ABSTRACT

LOWMAN, ZACHARY SCOTT. The Long Term Effects of In-Ovo and Nutritional Manipulation on Growth Rate, Feed Conversion, Mineral Utilization and Reproductive Performance of Avians. (Under the direction of Christopher Ashwell).

Ensuring future production of safe, affordable, and healthy food is the goal of commercial agricultural producers and researchers. Animal agriculture has drastically improved over the last 40 years in all aspects of production. This significant change in production demands novel approaches that compliment conventional practices. This research has explored multiple methods of early life manipulation (hypoxia, nutritional programming, immediate access to nutrition at hatch, dietary manipulation, and alterations in sanitation practices) as well including a very broad spectrum of avian species (Emu, Broilers, Layers, and Turkeys). It is apparent that the conditions an organism is exposed to during embryonic development and within the early portions of its life greatly impact phenotypic development and late life performance.

Reproduction in poultry has been studied for many years, and is a crucial factor to consider when selecting and raising parental lines. To explore the role of diet during grow out on reproductive traits in leghorn type males. Hy-line parent stocks were raised on 3 different diets Low (12%CP) Control (18% CP) or High (24% CP). As birds reached sexual maturity, body weight (BW), testis size, semen volume, sperm concentration, and histology were measured in males. This trial demonstrated significant differences in body weights, as well as testicle weights. Differences in semen volume and sperm concentrations and histological development were observed between the dietary treatment groups. In the female birds there were significant changes due to dietary treatment in albumen, yolk, and total egg weights,
shell thickness, porosity, and protein content of the albumin. The manipulation of the breeder diet significantly influences bird performance.

In animal production agriculture there is a critical need for antimicrobial products that are safe, effective and affordable to the food industry as well as acceptable for food production use by the consumer. Currently there are very limited options for approved products that are safe, effective, affordable, versatile, and capable of being utilized throughout most of the poultry industry that meet societal criteria. Spraying broiler breeder eggs with Bac-D™ significantly decreased aerobic bacteria on the exterior eggs. Bac-D™ sprayed eggs had reduced bacterial levels for up to 3 hours suggesting a moderately long residual kill-time. Bac-D™ washed eggs also had significantly higher conductance than control eggs. Turkey poults hatched from eggs washed with Bac-D™ showed differences in feed conversion, which were significantly lower when compared with the control birds at 21d. This improvement in FCR may be due to reduced levels of slightly pathogenic bacteria in the gut resulting from the Bac-D™ egg sanitization. External treatment of the egg significantly influences bird performance.

Emu production has had minimal amounts of research in all aspects of production compared to typical commercial poultry. From two field trials our results show that providing Hydrogel-95 in the hatcher significantly decreased weight loss in emu chicks during the first week of life. In a separate study, emu egg storage was explored. Eggs were stored for various amounts of time ranging from 1 to 8 weeks, subsequent effects on moisture loss and hatchability’s were recorded. Significant differences in hatchability and moisture loss of emu eggs due to storage time were observed. Manipulation of egg storage and hatching conditions significantly influences bird performance.
Calcium and phosphorus excretion in animal manures is of great environmental concern. Diets deficient in Ca and P for the first 72 hours of life have demonstrated increased absorption and utilization of P in chickens. Our studies evaluated this principal in turkey poults. In turkeys, the conditioned group had significantly higher retention levels of both Ca and P in comparison to the control group. Manipulation of early life nutrition significantly influences bird performance.

Specific manipulation of the early life environment of numerous avian models results in long-term impacts on performance and supports the concept that oviparous organisms have significant periods of plasticity. Specific conditioning leads to specific outcomes. By tailoring early life manipulation to targeted issues in poultry production it is possible to improve performance and provide positive economic conclusions.
The Long Term Effects of In-Ovo and Nutritional Manipulation on Growth Rate, Feed Conversion, Mineral Utilization and Reproductive Performance of Avians

by
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BIOGRAPHY

Zachary S. Lowman was born in Jacksonville, North Carolina on September, 23, 1989. He spent his childhood in Newton, North Carolina on his family cow farm. He has had a fondness for cattle and chickens since he was a child. He actively participates in breeding, raising, and showing the farms Angus and Shorthorn cattle. In addition to cattle Zack raises and shows light brown Leghorn and Sumatra chickens. Zack attended North Carolina State University where he received his bachelors in Poultry Science as well as his Masters in Poultry Science.
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LITERATURE REVIEW

Production agriculture has improved at an unmeasurable rate for the last few decades. The growth rate, feed conversion, and reproductive performance of chickens, turkeys, dairy cows, and swine, today are almost unfathomable to many older producers. The majority of these advances can be attributed to genetic selection (Havenstein et al. 2003; Anderson et al. 2013). However; researchers have discovered ways to make these elite animals perform at even more precise frequency. This is accomplished by manipulation of the organism both in-ovo and within the first few days of life by a menagerie of methods, including alteration in oxygen availability during development, implementation of improved methods of hygiene during incubation, alteration the nutritional status of the parents, and manipulation of available minerals and nutrients at various periods of development. All of these modest changes early in life can have a profound impact on the development, nutrient utilization, growth, and performance for the life of the animal.

Early Life Conditioning

In the 1970’s many producers were focusing on the growing and finishing time periods of the production period, the majority of input should be focused on those time periods (Thaxton and Parkhurst, 1976). However in light of current developments, there has been much research has focused on changes that occur during the first few days of life in regards to Nutrition, (Lowman and Parkhurst, 2014a; Noy, and Sklan, 1999; Thaxton and Parkhurst, 1976; Batal and Parsons, 2002) hypoxic conditions during incubation,(Dusseau and Hutchins, 1988, Druyan et al. 2007, 2012) presence of bacteria during incubation (Lowman
and Parkhurst, 2013, Arhienbuwa, et al 1980) and environmental factors affecting sex ratio (Pryke and Rollins 2012; Cichon, et al. 2005, Anderson et al, 1993). Data from studies using early life manipulation demonstrates that there are many simple sometimes unintentional, alterations that can be employed that result in life altering changes in the growth, development, performance, and efficiency of the organism. The basis for the majority of these conditioning or reprogramming events are the premise of compensatory growth. During the reprogramming or condition event, the organism is typically deficient, but the same theory applies when there is an excess of some nutrient, gas, or other manipulation. During this time period the organism adapts to the change, as a result of this adaptation the organism theoretically becomes more efficient in the utilization of the specific stressor. This environmental or dietary change or stressor that organism undergoes causes changes in expression of certain genes. Many people are of the belief that an organism either has a gene or does not have the gene, a basic Mendelian view, and that if the gene is present it is expressed. This concept is very easy to understand and believe. However it is well known that just because a gene is present, it does not mean that it is being expressed. There are many different “signals” that either up or down regulate genes. Many of these signals are dietary or environment related. There is much research describing signals that affect expression of genes (Lowman and Ashwell, 2014; Ashwell and Angel, 2010). The study of these external changes and the effect on gene expression has been termed epigenetics, which has been further broken down into various other subcategories such as nutrigenomics, the study of how the diet of an organism affects the resultant gene expression within an organism (Ashwell, 2010).
The gut of an embryonic chick undergoes much change during the first days of life, the growth of the villi in the duodenum is said to be almost complete at 7 days (Uni et al. 1998). During this time of rapid development it makes sense that this would be the opportune time to condition this developing organ. There are reports of increased compensatory growth and efficiency in feed conversion in both broiler and turkeys as a result of restricting feed access to poult and chicks at various time points during the first week of life (Plavnik and Hurwitz, 1988a; Plavnik and Hurwitz, 1988b). Ashwell and Angel (2010) demonstrated that chicks that were challenged with a diet that was low in phosphorus for 90 hours immediately post hatch, that these chicks were then able to utilize P more efficiently later in life. These chicks also had increased expression of the Na/P co-transporter genes later in life first demonstrating the first example of neonatal programming of gene expression in oviparous organisms (Ashwell and Angel, 2010). Short term hypoxic incubation of broiler eggs has shown to increase hematocrit and hemoglobin levels. This increases the oxygen holding capacity of the blood and makes broilers more efficient at meeting the great oxygen demand presented from the rapid growth rate of the birds, predominately the breast muscle (Druyan, 2012).

There has been some discussion whether embryos are conditioned based upon the diets of the parents during embryonic development, or in the case of birds, during production of the egg. Armitage et al. (2005) discusses many of the phenotypic changes in the offspring of parents that were fed diets that had a surplus of nutrition during pregnancy. The resulting offspring had many issues ranging from abnormal glucose homeostasis, increased blood pressure, and increased adiposity. In contrast Burdge et al. (2007) reviewed literature describing changes
in the offspring that resulted from parents being fed nutrient deficient diets. The resultant offspring were shown to have increased expression of glucocorticoid receptors, altered timing of sexual maturation, and variable birth weights. Willems et al. (2013) demonstrated that albumin deprived pullets had lower body weights during rearing. However, during laying, these pullets out preformed the control and sham groups, suggesting a long lasting programming effect (Willems et al. 2013). There have been several papers that report environmental conditions, specifically the availability and quality of food resources, can bias the sex ratio of hatchlings in sexually dimorphic avian species (Pike and Petrie, 2005; Pryke and Rollins, 2012). Typically, the smaller of the two sexes will be produced in greater numbers when there are scarcer resources because their rearing is less nutritionally costly. The effect appears to be maternally derived; which is based on resource availability. The female will skew the sex ratio of her offspring towards the sex that is more likely to survive on lower quality egg contents during incubation and can be reared in a less favorable environment. Generally, this is the sex that consumes less and, on average, has a lower mature body weight (Nager et al. 1998; Ellegren, et al. 1996).

