INFLUENCE OF INCUBATION CONDITIONS ON TURKEY POULT INTESTINAL DEVELOPMENT AND SUSCEPTIBILITY TO POULT ENTERITIS

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ABSTRACT

Exposure to environmental conditions that impact organ growth and function and overall performance may increase poult susceptibility to poult enteritis complex (PEC). Temperature and hypoxic stress during embryonic incubation may impact organ growth and development, development of immunocompetency, post-hatch performance and may predispose poults to enteric disease. The objective of the first study was to provide a baseline of responses to incubation conditions so that further studies could be conducted on whether these stressors may increase susceptibility to post-hatch infection. Commercial Hybrid turkey eggs were incubated at standard (37.5°C) conditions from embryonic day (ED) 0 to ED24. At ED24, eggs were divided into thirds for incubation at 37.5°C, 36.0°C, or 39.0°C from ED24 until hatch at ED28. The objective of the second study was to evaluate the effects of incubation temperature conditions on intestinal development and susceptibility to challenge with turkey coronavirus (TCV). Commercial Hybrid eggs were incubated at standard (37.5°C) conditions from ED0 to ED24. At ED24, one-third continued incubation at 37.5°C, one-third were incubated at 36.0°C, and one-third were incubated at 39.0°C from ED24 until hatch at ED28. At d 5 (0 days post-infection, dpi) half of the poults were administered 0.1mL of TCV inoculum (3×10^3 EID_{50}/0.1 mL). The third study examined the effects of incubation temperature conditions on intestinal development and susceptibility to dual challenge with both TCV

and enteropathogenic *E. coli*. Commercial Hybrid eggs were incubated at standard (37.5°C) incubation conditions from ED0 to ED25. At ED25, eggs were randomized and half continued incubation at 37.5°C and half were incubated at 36.0°C from ED25 until hatch at ED28. At d 5 (0 dpi) half of the poults were administered 0.1 mL of TCV inoculum (4 x 10^3 EID_{50}/0.1 mL) and 0.1 mL of *E. coli* (2.4 x 10^8 CFU/mL) by oral gavage. Main effects ($P \leq 0.05$) of incubation temperature and challenge, as well as two-way interactions ($P \leq 0.05$) of temperature and challenge were observed for the parameters evaluated in each study. These studies suggest an influence of incubation temperature conditions or PEC-associated pathogens on intestinal development and early post-hatch turkey poult performance.

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CHAPTER I

INTRODUCTION

Enteric diseases negatively impact commercial turkey production by causing a reduction in body weight gain, poor feed conversion, and increased morbidity and mortality, all of which translate to reduced economic return for the producer. Poult enteritis complex (**PEC**) refers to infectious enteric diseases of young turkeys, and PEC-associated pathogens are generally characterized by decreased bird performance due to detrimental impacts on intestinal development and function and suppression of the immune system. The specific causative agent(s) of PEC have not been conclusively identified, but the disease typically involves several different infectious agents and their interactions. Turkey coronavirus (TCV) has been implicated in many cases of PEC and is associated specifically as an inducer of increased mortality and depressed growth. This virus suppresses both humoral and cell-mediated immunity through a reduction in B and T lymphocyte populations as a result of compromised immune tissue structure. Destruction of the intestinal epithelium and diminished enterocyte absorptive function lead to improper nutrient utilization, which directly affects bird growth and flock uniformity. The lasting systemic effects of TCV on survivors of PEC may have a tremendous influence on overall bird health and could increase susceptibility to additional opportunistic enteric infections.

Turkey embryos undergo rapid development during incubation as reflected by accelerated post-hatch muscle development and improved feed efficiency due to selection for high-yield turkey strains. The increased growth rate of turkey embryos induces a subsequent increase in metabolic heat production during incubation, resulting in temperatures within the incubator to exceed the industry standard incubation temperature of 37.5°C. In addition to this imposed embryonic heat stress during the last several days of incubation, the embryo begins to increase its consumption of oxygen, which creates a hypoxic environment. This environment may delay

intestinal maturation due to inefficient energy utilization. The challenge associated with this plateau phase of incubation is that not only are the embryos faced with the hypoxic conditions associated with the plateau phase, they are also simultaneously exposed to elevated temperatures due to the metabolic heat that they are producing, as well as a lack of adequate ventilation between the eggs. Collectively, the combination of these stressors during the last few days of critical embryonic development may negatively impact organ and tissue development and could make the newly hatched poult more susceptible to additional post-hatch stress.

There is evidence supporting the influence of non-optimal incubation conditions on bird performance, but much of this research has been conducted in broilers. It is possible that environmental conditions such as those experienced during incubation may increase susceptibility of turkey poults to enteric infection due to their effects on tissue and organ growth, as there appears to be a relationship between maturation of the intestine and predisposition to PEC infection. The immune response of neonatal broiler chicks exposed to non-optimal incubation conditions is modified as reflected by alterations in immune organ and intestinal development, bird performance, and peripheral blood cell populations. However, the exposure to these embryonic conditions and their influence on susceptibility to enteric pathogens encountered during the first several weeks of life have not been investigated in turkey poults.

CHAPTER II

LITERATURE REVIEW

Incubation Management

Modern turkey and broiler strains have undergone improvements in growth over the last five decades compared to classic poultry of the past. These changes in growth rate are not only observed post-hatch, but have an impact on development of the embryo and subsequent hatchery management. However, the changes observed in embryonic development and growth rate over time have not resulted in modifications and improvements in commercial incubators. The most common change in incubators has simply been an increase in automated control of the incubator environment (Deeming, 2002). Commercial hatcheries utilize one of two incubation systems. In single-stage incubation, all embryos are at the same developmental stage, whereas in multi-stage incubation, embryos of multiple developmental stages are incubated together. Multi-stage incubation systems employ more efficient energy utilization, as embryos that are more developmentally advanced produce more metabolic heat than younger embryos and may be used to help incubate the younger embryos (Hamidu et al., 2007). Interestingly, embryos from different genetic breeder lines may possess varying growth potentials and metabolic rates (Tona et al., 2004), thus resulting in inconsistencies with regard to heat production by the embryo, temperature requirements, and general incubation environment. It is not uncommon to observe higher temperatures near the last few egg racks in multi-stage incubators (Hamidu et al., 2007). These egg racks contain embryos that are more developmentally advanced, and as a consequence of these temperature discrepancies, establishing and maintaining a temperature that is appropriate to meet the needs of different embryonic ages becomes challenging. Regardless of the incubation system that is employed, in both single- and multi-stage turkey incubation programs,

eggs are set in the incubator and remain there for 25 days, at which point they are transferred to the hatcher. Once the poults have hatched, they are removed from the hatcher for sorting and processing, followed by distribution to farms.

Embryonic Growth and Development. Embryonic development during incubation may be divided into two distinct periods: differentiation and growth. The entire incubation period of turkey poults takes 28 d from the time of placement in the incubator until the poult hatches. The differentiation period involves the development of embryonic tissues, such that 90% of the organs are present by embryonic day (**ED**) 12 (Deeming, 2002). The differentiation stage also involves the development of extra-embryonic tissues that support overall developmental processes in the organism. The embryo will increase in size, and tissues will mature during the growth phase to prepare the poult for hatching (Deeming, 2002). The avian embryo develops from a round ball of cells that forms the zygote. Once zygote formation is complete, initial embryonic development begins in the oviduct during the process of egg formation. By the time the egg has been laid, the blastoderm has reached the gastrula stage and consists of approximately 60,000 cells (Deeming, 2002). Only about 500 of these cells will contribute to the embryo itself as the remainder of the cells will be allocated to formation of extra-embryonic structures (Stevens, 1996). Development beyond this point will not proceed until the onset of continuous incubation, which is dependent on temperature.

Temperature. Temperature is considered one of the most crucial factors in incubation management. Maximum hatchability (French, 1997) and poult quality may be achieved when an incubation temperature that will facilitate optimum embryo growth and development is selected. The most favorable incubation temperatures range between 37°C and 38°C; however, hatchability may be achievable between 35°C and 40.5°C (Wilson, 1991).

The most imperative hatchery priority is to obtain a high rate of hatch consisting of viable chicks and a narrow spread of hatch (Decuypere and Bruggeman, 2007). Good quality chicks must be clean, dry, alert, and free from deformities with cleanly sealed navels and normal leg conformation (Decuypere and Bruggeman, 2007). Non-optimal temperatures during incubation may result in poor chick quality, which may be equated to reduced lifetime bird performance. It is accepted that non-optimal high temperatures during incubation result from the difference between embryonic heat production and heat transfer in the machine (Meijerhof, 2002).

Variability Between Eggshell Temperature and Incubation Temperature. Appropriate environmental temperature surrounding the egg is extremely important in terms of facilitating normal embryonic development. Embryos are poikilothermic and are reliant on the surrounding environment to establish body temperature. The evaluation of the actual temperature experienced by the embryo is difficult to determine as it would employ destructive methods that would negatively impact embryonic growth and development. Therefore, the use of eggshell temperature (**EST**) as a reflection of embryonic temperature is commonly accepted. Infrared fever thermometers are commonly used to determine EST (Hulet et al., 2007). The temperature that the embryo experiences may be dependent on several factors, which include incubator temperature, the transfer of heat between the incubator and the embryo, and the production of metabolic heat by the developing embryo (French, 1997). Although an average EST of 37.8°C has been observed in single stage incubators, the EST may range anywhere between 5°C above or below average depending on stage of incubation and egg position within the incubator (Lourens, 2001).

One of the primary aims of commercial incubation management is to maintain an equal air temperature across all eggs at every location within the incubator. Air velocity may be used

as a tool to achieve this uniform temperature; however, most incubators are unable to distribute an equal air velocity across all eggs. This may result in heat transfer differences between eggs, and consequently, varying embryonic temperatures (Meijerhof, 2002). Airflow uniformity within an incubator is dependent on the ability of air to pass across the pores of the eggshell to eliminate heat (Hulet et al., 2007). The number and size of pores, egg size, and eggshell thickness are determinants of heat dissipation between the embryo and incubator environment (Hulet et al., 2007). Lourens (2001) reported that eggs located in a trolley in front of the ventilator experienced EST that were 0.3°C higher than the corner trolley at ED1. Significant EST differences were seen especially after ED10. At ED18, corner trolley eggs experienced EST of 40.5°C, which was 2.7°C higher than the optimum incubation temperature of 37.8°C. These substantial deviations in EST may very well be attributed to air flow across the eggs in differing locations within the machine.

When the environmental temperature in the incubator is reported at 37.3°C, the internal egg temperature may reach 38.2°C at ED14 (Leksrisompong et al., 2007). In order for an internal egg temperature of 37.9°C to be maintained at ED19, the incubator temperature had to be reduced by 1.6°C to a set point of 36.3°C. These discrepancies between EST and incubator temperatures support findings that embryo development and hatchability are more affected by actual embryo temperature than the surrounding environmental temperature (Lourens et al., 2005). High EST have been reported during the latter part of incubation in both single- and multi-stage incubators (Joseph et al., 2006). During the third week of incubation, it is possible for environmental temperatures to reach as high as 40°C when airflow between trays is less than 0.1 m/s (Lourens, 2001).

High Temperatures During Late Incubation. Significant metabolic heat production by the embryo begins around ED4, and by ED9, embryonic temperature may be significantly higher than the surrounding environmental temperature (Hulet et al., 2007). Therefore, dissipation of heat by the embryo is a critical factor influencing overall embryonic development, hatchability, chick quality, and subsequent post-hatch bird performance. The detrimental effect of high incubation temperature on hatchability often increases with an increase in temperature and exposure time (Wilson, 1991). Additionally, the effects of high incubation temperatures are very much dependent on the developmental stage of the embryo (French, 2000). Subcellular, cellular, tissue, or organ changes during critical phases of embryological development can impact growth, reproduction, and performance as well as thermoregulation and the subsequent acquisition of thermotolerance (Decuypere, 1984).

Development of the thermoregulatory system begins during embryonic development as endothermic reactions that may ultimately influence thermoregulatory efficiency (Nichelmann and Tzschentke, 2002). Towards the end of incubation, the reaction of developing embryos to changed environmental conditions change from uncoordinated and non-adaptive to coordinated and adaptive responses (Tzschentke et al., 2004). In addition, the end phases of maturation of the critical players in metabolism, thermoregulation, and the stress response - the hypothalamus-pituitary-thyroid axis and hypothalamus-pituitary-adrenal axis – occurs during late phase incubation (Willemsen et al., 2010). Therefore, exposure of developing embryos to non-optimal temperatures during late incubation may influence general thermoregulation as well as response to environmental changes.

Incubation temperature greatly influences embryonic rate of development. Broiler embryos exposed to a high temperature of 40.6°C from ED16 -18 had a reduced relative embryo

weight and reduced relative yolk sac weight at ED18, at internal pipping, and at hatch compared to low (34.6°C) and standard (37.6°C) temperatures (Willemsen et al., 2010). Similar results of reduced relative embryo weight were reported when broiler embryos were exposed to constant thermal manipulation (39.5°C) from ED7-16 (Piestun et al., 2009). Additionally, carbohydrate and lipid metabolism were altered in embryos exposed to elevated incubation temperature, as reflected by levels of blood glucose, lactate, liver glycogen, and plasma triglycerides (Willemsen et al., 2010). Results from these studies suggest that exposure to an incubation temperature that is 3°C higher than optimal temperature during the last several days of incubation may actually negatively influence embryonic development.

Incubation temperatures higher than the standard and their effects on organ and body weights in male broiler chicks have been demonstrated (Leksrisompong et al., 2007). Heart and body weights were significantly smaller, and liver weights were significantly higher at hatch when chicks were exposed to 40.0°C from ED19-20 compared to 38.2°C. When egg temperatures of 38.4°C or 40.3°C were maintained from ED19-20, heart, gizzard, proventriculus, small intestine, and overall body weight were significantly smaller, and yolk sac weight was higher at hatch in chicks from the high incubation temperature. These findings are in agreement with other studies that have reported decreased yolk-free body weight and decreased heart size and heart weight in overheated embryos compared to controls (Wineland et al., 2000a,b). Hulet et al. (2006) reported that eggs exposed to temperatures between 37.8°C and 38°C during the last 5 or 7 d of incubation had a two percent greater hatch compared to eggs incubated at a temperature of 39.7°C and six percent greater than heat stressed embryos at 40.3°C. Heat stress in the hatcher also induced the incidence of dead embryos late in incubation, malpositions, red beaks, and enlarged yolk sacs (Hulet et al., 2006). High incubation temperatures negatively

impact chick quality by inducing poor coloring of the chicks, excessive blood in the eggshell, blood on the down and feathers, red hocks, unhealed navels, ectopic viscera, weakness, and a generally unthrifty appearance (Leksrisompong et al., 2007).

The effects of high temperature on embryonic development reported by Lourens et al. (2005) suggested that high EST (38.9°C) during the third week of incubation caused a significant decrease in embryo length and yolk free body weight at 21.5 d of incubation as compared to a constant EST of 37.8°C. Embryos heated to 39.6°C for 6 h daily from ED10-18 had lower body weight, higher heart weight, and shorter tibia length at ED18 and longer shank length and decreased lung weight at ED21 relative to control embryos incubated at 37.8°C (Yalcin and Siegel, 2003). Thermal manipulation of embryos at 38.5°C for 3 h during ED16, ED17, and ED18 resulted in decreased body temperature and reduced plasma T_3 and T_4 levels at hatch compared to controls at 37.8°C (Yahav et al., 2004). The reduction in plasma T_4 concentration reduction suggests a decline in thyroid activity in thermally stressed embryos, while the T_3 decrease indicates reduced deiodination activity (Yahav et al., 2004). The decreased body temperature together with lower thyroid hormone concentrations in heat stressed eggs suggests a reduction in metabolic rate and may later impact the ability of these birds to respond to additional heat stressors during the post-hatch period. Body weights of broilers at 44 d of age were lower after incubation temperatures of 39.7°C compared to 38.6°C and 37.5°C (Gladys et al., 2000). Additionally, feed conversion from 1 to 21 d post-hatch in the 39.7°C group was significantly higher (less efficient) compared to the other two incubation temperatures (Gladys et al., 2000).

Elevated incubation temperatures not only impact broilers but have been demonstrated in turkey embryos as well. Incubation temperatures exceeding 37°C from ED27-ED28 in turkey

embryos impacted gut development as indicated by depressed embryonic jejunum weight (Christensen et al., 2004). Intestinal function was evaluated by measuring alkaline phosphatase (**ALP**), an enzyme that serves as an indicator of overall growth and maturation activity in the body. Total ALP activity was higher in poults exposed to 39.0°C than those exposed to 36, 37, or 38°C, which suggests a relationship between elevated incubation temperature and intestinal metabolism. Increased mortality in turkey embryos has been reported when poults are exposed to high incubation temperatures between ED15-ED20 and ED24-ED28. Additionally, these high temperatures may induce increased incidence of excess albumin, ruptured yolk sacs, edematous heads, eye cataracts, and swollen down plumules (French, 1994). Hatchability in turkey poults was depressed when embryos were exposed to 38.5°C for different time periods throughout incubation relative to hatchability of the control temperature of 37.5°C (French, 2000).

Plateau in Oxygen Consumption. Oxygen requirements of turkeys during late incubation undergo modifications around ED25-26, at which time embryos utilize more oxygen and dispose of more carbon dioxide than the eggshell is capable of supporting (Dietz et al., 1998). This creates a subsequent plateau in oxygen consumption (Rahn, 1981). The oxygen plateau causes the embryo to continue growth and metabolic processes under hypoxic conditions, and consequently, delays intestinal maturation due to improper supply of energy under these conditions (Christensen et al., 2004). Intestinal maturation occurs during this plateau stage (Rahn, 1981), suggesting that if the embryo endures these conditions, development and resulting tissue function may be compromised. Additional challenges during the plateau could cause competition between growth and function for resources, and may result in a further increased demand for energy. Body weight of turkey embryos at ED27 was higher in birds exposed to more concentrated oxygen levels (23%) compared to lower (17% and 19%) concentrations, and

oxygen concentrations of 17% resulted in depressed jejunum weight at ED27 compared to 23% oxygen (Christensen et al., 2004). An overall reduced availability of oxygen at the end of incubation impairs the ability of the embryo to efficiently utilize yolk lipids for energy (Willemsen et al., 2011) and forces the embryo to refer to gluconeogenesis for glucose production (Moran et al., 2007). Additionally, low oxygen availability affected long bone development in broiler embryos at ED21, resulting in lighter and shorter tibias, lighter shanks, and increased relative asymmetry of the femur bone compared to embryos exposed to an optimal oxygen concentration (Oviedo-Rondon et al., 2008). Elevated incubation temperature in combination with depressed oxygen concentration could detrimentally impact intestinal maturation and preparation of the bird for post-hatch life, as well as negatively influence skeletal development in the embryo; however, there was no interaction between incubation temperature and oxygen concentration specifically during the plateau stage of incubation (Christensen et al., 2004; Oviedo-Rondon et al., 2008). Such a response suggests that incubation temperature and oxygen concentration may possibly act independently with regard to impact on intestinal maturation. However, a study evaluating the combination effects of incubation temperature and oxygen concentration on broiler embryo development reported that high EST (38.9°C) and low oxygen concentration (17%) from ED7-19 resulted in reduced hatchability and high embryo mortality (Molenaar et al., 2011). Whether oxygen concentration and incubation temperature interact or not, it should be noted that if incubation conditions are not modified to reflect the changing needs of the developing avian embryo, these heat and hypoxic stressors may impact organ and tissue development in terms of decreased intestinal weights and increased yolk sac weights (Leksrisompong et al., 2007). Such conditions may also detrimentally affect hatchability, poult quality, and survival rates (French, 1997), ultimately impacting overall post-

hatch performance and may make the new hatchling more susceptible to additional post-hatch stressors.

Poult Enteritis Complex

One of the most costly diseases affecting commercial turkey production in the U.S. is poult enteritis complex (PEC) (Edens et al., 1998). Clinical symptoms of PEC-affected poults include diarrhea, dehydration, increased mortality (Barnes and Guy, 1997), panting, increased vocalization, anorexia, and hypothermia (Odetallah et al., 2001). Increased mortality from PEC, defined as mortality exceeding 9% from 7 to 28 d of age with at least 3 consecutive days of mortality greater than 1% (Barnes and Guy, 1995), often occurs during the spring and summer months with estimates as high as a quarter million turkeys (Vukina et al., 1998). The growth depression resulting from PEC equates to an estimated $300 to $400 million in economic losses to the U.S. poultry industry annually (Barnes et al., 2000).

