MICROBIAL REDUCTION ON EGGSHELL SURFACES BY THE USE OF HYDROGEN PEROXIDE AND ULTRAVIOLET LIGHT

A Thesis

by

STEVEN MICHAEL GOTTSELIG

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

MASTER OF SCIENCE

August 2011

Major Subject: Poultry Science

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Approved by:

Chair of Committee,	Craig Coufal
Committee Members,	Tri Duong
	Matthew Taylor
Head of Department,	John Carey

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ABSTRACT

Microbial Reduction on Eggshell Surfaces by the Use of Hydrogen Peroxide and Ultraviolet Light. (August 2011)

Steven Michael Gottselig, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Craig Coufal

The effect of hydrogen peroxide (H_2O_2) in combination with ultraviolet light (UV) as an egg sanitization process on eggshell surfaces was studied. Preliminary experiments were conducted to develop an optimized methodology for eggshell disinfection that will be an effective and efficient way to reduce microorganisms on hatching eggs. Several experiments were conducted to reduce the natural flora found on the eggshell surface. Hatching eggs were collected from White Leghorn hens housed in floor pens with nest boxes. Eggs had no adhering organic material present. Results from these experiments led to the modification of the prototype equipment as well as the treatment application methodology. Following the experiments to optimize the methodology for H_2O_2 spraying and UV exposure time, the methodology was applied to eggs inoculated with *Salmonella* Typhimurium. Eggshell crush and rub methodology was used to enumerate bacteria within the pores and membranes of the egg. The optimized H_2O_2 and UV combination treatment process was then applied to commercial broiler breeder hatching eggs to evaluate the effects on hatchability.

Based on the parameters tested, results indicate that two applications of 3% H_2O_2 followed by 5 sec of UV exposure after each application produced the most consistent microbial reductions on eggshells. To enhance these effects, the addition of a 180° rotation between the two applications showed to be effective at further reducing the natural flora found on the eggshell surface. Studies using this optimal methodology on eggs inoculated with *Salmonella* at 9 $\log_{10}$ CFU/egg yielded greater than 5 $\log_{10}$ CFU/egg reductions. However, this methodology had little to no effect on reducing bacteria found within the pores and membranes of the eggs inoculated with *Salmonella*. These findings indicate that the effects of the disinfection process are largely limited to the eggshell surface. Hatch studies showed significant reductions in eggshell microbial levels under field conditions with eggs having large amounts of organic material present on the shell surface. Hatchability was maintained after treatment when compared to untreated eggs. Additional studies are needed to develop advanced equipment to apply this technology under commercial conditions.

DEDICATION

This thesis is dedicated to my parents, Leon Gottselig and Glenda Gottselig, whose incredible support and faith through life's challenges have lead me to where I am today. Thank you for believing in me as well as for providing me with unconditional love.

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NOMENCLATURE

hv	Light
DI	De-ionized water
UV	Ultraviolet light
H_2O_2	Hydrogen peroxide
HO·	Hydroxyl radical
CFU	Colony forming unit
Log	Logarithmic
g	Gram
mL	Milliliter
L	Liter
NO	Novobiocin
NA	Nalidixic acid
TSA	Tryptic soy agar
TSB	Tryptic soy broth
XLT-4	Xylose lysine tergitol-4
PBS	Phosphate buffered saline
LOD	Level of detection
APC	Aerobic plate count
MAX	3% H_2O_2 in combination with 5 sec of UVC for two applications with one rotation between applications

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CHAPTER I

INTRODUCTION

Eggshell disinfection is an important intervention step to reduce the prevalence of pathogen production and virulence. Reducing microorganisms found on the eggshell surface is vital to prevent cross contamination of adjacent eggs as well as incubation and hatching equipment. Therefore, proper sanitation methods are needed to reduce microbial levels found on eggs (Coufal et al., 2003). Previous studies conducted at Texas A&M University and Mississippi State University have focused on the effects of hydrogen peroxide (H_2O_2) combined with ultraviolet light (UV) applied to eggshell surfaces to reduce microbial levels (Coufal et al., 2003; Wells et al., 2010). Additional research is needed to refine an effective and efficient use of this methodology that is commercially applicable when compared to previous studies.

Providing an effective disinfection program in the hatchery is important to achieve quality chicks as well as possibly increasing hatchability (Brake and Sheldon, 1990). Reducing microbial levels found on eggshell surfaces can potentially optimize broiler production by maintaining high hatchability and chick quality. This will not only increase profitability but potentially decrease the effects microorganisms have on embryonic development and growth after hatch. Within commercial operations, the opportunity to adopt an egg disinfection program at the breeder farm and/or hatchery could directly address these issues.

This thesis follows the style of Poultry Science.

Due to possible influences on the functionality of the egg during embryonic development, it is essential to determine the effects an egg disinfection chemical or procedure could have on the cuticle. The cuticle, or bloom, is a delicate protein layer that surrounds the shell. Protein is excreted onto the exterior of the egg just prior to oviposition and dries within minutes when exposed to the environment. This layer acts as a barrier to prevent bacteria from entering the interior of the egg. The cuticle also regulates the amount of carbon dioxide and water loss throughout the incubation period. It has been shown that an egg will lose approximately 12% of its weight during the incubation cycle (Peebles and Brake, 1986). If the cuticle is affected by any outside factor such as a disinfectant, it can directly have an effect on the shell's porosity (Scott et al., 1993). The use of H_2O_2 and UV, both known disinfectants, could reduce bacterial levels without removing the cuticle and affecting the viability of the egg.

Typical commercial breeder operations consist of houses equipped with conveyor belts that will collect eggs at various times during the day. Nest clean eggs are preferred as they have been shown to have lower bacteria levels compared to eggs that have fecal material present on the eggshell surface (Berrang et al., 1997). Once collected onto belts, eggs are transported into a sorting room to separate unsettable from settable eggs. Eggs are placed onto plastic incubator flats that vary in the quantity of eggs held depending on the type of incubation system used. These flats are loaded onto carts and stored in farm coolers until transported to the hatchery. Temperature is the primary consideration after oviposition because high temperature can promote embryonic development. It is essential that the cooler be maintained at a temperature below physiological zero to

prevent embryonic growth. Typical cooler temperatures are set at 65 to 70°F (18 to 23°C) and a relative humidity of approximately 75% to prevent excess moisture loss during storage. Another objective of storing eggs in a cooler before transportation is to discourage microbial growth. However, prolonged storage in coolers has been shown to have some effect on embryo mortality. It has been determined that this is due to excess gaseous diffusion that is vital for chick development (Lapão et al., 1999). At the hatchery, egg carts are transported to holding areas before entering the incubator. Egg carts are placed into the incubator for 18 days until eggs are transferred to the hatching cabinets. Once eggs have hatched, chicks are separated from shells and vaccination is carried out.

Cox et al. (2000) suggested that for a sanitation program to be implemented and considered effective, eggs should be treated as close to lay as possible. Intervention to break the cycle of contamination on hatching eggs must be addressed during these early stages of the hatch process (Berrang et al., 1995). The use of equipment and machinery found in hatching egg operations allow for minimal direct contact of the eggshell surface, thus reducing cross contamination between eggs. Minimizing contact to the eggshell surface would reduce the risk of recontamination following implementation of an egg disinfection treatment at or near initial egg collection at the breeder farm. Once the egg is treated and placed in incubator flats, it will not likely come into direct human contact for the duration of the hatch process.

The overall goal of this research was to evaluate the effectiveness of H_2O_2 in combination with UV to reduce the natural flora and *Salmonella* found on eggshell

surfaces. Hatchability and chick parameters were also assessed after applying this methodology to broiler breeder eggs.

The specific objectives are: 1) reduce UV exposure time to the eggshell surface during treatment compared to previous research; 2) establish the optimal H_2O_2 concentration applied to the eggshell surface prior to UV exposure; 3) determine the optimal applications and/or rotations needed to achieve the greatest bacterial reduction; 4) manipulate the UV lamps inside a prototype chamber to achieve maximum UV exposure with minimal shadowing on the entire eggshell surface; 5) evaluate the effects of H_2O_2 and UV on bacteria found within the pores and membranes of the egg; 6) determine the effect of H_2O_2 and UV exposure on eggs inoculated with *Salmonella* onto the entire eggshell surface; and, 7) assessing hatchability and chick quality at hatch after treatment of fertile eggs with H_2O_2 and UV prior to incubation.

CHAPTER II

LITERATURE REVIEW

Incidence of Hatching Egg Contamination

Hatcheries play a significant role in influencing the level of microbial challenge to hatchlings. Both the collection of fertile eggs from breeder farms and hatching chicks for commercial applications can lead to the contamination of eggs and the hatchery environment if not properly managed (Kim and Kim, 2010). Hatchery hygiene is important to consider for reducing the risk of egg, equipment, and overall facility contamination, as well as the impacts such contamination could potentially have on the hatchlings. It was reported that microorganisms such as *Salmonella* that are found on hatching egg surfaces could be distributed throughout the facility, potentially affecting other chicks within the hatchery (Chute and Gersham, 1978; Berrang et al., 1998). Studies have shown that the environment eggs are exposed to can be a source of a variety of microorganisms and cause disease in the poultry industry (Sheldon and Brake, 1991). Examination of chick fluff has been assessed as a way to determine hatchery hygiene (Chen et al., 2002). Others have sampled the air quality to investigate the level and range of microorganisms found throughout the facility (Magwood and Marr, 1964). Results of their studies indicate that hatchery facilities show to have high levels of contamination that could potentially affect embryonic development and bird health as well as reducing hatch. Studies have shown that bacteria found on the final product often

originate from hatcheries and breeder flocks unless eliminated from flocks or from freshly laid fertile eggs (Cox et al., 2002).

Selection of nest clean eggs has been thought of as an industry method of reducing the chance of high bacterial contamination compared to eggs that have organic material present (Berrang et al., 1997; Cox et al., 2000). Nest clean eggs can be defined as those eggs that have no adhering organic material present on the eggshell surface. Studies have shown that nest clean eggs have an increased rate of hatch over dirty eggs as indicated by late embryo mortality, most likely from an increase in bacterial invasion (Buhr and Mauldin, 1994; Berrang et al., 1999). In attempts to clean dirty eggs, washing methods have been used in some facilities to salvage eggs that are contaminated with fecal material. Previous industry methods suggest that wetting or washing of hatching eggs can drastically reduce the ability to hatch. Nevertheless, studies have shown that with proper application, wet egg disinfection methods will not have adverse effects on hatchability (Berrang et al., 1997).

Methods of Disinfection

Traditional use of disinfectants on hatching eggs throughout the poultry industry consisted predominantly of fumigation with formaldehyde gas (Williams, 1970; Patterson et al., 1990; FDA, 2007). Formaldehyde has been used successfully for many years to limit and control microorganisms by acting on the surface of the eggshell without penetrating the interior of the egg (Williams, 1970). Research conducted by Graham and Michael (1931) showed that to effectively reduce organisms on eggs with formaldehyde required proper gas concentration, humidity and absence of any organic

material on the shell. The application of formaldehyde is generally as a spray or fogging method that can adequately disinfect equipment as well as the surface of eggs within the incubator. Formaldehyde is generated and released in a poultry incubator or hatcher by adding 1.2 mL of formalin to 0.6 g of potassium permanganate per cubic feet to produce a disinfecting gas (approximately 40 percent formaldehyde) (Williams, 1970). However, this involves handling of hazardous chemicals by employees who can possibly be exposed to the gas after mixing. Formaldehyde has been shown to be effective, though it is now decreasingly used throughout the industry due to regulations by the U.S. Occupational Safety and Health Administration (OSHA) (OSHA, 1987). The use of formaldehyde must be highly regulated, as well as management practices put in place that include properly ventilating incubators and the hatchery environment to prevent possible exposure to the toxic fumes released (Sheldon and Brake, 1990). Later research has shown that eggs treated with formaldehyde during embryonic development have an increased risk of hatched chicks developing respiratory issues (Nihgot, 2002). Additional research has confirmed that eggs treated with formaldehyde resulted in a reduction in hatchability when applied in incubators compared to those incubators that were not fumigated (Sander et al., 1995).

Quaternary ammonium compounds have also been used as sanitizers for hatching eggs. Brake and Sheldon (1990) showed that fertile eggs sprayed with a Hatching Egg Sanitizer Spray (quaternary ammonium compound) at both 1.5% and 3% resulted in significant reduction in bacteria counts. A factor to consider when applying chemical compounds to the eggshell surface is the effect it will have on the cuticle. The

application of foreign sanitizers could play a role in regulating gas exchange/escape between egg and the environment by removing this barrier (Brake and Sheldon, 1990). For this reason, quaternary ammonium applications have not been widely adopted throughout the industry. Further research has been recommended to determine a method to effectively apply quaternary ammonium to eggshell surfaces without removing the cuticle layer and potentially changing the functionality of the egg (Brake and Sheldon, 1990).

Studies have also investigated the use of hydrogen peroxide (H_2O_2) as a liquid disinfectant in an attempt to reduce bacteria on eggshell surfaces. Padron (1995) dipped eggs inoculated with *Salmonella* Typhimurium into a solution of 6% H_2O_2 to study its effectiveness at reducing bacteria as well as its impact on hatchability. This method reduced *Salmonella* by 95% when compared to untreated eggs. This study also indicated that using H_2O_2 as a disinfectant on eggshell surfaces does not reduce hatchability and may have some effect on bacteria other than *Salmonella* as well. Sheldon and Brake (1991) evaluated the effects of H_2O_2, formaldehyde, and water application to eggshell surfaces while in incubators. Their study specified that a 5% H_2O_2 solution was as effective as 3X formaldehyde (119.8 mL of formalin and 59.9 g of potassium permanganate per 2.83m^3) and surpassed the effectiveness of water to reduce microbial levels on the eggshell surface. Several studies have shown that the use of H_2O_2 did not affect hatchability by having a limited effect of the functionality of the egg with respect to water loss and gas exchange after the application (Sheldon and Brake, 1991; Scott and Swetnam, 1993b; Padron, 1995; Cox et al., 1999).