**Early Life Nutrition**

Nutrition is a crucial factor in all organisms, so it should come as no surprise that chicks, poults, and emu chicks, have all been shown to have increased performance and growth rates when provided nutrients immediately at hatch (Lowman and Parkhurst, 2014a; Noy, and Sklan, 1999; Thaxton and Parkhurst, 1976; Batal and Parsons, 2002). Nevertheless, in a commercial hatchery chicks are hatched over a 2 day period, and are not pulled from the
hatcher until the majority of eggs have hatched. So the chicks that hatch early are in the hatcher with no feed or water. Once the birds are removed they have to be vaccinated, sexed, packaged, and shipped. These processes can take large amounts of time and the birds can be with no food or water for up to 48 hours after hatch (Batal and Parsons, 2002; Noy and Sklan, 1999). As a result, many researchers have been exploring methods that allow increased access to nutrition and hydration for these birds. There are numerous types of supplemental feeds and additives that have been explored. Thaxton and Parkhurst (1976) reported beneficial effects from the addition of sucrose to the water at placement. Lowman and Parkhurst (2014a) reported increases in weight gain of emu chicks that were supplemented with a hatching gel supplement in the hatching baskets. Noy and Sklan (1999) utilized several types of hatching supplements including a hatching gel supplement, a liquid form of the hatching gel, and early access to feed, all of these methods of supplementation were shown to cause increased growth of the poults and chicks when compared to the control group. There are several possible mechanisms that could be responsible for the increased growth and performance observed in the early supplemented birds. Noy and Sklan (1999) suggested differences in yolk sack absorption, changes in gastrointestinal tract (GIT) development, or some factors associated with compensatory growth. Increased performance from chicks or poults that were supplemented early in life has been shown to have significantly increased GIT growth and development. Uni et al. (1998) demonstrated that chicks that were immediately provided with feed had significantly increased villi height and area when compared to fasted chicks as well as many changes in the production of digestive enzymes. Noy, et al. (1996) demonstrated that chicks allowed access to feed utilized their
yolk sac much more quickly than did fasted group. This is in agreement with past work in
chicks (Bierer and Eleazer, 1966) and in poult (Moran and Reinhart, 1980). Noy, et al.
(1996) states that the absorption of the yolk lipids was probably not influenced by the actual
presence of food in the GIT, but the increased yolk utilization is due to the increased
gastrointestinal activity.

**Nutritional effects on Growth and Performance**

Nutrition has long been known to be a crucial factor in the development of any
organism. Genetics are a major deciding factor in the developmental potential of an
organism (Havenstien et al, 2003). However, regardless of the quality of genetics an animal
possesses, if they do not receive sufficient nutrition throughout the growing period (Walsh
and Brake, 1997) or during the reproductive period (Walsh and Brake, 1997; Wilson et al.
1987) the animals will never be able to reach their full genetic potential.

Broiler breeders reared on low protein diets exhibit decreased fertility as compared to
broiler breeder females that were reared on higher levels of crude protein (CP) during grow-
out (Walsh and Brake, 1997 and 1999). It was suggested that the low CP group did not
receive an adequate amount of CP during growing, thus not allowing for optimum
development of the oviduct nor the spermatozoal storage ducts yielding decreases in fertility
(Walsh and Brake, 1997). In broiler breeders a negative correlation has been found to exist
between the body weight (BW) and sexual activity of the male (Burke and Mauldin, 1985)
Low CP diets during rearing have been shown to greatly influence the growth rate of broiler
breeders. Low CP diets produce males with lower body weights than males reared on diets
that contain a higher CP content (Romero-Sanchez et al. (1),2007, Zhang et al. 1999). Caged broiler breeder males that were raised on 16% CP diets had significantly lower semen concentrations as compared to males raised on 12% CP diets (Zhang et al. 1999), an inverse relationship as described in female broiler breeders. Broiler breeder growers commonly experience a noticeable decrease in fertility of flocks once they reach 50 weeks of age (Kirk et al., 1980). This is commonly attributed to the fact that broiler breeder males are too large to reproduce (Hocking and Duff, 1989) However, Romero-Sanchez et al, (1) 2007, theorized that this decline in fertility is due to a deficiency in nutritional intake, and the male can no longer support growth and maintenance of the body and appropriate reproductive performance. Wilson et al. 1988 reported that that broiler breeder males fed diets containing crude protein levels from 9-15% showed no significant difference in semen volume or concentration when collected at 48 and 49 weeks of age. Hocking and Benard (1997) reported that feeding broiler breeder males on higher levels of CP (16%) did not change semen volume or concentration as compared to the low CP (12%) group, and that the average testis size was smaller in the birds fed the higher CP diet than the low CP diet (Hocking and Benard, 1997). Decreases in protein intake have been shown by researchers to have no negative impact on fertility or semen volume in White leghorn males (Arscott, and Parker 1963).

Crude protein is not the only nutrient of importance in feeding poultry, total caloric intake or ME has been shown to also be a very important factor to consider when developing diets for poultry. Decreases in semen production have been observed in broiler breeder males that have been placed on lower caloric diets; it has been shown the decrease in semen
production becomes more evident when caloric intake drops below 330 kcal/day (Bramwell, et al. 1996). Sexton et al. (1989b) also reported similar results in broiler breeder males that were fed low caloric diets. The birds showed reductions in BW, semen weight, and sperm concentrations (Sexton et al. 1989b). Broiler breeders on low calorie diets have been shown to have decreased sperm penetration ability when compared to males on higher caloric diets (Bramwell et al. 1996).

**Nutritional influence on Sex Ratios**

Avian species are unique when compared to mammalian species in many ways. One major difference in avians is that the female is the heterogametic sex (Klein and Grossmann, 2008). The genetic sex of the embryo is said to be determined during the first meiotic division in the follicle, which is typically 1 hour just prior to ovulation (Olsen and Fraps, 1950). In the commercial poultry industry the sex ratio of a typical hatch has long been considered 1:1 (Szalay et al. 1989). However it has been demonstrated that wild birds by some unknown mechanism, possess the capability to alter the sex-ratio of the offspring in times of environmental stressors (Pryke and Rollins 2012). There have been several theories proposed to explain this phenomenon. The predominating theory is based on nutrient availability in the environment. Many researchers have found that in times of famine, the less nutrient demanding sex will thrive (typically the smaller sex), and the larger sex that requires higher nutrients will die (Cichon, et al. 2005, Anderson et al, 1993b). Pryke and Rollins, 2012 found that when female parrot finches were fed low quality diets the percentage of male offspring significantly increased to 72.9% males, but when birds were fed
a high quality diet the sex ratio was not significantly different with 48.6% of the chicks being male. This phenomenon is thought to be an a adaptive response due to the fact that males seem to be less affected by nutritional stressors as compared to female offspring. Nager et al. 1998 demonstrated with lesser black-backed gulls, that there was a direct link in maternal condition and sex biased survival of the offspring, as well as adaptive adjustments in the sex ratio of the resulting offspring.

Another plausible theory is that the female changes the sex ratio at hatch, and to some extent throughout the growth period due to changes in stress hormones such as corticosterone. The mechanism of adjustment has not been definitively identified but, increased corticosterone levels in the bloodstream of the mother results in the reciprocal rise in deposition in the yolk of the egg. There was a significant increase in the number of female offspring from mothers that had been implanted with corticosterone implants. This study not only revealed changes in primary sex ratio, but the males from the implanted group had significantly lighter body weights at hatch, slower growth rates when compared to non-implanted control groups, and slower cell-mediated immune responses. Conversely, female offspring appeared to be unaffected (Love, et al. 2005). Klein and Grossmann (2008) demonstrated male biased ratios in chickens at the beginning of lay. The skewing was only evident in a line of non-commercial fancy type chickens and only at the start of reproduction, the authors also state that since commercial layer type hens have undergone such increased selection that they have seem to have lost the natural skewing toward males as seen in the non-selected lines. The skewing toward male offspring at the start of lay is thought to be common, and is explained by the timing of hormone productions of androgens, progesterone, and corticosterone. As
chickens come into lay, their progesterone levels are low, which favors males, but as production continues, progesterone levels rise. It is believed that since layer type pullets mature so early due to selection, this causes them to no longer possess male bias (Klein and Grossmann, 2008). Correa et al. (2005) demonstrated that chickens with high progesterone levels produce a larger percentage of female chicks (75%) than the control group, and the low progesterone and control group were male biased (60%). High plasma corticosterone levels and low plasma testosterone levels have been found in peafowl to be associated with female biased primary sex ratios (Pike and Petrie, 2005).