The specific causative agent of PEC has not been identified, but the disease typically involves the interaction of several infectious agents, such as enteropathogenic viruses (coronavirus, rotavirus, astrovirus, adenovirus), bacteria (*Salmonella, E. coli*, *Campylobacter*, Clostridia), and/or protozoa (*Cryptosporidia*) (Odetallah et al., 2001). Regardless of the infectious agent responsible for PEC infection, symptoms are often recognizable between 7 and 12 d post-hatch, and birds may be symptomatic up to 5 weeks of age (Odetallah et al., 2001). Turkey coronavirus (TCV) has been implicated in many cases of PEC and is associated specifically as an inducer of high mortality and growth depression (Guy, 1998). Morbidity in TCV-infected birds is near 100%; however, mortality may range anywhere from 5 to 50% (Guy, 1998). The virus is shed in the feces and is transmitted by the fecal-oral route. Viral shedding may continue up to 7 weeks post-recovery (Breslin et al., 2000; Gomaa et al., 2009a); therefore,

older birds may serve as a reservoir and infection source for younger birds (Pomeroy and Nagaraja, 1991). Additionally, TCV may be spread through the movement of people and equipment, so clinical sign recognition, bird management, and biosecurity are crucial players in minimizing potentially negative effects of the virus on bird performance and overall production costs.

Pathobiology. Infected poults begin to exhibit clinical signs between 24 and 36 hours post-exposure (Barnes and Guy, 2003). Gross lesions of infected poults include a thin-walled, gas-filled, and distended intestine, minimal feed in the gastrointestinal tract, thymic, bursal, and splenic atrophy, dehydration, and emaciation (Barnes and Guy, 2003). It is not uncommon for accumulation of caseous exudate in the bursa to form a "bursal core" in 10-20% of infected birds during late infection (Barnes and Guy, 2003). Microscopic lesions are found in the bursal mucosa and intestine. Infected birds typically exhibit villous atrophy, crypt hyperplasia, and epithelial cell sloughing from villi tips (Barnes and Guy, 2003). Bursal epithelial cells also undergo sloughing, which contributes to bursal core formation, as well as apoptosis in bursal follicles, leading to lymphoid depletion and atrophy (Barnes and Guy, 2003).

Turkey Coronavirus

Turkey coronavirus is a positive-strand enveloped RNA virus with a nonsegmented genome containing about 30,000 nucleotides classified within the *Coronaviridae* family (Siddell, 1995). Coronaviruses are spherical in shape, pleomorphic (variable in size and shape), and contain long, club-shaped peplomers which give these viruses their distinctive and recognizable shape (Guy, 2003). The coronavirus genome is around 30 kilobases (kb) in length and is comprised of four structural proteins: a large surface (S) glycoprotein (90-180 kilodaltons (kDa)), the integral membrane (M) protein (20-35 kDa), the small envelope protein (12.5 kDa),

and a nucleocapsid (N) protein (50-60 kDa) (Siddell, 1995). Some coronaviruses contain a fifth structural protein, the hemagglutinin-esterase (HE). Immunofluorescence, enzyme-linked immunosorbent assay (ELISA), immunoblotting, and other serological assays have been conducted to determine antigenic relationships among different coronaviruses, and four antigenic groups have been established. Mammalian coronaviruses comprise antigenic groups 1 and 2, and group 3 encompasses TCV and infectious bronchitis virus (IBV) (Guy, 2000).

Virus Replication. There are several different classes of enteropathogenic viruses that vary in their pathogenesis; however, the shared commonality amongst the various classes is that they infect the enterocytes of the intestinal villi and/or crypts, thus disrupting normal intestinal integrity and inducing diarrhea. Coronavirus is a Type I enteropathogenic virus that undergoes fecal-oral transmission and directly infects the epithelial cells of intestinal villi. It is not fully understood why Type I viruses target villi enterocytes. It is possible that cell receptors and brush border enzymes may play a role; specifically, these enzymes may be required for uncoating of the virus as well as exposing cell receptors (Saif, 1990). The incubation period for such enteropathogenic viruses is relatively short, and fecal excretion of the virus occurs in large quantity and rapidly spreads to other individuals (Saif, 1990).

Immunofluorescence and immunoperoxidase staining studies have shown TCV replication in jejunum and ileum enterocytes (Adams et al., 1972; Patel et al., 1975; Pomeroy et al., 1978; Nagaraja and Pomeroy, 1980; Breslin et al., 2000), particularly the enterocytes along the upper half of the villus (Guy et al., 1997; Breslin et al., 2000). Viral antigen has also been identified in the interfollicular and follicular epithelium of the bursa of Fabricius (Guy et al., 1997).

Pathogenesis and Pathogenicity. The tropism of Type I enteropathogenic viruses to infect villi enterocytes results in atrophy of the intestinal villi as well as villi fusion. Villi atrophy, which occurs as a result of intestinal cell sloughing, yields a surface that is greatly impaired in terms of absorptive capacity. An intestine that is generally malabsorptive will result in diarrhea along with dehydration, and in severe cases, death (Saif, 1990). Infectious agents cause enteric disease by several mechanisms reviewed by Moon (1978). Moon specifically elucidates the physiological events responsible for inducing diarrhea, which include hypermotility, increased permeability, hypersecretion, and malabsorption. Diarrhea and fluid loss caused as a result of enteric viruses is usually reflective of the impaired function and damage to the intestinal tissue as compared to secretory diarrhea that is typically associated with bacterial infections (Moon, 1978). Thus, the classic mechanism employed by TCV is malabsorption. Due to the destruction of the intestinal villi during TCV infection, intestinal contents that cannot be absorbed in the small intestine as a result of intestinal damage will move on to the colon where it will continue to retain and hold water due to osmotic effect. However, once the absorptive capacity of the colon has been met or exceeded, diarrhea will ensue. Malabsorption is also associated with a reduction in brush border enzymes, such as disaccharidases, which could result in a buildup of undigested lactose in the gut. When the undigested lactose reaches the distal intestine, it is fermented by colonic bacteria and further contributes to diarrhea. Since the absorptive function of the enterocytes is greatly reduced, if not mostly eliminated in TCV infection, there is an increase in proliferation of crypt cells in order to replace the damaged enterocytes. This compensatory crypt hyperplasia may result in increased secretion. The crypt cells are proliferating rapidly, and in doing so, sometimes migrate up the villus prior to completion of cellular differentiation and maturation. As such, some crypt cells maintain

secretory capacity thus leading to hypersecretion, another mechanism by which enteric pathogens may disturb normal gut function. If the amount of solute secreted exceeds the amount of intestinal contents absorbed, diarrhea results. Another result of enteric viruses is hypermotility, which is reflective of increased rate of passage or change in intensity of passage. This alteration in intestinal passage rate precludes intestinal contents from having sufficient epithelial contact time for absorption as a result of amplified peristalsis.

Under normal circumstances, secretory (movement from blood to the intestine) and absorptive (movement from intestine to blood) fluxes occur at the same time, with absorptive fluxes exceeding secretory fluxes, resulting in net absorption. If secretion exceeds absorption, or if absorption is hindered, net secretion occurs but the excess is eliminated, causing diarrhea. Intestinal inflammation may result in a "leaky" intestine as a result of increased intestinal flow and increased hydraulic pressure. If the concentration of solutes that are extruded exceeds the amount of material absorbed, diarrhea will result.

Host Susceptibility to Enteric Viruses. Interactions amongst the host, environment, and infectious agent play a critical role in dictating the severity of infection. Age of the individual is a factor, as cellular turnover rate in young animals is slower than that of mature animals. This reduced turnover rate causes repair of intestinal damage to be slower and thus increase susceptibility of younger individuals to viral diarrhea. Interestingly, viruses that replicate only in specific portions of the intestine or that infect intestinal enterocytes that are scattered tend not to result in villi atrophy or diarrhea; however, viruses that replicate throughout the intestine or infect a large number of intestinal enterocytes will often result in more severe diarrhea and villi atrophy (Saif, 1990). Additionally, the specific location of lesions induced by enteric viruses may impact the severity of consequential diarrhea. Damage to the proximal and middle portion

of the jejunum may elicit very severe diarrhea because glucose and sodium absorption are greatest in these areas (Shepherd et al., 1979; Bachman and Hess, 1985). If the ileum is severely infected, absorption of bile salts could be impaired, which proceed to pass on to the large intestine or colon, stimulating further secretion and contributing to diarrhea (Moon, 1978).

Normal intestinal microflora are necessary in providing colonization resistance to enteric pathogens while also functioning in host immunity. A disruption in the microbial population of the host could be beneficial to invading pathogens in that they may be able to invade and colonize the host intestine (Sekirov and Finlay, 2009). Interestingly, some enteric pathogens are able to evade the inflammatory response elicited by the host that is directed at eliminating them, but the integrity of intestinal microflora may be compromised through this host immune response potentially increasing host susceptibility to infection (Sekirov and Finlay, 2009).

Turkey Coronavirus and Immune Function. Since TCV replicates in mature small intestinal epithelial cells (Deshmukh et al., 1975), protection against TCV is mainly provided by the immune response active at the mucosal surface of the intestine (Loa et al., 2002). However, overall immune system function is often suppressed in PEC-affected poults as indicated by decreased resistance to secondary infections and cytokine induction that not only results in intestinal inflammation (Heggen et al., 2000) but also compromises immune tissue development and function. Immune system dysfunction as a result of experimental TCV inoculation may be manifested in the form of increased eosinophil cell populations in whole blood (Edens et al., 1997), which may be reflective of a reduced cell-mediated response (Doerfler et al., 1998). Further, PEC-affected poults have shown reduced swelling in response to phytohemagglutinin-P (**PHA-P**) injection, which is indicative of reduced proliferation of T lymphocytes (Qureshi et al., 1997).

PEC-infection yields not only a modified cell-mediated response but also has a direct effect on humoral immunity. In a study evaluating the effects of PEC on immune system function, poults were exposed to a PEC-positive flock at 4 d post-hatch, and once exhibiting clinical signs of the disease, birds were evaluated for lymphoid organ integrity at several time intervals post-exposure. At 6, 9, 16, and 23 d following exposure, PEC-exposed poults had significant reductions in bursa, thymus, and spleen weights compared to non-infected controls (Qureshi et al., 1997). Morphologically, PEC-infected poults showed thymic atrophy and tissue hypoplasia (Barnes, 2002), as well as heterophil infiltration indicative of inflammation (Jindal et al., 2009), bursal follicle lymphocyte depletion 8 d post-exposure (Barnes, 2002), bursal epithelial cysts (Jindal et al., 2009), and splenic lymphocyte depletion 14 d post-exposure (Barnes, 2002). Poults injected with sheep red blood cells (SRBC) at 7 d following PEC exposure showed lower total antibody titers as well as lower mercaptoethanol-sensitive (presumably reflective of B lymphocyte surface protein, IgM) values in PEC-affected poults relative to control birds at both 4 and 8 d post-SRBC challenge (Qureshi et al., 1997). Cumulatively, the findings of these studies strongly suggest that PEC-affected poults may be incapable of mounting an effective immune response through several weeks post-exposure compared to age-matched uninfected counterparts.

Intestinal Integrity and Bird Performance. Poults infected with PEC often exhibit reduced growth and digestive function, which typically leads to stunting of the birds and a subsequent lack of overall flock uniformity (Culver et al., 2006). It is believed that PEC survivors suffer impaired nutrient utilization due to damage of the absorptive surface of the gastrointestinal tract (Odetallah et al., 2001). At 5 d of age, poults were exposed to a PEC-infected flock, and birds that survived this exposure were divided into groups that represented

three degrees of stunting (small, medium, and large) as a result of infection (Odetallah et al., 2001). All three groups exhibited a reduction in apparent nitrogen retention, apparent fat absorbability, and nitrogen corrected apparent metabolizable energy at 49 d compared to non-infected controls (Odetallah et al., 2001). These results suggest that protein digestion, amino acid utilization, and fat absorption are compromised even after recovery from an active PEC infection regardless of the degree of infection severity. Plasma D-xylose concentration is further indicative of intestinal absorptive function, and birds inoculated with TCV showed a reduction in plasma D-xylose at 24, 72, and 120 h post-inoculation and at both 30 and 90 minutes following oral D-xylose administration compared to controls (Ismail et al., 2003). Additionally, PEC-exposed poults at 5 d post-hatch showed decreased serum glucose values at 6 d following exposure, and the values remained depressed through 16 d post-exposure (Doerfler et al., 1998). The inability to efficiently utilize glucose for energy metabolism often results in the use of muscle protein, which further contributes to a reduction in weight gain and overall muscle wasting in affected poults (Doerfler et al., 1998).

Collectively, these responses are reflective of compromised intestinal structure and integrity following PEC infection. By 5 d post-infection, epithelial cell sloughing and villi fusion were evident (Gomaa et al., 2009b). In general, there were fewer villi in infected poults, and the remaining villi were broader and possessed denuded tips that lacked overlying enterocytes (Gomaa et al., 2009b) or appeared pleated due to contraction from epithelial cell sloughing (Barnes, 2002). Duodenum villus height was significantly reduced, and crypt depth increased at 7 d and 5 d post-infection, respectively (Gomaa et al., 2009b). Within the crypt epithelium at both 5 and 7 d post-infection, hyperplasia and crypt enterocyte division were observed (Gomaa et al., 2009b). This response was elicited most likely as a compensatory effort

due to the sloughing of mature enterocytes and to somewhat preserve intestinal absorptive capacity. Enterocytes may also present as pale and swollen as a result of these compensatory processes (Barnes, 2002).

These intestinal aberrations as a result of infection have a direct effect on bird performance. Poults infected at 2 d post-hatch showed a reduction in mean body weight compared to uninfected control poults, starting at 6 d post-infection and persisting through 44 d of age (Gomaa et al., 2009b). Poults challenged at 28 d post-hatch showed a similar pattern in body weight reduction. The 28 d old poults exhibited a body weight reduction compared to uninfected controls at both 35 and 44 d, and at 44 d, the mean body weights of the infected poults were only 77.7% of uninfected control weights (Gomaa et al., 2009b). These findings closely resemble the response reported by Doerfler et al. (1998) that showed a depression in body weight at 9, 16, and 23 d after PEC exposure at d 5. Correspondingly, cumulative mortality for a series of 8 experimental PEC studies showed a sustained increase from 6 d post-exposure (11 d of age) through 19 d of age, after which point a plateau was reached. Cumulative mortality rate as a result of PEC exposure reached nearly 35% compared to less than 4% in uninfected control poults (Doerfler et al., 1998).

Enteric infection not only provokes an immediate response in the bird, but the consequences of PEC exposure may be long term. The depletion of lymphocytes in the lymphoid tissue of infected birds may have tremendous implications with regard to predisposition for secondary infections, which may further impact overall poult health. The gastrointestinal tract of poultry is considered anatomically complete early in embryonic development; however, it is functionally immature at hatch. This immature system has a somewhat limited capacity for absorption of lipids, proteins, and carbohydrates initially during

early brooding. A reduced nutrient absorptive capacity combined with the susceptibility of the intestine to invading pathogens due to its early immaturity is further exacerbated by PEC exposure, especially TCV infection. Malabsorption of nutrients results in suboptimal growth, increasing the chance for nutritional deficiencies. These conditions could contribute to a further reduction in body weight and growth potential and lead to an inability of birds to adequately reach feeders and waterers. Management factors and reduction of environmental and pathogenic stressors upon the birds are especially effective in reducing the incidence of PEC in healthy turkeys and in mediating active infection in PEC-exposed flocks.

Enteropathogenic E. coli

Enteropathogenic strains of *E. coli* have been implicated in PEC infections and are characterized by adherence of the bacteria to the intestinal epithelium, resulting in enteric disease without the expression of diarrhea-inducing heat labile and heat stable toxins, such as those produced by enterotoxigenic *E. coli* strains (Guy et al., 2000). Enteropathogenic *E. coli* strains produce intestinal lesions referred to as "attaching and effacing" lesions as described by Moon et al. (1983). Attaching and effacing lesions typically cause loss of microvilli and loss of enterocytes and degeneration of cytoplasm in intestinal epithelial cells that remain, thus resulting in hindered digestion and absorption and consequential malabsorptive diarrhea (Moon et al., 1983). Turkeys infected with only *E. coli* strain R98/5 failed to develop clinical disease, and TCV-only infected birds developed mild disease and growth depression; however, turkeys infected with both TCV and *E. coli* R98/5 developed severe enteritis, high mortality, and growth depression (Guy et al., 2000). It is believed that TCV infection promotes colonization of the intestine by *E. coli*, and the two pathogens synergistically have a detrimental effect on bird

performance, mortality, intestinal structure and function, and cause lymphoid tissue atrophy (Guy et al., 2000).

Gastrointestinal Tract

Rapid post-hatch development of the small intestine occurs when the bird is transitioning from the nutritive yolk sac to an exogenous feed source. The initial post-hatch period is critical in terms of intestinal structure and function as the chick is transitioning from dependence on yolk as the primary nutrient source to carbohydrate and protein-rich feed (Noy and Sklan, 2001), and this dietary transition period fuels intestinal development. Utilization of the yolk occurs via two routes, the first of which involves direct transfer through circulation, and the second in which the yolk is transferred via the yolk stalk into the small intestine (Noy and Sklan, 2002). During this transitional period, the growth rate of the small intestines is greater than total body weight in turkey poults (Uni et al., 1999). From 0 to 12 days post-hatch, the length of both the jejunum and ileum increased more rapidly compared to the duodenum; however, mass increased more rapidly in the duodenum and jejunum compared to the ileum (Uni et al., 1999). This rapid post-hatch intestinal development has been quantified by observation of an increased number of intestinal enterocytes as a result of increased villus length (Geyra et al., 2001). At hatch, the intestinal villi are very small, and crypts are not detectable (Bar-Shira and Friedman, 2005). Crypts become well-defined by 2 to 3 d post-hatch and continue to increase in number until a plateau stage at 48 to 72 h after hatch (Bar-Shira and Friedman, 2005). In general, digestive and absorptive function in poults develops at a slower rate compared to chicks (Sklan and Noy, 2003).

The post-hatch period is crucial not only from a dietary transition standpoint and subsequent establishment of intestinal microflora, but also in the maturation of the gut-associated

lymphoid tissue (**GALT)**, which is imperative for the induction of the immune response to intestinal pathogens. The maturation of GALT occurs in two distinct phases, occurring during the first and second week post-hatch, respectively. Exposure to environmental antigens and the transition to feed induces the onset of GALT maturation, which is initiated by an increase in lymphocytes and natural killer cells in the intraepithelial lymphocyte compartment in the newly hatched chick (Bar-Shira et al., 2003). New T lymphocytes arrive at 3 d post-hatch and promote the maturation of the lamina propria. The GALT B lymphocyte population begins to increase around 4 d after hatch and continues to increase throughout the first 2 weeks of life. Bar-Shira et al. (2003) further suggested that cellular responses mature earlier and are required in order for a humoral response to occur. It is understood that the innate immune response reaches maturity around 5 d post-hatch, while the adaptive response does not mature until the second week of life. Maternal antibodies play a crucial role in providing initial immune protection until the immune system has developed and matured.

During the last three days of incubation, intestinal weight as a proportion of body weight increases from 1% at ED17 to nearly 3.5% at hatch (Uni et al., 2003). However, small intestine development may be impacted by a variety of environmental factors, one of which is exposure to thermal stress. Twenty-eight day old male broilers reared in climatic chambers for two weeks and exposed to thermal stress temperatures of 30°C had decreased small intestinal weight relative to birds that were maintained at a non-stressful rearing temperature of 20°C (Garriga et al., 2006). Twenty-four hour exposure to elevated temperatures (30°C) in 44 d broilers caused decreased crypt depth in the ileum compared to control temperatures (Burkholder et al., 2008). Uni et al. (2001) evaluated the effects of early post-hatch thermal stress and its influence on intestinal morphology and proliferation of intestinal epithelial cells. After 24 h exposure of 3 d

old male broiler chicks to 36.0°C, the jejunal mucosa exhibited narrow villi with large spaces between them and decreased villus volume 24 h post treatment. Immediately after heat exposure, the percentage of proliferating cell nuclear antigen (**PCNA**)-positive crypt cells was reduced in heat stressed chicks. Stress induced imbalances in the gastrointestinal tract can impact normal microbial populations and intestinal integrity and thus reduce innate protective mechanisms and increase subsequent susceptibility to pathogenic infections (Burkholder et al., 2008).