Hydrogen Peroxide

The application of hydrogen peroxide (H_2O_2) solution as a disinfectant can be a safe and effective way to reduce microbial levels with low concentrations of H_2O_2. Hydrogen peroxide is a known oxidizer, can be corrosive at high concentrations, and is an irritant to the skin and eyes (Sullivan and Krieger, 1992; Scott and Swetnam, 1993a). A potential carcinogenic effect is possible at high concentrations; however, 3% is the most widely used concentration due to its low toxicity (Gosselin et al., 1984). Hydrogen peroxide has a two-electron reduction state which has the ability to diffuse across cell membranes. The degradation of H_2O_2 results in water and molecular oxygen (1/2 O_2), which will not leave a toxic residue on the eggshell surface after application. Hydrogen peroxide is a colorless liquid that is miscible in water. It has been shown that disinfectants such as H_2O_2 can easily be applied in a variety of areas throughout poultry breeder operations (Sheldon and Brake, 1990). Sheldon and Brake (1990) showed that eggs treated with 5% H_2O_2 during the transfer from storage to setters as well as during storage periods can be beneficial by reducing microbial levels found on eggs. Hydrogen peroxide can be considered a stand-alone disinfectant and has the ability to reduce the levels of many of the bacteria that are associated with poultry. Sander and Wilson (1999) have shown that eggs exposed to H_2O_2 fogging within incubators had a reduction of bacterial counts compared with water-fogged machines.

There are several advantages of using H_2O_2 as an eggshell disinfectant. Research shows that lower concentrations of H_2O_2 can effectively reduce microbial levels (Bayliss and Waites, 1982; Padron, 1995; Wells et al., 2010) and still be deemed safe enough for

skin contact. Hydrogen peroxide is relatively inexpensive and is easy to incorporate into existing equipment that utilize sprays. Higher concentrations of H_2O_2 can be purchased and diluted at use to save on costs of purchasing large amounts of low concentrate and limit the space needed in the facility to accommodate large quantities of H_2O_2.

Ultraviolet Light

Irradiation with ultraviolet light (UV) is widely used for various food and water sanitation processes. It is a disinfection method that at low intensities will not alter organoleptic attributes or result in a decrease in the nutritional properties of food (Bintsis et al., 2000). The sun emits UV at different wavelengths throughout the electromagnetic spectrum. Most UV radiation is blocked by the Earth's ozone layer which keeps it from penetrating the Earth's atmosphere. The absorption of UV by living tissue causes a photochemical reaction that has the ability to alter the genetic material of a cell, thus, its antimicrobial power (mutagenesis). The mechanism of action of UV can prevent microorganisms from successfully replicating. Since the cell cannot reproduce it is unable to infect, and thereby deemed inactivated (Harm, 1980; Koutchma et al., 2009). The amount of cell damage is conditional to the dose that could potentially be absorbed by the microorganism. Ultraviolet dose requirements for destroying bacterial cells are relatively low and dependent on the microorganism, intensity and exposure time. Ultraviolet dose is the product of UV fluence rate (I) and exposure time (T) and is typically shown as milli-Watt second per square centimeter (mW/cm^2) (Koutchma et al., 2009). The impact of various obstacles can affect the optimal dose of UV since light emitted cannot be absorbed by most components (Figure 1).

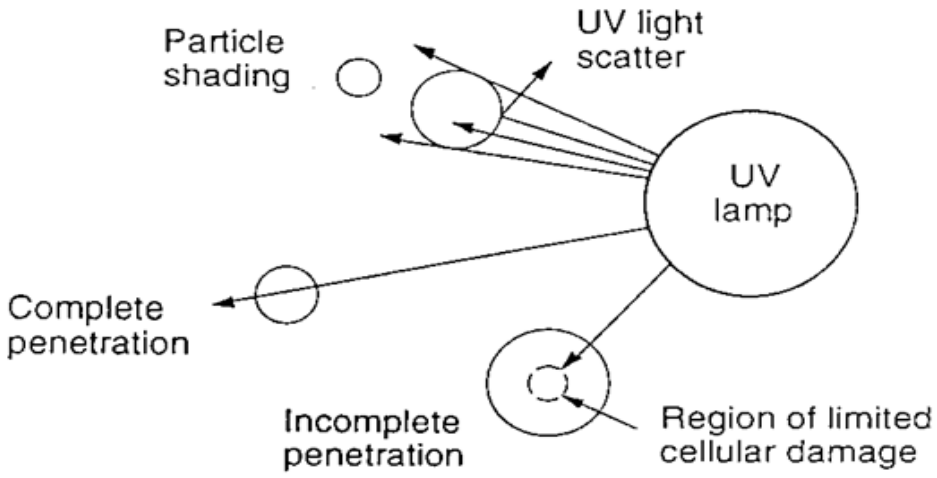


Figure 1. Interactions that impact UV effectiveness (Source: United States Environmental Protection Agency, 1999).

Figure 1 depicts the challenges of working with UV due to ineffectiveness if the target object is shadowed. It has been shown that for optimal reduction of bacteria, organisms should be in close proximity to the UV source and have direct contact with the UV (Koutchma et al., 2009). The UV spectrum can be divided into three primary bands based on wavelength in nanometers (nm); UVA (320-400 nm), UVB (280-320 nm), and UVC (100-280 nm). UVA rays with a wavelength greater than 320 nm are not considered very hazardous to cells. The spectrum for concern ranges from 180-320 nm, including UVB and UVC (Kowalski, 2009). A portion of the electromagnetic spectrum including UV is presented in Figure 2.

Direct exposure to UVB for extended time periods will have a direct effect on DNA damage and can cause a sunburn or skin cancer (Kowalski, 2009). UVC consists of rays with short wavelengths compared to other bands. This type of UV is germicidal

and most commonly used for disinfection (Kowalski, 2009). Since UVC is largely filtered out by the atmosphere, it is considered harmless in the environment. However, UVC lamps are manufactured to produce artificial UVC for disinfection purposes. These lamps use quartz glass containing a coating to block some wavelengths. The UVC lamps are still considered high power even with the use of filters and are marked by causing the most degree of damage to DNA cells.

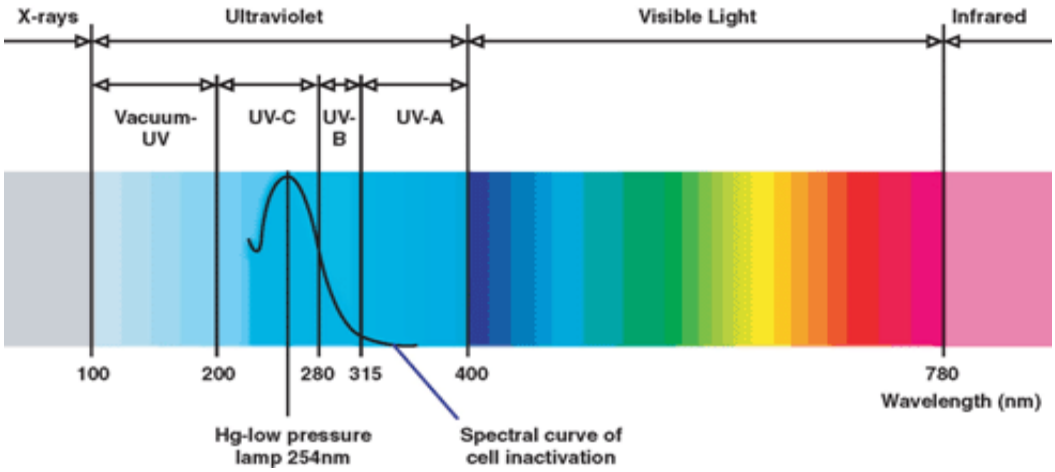


Figure 2. Ultraviolet/visible light spectrum (Source: Eclogiteskincare, 2009).

Development of UV lamps has increased in demand because of UV's versatility. Forms of UV can be used for forensics, photo-chemotherapy, air purification, and analyzing minerals. Eggshell disinfection with UVC has been researched in recent years because of its germicidal properties on the eggshell surface and will not remove the cuticle (Coufal et al., 2003).

Advantages of using UV are that it is environmentally friendly and will not require the storage of dangerous chemicals. The initial investment capital is low in comparison to similar technologies such as thermal disinfection (pasteurization) used in the table egg industry. The UV treatment process can be easily implemented in facilities without a long period of down time for installation. Ultraviolet light operations can have low power consumptions and very little operating costs while easily adapting to in-line processes and other processing equipment (Atilgan, 2007).

Research has shown that aerobic microorganisms, yeast, and mold populations found on the eggshell surface can be significantly reduced by 15 min of UV exposure at 254 nm (Kuo et al., 1997). Ultraviolet light has shown to also be effective at reducing *Salmonella* on the eggshell surface (Berrang et al., 1995; Gao et al., 1997; De Reu et al., 2006b). In past studies, Coufal et al. (2003) found that the optimal time to treat eggs with UV is before hatching eggs are placed into the cooler. After this point, eggs will not be directly handled again, thus reducing the chance of additional eggshell surface contamination. Studies conducted by Coufal et al. (2003) showed no difference in embryo mortality or hatchability when eggs are exposed to UV compared to those that were not exposed to UV. Experiments conducted by Gao et al. (1997) showed that UV cannot penetrate the shell of the egg; thus, it cannot directly have an effect on the embryo.

Hydrogen Peroxide and Ultraviolet Light as an Egg Disinfection Process

The combination of H_2O_2 and UV was first studied by Berglind et al. (1972) to oxidize substances in an aqueous solution. The principle behind the combined use is the generation of hydroxyl radicals (·OH) produced through UV photolysis of H_2O_2. When UV comes into contact with H_2O_2, UV will split the covalently bound H_2O_2 molecule into two hydroxyl radicals. The production of hydroxyl radicals was first investigated by Haber and Willstätter in 1932 as cited by Kehrer, (2000). The hydroxyl radical is an ion that contains an oxygen atom that is covalently bound with a hydrogen atom with an unpaired electron in the outer orbital. Oxygen is very reactive in that it will pull electrons away from molecules, an action termed oxidation (Block, 2001). A hydroxyl radical is an example of a reactive oxygen species, and has one unpaired electron in its structure that will deprive other substances of an electron, which drives its ability to oxidize other substances. This mechanism of action causes a chain reaction towards the destruction of bacteria (Sander and Wilson, 1999).

The photolysis of H_2O_2 is a simple process involving only H_2O_2 itself (Ikai et al., 2010). Hydroxyl radicals are short lived, approximately a nanosecond, with the capability of oxidizing membrane lipids, DNA and essential cell components to reduce microbial levels (Fredovich, 1978; Kehrer, 2000). The ability of H_2O_2 and UV to generate a hydroxyl radical is illustrated by the following reaction which has been widely exploited (Baxendale and Wilson, 1956; Alnaizy, 1999; Kehrer, 2000).

$$H_2O_2 + h\nu \longrightarrow HO\cdot + HO\cdot$$

It has been shown that gram-negative obligate anaerobes are highly sensitive to hydroxyl radicals (Block, 2001). Because gram-negative bacteria have a thin cell wall this makes the bacteria more susceptible to damages to the cell via hydroxyl radicals and an exposed outer membrane composed of phospholipids and surface-express lipopolysaccaride. Anaerobic organisms themselves do not produce a catalase to breakdown peroxide before cellular damage can occur. Hydrogen peroxide could be considered a harmful by-product of normal metabolic processes found within the body. However, the production of a catalase can quickly convert H_2O_2 into a less dangerous substance.

Salmonella

The presence of *Salmonella* on breeder hatching eggs is a critical point in preventing contamination in the poultry production system and can cause infection in chicks younger than 1 week of age (Padron, 1990). *Salmonella* is a bacteria of great concern that affects the poultry industry through its ability to be transmitted throughout commercial production and processing facilities. *Salmonella* is a facultatively anaerobic gram-negative bacterium within the family *Enterobacteriaceae*. The cells are motile, producing peritrichous flagella, and measure approximately 0.5 µm (length) by 0.2 µm (width) in size (Cox et al., 2000). These are important characteristics that enhance *Salmonella* proliferation on the interior and exterior of the egg. Its production of gas as well as the fermentation of glucose aids in the differentiation of this bacterium from other members of the *Enterobacteriaceae* family. Two serovars of *Salmonella* of great concern in poultry production and processing are *Salmonella* Enteriditis and *Salmonella*

Typhimurium. For decades, *Salmonella* Typhimurium was the largest bacterium found affecting poultry (Padron, 1995). However, since 1990, *Salmonella* Typhimurium was replaced with *Salmonella* Enteriditis (Miyamoto et al., 1998; Petter, 2001; Keklik et al., 2010). It was later confirmed that the cause of this increased outbreak was the incorporation of eggs into a variety of products and increased direct consumption of eggs, which is still seen today.