Nutritional (Mineral) Effects on Future Utilization

Calcium sources

The calcium that hens use to form egg shells comes from two main sources; absorption from the intestines, or reabsorption from the bones of the bird. All calcium is transported via the blood system. A large portion of calcium comes from reabsorption from the medullary bone and occasionally from the cortical bones under times of great deficiency. This reabsorption of calcium from the medullary bone is regulated by parathyroid hormone, and absorption from the intestines is regulated by 1,25-dihydroxyvitamin D₃ (Johnson, 2000, and Sugiyama, et al. 2004).

Calcium’s effects

Calcium is a very important nutrient, especially in laying hens, a deficiency in calcium is said to cause the fastest adverse signs in reproduction of laying hens (Roland et al. 1985). Cason and Britton (1981) demonstrated that exposing a laying hen to a low calcium diet for just
several hours can cause a significant decrease in shell quality of the eggs laid the following day. Feeding low levels of calcium to layer pullets during grow out causes an increase in body weight, liver weight, and fat pad weight, as well as a decrease in reproductive performance of the bird throughout life (Roland et al. 1985). These pullets also consumed a significantly larger amount of feed, with no benefit to production or egg size or quality, increasing production costs to the producers (Roland et al. 1985, Frost and Roland, 1991). As one would expect, increasing dietary calcium significantly increase the breaking strength of the tibia, as well increases tibia weight, and bone mineral content (Frost and Roland, 1991). Turkey poults have been shown to have significantly lower growth rates when raised on low calcium diets (0.37%), these birds also demonstrated decreases in feed conversion (Formica, et al. 1962).

**Calcium Phosphorus Ratio**

There is an antagonistic relationship between Ca and P in the guts of organisms, the digestibility of phosphorus is affected by the levels of both Ca and P present. (Liu, et al, 2013; Driver et al, 2005). Researchers have been studying the delicate balance of calcium to phosphorus in the diet for many years (Sullivan, 1962). Still after 50 + years of research, there is still some debate as to the proper ratio. This ratio is important due to the fact that having a surplus or deficiency of either can cause serious developmental and performance issues. Much of the total phosphorus in vegetable based diets is bound to phytate molecules which make it difficult for the organism to absorb in the gut (Eeckhout and Paepe, 1994; Liu et al, 2013). To overcome this issue a surplus of phosphorus is typically added to the diet.
This however results in an increased amount of phosphorus in the feces of the bird which is then spread on to fields (Honeyman, 1993). Phosphorus has been shown to accumulate in higher quantities than other nutrients on land where poultry litter is applied (Chapman, 1996). These high levels of phosphorus in the soil have been shown to increase the amount of phosphorus that is present in run-off water, which eventually runs in to lakes and streams and other surface water sources and causes eutrophication (Chapman, 1996; Sharpley et al. 1993). Runoff rates of P has been shown to range from 2.2% up to 7.3% of the P from litter spread on the fields (Chapman, 1996).

**Hypoxia**

The current selection process that has resulted in the elite performing broiler currently being produced come with a downfall; the increased selection for rapid growth has resulted in birds which have less than superior development of visceral systems (Havenstein et al., 2003b) and this has contributed to the difficulties of broiler chickens in regulating O2 supply to match O2 demands under suboptimal environmental conditions, especially at higher BW when the metabolic demands are high. In growing broilers, the inability of the cardiovascular system to efficiently maintain O2 supply leads to cardiovascular disorders and ultimately death, and results in significant economic losses (Julian, 1993, 2000; Balog, 2003). The effects of hypoxia on avian embryotic development has been well studied (Duran et al. 2007). When a bird is exposed to hypoxic conditions, there is a whole host of physiological changes that occur as the bird tries to cope with the stressful condition. Almost all of these changes are attempts by the bird to increase the oxygen level in the blood such as:
angiogenesis, polycythemia, and modification of hemoglobin isoforms (Dusseau and Hutchins, 1988, Druyan et al. 2007, and 2012). This was demonstrated by exposing chick embryos to 15% oxygen during incubation from E7 to E14 caused a significant increase in vascularity, with the vascular density index increasing by 34% to 41% (Dusseau and Hutchins, 1988). However, increasing oxygen demand by exposing chicks to ascites-inducing conditions (AIC) caused an increase in their hematocrit levels (Druyan et al., 2007b) due to increased erythropoiesis (Luger et al., 2003). The combination of increased blood vessel density as well as increases in red blood cells could potentially result in sufficient oxygen availability to the tissues.

When chickens are exposed to a stressor (hypoxia), it has been shown to elicit several physiological changes in blood composition. When an organism is subjected to a stressor such as heat or hypoxic conditions, this elicits a stress response within the organism which leads to an increase in corticosterone (De Smith et al. 2008). Corticosterone has been shown to initiate numerous physiological changes within birds, especially with metabolic function. One main function of corticosterone is that it causes the mobilization or production of glucose in order to help provide the energy for the organism to combat the stressor (Virden and Kidd, 2009). Glycogen levels have been shown to decline rapidly during hypoxic conditions leading to a dramatic increase in blood glucose levels (Beattie, 1964). Stress has also been shown to cause dramatic reductions in growth rates and development, which may be attributed to a decrease in resting metabolic rate (RMR) (Virden and Kidd 2009, Edens 1977). Hypoxia has also been shown to cause changes in blood gas levels of chickens, embryos exposed to hypoxic conditions have been shown to have decreased levels of $\text{HCO}_3^{-}$.
(Tazawa et al. 2012) which has been shown to have some association with a decrease in blood pH in the embryo (Tazawa et al. 2012, Tazawa, 1981) In contrast, other researchers reported very little or no change in blood pH (Everaert et al., 2008, Bruggeman et al. 2007). However, Tazawa et al, does state that the drop in pH in relationship to HCO$_3$ is much smaller than estimated, suggesting there is some other interaction taking place (Tazawa et al. 2012). These decreased levels of HCO$_3$ of the embryos exposed hypoxia is thought to attributed to the metabolic down regulation of the embryos, and that anaerobic glycolysis was a causative agent in this down regulation (Tazawa et. al. 2012).

Hatchery Sanitation

It has long been know that hatchery sanitation is of the up most importance to ensure the production of healthy hardy chicks and to maintain maximum hatchability rates. There are many sanitation methods employed to maintain the cleanest facilities possible. However, even if the machines and hatchery are completely bacteria free, the eggs that are being brought into the hatchery are not. There are large amounts of bacteria on unwashed broiler breeder eggs (Lowman and Parkhust, 2013; Brake and Sheldon, 1990). Gentry and Quarles 1972 reported bacterial counts on the exterior of eggs ranged from 190 to 430,000 per egg. Bacteria on the surface of the egg can cause significant decreases in hatchability, poor chick quality, and increased rates of infection in chicks (Arhienbuwa, et al 1980). There have been many studies exploring the possibility of sanitizing broiler breeder eggs for over 50 years, these trials range from the use of Quaternary Ammonium Sanitizers (Brake and Sheldon, 1990) Formaldehyde Fumigation (Funk,1954) Fumigation with Ethylene Oxide (Lorenz}
Dipping eggs in antibiotics (Miller 1956) Hydrogen peroxide (Sheldon and Brake, 1990). Even though many of these methods successfully decrease bacterial levels, there appears to be some drawback to each method, which make them not commercially applicable. These issues range from changing conductance of the eggs which can alter embryo development and decrease hatchability rates of older flocks (Vick and Brake, 1986). Others such as formaldehyde gas is dangerous to human, and has been found to be carcinogenic (Frasenko, et al 1990)

**Bac-D™**

Bac-D™ is a novel sanitizer that shows great promise in the commercial poultry industry. Bac-D contains Benzalkonium chloride (BZK) in very low levels. BZK compounds have been shown to significantly decrease bacterial levels on hatching egg, while causing no significant change in hatchability (Lowman and Parkhurst, 2013). Many disinfectants do not have a residual kill time, however Bac-D™ has been shown to be effective for up to 3 hours after application (Lowman and Parkhurst, 2013). BZK has been utilized as an antiseptic solution for humans for numerous years (Dyer, 1998) However, Bac-D™ also utilizes the use of a proprietary process of adding hydrogen ions to the solution. When used in combination with BZK makes it a truly novel, and potentially an extremely valuable, tool for the poultry industry. In preliminary trials poults that hatched from eggs that were misted with Bac-D™ showed a significant improvement in feed conversion ratio (FCR) (Lowman and Parkhurst, 2014b). This is potentially a huge breakthrough for producers, due to the fact that poultry are grown on such a large scale, it has been estimated that changing the FCR by
one point could potentially result in over a gain of one million dollars in just North Carolina complexes (Mahmoud and Edens, 2003).