Avian Immune System

In vitro studies have shown that the fundamental mechanisms of the immune response are identical in avian species, mammals, and most other vertebrates (Jeurissen et al., 1994). The primary lymphoid organs in avian species consist of the bursa of Fabricius and the thymus. Secondary lymphoid organs include the spleen, bone marrow, and mucosa-associated lymphoid tissues. Within each respective lymphoid organ, there exists specialized sections in which antigen presentation to T cells occurs, T and B cells interact, or immunoglobulins (**Ig**) are produced (Jeurissen et al., 1994). Immunoglobulins, the proteins that constitute antibodies, are present on the surface of cells and serve as receptors for antigen recognition by B cells. When antigen stimulation occurs, B cells respond by differentiating into plasma cells that produce antibodies that are antigen-specific. Three main classes of antibodies are produced by avian species: IgM, IgA, and IgG, the latter of which may be referred to as IgY in avians. The primary antibody response is elicited by IgM, followed by subsequent IgA and IgY production. Immune system development begins early during embryogenesis. Bursal precursor cells may be detected by ED7, and cells expressing surface IgM, IgG, and IgA may be detected at ED10. In general,

the primary goal and function of the immune system is to distinguish between self and nonself and to recognize and elicit an appropriate response to each.

Innate Immunity. Innate immunity is the first line of defense against foreign invaders. It prevents pathogen entry through two critical barriers, the skin and mucosal surfaces. Foreign invaders and antigens that cross these barriers are exposed to macrophages, heterophils, dendritic cells, natural killer cells, and lymphocytes. These cells are responsible for recognizing foreign pathogens, antigen presentation, and activation of the acquired immune response (Korver, 2006).

Acquired Immunity. Acquired, or adaptive, immunity must develop in avian species. Acquired immunity is unique from innate immunity in that it is specific and also has memory. This type of specific immunity consists of two different types of responses: cell-mediated and humoral. In cell-mediated immunity, infected cells are destroyed through their interaction with an effector cell, such as a T lymphocyte (Korver, 2006). Humoral immunity involves the production of antibodies by B lymphocytes in response to presence of foreign antigen.

Bursa of Fabricius. The bursa of Fabricius appears around ED3-4. It contains the following layers from exterior to interior: thin serosa, muscularis, mucosa, and epithelium of cylindrical or cuboidal cells. The thickest portion, the mucosa, establishes a structural foundation for the bursal follicles, which are embedded in the mucosa. The immunological role of the bursa of Fabricius in antibody production was first documented by Glick and colleagues (Glick et al., 1956). It serves as the primary lymphoid organ for B cell maturation and the generation of antibody diversity. The bursal follicle, the functional unit of the bursa, originates from epithelial buds that appear at ED12 (Glick, 1983). The follicles arise from the epithelial cells in the lamina propria and are colonized by lymphoid cells. The follicles may be divided into both an inner medulla and an outer cortex separated by a basal membrane and epithelial cells. The medulla

possesses epithelial cells, secretory cells, macrophages, plasma cells, and lymphocytes. The cortex region, lacking secretory cells, becomes much more apparent post-hatch (Glick, 1993). The cells of the medulla are separated from the bursal lumen by follicle-associated epithelium (FAE), which is derived in the embryonic bursa from mucosal surface epithelium. According to Toivanen and colleagues (1986), pre-bursal stem cells enter the bursa between ED8 to 14. By ED12, Ig genes undergo rearrangement, and beginning on ED12, bursal stem cells with surface IgM appear. Antigen-independent production of the antibody repertoire occurs between ED12 to 21. The bursa experiences its most rapid growth between 2 and 3 wks of age and plateaus between 3 and 8 wks, followed by involution and regression (Glick, 1983).

Spleen. During embryonic development, the spleen plays a critical role in erythropoiesis and granulocytopoiesis. It appears around ED5 in the developing embryo. During a brief period after hatch, the spleen is the primary organ to receive early post-bursal stem cells from the bursa (Toivanen, 1986). The spleen serves as a secondary lymphoid organ in the chicken and is surrounded by collagen and reticulum fibers. It is comprised of both red and white pulp, which contains both scattered lymphoid and non-lymphoid cells and densely packed lymphoid and non-lymphoid cells, respectively. Peri-arteriolar lymphoid sheaths (**PALS**) are found surrounding veins and venules and are made up of T cells. During a humoral response, germinal centers form in the PALS regions and consist primarily of B lymphocytes. Additional B cell areas exist in the peri-ellipsoid lymphocyte sheaths (**PELS**), as do macrophages and plasma cells. B cells originating from PELS in the spleen may selectively bind antigen and assist in the initiation of the humoral immune response (Jeurissen, 1993). The lymphoid organs work in concert to maintain a functional and efficient system prepared to combat any adverse physiological changes and to identify and eliminate invading pathogens. However, if the onset of development of these

organs is either delayed or accelerated due to environmental stressors, the competency of the lymphoid tissues and the capability of the immune system to elicit a response may be impacted.

Stress

Stress may be defined as a disruption of an organism's physiological homeostasis or well-being. Threats to an organism's homeostasis are referred to as stressors. Stressors include physical, chemical, and infectious factors that may be modified by stressor duration, severity, novelty, status of the immune system, and host genetics (Dohms and Metz, 1991). The success of an organism's response to a stressor is dependent on the severity of the stressor and the organism's physiological response to it (Siegel et al., 1980). The response to physical stressors is dependent on both the neural and endocrine systems. The neurogenic system (**NS**), consisting of the central nervous system and adrenal medulla, works in concert with the hypothalamus-pituitary-adrenal axis (**HPA**) to elicit a stress response. Response by the NS is rapid and is primarily mediated by the catecholamines, epinephrine and norepinephrine, which are critical players in the "fight or flight" response. These catecholamines ultimately induce the production of cyclic adenosine monophosphate (**cAMP**) which is crucial in energy reactions, but they also increase antibody formation, suggesting the impacts of stress on immune response efficiency. Activation of the HPA axis is typically induced in response to chronic stress. It begins with hypothalamic stimulation and induces an increase in adrenocorticotropic hormone (**ACTH**) from the anterior pituitary, which subsequently causes increased production of adrenal cortical steroids, such as corticosterone. The effects of corticosteroid production include decreases in bursa, thymus, and spleen tissue, a reduction in circulating lymphocytes, and an increase in heterophils (Siegel, 1985).

Blood System Response to Stress. According to Yahav and colleagues (1997), animals may be faced with acute cold or heat stress, seasonal temperature changes, and diurnal temperature cycle changes. Changes in the blood may be observed in response to these stressors, but it remains unclear as to whether these changes are attributed to the acclimation to environmental temperature or to the temperature stress itself. Yahav et al. (1997) reported that between 5 to 8 weeks post-hatch, male broilers exposed to a constant ambient temperature of 10.0°C had significantly higher blood volume values represented as percent body weight than those exposed to 20.0°C. Hematocrit and hemoglobin concentrations decreased with increased ambient temperature (10.0°C, 20.0°C, or 30.0°C). The heterophil:lymphocyte ratio (**H:L**) is a good indicator of stress in chickens. A continuous dose of ACTH caused an increase in H:L ratios 4 and 7 d following the onset of ACTH administration (Puvadolpirod and Thaxton, 2000). Heterophil:lymphocyte ratios have been shown to increase during crating (Zulkifli et al., 2009) and after exposure to environmental heat stress, and increase further after exposure to multiple stressors (Macfarlane and Curtis, 1989). Birds exposed to heat stress lasting 3 h at 36 and 37 d of age had increased H:L ratios (Altan et al., 2003). Environmental stressors appear to induce changes in cell dynamics in the blood system as a protective mechanism. Heat stress, especially, may result in elevated cell counts, making evaluation of these parameters a valuable indicator of homeostasis disruption and initiation of a defensive response.

Immune Response to Stress. A variety of environmental or physiological stressors may induce a lymphoid tissue response, and to demonstrate this, exogenous administration of ACTH may be utilized to mediate the adrenal gland response. Continuous administration of ACTH via mini-osmotic pumps caused decreased relative bursa, thymus, and spleen weights at 4 and 7 d following pump-implantation compared to control (Puvadolpirod and Thaxton, 2000). Oral

corticosterone administration retarded spleen weights in 21 d old broilers (Post et al., 2003). Heat stress in laying hens decreased antibody production to SRBC at 1 and 4 wks following heat exposure (Mashaly et al., 2004). These findings are in agreement with previous studies that have reported significantly lower antibody titers in heat stressed broilers (Khajavi et al., 2003). Additionally, in vitro analysis of effects of hyperthermic conditions on T cell proliferation suggested that helper T cells are targeted during heat stress and increase in number in response to these hyperthermic conditions (Jampel et al., 1983).

Summary

It is evident that exposure to non-optimal incubation conditions may have detrimental effects on embryonic and post-hatch development, bird performance, acquisition of thermotolerance, and immunocompetency in both broilers and turkeys. Exposure to non-optimal conditions during incubation may also cause increased sensitivity to subsequent post-hatch stressors. Specifically, it is possible that such conditions may increase susceptibility of turkey poults to enteric infection due to their effects on tissue and organ growth potential, as there appears to be a relationship between maturation of the intestine and predisposition to PEC infection.

The objectives of these experiments were to evaluate the effects of incubation conditions on turkey poult intestinal and immune system development and poult enteritis susceptibility induced by TCV or dual TCV/*E. coli* challenge. These objectives were tested by exposing turkey embryos to non-optimal conditions during the oxygen consumption plateau phase during late incubation followed by post-hatch challenge with PEC-associated pathogens. The effects from these parameters of interest were evaluated through peripheral blood cell profiles, intestinal morphology, immune organ development, and bird performance.

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CHAPTER III

Influence of Incubation Conditions on Turkey Poult Intestinal Development and Indicators of Bird Development

ABSTRACT Early mortality, grow-out performance, and health status of commercial turkeys can be largely impacted by stressors during early development, possibly including stress induced during incubation. Non-optimal incubation conditions may detrimentally affect intestinal and immune system development and maturation and can result in impaired intestinal integrity, thereby impacting digestion, absorption, and immune function. The objective of this study was to provide a baseline of responses of the bird to incubation stressors so that further studies could be conducted on whether these stressors may increase potential susceptibility to post-hatch infection. Commercial Hybrid turkey eggs from a 52 week-old breeder flock were incubated at standard (37.5°C) incubation conditions from embryonic day (ED) 0 to ED24. At ED24, eggs were divided into thirds for incubation at 37.5°C, 36.0°C, or 39.0°C from ED24 until hatch at ED28. Rectal temperatures were obtained at hatch, and poults were weighed at hatch and at d 7. Birds incubated at 39.0°C demonstrated a higher ($P<0.0001$) rectal temperature than those from 37.5°C or 36.0°C, which were different from each other. At hatch, birds that were incubated at 36.0°C had a higher ($P<0.0001$) body weight than birds incubated at 37.5°C or 39.0°C. At d 7, birds incubated at 39.0°C had a lower ($P=0.0173$) body weight than birds incubated at 37.5°C, and the body weight of birds incubated at 36.0°C was comparable to both 39.0°C and 37.5°C. At d 7, intestinal samples were obtained from the jejunum and ileum to evaluate intestinal morphology, whole blood was collected for blood smears to evaluate heterophil:lymphocyte ratios, and the spleen, bursa, yolk sac, and intestine were weighed. There were no effects of incubation temperature treatment on any of those parameters. Results from this study suggest

that exposure to an elevated temperature during late incubation negatively impacts post-hatch body weight, and it is possible that there may be an advantage to low temperature exposure during the last several days of incubation of turkey embryos.

Keywords: turkey, incubation, temperature

INTRODUCTION

A crucial factor in maximizing chick or poult hatchability (French, 1997) is managing an optimum incubator temperature that allows for efficient growth, development, and maturation of the embryo. The desired temperature in commercial hatcheries is between 37.0°C and 38.0°C (Hulet et al., 2007); however, several factors may influence the temperature that is actually experienced by the embryo. Eggshell temperature (**EST**) is the most reliable indicator of true embryonic temperature, and it may be obtained without compromising the structure of the eggshell and the developing embryo. It is not uncommon to observe elevated temperatures during late incubation in both single- and multi-stage incubation systems (Joseph et al., 2006). These elevated temperatures are reflective of genetic selection for rapid growth, and with this accelerated growth rate occurs an increase in metabolic heat production by the embryo. Embryonic heat that is expelled typically accumulates in the machine; however, most incubation and hatching equipment have not been modified to reflect the demands of current lines of poultry, and the build-up of excess embryonic heat may impose heat stress upon the bird. In fact, there is much concern that the production of heat by modern poultry exceeds that of poultry from several years ago (Hamidu et al., 2007). The physiological demands of turkey embryos selected for increased meat yield and the embryo's responses to current incubation management are generally not well understood (Hamidu et al., 2011). Heat stress during late incubation has resulted in increased incidence of late dead and malpositioned embryos, red beaks, and enlarged

yolk sacs (Hulet et al., 2006). Elevated incubation temperatures negatively impact chick quality by inducing poor coloring of the chicks, excessive blood in the eggshell, blood on the down and feathers, red hocks, unhealed navels, ectopic viscera, weakness, and a generally unthrifty appearance (Leksrisompong et al., 2007).

Oxygen requirements of turkeys increase during the last several days of incubation, and the amount of oxygen that the embryo requires exceeds what is available, creating a plateau in oxygen consumption. The plateau stage is followed by the actual hatching process in which the poult internally pips into the air cell and begins pulmonary respiration, externally pips the eggshell, and finally hatches. The exchange of oxygen and carbon dioxide during incubation impacts development of the embryo, hatchability, and bird quality (Decuypere et al., 2001; Tona et al., 2005). The oxygen plateau causes the embryo to continue growth and metabolic processes under hypoxic conditions, and consequently, delays intestinal maturation due to improper supply of energy under these conditions (Christensen et al., 2004). The reduction in available oxygen to the embryo during late incubation results in a reliance of the embryo on anaerobic metabolism for energy (Christensen et al., 1997) as provided by blood glucose and tissue glycogen stores (Freeman, 1965). If stress is incurred during the oxygen consumption plateau, growth and organ function may oppose each other, and the embryo may require even more energy in an effort to adapt to the stress exposure (Wineland et al., 2006).

The objective of this study was to evaluate the effects of non-optimal late incubation temperatures during the oxygen consumption plateau in embryonic turkeys. Previous research has suggested that incubation temperature and oxygen concentration impaired development of the intestine in turkeys (Christensen et al., 2004) and broilers (Wineland et al., 2006). The purpose of this experiment was to provide a general baseline of the poult's response to late

incubation conditions to further elucidate how such conditions impact late-term embryo maturation and consequential post-hatch development.

MATERIALS AND METHODS

Animal Welfare

This experiment was approved and conducted under the guidelines set forth by the Institutional Animal Care and Use Committee at Virginia Tech.

Incubation Temperature

Commercial Hybrid turkey eggs (n=700) were obtained from a 52-week-old breeder flock. Eggs were incubated in NOM 45/90/120 incubators[1] at the hatchery facility of the Virginia Tech Turkey Research Center. To simulate a commercial incubation environment, temperatures were set at the industry standard temperature (37.5°C) from embryonic day (**ED**) 0 to ED24. From ED24 to ED28, eggs were incubated in one of three incubators each set at a different temperature during the plateau of oxygen consumption. Following candling at transfer (ED24), one-third of the eggs were maintained at 37.5°C (n=219), one-third of the eggs were incubated at a low temperature (36.0°C; n=219), and one-third of the eggs were incubated a high temperature (39.0°C; n=219). Incubation temperature was regulated by thermistors connected to microprocessors with a temperature sensitivity of ± 0.05°C. Humidity was controlled with a comparable system by humidity sensors. Two temperature data loggers were placed in each incubator to monitor temperature. Incubation temperature and humidity were evaluated and logged daily. Eggshell temperatures were taken at the equator of the egg once daily and were recorded from 16 eggs per incubator at various locations within the incubator using an infrared

[1] NatureForm, Inc., Jacksonville, FL

thermometer[2]. Rectal temperatures were obtained at hatch from 50 randomly selected poults/incubation temperature.

Rearing and Diets

Poults were randomized within treatment and assigned to battery brooder pens (n=10 replicate pens/treatment with 8 poults/pen). The birds were housed in an environmentally controlled room under a lighting program recommended for Hybrid turkeys. Birds had ad libitum access to water and a corn-soybean based diet formulated to meet or exceed Hybrid Converter nutritional requirements. A pre-starter diet was administered from day of hatch (**DOH**) until the termination of the study at d 7.

Performance Parameters

All poults were weighed at DOH prior to placement in pens (n=128 poults/ incubation temperature). At d 7, poults (n=8 poults/treatment) were randomly selected for measurement of weight of bursa, spleen, yolk sac, and intestinal tract from the duodenum to ileocecal junction. Birds were weighed, euthanized by cervical dislocation, and organs were collected. Organ weights are presented as a percentage (%) of body weight (**BW**).

Heterophil/Lymphocyte Ratios

Whole blood (n=8 birds/treatment) was collected from the same birds from which organ samples were obtained. Blood was collected from the jugular or brachial vein at d 7 for evaluation of heterophil to lymphocyte (**H:L**) ratios. Blood samples were collected in 1.8 mL tubes containing 0.105 M buffered sodium citrate. A blood smear was made for each blood collection tube using a Morf slide spinner[3]. Smears were stained with a modified Wright-

[2] Raynger ST20 Pro Standard, Santa Cruz, CA
[3] Salem Specialties, Inc., Salem, VA

Giemsa stain using the protocol provided by the manufacturer[4]. Smears were evaluated by counting a total of 60 lymphocytes and heterophils per slide under 100X oil immersion. Heterophil:lymphocyte ratios were calculated by dividing the total number of heterophils by the total number of lymphocytes for each blood smear.

Intestinal Morphology

Eight birds per treatment (same sampling birds from which organ samples and whole blood were obtained) were randomly selected for evaluation of intestinal morphology at d 7. Tissue samples (3cm) were collected from the jejunum (mid-point from the pancreatic duct to Meckel's diverticulum) and ileum (Meckel's diverticulum to ileocecal junction). Intestinal segments were flushed with cold PBS and fixed in 10% neutral buffered formalin. Each segment was cut into 5 (1cm) sections and placed in a tissue cassette. Tissues were processed, embedded in paraffin, cut into 5µm sections, and mounted onto slides. Slides were stained using routine procedures for hemotoxylin and eosin (**H&E**) and were evaluated using a light microscope (Luna, 1968). Measurements were made using SigmaScan Pro 5 software[5]. Three of the five total intestinal tissue sections were evaluated per slide for villus height (from villus tip to opening of crypt) and crypt depth (from the opening of the crypt to the base). Villus height-to-crypt depth ratios were also calculated. Four villi and 4 crypts were evaluated for each of the 3 intestinal tissue sections. The average villus height, crypt depth, and villus height-to-crypt depth ratio per slide were analyzed (n=12 measurements/bird, 8 birds/treatment).

Statistical Analysis

Data were evaluated as a completely randomized experimental design. Analysis of variance was performed using the GLM procedure of SAS. Values are reported as least

[4] Sigma-Aldrich, St. Louis, MO
[5] Olympus America Inc., Melville, NY

squares (LS) means ± SEM. Bird served as the experimental unit for organ weights, heterophil:lymphocyte ratio, intestinal morphology, and rectal temperatures. Data analyzed as a percentage were transformed prior to analysis using arc-sine (square root of percent).

RESULTS

There were no differences in % hatch between temperature treatments (data not shown). There was no effect of incubation temperature on intestinal morphology, heterophil:lymphocyte ratio, or weight of the bursa, spleen, yolk sac, or intestine relative to body weight. There was a main effect ($P<0.0001$) of incubation temperature on rectal temperature at DOH. Birds incubated at 39.0°C had a higher rectal temperature compared to birds incubated at 37.5°C and 36.0°C, and birds incubated at 36.0°C had a higher rectal temperature compared to birds incubated at 37.5°C (Figure 3.1). Incubation temperature impacted body weight at DOH. Birds incubated at 36.0°C had a higher ($P<0.0001$) body weight compared to birds incubated at 37.5°C and 39.0°C (Figure 3.2). Body weight at d 7 was also impacted by incubation temperature, with birds incubated at 37.5°C exhibiting higher ($P=0.0173$) body weight compared to birds incubated at 39.0°C, and birds incubated at 36.0°C had comparable body weight to both 37.5°C and 39.0°C (Figure 3.2).