Since birds often do not show any sign of illness when colonized with *Salmonella*, it is difficult to detect the presence and inhibit the transmission of the bacteria (Petter, 2001). Fecal contamination is a potential sign that the exterior of the egg has come into contact with a potential contaminant. However, high levels of *Salmonella* have been recovered from clean intact eggs (Humphrey, 1994). Research confirms that egg age can play a factor in the number of bacteria found on the eggshell surface. Humphrey and Whitehead (1993) showed that some bacteria are present when the egg is first laid, primarily resulting from cells being deposited in the interior or on the exterior of the egg during oviposition. These bacteria will multiply as the egg goes through the hatching process. It has been shown that the transmission of *Salmonella* can occur rapidly through the cuticle, shell and shell membranes, and is influenced by moisture on the eggshell surface (Williams et al., 1968; Sparks and Board, 1984).

The transmission of *Salmonella* can be categorized as horizontal and vertical. Horizontal transmission occurs as bacterial cells invade the egg through the shell after being laid, while vertical transmission of bacteria results from the contamination of the egg while in the reproductive tract or in contact with fecal material of the hen (Cox et al.,

2000; De Reu et al., 2006a). Farm management practices have been shown to contribute to decreased bacterial loads by maintaining clean nest boxes, egg coolers and the facilities environment, which are known transmitters of *Salmonella* (Cox et al., 2000). Contamination can occur when hens bring organic material into nest boxes and directly contact the exterior of the egg. Bacteria that are in contact with the shell surface have the opportunity to invade the egg. Incubators are set at approximately 37°C which allows *Salmonella* to proliferate and increase in number (Bierer et al., 1961). Once the interior of the egg has become contaminated, especially the yolk sac, it is possible the embryo would become infected when the yolk sac is absorbed during incubation (Padron, 1990).

Studies have been conducted to observe the effects of hens inoculated with *Salmonella* to determine the rate of transmission throughout the ovary and oviduct. Timoney et al. (1989) observed that samples recovered from eggshell surfaces after lay from infected hens were not consistently contaminated with *Salmonella*. Despite the origin of the infection, *Salmonella* could be able to cross contaminate areas where the egg may come into contact with equipment and employees within the hatchery. *Salmonella* contamination is a concern to the poultry industry because infected embryos can hatch without significant signs of infection. Potentially infected chicks will further contaminate the hatchery and the farms where birds will be reared. This can directly result in an increase in mortality in newly hatched chicks and throughout the growth of the flock.

CHAPTER III

OPTIMIZATION OF A HYDROGEN PEROXIDE AND ULTRAVIOLET LIGHT DISINFECTION PROCESS TO REDUCE THE NATURAL FLORA FOUND ON EGGS

Introduction

To decrease hatchery contamination and reduce microbial levels found on eggs, the implementation of an egg disinfection method must be used. Previously reported methods of egg disinfection have been largely unsuccessful, and have not been widely implemented because of decreases in hatchability or the associated costs and dangers with chemical use. Currently the poultry industry does not routinely implement eggshell disinfection. As a result the lack of egg disinfection increase the challenges associated with hatchery sanitization. Infected or contaminated eggs show to have an increased amount of rotten or un-hatched eggs associated (Scott and Swetnam, 1993b). Research has shown that effectively sanitizing eggs is essential to achieve high hatchability as well as high quality chicks (Kuo et al., 1996). Breeder flocks and hatcheries are considered critical points for controlling microorganisms that enter poultry facilities (Cox et al., 1998).

It has been shown that a novel method of eggshell disinfection using H_2O_2 and UV can effectively reduce microbial levels on eggs (Wells et al., 2010). The objectives of this research are to refine the previously used methods in order to develop an

optimized methodology for hatching egg disinfection that has the potential to be commercially used.

Materials and Methods

Equipment Design and Usage

To expose the eggshell surface to germicidal ultraviolet light (UVC), a chamber containing 20 UVC (Sankyo Denki G30T8-Germicidal) lamps was used as previously described by Wells et al. (2010) (Figure 3). Eggs were first misted with a specified concentration of H_2O_2 prior to entering the UV chamber. A fine mist was sprayed on all surfaces of the egg with a minimal amount of application to prevent a washing effect. Once sprayed, eggs were immediately transferred into the chamber by manually pushing a wire flat or plastic incubator flat on a rail system. The wire flat used was constructed so that eggs rest on their side with the small end pointing towards the entrance of the UV chamber. The prototype wire flat held 32 eggs at a time and was designed to prevent eggs from having direct contact with one another, thus allowing for maximal exposure to all parts of the egg with minimal shadowing effects. Plastic incubator flats were also used from commercial hatchery systems. An aluminum frame was constructed to rest on the rails of the chamber and keep the plastic flat in place while entering and exiting the chamber. A fan located on the side of the chamber pulled air from the outside through a filter to aid in cooling of the internal environment of the chamber and lamps. The fan also created positive pressure air flow out the entrance and exit doors of the chamber, thus preventing airborne contaminates from entering the chamber. Switches allowed the operator to turn on/off separate sections of the lamps in the chamber at a time. The UVC

lamps were manipulated in various ways throughout the experiments to create the optimal exposure configuration to effectively reduce microbial levels on the eggshell surface. UV intensity was measured using a UVP radiometer (UVP, Inc. Upland, CA; P/N: 81-0064-01) at egg level and position on the flat within the UV chamber. Figure 4 is a cut-away view of the UV chamber with the optimal 16 UVC lamp configuration.

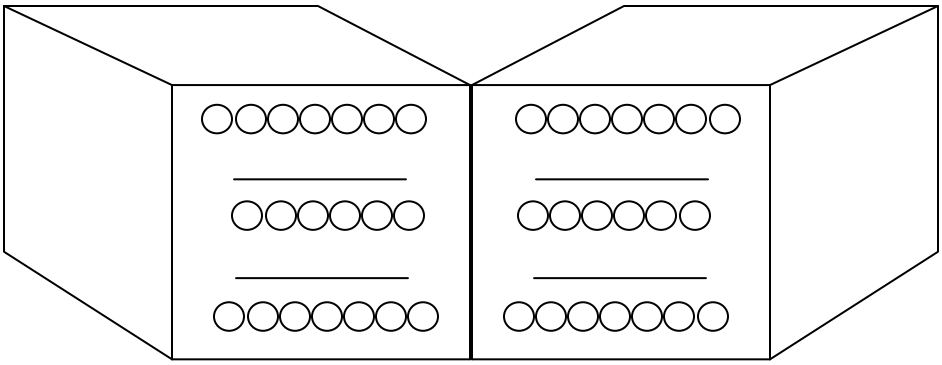


Figure 3. Cut-away view of the original UV chamber (20 Lamps).

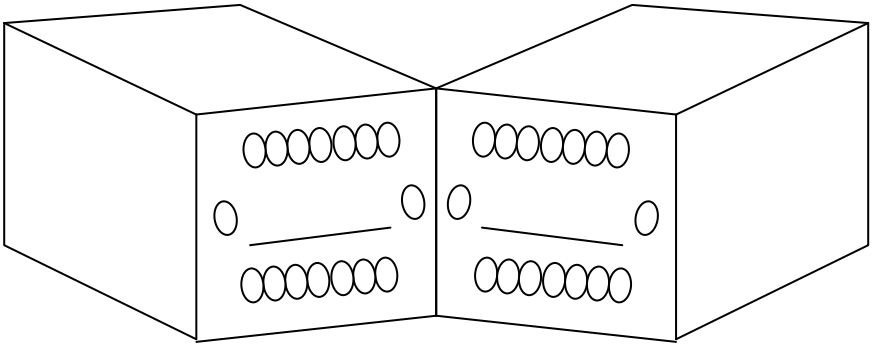


Figure 4. Cut-away view of optimal UV chamber (16 Lamps).

Eggshell Microbial Enumeration

Untreated and treated eggs were placed in sterile Whirl-pak bags (Nasco, Fort Atkinson, WI) containing either 25 or 50 mL of phosphate buffered saline (PBS). Each egg had an individual bag that was sealed and massaged vigorously for 1 min to dislodge bacterial cells from the eggshell surface. For untreated control eggs, serial dilutions were performed, and consisted of plating 0.2 mL of the rinse onto Tryptic Soy Agar (TSA; Becton Dickinson Co., Sparks, MD) in duplicate or triplicate plates for each sample. For treated samples, direct plating of the solution from the rinse bag onto the plate was performed in duplicate or triplicate. Sample size consisted of 0.2 mL plated to increase sensitivity of the procedure compared to plating only 0.1 mL. Aerobic plate counts (APC) were conducted to indicate the level of microorganisms found within the rinse (FDA, 2001). Plates were incubated at 37°C for 24 h. Bacterial colonies were hand counted and recorded. Microbial counts were expressed as $\log_{10}$ CFU/egg.

The level of detection (LOD) was dependent on the use of various quantities of PBS in the rinse bags. The detection limit was calculated by multiplying the amount of rinse used (mL) by the lowest detectable number of colonies per mL based on the amount of rinsate plated. Since 0.2 mL was directly plated, the lowest detectable number of colonies per 1 mL of rinsate is 5. The fewest number of cells that could be detected in the rinse for the eggshell was converted to a logarithmic value. For techniques using 25 or 50 mL of PBS, the calculated LOD is as follows:

25 mL (PBS) * 5 = 125 CFU/egg. log (125) = 2.1 $\log_{10}$ CFU/egg

50 mL (PBS) * 5 = 250 CFU/egg. log (250) = 2.4 $\log_{10}$ CFU/egg

Plates yielding no colonies were assigned a logarithmic value slightly lower than the LOD. For a 25 mL rinse, a value of 2.0 $\log_{10}$ CFU/egg was assigned to eggs, and for eggs rinsed in 50 mL of PBS and yielding 0 counts, a value of 2.2 $\log_{10}$ CFU/egg was assigned. These procedures were used for all eggs sampled in Experiments 1-11.

The criteria for selecting eggs used for each experiment included: (1) eggs were not selected based on size so as to develop a methodology to treat a variety of egg sizes that maybe found in typical breeder operations and (2) eggs had no organic material present on the eggshell surface since the methodology being developed was intended to be used on nest clean eggs only.

Project 1

Experiment 1 used 48 eggs over two trials. Each trial consisted of 8 untreated controls and 16 treated eggs randomly selected. A mist of 3% H_2O_2 (Topical Solution, United States Pharmacopeia (U.S.P.)) was sprayed on all surfaces of the egg followed by 8 min of UVC exposure to authenticate results found by Wells et al. (2010). Four rows of eggs containing eight eggs per row were positioned on the wire flat. The three outside rows were used for the purpose of creating shadowing effects on one of the center rows, which acted as the sample group (Figure 5).

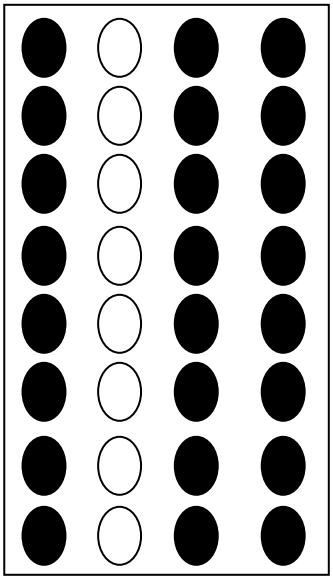


Figure 5. Overhead-view of 32-egg wire flat used to expose eggs to UVC in the prototype chamber. Eggs were sampled from one of the center rows (indicated as white).

Experiment 2 was conducted to evaluate the effectiveness of different H_2O_2 concentrations in combination with different UVC exposure times. Treatment groups consisted of misting eggs with 3%, 4.5% or 6% (Wyn's Water Hydrogen Peroxide Food Grade Quality; The Dancing Algae Company) H_2O_2 concentrations and exposing the surface of the egg to 1 or 2 min of UVC. This experiment used both wire and plastic flats to compare flat type. A total of 78 eggs were used in which 6 eggs served as untreated controls while treated eggs were divided into 6 groups of 12 eggs for each H_2O_2 concentration combined with each UVC exposure time.

Experiment 3 used the optimal UVC exposure time found in the previous experiment combined with 3%, 4.5% or 6% H_2O_2 concentrations on both wire and plastic flats. The experiment evaluated the effects of time between H_2O_2 application and

UVC exposure by misting eggs with the corresponding H_2O_2 concentration and allowing the H_2O_2 to sit on the surface of the egg for 0 min (eggs immediately exposed to UVC after spraying) or sprayed 5 min prior to exposure of 1 min of UVC. A total of 78 eggs were used, 6 of which served as untreated controls and 6 groups of 12 eggs were treated by different H_2O_2 concentrations combined with H_2O_2 application times.

Observed effects from previous experiments led to the manipulation of the UVC lamps, segregating the bottom half of the chamber from the top with aluminum to create a smaller chamber with a reflective surface directly beneath the bottom lamps. In an attempt to increase UV exposure to the eggshell surface, 20 UVC lamps were placed in close proximity to irradiate on the top and bottom of the egg flat.

Experiment 4 adopted the methods of Kuo et al. (1997) that found rotation of the egg during exposure would increase reduction of bacteria found on the surface. For this experiment eggs were rotated 180° between repeated applications of 3% H_2O_2 and 1 min of UVC exposure. Rotation was accomplished by use of tongs sterilized between rotations for each egg after each application. After rotation, the eggs were immediately misted with H_2O_2 and exposed to UVC depending on the number of applications. One hundred and forty eggs were used over two trials. Trials used 6 eggs assigned as untreated controls and 6 groups of 12 eggs for each application treatment. Treatment groups consisted of 1, 2 or 4 H_2O_2/UVC applications on wire and plastic flats.