**EMU** (*Dromaius novaehollandiae*)

Emu (*Dromaius novaehollandiae*) are large flightless birds and are members of the Ratite family. They originated in Australia and have long been raised for their meat and oil. Emu meat is extremely lean and is low in cholesterol (Davis, 2007) and the fat and oil is highly valued because of its antibacterial, anti-inflammatory, and burn healing properties (Beckerbauer et al. 2001; O’Banion and Griswold, 1998).

Emu females typically reach sexual maturity at around 2 years of age. Emu typically lay between 25-30 eggs a year, then the eggs are incubated by the male emu (Davis, 2007; Davies, 1975). Incubation times for emu eggs vary greatly and is very temperature dependent, typically ranging from 48-56 days (Davis, 2007; Minnaar and Minnaar, 1993). The hatch rate of emu is much lower than that of other commercial avian species with hatch rates reported ranging from 56% to 68% (Szczerbinska et al 2004; Majewska, 2001). These very low hatch rates are a great issue for emu producers due to the fact that emu are much more expensive to raise in comparison to current commercial poultry. Emu production is costly due to the fact that emu are such large birds, have a very slow growth rate, poor feed conversion, a long incubation period, and have a very limited season of lay.

**Egg Storage**

There has been numerous papers published focusing on egg storage in both turkeys and chickens. Brake et al. (1997) published a very thorough review addressing many of the key
factors involved with storage of avian eggs. It is suggest that there is not one single

temperature, humidity, or length that is optimal for all situations. Some of the most

important factors presented that must be taken in to consideration before deciding on a

storage protocol are age of the flock, the genetics of the flock, and weight of the egg. Egg

storage is a very crucial consideration factor when setting up an incubation program. It has

been found in poultry that there is not a single set storage time, that there are many

contributing factors that must be considered. Extended storage of chicken and turkey

hatching eggs has been shown to cause increased embryo mortality (Elibol and Brake, 2007;

Brake et al. 1997), decreased hatchability (Elibol, and Brake, 2007; Brake et al, 1997)

variation in hatch time (Mayes and Takeballi, 1984), decreased egg quality (Brake et al.

1997), and increases in pH of the albumen to above optimal levels (Goodrum et al. 1980).
There are many factors that can alter moisture loss. The age of the flock has been shown to

have a significant impact on shell thickness, pore length, and pore numbers (Brake et al.

1997). Females from young flocks have been shown to produce thicker egg shells, and as the

hen progresses in her lay cycle the shells become thinner (Peebles and Brake, 1987; Roland,

1976) The porosity of eggs is smaller in younger birds, but as production increases so does

porosity of the egg (Peebles and Brake, 1987).

Storage time

Therefore, It should come as no surprise that the longer an egg is stored the more it decreases

the chances of it hatching, however it is also logical to think that an egg is at its maximum

hatching potential as soon as it is laid (Wilson, 1991). However, chicken eggs have higher
hatchability after 1-2 days of storage than fresh set eggs (Funk et al., 1950). This increase is thought to be attributed to the breakdown of the albumin in the egg. The albumin holds the yolk in the middle of the egg, but as albumin quality decreases it allows the blastoderm on the yolk to move closer to the side of the egg (Hurnik, et al, 1978). This allows for increased gas exchange for the developing embryo (Brake and Rosseland, 1995).

Most commercial hatcheries try and set eggs after 3-4 days of storage, and rarely if ever store beyond 7 days (Mayes and Takeballi, 1984). Prolonged storage of eggs have been shown to cause increases in incubation times of eggs (Kaufman, 1939, Bohren et al, 1961) It has been reported that every day that an egg is stored, that it ads approximately one hour to the hatching time (Becker et al. 1968). There are numerous reports that discuss the time at which egg storage starts to cause decreases in hatchability. Byng and Nach (1962) reported decreased hatchability from just 2-3 days of storage, however Asmundson and McIlratith (1948) found that turkey eggs hatch better after 2-3 days of storage, chicken eggs were shown to hatch better after 1-2 days of storage as compared to freshly laid eggs (Funk et al. 1950). Funk (1934) and Scott (1933) reported that hatchability did not decrease until 7 days of storage. The storage times of emu eggs appear to be more variable than other commercially raised avian species. A 28 day storage period is thought to be the standard storage time for most emu producers; however there have been reports of storage for up to 44 days before incubation (Minnaar and Minnaar, 1998). Egg storage even if just over night is recommended on all emu eggs even freshly laid eggs. Brake suggest that fresh eggs be stored at 70-75F overnight to allow for the breakdown of the albumen (Brake and Rosseland, 1995). There are some sources that recommend that emu eggs not be stored longer than a 7
day period (Christianson, 1993) yet; there are other sources that suggest that emu eggs can be stored up to 21 days with minimal decreases in hatchability. The 21 day storage time is thought to be of similar duration that the eggs undergo in the wild (Brake and Rosseland, 1995).
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MANUSCRIPT 1

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The Effect of Bac-D™ on hatchability, conductance, growth rate, and feed conversion on turkey poults

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Primary Audience: Researchers, Turkey Producers, Hatchery Managers
SUMMARY

The major factor facing the commercial poultry industry today is the cost of feed. Breeding companies have put great emphasis on selecting their lines for rapid growth and low feed to gain to increase the efficiency of production. Even though genetics have made drastic differences in growth parameters, evident from the last 45 years of genetic selection, producers still employ other methods to help birds perform more efficiently including: feed additives, temperature control, incubation, ventilation, hatchery sanitation, and egg disinfection. Bac-D™ is a novel disinfectant, which is currently being used as a wound wash for humans and animals. The product utilizes benzalkonium chloride, a well-studied quaternary ammonium compound, which has been generally regarded as safe (GRAS). Bac-D™ has been effectively used as a human first aid antiseptic for many years. The objectives of this trial were to determine the effects of Bac-D™ on egg conductance, hatchability, body weight, and feed conversion (FC) in turkey eggs and poults. Turkey eggs were washed with Bac-D™ then incubated under standard conditions while recording egg conductance and hatchability. Body weights were measured at hatch, 21d, and 42d. The results showed that egg conductance, hatchability, or body weights of chicks hatched from Bac-D™ washed eggs did not differ significantly from water washed controls. However, mean feed conversion was significantly lower (p<0.0062) in Bac-D™ poults (FCR=1.10) compared with the control birds (FCR=1.17) at 21d. The improvement in FCR may be due to reduced levels of slightly pathogenic bacteria in the gut resulting from the Bac-D™ egg sanitization. The potential reduction in FCR will be of significant economic impact to poultry producers.
DESCRIPTION OF PROBLEM

The main goal of the commercial poultry industry is to produce the largest bird on the smallest amount of feed. To do this companies have put great emphasis on selecting their lines for rapid growth rate while also selecting for feed conversion. This genetic progress has been demonstrated by Havenstein et. al 1994, 2003. Where the current commercial broiler was compared to a random breeding meat line that is representative of the broiler lines of 1957. From these studies the dramatic effects of over 45 years of genetic selection is very evident, 1957-2003. Even though genetics have made such a drastic difference in growth parameters, there are still other methods that producers employ to help the birds perform even more efficiently. Feed additives, temperature control, incubation, ventilation, hatchery sanitation, egg disinfection.

There are many methods that have been employed by producers to reduce bacteria on eggs including: ethylene oxide (Lorenz et al. 1950), hydrogen peroxide (Sheldon and Brake, 1990), quaternary ammonium sanitizers (Brake and Sheldon, 1989), antibiotics (Miller, 1956), UV light exposure (Coufal et al, 2003), and heat treatment (Funk et al, 1954). Many of these disinfectant methods are very effective at reducing bacterial counts; however, they also can have detrimental effects on the developing embryo due to the fact that some sanitizers may remove the cuticle of the egg, a protective thin layer that seals the egg, which
allows for increase in loss of moisture from the egg, which directly affects the embryo by interfering with respiration (Peebles and Brake, 1985).

Formaldehyde fumigation has been the most commonly used method for egg sanitation in the industry (Coufal, et al, 2003). The main issues then industry face with formaldehyde is inhalation of the vapors by workers (Walker, 1944) as well as its carcinogenic properties and decrease in hatchability (Fasenko et al., 2009). Since there are many drawbacks associated with formaldehyde use, there has been much effort aimed at finding alternative approaches to the use of formaldehyde (Brake and Sheldon, 1989).

The effect the product has on hatchability is a major consideration that hatchery managers must consider when searching for alternative hatching egg sanitizers. Coufal et al, 2003 explored the use of UV light as a disinfectant method. Ultra violet light was shown to yield the same hatchability results when compared to untreated eggs (Coufal, et al, 2003). Broiler eggs exposed to ozonated water for 30 min resulted in reduced hatchability rates, while eggs exposed to triple strength formaldehyde remained unaffected (Whistler and Sheldon, 1988). Eggs washed with electrolyzed water showed very little difference between the treatment and control groups (Fasenko et al., 2009).