DISCUSSION

Previous studies have suggested that incubation temperatures greater than 37.0°C and oxygen concentrations less than 21% during late incubation of broiler embryos negatively impact intestinal development (Wineland et al., 2006). In the current study, an incubation temperature of 39.0°C during the last several hypoxic days of incubation resulted in no effect on intestinal morphology. Final intestinal maturation occurs during the last several days of incubation, and it was hypothesized that exposure to environmental stressors during this maturation process could

result in delayed development. The commercial eggs utilized in this study were obtained from a 52 week-old breeder flock, which is considered a mature flock age. Embryos from young breeder hens (around 30 weeks) tend to develop at a slower rate compared to embryos from older parents (Christensen et al., 2001). As a result of this difference in growth rate between flock ages, embryos from older flocks typically reach the plateau in oxygen consumption earlier than embryos from younger flocks (Christensen et al., 1996) and may prematurely utilize available oxygen in the air cell at internal pipping (Hamidu et al., 2011). In turkeys, the plateau occurs around ED25. However, because of the breeder flock age in this study, it is possible that the plateau may have commenced earlier. If the plateau occurred several days, or even just hours, earlier the embryos may not have been initially stressed because all eggs were incubated at 37.5°C until ED24, at which point eggs that were in the high incubation temperature treatment group were exposed to 39.0°C. A majority of incubation research to date has been conducted in broilers, and research in the area of incubation temperature effects on turkey embryos is not well represented. Therefore, it is somewhat difficult to determine the appropriate timing of the oxygen consumption plateau as it relates to breeder flock age.

Exposure to a high incubation temperature from ED24 until ED28 resulted in a reduction in body weight at hatch compared to birds exposed to a lower incubation temperature in this study. Elevated temperature and reduced oxygen availability in the incubator during late incubation slow down the process of functional intestinal maturation (Wineland et al., 2006), which could explain the reduction in overall body weight observed at hatch in this experiment. The exposure to hypoxic conditions beginning at ED25 reduces the ability to metabolize lipids (Christensen et al., 2003). Eggs from a 37 week-old breeder flock had a higher eggshell conductance than eggs from a 45 or 53 week-old flock (O'Dea et al., 2004). With this in mind, it

has been suggested that a lower eggshell conductance, such as that found in eggs from older breeder flocks, delays development and reduces intestinal weight and function (Christensen et al., 2003). Elevated temperatures suppress thyroid hormone levels during the plateau stage, and there appeared to be a relationship between reduced thyroid hormone levels and delayed intestinal maturation (Wineland et al., 2006). Unfortunately in this study, there was no effect of incubation temperature on intestine weight or on intestinal morphology; however, the samples obtained to evaluate those parameters were not harvested for analysis until 7 days post-hatch. It is possible that incubation temperature may have impacted those specific parameters initially following hatch and the negative effects did not persist as indicated by the lack of differences in intestinal morphology and intestine weight at d 7 post-hatch. However, this speculation is difficult to determine without appropriately analyzed samples.

Interestingly, birds that were exposed to a low incubation temperature showed a heavier body weight at hatch compared to birds exposed to the high or standard temperatures. A previous study evaluating the effects of suboptimal incubation temperatures in broilers demonstrated that exposure to a low eggshell temperature resulted in reduced embryo weight, hatchability, and chick quality; however, the embryo was exposed to a reduced temperature during the first ten days of incubation (Joseph et al., 2006). Embryonic development is divided into two distinct phases: differentiation and growth. The differentiation period involves the development of embryonic tissues, such that 90% of the organs are present by ED12 (Deeming, 2002). Following this phase, the embryo will increase in size, and tissues will mature during the growth phase to prepare the birds for hatching (Deeming, 2002). Perhaps exposure to suboptimal temperatures during the differentiation phase of incubation has a more deleterious effect on bird quality and hatch weight than does exposure to a low temperature during late

incubation. The low temperature in this study was selected to determine if a reduced temperature during the plateau phase of incubation is advantageous due to the possible reduction in additive environmental condition factors (temperature plus hypoxia). It could be speculated that if the environmental temperature is reduced, then once the bird pips and is breathing on its own, the bird's respiratory rate would be less than if it was exposed to an elevated temperature. With this in mind, a higher concentration of oxygen in the incubator during late incubation has resulted in heavier body weight (Wineland et al., 2006). Although oxygen concentration was not monitored in the current study, it is possible that a lower late incubation temperature is advantageous prior to and during the hatch process by making oxygen more available and allowing for more efficient yolk utilization and thus heavier body weight. It is also possible that a low temperature during late incubation simply minimizes non-optimal temperature effects on the developing embryo, and the beneficial effects exhibited by birds exposed to a low temperature are unrelated to increased oxygen availability.

Birds exposed to an elevated incubation temperature continued to exhibit a lower body weight at 7 days post-hatch compared to birds that were incubated at a standard incubation temperature. Broiler embryos exposed to a high temperature of 40.6°C from ED16 -18 had a reduced relative embryo weight and reduced relative yolk sac weight at ED18, at internal pipping, and at hatch compared to low (34.6°C) and standard (37.6°C) temperatures (Willemsen et al., 2010). Similar results of reduced relative embryo weight were reported when broiler embryos were exposed to constant thermal manipulation (39.5°C) from ED7-16 (Piestun et al., 2009). Additionally, carbohydrate and lipid metabolism were altered in embryos exposed to elevated incubation temperature, as reflected by levels of blood glucose, lactate, liver glycogen, and plasma triglycerides (Willemsen et al., 2010). Results from these studies suggest that

exposure to an incubation temperature that is 3°C higher than optimal temperature during the last several days of incubation may negatively impact embryonic development. It is possible that the observed effects of high incubation temperature on the embryo are retained at least during the first week post-hatch.

Conclusions from this study are two-fold. First, it is difficult to provide definitive explanation for the physiological responses exhibited by the birds in this experiment when there is still much research to be conducted in the area of late incubation conditions on modern turkey embryos. The possible influences of eggshell conductance relative to breeder flock age, the timing of non-optimal conditions during late incubation and the specific onset of the oxygen consumption plateau, and the potential advantage of low temperature exposure and consequential increased oxygen availability all require further research to be fully understood. Second, the data from this study did demonstrate that turkey embryo exposure to elevated temperatures during the oxygen consumption plateau caused a reduction in body weight that was maintained until 7 days post-hatch. Additionally, birds exposed to a low temperature during the plateau period exhibited the heaviest body weight at hatch suggesting that hatchery management may need to be modified during late incubation with regard to reduced hatcher temperature in order to cater to the oxygen and temperature demands of current poultry.

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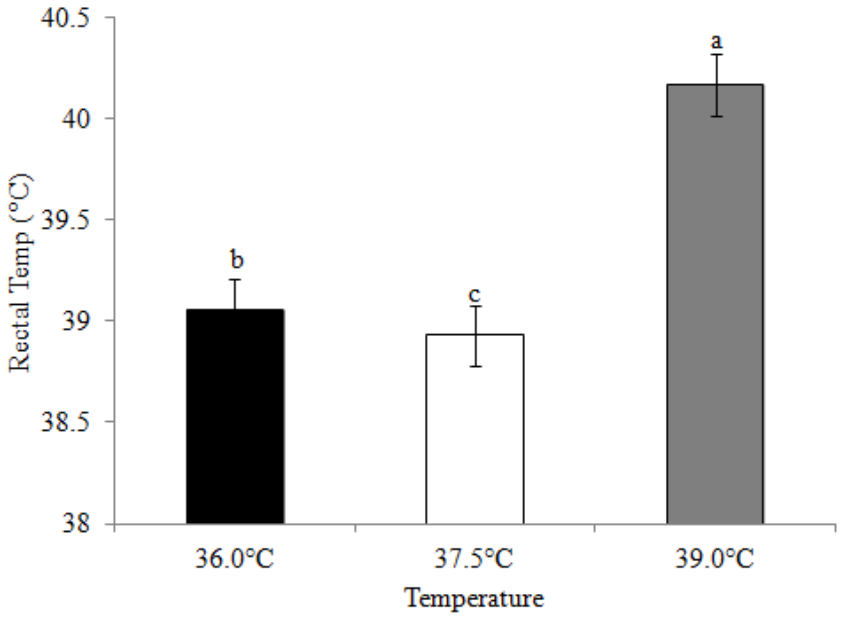

Figure 3.1 Effect of incubation temperature on rectal temperature of Hybrid turkey poults at DOH. All embryos were incubated at 37.5°C from ED0 to ED24, and from ED24 to ED28, treatments were designated as low (36.0°C), high (39.0°C), or standard (37.5°C). Data are presented as LS means ± SEM (n=50/treatment). There was a main effect ($P<0.0001$) of temperature.

[a-c] Data lacking a common superscript differ significantly

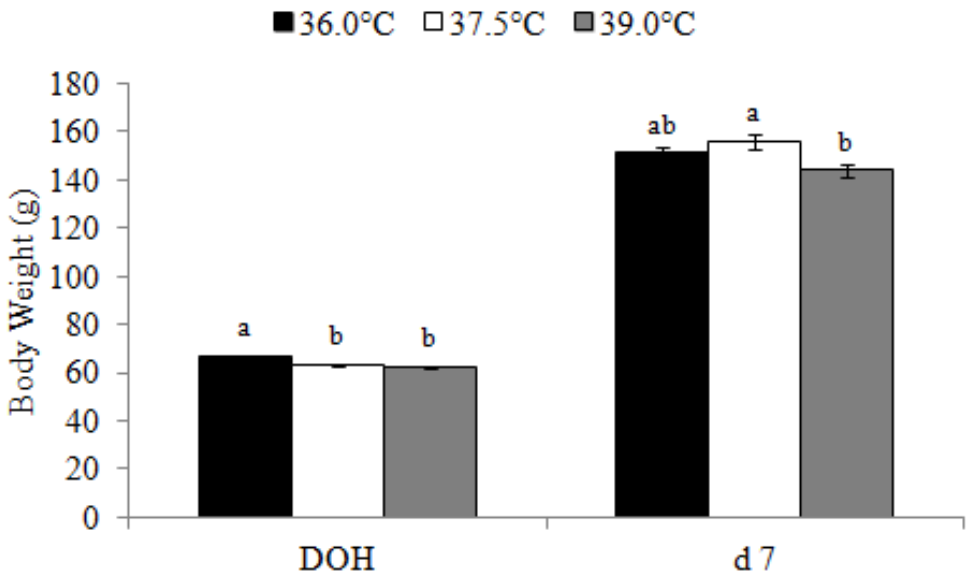

Figure 3.2 Effect of incubation temperature on body weight of Hybrid turkey poults. All embryos were incubated at 37.5°C from ED0 to ED24, and from ED24 to ED28, treatments were designated as low (36.0°C), high (39.0°C), or standard (37.5°C). Data are presented as LS means ± SEM (n=10/treatment). There was a main effect ($P<0.0001$; $P=0.0173$, respectively) of temperature at DOH and d7.

[a,b] Data lacking a common superscript differ significantly

CHAPTER IV

Influence of Incubation Conditions on Turkey Poult Intestinal Development and Susceptibility to Poult Enteritis Complex

ABSTRACT Poult enteritis complex (PEC) is an enteric disease of young turkeys associated with reduced bird performance as a result of negative effects on intestinal development and function, immune system suppression, and increased mortality. Turkey coronavirus (TCV) is a viral agent often associated with PEC. Exposure to stressful environmental conditions that impact organ growth and function and overall performance may increase poult susceptibility to PEC. Temperature and hypoxic stress during embryonic incubation may impact organ growth and development, acquisition of a fully competent immune system, post-hatch performance, and may predispose poults to enteric disease. Commercial Hybrid eggs from a 60 week-old breeder flock were incubated at standard (37.5°C) incubation conditions from embryonic day (ED) 0 to ED24. At ED24, eggs were randomized, and one-third continued incubation at 37.5°C, one-third were incubated at 36.0°C, and one-third were incubated at 39.0°C from ED25 until hatch at ED28. At d 5 (0 days post-infection, dpi) half of the poults from each temperature treatment were administered 0.1 mL of TCV inoculum (3×10^3 EID_{50}/0.1 mL). Body weight (BW) was evaluated at d 0, d 5 (0 dpi), d 11 (6 dpi), and d 19 (14 dpi) and relative bursa, spleen, yolk sac, and gastrointestinal tract weights, and morphology of the duodenum and jejunum were evaluated at d 5(0 dpi) and d 11(6 dpi). Blood was collected at d 5 (0 dpi), d 12 (7 dpi), and d 19 (14 dpi) for heterophil:lymphocyte ratios. Incubation temperature and TCV challenge resulted in a two-way interaction (P=0.0536) on BW at d 11. Incubation temperature impacted BW at d 0 (P<0.0001). Challenge reduced (P=0.0174) BW compared to BW of non-challenged birds at d 19 (14 dpi). Bursa weight was heavier (P=0.0128) in birds incubated at 36.0°C compared to

39.0°C at d 5. Birds incubated at 36.0°C exhibited a heavier (P=0.0066) gastrointestinal tract compared to both 37.5°C and 39.0°C. Challenge with TCV resulted in a reduction (P=0.0323) in heart weight at d 11 compared to birds that were not challenged, and TCV challenge increased (P=0.0001) gastrointestinal tract weight compared to non-challenged birds. Birds incubated at 37.5°C had shorter (P=0.0515) jejunum villi at d 5 (0 dpi) compared to birds incubated at 36.0°C. Birds incubated at 37.5°C had shallower (P=0.0031) jejunum crypts at d 5 compared to those incubated at 36.0°C and 39.0°C. Challenge with TCV resulted in deeper (P<0.0001) crypts in both the duodenum and in the jejunum at d 11. Villus height:crypt depth ratio was reduced (P<0.0001 and P=0.0025, respectively) in the duodenum and jejunum in challenged birds at d 11. Birds that were challenged exhibited a lower (P=0.0022) H:L ratio at 7 dpi compared to birds that were not challenged, while birds at 0 and 14 dpi exhibited no differences in H:L ratio between challenge and non-challenged birds. Results from this study suggest that there may be an advantage to the low temperature exposure during late incubation as reflected by post-hatch BW and intestinal development. Challenge with TCV resulted in reduced BW and altered intestinal morphology; however, incubation conditions did not appear to affect TCV susceptibility. A dual challenge with TCV and another PEC-associated pathogen could induce a more severe post-hatch enteritis.

Keywords: turkey, incubation, temperature, enteritis

INTRODUCTION

Enteric diseases have a negative impact on commercial turkey production by causing a reduction in body weight gain, poor feed conversion, and increased morbidity and mortality, all of which translate to reduced economic return for the producer. Poult enteritis complex (**PEC**) refers to infectious enteric diseases of young turkeys, and PEC-associated pathogens are

characterized by decreased bird performance due to detrimental impacts on intestinal development and function and suppression of the immune system. The specific causative agent(s) of PEC have not been conclusively identified, but the disease typically involves several different infectious agents and their interactions, such as enteropathogenic viruses (coronavirus, rotavirus, astrovirus, adenovirus), bacteria (*Salmonella*, *E. coli*, *Campylobacter*, *Clostridia*), and/or protozoa (*Cryptosporidia*) (Odetallah et al., 2001).

Turkey coronavirus (**TCV**) has been implicated in many cases of PEC and is associated specifically as an inducer of mortality and depressed growth. This virus has been shown to suppress both humoral and cell-mediated immunity through a reduction in B and T lymphocyte populations as a result of compromised immune tissue structure. Destruction of the intestinal epithelium and diminished enterocyte absorptive function lead to improper nutrient utilization, which directly affects bird growth and flock uniformity. The lasting systemic effects of TCV on survivors of PEC may have a tremendous influence on overall bird health and may increase susceptibility to additional opportunistic enteric infections.

Turkey embryos undergo rapid development during incubation as reflected by accelerated post-hatch muscle development and improved feed efficiency due to selection for high-yield turkey strains. The increased growth rate of turkey embryos induces a subsequent increase in metabolic heat production during incubation. Turkey eggs are incubated at the industry standard temperature of 37.5°C; however, air temperature surrounding the eggs at times exceeds 38.5°C (French, 1997). This may result in malpositioned embryos and ruptured yolk sacs, as well as increased mortality due to overheating, especially during the 3rd and 4th weeks of incubation. During the last several days of incubation (embryonic day (**ED**) 24 to ED 28), the embryo begins to increase its consumption of oxygen, which creates a hypoxic environment that may delay

intestinal maturation due to improper energy utilization (Christensen et al., 2004). The challenge associated with this plateau phase of incubation is that it occurs during the time of peak embryonic intestinal maturation (Rahn, 1981), and not only are the embryos faced with the hypoxic conditions associated with the plateau phase, they are also simultaneously exposed to elevated temperatures due to the metabolic heat that they are producing as well as a lack of adequate ventilation between the eggs. Collectively, the combination of these stressors during the last few days of critical embryonic development may have a negative impact on organ and tissue development and may make the newly hatched poult more susceptible to additional post-hatch stress.

There is evidence supporting the influence of non-optimal incubation conditions on bird performance, but much of this research has been conducted in broilers. It is possible that environmental conditions, such as those experienced during incubation, may increase susceptibility of turkey poults to enteric infection by effects on tissue and organ growth potential, as there appears to be a relationship between maturation of the intestine and predisposition to PEC infection. Work in our laboratory has shown that the immune response of neonatal broiler chicks exposed to non-optimal incubation conditions is modified as reflected by alterations in immune organ development and peripheral blood cell populations (Sottosanti, unpublished data). However, the response to incubation conditions and their influence on susceptibility to pathogens encountered during the first several weeks of life have not been investigated in turkey poults.

MATERIALS AND METHODS

Animal Welfare

This experiment was approved and conducted under the guidelines set forth by the Institutional Animal Care and Use Committee at Virginia Tech.

Incubation Temperature

Commercial Hybrid turkey eggs (n=500) were obtained from a 60-week-old breeder flock. Eggs were incubated in NOM 45/90/120 incubators[6] at the hatchery facility of the Virginia Tech Turkey Research Center. To simulate a commercial incubation environment, temperatures were set at the industry standard temperature (37.5°C) from ED0 to ED24 with all eggs contained in one incubator. From ED24 to ED28, eggs were incubated in one of three incubators each set at a different temperature during the plateau of oxygen consumption. One-third of the eggs were maintained at 37.5°C (n=166), one-third of the eggs were incubated at a low temperature relative to 37.5°C (36.0°C; n=167), and one-third of the eggs were incubated a high temperature relative to 37.5°C (39.0°C; n=167). These temperature combinations yielded three total incubation treatments: eggs incubated at 37.5°C from ED0 to ED24 and 37.5°C from ED24 to ED28, eggs incubated at 37.5°C from ED0 to ED24 and 36.0°C from ED24 to ED28, and eggs incubated at 37.5°C from ED0 to ED24 and 39.0°C from ED24 to ED28. Incubation temperature was regulated by thermistors connected to microprocessors with a temperature sensitivity of ± 0.05°C. Humidity was controlled with a comparable system by humidity sensors. Two temperature data loggers were placed in each incubator to monitor temperature. Incubation temperature and humidity were evaluated and logged daily.

Turkey Coronavirus

Turkey coronavirus (**TCV**: NC95) was isolated from infected turkeys as previously described (Guy et al., 1997), and TCV inoculum was prepared according to the methods of Guy et al. (2000). Inoculum was stored at -80°C until use. At d 5 post-hatch, half of the poults from

[6] NatureForm, Inc., Jacksonville, FL

each incubation treatment (n=108 total) were challenged with TCV and half were not challenged. Birds were administered 0.1 mL of inoculum ($3x10^3$ EID_{50}/0.1 mL) by oral gavage.

Rearing and Diets

Poults were randomized within treatment (n=36 poults/treatment) and assigned to battery brooder pens (n=4 replicate pens/treatment with 9 poults/pen). The birds were housed in an environmentally controlled room under a lighting program recommended for Hybrid turkeys. Birds had ad libitum access to water and a corn-soybean based diet formulated to meet or exceed Hybrid Converter nutritional requirements. A pre-starter diet was administered from DOH until the termination of the study at d 19.

Body Weight and Organ Development

All poults were weighed at DOH prior to placement in pens. At d 5 (0 days post-infection, **dpi**) and d 11 (6 dpi), poults were randomly selected for evaluation of bursa, spleen, yolk sac, gastrointestinal tract (gizzard to ileocecal junction), and heart weight (n=8 poults/treatment). Birds were weighed, euthanized by cervical dislocation, and organs were collected. Organ weights are presented as a percentage (%) of body weight (**BW**).