Experiment 5 used the optimal treatment parameters found from H_2O_2 concentration, UVC exposure time and number of applications with rotation between applications. The objective of this experiment was to focus on varying the intensity of

UVC received by the eggs. High, medium and low intensities were achieved by manipulating the lamps to 16, 8 or 4 lamps, respectively (Figure 6). Eggs were misted with 3% H_2O_2 and exposed to 1 min of UVC for two applications with a 180° rotation between applications on wire and plastic flats. For this experiment, two trials were conducted using a total of 112 eggs in which 16 eggs were assigned as untreated controls and 6 groups of 16 eggs were divided into intensity groups and then further subdivided into wire and plastic treated groups.

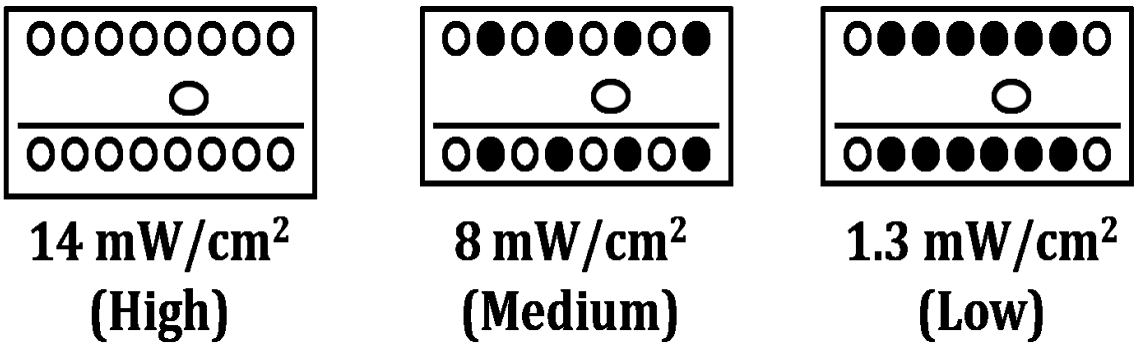


Figure 6. UV lamp manipulation within the chamber to vary the intensity received by the egg. ○ = lamp on, ● = lamps off.

The purpose of Experiment 6 was to compare UVB combined with H_2O_2 as a safer alternative to UVC combined with H_2O_2. Treatments of 3% H_2O_2 combined with 1 min of UV for two applications with one rotation between applications were administered. Additional treatment methods compared the effects of H_2O_2 combined with UV to 3% H_2O_2 applied to eggs alone for two applications with one rotation between applications. The first trial evaluated the above methods using 56 eggs, 8 of which were assigned as untreated controls, 16 eggs for each H_2O_2 combined with

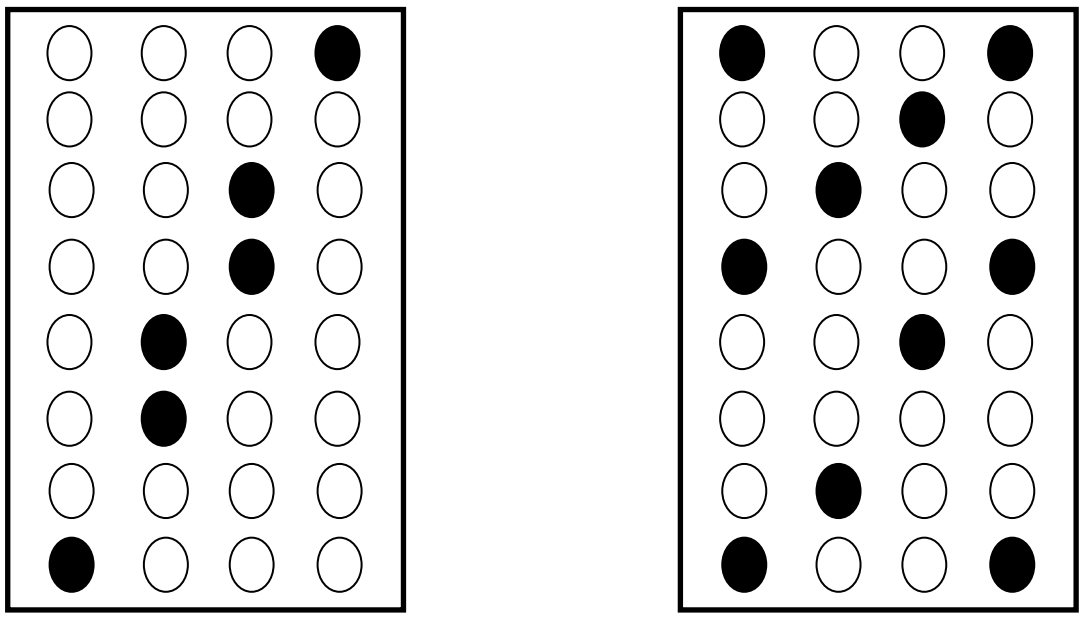
UVC/UVB and H_2O_2 alone treated groups. The second trial used 72 eggs, 8 of which were assigned as untreated controls, 16 eggs for each H_2O_2 combined with UVC/UVB for 1 min of UV exposure, 16 eggs for the H_2O_2/UVB group with 2 min of UV exposure and 16 eggs for the H_2O_2/ UVB group with 3 min of UV exposure.

In an attempt to reduce the exposure time needed, all 16 UVC lamps were used within the UV chamber and separated further apart to provide maximum exposure to the eggshell surface. Experiment 7 was conducted to assess the effectiveness of shorter UVC exposure times to attempt to make this methodology practical for commercial applications. Two trials were conducted using 3% H_2O_2 combined with 15, 30, 45 sec or 1 min of UVC exposure with one rotation between two applications. A total of 144 eggs were used in which 16 eggs were assigned as untreated controls and 32 eggs were exposed to 15, 30, 45 sec or 1 min of UVC per trial.

Experiment 8 focused on comparing multiple H_2O_2 and UVC applications with and without rotation. Two trials were performed using 3% H_2O_2 in combination with 5 sec or 1 min of UVC exposure with one rotation between two applications or two applications without rotation. A total of 80 eggs were used in which 8 were assigned as untreated controls and 16 eggs for each combination of rotation and UVC exposure time per trial on wire and plastic flats.

Experiment 9 was conducted to treat eggs in all 32 spaces of the wire flat system and sample eggs in various areas to confirm consistent microbial reductions across all positions (Figure 7). The experiment used 3% H_2O_2 combined with 5 sec of UVC exposure with one rotation between two applications. This optimal methodology will be

referred to as the MAX method throughout the rest of this text. Two trials were conducted. The first trial used a total of 35 eggs in which 5 eggs were assigned as untreated controls and 6 locations on the wire flat were sampled 5 times per location. After variations in microbial reductions were found in trial 1, further UVC lamp manipulation took place by transitioning one lamp from the top and the bottom sections of the UV chamber and placing the lamps on the sides of the interior of the chamber (Figure 4). The remaining lamps on the top and bottom sections were equally spaced to allow for maximum UV exposure to the eggshell surface. For the second trial, 55 eggs were sampled in which 5 eggs served as untreated controls and eggs from 10 locations at various areas on the wire flat were sampled 5 times per location.



(a) (b)

Figure 7. Position of eggs sampled from entire wire flats in trial 1 (a) and trial 2 (b).

The purpose of Experiment 10 was to evaluate the efficacy of H_2O_2 and UVC treatment using a Chickmaster 84-egg plastic flat (Figure 8) without rotation between applications compared to the MAX method. This flat design consisted of more plastic material than found in other traditional style incubator flats used in the broiler breeder industry. Since rotation of the eggs between applications may not be possible in all commercial settings, rotating eggs on the plastic flat was eliminated during this experiment to evaluate the effects of treatment using larger hatching egg flats. In addition, the effect of UVC exposure alone (without H_2O_2) was compared to H_2O_2 combined with UVC (MAX). The experiment consisted of the following groups: untreated control, 5 sec of UVC alone on the wire flat for two applications with one rotation between applications, the MAX method, and 3% H_2O_2 combined with 5 sec of UVC exposure for two applications without rotation on the 84-egg plastic flats. Treatments consisted of 8 eggs assigned as untreated controls and 16 eggs per treated groups.

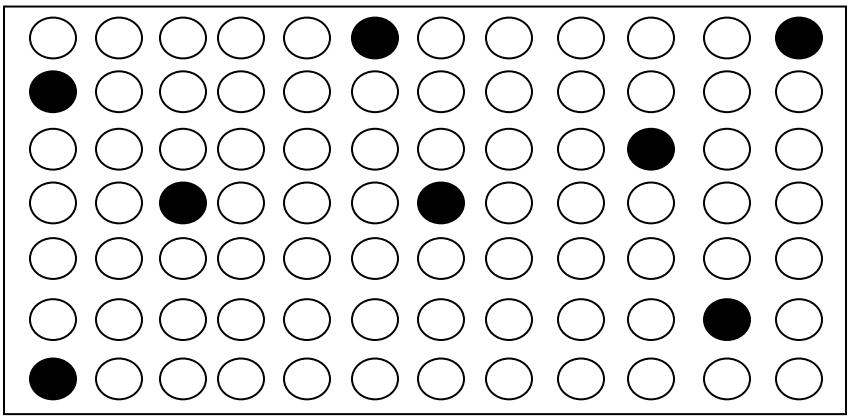


Figure 8. Sampled eggs using the Chickmaster plastic flat (Experiment 10).

Experiment 11 was conducted to compare the effects of light and heavy spray applications of H_2O_2 combined with UVC on Chickmaster 84-egg plastic flats using the same method as shown in Experiment 10 (Figure 9). The purpose of this experiment was to determine if applying a greater volume of H_2O_2 than that used in previous trials would result in greater microbial reductions on egg surfaces when using a plastic flats. Prior to treatment, the Chickmaster egg flat was filled with 84 eggs and misted on all sides with a light application of H_2O_2 as was done in all previous experiments. The flat was inserted into the UV chamber for 5 sec, removed, then misted with the same amount as the first application, and exposed to UVC again. The heavy application of H_2O_2 used twice the volume of H_2O_2 as the light application, and was performed in the same manner as the previously described light application of H_2O_2.

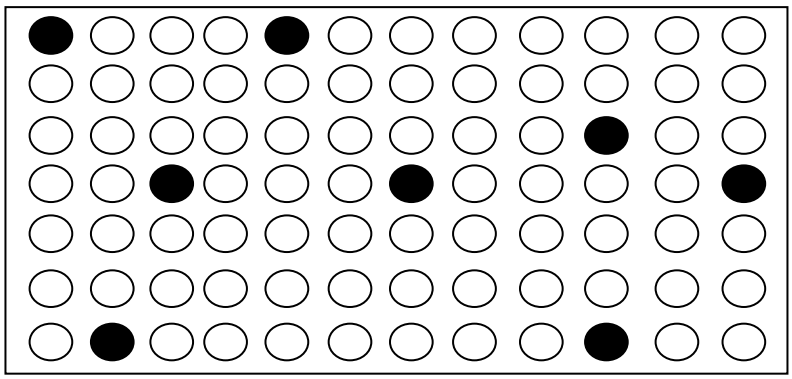


Figure 9. Sampled eggs using the Chickmaster plastic flat (Experiment 11).

Crush and Rub Analysis

In experiments numbers 6 and 7 incorporated a methodology for sampling bacterial cells to evaluate the effects of H_2O_2 and UVC on bacteria found within the eggshell pores and membranes. Musgrove et al. (2005b) used a sampling method known as the crush and rub method which extracted bacterial cells found in the pores and membranes of the eggshell that may not have been removed by the rinsing method (Figure 10). After the first rinsate was plated from the rinse bag, the egg was then aseptically removed and placed into a second rinse bag and massaged for 1 min to remove any remaining bacterial cells on the surface of the egg. The second rinse bag was sampled and plated using the same method as for the first rinse. After sampling the second rinse bag, the egg was aseptically removed and broken out to expel all internal egg contents. The interior of the eggshell was then rinsed with sterile DI water to remove any adhering material. The eggshell was then placed into a sterile conical tube containing 20 mL of PBS rinse. A sterile glass wand was used to crush the shell with

membranes until the eggshell was thoroughly macerated into the solution. Rinse solutions were then directly plated onto TSA. A total of 32 eggs from two separate experiments were used in which each of these experiments consisted of two separate trials within. Eggs were selected from experiments that were treated with 3% H_2O_2 combined with 1 min of UVC for two applications with one rotation between applications. The LOD for crush and rub analysis used the same calculations found in Experiments 1 to 11, in addition to the conical tube which was calculated by multiplying the amount of PBS (20 mL) by the lowest detectable number of colonies in a 1 mL sample shown above. The logarithmic value was calculated as 2.0 $\log_{10}$ CFU/egg as the LOD for calculation of means for the conical tube.

