EVALUATION OF POULTRY HOUSE HEAT TREATMENT AS AN ALTERNATIVE TO POST OUTBREAK WET CLEANING AND DISINFECTION

by

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ABSTRACT

Management and control of fast-moving poultry diseases such as highly pathogenic avian influenza virus (HPAIV) infections require a combination of steps including biosecurity, surveillance, quarantine, depopulation, disposal, and cleaning and disinfection. The latter are applied at multiple stages of the process to clean and disinfect personnel, equipment and facilities. Traditionally, post-outbreak cleaning and disinfection of poultry facilities has required a combination of wet and dry cleaning, including pressure washing, rinsing and chemical disinfection treatment. Because of the difficulties associated with cleaning and disinfection, a heat treatment program was used during the 2014 – 2015 HPAI outbreak. To obtain data on the efficiency of such a treatment for a surrogate virus and a bacterial pathogen, poultry litter was placed in modified large colony houses. Steel coupons were inoculated with a lentogenic strain, LaSota, of Newcastle disease virus (NDV) and *Salmonella enterica* subspecies enterica serovar Enteritidis to test persistence at time points (TP) over 10 days and at different chicken litter depths: surface or 0 cm, 0.6 cm, 2.5 cm, and 10 cm at temperatures between 38 °C to 48 °C. In total, three trials were carried out. Despite solid recovery of control samples for both the treatment and control houses, virus was recovered only from the treatment house at TP-2 and TP-9 in the first trial, at TP-2 in the second trial, and no recovery in the third trial. Viral recovery from the control house occurred at TP-1 for the first trial, TP-1 and TP-4 for the second trial, and TP-1

during the third trial. When compared to the number of overall samples, recovered samples in the treatment house, resulted in 0.07% positive; recovered control samples resulted in 0.12% positive. In the first trial, the *Salmonella* strain was completely inactivated after four days, likely due to a combination of the heat treatment and ammonia that was emitted by the litter (>100 ppm). In subsequent trials, ammonia concentrations in the treatment house were lower (beginning value for trial 2 was 82 ppm; ending value for trial 3 was 5 ppm). *Salmonella* inactivation was 2.23 log units after 10 days for trial 2 and 1.49 log units after 10 days for trial 3. Results with respect to litter depth for both biological agents were inconclusive as there was no clear-cut depth effect on each. More studies should be conducted to further evaluate the persistence of each organism at prescribed depths of material, temperature, humidity and time during heat treatment programs.

Chapter 1

INTRODUCTION

1.1 Background

Since the late 1800s, when avian influenza (AI) was described in Italy, albeit then known as “fowl plague” and later identified in 1955 as an influenza type A virus, it has struck with devastating severity among avian populations (Lupiani & Reddy, 2009). However, when human infections of avian H5N1 were reported in Hong Kong in 1997, people began to take notice as the World Health Organization (WHO) tracked more cases from Asia to Europe and Africa (Sandrock & Kelly, 2007; WHO, 2020). Since then, human infections with other strains such as H7N7, H7N9 and H9N2 have occurred. H5N1, perhaps the most pathogenic of the group, has infected 861 persons with 455 fatalities for a 53% mortality rate (WHO, 2020). A newer strain, H7N9, has had a quicker impact on the human population with 1,568 confirmed cases and 615 deaths following its identification in 2013 (WHO, 2020). Strains such as H7N7 and H9N2 can be severe in humans but rarely result in death. Human cases of avian influenza virus (AIV) are tracked by the World Health Organization and the Center for Disease Control (CDC) in the U.S (CDC, 2017).

The World Organization for Animal Health (OIE) and the U.N. Food and Agriculture Organization (FAO) track and report high pathogenic AI (HPAI) cases worldwide, as does the United States Department of Agriculture (USDA) for the U.S. (USDA, 2020b). From 2013 to 2018, there were 7,122 reported outbreaks of AIV in poultry and wild birds in 68 different countries. Twelve

different subtypes of the virus were responsible for over 122 million domestic bird losses during this time (OIE AIV Portal, 2020). Although most sources list H1N1 as a swine flu, it is also capable of infecting turkeys which makes it an AIV as well and raises the question if turkeys are also vessels like swine for reassortment of type A viruses (Berhane et al., 2016). Not only are AIV cases tracked but the organizations listed above monitor the major flyways and conduct AIV testing on migratory species (FAO, 2020). Crisscrossing the globe, there are several intersecting points where various AIV strains are deposited and reacquired by different species only to have the disease strains deposited thousands of kilometers away. The migratory flyways help to explain how three different strains: H5N1, H5N2 and H5N8, arrived in the U.S in 2014 and initiated the largest foreign animal disease outbreak of any species in U.S. history (Krauss et al., 2016).

The U.S. highly pathogenic avian influenza virus outbreak of 2014-2015 affected 232 separate premises spanning 21 states with these cases ranging from backyard flocks to one of the country's largest egg production facilities. This outbreak resulted in bird losses of 50 million at a cost of nearly $1.6 billion and with added trade losses, total economy-wide losses were estimated at least at $3.3 billion, placing this outbreak on par with other U.S. natural disasters (Greene, 2015).

Avian influenza can cause high mortality in animals and as a result, tremendous economic losses for their producers. Today, when a poultry grower learns that his flock is infected with AIV, it sets into motion a plan that was revamped largely in part from lessons learned during the 2014-2015

outbreak and involves local, state and federal agencies. This plan is outlined in the United States Department of Agriculture Animal and Plant Health Inspection Service (USDA APHIS) HPAI Response Plan: The Red Book, which covers the strategic elements of the outbreak response process (USDA, 2017a). While this plan lists many actions that must be conducted following an outbreak, there are eight key emergency response steps for any outbreak: detection and quarantine; appraisal and compensation; depopulation; disposal; virus elimination (cleaning and disinfection); testing; restocking; and maintaining biosecurity (USDA, 2017a). In the case of HPAI, entire flocks must be depopulated to stop the spread of the virus and to protect other animals as there is no effective vaccine or treatment upon positive confirmation of infection.

After depopulation and subsequent carcass disposal, traditional cleaning and disinfection (C&D) of all structures contaminated by the virus can take months to accomplish for them to be declared virus-free and production can resume. The U.S. response plan calls for a dry cleaning to remove dried and caked-on organic matter followed by a wet cleaning with water and detergent to get surfaces ready for disinfection (USDA, 2017a). Physically scrubbing all areas during this step greatly reduces the amount of contamination and increases the surface areas on which chemical disinfectants will work. Disinfection follows, and this process may be either with chemicals, heat or via fallowing which is leaving the structure out of production for a minimum of 120 days or longer. Chemicals used for this task must be approved by the Environmental Protection Agency (EPA) specifically

for the elimination of the HPAI virus. Chemicals must be properly mixed or diluted, allowed to set for the required amount of contact time, rinsed properly and areas be allowed to dry. Additionally, efforts must be made to collect any chemical run-off as it could be contaminated or at least, dangerous for the environment.

Heat treatment is the latest addition to the C&D arsenal per USDA guidance, and it could become the method of choice to render premises safe following an AIV infection (USDA, 2016). It is up to the Incident Commander, State Animal Health Officials, and USDA-APHIS if this is the method to be utilized for decontamination and elimination of AIV on infected premises. After dry cleaning, the poultry house is heated between 38 °C to 48 °C for seven days with at least three consecutive days in this range. The advantages of pursuing this method are, less labor required as compared to traditional chemical disinfection, a reduced amount of contaminated chemical waste requiring disposal and lower costs than traditional chemical clean up.

Based upon documented evidence on Newcastle disease virus (NDV) persistence and decontamination studies, the same methods outlined in the USDA document could theoretically be applied to eliminate NDV from premises following an infection. Additionally, it is theorized that *Salmonella* numbers could potentially be reduced proportionally to the temperature and duration of heat applied.

1.2 Avian Influenza Virus

The avian influenza virus belongs to the *Orthomyxoviridae* family and infects over 90 different species of birds worldwide (Guan et al., 2008). AIV is

an enveloped, negative-sense, single-stranded RNA virus composed of eight gene segments which can present in either of two disease forms: HPAI or low pathogenic avian influenza (LPAI). Additionally, the virus can mutate from a LPAI form to a HPAI form. Its effects can range from subclinical to severe in poultry, including respiratory tract disease, reduced egg production and a multi-organ systemic disease with close to a 100% mortality rate (Swayne & King, 2003).

Persistence of AIV depends on many variables. Studies on the effect of pH on infectivity and inactivation of the virus vary in their results; one possible reason for this is because of the number of different viral strains. Most experts agree AIV is affected by acidic or alkaline conditions, but it is unclear how the virus responds to variations in pH and for how long are still inconclusive (De Benedictis et al., 2007). In water, AIV can persist for more than 100 days at 28 ^{o}C, 200 days at 17 ^{o}C and an estimated 1,300 days at 4 ^{o}C (Stallknect et al., 1990; De Benedictis et al., 2007). Temperature has an effect on virus survival. In one study at 28 ^{o}C, AIV lost its infectivity after 24 h. (Shahid et al., 2009). In naturally infected chicken meat, another study exposed breast and thigh meat to temperatures up to 70 °C. The initial temperature was 25 °C and the run-up to attain 70 °C took less than 40 s. Virus titers only began to drop after 27 s at 60 °C. No virus was isolated at 70 °C after 1 s in breast meat and 5 s in thigh meat (Swayne, 2006). A study incorporating various levels of temperature, relative humidity and simulated sunlight directed at AIV deposited on coupons made from basswood, concrete, galvanized metal, glass, and pinewood, sought to determine the level of persistence over a

course of time. Although this study concluded after 13 days, AIV was still persistent in many of the different conditions (Wood et al., 2010). In the case of water, manure and feathers associated with infected birds, feathers were found to harbor infectious virus up to 160 days at 4 ^{o}C and for 15 days at 20 ^{o}C (Yamamoto et al., 2010). When AIV was mixed with chicken manure under different environmental conditions, it was concluded that at a temperature of 32-35 ^{o}C, the virus was completely inactivated after 30 minutes in direct sunlight but remained viable after four days in shade at 25-32 ^{o}C (De Benedictis et al., 2007). However, another study that tested both the effects of temperature and UV light on the infectivity of AIV in manure found that UV light failed to destroy virus infectivity even after four hours. It did confirm, however, that AIV is highly susceptible to heat and desiccation. At 25 ^{o}C, the virus was inactivated within 24 h and at 40 ^{o}C, it was inactivated after 15 minutes (Chumpolbanchorn et al., 2006). When small amounts of dry and wet manure were compared against temperature, AIV survived up to five days at 24 ^{o}C and eight weeks at 4 ^{o}C with no discernable difference between the amount of moisture in the samples (Kurmi et al, 2013). In a recently published study by Stephens & Spackman (2017), the time required to inactivate AIV in poultry litter at different temperatures was investigated. Eight different temperatures, ranging from 10 to 49 ^{o}C were tracked for LPAI and four temperatures, ranging from 10 to 43.3 ^{o}C were studied for HPAI. While heat was expected to have the same effect on both virus types, a subset of this study was to determine if this was true. Results showed that the number of days required to inactivate the viruses are inversely related to the temperature required; i.e. the lower the

temperature, the greater number of days required and both viruses react to heat in much the same manner.