Intestinal Morphology

Poults that were sampled for organ weights and whole blood were also used for evaluation of intestinal morphology at d 5 (0 dpi) and d 11 (6 dpi) (n=8 poults/treatment). Tissue samples (3cm) were collected from the duodenum (mid-section of the ascendant loop) and jejunum (mid-point from the pancreatic duct to Meckel's diverticulum). Intestinal segments were flushed with cold PBS and fixed in 10% neutral buffered formalin. Each segment was cut into 5 (1cm) sections and placed in a tissue cassette. Tissues were processed, embedded in paraffin, cut into 5μm sections, and mounted onto slides. Slides were stained using routine

procedures for hemotoxylin and eosin (**H&E**) and were evaluated using a light microscope (Luna, 1968). Measurements were made using SigmaScan Pro 5 software[7]. Three of the five total intestinal tissue sections were evaluated per slide for villus height (from villus tip to opening of crypt) and crypt depth (from the opening of the crypt to the base). Villus height-to-crypt depth ratios were also calculated. Four villi and 4 crypts were evaluated for each of the 3 intestinal tissue sections. The average villus height, crypt depth, and villus height-to-crypt depth ratio per slide were analyzed (n=12 measurements/bird, 8 birds/treatment).

Heterophil/Lymphocyte Ratios

Whole blood was collected (n=8 poults/treatment) from the same birds from which organs were collected at d 5 (0dpi) and at d 12 (7dpi) and d 19 (14dpi). Blood was collected from the jugular or brachial vein at d 5 (0 dpi), d 12 (7 dpi), and d 19 (14 dpi) for evaluation of heterophil to lymphocyte (**H:L**) ratios. Blood samples were collected in 1.8 mL tubes containing 0.105 M buffered sodium citrate. A blood smear was made for each blood collection tube using a Morf slide spinner[8]. Smears were stained with a modified Wright-Giemsa stain using the protocol provided by the manufacturer[9]. Smears were evaluated by counting a total of 60 lymphocytes and heterophils per slide under 100X oil immersion. Heterophil:lymphocyte ratios were calculated by dividing the total number of heterophils by the total number of lymphocytes for each blood smear.

TCV ELISA

Antibody titers to TCV were evaluated at d 5 (0 dpi), d 12 (7 dpi), and d 19 (14 dpi). Whole blood was collected from the same birds from which organs were collected plus birds

[7] Olympus America Inc., Melville, NY
[8] Salem Specialties, Inc., Salem, VA
[9] Sigma-Aldrich, St. Louis, MO

representative from an additional replicate/treatment (n=12 birds/treatment). Approximately 1 mL of blood was collected from the jugular or brachial vein, and the blood sample was placed in a polypropylene culture tube and sealed. Samples remained at room temperature until centrifugation. Samples were centrifuged at 1000 RPM for 10 minutes, and serum was removed and aliquoted to 1.5mL microcentrifuge tubes. Serum was stored at -20°C until further analysis. A competitive enzyme-linked immunosorbent assay (ELISA) was conducted to detect presence of TCV antibodies as previously described (Guy et al., 2002).

Statistical Analysis

Data were evaluated as a completely randomized experimental design arranged as a 3 x 2 factorial (3 incubation temperature treatments and 2 challenge treatments). Analysis of variance was performed using the GLM procedure of SAS. Values are reported as least squares (LS) means ± SEM. Data reported as a percentage were transformed prior to analysis using arc-sine (square root of percent). Pen served as the experimental unit for body weight. Bird served as the experimental unit for histology, heterophil:lymphocyte ratios, and organ weights.

RESULTS

There were no differences in % hatch between incubation temperature treatments (data not shown).

Body Weight and Organ Development

Incubation temperature and TCV challenge resulted in a two-way interaction (P=0.0536) on body weight at 6 dpi (Table 4.1). Birds incubated at 37.5°C or 36.0°C showed no difference in body weight regardless of challenge. However, birds that were incubated at 39.0°C had a reduced body weight when challenged compared to body weight of non-challenged birds.

Incubation temperature impacted body weight at d 0 (Table 4.1: P<0.0001). Birds incubated at 36.0°C exhibited a higher body weight compared to birds incubated at 37.5°C and 39.0°C, and birds incubated at 37.5°C showed a higher body weight than birds incubated at 39.0°C. Challenge with TCV resulted in a reduction (P=0.0174) in body weight compared to body weight of non-challenged birds at d 19(14 dpi) (Table 4.1).

Bursa weight was influenced by incubation temperature at d 5, with birds incubated at 36.0°C possessing on average heavier (P=0.0128) bursae compared to birds incubated at 39.0°C (Table 4.2). Birds incubated at 37.5°C had a comparable bursa weight to those incubated at 36.0°C or 39.0°C. Birds incubated at 36.0°C exhibited a heavier (P=0.0066) gastrointestinal tract weight compared to both 37.5°C and 39.0°C, in which gastrointestinal tract weights were comparable to each other (Table 4.2). Challenge with TCV resulted in a reduction (P=0.0323) in heart weight at 6 dpi compared to birds that were not challenged (Table 4.2), and TCV challenge caused an increase (P=0.0001) in gastrointestinal tract weight compared to non-challenged birds (Table 4.2). No effect of incubation temperature was observed on TCV susceptibility of poults exposed at 5 d of age on body weight and organ development.

Intestinal Morphology

Incubation temperature had a main effect (P=0.0515) on intestinal villus height in the jejunum at d 5(0 dpi) (Table 4.3). Birds incubated at 37.5°C had shorter jejunum villi compared to birds incubated at 36.0°C, which showed comparable villi height to those incubated at 39.0°C. Crypt depth in the jejunum was impacted by incubation temperature at d 5 (Table 4.3). Birds incubated at 37.5°C had shallower (P=0.0031) crypts compared to those incubated at 36.0°C and 39.0°C. Challenge with TCV resulted in deeper (P<0.0001) crypts in both the duodenum and in the jejunum (Table 4.3) at 6 dpi compared to the non-challenged group. Villus height:crypt

depth ratio was significantly reduced (P<0.0001 and P=0.0025, respectively) in the duodenum and jejunum as a result of TCV challenge at 6 dpi (Table 4.3). No effect of incubation temperature was observed on TCV susceptibility of poults exposed at 5 d of age on intestinal morphology.

Heterophil:Lymphocyte Ratio

There was a main effect (P=0.0022) of TCV challenge on heterophil:lymphocyte ratio at 7 dpi (Table 4.4). Birds that were challenged exhibited a lower H:L ratio at 7 dpi compared to birds that were not challenged, while birds at 0 and 14 dpi exhibited no differences in H:L ratio between challenge and non-challenged birds. No effect of incubation temperature was observed on TCV susceptibility of poults exposed at 5 d of age on H:L ratios.

TCV ELISA

There was no presence of TCV specific antibodies at 0 dpi or 7 dpi (Table 4.5). At 14 dpi, TCV positive sera was detected in one bird from those incubated at 37.5°C and non-challenged, 39.0°C and challenged, and 39.0°C and non-challenged. ELISA results suggest that incubation temperature does not increase susceptibility to TCV challenge.

DISCUSSION

Exposure of commercial turkey embryos to non-optimal temperatures during the plateau stage of incubation had an impact on intestinal development in the present study. There is a large increase in number of embryonic enterocytes during the last few days of incubation, and as a result, villi height subsequently increases, as does absorptive surface area (Uni et al., 2003). The development of the intestine is crucial to general bird health and performance because of its involvement in nutrient absorption and utilization (Kawalilak et al., 2010). In this study, the jejunum was affected by incubation temperature. Villus volume changes minimally during the

first 2 d post-hatch; however, after this time point, it increases rapidly and is complete by d 7 in the duodenum and by d 14 in the jejunum (Uni et al., 1998). It is possible that the jejunum may have been more sensitive to the effects of incubation temperature for a greater period of time post-hatch due to its dynamics of development compared to the duodenum. The deeper crypts in birds exposed to the 36.0°C or 39.0°C temperatures may suggest compensatory, or possibly even delayed, cellular maturation as a result of exposure to non-optimal temperatures during the period of incubation in which intestinal maturation occurs.

Infection with poult enteritis resulted in increased duodenum crypt depth 5 dpi as a result of crypt hyperplasia (Gomaa et al., 2009). A similar response in the intestinal crypts was observed in this study in both the duodenum and jejunum. Functional integrity of the intestine is compromised during TCV infection due to epithelial cell sloughing from the intestinal villi, and cellular proliferation, and thus crypt depth, are increased to replace the sloughed enterocytes. Villus height to crypt depth ratio was reduced in TCV challenged birds in the duodenum and jejunum. In general, this reduced ratio is reflective of a shorter villus and deeper crypt common with enteric infection. A typical clinical sign of enteritis in poults is diarrhea, and the intestinal response observed in this study as a result of TCV infection is a likely contributor. Crypt hyperplasia may result in increased secretion, causing crypt secretion to exceed villous absorption, contributing to both diarrhea and malabsorption (Moon, 1978).

Birds that were exposed to the high temperature during late incubation had a reduction in BW at hatch compared to birds incubated at the standard or low temperature. Reduced BW following exposure to high incubation temperature conditions have been previously reported (Leksrisompong et al., 2007). Exposure to temperatures lower than industry standard during late incubation may be advantageous. At hatch, birds incubated at 36.0°C had higher body weights

compared to birds incubated at 37.5°C or 39.0°C. A study by Geers et al. (1983) reported that reduced incubation temperature resulted in compensatory growth due to increased metabolic heat production, which may explain why the low temperature exposure in this study elicited a similar response. The interaction between incubation temperature and TCV challenge resulted in comparable body weight between challenge and non-challenged birds exposed to 36.0°C at d 11, and a similar response was seen between challenge and non-challenged birds incubated at 37.5°C. However, birds incubated at 39.0°C had a reduction in body weight in birds that were challenged compared to non-challenged. Previous studies have indicated that TCV challenge causes a decrease in body weight following infection (Doerfler et al., 1998; Gomaa et al., 2009). Exposure to the higher temperature seemed to increase the challenge impact on the birds, as reflected by reduced body weight when challenged; however, exposure to the lower temperature did not have any effect on body weight associated with challenge. Perhaps the combination of a reduced temperature in an oxygen-deprived late incubation environment is more beneficial in preparing the bird for post-hatch life and potential post-hatch challenges to which it may be exposed, as opposed to a high temperature plus reduced oxygen availability which may have additive stress effects upon the bird.

Although not statistically significant, there was a biological effect of yolk sac weight that corresponded to the differences in small intestine weight, with birds incubated at 36.0°C exhibiting numerically lighter yolk sacs compared to both 37.5°C and 39.0°C. Broiler embryos exposed to 39.5°C for 6 h daily from ED10 to ED18 showed reduced yolk sac utilization (heavier yolk sac) compared to embryos incubated at standard conditions (Yalcin et al., 2008; Aksit et al., 2010). Decreased yolk utilization as a result of high incubation temperature is also in agreement with results reported by Wineland et al. (2000a,b). Exposure to high incubation

temperature has previously resulted in reduced liver weight (Yalcin et al., 2008), which could explain the reduced utilization of yolk sac contents when exposed to non-optimal conditions. Additionally, the combination of high incubation temperature with a reduction in available oxygen from ED7 to ED19 resulted in large residual yolk sacs compared to standard incubation conditions (Molenaar et al., 2010). Embryos incubated under such conditions tend to have poor navel condition as a result of delayed closure of the body cavity around the yolk sac during late incubation (Piestun et al., 2008), thus possibly resulting in poor yolk absorption. In the current study, relative weight of the small intestine was heaviest in birds incubated at 36.0°C compared to 37.5°C and 39.0°C at d 5. There are substances contained in the yolk that contribute to the development of the small intestine, which could explain the heavier small intestine weight along with the corresponding lighter yolk sac in the 36.0°C treatment group in this study. Ablation of the yolk sac of chicks at hatch resulted in a decrease in villus volume and crypt depth throughout the small intestine (Uni et al., 1998). Following yolk sac ablation, intubation of chicks with yolk contents resulted in increased body weight and increased pancreatic and liver development (Nitsan et al., 1995). Results from these previous studies indirectly demonstrate the involvement of the yolk sac in intestinal maturation and growth, which could explain the differences in intestinal weight and corresponding yolk sac weights at d 5 in the current study.

The hypothalamic-pituitary-adrenal (HPA) axis is activated as a result of exposure to stress or disruption of homeostasis (McEwen, 2000). Exposure to post-hatch heat stress resulted in altered bird performance and intestinal integrity, and these responses were attributed to modifications in HPA function (Quinteiro-Filho et al., 2010). In the present study, the high incubation temperature caused a reduction in relative bursa weight compared to the low incubation temperature. Post-hatch heat stress resulted in reduced relative bursa, spleen, and

thymus weights (Quinteiro-Filho et al., 2010), and a glucocorticoid-dependent mechanism induced involution of lymphoid organs in broilers (Puvadolpirod and Thaxton, 2000). It is suggested that heat stress activates the HPA axis, increases serum corticosterone levels, and subsequently decreases relative lymphoid organ weight (Quinteiro-Filho et al., 2010). Exposure to environmental stressors may impair immune function, specifically cell-mediated immunity (Zulkifli et al., 1994) as a result of increased inflammatory cytokines due to stress (Ogle et al., 1997), which in turn stimulates the production of corticotropin releasing factor. Exposure to temperatures of 38.8°C beginning at ED10 resulted in decreased relative bursa weight, and the developing lymphoid follicles were smaller and follicle associated epithelial cells were depressed (Oznurlu et al., 2010). High incubation temperatures may induce immunosuppression as reflected by decreased relative lymphoid organ weights.

There were no apparent effects of incubation temperature resulting in increased susceptibility to TCV infection in this study. Results from this study suggest that there may be benefits to the lower temperature exposure during late incubation as reflected by post-hatch body weight and intestinal development. Exposure to a lower incubation temperature may be beneficial to the embryo during the last several days of incubation, allowing for improved poult quality and potentially reduced susceptibility to post-hatch challenges. Challenge with TCV resulted in reduced body weight and altered intestinal morphology as expected; however, this experiment employed only a mild viral challenge and lacked a bacterial challenge. A combined viral and bacterial challenge could likely have a greater impact on intestinal integrity and function, as it is common for interactions between PEC-associated infectious agents (Odetallah et al., 2001), thus causing more severe enteritis.

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Treatment		DOH	d 5(0dpi)	d 11(6dpi)	d 19(14dpi)
36.0°C		64.13[a]	129.22	190.99	313.90
37.5°C		62.21[b]	126.84	180.17	321.74
39.0°C		59.07[c]	121.13	187.76	331.07
Pooled SEM		0.53	2.90	8.60	12.08
Challenge (C)		---	---	176.93	306.18[b]
Non-Challenge (NC)		---	---	195.68	338.30[a]
Pooled SEM		---	---	6.90	9.90
36.0°C	C	---	---	189.05[ab]	281.33
36.0°C	NC	---	---	192.93[ab]	346.48
37.5°C	C	---	---	180.81[ab]	318.33
37.5°C	NC	---	---	179.53[ab]	325.14
39.0°C	C	---	---	160.93[b]	318.88
39.0°C	NC	---	---	214.6[a]	343.27
Pooled SEM		---	---	12.11	17.04
P-value					
Temp		<0.0001	0.1410	0.6590	0.6070
Challenge		---	---	0.0650	0.0174
Temp*Challenge		---	---	0.0536	0.1995

Table 4.1 Effect of incubation temperature and TCV challenge on body weight (g) of Hybrid turkey poults. All embryos were incubated at 37.5°C from ED0 to ED24, and from ED24 to ED28, treatments were designated as low (36.0°C), high (39.0°C), or standard (37.5°C). Poults were inoculated with TCV (3×10^3 EID_{50}/0.1mL) at d 5. Data are presented as LS means ± SEM (n=4 reps/treatment).

[a-c] Data within a column lacking a common superscript differ significantly

Treatment		d 5(0dpi)					d 11(6dpi)			
		Bursa	Spleen	Yolk	Heart	GIT	Bursa	Spleen	Heart	GIT
36.0°C		0.14[a]	0.05	0.17	0.91	14.41[a]	0.14	0.09	0.75	13.83
37.5°C		0.13[ab]	0.06	0.88	0.93	13.41[b]	0.17	0.09	0.79	12.64
39.0°C		0.11[b]	0.05	0.85	0.96	13.52[b]	0.14	0.09	0.82	11.98
Pooled SEM		0.001	0.001	0.018	0.002	0.005	0.002	0.002	0.003	0.017
Challenge (C)		---	---	---	---	---	0.15	0.09	0.74[b]	14.71[a]
Non-Challenge (NC)		---	---	---	---	---	0.16	0.09	0.84[a]	10.35[b]
Pooled SEM		---	---	---	---	---	0.002	0.002	0.003	0.017
36.0°C	C	---	---	---	---	---	0.12	0.10	0.72	15.87
36.0°C	NC	---	---	---	---	---	0.17	0.07	0.78	9.75
37.5°C	C	---	---	---	---	---	0.18	0.07	0.73	15.00
37.5°C	NC	---	---	---	---	---	0.16	0.10	0.86	10.28
39.0°C	C	---	---	---	---	---	0.14	0.09	0.76	13.26
39.0°C	NC	---	---	---	---	---	0.14	0.09	0.89	10.71
Pooled SEM		---	---	---	---	---	0.002	0.002	0.003	0.017
P-value										
Temp		0.013	0.139	0.077	0.567	0.007	0.426	0.984	0.460	0.330
Challenge		---	---	---	---	---	0.494	0.906	0.032	0.0001
Temp*Challenge		---	---	---	---	---	0.390	0.220	0.793	0.382

Table 4.2 Effect of incubation temperature and TCV challenge on relative organ weight (% BW) of Hybrid turkey poults. All embryos were incubated at 37.5°C from ED0 to ED24, and from ED24 to ED28, treatments were designated as low (36.0°C), high (39.0°C), or standard (37.5°C). Poults were inoculated with TCV (3×10^3 EID_{50}/0.1mL) at d 5. Data are presented as LS means ± SEM (n=8/treatment).

[a,b] Data within a column lacking a common superscript differ significantly

		d 5(0dpi)						d 11(6dpi)					
Treatment		Duo VH[1]	Duo CD[2]	Duo VCR[3]	Jej VH	Jej CD	Jej VCR	Duo VH	Duo CD	Duo VCR	Jej VH	Jej CD	Jej VCR
36.0°C		1.440	0.088	16.550	0.65[a]	0.070[a]	9.200	1.310	0.137	10.558	0.712	0.127	5.970
37.5°C		1.330	0.100	14.830	0.546[b]	0.061[b]	8.890	1.220	0.146	8.949	0.697	0.130	5.720
39.0°C		1.390	0.092	15.100	0.581[ab]	0.068[a]	8.520	1.310	0.152	9.251	0.711	0.141	5.200
Pooled SEM		0.038	0.009	0.570	0.029	0.002	0.310	0.059	0.007	0.664	0.041	0.006	0.486
Challenge (C)		---	---	---	---	---	---	1.261	0.170[a]	7.63[b]	0.686	0.148[a]	4.729[b]
Non-Challenge (NC)		---	---	---	---	---	---	1.301	0.119[b]	11.55[a]	0.728	0.117[b]	6.532[a]
Pooled SEM		---	---	---	---	---	---	0.049	0.006	0.542	0.033	0.005	0.397
36.0°C	C	---	---	---	---	---	---	1.229	0.167	7.520	0.611	0.144	4.276
36.0°C	NC	---	---	---	---	---	---	1.390	0.106	13.596	0.813	0.111	7.669
37.5°C	C	---	---	---	---	---	---	1.314	0.167	8.163	0.717	0.150	5.006
37.5°C	NC	---	---	---	---	---	---	1.132	0.125	9.735	0.677	0.109	6.425
39.0°C	C	---	---	---	---	---	---	1.239	0.177	7.194	0.729	0.152	4.905
39.0°C	NC	---	---	---	---	---	---	1.382	0.128	11.309	0.693	0.130	5.502
Pooled SEM		---	---	---	---	---	---	0.084	0.010	0.939	0.057	0.008	0.687
P-value													
Temp		0.1466	0.5923	0.0876	0.0515	0.0031	0.3138	0.4955	0.3213	0.2023	0.9607	0.2241	0.5277
Challenge		---	---	---	---	---	---	0.5559	<0.0001	<0.0001	0.3735	<0.0001	0.0025
Temp*Challenge		---	---	---	---	---	---	0.0837	0.6404	0.0666	0.0643	0.5148	0.1249

[1] VH = villus height (mm)

[2] CD = crypt depth (mm)

[3] VCR = villus height:crypt depth ratio

Table 4.3 Effect of incubation temperature and TCV challenge on intestinal morphology of Hybrid turkey poults. All embryos were incubated at 37.5°C from ED0 to ED24, and from ED24 to ED28, treatments were designated as low (36.0°C), high (39.0°C), or standard (37.5°C). Poults were inoculated with TCV (3×10^3 EID_{50}/0.1mL) at d 5. Data are presented as LS means ± SEM (n=8/treatment).