Bac-D™ is a novel disinfectant, which is currently being used as a wound wash for humans and animals. Bac-D™ utilizes a well-studied quaternary ammonium compound benzalkonium chloride. Benzalkonium chloride which has commonly been recognized as safe, and has been effectively used as a first aid antiseptic developed for humans for many years (Dyer et al, 1998; Gerald et al., 2011). Recently, researchers have begun to study the effects of different benzalkonium chloride compounds on hatching eggs (Aygun and Sert,
2012a, 2012b). There is still a great amount of investigation needed in order to determine the specific biocidal effects of Bac-D™ on different bacteria and viruses in order that the killing potential may be maximized. The mode of action of benzalkonium chloride consists on the modification of cell membrane permeability which results in the leakage of the contents of the cell (Fazlara and Ekhtelat, 2012; Gradel et al, 2005). Bac-D™ is a novel disinfectant, which is currently being used as a wound wash for humans and animals, and the active ingredient is benzalkonium chloride. Bac-D™ is a safe, potential substitute to harsh chemicals.

Therefore, the objectives of this trial were to determine the effect of washing eggs in Bac-D™ on the conductance, hatchability, livability, growth rates, and feed conversion in turkey poults.

MATERIALS AND METHODS

General procedures

The experimental procedure used in this investigation was approved by the North Carolina State University Animal Care and Use Committee. Six hundred eggs from a commercial turkey company (Butterball, Garner, NC) were randomly divided into 2 groups, application at hatch and application prior to hatch. These two groups were further divided in to Bac-D™ or PBS at hatch or Bac-D™ and water prior to hatch. In the first group 150 eggs were sprayed before incubation with Bac-D™, 150 were sprayed with water, prior to spraying 50 eggs from each treatment were labeled with a number and were then weighed. The eggs were sprayed with ~20 mL per flat of eggs, with a manual hand pump sprayer (Fisher
Scientific, Waltham, MA) for both the Bac-D™ and water sprayed eggs. The 300 eggs for the post-hatch application were incubated in the same incubator as the pre-incubation treated eggs. The eggs were incubated under standard conditions 99.5°F 55% RH; on day 7 the eggs that were numbered were weighed again to calculate relative moisture loss and conductance. On day 25 all of the eggs were moved to the same hatcher. Upon pip the 300 post hatch poult eggs were then split into their 2 treatment groups and half were misted with PBS and the other half misted with Bac-D™ just prior to removal from hatcher. Upon hatching the chicks were individually tagged and weighed.

**Conductance and Hatchability**

The egg weights from day 0 and day 7 of incubation were used to calculate the relative moisture loss, as well as egg shell conductance. The average conditions for the 7 days were temperature of 99.5°F, barometric pressure of 30.11, and relative humidity of 50%. The conductance was calculated using the equation of Paganelli et. al. 1974 [19]. Hatchability was calculated at hatch, the un-hatched eggs were broken out to determine if the eggs were infertile or time at which they had died.

**Brooding**

The poultts were placed in groups of 10 poultts in each pen. Birds were reared at North Carolina State University on the Turkey Education Unit (Lake Wheeler Road, Raleigh, NC). The house contained 48 pens, each pen had approximately 5 inches of pine wood shavings as bedding. Each pen contained one feeder and one plasson™ waterer, and for the first week a second small hand waterer, as well as a cardboard scratch feed pan. After the first week the
small waterer and cardboard scratch pan were removed. The house was kept at a constant 30°C for the first week, reduced to 28°C for the second week, reduced to 26°C for the third week, and reduced to 24°C for the remainder of the trial. The poults were kept on 24 hours of incandescent light for the first 3 weeks and then were placed on 23 hours of light for the last 3 weeks of grow out. The feed and water were checked twice daily, feed was dispensed from hanging tube feeders. The waterers were washed once daily to keep them clean. The birds were fed 2 diets, a starter diet for the first 21 days and grower diet for the last 21 days of the trial. The starter diet was composed of 29.5% CP and 1481 kcal/lb. The grower diet consisted of 25.09% CP and 1481 kcal/lb. All feeds were produced by the North Carolina Agriculture Research Service Feed Mil.

**Sampling, Body Weights, and Feed Conversion**

Individual body weights were recorded at hatch, 7, 21, 42 days. Feed conversion was recorded for the entire duration of the trial.

**Statistical Analysis**

The experimental data were analyzed as a 2x2 design. Data were analyzed using ANOVA (JMP 10, SAS, Cary, NC) using Tukey-Kramer comparison of means. Eggs and Poults were used as the experimental units and an alpha of 0.05 was used to establish significances.

**RESULTS AND DISCUSSION**

Bac-D™ showed no significant effect on the hatchability of turkey eggs. The hatchability of the control group and treatment group varied only slightly from each other and were
comparable with industry standards that week (Table 1). Bac-D™ also had no significant difference on the conductance of the turkey eggs. Eggs from Bac-D™ treated group had a mean conductance of 0.0176, and the control eggs had a mean conductance of .0169 (Table 2). Lowman and Parkhurst (2014) reported Bac-D™ to cause a small yet significant change in conductance of broiler breeder eggs [20].

**Body Weights**

Significant differences were seen at the initial hatch weighing, with the Bac-D™ misted eggs having a significantly higher (p<0.0001) mean weight of 60.35g when compared to the water washed control eggs which had a mean of 57.67g. The Bac-D™ and the PBS misted poults means did not differ significantly from each other (Table 3). There were no significant differences between any of the 4 treatment groups neither for the D21 weights nor for the D42 weights (Table 3).

**Feed Conversion**

There were significant differences (p<0.0062) in feed conversion at the 21 day weighing. The poults from the eggs washed with Bac-D™ prior to incubation had a mean FCR of 1.1 where the poults from the eggs washed in water had a mean FCR of 1.17(Table 4). This resulted in 6 points in improved feed conversion. There were no significant differences in feed conversion at the 42 day weigh back between the two groups (Table 4). There were no significant differences observed at Day 21 or 42 between the poults that were misted with Bac-D™ or PBS at pip (Table 5).
From the data collected from this trial Bac-D™ appears to have no negative effects on the hatchability or growth rates of pouls that were hatched out of eggs that had been misted with Bac-D™. This lack of significant difference in hatchability and production parameters were all in agreement with Ayugun et al. 2012a, which demonstrated that Benzalkonium Chloride had no effect on the growth or hatchability in Japanese quail. The initial difference at hatch may be attributed to several potential causes. This could be due to the decreased bacterial load on the eggs during incubation, which resulted in increased growth. Even though bacterial counts were not measured in this trial, Lowman and Parkhurst (2013) have demonstrated that Bac-D significantly lowers exterior bacterial counts on the egg which could serve as a possible explanation to the changes observed from this trial (Lowman and Parkhurst, 2013). One other very plausible explanation could be change in conductance, even though not significantly different could have had a beneficial effect on the development of the embryo during incubation resulting in a larger chick at hatch as compared to the control group. Bac-D™ has been reported to significantly change the conductance of broiler breeder eggs (Lowman and Parkhurst, 2013). Duryan et al. (2012) demonstrated that changes in oxygen levels at various time points thorough out incubation cause a significant impact on the development of the embryo. The improvement in feed conversion could be due to the decrease in bacterial loads embryonically resulting in a change in the gut microflora as a result of the Bac-D™ egg sanitation. This change in FCR could be of great economic benefit to poultry producers. In North Carolina alone, even a one point in FCR could yield over one million dollars of monthly revenue (Mahmoud and Edens, 2003).
CONCLUSIONS AND APPLICATIONS

1. Washing eggs in Bac-D™ has no significant effect on hatchability or overall growth rate of turkey poults.

2. Spraying eggs with Bac-D™ has some significant effect on feed conversion of poults.

3. This improvement in feed conversion could be of great economic benefit to the poultry industry.

4. Spraying eggs with Bac-D™ does not significantly change conductance or gas exchange of turkey eggs.

ACKNOWLEDGEMENTS

The authors would like to thank RDM Products for providing the Bac-D™ used for the trial. We are also greatly indebted to the Charles Lee Guy foundation for financial support, as well as Butterball for supplying the turkey eggs.
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### Table 1. Hatchability of eggs, and current industry average

<table>
<thead>
<tr>
<th></th>
<th>Hatchability</th>
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<tr>
<td></td>
<td>% hatch</td>
<td>Infertile</td>
</tr>
<tr>
<td>Bac-D</td>
<td>83.89</td>
<td>9</td>
</tr>
<tr>
<td>Water</td>
<td>86.3</td>
<td>8</td>
</tr>
<tr>
<td>Industry</td>
<td>83.18 *</td>
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</tr>
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</table>

Hatchability of Turkey eggs from Bac-D treatment and Water treatment, Breakout for eggs that did not hatch, infertile, early dead (1D-9D) Mid-dead (10D-19D) late dead (20D-28D). *denotes number from personal communication with Butterball staff.
Table 2. Conductance of turkey eggs by treatment