Highly pathogenic AIV is classified as a biosafety level-3 (BSL-3) organism and given the possible risk to other animals, NDV was used as a surrogate for all phases of the current study. There are several reasons for selecting NDV as a surrogate. Both NDV and AIV are single-stranded, negative-sense RNA viruses with helical capsid structures enclosed within a viral envelope. The lipid envelope, surrounded by hemagglutinating and neuraminidase protein structures projecting from it, makes both viruses susceptible to heat and changes in pH. Their structural similarities along with their persistence under similar environmental conditions, make NDV a suitable surrogate for AIV (Benson et al., 2008).

1.3 Newcastle Disease Virus

Newcastle disease is an avian virus-induced disease caused by an avian paramyxovirus-1 (APMV-1) that infects over 250 species of birds. Lentogenic, or low pathogenic, strains of NDV, occur worldwide and often have a much lower mortality rate than the velogenic or virulent (vNDV) strains that are endemic in Asia, Africa and in some South American countries. In 2002, the U.S. experienced its worse outbreak of vNDV that resulted in the death or depopulation of close to four million birds and economic losses of close to $121 million (Brown & Bevins, 2017). As of March 2020, a current outbreak of vNDV in California that began in 2018 has resulted in 476 cases (USDA, 2020). It is particularly devastating to chickens and it is not uncommon for 100% of the flock to succumb to these strains within two to four

days (CFSPH, 2016; Cattoli et al., 2011). NDV is an enveloped, negative-sense, single-stranded RNA virus whose genome is comprised of six genes. Symptoms of the disease in birds include respiratory distress, diarrhea and neurological impairment (Nanthakumar et al., 2000)

Survival data for NDV widely varies. Newcastle disease virus can be inactivated by heat at three hours at 56 ^{o}C or at 30 minutes at 60 ^{o}C. It is destroyed by acids with a pH of 3 or less. Ether and formalin compounds will inactivate it, although formalin is affected by temperature (CFSPH, 2016). Newcastle disease virus has survived in carcasses of frozen poultry for over 730 days and in buried carcasses for up to 121 days (Pirtle & Beran, 1991). At temperatures slightly above freezing, 1-2 ^{o}C, it was isolated from poultry skin at 160 days. One study reported that its survivability in contaminated poultry houses fluctuated with the temperature; seven days in summer, 14 days in spring and 30 days in colder winter temperatures (Kinde et al., 2004). While one study claimed isolation of NDV at 16 days post-depopulation, another study found the virus viable at 255 days indoors at ambient temperatures from -11 ^{o}C to 36 ^{o}C. Finally, at 23-29 ^{o}C it has survived in contaminated litter for 10-14 days and at 20 ^{o}C in soil for 22 days and experimentally contaminated lake water for 11-19 days (CFSPH, 2016). Eggshells, feed, litter, feces and other fomites easily transmit AIV and NDV.

In addition to studies that tested each organism separately, there are several that compared the two viruses during the same test conditions. Both AIV and NDV were suspended in allantoic fluid and dried on glass and stainless steel. Both were reduced to a titer of zero after 30 minutes exposure

to vaporized hydrogen peroxide (Heckert et al., 1997). Another study tested each virus against nine commercially available disinfectants in the following categories: phenolic compounds, quaternary ammonium compounds (QACs), glutaraldehyde, peroxyacetic acid, sodium hypochlorite, and peroxygen. Results showed that phenolics were the most effective, followed by glutaraldehyde and peroxygen against both viruses. QACs were effective to a lesser extent against both, but results improved slightly when used with sodium carbonate for NDV. Sodium hypochlorite worked only on NDV. Of the nine compounds tested, NDV showed a greater percent of inactivation in all but two categories; phenolic compounds and one of the QAC disinfectants worked better on AIV (Patnayak et al., 2008). Peroxygen, bleach and surface decontamination foam (SDF) proved effective in another study against NDV at temperatures below 0 ^{o}C at varying contact times (Guan et al., 2014). However, a separate study comparing five different disinfectants found that Virkon-S™ and H_2O_2 (50% H_2O_2 & Dihydroxybenzole) did not perform well under organic load (Genhan et al., 2009). Commonly available chemicals including acetic acid, sodium hydroxide, calcium hydroxide, sodium carbonate and powdered laundry detergent without bleach were tested against AIV. The virus was dried on steel coupons and treated with each disinfectant for 10 min. The first three chemicals performed well; the last two were not effective in eliminating LPAI (Alphin et al., 2009). A study on the treatment of lab effluent containing HPAIV, LPAIV, vNDV and lentogenic NDV determined that both HPAIV and vNDV could be reduced by 6 $\log_{10}$ in 30 s at 82 ^{o}C and the lower pathogenic versions of these viruses required three minutes to attain the same

6 $\log_{10}$ reduction (Chmielewski et al., 2011). In the absence of dust, glass or other materials that can block UV radiation, UV light deactivates NDV twice as easily as AIV (Sutton et al., 2013).

While these different disinfection studies are important, no study has identified a primary disinfection method of choice following an outbreak. Cleaning and disinfection using harsh chemicals may inactivate AIV but several of the chemicals are not environmentally friendly and proper disposal of the run-off is required. Many of these chemicals and methods are expensive especially in the amounts required to disinfect large areas or facilities and therefore are cost-prohibitive for most farmers. Some of the studies involve methods that, while achievable in a laboratory setting, are not feasible in a large-scale environment. Of all the C&D methods studied, heat disinfection appears to be the most effective and the most suitable for large-scale disinfection.

1.4 *Salmonella*

Occurring world-wide, *Salmonella enterica* causes disease in humans and animals. Some poultry are persistent carriers and shed the bacteria without showing signs of illness themselves. While most healthy adults do not get severely ill from the bacteria, *Salmonella* can be deadly for young children, the elderly and those who are immunocompromised. Signs of infection include nausea, vomiting, cramping, diarrhea, fever, chills, headache and blood in stool. Since 1962, CDC has tracked U.S. *Salmonella* cases and according to CDC estimates, there are 1.35 million infections leading to 26,500 hospitalizations and 420 deaths yearly in the US (CDC, 2020). In 2019, 1,134

of these reported cases were directly linked to contact with backyard poultry (CDC, 2019). In 2010, economic costs as a result of *Salmonella* infections were estimated at $2.71 billion for 1.4 million cases (Andino & Hanning, 2015). More than 2,500 serotypes have been identified for *Salmonella,* but most human infections are caused by less than 100 of them.

Salmonella are ubiquitous as they are relatively hardy and live in a wide range of environments. Host environment for *Salmonella* is not selective as it infects everything from humans to reptiles to birds to even flies which suggest it has a two-stage lifecycle: host and external environment. Factors in the environment such as sunlight, low nutrients, temperature, humidity and pH certainly affect its survival. In soil samples from both agricultural and recreational areas, the organism has survived and multiplied for over a year. This is also true for samples obtained from poultry houses thought to be disinfected (Winfield & Groisman, 2003).

Another attribute of *Salmonella* which enables its hardiness is its ability to form biofilms. Biofilms are essentially clusters of cells that have formed a protective slime or film to protect themselves from harsh environments including disinfectants. When in a biofilm, *Salmonella* is next to impossible to remove without harsh scrubbing as its film layer is virtually impenetrable. Because the bacteria are easily able to form biofilms it becomes a great risk if established in drinkers, waterlines, feeders or tubing (Vestby et al., 2009). Once the bird acquires *Salmonella*, it may not be discovered until it is passed along to consumers as birds do not always show signs of infection. For infections occurring in layer facilities, a few birds become carriers of the

organism and may lay eggs that are internally contaminated with *Salmonella* (Davies & Breslin, 2003). It is thought but not yet proven that the organism can survive for long periods in a viable but nonculturable (VBNC) state (Winfield & Groisman, 2003). If this is true, then it may change the level of persistence growers and producers once thought they had to overcome. Even if a VBNC state is proven false, *Salmonella* species can survive in both fresh and saltwater for up to two months, in septic systems for 15 days and in toilet bowl biofilm samples for up to 50 days.

How then does one eliminate or, more aptly, reduce the numbers of *Salmonella* in poultry-related facilities? Cleaning and disinfection are crucial processes to eliminate *Salmonella* from facilities. It is challenging particularly in caged layer facilities because of the cages, egg and manure belts, feed troughs and drinkers. While studies have shown that formaldehyde is the most effective disinfectant, it is prohibited for use in the U.S. in livestock units because of personnel safety considerations (Davies & Wray, 1995). Following formaldehyde, peroxygen compounds with organic acids and tar oil mixtures were all tried with varying success to eliminate *Salmonella*. In another study, phenolics, quaternary ammonium (QAT), a QAT/formaldehyde product, and a sodium hypochlorite detergent product were used with success. A further recommendation of this study included rotating the disinfectants used so organisms do not build up resistance to any one of them (White et al., 2018). All disinfectants work better after a thorough gross cleaning to remove as much organic load as possible. Finally, compressed air foam systems apply

disinfection products with foam to increase contact time which is useful for transportation cages and layer facilities (White et al., 2018).

Litter treatment by composting and windrowing is another way to reduce numbers of *Salmonella* in poultry houses. Temperatures higher than 65 °C resulted in complete elimination of *Salmonella* in laboratory settings. Moisture content does not have a great effect on pathogens in the middle of the windrow but contributes to organism survival along the edges of the windrows. In comparisons involving turned vs. unturned windrows, turned windrows left *Salmonella* undetectable after six weeks but it was viable after 12 weeks in unturned windrows (Wilkinson et al., 2011).

Although it is desirable for poultry facilities to be completely *Salmonella*-free, it is highly unlikely this can be achieved. High environmental persistence and the ability to form biofilms along with a non-preference for a specific host, make *Salmonella* difficult to eliminate from poultry operations. Layer facilities have the additional burden to keep both layers and eggs free from *Salmonella* as it may be passed directly into the egg before the egg is even laid. Not all birds with *Salmonella* are symptomatic which makes this task even more difficult. By utilizing the methods listed above in combination, the amount of *Salmonella* within poultry facilities may be greatly reduced.

Avian influenza virus, vNDV, and *Salmonella* are capable of infecting humans as evidenced by numbers cited previously. Human infections caused by foodborne *Salmonella* are three orders of magnitude greater than *Salmonella* infections related to poultry; however, the organism remains a significant concern for the poultry industry. Once an outbreak occurs and

dead birds are removed from the facilities, all structures must be cleaned and disinfected prior to them being declared safe. Until recently, the primary means of cleaning and disinfecting poultry facilities following an outbreak included wet and dry cleaning followed by pressure washing, rinsing and disinfecting with chemicals. As previously described, this method of cleaning and disinfection comes with drawbacks. The alternative method of heat treatment was tried during the Spring-Summer HPAI outbreak of 2015. Following successful results, proponents of this method wish to expand its applicability.

