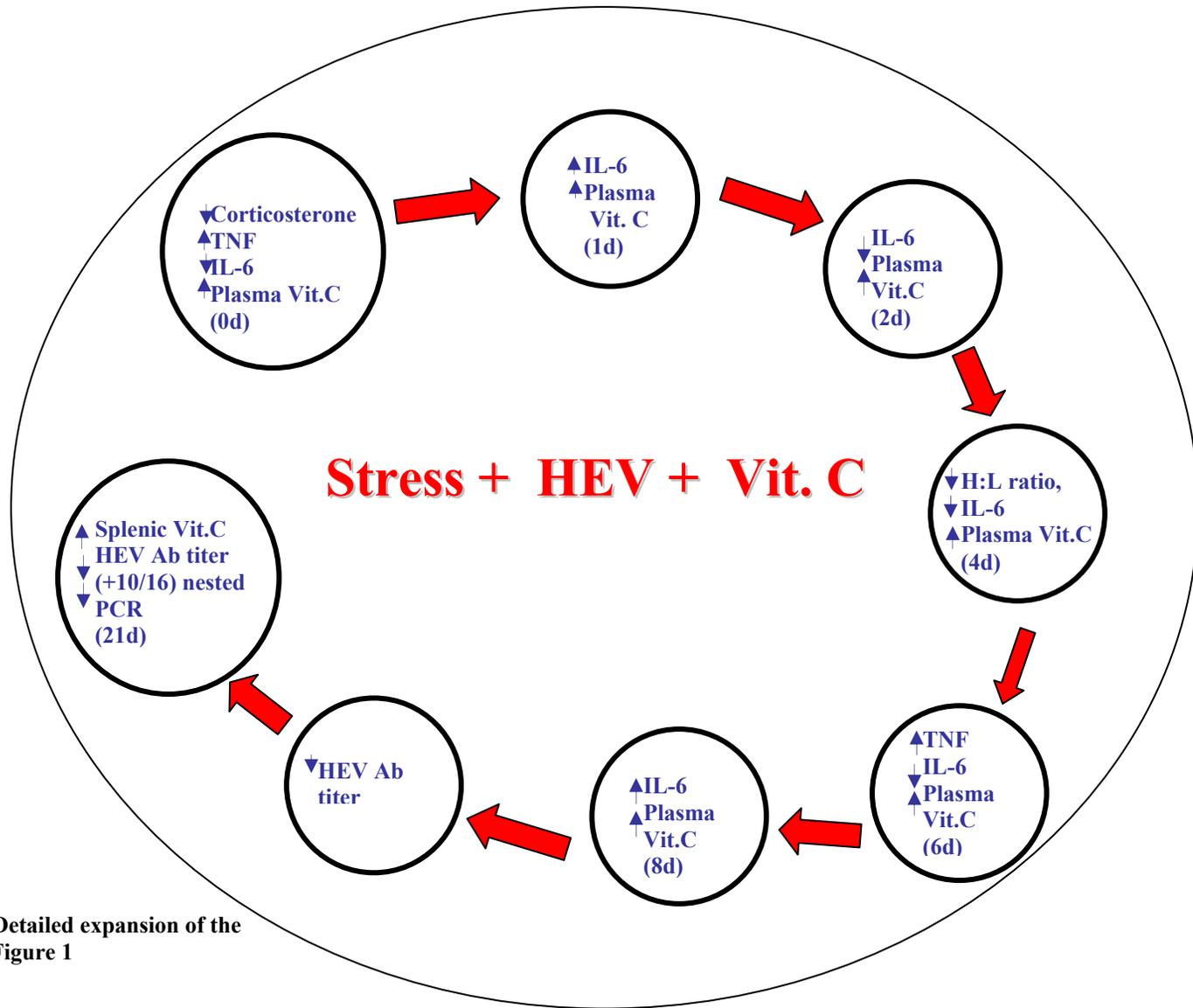


Figure 2. Detailed expansion of the center of Figure 1

## Appendix 1

### Guaranteed Analysis Southern State Cooperative, Inc., Richmond, VA

#### Feed Composition

Composition of medicated (Avatec) basal diet (Sporting Bird Starter- SSC-26-236207) from Southern States (as fed) was as follows:

Crude protein.....	(min.)	28.00%
Lysine.....	(min.)	1.40%
Methionine.....	(min.)	0.75%
Crude fat.....	(min.)	4.50%
Crude fiber.....	(max.)	4.00%
Calcium (Ca) (min.) 1.00%.....	(max.)	1.50%
Phosphorus (P).....	(min.)	0.75%
Salt (NaCl) (min.) 0.20%.....	(min.)	0.70%

## Appendix 2

### Protocol for nested HEV PCR

#### First PCR reaction:

Each PCR reaction contained 2µg of template DNA, 1.5mM MgCl<sub>2</sub> (GibcoBRL, Life Technologies, Gaithersburg, MD), 1x PCR Buffer (MgCl<sub>2</sub> (GibcoBRL, Life Technologies, Gaithersburg, MD)), 0.05mM each nucleotide tri-phosphate (NTP), 1pmol/µl of each primer (HEV 14122: GTT, CCT, TCA, CCT, AAT, ACT, GG and HEV 14521: CTG, TAG, CCA, AGA, ACC, ACT, A, Operon Technologies, Alameda, Ca) and 0.025U/µl Taq DNA Polymerase MgCl<sub>2</sub> (GibcoBRL, Lie Technologies, Gaithersburg, MD) in a total volume of 20µl.

#### Second PCR reaction:

For the second PCR reaction, 0.5-1µl of the first reaction was used as a template, total volume 20µl. Each PCR reaction contained 1.5mM MgCl<sub>2</sub> MgCl<sub>2</sub> (GibcoBRL, Lide Technologies, Gaithersburg, MD), 1x PCR Buffer MgCl<sub>2</sub> (GibcoBRL, Lide Technologies, Gaithersburg, MD), 0.05mM each NTP, 1pmol/µl of each primer (nHEVF: GTG, GTT, CAG, CAG, AAA, GTT, CTT and nHEVR: CAG, TAG, ACT, CAT, AAG, CAA, CTA, T, Operon Technologies, Alameda, Ca), AND 0.025u/MI Taq DNA polymerase (GibcoBRL, Lide Technologies, Gaithersburg, MD).

## Appendix 3

### Vitamin C Biological Activity from Craft Technologies (Wilson, NC)

#### A. Virginia Polytechnic Institute Stay-C Analysis

Track No. 2002-629  
2003-657

Work Performed for Sharonda Meade  
Analysis Date: December 13, 2002  
October 30 - November 10, 2003

Product #	delta-Tocopherol	gamma-Tocopherol	alpha-Tocopherol	alpha-Tocopherol Acetate	beta-Carotene	Sodium Ascorbate	Vit C (Stay-C) expressed as Ascorbic Acid
	mcg/g					mcg/g	
Turkey Feed	nr	nr	nr	nr	nr	nr	313
Turkey Feed	nr	nr	nr	nr	nr	nr	291

nr - not requested

## Vita

Sharonda Meade was born on May 23, 1974, in Edison, New Jersey. After finishing high school, she attended Rutgers University Cook College where she graduated with a B.S in Animal Science and Equine Science. She worked as a veterinary technician until August 1997, when she enrolled in a Master of Science program in Animal and Poultry Science under the supervision of Dr. Mike Denbow. In 1999, she enrolled in a Doctor of Philosophy program at the VA-MD Regional College of Veterinary Medicine, Department of Large Animal Clinical Sciences under Dr. Bill Pierson in avian immunology. Sharonda is the daughter of Joycelyn-Duncan-Meade, RN and Russell Meade, chemical engineer. Sharonda will be entering into a NIH Postdoctoral Training program in tissue engineering and implant science at NJ Center for Biomaterials upon completion of her degree.

Sharonda has several honors and awards. The list includes and but is not limited to: Minority Academic Opportunities Program Certificate of Appreciation Intern Science Advisor, Virginia Polytechnic Institute and State University, 1998, Phi Sigma, Biological Sciences Honor Society, Virginia Tech, 1999, Graduate Student Award for Best Paper, 72nd Northeastern Conference on Avian Diseases, Newark, Delaware, 2000, Who's Who Among American Colleges and Universities, Virginia Tech, 2001, Omicron Delta Kappa, The National Leadership Honor Society, 2002, Graduate Student Assembly Community Award, Virginia Tech, 2004, MANRRS (Minorities in Agricultural, Natural Resources and Related Sciences) Outstanding Graduate Student Award, Virginia Tech, 2004, Graduate Student Award for Best Paper, 76th Northeastern Conference on Avian Diseases, State College, Pennsylvania, 2004.