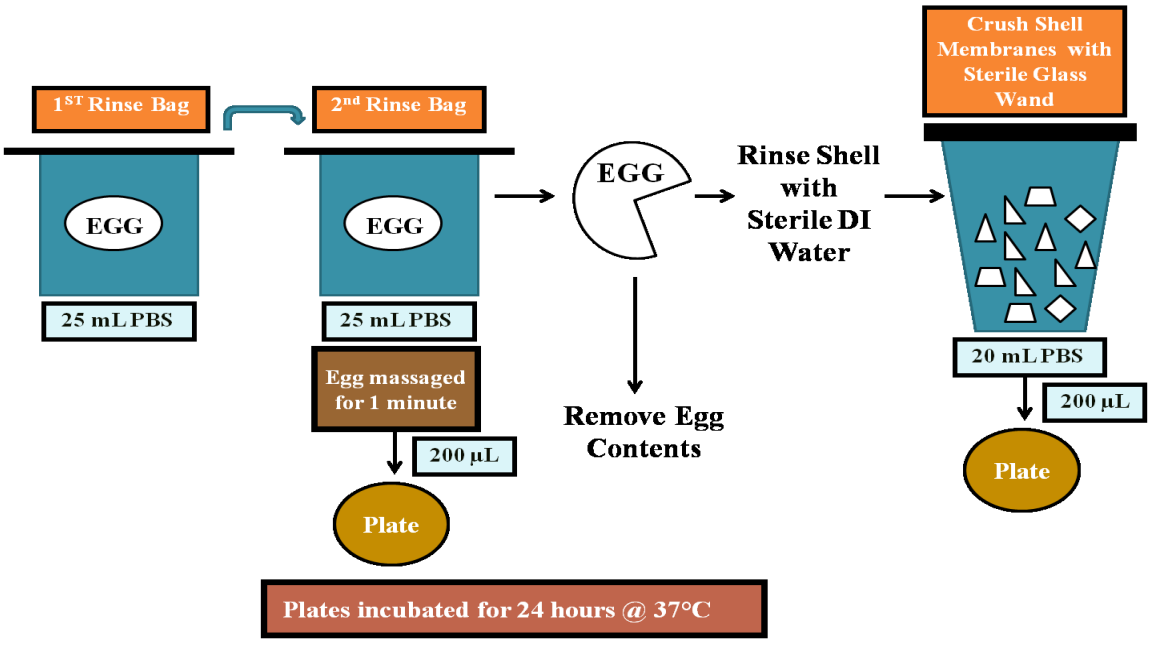


Figure 10. Crush and rub eggshell sampling method.

Statistical Analysis

Individual eggs served as the unit of replication within each treatment. Means were compared by analysis of variance (ANOVA) using the general linear model (GLM) procedures of SPSS and means separated by Duncan's Multiple Range Test. Means were considered statistically different at $P \leq 0.05$.

Results and Discussion

The purpose of Experiment 1 was to replicate the bacterial reduction found by Wells et al. (2010) as a starting point for current investigations. This was accomplished by applying the same methodology and conducting the experiment with the same equipment used with no initial manipulation of the UVC lamps or chamber. Results indicate similar bacterial reductions when 3% H_2O_2 was misted on the eggshell surface and exposed to 8 min of UVC at 11 mW/cm^2. Aerobic plate counts of treated eggs showed a 2.62 $\log_{10}$ CFU/egg reduction compared to untreated control eggs (Table 1). This reduction in bacteria found on the eggshell surface is statistically significant; however, the use of 8 min of UVC exposure does not appear to be commercially practical. Most breeder facilities collect thousands of eggs per day with few workers. Treatment of eggs must be expedited to maintain a minimum amount of time between egg collection and placement into the egg cooler. Over-exposure of eggs to high ambient temperatures, specifically in summer months, can result in decreased hatchability as well as increased microbial growth in contaminated eggs. Nevertheless, the results of Experiment 1 established a standard for microbial reduction for future experiments.

Table 1. Effects of 3% H_2O_2 spray and 8 min of UVC exposure on aerobic plate counts found on the eggshell surface (Experiment 1).

Treatment	n/trial	$\log_{10}$ CFU/egg
Control	8	4.97 ± 0.11^{a}
Treated	16	2.35 ± 0.10^{b}

[a, b] Means within columns with different superscript differ significantly ($P \leq 0.05$)

To reduce UVC exposure time, Experiment 2 incorporated various concentrations of H_2O_2 including: 3%, 4.5% or 6% applied to the eggshell surface in combination with UVC exposure. Ultraviolet light exposure time was selected to be 1 or 2 min in attempts to make this methodology more commercially feasible. The use of plastic egg flats were incorporated into this experiment to compare against treatment on the wire flat (best case scenario). Successful treatment of eggs located on a plastic flat would allow this methodology to be more commercially feasible. The plastic flats used were 42-egg flats used in a Jamesway incubation system. Results from this experiment showed that 3%, 4.5% or 6% H_2O_2 concentrations in combination with 1 min of UVC using plastic flats produced reductions in APC of 1.97, 1.32 and 1.41 $\log_{10}$ CFU/egg, respectively. Applying 3%, 4.5% or 6% H_2O_2 in combination with 2 min of UVC exposure using plastic flats produced reductions of 1.56, 1.45, and 1.44 $\log_{10}$ CFU/egg, respectively. Treatment on the wire flat yielded a 2.03 $\log_{10}$ CFU/egg reduction (Table 2). Results indicated that 2 min of UVC exposure time provided no additional benefit in decreasing microbial levels compared to 1 min of UVC exposure time. Concentrations of H_2O_2 above 3% also showed no additional reduction in APC regardless of duration of

exposure to UVC. This agrees with Wells et al. (2010) that higher concentrations of H_2O_2 greater than 2.5% did not yield additional microbial reductions. When plastic flat treatments were compared to the wire flat treatment, the plastic flat showed comparable results with the wire flat. This suggests that treatment on the plastic incubator flat could yield acceptable results, thus making the H_2O_2 and UV treatment process easier to implement in a breeder facility.

Table 2. Effects of various concentrations of H_2O_2 combined with 1 or 2 min of UVC on plastic flats at 10.37 mW/cm^2 compared to untreated control and treatment of 3% H_2O_2 combined with 1 min of UVC on a wire flat (Experiment 2).

Treatments	% H_2O_2	UV Exposure	n	$\log_{10}$ CFU/egg
Control			6	5.33 ± 0.15^{a}
Wire	3	1 min	12	3.30 ± 0.25^{c}
Plastic				
	3	1 min	12	3.36 ± 0.13^{c}
	4.5	1 min	12	4.01 ± 0.13^{b}
	6	1 min	12	3.92 ± 0.12^{b}
Plastic				
	3	2 min	12	3.77 ± 0.14^{bc}
	4.5	2 min	12	3.88 ± 0.16^{b}
	6	2 min	12	3.89 ± 0.11^{b}

[a-c] Means within columns with different superscript differ significantly ($P \leq 0.05$)

In Experiment 3, the UVC exposure time of 1 min was used for all treatments. The objective of this experiment was to determine if a greater APC reduction could be accomplished by extending the time between application of H_2O_2 and UVC exposure.

Results obtained from this experiment using 3%, 4.5% or 6% H_2O_2 concentration and immediately (0 min of wait) exposed to 1 min of UVC using plastic flats showed a 1.20, 1.70 and 2.07 $\log_{10}$ CFU/egg reduction, respectively. Treatment with 3%, 4.5% or 6% H_2O_2 concentrations for 5 min of application time in combination with 1 min of UVC on plastic flats showed a 1.73, 1.66, and 1.64 $\log_{10}$ CFU/egg reductions, respectively. Treatment on the wire flat (best case scenario) using 3% H_2O_2 immediately exposed to 1 min of UVC yielded a 2.72 $\log_{10}$ CFU/egg reduction (Table 3). Data suggests no significant statistical differences between bacterial reductions for eggs misted with 3% H_2O_2 and allowed to reside on the eggshell surface for 5 min with 1 min of UVC compared to eggs sprayed with H_2O_2 and immediately exposed to UVC. Similar results were obtained by Jones et al. (1993), using H_2O_2 to decontaminate the interior of bio-safety cabinets. It was shown that shorter time periods could eliminate organisms as long as H_2O_2 was well distributed. Further, results suggest that there is no substantial benefit in using higher concentrations of H_2O_2 above 3%. Comparisons between wire and plastic flats suggest that wire flats allow for a greater reduction in microbial levels when compared to plastic because of the minimal amount of material in contact with the eggshell surface during treatment. Results observed in Experiment 2 suggested plastic flat treatments could be comparable to wire flat treatment; however, natural variation of the egg could have allowed eggs located on the plastic flat to be treated at a greater extent than eggs located on the plastic flat in this experiment. The most effective methodology tested to reduce microbial levels was shown to be 3% H_2O_2 immediately exposed to 1 min of UVC on a wire flat.

Table 3. Effects of various concentrations of H_2O_2 for 0 or 5 min before exposure to 1 min of UVC on wire and plastic flats at 10.9 mW/cm^2 (Experiment 3).

Treatment	% H_2O_2	Delay[1]	n	$\log_{10}$ CFU/egg
Control			6	5.89 ± 0.22^{a}
Wire	3	0 min	12	3.17 ± 0.21^{d}
Plastic				
	3	0 min	12	4.69 ± 0.14^{b}
	4.5	0 min	12	4.19 ± 0.15^{bc}
	6	0 min	12	3.82 ± 0.23^{c}
Plastic				
	3	5 min	12	4.16 ± 0.15^{bc}
	4.5	5 min	12	4.23 ± 0.09^{bc}
	6	5 min	12	4.25 ± 0.14^{bc}

[1] The time between H_2O_2 spray application and UV exposure
[a-c] Means within columns with different superscript differ significantly ($P \leq 0.05$)

Experiment 4 incorporated rotation(s) between multiple applications to further reduce microbial levels found on the eggshell surface with the new configuration of UVC lamps within the UV chamber producing an overall intensity of 10.37 mW/cm^2. Results using wire flats indicated that one application (H_2O_2 and UVC) with no rotation produced a 2.57 $\log_{10}$ CFU/egg reduction, and two applications with one rotation between applications resulted in a 3.05 $\log_{10}$ CFU/egg reduction in APC, while four applications with three rotations between applications resulted in a 3.08 $\log_{10}$ CFU/egg reduction. Treatments on plastic flats using one, two or four applications with rotation yielded microbial reductions of 1.91, 3.17 and 3.38 $\log_{10}$ CFU/egg, respectively (Table 4). These results indicate that multiple H_2O_2 and UVC applications with rotation

between applications yield a greater microbial reduction than a single application without rotation on both types of flats. These results are similar to previous findings reported by Kuo et al. (1997) in which the rotation of the egg enhanced microbial reductions by effectively exposing the entire eggshell surface to treatment. This includes treating those areas of the eggshell that were shadowed or in contact with the flat. Due to their design, plastic egg flats possess areas that cannot be fully exposed to treatment with H_2O_2 and UVC without the use of rotation. It was also concluded that one rotation between two applications yielded similar results to three rotations between four applications. Results from this experiment indicate that 3% H_2O_2 combined with 1 min of UVC exposure for two applications with one rotation between applications was the most effective methodology compared to the other treatments utilized.

Table 4. Effects of rotations between applications of 3% H_2O_2 combined with 1 min of UVC on wire and plastic flats at 11 mW/cm^2 (Experiment 4).

Treatment	Applications	Rotations	n/trial	Trial 1	Trial 2
				$\log_{10}$ CFU/egg	
Control			6	5.72 ± 0.19^{a}	5.73 ± 0.27^{a}
Wire					
	1	0	12	2.70 ± 0.25^{bc}	3.16 ± 0.25^{c}
	2	1	12	2.19 ± 0.06^{c}	3.15 ± 0.24^{c}
	4	3	12	2.77 ± 0.20^{bc}	2.51 ± 0.26^{cd}
Plastic					
	1	0	12	3.61 ± 0.17^{b}	4.02 ± 0.13^{b}
	2	1	12	2.46 ± 0.11^{c}	2.64 ± 0.13^{cd}
	4	3	12	2.43 ± 0.10^{c}	2.25 ± 0.12^{d}

[a-c] Means within columns with different superscript differ significantly ($P \leq 0.05$)

Experiment 5 was conducted to further refine the H_2O_2 and UVC disinfection technique by evaluating the intensity required for the most effective methodology identified during Experiment 4. Various intensities were achieved within the chamber to create a high, medium and low UVC intensity at egg level on the flat. Each treatment consisted of 3% H_2O_2 combined with 1 min of UVC exposure for two applications with one rotation between applications. The use of 16, 8 or 4 lamps produced UV intensities of 14.0, 8.0 and 1.3 mW/cm^2, respectively. Each treatment used both wire and plastic flats. Results indicate that high, medium or low UVC exposure produced a 2.70, 2.80 and 2.43 $\log_{10}$ CFU/egg reduction using the wire flat (Table 5). Intensity treatments using high, medium and low UVC on plastic flats reduced APC by 2.94, 2.68, and 2.47 $\log_{10}$ CFU/egg, respectively. These results indicate that high UVC intensity exposure is not necessary to effectively reduce microbial levels when H_2O_2 is combined with UVC for two applications with one rotation between applications. High and medium intensities yielded the most consistent numerical values compared to low intensity which yielded the two highest numerical values out of all three intensities. Results from Chavez et al. (1999) used UVC (7.5 mW/cm^2) alone on eggs showing a 1.0 to 2.0 $\log_{10}$ reduction per egg. Coufal et al. (2003) confirmed these results, concluding that the use of intensities from 4 to 14 mW/cm^2 resulted in reductions in APC by 1.3 $\log_{10}$CFU/egg. Results also indicated that eggs treated on the plastic flat under these conditions could yield similar results to eggs treated on the wire flat.

Table 5. Effects of 3% H_2O_2 combined 1 min of various UVC intensities with one rotation between two applications on wire and plastic flats (Experiment 5).