Thus, the objectives of the present project were: assess the range of depth, temperature, humidity and time profiles that would have a positive impact on inactivation of organisms; evaluate the suitability of a facility heat treatment process on *Salmonella* and NDV in the laboratory; and evaluate the suitability of a facility heat treatment process on *Salmonella* and NDV in floor reared poultry facilities.

Chapter 2

MATERIALS AND METHODS

2.1 General Set-up

The experiment entailed the use of two large colony houses on the University of Delaware (UD) Farm in Newark, DE. The first large colony house served as the treatment house and the second was the control house. In this project, galvanized steel coupons were inoculated with Newcastle disease virus (NDV) to test virus persistence at various time points (TP) over a period of 10 days and at different depths: surface or 0 mm, 0.6 cm, 2.5 cm, and 10 cm within poultry litter at temperatures over 38 °C. Two replications (A and B) per time point were processed for viruses. In parallel, stainless steel coupons were inoculated with *Salmonella enterica* Enteritidis and persistence was tested with three replicates at the same time points and at the same depths of litter.

To ensure conditions were as similar as possible to those of a commercial poultry house, trials were conducted with previously used litter from the UD farm. While the litter consisted of wood shavings, manure and feathers, care was taken to remove any cake and that the mixture was uniform throughout. The total depth of litter used in the treatment house was 20 cm with a minimum of 10 cm of litter beneath all test coupons to provide a thermal barrier or buffer within each house. Moisture content values were obtained before and after the conclusion of each trial; litter nutrient values before the first trial and after the third trial. To calculate litter moisture values, litter samples were weighed, then heated at 105 °C until no further mass was lost

and reweighed. Litter moisture values were adjusted by adding water and turning the litter until the moisture content was between 25% and 35% which are levels typically found in houses with mature birds. Once in this range and after the litter was uniformly mixed, trials commenced.

The litter test area in the treatment house, H5, measured 2.7 m x 3.35 m x 0.2 m deep. A total of 20 test containers were required, allowing for a four by five row matrix of evenly spaced containers. Once the litter was at optimal moisture content levels, sleeves slightly larger in diameter than the test containers and made from polyvinyl chloride (PVC) pipe were placed into the litter. Most of the litter inside each sleeve was removed with only 5 cm remaining in the bottom of each hole. This was done within 48 h of the trial start to avoid altering litter moisture during the time the house was brought up to starting temperature.

The litter test area in the control house, H6, was maintained in one 76-L PVC concrete mixing tub measuring 61 cm x 91 cm x 19.8 cm deep (The Home Depot). The tub was filled with 10 cm of moisture-adjusted litter from the treatment house and moved to the control house prior to preheating of the treatment house. Another 76-L tub was filled with the same litter for use in filling the containers when the coupons were placed and for a 2.5-cm cover layer for control coupons.

Four heaters were used to achieve the required temperatures in the treatment house. Three heaters were electric infrared tube heaters (Fostoria: OCH-46-120v, Johnson City, TN) and the fourth was an electric forced hot air heater (QMark PT268, 4,000W, Marley Eng. Prod., Bennettsville, SC). All four

were controlled by the central controller (Chore-Time: Model 8, Milford, IN). The electronic controller cycled the heaters in accordance with the pre-set temperature range of 38 - 49 ^{o}C as measured by two independent temperature sensors (Chore-Time: Model 8, Milford, IN); one at litter surface and one at ceiling height. Ventilation to the outside was turned off and shielded for the duration of the trial. As for the control house, one electric forced hot air heater was set to 10 ^{o}C and controlled by the same type of central controller. Even during winter trials, house temperatures reached starting temperatures within 12 h.

Hobo data loggers (Models: MX100 and MX2302, Onset Computer Corporation, Bourne, MA) were used in duplicate for all levels: surface or 0 mm, 0.6 cm, 2.5 cm, and 10 cm measured within the litter of the treatment house. The same type of sensors was also placed in the treatment house at ceiling height, surface, three feet above litter surface and outside. In the control house, Hobo data loggers (Models: MX100 and MX2302, Onset Computer Corporation, Bourne, MA) were placed in the tub of litter at surface and 2.5 cm and in the room at tabletop height. Relative humidity was measured only in the control house as the high ammonia levels in the treatment house interfered with the readings on the Hobo sensor. Sensors logged temperatures every 10 minutes. Ammonia levels in the treatment house were monitored during the second and third trials by Chillgard RT monitors (MSA, Pittsburg, PA).

2.2 Laboratory Set-up

Virus seed stocks were propagated in specific pathogen free embryonated eggs from a low-passage, LaSota strain of NDV from stocks originally propagated on 10/15/2009. Chorioallantoic fluid (CAF) from these eggs was aliquoted in 1.5-mL quantities into cryopreservation tubes and kept frozen at -80 °C until used. The virus, titrated using standard techniques as outlined by Reed and Muench, had a titer of $10^{9.5}$ embryo infective dose 50 (EID_{50})/mL (Reed & Muench, 1938). This same seed stock was used for all phases of the project.

Salmonella enterica Enteritidis Nal^R (*S. enterica*) was from the strains collection of the Department of Animal and Food Sciences, University of Delaware. This specific strain was chosen because it is resistant to nalidixic acid which will enable it to be distinguished from any other strains that might be in the litter. It was retrieved from frozen stock and plated onto Tryptic soy agar (TSA) plates. The plates were incubated overnight at 37 °C and a single colony was retrieved and streaked onto TSA and Xylose Lysine Deoxycholate (XLD) agar containing 40 μg/mL of nalidixic acid to verify the specific identity of the strain. A single colony was inoculated into 20 mL of Tryptic soy broth (TSB) and the culture was incubated overnight at 37 °C with shaking at 250 rpm. Colony counts were obtained by plating dilutions of cultures on XLD agar with 40 μg/mL of nalidixic acid. This same seed stock was used for all phases of the project.

Virus coupons were galvanized steel of a standard size of 2.2 cm x 2.2 cm x 0.2 cm. Stainless steel fender washers 2.5 cm in diameter were used for bacteria because preliminary trials revealed that the bacteria did not persist on

galvanized steel; possibly a result of its zinc content. All coupons were washed three times with detergent (Alconox^{R}, Fisher Scientific, product no. 16-000-104) and rinsed with deionized water prior to sterilization at 121 ºC for 30 minutes before use. The two distinct types of coupons facilitated processing of each organism because it was easier to distinguish between the two materials.

On the day of use, virus was thawed at room temperature and diluted 1:3, combining virus seed stock with phosphate buffered saline (PBS) to make virus working stock. This virus working stock was further diluted 1:20 with fetal bovine serum (FBS) to simulate an organic load and to stabilize the virus (OIE, 2018). This is also to maintain consistency with the procedure used throughout all phases of this project.

In a biosafety cabinet, autoclaved metal coupons were placed on sterilized aluminum foil for inoculation. A total of 408 galvanized steel coupons were inoculated with 100 μL of the virus suspension. Additionally, 408 stainless steel fender washers were inoculated with 25 μL of *S. enterica* Enteritidis Nal^{R} overnight culture containing approximately 7.5 x 10^{7} colony forming units (CFU) per mL. Once inoculated, all coupons were left to dry in the biosafety cabinet. For the second and third trials, coupons were placed four per strip onto strips of tape and then inoculated on aluminum foil. The tape assisted greatly with recovery of the coupons from the litter after heat treatment. Once dry, coupons were placed into 20 cm cardboard boxes lined with aluminum foil. The boxes were double bagged for transport to the control house.

Along with coupons, viral wet controls were prepared and frozen by adding 0.2 mL of viral inoculum plus 1.8 mL of PBS into the first tube. Ten-fold serial dilutions were performed from 10^{-1} to 10^{-10}; however, the tubes were not processed. The purpose of these wet controls was to measure the viability of the virus applied to the coupons before being subjected to test conditions outside of the laboratory. Wet controls would only be processed in the case of zero virus recovery post heat treatment and zero virus recovery for control samples.

2.3 Trial Set-up

Several different methods to contain bacteria-treated coupons during treatment were considered. Since the virus was a low pathogenic strain of Newcastle disease virus and all poultry on the farm are routinely vaccinated, use of this virus during trials did not hold the same concerns as the use of a pathogenic bacterium. A wide variety of options were considered but ultimately both the inoculated coupons and washers were loaded into hermit crab cages which were locally purchased. Each of the 20 cages was 20 cm in both diameter and in height and resembled one-gallon paint cans with handles. Each container had 0.6 cm wire screen secured to a solid plastic bottom. The upper edge of the wire was secured to a similar plastic ring with a removeable screw-type lid in the top center. The wire screen on the side allowed for air and moisture transfer to the coupons while the height of the cage allowed for all four depths for one day to be tested in a single container. The wire handle made it easy to emplace and remove the container from the litter test area. Prior to test day, containers were measured and marked for

the depths tested. Marks corresponding to each depth were placed on two opposing sides so that the coupons would be level during the test.

Figure 2.1 Hermit crab cage with depth marks corresponding to the levels at which the coupons are buried. Note: The litter has settled post heat treatment.

On test day in the control house the containers were loaded with the inoculated coupons in the following manner. Litter was placed in the bottom of the container up to a level of 5 cm deep (at the 4 in. mark in the photo). Two strips of coupons, one viral and one bacterial, were gently placed face up on top of the litter. Next, 7.5 cm of litter were layered on top of the coupons in the container. One more strip of each type of coupons was placed on top of this litter (at the 1 in. mark in the photo) and covered with 1.9 cm of litter. Another strip of each type of coupons was placed on top of this litter (at the ¼ in. mark in the photo) and covered with 0.6 cm of litter. Finally, one strip of each type

of microorganism was placed on the surface of the litter and the container lid was closed only for transport to the treatment house.

Once the containers were moved to the treatment house, they were placed inside the PVC sleeves in a random manner (Microsoft Excel Random Generator) with a new randomization pattern for each trial. Sleeves were removed and litter was filled in around the container and leveled to the top of the litter inside the container based upon the marks on each side of the container. The litter was not packed tightly around the containers, but large airspaces were eliminated. Lids were removed at this point and any stray litter was removed from surface coupons. Coupon strips serving as test controls were placed on top of the litter in the center of the treatment house and were removed after 1 h.

One strip for each microorganism for each time point was placed on top of the 10 cm of litter in the mixing tub to serve as controls. These coupons were covered with 2.5 cm of litter and again, one strip for each microorganism for each time point was placed on the surface of this layer.