<table>
<thead>
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<th></th>
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<tbody>
<tr>
<td>Bac-D</td>
<td>0.0175</td>
<td>49</td>
<td>0.00032</td>
</tr>
<tr>
<td>Water</td>
<td>0.0169</td>
<td>50</td>
<td>0.00032</td>
</tr>
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</table>

Conductance values of Turkey eggs washed in either Water or Bac-D prior to incubation. Lack of super scripts denotes no significant different.
Table 3. Poult body weights at D0, D21, D42

<table>
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<th>D42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bac-D Washed</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
</tr>
<tr>
<td>Bac-D Washed</td>
<td>60.35</td>
<td>0.431</td>
<td>574.30</td>
</tr>
<tr>
<td>Water Washed</td>
<td>57.68</td>
<td>0.431</td>
<td>598.18</td>
</tr>
<tr>
<td>Bac-D Misted at pip</td>
<td>59.28</td>
<td>0.422</td>
<td>581.34</td>
</tr>
<tr>
<td>PBS Misted at pip</td>
<td>58.44</td>
<td>0.429</td>
<td>581.57</td>
</tr>
</tbody>
</table>

Body weight of Pouls at hatch (D0), D21, and D42 in grams. \(^{ABC}\) denotes significance at the (p<0.005)
Table 4. Feed Conversion at 21D and 42D washed eggs

<table>
<thead>
<tr>
<th></th>
<th>D 21</th>
<th>D42</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean FCR</td>
<td>SE</td>
</tr>
<tr>
<td>Bac-D Washed</td>
<td>1.1^A</td>
<td>0.014</td>
</tr>
<tr>
<td>Water Washed</td>
<td>1.17^B</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Feed Conversion Ratios for Eggs washed with Bac-D or Water prior to incubation, ^AB denote significance at (p<0.005) with in each column.
Table 5. Feed Conversion at 21D and 42D of poults misted at pip

<table>
<thead>
<tr>
<th></th>
<th>D 21</th>
<th></th>
<th>D42</th>
<th></th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean FCR</td>
<td>SE</td>
<td>Mean FCR</td>
<td>SE</td>
<td></td>
</tr>
<tr>
<td>Bac-D at Pip</td>
<td>1.14 A</td>
<td>0.035</td>
<td>1.74 A</td>
<td>0.067</td>
<td>12</td>
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<tr>
<td>PBS at Pip</td>
<td>1.14 A</td>
<td>0.033</td>
<td>1.86 A</td>
<td>0.067</td>
<td>12</td>
</tr>
</tbody>
</table>

Feed Conversion Ratios for poults misted with Bac-D or PBS at pip, A^B denote significance at (p<0.005) with in each column.
The Effects of Bac-D™ on Total Aerobic Bacteria Naturally Found on Broiler Breeder

Eggs

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Running Headline: BAC-D – A NOVEL DISINFECTANT WASH FOR EGGS

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ABSTRACT

Hatchery sanitation is of the utmost importance in the poultry industry, and may have drastic economic effects within a company. It has been shown that eggs with increased total aerobic bacterial counts may cause a decrease in hatchability, performance and growth, as well as a decrease in overall chick quality. Several methods have been utilized to decrease bacterial load on the exterior surface of the egg such as the use of: hydrogen peroxide, quaternary ammonium compounds, antibiotics, and UV light exposure. Many disinfectants may effectively sanitize the egg; however, they have detrimental effects on the developing embryo due to the removal of the cuticle, allowing increased moisture loss from the egg. Benzalkonium chloride has been effectively used as a first aid antiseptic for humans. Bac-D™, a novel disinfectant with benzalkonium chloride utilized as the active ingredient. Bac-D™ is a safe, potential substitute to harsh chemicals. In this trial, eggs were sprayed with the same volume of either Bac-D™ or water. Eggs were sampled at 3 different time points after spray (0, 1.5, or 3 h). At the culmination of each time point, a portion of the eggs was inoculated with an endogenous bacterial inoculum. Eggs were placed in a bag with 1% PBS and the rinsate was promptly plated on TSA (Tryptic Soy Agar). There were significant decreases (p < 0.0001) in the log CFU/mL numbers at each time point (0, 1.5, 3 h). These results reveal the potential sanitizing effects of Bac-D™ on total aerobic bacterial counts on eggs.

Key words: eggs, egg wash, disinfectant, total aerobic bacteria, Bac-D™
INTRODUCTION

Hatchery sanitation is a crucial factor in today’s poultry industry. Since poultry are produced in such large numbers, even very small deviations can have drastic economical impacts. Eggs with increased total aerobic bacterial counts can cause a decrease in hatchability, performance and growth, as well as a decrease in chick quality (Coufal et al., 2003). Many different techniques have been explored to determine the correlation between reducing bacteria present on eggs and improving hatchability.

Some of the methods used to reduce bacteria on the shell of the egg include: ethylene oxide (Lorenz et al., 1950), hydrogen peroxide (Sheldon and Brake, 1990), quaternary ammonium sanitizers (Brake and Sheldon, 1989), antibiotics (Miller, 1956), UV light exposure (Coufal et al., 2003), and heat treatment (Funk et al., 1954). Many disinfectants may effectively sanitize eggs; however, they have detrimental effects on the developing embryo. This phenomenon occurs because sanitizers may remove the cuticle, a protective thin layer that seals the egg, which allows for an increase in loss of moisture from the egg, which directly affects the embryo (Peebles and Brake, 1985).

Currently, formaldehyde fumigation is the most commonly used intervention method for egg sanitation in the industry (Coufal et al., 2003). The main disadvantages associated with the use of formaldehyde are its carcinogenic properties as well as its effect on decreasing hatchability (Fasenko et al., 2009). As a result, there has been much effort aimed at finding alternative approaches to the use of formaldehyde (Brake and Sheldon, 1989).

Since rate of hatchability is a major consideration when searching for alternative hatching egg disinfectants, it may be considered as a valuable parameter to measure the
efficacy of different disinfecting compounds. The use of UV light as a sanitation procedure compared to water resulted in no significant differences in hatchability when compared to untreated eggs (Coufal et al., 2003). Broiler eggs fogged with ozonated water for 30 min caused significantly reduced hatchability rates, while eggs fogged with triple strength formaldehyde remained unaffected (Whistler and Sheldon, 1989). Electrolyzed water treated eggs showed similar hatching rates between the treatment and control groups (Fasenko et al., 2009). All approaches herein mentioned have shown mixed hatchability rates thereby eliciting the increasing need for a method, which has consistent and effective results.

Benzalkonium chloride, a quaternary ammonium compound, generally recognized as safe, has been effectively used as a first aid antiseptic for humans through the years (Dyer et al., 1998; Gerald et al., 2011). The mode of action of benzalkonium chloride consists on the modification of cell membrane permeability causing the leakage of cell contents (Gradel et al., 2005; Kuda et al., 2007; Fazlara and Ekhtelat, 2012). Recently, researchers have studied the effects of different benzalkonium chloride compounds on hatching eggs (Aygun et al., 2012a,b). This intervention practice is still much less frequently used in the US than formaldehyde fumigation. Bac-D is a novel disinfectant, which is currently being used as a wound wash for humans and animals, and the active ingredient is benzalkonium chloride. Bac-D is a safe, potential substitute to harsh chemicals and it may be capable of decreasing bacteria and improving hatchability. Much investigation is needed in order to determine the specific biocidal effects of Bac-D on different bacteria, viruses, and protozoa, so that a kill spectrum may be established.
Therefore, the objectives of this trial were to determine the effects of Bac-D on total aerobic bacterial counts and to explore the claim that Bac-D has an effective killing time of over 3 hours.

**MATERIALS AND METHODS**

**Experimental Design**

Broiler breeder eggs were obtained from the North Carolina State University poultry education unit (Raleigh, NC). The eggs were stored for 4 days in an egg cooler at 60 F and 70 % relative humidity. The experiment was designed as a 3 x 2 x 2 factorial. There were 3 sampling time points (0, 1.5, 3 h), 2 spray treatments (Bac-D and water), and inoculum (inoculated vs. uninoculated). This design resulted in 12 treatments consisting of 50 eggs each for a total of 600 eggs. The 4 treatment groups for each time point were: Bac-D, inoculated; Bac-D, uninoculated; DI water, inoculated; and DI, uninoculated.

**Inoculum**

Fresh inoculum was prepared on the day of sampling by placing 25 eggs from the same broiler breeder flock in a sterile container with 40 mL of PBS (Fisher Scientific, Waltham, MA). The eggs were shaken for 60 s, and then removed, and the inoculum was placed in a sterile 1 L bottle at room temperature until inoculation.