[a,b] Data within a column lacking a common superscript differ significantly

Treatment		d 5(0dpi)	d 12(7dpi)	d 19(14dpi)
36.0°C		0.0288	0.0164	0.0306
37.5°C		0.0269	0.0165	0.0354
39.0°C		0.031	0.0169	0.0249
Pooled SEM		0.0022	0.0023	0.0038
Challenge (C)		---	0.0123[b]	0.0316
Non-Challenge (NC)		---	0.0209[a]	0.0289
Pooled SEM		---	0.0019	0.0032
36.0°C	C	---	0.0161	0.0316
36.0°C	NC	---	0.0168	0.0296
37.5°C	C	---	0.0091	0.0379
37.5°C	NC	---	0.0239	0.0329
39.0°C	C	---	0.0117	0.0254
39.0°C	NC	---	0.0222	0.0244
Pooled SEM		---	0.0032	0.0055
P-value				
Temp		0.4887	0.9874	0.177
Challenge		---	0.0022	0.558
Temp*Challenge		---	0.0935	0.9313

Table 4.4 Effect of incubation temperature and TCV challenge on heterophil:lymphocyte ratio of Hybrid turkey poults. All embryos were incubated at 37.5°C from ED0 to ED24, and from ED24 to ED28, treatments were designated as low (36.0°C), high (39.0°C), or standard (37.5°C). Poults were inoculated with TCV (3×10^3 EID_{50}/0.1mL) at d 5. Data are presented as LS means ± SEM (n=8/treatment).

[a,b] Data within a column lacking a common superscript differ significantly

	Number Positive/Number Tested					
Days post-exposure	37.5°C - C[1]	37.5°C - NC[2]	36.0°C - C	36.0°C - NC	39.0°C - C	39.0°C - NC
0	0/12	0/12	0/12	0/12	0/12	0/12
7	0/12	0/12	0/12	0/12	0/12	0/12
14	0/12	1/11	0/12	0/12	1/12	1/11

[1] C = Challenge
[2] NC = Non-challenge

Table 4.5 Detection of TCV specific antibodies in sera of experimentally infected turkeys by cELISA. Poults were inoculated with TCV (3×10^3 EID_{50}/0.1mL) at d 5, and serum was collected at 0 dpi, 7 dpi, and 14 dpi.

CHAPTER V

Influence of Incubation Conditions on Turkey Poult Intestinal Development Following Dual Challenge with Turkey Coronavirus and Enteropathogenic *E. coli*

ABSTRACT Poult enteritis complex (PEC) refers to infectious agents inducing enteric disease in young turkeys, and is associated with reduced bird performance as a result of negative effects on intestinal development and function, immune system suppression, and increased mortality. Turkey coronavirus (TCV) and enteropathogenic *E. coli* are often associated with PEC. Exposure to stressful environmental conditions that impact organ growth and function and overall performance may increase poult susceptibility to PEC. Temperature and hypoxic conditions during embryonic incubation may impact organ growth and development, acquisition of a fully competent immune system, and post-hatch performance and may predispose poults to enteric disease susceptibility. Commercial Hybrid eggs from a 52 week-old breeder flock were incubated at standard (37.5°C) incubation conditions from embryonic day (ED) 0 to ED25. At ED25, eggs were randomized, and half continued incubation at 37.5°C and half were incubated at 36.0°C from ED25 until hatch at ED28. At d 5 (0 days post-infection, dpi) half of the poults were administered 0.1 mL of TCV inoculum (4×10^3 EID_{50}/0.1 mL) and 0.1 mL of *E. coli* (2.4×10^8 CFU/mL) by oral gavage. Body weight (BW) was evaluated at d 0, d 5 (0 dpi), d 11 (6 dpi), and d 17 (12 dpi), and relative bursa, spleen, yolk sac, and gastrointestinal tract weights, and morphology of the jejunum and ileum were evaluated at d 5 (0 dpi) and d 11 (6 dpi). Blood was collected at d 5 (0 dpi), d 12 (7 dpi), and d 17 (12 dpi) for heterophil:lymphocyte ratios. Mortality, feed intake, and feed conversion ratio were evaluated from d 0-d 5, d 5-d 11, d 11-d 17, and d 5-d 17. Birds incubated at 36.0°C exhibited a heavier BW compared to birds incubated at 37.5°C at d 0 (P=0.001), d 5 (P=0.0079), and d 11(P=0.012). Challenged birds had a lower

BW compared to non-challenged birds at d 11 and d 17 (P<0.0001). Non-challenged birds exhibited a higher BWG compared to challenged birds from d 5 to d 11, d 11 to d 17, and d 5 to d 17 (P<0.0001; P=0.0006; P<0.0001, respectively). Birds that were incubated at 36.0°C had a higher feed intake (FI) than birds incubated at 37.5°C from d 0 to d 5, d 5 to d 11, and cumulatively from d 0 to d 17 (P=0.0004; P=0.0227; P=0.0167, respectively). FI was reduced in challenged birds compared to non-challenged birds from d 5 to d 11 (6 dpi), d 11 (6 dpi) to d 17 (12 dpi), and d 5 (0 dpi) to d 17 (12 dpi) (P=0.0162; P<0.0001; P<0.0001, respectively). Challenged birds exhibited a higher (P<0.0001) percent mortality compared to non-challenged birds from d 11(6 dpi) to d 17(12 dpi), and d 5(0 dpi) to d 17(12 dpi). Relative bursa weight was increased (P=0.015) at d 5 (0 dpi) in the 36.0°C treatment compared to 37.5°C. Incubation temperature and challenge resulted in a two-way interaction (P=0.0555) at d 12 (7 dpi) on heterophil:lymphocyte ratio. At d 5 (0 dpi), jejunum villus height was higher (P=0.0437) in birds incubated at 36.0°C than 37.5°C. Challenged birds showed a reduction in jejunum villus height (P=0.0007) and villus height:crypt depth ratio (P=0.0123) at d 11 (6 dpi) compared to non-challenged birds. There was no effect on crypt depth in the jejunum at d 11 (6 dpi) due to incubation temperature or challenge. There was a two-way interaction of incubation temperature and challenge on ileum villus height (P=0.0353) and ileum crypt depth (P=0.0084). Ileum villus height:crypt depth ratio was impacted by the interaction (P=0.0012) of incubation temperature and challenge at d 11 (6 dpi). Incubation conditions did not appear to affect susceptibility to TCV/*E.coli* infection. Results from this study indicate that incubation temperature or dual challenge with TCV and *E. coli* may independently impact bird performance, intestinal development, organ development, and peripheral blood cell dynamics.

Keywords: turkey, incubation, temperature, enteritis

INTRODUCTION

Poult enteritis complex (PEC) is an enteric disease of young turkeys characterized by decreased performance due to detrimental impacts on intestinal development and function (Doerfler et al., 1998; Odetallah et al., 2001; Barnes, 2002; Gomaa et al., 2009), suppression of the immune system (Qureshi et al., 1997; Barnes, 2002; Jindal et al., 2009), and high incidence of mortality (Barnes and Guy, 1997). The specific causative agent(s) is unknown; however, the disease typically involves the interaction of several infectious agents, such as enteropathogenic viruses (coronavirus, rotavirus, astrovirus, adenovirus), bacteria (*Salmonella, E. coli, Campylobacter, Clostridia*), and/or protozoa (*Cryptosporidia*) (Odetallah et al., 2001).

Turkey coronavirus (TCV) is one of the viruses associated with PEC. This virus has been shown to repress both humoral and cell-mediated immunity through a reduction in B and T lymphocyte populations as a result of compromised immune tissue structure. Destruction of the intestinal epithelium and diminished enterocyte absorptive function lead to improper nutrient utilization, which directly affects bird growth and flock uniformity. The lasting systemic effects of turkey coronavirus on PEC survivors may have a tremendous influence on overall bird health and may increase propensity for additional opportunistic enteric infection.

Enteropathogenic strains of *E. coli* have been implicated in PEC infections and are characterized by adherence of the bacteria to the intestinal epithelium, resulting in enteric disease without the expression of diarrhea-inducing heat labile and heat stable toxins, such as are produced by enterotoxigenic *E. coli* strains (Guy et al., 2000). Enteropathogenic *E. coli* strains produce intestinal lesions referred to as “attaching and effacing” lesions as described by Moon et al. (1983). Attaching and effacing lesions typically cause loss of microvilli and enterocytes and degeneration or loss of cytoplasm in intestinal epithelial cells that remain, thus resulting in hindered digestion and absorption and consequential malabsorptive diarrhea (Moon et al., 1983).

Turkeys infected with only *E. coli* strain R98/5 failed to develop clinical disease, and TCV-only infected birds developed mild disease and growth depression; however, turkeys infected with both TCV and *E. coli* R98/5 developed severe enteritis, high mortality, and growth depression (Guy et al., 2000). It is believed that TCV infection promotes colonization of the intestine by *E. coli*, and the two pathogens synergistically have a detrimental effect on bird performance, mortality, intestinal structure and function, and cause lymphoid tissue atrophy (Guy et al., 2000).

Oxygen requirements of turkey embryos during the latter stage of incubation undergo modifications around ED25-26, at which time embryos utilize more oxygen and dispose of more carbon dioxide than the eggshell is capable of supporting (Dietz et al., 1998). This creates a subsequent plateau in oxygen consumption (Rahn, 1981). The oxygen plateau causes the embryo to continue growth and metabolic processes under hypoxic conditions, and consequently, delays intestinal maturation due to improper supply of energy under these conditions (Christensen et al., 2004). Intestinal maturation occurs during this plateau stage (Rahn, 1981), suggesting that if the embryo endures stress at this time, development and resulting tissue function may be compromised. Additionally, since turkeys are selected to undergo rapid development as early as during embryonic development, increased metabolic heat production in the incubator may impose heat stress upon the developing embryo. Turkey eggs are incubated at the industry standard temperature, 37.5°C; however, air temperature surrounding the eggs at times exceeds 38.5°C (French, 1997). Collectively, the combination of these stressors during the last few days of critical embryonic development may have a negative impact on organ and tissue development and may make the newly hatched poult more susceptible to additional post-hatch stressors.

There is evidence supporting the influence of non-optimal incubation conditions on bird performance and indicators of bird development, but much of this research has been conducted in broilers. It is possible that environmental conditions such as those experienced during late incubation may increase susceptibility of turkey poults to enteric infection due to their effects on tissue and organ growth, as there appears to be a relationship between maturation of the intestine and predisposition to PEC infection.

MATERIALS AND METHODS

Animal Welfare

This experiment was approved and conducted under the guidelines set forth by the Institutional Animal Care and Use Committee at Virginia Tech.

Incubation Temperature

Commercial Hybrid turkey eggs (n=700) were obtained from a 52-week-old breeder flock. Eggs were incubated in NOM 45/90/120 incubators[10] at the hatchery facility of the Virginia Tech Turkey Research Center. To simulate a commercial incubation environment, temperatures were set at the industry standard temperature (37.5°C) from embryonic day (**ED**) 0 to ED25. From ED25 to ED28, eggs were incubated in one of two incubators each set at a different temperature during the plateau of oxygen consumption. Following candling at transfer (ED25), one half of the eggs were maintained at 37.5°C (n=318), and one half of the eggs were incubated at a lower temperature of 36.0°C (n=318). Incubation temperature was regulated by thermistors connected to microprocessors with a temperature sensitivity of ± 0.05°C. Humidity was controlled with a comparable system by humidity sensors. Two temperature data loggers were placed in each incubator to monitor temperature. Incubation temperature and humidity were evaluated and logged daily. Eggshell temperatures taken at the equator of the egg once daily and

[10] NatureForm, Inc., Jacksonville, FL

were recorded from 16 eggs per incubator at various locations within the incubator using an infrared thermometer[11].

Turkey Coronavirus and Enteropathogenic E. coli

Turkey coronavirus (**TCV**: NC95) was isolated from infected turkeys as previously described (Guy et al., 1997), and TCV inoculum was prepared according to the methods of Guy et al. (2000). Inoculum was stored at -80°C until use.

Escherichia coli (R98/5) was isolated from infected turkeys as previously described (Guy et al., 2000). To prepare inoculum, R98/5 was propagated in Luria broth with aeration at 37°C to an optical density of 0.4 at 600nm. Previous growth curve experiments indicated that when R98/5 was grown to this density, the yield was approximately 5 x 10^8 colony-forming units (CFU)/mL. Inoculum was prepared by diluting with Luria broth and was used immediately following preparation. Challenge quantity was determined by preparing 10-fold dilutions of inoculum in Luria broth and streaking 0.1 mL per dilution onto MacConkey agar plates. Plates were incubated overnight at 37°C, and colonies were counted to determine exact CFU/mL.

At d 5, half of the poults (n=192) were administered a dual TCV/*E.coli* challenge, and half were not challenged. Birds were administered 0.1 mL of TCV inoculum (4 x 10^3 EID_{50}/0.1 mL) and 0.1 mL of *E. coli* (2.4 x 10^8 CFU/mL) by oral gavage. A total of four treatment groups resulted, designated as a combination of incubation temperature and challenge (Table 5.1).

Rearing and Diets

Poults were randomized within treatment (n=192 poults/incubation treatment, 96 poults/treatment) and assigned to battery brooder pens (n=12 replicate pens/treatment with 8 poults/pen). The birds were housed in an environmentally controlled room under a lighting

[11] Raynger ST20 Pro Standard, Santa Cruz, CA

program recommended for Hybrid turkeys. Birds had ad libitum access to water and a corn-soybean based diet formulated to meet or exceed Hybrid Converter nutritional requirements. A pre-starter diet was administered from d 0 until termination of the study at d 17.

Performance Parameters and Organ Development

Body weight (**BW**) and feed intake (**FI**) of birds were measured by pen on d 0, d 5 (0 days post-infection, **dpi**), d 11 (6 dpi), and d 17 (12 dpi) to calculate body weight gain (**BWG**) and feed conversion (**FC**) for the following periods: d 0-d 5, d 5-d 11, d 11-d 17, and cumulatively following challenge from d 5-d 17. Mortality was recorded daily, and birds removed as mortality or for sampling were weighed to adjust the FI and FC respective of the number of bird days. At d 5 and d 11, poults were randomly selected for evaluation of bursa, spleen, yolk sac, and gastrointestinal tract from the duodenum to ileocecal junction (n=8 poults/treatment). Birds were weighed, euthanized by cervical dislocation, and organs were collected. Organ weights are presented as a percentage (%) BW.

Heterophil/Lymphocyte Ratios

Whole blood (n=8 birds/treatment) was collected from the same birds from which organ samples were obtained at d 5, as well as at d 12 and d 17. Blood was collected from the jugular or brachial vein at d 5, d 12, and d 17 for evaluation of heterophil to lymphocyte (**H:L**) ratios. Blood samples were collected in 1.8 mL tubes containing 0.105M buffered sodium citrate. A blood smear was made for each blood collection tube using a Morf slide spinner[12]. Smears were stained with a modified Wright-Giemsa stain using the protocol provided by the manufacturer[13]. Smears were evaluated by counting a total of 60 lymphocytes and heterophils per slide under

[12] Salem Specialties, Inc., Salem, VA
[13] Sigma-Aldrich, St. Louis, MO

100X oil immersion. Heterophil:lymphocyte ratios were calculated by dividing the total number of heterophils by the total number of lymphocytes for each blood smear.

Intestinal Morphology

Eight birds per treatment (same sampling birds from which organ samples and whole blood were obtained) were randomly selected for evaluation of intestinal morphology at d 5 and d 11. Tissue samples (3cm) were collected from the jejunum (mid-point from the pancreatic duct to Meckel's diverticulum) and ileum (Meckel's diverticulum to ileocecal junction). Intestinal segments were flushed with cold PBS and fixed in 10% neutral buffered formalin. Each segment was cut into 5 (1cm) sections and placed in a tissue cassette. Tissues were processed, embedded in paraffin, cut into 5μm sections, and mounted onto slides. Slides were stained using routine procedures for hemotoxylin and eosin (**H&E**) and were evaluated using a light microscope (Luna, 1968). Measurements were made using SigmaScan Pro 5 software[14]. Three of the five total intestinal tissue sections were evaluated per slide for villus height (from villus tip to opening of crypt) and crypt depth (from the opening of the crypt to the base). Villus height-to-crypt depth ratios were also calculated. Four villi and 4 crypts were evaluated for each of the 3 intestinal tissue sections. The average villus height, crypt depth, and villus height-to-crypt depth ratio per slide were analyzed (n=12 measurements/bird, 8 birds/treatment).

TCV ELISA

Antibody titers to TCV were evaluated at d 5 (0 dpi), d 12 (7 dpi), and d 17 (12 dpi) in 8 poults/treatment. Approximately 1 mL of blood was collected from the jugular or brachial vein, and the blood sample was placed in a polypropylene culture tube and sealed. Samples remained at room temperature until centrifugation. Samples were centrifuged at 1000 RPM for 10

[14] Olympus America Inc., Melville, NY

minutes, and serum was removed and aliquoted to 1.5mL microcentrifuge tubes. Serum was stored at -20°C until further analysis. A competitive enzyme-linked immunosorbent assay (ELISA) was conducted to detect presence of TCV antibodies as previously described (Guy et al., 2002).

Statistical Analysis

Data were evaluated as a completely randomized experimental design arranged as a 2 x 2 factorial (2 incubation temperature treatments and 2 challenge treatments). Analysis of variance was performed using the GLM procedure of SAS. Values are reported as least squares (LS) means ± SEM. Organ weight data presented as a percentage were transformed prior to analysis using arc-sine (square root of percent). Bird served as the experimental unit for evaluation of intestinal morphology, organ weights, and heterophil:lymphocyte ratios. Pen served as the experimental unit for BW, BWG, FI, FCR, and mortality.

RESULTS

There were no differences in % hatch between incubation temperature treatments (data not shown).

Clinical Signs

Clinical signs in dual-infected poults began at 6dpi and included reduced feed and water consumption, depression, huddling, ruffled feathers, and diarrhea. No clinical signs were observed in the non-challenged birds.

Performance Parameters and Organ Development

Incubation temperature impacted body weight at d 0, d 5, and d 11 with birds incubated at 36.0°C exhibiting a heavier (P=0.001; P=0.0079; P=0.012, respectively) BW compared to birds incubated at 37.5°C (Table 5.2). There was also a main effect (P<0.0001) of challenge on BW at

d 11 (6 dpi) and d 17 (12 dpi) (Table 5.2). Birds that were challenged had a lower BW compared to non-challenged birds. There was no effect of incubation temperature on BWG; however, challenged birds had a lower BWG than non-challenged birds from d 5 (0 dpi) to d 11 (6 dpi) ($P<0.0001$), d 11 (6 dpi) to d 17 (12 dpi) ($P=0.0006$), and d 5 (0 dpi) to d 17 (12 dpi) ($P<0.0001$) (Table 5.3).

Feed intake was affected by incubation temperature from d 0 to d 5, d 5 to d 11, and cumulatively from d 0 to d 17 ($P=0.0004$; $P=0.0227$; $P=0.0167$, respectively). Birds that were incubated at 36.0°C consumed more feed than birds that were incubated at 37.5°C (Table 5.4). Feed intake was reduced in challenged birds compared to non-challenged birds from d 5(0 dpi) to d 11 (6 dpi), d 11 (6 dpi) to d 17 (12 dpi), and d 5 (0 dpi) to d 17 (12 dpi) (Table 5.4: $P=0.0162$; $P<0.0001$). Birds incubated at 37.5°C exhibited a more efficient FCR ($P=0.036$) compared to birds incubated at 36.0°C from d 0 to d 5 (0 dpi), and birds that were non-challenged exhibited a more efficient FCR ($P=0.0111$) compared to challenged birds from d 5 (0 dpi) to d 11 (6 dpi) (Table 5.5).

There were no differences in mortality as a result of incubation temperature. Mortality was higher in the challenged groups from d 11 (6 dpi) to d 17 (12 dpi), and d 5 (0 dpi) to d 17 (12 dpi) ($P<0.0001$) (Table 5.6).