2.4 Recovery and Sampling

The two containers for each time point were retrieved from the treatment house and taken to the control house. Coupon strips for both microorganisms were gently removed from each layer in the container and separated. Coupons were placed one per well in a row of a pre-labeled 6-well plate for a total of three coupons per depth. The additional coupon was recovered but not used unless one of the others was otherwise mishandled. Coupons from the “A” and “B” replicates of the same depth were not placed in

the same plate in case the entire plate was mishandled. The lid was securely taped, and the plates were double contained for transport to the laboratory.

2.4.1 Virus Recovery and Titer Determination

The virus trial consisted of the treatment trial with two replicates, "A" and "B" and a control trial. These trials required 408 coupons and a total of 1,570 specific pathogen-free (SPF) eggs to process samples for all timepoints. Recovered samples were diluted 10^{-3} and each dilution required five eggs for a total of 15 eggs per depth per TP. Additionally, samples from TP 0 from the treatment house and the control house were diluted to 10^{-8} respectively to serve as a test controls and positive controls.

Once in the lab, the plates containing virus-inoculated coupons were opened and three replicate coupons were processed at a time. The remaining row was covered with the lid of the plate to protect the coupons from cross-contamination during processing. Once covered, 2.0 mL of PBS was pipetted into each well and the coupons were scraped with the pipette. The fluid in each well was aspirated from the well and then released back onto the coupons to wash off additional adherent virus. This process was repeated three times. All fluids from the wells of a single time point were pooled into a single tube, vortexed and frozen at -20 ^{o}C until samples from all time points for the trial were collected and processed.

Since the day 0 TP for dried virus was of known titer, the sample from the control house served as a positive control. Three separate tubes of this sample were frozen at -20 ^{o}C so different time points could be processed at

different times to keep the number of eggs processed at a time to manageable numbers.

For the determination of virus titers in the frozen samples, the samples were thawed at room temperature. The fluid was vortexed and 10-fold serially diluted from 10^{-1} to 10^{-3} by adding 0.5 mL to 4.5 mL of PBS with antibiotics (450 mL of PBS with 150mL of Penicillin/Streptomycin, BioWhittaker, catalog no: 09-757F). Following dilution, 0.2 mL of solution from each dilution were inoculated into the chorioallantoic sacs (CAS) of five, 9-11 day-old, embryonated SPF chicken eggs and candled daily for five days. Mortality was recorded each day and on the fifth day, eggs were placed at 4 ˚C overnight.

2.4.1.1 NDV Hemagglutination Activity (HA)

Chorioallantoic fluid (CAF) was collected from each egg and tested for NDV hemagglutination activity (HA). Washed chicken red blood cells (RBC) were obtained by mixing whole blood with 50% Alsever's solution (Sigma-Aldrich, St. Louis, MO), centrifuging at 1,000 g for 10 minutes and re-suspending the cells in PBS three times. Finally, 0.5 mL RBCs were mixed in 99.5 mL of PBS to produce a final dilution of 0.5%.

HA tests were performed by adding 50 μL of PBS to each well of a 96-well, U-bottom microtiter plate. Next, 50 μL of CAF were added to the first well of each row allocating one row per fluid sample and ensuring there was one control per every five plates. Two-fold serial dilutions were made beginning at this well across the eight wells of the row. Once dilutions were complete, 50 μL were removed from the last well so that all wells contained equal amounts of fluid. Once 50 μL of 0.5% RBCs were added into each well, the plates were

covered, and the contents were allowed to settle for 40-50 minutes. Control RBCs (no virus) settled to a distinct “button” at the bottom of the well; wells that formed a button which indicated no agglutination were considered HA negative (Killian, 2014).

Samples that showed HA were considered positive for NDV and those that did not show any HA were considered negative for NDV. HA tests were performed on each egg individually and were used to determine a mean EID_{50} for all tests. At a minimum, if complete HA activity was evident by the third well in the series, then the egg was counted as positive for virus with a minimum HA titer of 1:8 (2^3 or $\log_2 3$ if expressed as the reciprocal) based upon the dilution.

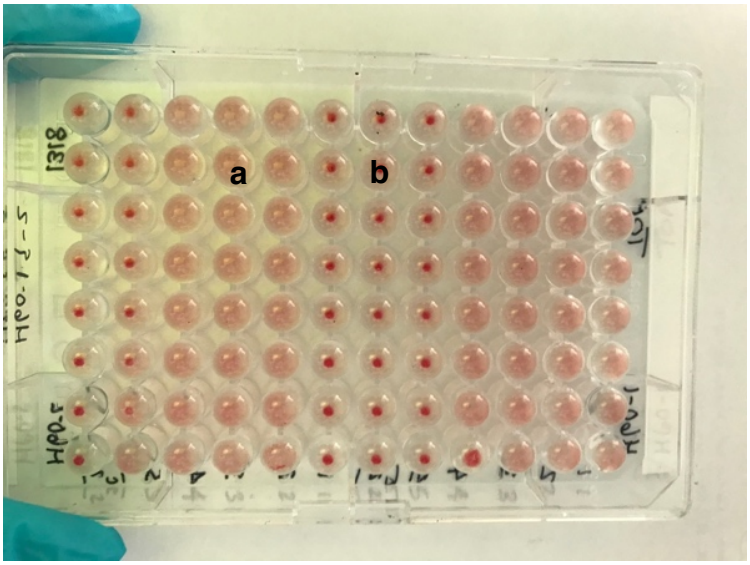

Figure 2.2 Ninety-six well microtiter plate depicting hemagglutination activity (HA) results from TP-0; (a) positive and (b) negative HA samples were processed from the control house (H6). Equal parts (50μL) of sample and phosphate buffered saline (PBS) were serially diluted before addition of red blood cells (RBC). In this Figure, sample rows run from bottom to top.

2.4.1.2 NDV Hemagglutination Inhibition (HI) Activity

All samples with a positive HA result were tested for hemagglutination inhibition (HI) with NDV anti-sera containing antibodies to the NDV virus or antigen to determine if NDV was the cause for agglutination. When present in the correct ratios, antibodies bind to the antigen and inhibit the RBCs from binding. If inhibited, the RBC settle to the bottom of the well and form buttons. This is read as a positive HI; a negative HI results from the virus' inability to bind with the antisera and its binding with the RBCs causing hemagglutination. NDV antisera (Charles River, Lot # C0112B, Norwich, CT) was diluted prior to HI testing. Two dilutions, 1:5 and 1:10 were tested against the virus seed stock to determine the best virus to antiserum (or antigen to antibody) ratio for

HI testing. Other protocols allow dilution of the antisera based upon the level of positive HA; however, the volume of samples for each run precluded this from happening. If the sample was a “high positive” HA, the 1:5 dilution was chosen: if a “low positive”, it was diluted 1:10.

The assay was performed by adding 25 μL of PBS to each well across 12 columns (one row) for each dilution. Then 25 μL of virus seed stock were added to the first well of three rows. This part was done in triplicate, i.e. three rows for 1:5 and three rows for 1:10. Two-fold serial dilutions were made across the rows, and 25 μL were removed from the last well. One row each for a positive (virus, antisera, RBCs & PBS) and negative (PBS and RBCs) control was added to the plate. Next, 25 μL of the 1:5 or the 1:10 diluted antisera were added to each well in the appropriate rows. The plate contents were allowed to settle for 30 minutes at room temperature. Finally, 50 μL of RBCs were added to each well (including controls) and gently swirled to mix. After the contents were allowed to settle for 40-45 minutes at room temperature, the plates were read in the same manner as the HA plates. Agglutination was assessed by tilting the plate. Only those wells in which the RBCs streamed at the same rate as the control wells were counted as showing inhibition.

CAF samples that were positive HA for NDV were next assessed for positive HI in the same manner as described above. Based upon the titer of the positive as assessed by the number of wells that agglutinate during the HA process, the appropriate dilution, either 1:5 or 1:10, of antisera was chosen to confirm the HI. If results were less than definitive, the assay was repeated

with both dilutions. Agglutination was assessed by tilting the plate. Only those wells in which the RBCs streamed at the same rate as the control wells were counted as showing inhibition or positive for NDV.

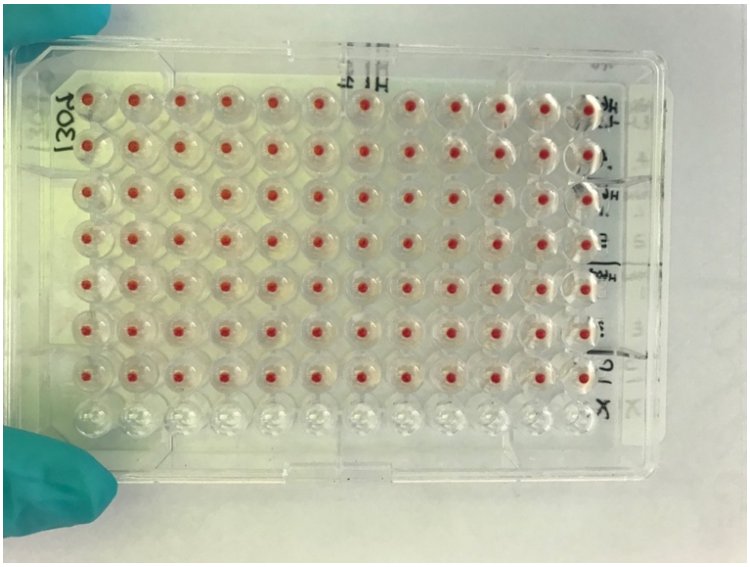

Figure 2.3 Ninety-six well microtiter plate depicting hemagglutination inhibition (HI) positive samples. Equal parts (25μL) of sample and phosphate buffered saline were serially diluted before addition of 25μL of NDV antiserum. Contents were allowed to settle for 30 min followed by addition of 50μL of red blood cells and another settling period of 40 – 45 min. In this Figure, sample rows run from right to left.

2.4.2 Bacteria Recovery

Bacteria trials required the same number of coupons per TP as the virus trials. Unlike virus TPs, bacteria TPs were processed daily to avoid potential inactivation of the bacteria as a result of freezing and thawing.

Coupons were processed separately, and each sample was diluted to the point where 30-300 colony forming units (CFUs) were visible on XLD plates. As the trials progressed, it was necessary first to revive injured cells with Salmonella Enrichment Broth (Sigma Aldrich, product # 84370) and then apply the more selective medium, Rappaport-Vassiliadis R-10 Broth, 3M, product # BP0288500) to determine if *Salmonella* was present or not.