**Sampling**

Eggs were placed in a sterile plastic egg flat. Each flat was then sprayed with approximately 60 mL of either sterile DI water, or Bac-D. The eggs were incubated at room
temperature for 0, 1.5, or 3 h. At the end of their corresponding time points, 50 eggs were inoculated with 40 μL of the endogenous inoculum (2.98 log CFU/mL), which was evenly spread over the surface of the egg with a sterile cotton swab. The inoculum was allowed to air dry for 10 minutes, and then the egg was placed in a 710 mL Whirl-pak bag (Nasco, Fort Atkinson, WI) that contained 20 mL PBS (Fisher Scientific, Waltham, MA). The bags were manually shaken for 60 s, then the egg was removed, 40 μL of rinsate were plated on TSA (Becton Dickinson, Sparks, MD), and spread with disposable L-spreaders (Fisher Scientific, Waltham, MA). Plates were incubated at 35 C for 24 h. At the end of the incubation period, colonies were enumerated and recorded, and log CFU/mL were calculated.

Conductance and Hatchability

A group of 50 eggs from each treatment were labeled and the eggs were weighed on day 0 prior to incubation and again on day 7 of incubation. These weights were used to calculate the relative moisture loss, as well as egg shell conductance. The average conditions for the 7 days were temperature of 99.5F, barometric pressure of 30.11, and relative humidity of 50%. The conductance was calculated using the equation of Paganelli et. al. 1974. Hatchability was calculated at hatch, the un-hatched eggs were broken out to determine if the eggs were infertile or time at which they had died.

Statistical Analysis

The experimental data were analyzed as a 3 x 2 x 2 design. The bacterial counts obtained as log CFU/mL based on the variables: sampling times (0, 1.5, 3 h), spray (Bac-D, water), and inoculum (inoculated vs. uninoculated) were analyzed by ANOVA (JMP 10,
RESULTS

The effect of Bac-D as a disinfectant is evident when comparing the mean log CFU/mL of Bac-D washed eggs to eggs washed with DI water only. There was a significant decrease in total aerobic bacteria (p < 0.0001). Bac-D washed eggs yielded a mean log CFU/mL of 2.08 and water washed eggs, a mean of 3.64 log CFU/mL. This was approximately a 1.5 log CFU/mL reduction in total aerobic bacterial counts (Figure 1).

Inoculated eggs treated with Bac-D had significantly lower mean log CFU/mL counts at the p < 0.0001 level for all 3 time points of the inoculated group when compared to the control. At the different sampling times (0, 1.5, 3 h) the bacterial counts were 1.96, 2.60, 1.37 log CFU/mL, respectively for Bac-D treated eggs. The total aerobic bacteria counts for control eggs at the same sampling times were 3.76, 3.66, 3.55 log CFU/mL (Table 1).

The same pattern was observed in uninoculated eggs. Bac-D treated eggs had significantly lower bacterial counts (p < 0.0001) at 1.5 and 3 h (2.36, 2.14 log CFU/mL). Eggs washed with DI water had significantly higher (p < 0.0001) bacterial counts when compared to Bac-D washed eggs: 1.5 h, 3.62; 3 h, 3.63 log CFU/mL (Table 2).

When samples from both treatments were pooled, Bac-D washed eggs had significantly lower total aerobic bacterial counts at all 3 sampling time points: 0, 1.5, and 3 h with an average of 1.96, 2.48, and 1.75 log CFU/mL. On the other hand, eggs washed with
DI water at the same sampling points produced 3.78, 3.65, and 3.59 log CFU/mL, respectively (Table 3).

There were no significant differences in hatchability or hatch residue counts, however there was a significant (p<0.0468) change in conductance of the eggs. The eggs that were washed with Bac-D had a slightly higher conductance rate than did the water washed eggs (Table 4).

**DISCUSSION**

Researchers have found benzalkonium chloride to be an effective disinfectant when used as a wound wash for animals and humans (Dyer et al., 1998). This may be due to its biocidal properties on different organisms found in the environment (Houari and Martine, 2007). Overall, when results for eggs both inoculated and uninoculated were pooled, averaged, and compared only between treatments (Bac-D vs. water), there was approximately a 1.5 log CFU/mL reduction of total aerobic bacteria on the exterior of eggs when Bac-D was used. This reduction was lower than a study performed by Romanova et al. (2007) who demonstrated a reduction of approximately 4 log CFU/mL when benzalkonium chloride was applied for 30 minutes at a rate of 1 mg/mL on *Listeria monocytogenes* biofilms. This may be due to increased sensitivity of *L. monocytogenes* to benzalkonium chloride, as well as, the different composition of the disinfectant tested. Similar results were found by Velazquez and coworkers (2009) who showed significant reductions of *Yersinia enterocolitica* and *Escherichia coli* O157:H7 in tomatoes and lettuce treated with a solution containing 0.1 mg/mL of benzalkonium chloride. Aygun and Sert (2012) tested the efficacy of
benzalkonium chloride on hatching eggs and determined that total aerobic mesophilic bacteria were significantly reduced compared to the control treatment (water) after 7 and 14 d of treatment. These results coincide with our findings; longer exposure to treatment yielded lower total aerobic bacteria counts. It may be worth noting that this investigation only compared 3 sampling time points (0, 1.5, and 3 h). Future work should focus on extended treatment times, which may result in greater total aerobic bacteria log CFU/mL reductions. Kuda et al. (2007) established that the presence of organic materials such as milk, and beef and tuna gravy inhibited the efficacy of benzalkonium chloride on biofilms of *E. coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. This is in agreement with results found by Klimek and Bailey (1955) and may be the reason why our work did not yield higher log CFU/mL reductions in unwashed eggs. Kuda et al. (2007) determined that using benzalkonium chloride after a wash resulted in non-detectable bacterial counts. Therefore, a wash and disinfectant process should be investigated in order to achieve lower bacterial loads in hatching eggs.

Bac-D is an intervention method worth exploring by the egg industry since it does not have the carcinogenic concerns of formaldehyde and other inconveniences of fumigation (Adler et al., 1979). Bac-D is a new product with disinfectant properties, which warrants more investigation. Future work must focus on testing different product concentrations, as well as, impact on hatchability. Furthermore, the development of Bac-D as a disinfectant for other poultry-related uses, such as house cleanouts is paramount in order to mitigate the need for alternative sanitizing solutions.
ACKNOWLEDGEMENTS

The authors would like to thank RDM Products for providing the Bac-D used for this trial. We are also greatly indebted to the Charles Lee Guy fund for financial support.
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Table 1. Mean log CFU/mL Comparison of Total Aerobic Bacteria on Inoculated Eggs Sprayed with Bac-D vs. Water at Different Sampling Times

<table>
<thead>
<tr>
<th>Sampling Times</th>
<th>0 h</th>
<th>1.5 h</th>
<th>3 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE</td>
<td>SE</td>
<td>SE</td>
<td></td>
</tr>
<tr>
<td>Bac-D</td>
<td>1.96 B</td>
<td>0.131</td>
<td>2.60 B</td>
</tr>
<tr>
<td>Water</td>
<td>3.76 A</td>
<td>0.142</td>
<td>3.66 A</td>
</tr>
</tbody>
</table>

A,B Means within columns with different superscripts differ significantly (p < 0.0001).
Table 2. Mean log CFU/mL Comparison of Total Aerobic Bacteria on Uninoculated Eggs Sprayed with Bac-D vs. Water at Different Sampling Times

<table>
<thead>
<tr>
<th>Sampling Times</th>
<th>1.5 h</th>
<th>3 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SE</td>
<td>SE</td>
</tr>
<tr>
<td>Bac-D</td>
<td>2.36^B</td>
<td>2.14^B</td>
</tr>
<tr>
<td></td>
<td>0.137</td>
<td>0.136</td>
</tr>
<tr>
<td>Water</td>
<td>3.62^A</td>
<td>3.63^A</td>
</tr>
<tr>
<td></td>
<td>0.146</td>
<td>0.141</td>
</tr>
</tbody>
</table>

^A,B^ Means within columns with different superscripts differ significantly (p < 0.0001).
Table 3. Mean log CFU/mL Comparison of Total Aerobic Bacteria on Inoculated and Uninoculated Eggs Sprayed with Bac-D vs. Water at Different Sampling Times

<table>
<thead>
<tr>
<th>Sampling Times</th>
<th>0 h</th>
<th>1.5 h</th>
<th>3 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Bac-D</td>
<td>Water</td>
<td>Bac-D</td>
</tr>
<tr>
<td>Mean CFU</td>
<td>1.96</td>
<td>3.78</td>
<td>2.48</td>
</tr>
<tr>
<td>SE</td>
<td>0.133</td>
<td>0.144</td>
<td>0.097</td>
</tr>
</tbody>
</table>

A, B, C Means with different superscripts differ significantly (p<0.0001).
Table 4. Hatchability, Conductance and hatch residue breakout from eggs washed in Bac-D or water.