Relative bursa weight was increased ($P=0.015$) at d 5 (0 dpi) in birds that were incubated at 36.0°C compared to birds that were incubated at 37.5°C (Table 5.7). Challenged birds had a reduced ($P=0.0271$) relative bursa weight at d 11 (6 dpi) compared to non-challenged birds (Table 5.7). No differences were observed due to treatment main effects or interactions on spleen, yolk sac, or gastrointestinal tract weight.

Heterophil:Lymphocyte Ratio

Incubation temperature and challenge resulted in a two way interaction (P=0.0555) at d 12 (7 dpi) on heterophil:lymphocyte ratio (Table 5.8). There was no difference in heterophil:lymphocyte ratio in non-challenged birds regardless of incubation temperature; however, birds that were exposed to 36.0°C resulted in a higher heterophil:lymphocyte ratio when challenged compared to birds incubated at 37.5°C and challenged.

Intestinal Morphology

At d 5, jejunum villus height was higher (P=0.0437) in birds incubated at 36.0°C than 37.5°C (Table 5.9). Crypt depth or villus height:crypt depth ratio were not impacted by incubation temperature at d 5 (0 dpi). Jejunum villus height and villus height:crypt depth ratio were affected by challenge at d 11 (6 dpi). Challenged birds showed a reduction (P=0.0007) in villus height and a reduced (P=0.0123) villus height:crypt depth ratio in the jejunum at d 11 (6 dpi) compared to non-challenged birds (Table 5.9). There was no effect on crypt depth in the jejunum at d 11 (6 dpi) due to incubation temperature or challenge. There was a two way interaction of incubation temperature and challenge on ileum villus height and crypt depth at d 11 (6 dpi) (Table 5.9). There was no difference in villus height between challenge and non-challenged birds; however, birds that were challenged and incubated at 36.0°C had longer (P=0.0353) villi compared to birds that were challenged and incubated at 37.5°C. There were no crypt depth differences between birds incubated at 36.0°C or 37.5°C that were non-challenged; however, birds that were challenged and incubated at 36.0°C had shallower (P=0.0084) crypts than birds that were challenged and incubated at 37.5°C. Due to these changes in ileal villus height and crypt depth, the ileum villus height:crypt depth ratio was also impacted by the interaction (P=0.0012) of incubation temperature and challenge at d 11 (6 dpi) (Table 5.9). Non-

challenged birds incubated at either 36.0°C or 37.5°C showed no difference in villus height:crypt depth ratio, but challenged birds incubated at 36.0°C exhibited a higher ratio compared to challenged birds incubated at 37.5°C.

TCV ELISA

There was no presence of TCV specific antibodies at 0 dpi or 7 dpi (Table 5.10). At 12 dpi, TCV positive sera was detected in five birds incubated at 37.5°C and challenged and three birds incubated at 36.0°C and challenged.

DISCUSSION

Incubation temperature conditions and post-hatch dual challenge had both independent and interactive effects on the evaluated parameters in this study. Exposure to an incubation temperature of 36.0°C as compared to 37.5°C was advantageous for body weight beginning at d 0 and maintaining through d 11 (6 dpi). Differential responses to suboptimal incubation temperatures have been previously reported. Much incubation research has been conducted in broiler chickens, and the predominant focus has been on temperature conditions as related to multi-stage incubation. In multi-stage incubators, it is not uncommon for low eggshell temperatures to be reported at the start of incubation (Joseph et al., 2006). Embryonic heat production is somewhat insignificant at the beginning of incubation; therefore, egg temperature is less than incubation temperature because embryonic heat loss due to evaporative cooling exceeds heat produced by the embryo (French, 1997). With these factors in mind, low eggshell temperatures are not naturally occurring during late incubation; however, they were evaluated in this study to determine if there is an advantage to manually reducing the incubator temperature during the last several days of incubation. Embryo length and yolk-free body weight, as indicators of broiler chick quality, were reduced at ED7, 14, 18, and 21.5 in embryos incubated

at a low incubation temperature of 36.7°C (Lourens et al., 2005). At 3 and 6 wk post-hatch, broiler chicks that were incubated at low eggshell temperatures had lower body weights and decreased body weight gain compared to chicks that had been exposed to standard temperatures (Joseph et al., 2006). Turkey poults demonstrated decreased body and pectoral weight and number of semitendinosus muscle fibers at 16 d post-hatch after exposure to 35.5°C from ED5 to ED8 (Maltby et al., 2004). In contrast, earlier studies have shown that temperatures below 37°C during the first 10 days of incubation resulted in increased body weight, gastrointestinal tract weight, and liver weight at 2 and 3 wk post-hatch, which is suggestive of increased heat production due to compensatory growth (Geers et al., 1983). Although most reports in the literature have indicated that low incubation temperature yields a contrasting response to what is reported in this study, it is critical to note that none of those studies evaluated low incubation temperature during late incubation of turkeys. During the last several days of incubation, embryos are not only experiencing potential temperature stress as a result of metabolic heat expulsion, but they are also undergoing the energy expensive process of hatching under conditions in which oxygen availability is limited. Previous results have indicated that low temperature exposure during the last several days of incubation resulted in a higher body weight at hatch compared to both standard and high temperatures (Chapter 3). It may be speculated that if the environmental temperature is reduced in the incubator, once the bird pips and is breathing on its own, the bird's respiratory rate would be less than if it was exposed to an elevated temperature. With this in mind, a higher concentration of oxygen in the incubator during late incubation has resulted in heavier body weight (Wineland et al., 2006). Although oxygen concentration was not monitored in the current study, it is possible that a lower late incubation temperature prior to and during the hatch process makes oxygen more available and allows for

more nutrient utilization and thus contributes to heavier body weight post-hatch. It is also possible that the beneficial effects exhibited by birds exposed to a low temperature are unrelated to increased oxygen availability and may be related simply to exposure to a more appropriate late incubation temperature for high-yield turkey embryos.

Feed intake data from this study correspond with the differences in body weight between the low and standard incubation temperature groups, in that birds incubated at 36.0°C consumed more feed compared to birds incubated at 37.5°C from d 0 to d 5, from d 5 to d 11, and also cumulatively from d 0 to d 17. The effects of incubation temperature on feed consumption have been previously reported. Feed intake of broilers exposed to a high incubation temperature from ED16 until hatch showed a lower feed consumption from d 0 to d 7, d 7 to d 14, and d 14 to d 21 than birds exposed to a standard incubation temperature (Leksrisompong et al., 2009). Although this previous study was conducted in broilers and not in turkeys, and there was no low incubation temperature treatment, the response to incubation temperature may still be compared to the results of the current study. In the study conducted by Leksrisompong et al., the birds that were exposed to elevated incubation temperature consumed less feed than birds from the standard temperature treatment. Likewise, in the current study, birds that were exposed to potentially more appropriate late incubation conditions (36.0°C) had a greater feed intake in comparison to birds incubated at 37.5°C.

As expected, challenge with TCV and *E. coli* resulted in a reduction in body weight compared to non-challenged birds at both 6 and 12 dpi, as well as reduced body weight gain from d 5 (0 dpi) to d 11 (6 dpi), d 11(6 dpi) to d 17(12 dpi), and d 5(0 dpi) to d 17(12 dpi). Poults infected at 2 d post-hatch showed a reduction in mean body weight compared to non-infected control poults, starting at 6 d post-infection and persisting through 44 d of age (Gomaa

et al., 2009). Poults challenged at 28 d post-hatch showed a similar pattern in body weight reduction. The 28 d old poults exhibited a body weight reduction compared to non-infected controls at both 35 and 44 d, and at 44 d, the mean body weights of the infected poults were only 77.7% of uninfected control weights (Gomaa et al., 2009). These findings closely resemble the response reported by Doerfler et al. (1998) that showed a depression in body weight at 9, 16, and 23 d after exposure at d 5. A study by Guy et al. (2000) reported significantly reduced body weight gain and average daily gain in poults infected with both TCV and *E. coli* compared to sham-inoculated controls. Poults infected with TCV often exhibit reduced growth and digestive function, which typically leads to stunting of the birds and a subsequent lack of overall flock uniformity (Culver et al., 2006). Additionally, TCV infects epithelial cells of the intestinal villi, which results in its atrophy, as well as causing villi fusion. Villi atrophy, which occurs as a result of intestinal cell sloughing, yields a surface that is greatly impaired in terms of absorptive capacity. Naturally, a generally malabsorptive intestine will cause a reduction in overall body weight, which is supported by the feed intake data in this study that showed consumption reduction in challenged birds at all time phases following challenge.

There were no differences in mortality between birds that were incubated at 36.0°C compared to birds incubated at 37.5°C. However, birds that were challenged demonstrated a significantly higher percent mortality from d 11 (6 dpi) to d 17 (12 dpi), and d 5 (0 dpi) to d 17 (12 dpi). Sham-inoculated control poults expressed 4% mortality compared to poults dually challenged with both TCV and *E. coli*, in which mortality was 79% (Guy et al., 2000). It is possible that the high mortality exhibited in poults co-infected with these pathogens may be attributed to disease-associated diarrhea. An intestine that is generally malabsorptive will result in diarrhea along with dehydration, and in severe cases, death (Saif, 1990). Infectious agents

cause enteric disease by several mechanisms as reviewed by Moon (1978). Moon specifically elucidates the physiological events responsible for inducing diarrhea, which include hypermotility, increased permeability, hypersecretion, and malabsorption. Diarrhea and fluid loss caused as a result of enteric viruses is usually reflective of the impaired function and damage to the intestinal tissue as compared to secretory diarrhea that is typically associated with bacterial infections (Moon, 1978). Thus, the classic mechanism employed by TCV is malabsorption. Due to the destruction of the intestinal villi during TCV infection, intestinal contents that cannot be absorbed in the small intestine will move on to the colon where it will continue to retain and hold water due to osmotic effect. However, once the absorptive capacity of the colon has been met or has been exceeded, diarrhea will ensue. It is believed that mortality associated with TCV and *E. coli* may occur as a result of this diarrhea that causes dehydration as well as electrolyte imbalance (Guy et al., 2000).

A variety of environmental or physiological stressors may induce a lymphoid tissue response. The neurogenic system, consisting of the central nervous system and adrenal medulla, works in concert with the hypothalamus-pituitary-adrenal axis (**HPA**) to elicit a stress response. Activation of the HPA axis is typically induced in response to chronic stress. It begins with hypothalamic stimulation and induces an increase in adrenocorticotropic hormone (**ACTH**) from the anterior pituitary, which subsequently causes increased production of adrenal cortical steroids, such as corticosterone. The effects of corticosteroid production include decreased bursa, thymus, and spleen tissue, a reduction in circulating lymphocytes, and an increase in heterophils (Siegel, 1985). A study evaluated the effects of continuous administration of ACTH via mini-osmotic pumps and reported decreased relative bursa, thymus, and spleen weights 4 and 7 d following pump-implantation compared to control (Puvadolpirod and Thaxton, 2000). In the

present study, birds that were incubated at 36.0°C showed a higher relative bursa weight compared to birds that were incubated at 37.5°C. As previously suggested, it is possible that incubation temperature reduction during late incubation has advantages. If the embryos had experienced chronic stress as a result of low temperature exposure during incubation, relative immune organ weights would have reflected that stress exposure as in previous studies. However, the relative weight of the bursa in the low temperature group exceeded that of the standard, possibly suggesting that low incubation temperature exposure mediates or potentially reduces late incubation stress on the embryo as it relates to immune organ development.

Heterophil:lymphocyte ratios are considered a reliable indicator of stress in chickens (Siegel, 1995). Typically, a higher H:L ratio suggests a higher stress level. Immune cells are influenced by the release of glucocorticoids, such as corticosterone, in response to environmental stressors and pathogen exposure (Shini et al., 2008). This modulation of corticosterone production and secretion causes a reduction in lymphocyte circulation and an increase in heterophils (Siegel, 1985). There was an interaction of incubation temperature and TCV/*E. coli* challenge on H:L ratio at d 12 (7 dpi), with birds incubated at 36.0°C exhibiting a higher H:L ratio compared to challenged birds from the 37.5°C incubation temperature group and non-challenged birds from both incubation temperature groups. In general, overall immune system function is often suppressed in TCV-affected poults as indicated by decreased resistance to secondary infections and cytokine induction that not only results in intestinal inflammation (Heggen et al., 2000), but also compromises immune tissue development and function. It is then logical that the challenged birds would express a higher H:L ratio compared to the non-challenged. This response was only observed one week following challenge, suggesting that the elevated stress level associated with challenge is not maintained long-term with regard to H:L

ratio. Although a low incubation temperature appears to be advantageous with regard to post-hatch body weight, it is possible that the immunocompetency is somewhat compromised in birds exposed to a low incubation temperature and then exposed to pathogenic challenge, as indicated by this specific parameter.

Incubation temperature, challenge, and the interaction of incubation temperature and challenge influenced intestinal morphology in both the jejunum and ileum at various time points. In the jejunum, exposure to a low incubation temperature was advantageous with regard to villus height at d 5. Birds exposed to 36.0°C demonstrated longer villi compared to birds exposed to 37.5°C, and this response is equivalent to previous results at the same age (Chapter 4). Intestinal maturation occurs during the last several days of incubation during the oxygen consumption plateau (Rahn, 1981), during which time the embryo experiences both temperature and hypoxic conditions. Perhaps exposure to a low incubation temperature during this time period does not delay or impede normal maturation processes of the intestine as the embryo prepares to hatch.

Challenge resulted in a reduction in villus height:crypt depth at 6 dpi in the jejunum compared to non-challenged birds, and these results are in agreement with a previous study (Chapter 4). Functional integrity of the intestine is compromised during TCV infection due to epithelial cell sloughing from the intestinal villi, and cellular proliferation, and thus crypt depth is increased to replace these sloughed enterocytes. A reduced villus height:crypt depth ratio is reflective of a shorter villus and deeper crypt common with enteric infection. A typical clinical sign of enteritis in poults is diarrhea, and the intestinal response observed in this study as a result of TCV infection is a likely contributor. Crypt hyperplasia may result in increased secretion, causing crypt secretion to exceed villous absorption, resulting in both diarrhea and malabsorption (Moon, 1978).

The ileum demonstrated longer villi 6 dpi in challenged birds from the 36.0°C incubation temperature compared to birds that were exposed to 37.5°C and challenged. In fact, the villus height of birds exposed to 36.0°C and challenged was comparable to villus height of birds that were non-challenged and exposed to either incubation temperature. This response suggests that reduction of temperature during late incubation may be beneficial to the maturation process of the intestine. In contrast, however, challenged birds exposed to 36.0°C had shallower crypts compared to challenged birds exposed to 37.5°C. A shallower crypt may be indicative of reduced proliferation of the intestinal enterocytes, but also may indicate reduced cellular turnover. If cellular turnover is reduced, crypts may be shallower, and villi may be longer and consist of more mature intestinal enterocytes. Although the villi are longer, if the enterocytes are not being replaced, then their absorptive capacity may be reduced. It seems logical to suggest that exposure to a reduced incubation temperature may allow the gut to become more developmentally advanced to the point that the bird may be more equipped to handle enteric infection compared to counterparts that are exposed to a standard incubation temperature. However, a decrease in crypt depth seems to indicate that intestinal function, as it relates specifically to epithelial cell proliferation or turnover, may be somewhat compromised. Interestingly, an increased villus height would indicate an increase in number of mature enterocytes. A greater number of mature enterocytes provides more target cells for TCV to infect, which would presumably lead to increased challenge impact. In the current study, however, longer villi as a result of incubation temperature did not lead to increased susceptibility to infection. The reason for this response in the current study is not understood.

Results from this study indicate that there is no apparent link between incubation conditions and post-hatch susceptibility to poult enteritis as induced by both TCV and *E. coli*

based on the evaluated parameters. As expected, dual challenge with TCV and *E. coli* resulted in negative effects on bird performance, intestinal morphology, and mortality. There appears to be a beneficial effect on bird performance, bursa development, and intestinal development in birds exposed to a low temperature compared to a standard temperature during late incubation during the first several days post-hatch. The current study was only conducted until 17 d post-hatch, so long-term advantages of a low incubation temperature are somewhat difficult to determine; however, broilers that were heavier at an earlier age tended to maintain heavier weight advantage until market age (Willemsen et al., 2008). If this study had been conducted for a longer duration, it is possible that the advantageous effects of exposure to the low incubation temperature may have been maintained to a later post-hatch age. Future studies should perhaps evaluate the effects of low incubation temperature in combination with various oxygen concentrations during late incubation to determine the effects on post-hatch performance as well as impacts on intestinal morphology. It would be beneficial to evaluate the effects of incubation temperature and post-hatch pathogen susceptibility in broilers, in addition to exploring the susceptibility of turkeys to other PEC-associated viruses (such as astrovirus) alone and in combination with PEC-associated bacteria. If enteric viruses infect the intestine through various mechanisms and possess differing tropisms, perhaps a connection between a low incubation temperature advantage with regard to pathogen exposure compared to standard or high temperatures may be determined through examination of different temperature and pathogen combinations in future studies.

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Incubation Treatment	Challenge Treatment	n
36.0°C	Challenge	96
36.0°C	Non-Challenge	96
37.5°C	Challenge	96
37.5°C	Non-Challenge	96

Table 5.1 There were four total treatment groups consisting of a combination of incubation temperature and challenge. All embryos were incubated at 37.5°C from ED0 to ED25, and from ED25 to ED28, treatments were designated as low (36.0°C) or standard (37.5°C). Poults were inoculated with TCV (4×10^3 EID_{50}/0.1mL) and *E. coli* R98/5 (2.4×10^8 CFU/ml) at d 5.

Treatment		Day 0	Day 5 (0dpi)	Day 11 (6dpi)	Day 17 (12dpi)
36.0°C		62.13[a]	121.66[a]	230.34[a]	334.14
37.5°C		59.69[b]	114.73[b]	218.24[b]	327.77
Pooled SEM		0.50	1.76	3.26	9.43
Challenge (C)		---	116.81	211.47[b]	289.49[b]
Non-Challenge (NC)		---	119.58	237.11[a]	372.43[a]
Pooled SEM		---	1.76	3.26	9.32
36.0°C	C	---	121.07	218.78	297.55
	NC	---	122.26	241.9	370.73
37.5°C	C	---	112.56	204.16	281.42
	NC	---	116.91	232.33	374.12
Pooled SEM		---	2.49	4.61	13.12
P-value					
Temp		0.001	0.0079	0.012	0.636
Challenge		---	0.272	<0.0001	<0.0001
Temp*Challenge		---	0.529	0.586	0.469

Table 5.2 Effect of incubation temperature and TCV/*E. coli* challenge on average body weight (g) in Hybrid turkey poults. All embryos were incubated at 37.5°C from ED0 to ED25, and from ED25 to ED28, treatments were designated as low (36.0°C) or standard (37.5°C). Poults were inoculated with TCV (4×10^3 EID_{50}/0.1mL) and *E. coli* R98/5 (2.4×10^8 CFU/ml) at d 5. Data are presented as LS means ± SEM (n=12/treatment).

[a,b] Data within a column lacking a common superscript differ significantly

Treatment		d0 - d5(0dpi)	d5(0dpi) - d11(6dpi)	d11(6dpi)-d17(12dpi)	d5(0dpi) - d17 (12dpi)	d0 - d17 (12dpi)
36.0°C		59.53	108.68	105.83	214.62	271.99
37.5°C		55.04	103.51	111.02	215.05	268.45
Pooled SEM		1.79	2.56	9.99	9.07	9.35
Challenge (C)		---	94.65[b]	81.53[b]	176.83[b]	---
Non-Challenge (NC)		---	117.53[a]	135.31[a]	252.84[a]	---
Pooled SEM		---	2.56	9.88	8.97	---
36.0°C	C	---	97.71	82.82	180.76	---
	NC	---	119.64	128.83	248.47	---
37.5°C	C	---	91.6	80.24	172.89	---
	NC	---	115.42	141.79	257.21	---
Pooled SEM		---	3.61	13.96	12.68	---
P-value						
Temp		0.0835	0.159	0.716	0.973	0.79
Challenge		---	<0.0001	0.0006	<0.0001	---
Temp*Challenge		---	0.794	0.586	0.522	---

Table 5.3 Effect of incubation temperature and TCV/*E. coli* challenge on average body weight gain (g) in Hybrid turkey poults. All embryos were incubated at 37.5°C from ED0 to ED25, and from ED25 to ED28, treatments were designated as low (36.0°C) or standard (37.5°C). Poults were inoculated with TCV (4×10^3 EID_{50}/0.1mL) and *E. coli* R98/5 (2.4×10^8 CFU/ml) at d 5. Data are presented as LS means ± SEM (n=12/treatment).