The *Salmonella*-inoculated washers were processed in the same manner; however, 3.0 mL of PBS was used for each well. A new serological pipette was used for each coupon since each was a separate replicate for a total of three per depth. From the liquid in each well, 100 μL were serially diluted from 10^{-1} to 10^{-6} with 900 μL of PBS serving as dilution medium in each tube. Four spots containing 2.5 μL each (total of 10 μL) for each dilution were spotted onto XLD agar plates containing nalidixic acid. One hundred microliters of undiluted wash solution were also plated on XLD agar to ensure the detection of *Salmonella*. The remaining liquid in each well was mixed with 3 mL of *Salmonella* Enrichment broth (Sigma Aldrich, product # 84370) and incubated at 37 ºC overnight. Following incubation, a 50-μL sample from the enriched culture was plated onto XLD agar containing nalidixic acid to confirm the presence of *Salmonella* and 100 μL of this same culture were added to 900 μL of Rappaport-Vassiliadis R-10 broth (3M product # BP0288500) and incubated overnight at 37 ºC. The next day, 50 μL of this culture were plated on XLD agar plates containing nalidixic acid to confirm the presence of *Salmonella*. The endpoint for serial dilutions was adjusted to colony counts achieved from the previous sampling TP. As the titer decreased, so did the

number of dilutions of the next sample. Along with this decrease in titer, two plates of 100 μL, instead of 10 μL, for each target dilution were often required. As for the original titer, a 10-fold serial dilution from 10^{-1} to 10^{-6} using PBS was made from the coupon inoculum. Aliquots of the dilutions were plated onto XLD agar plates and incubated overnight at 37 °C to determine the titer. Day 0 TP samples from the test house and the control house served, respectively, as test and positive bacteria controls as they were processed 1 h after beginning heat treatment.

Chapter 3

RESULTS

3.1 Environmental Conditions

3.1.1 Temperature

For the duration of all three trials, temperatures within the treatment house achieved and maintained the applicable test range of 38 - 49 °C. Thermal imaging (FLIR Model E8, FLIR Systems Inc., Arlington, VA) was used to measure surface temperatures of the litter and coupons. Trials were conducted during October, January and February with outdoor temperatures ranging from 2 ºC – 23 ºC; -8 ºC – 17 ºC; and -1.5 ºC – 13 ºC during the respective trial periods. The following Figures depict average daily temperatures within the treatment house and outside.

The 10-cm depths did not maintain the test temperatures throughout the trial. Temperatures at this level were at the appropriate level at the beginning of each trial because of the exposed holes prior to emplacement of the test containers. Once the containers were emplaced, these temperatures decreased to and remained at an average of 35 ºC; 33 ºC; and 29 ºC among the trials.

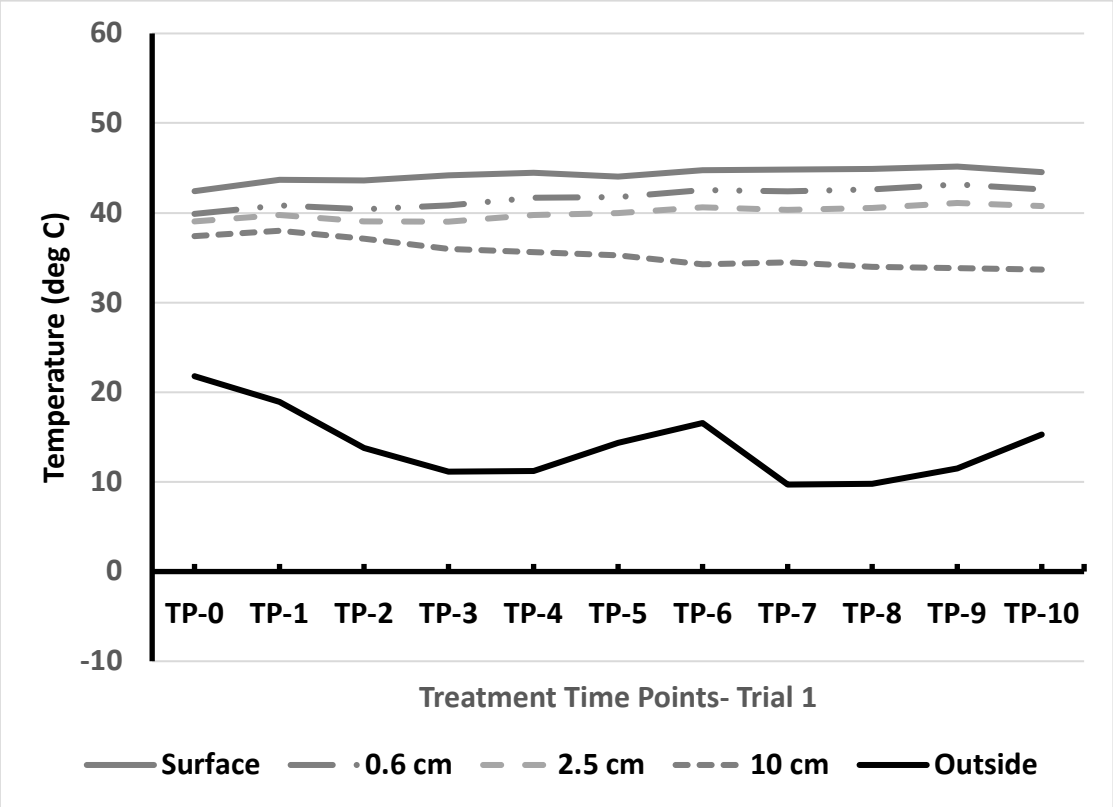

Figure 3.1 Trial 1 (October) mean daily outside temperatures compared with mean temperatures per depth within the treatment house. Hobo data loggers (Models: MX100 and MX2302, Onset Computer Corporation, Bourne, MA) were used in duplicate for all levels.

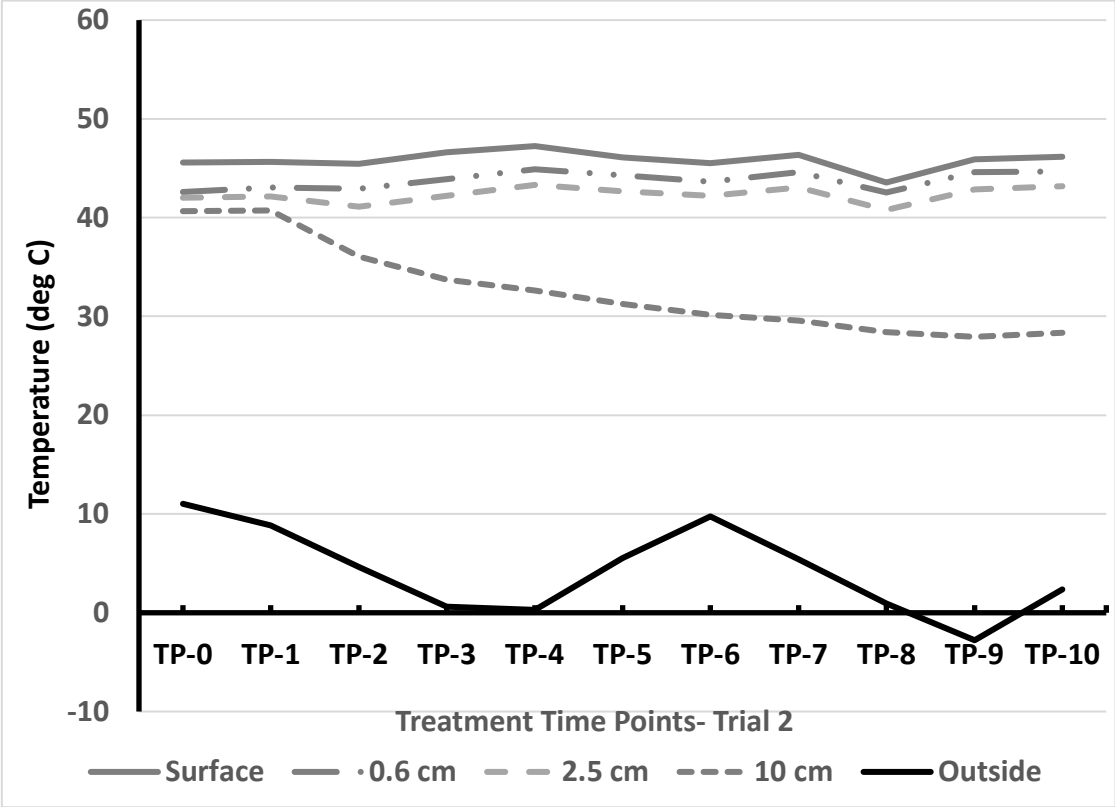

Figure 3.2 Trial 2 (January) mean daily outside temperatures compared with mean temperatures per depth within the treatment house. Measurements were obtained as described for Figure 3.1.

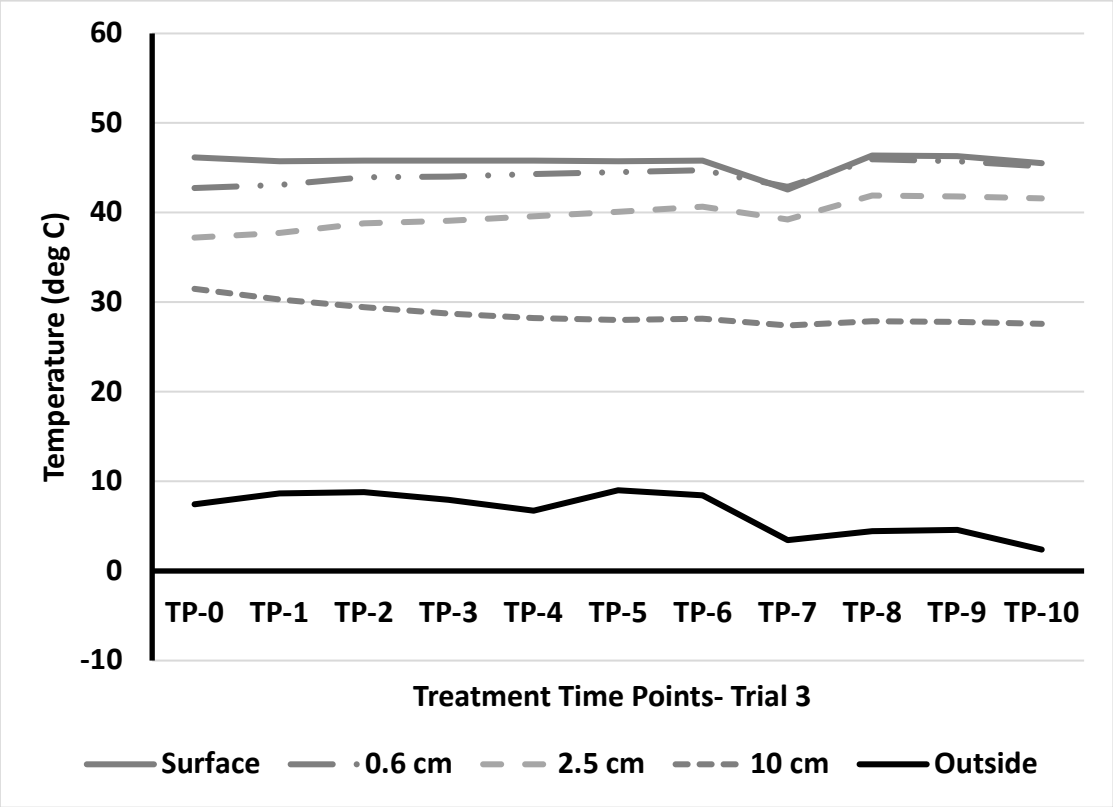

Figure 3.3 Trial 3 (February) mean daily outside temperatures compared with mean temperatures per depth within the treatment house. Measurements were obtained as described for Figure 3.1.