Treatment	UV Intensity	n/trial	Trial 1	Trial 2
			$\log_{10}$ CFU/egg	
Control		8	5.37 ± 0.14^{a}	5.13 ± 0.28^{a}
Wire				
	High[1]	16	2.62 ± 0.22^{bc}	2.48 ± 0.13^{bc}
	Medium[2]	16	2.18 ± 0.00^{c}	2.73 ± 0.25^{bc}
	Low[3]	16	3.22 ± 0.39^{b}	2.41 ± 0.12^{c}
Plastic				
	High	16	2.29 ± 0.10^{c}	2.33 ± 0.10^{c}
	Medium	16	2.71 ± 0.25^{bc}	2.43 ± 0.10^{c}
	Low	16	2.58 ± 0.17^{bc}	2.98 ± 0.15^{c}

[1] High = 14 mW/cm^2 (16 lamps)
[2] Medium = 8 mW/cm^2 (8 lamps)
[3] Low = 1.3 mW/cm^2 (4 lamps)
[a-c] Means within columns with different superscript differ significantly ($P \leq 0.05$)

Experiment 6 implemented the most effective methodology from Experiment 5 of 3% H_2O_2 in combination with 1 min of UVC exposure for two applications with one rotation between applications at medium (8 mW/cm^2) intensity. This methodology was compared to the use of H_2O_2 combined with UVB lamps and H_2O_2 alone. Treatment of eggs with H_2O_2 alone resulted in a 1.38 $\log_{10}$ CFU/egg reduction in APC compared to controls (Table 6). The antimicrobial capabilities of H_2O_2 were observed in this experiment; however, data suggest that H_2O_2 combined with UV will allow for an enhanced microbial reduction from the formation of hydroxyl radicals. Results observed from H_2O_2 combined with UV compared to H_2O_2 alone suggest that the microbial reduction shown during enumeration by the combination of components was from the

initial treatment of eggs and not a residual effect of the H_2O_2 on the eggshell surface while sampling took place. It can be determined that the amount of H_2O_2 applied to the egg was diluted by the volume of PBS in the sample rinse bag. Results incorporating 3% H_2O_2 application combined with 1, 2 or 3 min of UVB yielded 1.96, 2.06, and 2.21 log_{10} CFU/egg reduction, respectively, compared to UVC treated group which yielded a 2.67 log_{10} CFU/egg reduction. These results suggest that UVB exposure in combination with H_2O_2 has antimicrobial properties associated, though it is not as effective as UVC combined with H_2O_2.

Table 6. Effects of 3% H_2O_2 combined with ultraviolet UVC or UVB exposure compared to eggs solely treated with H_2O_2 (Experiment 6).

Treatment	UV Exposure Time	n[1]	log_{10} CFU/egg
Control		16	4.99 ± 0.12^{a}
H_2O_2 Alone (3%)		16	3.91 ± 0.13^{b}
UVC Wire	1 min	32	2.32 ± 0.05^{d}
UVB Wire			
	1min	32	3.03 ± 0.10^{c}
	2 min	16	2.93 ± 0.16^{c}
	3 min	16	2.78 ± 0.15^{c}

[1] Trial 1 = 8 control, 16 H_2O_2 for 1 min of UVC/UVB and H_2O_2 alone. Trial 2 = 8 control, 16 H_2O_2 for 1 min UVC/UVB and H_2O_2 for 2 and 3 min UVB.
[a-d] Means within columns with different superscript differ significantly ($P \leq 0.05$)

Experiment 7 used 3% H_2O_2 in combination with UVC at an intensity of 13.86 mW/cm^2 with one rotation between two applications to assess the amount of UVC exposure time needed to effectively reduce microbial levels. Previous experiments had

found that 1 min of UVC was sufficient compared to longer exposure periods; however, if the exposure time was further reduced it would increase the opportunity for this methodology to be adopted into commercial applications. Results showed that treatment with H_2O_2 combined with 15, 30, 45 sec or 1 min of UVC yielded bacterial reductions of 3.11, 3.05, 2.92 and 3.10 $\log_{10}$ CFU/egg, respectively (Table 7). These results indicate that shorter UVC exposure times can effectively reduce microbial levels when applied under these conditions. This suggests that hydroxyl radicals form rapidly when H_2O_2 is exposed to UVC. Shifting the UVC lamps over a larger area of the chamber allowed for maximum exposure of the eggshell surface to allow for the greatest amount of UVC exposure to the eggshell surface as possible.

Table 7. Effects of 3% H_2O_2 combined various UVC exposure times with one rotation between two applications on wire (Experiment 7).

Treatment	UV Exposure Time	n[1]	$\log_{10}$ CFU/egg
Control		16	5.49 ± 0.15^{a}
Wire Flat			
	15 sec	32	2.38 ± 0.07^{b}
	30 sec	32	2.44 ± 0.12^{b}
	45 sec	32	2.57 ± 0.14^{b}
	1 min	32	2.39 ± 0.08^{b}

[1] n = number of eggs per trial (8 controls and 16 eggs per treated groups).
[a, b] Means within columns with different superscript differ significantly ($P \leq 0.05$)

Experiment 8 was conducted to refine the most effective methodology found in Experiment 7. Further studies were conducted to evaluate the effect of applying multiple applications of H_2O_2 and UVC treatment with or without rotation on wire flats. To reduce UVC exposure time further than what was used in Experiment 7, an exposure time of 5 sec was compared to 1 min. It was determined that 5 sec was the minimal amount of time needed to consistently insert the wire egg flat manually into the UV chamber and remove from the opposite end. Results indicate that 3% H_2O_2 combined with 5 sec of UVC exposure applied with two applications and one rotation between applications yielded a 2.71 $\log_{10}$ CFU/egg reduction in APC compared to 2.71 $\log_{10}$ CFU/egg following two applications without rotation. These data suggest that the formation of hydroxyl radicals from H_2O_2 after exposure to UVC could be nearly instantaneous. Therefore, possible commercial application would be substantially simplified. The results using 1 min of UVC treatment with two applications with one rotation between applications produced a 2.09 $\log_{10}$ CFU/egg reduction compared to two applications without rotation showing a 2.88 $\log_{10}$ CFU/egg reduction (Table 8). These results indicate that two applications without rotation can reduce microbial levels found on eggshell surfaces. However, under higher initial microbial loads (trial 1), rotation of the egg during treatment further reduced microbial levels compared to no rotation. It was also determined that 5 sec under these circumstances is comparable to 1 min of UVC exposure.

Table 8. Effects of 2 applications of 3% H_2O_2 with 5 sec or 1 min of UVC exposure with or without rotation between applications (Experiment 8).

Treatment	UV Exposure	Rotations	n	Trial 1	Trial 2
				$\log_{10}$ CFU/egg	
Control			16	5.34 ± 0.16[a]	4.49 ± 0.15[a]
Wire Flat					
	5 sec	0	16	2.40 ± 0.18[b]	2.00 ± 0.03[b]
	1 min	0	16	2.29 ± 0.20[b]	2.11 ± 0.03[b]
	5 sec	1	16	2.00 ± 0.01[b]	2.02 ± 0.09[b]
	1 min	1	16	2.00 ± 0.03[b]	2.06 ± 0.20[b]

[a-c] Means within columns with different superscript differ significantly ($P \leq 0.05$)

Experiment 9 was conducted to use the MAX methodology determined in Experiment 8. The sample group for this study was increased to treat wire flats containing a total of 32 eggs to establish if the methodology observed from previous experiments was applicable for whole flat treatments. The first trial of this experiment sampled eggs from 6 locations on the wire flat. Each location was sampled 5 times over the course of the trial to observe an average bacterial reduction for that location. Results indicated that eggs located in the center of the flat yielded an average of 2.02 $\log_{10}$ CFU/egg reduction after treatment with H_2O_2 and UVC compared to those eggs located near the sides of the flat which averaged a 1.64 $\log_{10}$ CFU/egg reduction. Data suggests that eggs located near the edge of the flat near the entrance and exit of the UV chamber are not as effectively treated as those found towards the middle of the UV chamber. Further lamp manipulation took place between trial 1 and trial 2 of this experiment. The

second trial sampled 10 separate egg locations sampling 5 eggs per location. Results for this experiment indicate that eggs found in the middle of the UV chamber averaged 2.81 log_{10} CFU/egg APC reduction while those found on the outside edges of the UV chamber averaged APC reductions of 2.94 log_{10} CFU/egg (Table 9). This suggests that the MAX methodology found for eggshell disinfection can be applied to treat the entire wire flat and consistently provide similar log reductions on eggs from various areas of the flat.

Table 9. Effects of 3% H_2O_2 combined with 5 sec of UVC with one rotation between two applications on full treated wire flats (Experiment 9).

Treatment	Trial 1[1]	Trial 2[2]
	log_{10} CFU/egg	
Control	4.11	5.05
Treated		
	2.21	2.00
	2.04	2.33
	2.00	2.06
	2.01	2.00
	2.73	2.26
	TNTC*	2.22
		2.00
		2.00
		2.29
		2.23

n^1= 35 eggs (5 untreated control, 5 per location/6 locations)
n^2= 55 eggs (5 untreated control, 5 per location/10 locations)
* Too numerous to count

The objective of Experiment 10 was to evaluate various treatment methods on the eggshell surface to reduce microbial loads. It was shown in previous experiments that H_2O_2 alone has antimicrobial capabilities; however, when combined with UV exposure, microbial levels were further reduced. The first treatment in Experiment 10 was conducted to evaluate UVC alone to show proof of concept of the effects of using both H_2O_2 and UVC combined. This experiment further evaluated the use of the Chickmaster egg 84-plastic flat, which has more plastic material for structural strength. This additional material would potentially cause a decreased efficacy of the MAX methodology found on wire flats. Ultraviolet intensity measured at egg level on wire flats was 12.7 mW/cm^2 and 9.08 mW/cm^2 on the Chickmaster flat. Results indicated that UVC alone yielded a 1.70 log_{10} CFU/egg reduction in APC (Table 10). While eggs treated with H_2O_2 and UVC on the Chickmaster egg flat yielded a 2.38 log_{10} CFU/egg reduction in APC. The MAX methodology yielded a 2.91 log_{10} CFU/egg reduction in APC. These results indicate that UVC alone has antimicrobial capabilities, however, does not reduce bacterial levels as effectively as when combined with H_2O_2. Similar results were obtained by Bayliss and Waites (1979 and 1982), in which they showed that combining H_2O_2 and UVC can yield a 2000-fold greater reduction in bacteria than the components used separately. Results observed indicated that treatment on the Chickmaster plastic flat was comparable to the MAX treatment on wire flats, however, the use of the wire flat and addition of rotation within the treatment allow for an enhanced reduction in bacterial levels.

Table 10. A comparison of untreated control, MAX (3% H_2O_2 combined with 5 sec of UVC for two applications with one rotation between applications on wire), UVC alone for 5 sec with two exposures and treatment on the Chickmaster egg flat with 3% H_2O_2 combined with 5 sec of UVC for two applications without rotation (Experiment 10).

Treatment	n	log_{10} CFU/egg
Control	8	5.09 ± 0.27^{a}
UVC Alone	16	3.39 ± 0.20^{b}
Plastic (Chickmaster)	16	2.71 ± 0.22^{c}
Wire (MAX)	16	2.18 ± 0.11^{c}

[a-c] Means within columns with different superscript differ significantly ($P \leq 0.05$)

Experiment 11 was conducted to evaluate if applying heavier than previously used volumes of 3% H_2O_2 in combination with UVC could increase effectiveness of the process on a plastic flat. The 84-egg Chickmaster flat appears to have an increased amount of plastic surface area covering the eggshell surface compared to Jamesway plastic flats and the wire flat. Since some companies in the breeder industry use the Chickmaster system or systems with similar large flat styles, information was needed to determine what potential bacterial reduction could be achieved. After applying H_2O_2 to the eggs in the experiment, the volume used was determined. The light application (used in all previous methods) used 0.89 mL/egg while the heavy application used 1.9 mL/egg. The overall bacterial reduction for light application compared to heavy application was 2.54 versus 3.09 log_{10} CFU/egg, respectively (Table 11). This indicates that the greater volume applied to the eggshell surface was able to wet more of the eggshell surface that may have been covered or shadowed by the flat itself or other eggs. It is important to also note that this amount of volume did not cause a washing effect on the egg. There

was minimal amount of liquid on the heavy application that dripped from the bottom of the egg before entering the chamber. This is a positive attribute since it has been shown that any moisture residing on the eggshell surface can directly cause microbial penetration into the interior of the egg (Berrang et al., 1999). Overall observations suggest that treatment with higher volumes of H_2O_2 yield similar results that could be seen in previous experiments using the MAX methodology.

Table 11. Effect of light and heavy application of 3% H_2O_2 combined with 5 sec of UVC for two applications with no rotation between applications on 84-egg plastic egg flats (Experiment 11).

Treatment	n	$\log_{10}$ CFU/egg
Control	8	5.46 ± 0.12^{a}
Light Application	16	2.92 ± 0.20^{b}
Heavy Application	16	2.37 ± 0.13^{c}

[a-c] Means within columns with different superscript differ significantly ($P \leq 0.05$)

During Experiments 6 and 7 the crush and rub method adopted from (Musgrove et al., 2005b) was used to determine if the H_2O_2 and UVC treatment methodology had a residual effect on bacteria found within the shell pores and membranes of the egg. Results indicated that the second rinse bag removed additional microorganisms from the eggshell after the first rinse was performed. This allowed for a more accurate enumeration of the microorganisms present within the pores and membranes of the egg. Crush and rub analysis results indicated no consistent reduction in bacterial levels

between control and treated groups, indicating that there is likely little to no effect of treatment on microbes found within the pores and membranes of the eggshell (Table 12).