[a,b] Data within a column lacking a common superscript differ significantly

Treatment		d0 - d5(0dpi)	d5(0dpi) - d11(6dpi)	d11(6dpi)-d17(12dpi)	d5(0dpi) - d17 (12dpi)	d0 - d17 (12dpi)
36.0°C		62.66[a]	133.5[a]	165.4	283.98	342.37[a]
37.5°C		54.47[b]	121.93[b]	155.21	264.18	313.90[b]
Pooled SEM		1.50	3.47	6.31	8.49	8.10
Challenge (C)		---	121.59[b]	97.59[b]	213.81[b]	---
Non-Challenge (NC)		---	133.84[a]	223.01[a]	334.35[a]	---
Pooled SEM		---	3.47	6.31	8.49	---
36.0°C	C	---	128.71	110.16	230.9	---
	NC	---	138.29	220.64	337.05	---
37.5°C	C	---	114.47	85.02	196.71	---
	NC	---	129.39	225.39	331.64	---
Pooled SEM		---	4.90	8.93	12.01	---
P-value						
Temp		0.0004	0.023	0.260	0.106	0.0167
Challenge		---	0.016	<0.0001	<0.0001	---
Temp*Challenge		---	0.589	0.101	0.237	---

Table 5.4 Effect of incubation temperature and TCV/*E. coli* challenge on feed intake (g) in Hybrid turkey poults. All embryos were incubated at 37.5°C from ED0 to ED25, and from ED25 to ED28, treatments were designated as low (36.0°C) or standard (37.5°C). Poults were inoculated with TCV (4 x 10^3 EID_{50}/0.1mL) and *E. coli* R98/5 (2.4 x 10^8 CFU/ml) at d 5. Data are presented as LS means ± SEM (n=12/treatment).

[a,b] Data within a column lacking a common superscript differ significantly

Treatment		d0 - d5(0dpi)	d5(0dpi) - d11(6dpi)	d11(6dpi)-d17(12dpi)	d5(0dpi) - d17 (12dpi)	d0 - d17 (12dpi)
36.0°C		1.069[a]	1.25	2.017	1.388	1.289
37.5°C		0.992[b]	1.204	1.608	1.272	1.17
Pooled SEM		0.025	0.047	0.175	0.064	0.044
Challenge (C)		---	1.316[a]	1.944	1.333	---
Non-Challenge (NC)		---	1.139[b]	1.681	1.328	---
Pooled SEM		---	0.047	0.175	0.063	---
36.0°C	C	---	1.346	2.284	1.416	---
	NC	---	1.155	1.749	1.36	---
37.5°C	C	---	1.285	1.603	1.249	---
	NC	---	1.123	1.612	1.295	---
Pooled SEM		---	0.067	0.245	0.089	---
P-value						
Temp		0.036	0.491	0.109	0.207	0.0615
Challenge		---	0.011	0.297	0.956	---
Temp*Challenge		---	0.829	0.282	0.577	---

Table 5.5 Effect of incubation temperature and TCV/*E. coli* challenge on feed conversion (g/g) in Hybrid turkey poults. All embryos were incubated at 37.5°C from ED0 to ED25, and from ED25 to ED28, treatments were designated as low (36.0°C) or standard (37.5°C). Poults were inoculated with TCV (4 x 10^3 EID_{50}/0.1mL) and *E. coli* R98/5 (2.4 x 10^8 CFU/ml) at d 5. Data are presented as LS means ± SEM (n=12/treatment).

[a,b] Data within a column lacking a common superscript differ significantly

Treatment		d0 - d5(0dpi)	d5(0dpi) - d11(6dpi)	d11(6dpi) - d17(12dpi)	d5(0dpi) - d17(12dpi)	d0 - d17(12dpi)
36.0°C		2.083	0.595	33.656	31.027	31.77
37.5°C		4.688	2.679	32.847	30.863	34.9
Pooled SEM		1.77	0.77	3.77	3.38	3.63
Challenge (C)		---	2.679	66.503[a]	61.295[a]	---
Non-Challenge (NC)		---	0.595	0[b]	0.595[b]	---
Pooled SEM		---	1.28	3.78	3.38	---
36.0°C	C	---	1.19	67.312	62.054	---
	NC	---	0	0	0	---
37.5°C	C	---	4.167	65.69	60.536	---
	NC	---	1.19	0	1.19	---
Pooled SEM		---	1.81	5.34	4.79	---
P-value						
Temp		0.305	0.256	0.880	0.973	0.545
Challenge		---	0.256	<0.0001	<0.0001	---
Temp*Challenge		---	0.624	0.880	0.779	---

Table 5.6 Effect of incubation temperature and TCV/*E. coli* challenge on mortality (%) in Hybrid turkey poults. All embryos were incubated at 37.5°C from ED0 to ED25, and from ED25 to ED28, treatments were designated as low (36.0°C) or standard (37.5°C). Poults were inoculated with TCV (4 x 10^3 EID_{50}/0.1mL) and *E. coli* R98/5 (2.4 x 10^8 CFU/ml) at d 5. Data are presented as LS means ± SEM (n=12/treatment).

[a,b] Data within a column lacking a common superscript differ significantly

Treatment		d5(0dpi)				d11(6dpi)		
		Bursa	Spleen	Yolk	GIT	Bursa	Spleen	GIT
36.0°C		0.153[a]	0.066	0.153	7.421	0.159[a]	0.100	7.002
37.5°C		0.120[b]	0.063	0.449	7.165	0.127[b]	0.109	6.677
Pooled SEM		0.0017	0.0009	0.0128	0.0057	0.0013	0.0017	0.0076
Challenge (C)		---	---	---	---	0.132[b]	0.099	7.216
Non-Challenge (NC)		---	---	---	---	0.154[a]	0.111	6.463
Pooled SEM		---	---	---	---	0.0013	0.0017	0.0076
36.0°C	C	---	---	---	---	0.148	0.102	7.593
36.0°C	NC	---	---	---	---	0.171	0.098	6.411
37.5°C	C	---	---	---	---	0.117	0.095	6.838
37.5°C	NC	---	---	---	---	0.136	0.123	6.515
Pooled SEM		---	---	---	---	0.0013	0.0017	0.0076
P-value								
Temp		0.015	0.331	0.160	0.583	0.0018	0.5679	0.4202
Challenge		---	---	---	---	0.0271	0.4104	0.0661
Temp*Challenge		---	---	---	---	0.984	0.200	0.306

Table 5.7 Effect of incubation temperature and TCV/*E. coli* challenge on relative organ weight (% BW) of Hybrid turkey poults. All embryos were incubated at 37.5°C from ED0 to ED25, and from ED25 to ED28, treatments were designated as low (36.0°C) or standard (37.5°C). Poults were inoculated with TCV (4 x 10^3 EID_{50}/0.1mL) and *E. coli* R98/5 (2.4 x 10^8 CFU/ml) at d 5. Data are presented as LS means ± SEM (n=8/treatment).

[a,b] Data within a column lacking a common superscript differ significantly

Treatment		d5(0dpi)	d12(7dpi)	d17(12dpi)
36.0°C		0.0307	0.0304[a]	0.0335
37.5°C		0.0291	0.0229[b]	0.0371
Pooled SEM		0.0024	0.0022	0.0023
Challenge (C)		---	0.0329[a]	0.0371
Non-Challenge (NC)		---	0.0203[b]	0.0336
Pooled SEM		---	0.0022	0.0023
36.0°C	C	---	0.0397[a]	0.0366
36.0°C	NC	---	0.0210[b]	0.0305
37.5°C	C	---	0.0261[b]	0.0376
37.5°C	NC	---	0.0196[b]	0.0366
Pooled SEM		---	0.0030	0.0032
P-value				
Temp		0.6352	0.02	0.2787
Challenge		---	0.0003	0.2805
Temp*Challenge		---	0.0555	0.4415

Table 5.8 Effect of incubation temperature and TCV/*E. coli* challenge on heterophil:lymphocyte ratio of Hybrid turkey poults. All embryos were incubated at 37.5°C from ED0 to ED24, and from ED24 to ED28, treatments were designated as low (36.0°C) or standard (37.5°C). Poults were inoculated with TCV (4 x 10^3 EID_{50}/0.1mL) and *E. coli* R98/5 (2.4 x 10^8 CFU/ml) at d 5. Data are presented as LS means ± SEM (n=8/treatment).

[a,b] Data within a column lacking a common superscript differ significantly

		d5(0dpi)						d11(6dpi)					
Treatment		Jej VH[1]	Jej CD[2]	Jej VCR[3]	Ile VH	Ile CD	Ile VCR	Jej VH	Jej CD	Jej VCR	Ile VH	Ile CD	Ile VCR
36.0°C		0.8521[a]	0.081	10.697	0.551	0.071	7.893	0.979	0.174	5.802	0.706	0.134	5.334[a]
37.5°C		0.7783[b]	0.076	10.290	0.531	0.070	7.586	1.019	0.173	5.951	0.655	0.141	4.695[b]
Pooled SEM		0.0248	0.0018	0.3899	0.0174	0.0022	0.2782	0.038	0.008	0.287	0.026	0.003	0.207
Challenge		---	---	---	---	---	---	0.896[b]	0.172	5.334[b]	0.661	0.134	5.009
Non-Challenge		---	---	---	---	---	---	1.102[a]	0.174	6.419[a]	0.700	0.140	5.020
Pooled SEM		---	---	---	---	---	---	0.038	0.008	0.287	0.026	0.003	0.207
36.0°C	C	---	---	---	---	---	---	0.828	0.174	4.970	0.728[a]	0.124[b]	5.855[a]
36.0°C	NC	---	---	---	---	---	---	1.130	0.174	6.633	0.684[ab]	0.144[a]	4.812[ab]
37.5°C	C	---	---	---	---	---	---	0.964	0.171	5.698	0.595[b]	0.144[a]	4.162[b]
37.5°C	NC	---	---	---	---	---	---	1.074	0.175	6.205	0.716[ab]	0.137[ab]	5.229[ab]
Pooled SEM		---	---	---	---	---	---	0.054	0.012	0.406	0.037	0.005	0.293
P-value													
Temp		0.0437	0.0805	0.4665	0.4122	0.8801	0.4417	0.4681	0.9485	0.7150	0.1879	0.1546	0.0378
Challenge		---	---	---	---	---	---	0.0007	0.8680	0.0123	0.3037	0.1960	0.9678
Temp*Challenge		---	---	---	---	---	---	0.0875	0.8553	0.1652	0.0353	0.0084	0.0012

[1] VH = villus height (mm)
[2] CD = crypt depth (mm)
[3] VCR = villus height:crypt depth ratio

Table 5.9 Effect of incubation temperature and TCV/*E. coli* challenge on intestinal morphology of Hybrid turkey poults. All embryos were incubated at 37.5°C from ED0 to ED25, and from ED25 to ED28, treatments were designated as low (36.0°C) or standard (37.5°C). Poults were inoculated with TCV (4 x 10^3 EID_{50}/0.1mL) and *E. coli* R98/5 (2.4 x 10^8 CFU/ml) at d 5. Data are presented as LS means ± SEM (n=8/treatment).

[a,b] Data within a column lacking a common superscript differ significantly

	Number Suspect/Number Tested			
Days post-exposure	37.5°C-C	37.5°C -NC	36.0°C - C	36.0°C - NC
0	0/8	0/8	0/8	0/8
7	0/8	0/8	0/8	0/8
12	5/8	0/8	3/8	0/8

[1] C = Challenge
[2] NC = Non-challenge

Table 5.10 Detection of TCV specific antibodies in sera of experimentally infected turkeys by cELISA. Poults were inoculated with TCV (4 x 10^3 EID_{50}/0.1mL) at d 5, and serum was collected at 0 dpi, 7 dpi, and 12 dpi.

CHAPTER VI

EPILOGUE

Non-optimal incubation temperatures have been shown to impose challenges upon the bird during both embryonic development and post-hatch. Incubation temperatures that do not fall within the optimal range of 37°C to 38°C required by the developing embryo have been shown to impact hatchability, bird quality, post-hatch development, and bird performance. The exposure of developing embryos to high incubation temperatures is reflective of the increased metabolic rate employed by fast-growing and high-yield birds due to genetic selection. Selection for such traits results in increased metabolic heat production by the embryo. Currently, few modifications in hatchery equipment and management have occurred to compensate for the changed needs of the high-yield embryo, and consequently, many conditions in commercial incubators are reflective of environmental requirements for classic poultry. Studies have been conducted to evaluate the effects of non-optimal incubation temperatures and the timing of these temperatures on organ development, thermoregulation, performance, and other growth parameters in broilers and turkeys; however, no studies to date have examined the effects of non-optimal late-stage incubation temperatures on development of intestinal and immune function and subsequent susceptibility to post-hatch enteric infection in turkeys.

Results from the presented studies indicate that incubation temperature stress alone or post-hatch challenge with TCV or TCV and enteropathogenic *E. coli* influences turkey poult development. The oxygen consumption plateau begins around ED25, and intestinal maturation occurs during this time (Rahn, 1981), so it seems logical to examine non-optimal temperature effects during this time period, especially as it relates to intestinal function and competency of the gut to respond to enteric challenge during the post-hatch period. Regulating oxygen concentration during this plateau phase could have been beneficial. Other studies have

monitored atmospheric gases with infrared detectors for both carbon dioxide and oxygen, and gases were manually infused into the experimental incubator cabinets to create the desired concentrations (Oviedo-Rondon et al., 2008). It is understood that oxygen availability during the plateau phase is compromised; however, monitoring and measuring the oxygen levels in the incubator in these studies would have provided a definite knowledge that the birds were in fact exposed to hypoxic stress in addition to temperature stress. Elevated temperatures or thermal conditioning at different incubation time points have caused reduced bird weight (Willemsen et al., 2010), heavier yolk sacs, and reduced relative weight of the small intestine (Leksrisompong et al., 2007), while reduced oxygen concentration resulted in decreased jejunum weight and overall body weight (Christensen et al., 2004). It appears unclear as to whether temperature or oxygen concentration elicits the most detrimental effect upon the bird, and no interactions between incubation temperature and oxygen concentration have been reported in the literature to date. In avian embryo incubation, temperature deviations of even tenths of a degree may impact embryological development. Perhaps a slightly higher elevated temperature (39.5°C to 39.8°C) may have had a more dramatic impact on embryo development. Another beneficial parameter to consider examining could have been the timing of internal and external pipping. Does the combination of temperature and hypoxic stressors result in earlier pipping so that the embryo can escape from the confinement of the eggshell, or does it actually result in delayed pipping due to the impacts of those stressors on timing of hatch as well as final maturation and preparation of the embryo to emerge from the shell?

The effects of egg storage in combination with temperature stress and susceptibility to post-hatch challenge could be interesting to examine. Storage of fertile eggs is a common practice in the poultry industry; however, the timing and temperature of storage could have

major impacts on embryo viability and bird quality. It is not uncommon for hatching eggs to be stored for periods beyond 7 days due to limitations regarding incubator capacity (Hamidu et al., 2011). Storage conditions for longer than 7 days may reduce hatchability, and after 14 days of storage, broilers showed a reduced growth rate and poor chick quality (Fasenko et al., 2001). Long term storage of hatching eggs may decrease the number of healthy embryonic cells and impact embryonic development (Fasenko et al., 1992). Hamidu et al. (2011) suggested that a reduction in number of blastodermal cells may result in decreased oxygen availability that the embryo requires for metabolic function, which in turn may cause the embryo to inefficiently utilize available oxygen to break down carbohydrates, fats, and proteins needed for growth. It could be interesting to examine the effects of egg storage, both short and long term, followed by temperature and hypoxic stress during late incubation. The effects of decreased oxygen availability during the last several days of turkey embryo incubation are already known; however, the additional effects of reduced embryonic cell number as a result of egg storage and the associated reduction in oxygen availability and how these factors may influence intestinal development and maturation have not been evaluated to date.

Current research in the area of incubation temperature stress effects on turkey embryonic development is lacking, as research has predominantly been conducted in broilers. In fact, a study was just recently conducted in the area of parent flock age and embryonic metabolism in modern turkeys (Hamidu et al., 2011). The embryonic metabolism of some turkey strains may be higher than others (Hamidu et al., 2011), and eggs from younger breeder flocks are smaller than eggs from older flocks. Embryos from younger parents tend to develop at a slower rate than embryos from older parents during the last week of incubation, even when incubated under identical conditions (Christensen et al., 2001; Applegate, 2002). Consequently, embryos from

older flocks may reach the plateau in oxygen consumption earlier than embryos from younger flocks (Christensen et al., 1996). With these factors in mind, it is possible that the onset of temperature stress during the oxygen consumption plateau in these studies may have been late considering the embryos were from somewhat older parent flocks. This is still somewhat difficult to determine though considering the limited research available related to incubation dynamics and turkey embryos.

The exchange of oxygen and carbon dioxide during incubation impacts development of the embryo, hatchability, and bird quality (Decuypere et al., 2001; Tona et al., 2005). Gas exchange, specifically oxygen availability, is greatly impacted by altitude. Eggs incubated at high altitude demonstrated increased egg weight loss, embryonic mortality, and reduced hatchability (Sahan et al., 2011). It is possible that as availability of oxygen is reduced with increased altitude, the amount of oxygen required by the embryo is reduced as an adaptational response and may result in a shorter incubation time (Julian, 2000). Oxygen availability and carbon dioxide concentration may actually influence the timing of hatch (Hassanzadeh et al., 2002). Would birds incubated at high altitude potentially be more susceptible to enteric disease due to the limited availability of oxygen and consequential shorter incubation time, which may equate to a less mature intestine? It has been suggested that oxygen supplementation during late incubation at high altitude may improve hatchability as a result of reduced stress and increased uptake of oxygen by the embryo (Sahan et al., 2011). Additionally, it is possible that birds incubated at high altitude that have limited availability of oxygen may have a possibly reduced metabolic rate, in which case nutrients would not be as efficiently utilized, which could impact intestinal development. A resulting immature intestine could increase bird susceptibility to post-hatch enteritis.

There may be potential for *in ovo* feeding to mediate some of the negative impacts that incubation temperature stress may have on gastrointestinal tract development. Elevated incubation temperatures during late incubation resulted in decreased gastrointestinal tract weight as a percentage of body weight at hatch compared to embryos exposed to a standard incubation temperature (Leksrisompong et al., 2007). This decreased organ weight likely relates to delayed maturation in the gut. Administration at ED17.5 of a solution containing maltose, sucrose, dextrin, and β-hydroxy-β-methylbutyrate resulted in increased body weights and higher pectoral muscle weights, both of which were maintained to d 25 post-hatch (Uni et al., 2005). Could *in ovo* feeding of turkey embryos help promote intestinal growth and development to compensate for the negative effects of incubation heat stress and therefore allow these temperature-stressed birds to achieve a better start post-hatch and be more equipped to respond efficiently to post-hatch challenge?

The presented series of studies were all conducted in a very regulated and controlled experimental environment. The incubators were not at complete capacity, they contained eggs from all the same flock and flock age, and at hatch the poults did not incur standard hatchery processing procedures (vaccination, sorting, transport). Birds were also reared in battery brooder pens with wire floors in a special facility because of the nature of the pathogens that were used. If these studies had been conducted as field trials in a live production setting, the results may have been different. Eggs set in a large scale, commercial incubator filled to capacity with eggs obtained from different flock ages may experience greater temperature stress than eggs in a small scale production incubator with representation from one breeder flock age. Although embryos may incur less temperature stress when one flock age is incubated at a time, it is presumably more cost effective for several flock ages to be set in the incubator at once. In a commercial

hatchery setting, is stress of the oxygen consumption plateau increased due to the incubator capacity and the increased amount of heat that may be contained within the machine? Once those embryos hatch and arrive at the farm after having incurred the stress of transportation, if they are placed on built-up litter one could speculate that pathogenic exposure would be higher than the scenario demonstrated in these experiments. In these studies, poults were inoculated by hand with TCV and *E. coli*, which are both known to be transmitted by the fecal-oral route. Birds were raised in battery pens in which feces fall into a drop pan below the wire floor of the pen. Birds raised on litter on the floor may be more severely impacted by such enteric challenges because of the direct access of pathogen-containing feces on the litter. The goal of these experiments is to create an industry applicable scenario and setup to examine industry issues. However, there are some parameters in an industry scenario that cannot be replicated in a research-type setting. Although results from these studies were interesting and there were certainly effects of both incubation temperature or challenge, there exists the likelihood of a stronger response to the tested parameters in an industry field trial.

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