3.1.2 Humidity, Litter Moisture and Ammonia

Relative humidity (RH) values were recorded during the assessment of depth, temperature, humidity and time profiles during the first phase of this project but were not measured during the trials. Litter moisture was closely monitored and despite extreme temperatures, overall losses in soil moisture in each trial were 15.6%, 8.6%, and 17.7%, respectively. The lowest trial endpoint moisture percentage of all the trials was 13% during the first trial. In trial 1, high ammonia levels were present and measured manually at TP-4 with a reading >100 ppm as depicted on the Draeger ammonia tube scale 0-100 ppm. A follow-up reading was taken at TP-5 using the Draeger ammonia tube scale of 2-600 ppm, but at this point, levels had fallen to approximately 80 ppm and were not tracked after this. For the second and third trials, ammonia sensors were employed. The following charts depict the ammonia levels in House 5 as recorded during the second and third trials for TP-0 through TP-10. Ammonia values were at their highest at the onset of the second trial with a value of 82 ppm and at TP-10, the value was 12 ppm. House 6 values are not shown for either trial, however, the highest recorded value for both trials is 1 ppm. For the third trial, ammonia levels were markedly lower across all time points as shown below in Figures 3.4 and 3.5.

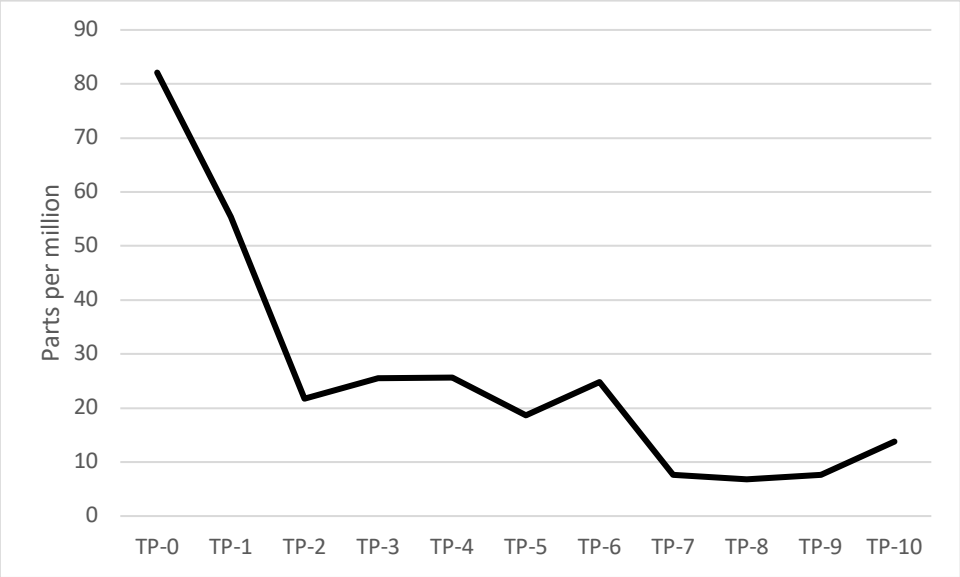

Figure 3.4 Trial 2 (January) treatment house ammonia levels as measured 0.5 m above the litter. Measurements were obtained using Chillgard RT monitors (MSA, Pittsburg, PA) and recorded at the time of coupon retrieval.

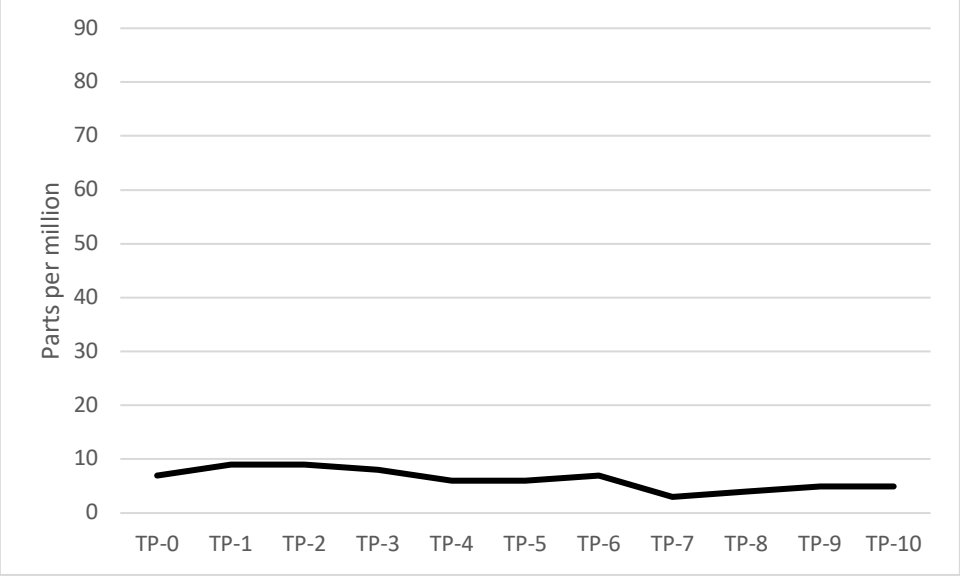

Figure 3.5 Trial 3 (February) treatment house ammonia levels measured as described for Fig. 3.4.

3.1.3 Litter Conditions

The litter was analyzed prior to the first heat treatment run and following the final run. The pH on average across all three trials was slightly basic and attributed to the presence of ammonia (NH_3) and ammonium (NH_4^+). Litter analysis confirmed NH_4^+, while NH_3 was detected through the use of a manual Draeger ammonia sensor. However, at the end of trial 3, most of the ammonia had volatilized from within the litter and measurements were well below that of the first trial. The following tables depict start and end values for key litter components.

Table 3.1 Physical and chemical properties of the litter samples on a wet weight basis at the start and end of trials. Data were generated by the Soil Testing Laboratory at the University of Delaware.

Sample ID	Moisture (%)	Solids (%)	Sample pH	Total N (%)	Total C (%)	C:N Ratio	NH4-N (mg/kg)	NO3-N (mg/kg)
Trial 1 -start	28.6	71.4	8.3	1.182	31.53	26.7	774.0	60.5
Trial 1 - end	13.0	87.0	8.5	1.565	35.55	22.7	657.0	76.5
Trial 2 - start	25.9	74.1	7.9	1.235	32.13	26.1	526.5	280.0
Trial 2 - end	17.3	82.7	8.1	1.273	26.48	20.8	433.5	121.5
Trial 3 - start	39.0	61.0	8.1	1.026	25.64	25.3	479.0	603.0
Trial 3 - end	21.3	78.7	7.8	1.755	31.36	18.1	281.4	971.3

Table 3.2 As above, litter samples on a dry weight basis.

Sample ID	Total N (%)	Total C (%)	C:N Ratio	NH4-N (mg/kg)	NO3-N (mg/kg)
Trial 1 -start	1.655	44.16	26.7	1084.0	84.7
Trial 1 - end	1.799	40.86	22.7	755.2	87.9
Trial 2 - start	1.665	43.33	26.1	710.1	377.6
Trial 2 - end	1.549	32.19	20.8	525.5	147.3
Trial 3 - start	1.681	42.00	25.3	784.7	897.8
Trial 3 - end	2.221	39.96	18.1	281.4	1282.7

3.2 Newcastle Disease Virus Results

The effect of heat treatment on the persistence of NDV was inconsistent throughout all three trials. Care was taken to retrieve samples at the same time each day they were pulled. Since each trial required 1,570 SPF eggs to process all the samples, each trial was divided into thirds, or runs, to keep the amount of required eggs to a manageable number. Storage times for each trial (T1, T2, or T3) and its three runs (R1, R2, or R3) are in Table 3.3.

Table 3.3 Storage times for recovered virus samples from trials 1 (T1), 2 (T2) and 3 (T3) at the various time points (TP)

Run ID	TP Sampled	Eggs Processed	Weeks Frozen
T1 R1	0, 1, 2, 4, & 6	670	1
T1 R2	3, 5, 7, & 8	600	11
T1 R3	9 & 10	300	18
T2 R1	0, 3-8 (Surface & 0.6 cm)	520	7
T2 R2	3-8 (2.5 & 10 cm); H6-0	490	10
T2 R3	1 & 12; H6-0	340	18
T3 R1	0, 3-8 (Surface & 0.6 cm)	520	9
T3 R2	3-8 (2.5 & 10 cm); H6-0	490	11
T3 R3	1 & 2; H6-0	340	16

Note: For trials 2 & 3, H6-0 controls were repeated for comparison during processing of R2 & R3.

3.2.1 Trial 1- October

Initial analysis was conducted for samples TP-0, TP-1, TP-2, TP-4, and TP-6 to allow for processing samples in smaller batches. The second batch of samples was from TP-3, TP-5, TP-7, and TP-8. The remaining days, TP-9 and TP-10 were processed last. The results from the control house, H6, showed recovery at the one-hour time point, otherwise referred to as TP-0,

and one sample from TP-1 but no further recovery after that day. These samples were highly positive through the -5 dilution. Treatment house, H5, controls also retrieved one hour after the start of the trial, rendered positive results through the -6 dilution. Additionally, two positive samples were recovered on day two and day nine: TP-2 – 0.6 cm and TP-9 – 10 cm. Table 3.4 provides a roll up of depths by time point of recovered positive samples for all three trials.

3.2.2 Trial 2- January

Samples for this trial were processed in the following groups or batches. The first group consisted of TP's 3 - 8 and surface and 0.6 cm levels along with TP-0 controls for H5 and H6. The second group consisted of the 2.5 cm and 10 cm samples for the same days. The final group of this trial included TP-1 and 2 for all depths; TP-9 and TP-10 samples were not processed as there were no positives for any days after TP-4 from the controls. Positive samples recovered from this trial were the TP-0 controls from both the control and treatment houses, through the -4 dilution and the -3 dilution, respectively. Two more positives from the control house, a TP-1 surface sample and a TP-4 2.5 cm sample were recovered. Additionally, a single 2.5 cm sample from TP- 2 from the treatment house was recovered.

3.2.3 Trial 3- February

Samples from this trial were processed in the same groups as with Trial 2. Again, TP-9 and TP-10 were not processed. Positive samples recovered for this trial were TP-0 controls from the treatment and control houses; both

only through the -3 dilutions. One additional sample from the control house at TP-1 surface level proved positive.