Table 12. Effects of 3% H_2O_2 combined with 1 min of UVC exposure for two applications with one rotation between applications using the crush and rub method.

Experiment	Trial #	Treatment	Rinse 1	Rinse 2	C&R
			log_{10} CFU/egg		
6	1	Control	5.06 ± 0.17^a	3.31 ± 0.18^a	3.07 ± 0.20^a
		Treated	2.26 ± 0.10^b	2.15 ± 0.02^b	2.27 ± 0.21^b
	2	Control	4.98 ± 0.43^a	2.80 ± 0.36	2.75 ± 0.25
		Treated	2.61 ± 0.30^b	2.67 ± 0.29	2.67 ± 0.81
7	1	Control	5.31 ± 0.18^a	4.12 ± 0.10^a	3.85 ± 0.35^a
		Treated	2.20 ± 0.00^b	2.25 ± 0.05^b	3.05 ± 0.54^b
	2	Control	5.49 ± 0.42^a	3.52 ± 0.26^a	3.32 ± 0.27^a
		Treated	2.58 ± 0.42^b	2.55 ± 0.05^b	3.83 ± 0.74^b

[a, b] Means within columns within trials significantly differ ($P \leq 0.05$)

Conclusion

The results of Experiments 1 through 11 indicate that an optimized method for the use of H_2O_2 and UVC was developed that could be implemented for commercial application. Experiments revealed that H_2O_2 concentrations in excess of 3% when combined with UVC are not necessary to achieve maximum microbial reductions. Other data gathered during these studies suggest that manipulating the UVC lamps within the

UV chamber and minimizing the dimensions of the chamber to create fewer shadows as well as increasing the UVC reflection off of confined reflective surfaces maximized UVC exposure to the eggshell surface. The action of rotating the egg between multiple treatments was also found to increase the efficacy of the treatment. This allows for the entire surface area of the egg to be exposed to H_2O_2 and UVC. It was further indicated that treatment on plastic flats could result in similar bacterial reductions observed using wire flats, allowing the ability of this methodology to be applied to the commercial industry. There was no conclusive indication of a reduction in bacteria levels within the pores and membranes of the eggshell, suggesting that this is a surface treatment. The optimal methodology to reduce the natural flora found on eggshell surfaces appears to be a mist of 3% H_2O_2 combined with 5 sec of UVC for two applications with one rotation between applications (i.e. MAX).

CHAPTER IV

EFFECT OF HYDROGEN PEROXIDE AND ULTRAVIOLET LIGHT ON EGGS INOCULATED WITH *SALMONELLA* TYPHIMURIUM

Introduction

The elimination of *Salmonella* serovars on eggs can be difficult since contamination is found essentially in all areas of the hatching egg operation (Bailey et al., 1996). Currently, *Salmonella* is the most prolific pathogenic bacterium associated with the contamination of eggs (Kuo et al., 1997 and Cox et al., 2000). The main source of this contamination is the result of the lack of egg disinfection, improper sanitization of equipment and facilities, and management practices at the farm (Bailey et al., 1996). Once *Salmonella* has colonized the gastrointestinal tract of the chicken, it has the ability to replicate and be shed into the environment. The infection found within one bird could cause any bird that is housed within the flock to become infected (Byrd et al., 1998). Vertical transmission of *Salmonella* from the hen to the egg can occur by contamination of egg components in the reproductive tract or by contact with fecal material (Cox et al., 2000). *Salmonella* can spread from the egg to the embryo potentially decreasing hatchability (Bailey et al., 1996). Viable chicks that are contaminated will likely show no sign of infection and be reared for human consumption (Byrd et al., 1998). These carcasses could be directly associated with an outbreak of food-borne illness (Cox et at., 2000). The ability to decrease or eliminate contaminated environments could reduce the frequency with which birds would come into contact with the bacteria. To successfully

achieve a reduction in egg contamination, an eggshell disinfection process must be implemented. This should be conducted as close to lay as possible to decrease the chance for contamination to occur from other environmental sources (Coufal et al., 2003). Assuming hens are free of *Salmonella*, the adaptation of an eggshell disinfection process could allow the egg to enter the hatchery environment potentially free of contamination.

The MAX methodology described in Chapter III was used to evaluate the effectiveness of treatment of eggs inoculated with *Salmonella enterica* serovar Typhimurium. Experiments were conducted using the crush and rub method on eggs inoculated with *Salmonella* to determine if there is a residual effect on bacteria located within the pores and membranes of the egg.

Materials and Methods

Artificial Contamination with Salmonella

In order to differentiate intentionally inoculated *Salmonella* from background microbiota, artificial contamination of eggs was performed using a *Salmonella* enterica serovar Typhimurium isolate resistant to both novobiocin (NO) and nalidixic acid (NA) (Byrd et al., 1998). *Salmonella* was propagated aerobically using Tryptic Soy Broth (TSB) (Difco, Detroit, MI) and enumerated using XLT-4 Agar (Difco). Both media were supplemented with 20 ug/mL NO (Calbiochem, La Jolla, CA) and 25 ug/mL NA (Fischer BioRegeant, Fair Lawn, NJ). An individual egg was placed in a WhirlPak bag and a 10 mL suspension of *Salmonella* culture of ~10^9 CFU/mL was added. Eggs were massaged for 1 min, removed from the WhirlPak bag and allowed to dry for 30 min. Eggs were then randomly selected for untreated control and treatment groups.

Project 2

In Experiment 1, two trials were conducted to evaluate the effectiveness of the MAX methodology discussed in Chapter III on eggs inoculated with *Salmonella*. Treated eggs consisted of using a 3% H_2O_2 solution combined with 5 sec or 1 min of UVC exposure with two applications with one rotation between applications. The incorporation of 1 min was used to verify the effectiveness of the MAX methodology. For this experiment eggs, were placed on the wire flat and misted with 3% H_2O_2 on all surfaces. The flat was immediately manually inserted into the UVC chamber for 5 sec or 1 min of exposure time. After the exposure to UVC, the egg flat was slid out of the opposite end of the UV chamber and brought back to the entrance. Eggs were aseptically rotated 180° on the egg flat and treated by the same method previously described. A total of 18 eggs were used in which 6 were assigned for each treatment group. Treatment eggs were placed into sterile plastic sample bags containing 25 mL of PBS. Untreated control eggs were sampled by suspending 1 mL of the rinse into a sterile culture tube containing 9 mL of PBS and preparing serial dilutions. Each dilution was sampled by plating 0.01 mL onto XLT-4 agar with a 0.01 mL sterile loop into respective quadrants. One mL from the treated sample rinse bag was suspended into a culture tube containing 9 mL of PBS. Sampling from the culture tubes consisted of plating 0.2 mL onto XLT-4 agar and spread plating with a sterile plastic "L" rod. Plates were incubated for 24 h at 37°C. Enumeration was conducted by hand counting colonies, and results were reported as $\log_{10}$ CFU/egg.

Experiment 2 further tested the MAX methodology over multiple trials using eggs inoculated with *Salmonella* Typhimurium. Eggs were first inoculated with a *Salmonella* concentrate and then treated with the MAX methodology. Three separate trials consisting of a total of 92 eggs were conducted assigning 46 eggs as untreated controls and 46 eggs as treated. Enumeration procedures used were the same as described for Experiment 1.

Crush and Rub Analysis

An additional eggshell microbial analysis was performed during the 2nd and 3rd trial in Experiment 2. The crush and rub methodology reported by Musgrove et al. (2005a, 2005b) was used to determine if the MAX methodology had any residual effect on *Salmonella* cells trapped within the pores and membranes of the egg. The experiment sampled a total of 60 eggs in which 30 eggs were selected from the untreated controls and 30 eggs from the treated. After plating a sample from the first rinsate bag, the egg was aseptically removed and placed into a second rinsate bag containing 25 mL of PBS. The rinsate bag was massaged for 1 minute. Plating and enumeration was performed as described for the first rinse bag. The egg was then aseptically removed and the contents of the egg broken out to discard albumen and yolk. Sterile DI water was then used to rinse any remaining adhering material from the interior of the eggshell. The remaining shell was then placed into a sterile conical tube containing 20 mL of PBS and crushed with a sterile glass wand until the shell was finely pulverized. Sampling, plating and enumeration of the conical tube rinsate was performed as previously discussed.

Statistical Analysis

Means were compared by analysis of variance (ANOVA) using the general linear model (GLM) procedure of SPSS and means separated by Duncan's Multiple Range Test. Means were considered statistically different at $P \leq 0.05$.

Results and Discussion

In experiments discussed in Chapter III, results indicated that 5 sec of UVC exposure was equal to 1 min of exposure for effective bacterial inactivation. Experiment 1 results indicate that 3% H_2O_2 combined with UVC exposure of 5 sec or 1 min yielded a 5.94 or 5.76 $\log_{10}$ CFU/egg reduction of *Salmonella*, respectively (Table 13). These data indicate that the MAX methodology (5 sec) has the ability to reduce *Salmonella* Typhimurium as effectively as 3% H_2O_2 combined with 1 min of UVC exposure, indicating that the production and effects of hydroxyl radicals occur in a short amount of time under these circumstances.

Table 13. Effects of 3% H_2O_2 combined various UVC exposure times with one rotation between two applications to reduce *Salmonella* (Experiment 1).

Treatment	n	$\log_{10}$ CFU/egg
Control	6	7.94 ± 0.25^{a}
5 sec	6	2.00 ± 0.00^{b}
1 min	6	2.17 ± 0.17^{b}

[a, b] Means within columns with different superscript differ significantly ($P \leq 0.05$)

Experiment 2 was conducted to evaluate the effects of the MAX methodology found optimal in previous experiments performed over multiple trials. Results for this

experiment indicate that the MAX method treatment yielded a 5.18 $\log_{10}$ CFU/egg reduction of *Salmonella* (Table 14). These results indicate that the methodology used can effectively reduce *Salmonella* Typhimurium found on the eggshell surface consistently.

Table 14. Effects of 3% H_2O_2 combined with 5 sec of UVC exposure times compared with one rotation between two applications to reduce *Salmonella* (Experiment 2).

Treatment	n	$\log_{10}$ CFU/egg
Control	46	7.48 ± 0.27^{a}
Treated	46	2.30 ± 0.09^{b}

[a, b] Means within columns with different superscript differ significantly ($P \leq 0.05$)

Crush and rub during trials 2 and 3 were conducted to evaluate any residual effect of treatment on *Salmonella* Typhimurium found in the pores and membranes of the egg. The second rinse bag for the untreated control group indicated a significant amount of *Salmonella* cells still adhering to the surface following the first rinse. These results suggest that the washing method used on eggs to remove bacterial cells from the surface does not recover all viable organisms. The second rinse conducted on treated eggs indicated that viable *Salmonella* cells were removed during the first rinse. The use of the crush procedure showed that 5.60 and 5.66 $\log_{10}$ CFU/egg of inoculated *Salmonella* were viable within the pores and membranes of both untreated and treated eggs, respectively (Table 15). It can be concluded from this analysis that the dipping method of applying the inoculum to the eggshell surface forced cells within the egg. The crush and rub methodology used indicated that treatment of eggs under these conditions did not result in any apparent residual effect on bacterial cells found within the pores and

membranes of the egg. Results indicate that treatment using the MAX methodology is most likely only a surface disinfection process.

Table 15. Effects of 3% H_2O_2 combined with 5 sec of UVC exposure with one rotation between two applications to reduce *Salmonella* within the pores and membranes of the eggshell using the crush and rub enumeration method.

Trial #	Treatment	Rinse 1	Rinse 2	C&R
			$\log_{10}$ CFU/egg	
2	Control	6.41 ± 0.65^{a}	4.36 ± 0.21^{a}	5.00 ± 0.20
	Treated	2.16 ± 0.12^{b}	2.36 ± 0.18^{b}	5.01 ± 0.08
3	Control	7.44 ± 0.19^{a}	6.33 ± 0.13^{a}	6.20 ± 0.21
	Treated	2.36 ± 0.23^{b}	2.32 ± 0.01^{b}	6.32 ± 0.40

[a, b] Means within columns with different superscript differ significantly ($P \leq 0.05$)

Conclusion

The results from these experiments indicate that using the MAX methodology can be effective at reducing *Salmonella* Typhimurium on eggs. Eggs that were inoculated to high levels showed an average of 5.0 $\log_{10}$ CFU/egg reduction in bacterial levels on the eggshell surface. However, the data show that this methodology has no effect on bacteria found within the pores and membranes of the shell. The ability to reduce *Salmonella* on eggshell surfaces is substantial to the overall hatching egg industry. *Salmonella* has the ability to transfer on and into the interior of the egg in a relatively short amount of time. Sanitization of hatching eggs soon after laying can potentially reduce the incidence of contaminated eggs, chicks and hatching facilities

(Coufal et al., 2003). Contamination of hatching eggs can lead to increased embryo mortality and decreased bird performance. Such contamination can be transmitted through grow out of the bird and potentially enter processing facilities (Berrang et al., 1999). This could pose industry concerns related to contaminated carcasses that could lead to possible food safety hazards. By implementing the MAX methodology, the potential for *Salmonella* presence can be reduced assuming no further contamination occurred during ovulation or through hatchery procedures.