Table 3.4 Detection of NDV by hemagglutination activity (HA) from coupons located at various depths within the litter and retrieved at indicated days (TP)- Depth by time point by trial.

Time Point-> Depth	TP 0	TP 1	TP 2	TP 3	TP 4	[4]	TP 9	TP 10
H5-Surface	+[1,2,3]	-	-	-	-		-	-
H5-0.6 cm		-	+[1]	-	-		-	-
H5-2.5 cm		-	+[2]	-	-		-	-
H5-10 cm		-	-	-	-		+[1]	-
H6-Surface	+[1,2,3]	+[1,2,3]	-	-	-		-	-
H6-2.5 cm		-	-	-	+[2]		-	-

Note: All three trials are depicted. [1.] Trial 1. [2.] Trial 2. [3.] Trial 3. [4.] TP's 5 – 8 are omitted from this table as there were no positive samples recovered during these periods.

3.3 Salmonella Results

As with NDV, samples were retrieved at the same time each day of all three trials. Heat treatment had a significant effect on the persistence of the bacteria, particularly for the first trial. Starting titers for all three trials were above 10^8 and were reduced throughout the duration of all three trials in the treatment house. In the control house; however, positive samples were recovered through TP-10 from all depths. Like viruses, results exhibited high variability across all three trials. As recovery became more difficult as a result of drops in titer for certain samples, it became necessary to enrich the recovery medium overnight to revive injured but viable cells prior to plating as

explained in the methods chapter. If cells were revived via this process, then the samples were assigned counts based on the detection limit for graphing.

3.3.1 Trial 1- October

The initial titer for this trial was 10^8 CFU/mL. Positive samples were recovered from both depths, surface and 2.5 cm, from the control house through TP-10. Treatment house recovery was lower with no positive samples recovered post Day 3.

3.3.2 Trial 2- January

Initial titer for this trial was 10^{11} CFU/mL. Based upon the results from the first trial, the titer for this trial was as high as possible to attempt to obtain results from as many days as possible. Every depth experienced a sharp decline in log (CFU) from TP-1 – TP-3 with the exception of the 2.5-cm level in the control house. This level remained within one log (CFU) value through Day 9.

3.3.3 Trial 3- February

Initial titer for this trial was 10^9 CFU/mL. Titers for recovered samples also declined in titer at a slower rate than those of the first trial. The 10 cm level showed the greatest drop in titer consisting of approximately three Log (CFUs) which directly corresponds to the depth which required the most enrichment to revive enough cells to obtain counts.

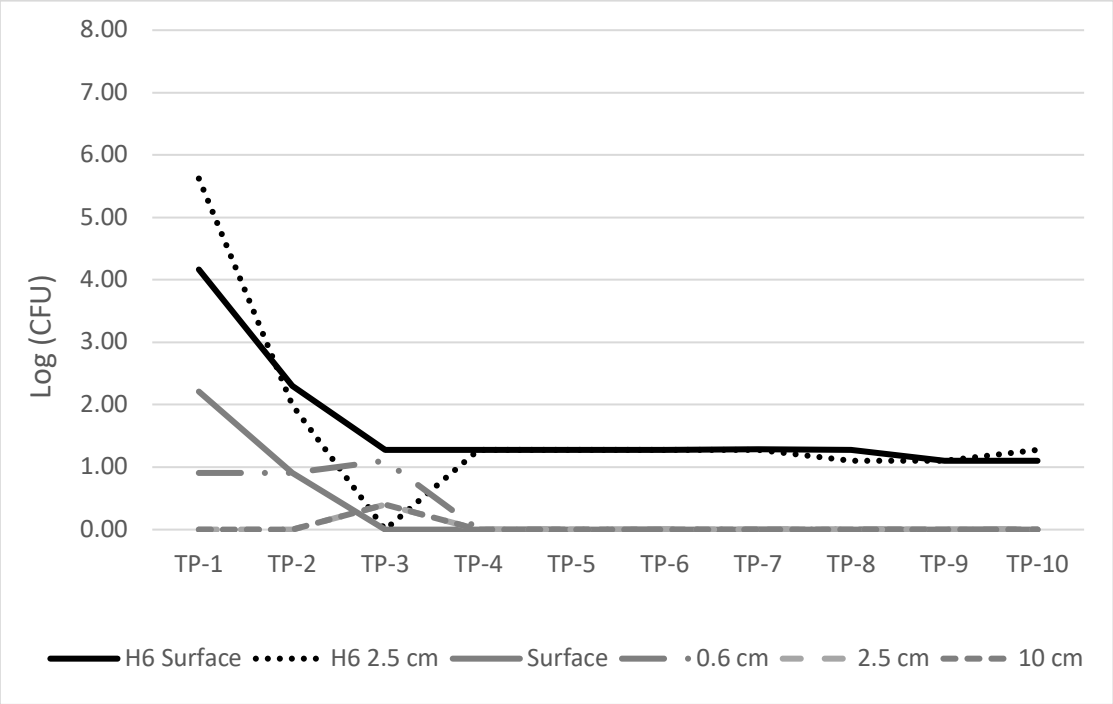

Figure 3.6 Trial 1 (October) Log (CFU) of *Salmonella* recovered of days 1 through 10 (TP-1 to TP-10) from coupons located at various depths in the litter. Counts were obtained by direct plating. Samples that did not provide direct counts but were positive for *Salmonella* after enrichment in *Salmonella* broth and culture in Rappaport-Vassiliadis medium were assigned counts of 1.99×10^2 CFU/mL.

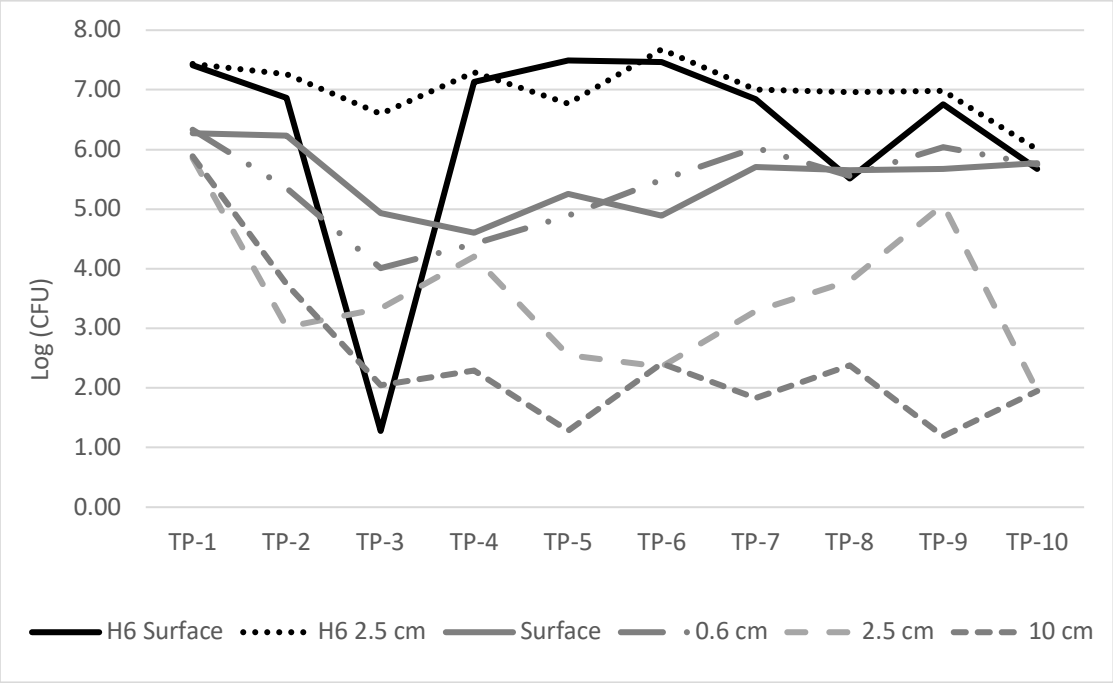

Figure 3.7 Trial 2 (January) Log (CFU) of *Salmonella* recovered at days 1 to 10 as described for Fig. 3.6.

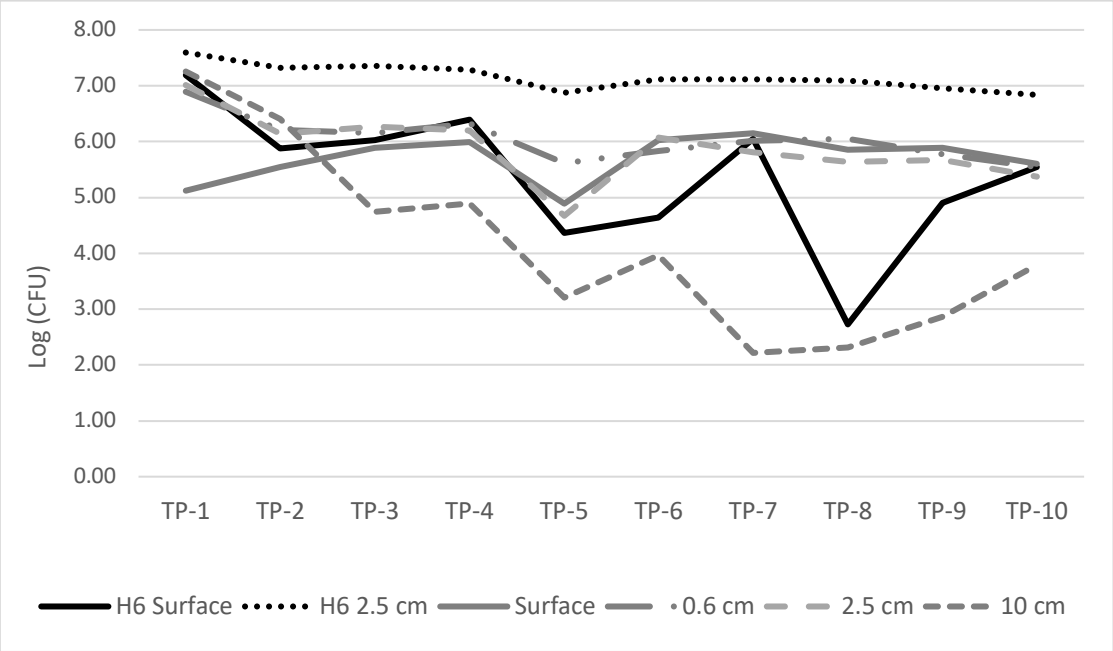

Figure 3.8 Trial 3 (February) Log (CFU) of *Salmonella* recovered at days 1 to 10 as described for Fig. 3.6.

Chapter 4

DISCUSSION

4.1 Environmental Conditions

4.1.1 Temperature

Despite cold winter temperatures, it was not difficult to attain the required temperatures to conduct the trials. However, once the pre-dug holes were filled with the test containers, the litter temperatures failed to remain between 38 °C to 48 °C at the 10 cm level. At lower temperatures, viability counts of *Salmonella* recovered from that level were significantly lower than those from other depths. It appeared that the concrete floor in the treatment house had an effect on lower depth temperatures during the last two trials when outside temperatures were the coldest. In the treatment house, the concrete floor was at ground level with the slab extending past the walls of the house. This configuration left it exposed to the outside at ground level. Therefore, this slab did not have an insulation barrier to the elements and was unable to maintain the required temperature at 10 cm depth. It was unclear during this project how much of an effect the type of concrete floor of the treatment house had on overall litter temperatures and if the same results would be realized in typical poultry barns with a concrete floor without exposed edges or a dirt floor. According to one study, general thermal comfort conditions during a grow-out period were not different between concrete and hard-packed floors (Abreu et al. 2011). It would be a worthwhile temperature study for future endeavors.

4.1.2 Humidity, Litter Moisture and Ammonia

As previously noted, increased temperatures did have an effect on the reduction of *Salmonella* for the entire project with the first trial resulting in the lowest *Salmonella* recovery of all the trials. It was initially thought that increased temperatures resulted in increased ammonia levels coming from the litter. It may not just be a result of increased temperatures releasing ammonia but a combination of moisture and high temperatures as this trial saw the largest drop in moisture percentage of the three trials. Research has shown that increased moisture levels in the litter when combined with higher temperatures, aid ammonia volatilization up to a certain level (Miles et al., 2011). Although this project reached the upper temperature level of 40 °C, none of the trials reached the moisture range of 47 – 51% for maximum NH_3 volatilization as mentioned in the study. It was unfortunate that relative humidity could not be recorded to assist with tracking moisture released from the litter and into the air. Increased ammonia levels at the end of the high temperature portion of the preliminary phase rendered the RH sensors inoperable. This is important in that while moisture is beneficial to bacteria survival; ammonia above certain levels is not (Abreu et al., 2009). It may be a coincidence but the mean outside temperatures in trials two and three bear a slight resemblance to the plots of the ammonia levels recorded in the same trials despite the house temperatures. The interaction of moisture, heat and ammonia concentration warrants further study to provide growers a better means to reduce *Salmonella* between flocks.

4.1.3 Litter Conditions

Litter pH levels were slightly basic throughout all three trials with the pH ranging from 7.6 – 8.5 throughout all runs, with the highest pH at the end of Trial 1. Beginning at a pH of 7.0, increases in pH serve to release ammonia from the litter with high levels released at a pH of 8.0 and higher. Ideally, the pH should be below 8.0 with a moisture content <30% to avoid volatilizing ammonia. Lowered pH levels tend to decrease bacteria levels as well (Abreu et al., 2011).

4.2 NDV Persistence

It had been hoped to achieve a large reduction in virus persistence as a result of increased temperatures and at all depths tested. It has been proven that at surface and 0.6 cm, heat will eliminate virus, and this project was to replicate that work and possibly add new depth levels to the heat treatment protocol (USDA, 2016). At best, the results are inconclusive as there was no clear-cut depth effect for this project. Because each trial required 1,570 SPF eggs, trials were divided into thirds to effectively manage the number of eggs for processing. Additionally, the days chosen for processing were in hopes of avoiding analyzing days where previous time points were negative for recovery. Variability within each trial, however, led to processing almost all samples. During the time it took to process a complete trial, storage time of recovered virus samples may have been a factor. Until this project, recovered virus had not been stored in this lab for the same amount of time and without organic material until processing in eggs. Previous research by the group stored recovered NDV samples for up to five days with an organic load (breast

meat) at -20 ºC with a minimum drop in titer (Benson et al., 2008). It is noted that NDV is best stored at -70 to -80 ºC for long-term or at 4 ºC for up to two weeks, and storage at -20 ºC is not recommended as the virus will lose infectivity over time (Grimes, 2002). Based upon previous studies by the group, the protocol for this experiment called for freezing recovered NDV in PBS at -20 ºC and in the past, this method has been adequate for short but undetermined, amounts of time. No studies have been found to provide an acceptable time period for NDV storage without organic material or if there is an acceptable organic that may be added post recovery to effectively freeze samples until they may be processed in SPF eggs. There was no evident difference between the lack of positive samples recovered from the same timepoints over the course of the three trials as compared to the amount of time each spent in frozen storage. For Trials 2 and 3, additional positive controls were frozen and across the time it required to process all eggs, 18 weeks and 16 weeks, respectively, each set of controls from the control house experienced an approximate drop in titer of one log or less per month of storage. These samples were thawed on the day they were processed as it is well recognized that each freeze-thaw cycle could yield a drop in titer (Santry et al., 2017). Further work should be done to determine the storage times and organic load best suited for preservation at each storage temperature. Additionally, another approach for future studies would be to increase processing capabilities or to perform this study on a smaller scale that will allow for processing of all coupons and samples without any freezing involved. This would require daily inoculation into SPF eggs and HA/ HI processing as

incubation times are met but would eliminate all frozen storage. Sample and egg management would become slightly more difficult, but results would be better able to assess the true effects of temperature on the virus.

Another factor that bears on this project is that of NDV persistence in litter. During phase two of this project, persistence of NDV was determined in a laboratory setting via inoculation onto and recovery from coupons without the use of litter. In a study to determine the effects of treatments for recycled broiler litter on microorganisms, it was discovered that NDV did not survive longer than five days in the litter even in the absence of treatment; AIV remained viable for only three days (Voss-Rech et al., 2017). A similar study found NDV was inactivated in litter quicker than other materials with only a seven-day survival for ambient temperature specimens (Guan et al., 2009). Further replications of this study should involve retesting in litter under controlled conditions in the laboratory.

Initially there was difficulty on interpreting HA and later, HI results. The single most important factor to make these assays less ambiguous and easier to interpret is that of RBC concentration. If RBCs are not present in the correct ratio, results may be misread as positive HA or negative HI. If this occurs, a new RBC solution should be made, and the assay repeated. To correctly ensure the ratio of RBCs to PBS, a cell counter or a hemocytometer and a microscope should be used until laboratory personnel become experienced in the task as the current method is not as precise. All positive test results as well as any that were suspect, were rerun to verify the results.

4.3 Salmonella Persistence

This protocol called for three samples per replicate, A or B, to be sampled for each timepoint. Given the size of the two houses, treatment and control, results would be better served if more samples were pulled from each house for each timepoint. The range of CFU values for the six samples (A & B replicates) for each timepoint was quite large. One suggestion to improve results would be to add more samples per timepoint. Perhaps replacing the scraping process would serve to liberate more bacteria from the coupons. Techniques such as sonication and vortexing could assist in recovery of bacteria from coupons if used for short periods. However, care was taken to monitor and ensure all sections of the house were within the required temperature and moisture ranges, therefore these variables were not likely to contribute to the range of results.

Moisture, ammonia, and pH levels increase during a grow-out. The litter becomes bactericidal as moisture and pH levels increase and as a result ammonia is volatilized from the litter (Turnbull & Snoeyenbos, 1973; Ottoson et al., 2007). However, no single factor is completely responsible for a long-term reduction in bacteria. For example, if pH is raised, *Salmonella* will decrease but return as the pH is lowered (Ottoson et al., 2007). When the pH is raised and the moisture level is also raised, then cell counts are reduced often to the point of no recovery (Turnbull & Snoeyenbos, 1973). The same effect is realized when pH levels and ammonia levels increase. When both of these factors are high, *Salmonella* survival is greatly reduced (Turnbull & Snoeyenbos, 1973). On the other end of the scale, low values for moisture and pH, can also inhibit *Salmonella* viability (Payne et al, 2006). In a study

comparing drying of manure to 10% water content and then gassing with 1% ammonia, *Salmonella typhimurium* experienced an 8-log reduction (Himathongkham & Riemann, 1999). Moreover, temperature alone will not eliminate bacteria from heat treatment. It is realized that increased temperatures will reduce the amount of *Salmonella* in a poultry house by releasing ammonia from the litter. Based upon results from subsequent trials, this lack of viability in the first trial may be more of a result of the high ammonia concentration and less because of the high temperatures alone. Because the litter was fresh, high amounts of ammonia were present to begin with which could result in large amounts of ammonia emissions from the litter during heat treatment (Chen, 2015). Upon discovery of the high concentration of ammonia, subsequent trials were monitored, and it was shown that the ammonia concentration continued to decrease as temperatures were maintained at testing levels of 37 - 49 ^{o}C. Temperature along with moisture content has an effect on bacterial survivability. One study compares moisture contents of 30% and 65% during heating at 45 ^{o}C. Fifty times more *Salmonella typhimurium* were enumerated from the higher water content sample (Wilkinson et al., 2011). If litter is to be recycled, it is suggested, therefore that growers capitalize on the higher moisture, pH and ammonia levels at the end of the grow-out and raise the temperature in poultry houses between flocks as a potential means to reduce bacteria and ammonia levels.

Trial 2 showed a sharp decline in log values for every timepoint and depth with the exception for the 2.5 cm level in the control house. This may be a result of less heat in the control house to volatize ammonia from the litter

and the protective one-inch layer of litter to prevent desiccation of the bacteria. In the treatment house, the 10 cm level dropped below 37 ^{o}C by TP-2 and continued to drop which had an adverse effect on recovery from this depth for both Trials 2 and 3. The sharp decline of the surface samples also from the control house on TP-3 may be simply a result of having a small sample representation as that level behaved in the same manner as the other control house sample for all the other days within the trial. Additionally, since there was an approximate log reduction of one and a half logs on average for the third trial, this may be attributed to less ammonia in the treatment house after two other trials.

Chapter 5

CONCLUSIONS

This project evaluated the use of heat as an alternative cleaning and disinfectant procedure to dry and wet cleaning followed by a chemical disinfectant. While heat is already accepted by the USDA as a disinfection method, the current protocol requires that high risk areas be cleaned to less than 0.6 cm and low risk areas should be cleaned to less than 1.2 cm of organic matter. Newcastle disease virus served as a surrogate for AIV and *Salmonella* served as a representative bacterium of interest. Both organisms were affected by heat, although *Salmonella* counts experienced a greater reduction during the first trial utilizing this method. This may be in part due to the high ammonia concentration within the litter and possibly in conjunction with the moisture content and pH of the litter.

Because of low virus recovery rates possibly as a result of freezing temperature, storage times, or interactions between the litter and NDV, results were inconclusive in this study on the impact of high temperature and depth on the survivability of NDV in chicken litter.

Salmonella samples showed a direct link between temperature and reduction in bacteria numbers across the course of the trials. Results did vary among the trials and factors that led to this outcome were sample size, moisture, pH, and ammonia levels. It may prove to be a practice worth adopting by growers between flocks to utilize increased temperatures in conjunction with moisture levels, to force increased ammonia volatility which would result in lowered bacteria counts in preparation for the subsequent flock.

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