CHAPTER V

EFFECT OF HYDROGEN PEROXIDE AND ULTRAVIOLET LIGHT APPLIED TO FERTILE EGGS TO EVALAUTE HATCHABILITY AND CHICK PARAMETERS

Introduction

Microorganisms can penetrate the shell during several stages of embryonic development causing an increase in contamination and also decreased hatchability and poor chick quality (Scott and Swetnam, 1993a). Poor hatching egg sanitation can play a major role in this occurrence. At the time of lay a potentially clean eggshell can be contaminated by microorganisms that are found throughout the environment (Buhr and Mauldin, 1994). Horizontal and vertical transmission of bacteria can contaminate the exterior and/or interior of the egg. Therefore, it is essential to disinfect all eggs that are transferred to the hatchery (Humphrey, 1994; Chavez et al., 2002). As shown in Chapters III and IV, the MAX methodology was found to function mainly an eggshell surface disinfectant. The implementation of this treatment has been shown to significantly decrease microorganisms found on eggs. To investigate the effectiveness of the MAX methodology in a commercial setting, a field experiment was conducted using fertile eggs at a local broiler breeder operation. The objective of this field experiment was to determine if this methodology could maintain or increase hatchability while effectively reducing the bacteria on the eggshell surface. The MAX methodology described previously was applied to eggs that were sorted by the breeder farm employees

to evaluate the effect of treatment on eggs that would typically enter the hatchery environment.

Materials and Methods

Project 3

The breeder operation where the experiment was initiated produced eggs from four houses joined by a common hallway. Eggs from 63 week old hens were collected from conveyor belt style houses. Eggs were collected by the grower and placed into plastic incubator flats which held 42 eggs/flat. Collection of eggs took place from 8:00 am to 1:30 pm to obtain the necessary amount of eggs needed to fill 1 incubator cart (total of 7,560 eggs). The total eggs used for this experiment was divided in half, assigning 3,780 eggs as untreated controls and 3,780 eggs as treated. Each farm rack held 120 plastic egg flats within 30 shelves holding a total of 5,040 eggs/rack. To fill 1 incubator cart, 1.5 farm racks were needed. Flats were numbered from 1 to 180. Odd numbered flats were selected as untreated control groups. Control eggs were weighed after collection and then directly placed into the farm rack. Even numbered flats were selected for treatment with H_2O_2 and UVC. Once treated on the wire flat used for the MAX methodology, eggs were aseptically placed back onto plastic flats and weighed. The flat was then transferred to the farm rack along with control flats. The experiment used eggs that were sorted by settable and unsettable size and/or staining that was present on the eggshell surface. Only eggs that showed high organic contamination on the surface or cracked eggs were discarded by the breeder farm employees during collection. Once a substantial number of eggs were placed onto the farm rack, it was

then placed into egg cooler at 65°F (18°C) with 75% relative humidity until transported to the hatchery two days later. At the hatchery, plastic flats were transferred to an incubator cart prior to setting in the incubators for 18 days. On day 18, eggs were candled to remove infertile, eggs containing dead embryos, cracked or rotten eggs before transfer to the hatchers. All eggs removed during candling were classified by break-out. Each incubator flat that contains eggs was weighed again before transfer to the hatching cabinet to determine an average egg moisture loss. Eggs were then transferred to hatching trays and placed into the hatching cabinet until day 21. On day 21, hatched chicks were counted and weighed. Dead chicks and piped eggs were counted and discarded, and all remaining unpipped eggs were broke-out and classified as previously described. To calculate the number of fertile eggs set, infertile, farm cracked, transfer cracked, eggs sampled for enumeration and rotten eggs were subtracted from the initial egg count. Hatchability was reported as a percentage of fertile eggs.

Eggshell microbial samples were taken by selecting random eggs from the plastic flats of each treatment group. Sampled eggs were collected prior to eggs being placed in the farm cooler at the breeder facility (Day 1) and 3 days later before the incubator cart was placed into the incubator (Day 2).

Sampling and enumeration was performed by aseptically placing eggs into a sterile plastic bag containing 25 mL of PBS. The bag containing the egg and PBS was massaged for 1 min to remove microorganisms from the eggshell surface. Control rinse bags were serially diluted by suspending 1 mL of the rinse into 9 mL of PBS. Plating from each control culture tube was performed by spreading 0.01 mL of rinse onto TSA

with a sterile loop. Treated rinse bags were serially diluted by suspending 1 mL of the rinse into 9 mL of PBS. Sampling from each culture tube was performed by pippetting 0.2 mL of the rinse onto TSA and spread with a sterile "L" rod. Plates were incubated for 24 h at 37°C. Colonies were hand counted and data recorded. Results were reported in $\log_{10}$ CFU/egg. Level of detection was calculated as shown in Chapter III. Plates yielding 0 colonies were assigned a LOD of 2.0 $\log_{10}$ CFU/egg.

Statistical Analysis

Aerobic plate counts and chick weight means were compared by analysis of variance (ANOVA) using the general linear model (GLM) procedure of SPSS. Hatchability and embryonic mortality of control vs. treated groups were compared using the Test of Binomial Proportions at a significance level of $P<0.05$.

Results and Discussion

Results of the initial hatching egg treatment (Day 1) resulted in a 3.36 $\log_{10}$ CFU/egg reduction compared to the control group. Microbial samples taken at the hatchery facility immediately before eggs entered the incubator (Day 2) yielded a 3.17 $\log_{10}$ CFU/egg reduction in bacteria levels compared to the control group (Table 16). The experiment indicates that high bacteria levels were present on the eggshell surface following the selection criteria maintained by the breeder farm. After treatment, significant bacterial reductions were observed even in the presence of organic material on the eggshell surface. Colder temperatures found in the cooler had some effect from initial treatment (Day 1) and before eggs entered the incubator (Day2) by retarding the growth of microbes found on the eggshell surface. Thus, the results show that

implementing this treatment at the day of collection will reduce microbial levels found on the eggshell surface until entering the incubator. Proper management of clean equipment and transportation vehicles may be able to keep contaminants from transferring to the disinfected eggshell. Egg moisture loss did not differ significantly between control and treated groups, indicating that the MAX methodology does not negatively impact the functionality of the egg during embryonic development. Results indicate that chick weight did not differ significantly between control and treated groups, suggesting that treatment did not affect the efficacy of viable chicks.

Table 16. Eggshell APC, egg moisture loss during incubation, and chick weight at hatch for untreated control and treated (3% H_2O_2 combined with 5 sec of UVC for two applications with one rotation between applications) groups.

Treatment	Control	Treated
Day 1[1] (log_{10} CFU/egg)	5.57 ± 0.18^{a}	2.21 ± 0.15^{b}
Day 2[1] (log_{10} CFU/egg)	5.34 ± 0.25^{a}	2.17 ± 0.11^{b}
% Moisture Loss[2]	17.01	18.01
Chick wt. (g)[3]	46.89 ± 0.22	48.72 ± 0.47

[1] n = 39 eggs (13 controls, 26 treated)
[2] n = 180 plastic incubator flats with 42 eggs in each (90 controls, 90 treated)
[3] n = 44 chick trays with chicks (22 controls, 22 treated)
[a, b] Mean within a row with different superscript differ significantly ($P \leq 0.05$)

Results indicate that H_2O_2 and UVC treatment of eggs immediately after collection at the breeder facility showed no negative effect on hatchability compared to the control eggs (Table 17). Based on no significant differences in egg moisture loss, H_2O_2 and UVC treatment showed to have no affect on eggshell quality that could

potentially reduce hatch. However, similar numbers of rotten eggs were observed between treated and control groups.

Table 17. Egg fertility, embryonic mortality, and hatchability for untreated control and treated (3% H_2O_2 combined with 5 sec of UVC for two applications with one rotation between) groups[1].

	Control	**%**	**Treated**	**%**
All Set Eggs	**3870**		**3780**	
Infertile	544	14.39	546	14.44
Farm Crack	61	1.61	42	1.11
Transfer Crack	15	0.40	20	0.53
Rotten Eggs	37	0.98	30	0.79
TOTAL	657		638	
# of Fertile Set	3102		3106	
Unaccounted for at Hatch	-25		-60	
# of Fertile	**3077**		**3046**	
Early Dead	187	6.08	196	6.43
Mid Dead	13	0.42	16	0.53
Late Dead	118	3.83	100	3.28
PIP	15	0.49	13	0.43
Cull Chicks	70	2.27	80	2.63
TOTAL	403	13.10	405	13.30
Chicks (HOF)[2]	2674	**86.90**	2641	**86.70**

[1] No statistical differences were found between control and treated eggs for any of the parameters measured.
[2] HOF= Hatch of Fertile

Conclusion

Results found in this experiment indicate that treated eggs entered the incubator with minimal contamination on the eggshell surface. Further observations from the analysis of hatch residue indicate that treatment did not affect embryo mortality when compared to untreated controls. Treatment of hatching eggs using H_2O_2 combined with UVC showed to be an inexpensive, rapid and effective treatment method applied to the exterior of the eggshell. Previous results found in Chapter IV showed that this methodology can be effective against pathogens such as *Salmonella*. Reduction of such pathogens on the surface of eggs bound for the hatchery could decrease the transfer rate of bacteria from the breeder farm to the hatchery. If pathogens had been present on the eggshell surface at the time of egg collection in this experiment, the elimination of the microorganisms occurred without impacting hatchability. Hydrogen peroxide combined with UVC appears to be a safe methodology that could be easily adopted into the poultry industry. The overall goal of implementing this methodology is to reduce the microbial load found on hatching egg surfaces, thus potentially improving hatchery sanitation and preventing chick contamination at hatch.

CHAPTER VI

CONCLUSION

The combination of H_2O_2 and UVC was studied to optimize the reduction of microbial levels found on eggshell surfaces. Each component separately will act as a disinfectant and has been shown to reduce microbes. However, used in combination H_2O_2 and UVC will produce hydroxyl radicals that inactivates bacterial cells by oxidizing and mutating the DNA of the organism, preventing subsequent replication.

The reduction of the natural flora found on the eggshell surface was conducted using eggs collected from a typical nest box environment. The inactivation of bacteria found on these eggs was made possible with the manipulation of H_2O_2 application, UVC exposure, incorporation of rotation between applications, as well as direct manipulation of UVC lamps located inside the chamber to effectively irradiate the entire eggshell surface. Experiments also compared wire flat versus plastic incubator flats to show proof of concept using incubator flats used throughout the poultry industry. Further studies used the crush and rub method on eggs to determine if the MAX methodology reduced microbial loads found in the pores and membranes of the eggshell.

Results indicated that increasing H_2O_2 concentrations above 3% showed no further reduction in bacterial levels. The addition of rotation of the egg between multiple applications showed enhanced microbial reductions by eliminating microbes that may have been injured or survived a single treatment of UVC exposure. Treatment of eggs showed to be highly effective using fewer lamps than Wells et al. (2010). Results

indicated that high UVC intensities were not needed to reduce microbes found on the eggshell surface. Each of these factors suggest that a fine mist of 3% H_2O_2 combined with 5 sec of UVC with one rotation between two applications yielded the greatest reduction over other combinations. This methodology shows to be feasible for commercial application compared to the original methodology of H_2O_2 combined with 8 min of UVC exposure. Eggs placed on the Chickmaster incubator flat showed that applying twice the volume of H_2O_2 can effectively reduce microbial levels without rotation being required. Results indicate that the MAX methodology could be applied to plastic incubator flats and yield comparable reduction in microbial loads compared to wire flats. Crush and rub enumeration conducted on eggs treated with H_2O_2 and UVC showed inconsistent results, suggesting that the methodology did not influence bacteria found within the pores and membranes of the egg.

The MAX methodology was directly applied to eggs inoculated with high concentrations of *Salmonella* Typhimurium. The objective of experiments outlined in Chapter IV was to test the methodology with higher levels of the pathogen than what would typically be found in commercial settings. This allows for the opportunity to test the limits of this methodology by showing the maximum reduction possible on eggshell surfaces. Results indicated that the MAX methodology applied to eggs inoculated with *Salmonella* Typhimurium produced 5 $\log_{10}$CFU/egg reductions consistently on the entire egg surface. Implementing crush and rub methods suggest that the MAX methodology does not reduce *Salmonella* Typhimurium found in the pores and membranes of the eggshell when applied in an inoculum wash to the surface of the egg.

To determine if the MAX methodology developed can be applied to commercial settings, a field trial was conducted to evaluate treatment on eggs typically found in day to day breeder operations. It was noted during this experiment that eggs were successfully treated at time of collection at the breeder farm and held for several days in a cooler setting before placement into the incubator. At this point no indication of additional contamination on the eggshell surface occurred. There was no significant relationship found between treatment and hatchability. However, the reduction in bacterial levels shows promise to reduce contamination on eggs entering a hatchery environment. In addition, possible decreases in the amount of rotten eggs found could reduce the occurrence of exploding eggs contaminating the hatch environment.

Further research needs to be conducted using equipment that allows for mechanical treatment of eggs on a continuous belt system to determine if the photolysis of H_2O_2 and UVC and subsequent bacterial inactivation is instantaneous. Further reducing the UVC exposure time needed to effectively reduce microbial levels will increase the opportunity for this methodology to be adopted into commercial applications. The incorporation into everyday hatch facilities must also take place to determine if this methodology is commercially feasible. Research should also incorporate full grow out of chicks that are hatched from eggs treated with this methodology and compare levels of contamination of equipment, houses, the intestinal tract as well as reproductive system to analyze if there is a prolonged benefit for treatment beyond the initial application of disinfected eggs. The implementation of H_2O_2 and UVC should also be studied with respect to the table eggs industry as an additional

treatment after traditional washing of eggs has occurred. The added treatment could further reduce food-borne safety issues that may be associated with the consumption of eggs.

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