<table>
<thead>
<tr>
<th></th>
<th>Infertile</th>
<th>Early Dead</th>
<th>Mid Dead</th>
<th>Late Dead</th>
<th>Hatchability</th>
<th>Conductance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bac-D</td>
<td>16</td>
<td>4</td>
<td>2</td>
<td>13</td>
<td>76.6%</td>
<td>0.0156</td>
</tr>
<tr>
<td>Water</td>
<td>24</td>
<td>2</td>
<td>2</td>
<td>11</td>
<td>74.0%</td>
<td>0.0147</td>
</tr>
</tbody>
</table>

Means within columns with different superscripts differ significantly (p < 0.0001).
Figure 1. Overall Comparison of Mean log CFU/mL by Treatment (Bac-D vs. Water) of Inoculated and Uninoculated Eggs at all Sampling Time Points (0, 1.5, 3 h) "Means differ significantly (p < 0.0001).
Manuscript III

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The Effect of Feeding Hydrogel-95 to Emu Chicks at Hatch

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Primary Audience: Researchers, Emu Producers
SUMMARY

There is very little research conducted on emu (*Dromaius novaehollandiae*) in comparison to other types of poultry. Much of the information that is available to producers is very conflicting. Feed restriction for the emu chick for the first 4 days of life is a common practice amongst the emu industry; however, there has not been scientific research found to support this practice. To the best of our knowledge, the effects of feed supplements such as Hydrogel-95 have not been investigated in the emu. Therefore, weights were recorded on emus fed Hydrogel-95 and were compared to emus that were not fed any supplements to determine if the supplement had any effect on the growth parameters of emu chicks. From these 2 field trials our results show that feeding Hydrogel-95 significantly decreases weight loss in emu chicks during the first week of life.

Key words: emu, body weight, hydrogel-95

DESCRIPTION OF PROBLEM

Emu (*Dromaius novaehollandiae*) are a flightless bird from the ratite family. Emu are typically raised for either meat or oil (Beckerbauer et al. 2001). Emu oil has been shown to possess anti-inflammatory, antiviral and antibacterial; as well as burn wound healing properties (Beckerbauer et al., 2001; O’Banion and Griswold, 1998). Emu literature is very limited compared to other types of commercial poultry and even other ratite groups. There is much debate and conflicting data on management practices for emu ranging from incubation temperatures, humidity, and egg storage time and temperature, all the way to what day chicks should be allowed first access to feed (Majewska, 2001; Nelson, 1992). The data pertaining
to feeding emu chicks is quite contradictory; many sources recommend not providing chicks feed until they are 4 days old (Jodoin, 1995); however, there are also recommendations to feed chicks as soon as they are pulled from the hatcher (Beckerbauer et al, 2001). Currently, there are no published studies analyzing the effects of immediate feeding compared to the fasting method. Pre-feeding products such as Oasis, a hatching supplement, and similar products have been utilized heavily in both chicken and turkey hatcheries. There have been many reports that show that these products provided to chicks and poults increased weight gain and other growth parameters (Batal and Parsons, 2002; Knight and Dibner, 1998).

Hydrogel-95 (Clear H2O, Portland, OR) is a novel green hatching gel supplement (HGS), it is 95% water and provides chicks with crucial hydration and extra nutrients, to allow for an optimal start to life. It has been successfully used in chick and poult boxes during shipment to farms.

**MATERIALS AND METHODS**

The following trial was conducted with a local emu producer in North Carolina. The emu chicks were handled and reared in accordance with proceedings described in the “Guide for the care and use of Agricultural Animals in Research and Teaching” (Federation of Animal Science Societies, 2010). The eggs were obtained from several different emu farms, and two trials were conducted. Trial 1 used a temperature of 97.5°F and 30% relative humidity for 53 days during incubation. The hatchery manager changed the incubation parameters slightly in the incubator for the second trial due to a decision by the farm owners after reading literature pertaining to ratite incubation. In Trial 2, eggs were incubated at a temperature of 97°F and a
relative humidity of 33%. The eggs from each trial were separated into two groups: one group was given a HGS [9] in the hatcher for immediate access, the other group was not provided with HGS (Control). At hatch, chicks were weighed as soon as they had dried and leg banded for identification. Chicks were left in the hatcher for 24 hours and then transferred to brooding pens (3ft X 6ft) with 10-12 birds per pen. The temperature was kept at 29°C for all 7 days of the trial. There were 38 birds that hatched from the HGS group, and 24 birds from the control group, for a total of 62 emu chicks for Trial 1. For Trial 2, there were 18 chicks that hatched from the control group and 23 from the HGS group for a total of 41 emu. The experimental group was provided HGS in addition to common starter feed for 7 d after placement. The control group was provided with only the starter feed. Birds were weighed at 7 days of age. It is worth noting that birds on the HGS appeared to be more active and produced noticeably more manure than the control group.

**RESULTS AND DISCUSSION**

The weights were analyzed using JMP Pro 10 One-Way ANOVA. There was an overall decrease in chick body weights for the 7 d period for both groups in Trial 1. Majewska, 2001 and Nelson 1992 reported similar results in that emu have very low growth rates and commonly even reduction in weight occur during the first week of life. Majewska, 2001 and Nelson 1992, attributed this decrease to chicks mainly relying on nutrients from the yolk. In this trial, both the control and experimental groups had decreases in body weight; however the birds fed the HGS had significantly less (p < 0.0427) weight loss than birds fed the starter ration (Table 1).
In Trial 2 the HGS group performed better than control birds. However, unlike the first trial the birds on the HGS actually gained a significantly different amount of weight, on average 38.76 g, whereas control birds lost on average 20.19 g (p < 0.0001; Table 2). This improvement in growth is thought to be due to the increased availability of nutrients and hydration that Hydrogel-95 provided to the newly hatched chicks. The beneficial effects of providing extra nutrients to chicks immediately post hatch have been recognized for nearly 40 years (Thaxton and Parkhurst, 1976). There have been several observations made as to why these birds perform more efficiently. Birds that are not allowed access to feed have been shown have decreased rates of yolk utilization (Noy and Sklan, 1999). This lack of feed causes slower maturation of the GI tract, as demonstrated by decreased height and area of villi in the small intestine (Uni et al, 1998). Thus it has been suggested that similar products such as Oasis may stimulate the gastrointestinal tract, or even stimulate energy metabolism and utilization leading to the beneficial effects that result from the use of these supplemental products (Batal and Parsons, 2002). To determine the actual mechanism by which Hydrogel-95 facilitates increased growth and performance will require further research.

**CONCLUSIONS AND APPLICATIONS**

1. Providing Hydrogel-95 may serve as a beneficial practice when hatching and brooding emu.

2. Feeding Hydrogel-95 to emu chicks causes beneficial effects on growth performance as similar products in other poultry.
ACKNOWLEDGEMENTS

The authors are greatly indebted to the Great American Emu Company for their funding and contributions to this trial.
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Majewska, D. 2001. The Influence of Emu (Dromaius Novaehollandiae) Egg storage time on hatchability and chick survival. EJPAU 4(2)


Table 1. Mean weight loss (g) of emu chicks in 7 days by treatment with Hydrogel-95.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-39.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.5196</td>
</tr>
<tr>
<td>Hydrogel-95</td>
<td>-27.50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.5918</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> Denotes significant difference at p < 0.0427
Table 2  Trial 2 Mean weight gains

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Mean</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18</td>
<td>-20.198b</td>
<td>7.864</td>
</tr>
<tr>
<td>Hydro-gel</td>
<td>24</td>
<td>38.757a</td>
<td>6.957</td>
</tr>
</tbody>
</table>

Table 2. Mean weight loss/gain (g) of emu chicks in 7 days by treatment with Hydrogel-95
a,b Denotes significant difference at p < 0.05
The Length of Storage of Emu Eggs and Resultant Effects on Hatchability

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North Carolina State University, Raleigh, NC, 27606

Primary Audience: Researchers, Emu Producers, Hatchery Managers
ABSTRACT

Emu (*Dromaius novaehollandiae*) are a large flightless bird and are members of the Ratite family. Emu originated from Australia and have long been raised for their meat and oil. The cost associated with raising emu is high which makes emu meat and products expensive. Emu have a very low hatchability rates when compared to other commercial poultry. Little research has been published on egg storage conditions of emu eggs which may contribute to the decreased hatchability of the eggs. In this study eggs were stored for various amounts of time ranging from 1 to 8 weeks, subsequent effects on moisture loss and hatchability’s were recorded. In this trial there were significant differences (p<0.0104) in hatchability of emu eggs due to storage time. Significant differences (p<0.0001) were also found in moisture loss due to egg storage.

INTRODUCTION

Emu (*Dromaius novaehollandiae*) are a large flightless bird and are members of the Ratite family. They originated in Australia and have long been raised for their meat and oil. Emu meat is said to be low in cholesterol and fat (Davis, 1997) and the oil is highly valued because of its antibacterial, anti-inflammatory, and burn wound healing (Beckerbauer et al, 2001; O’Banion and Griswold, 1998). The hatch rate of emu is much lower than that of other commercial avian species with hatch rates reported ranging from 56% to 68% (Szczerbinska, et al, 2004; Majewska, 2001). These very low hatch rates are an issue for emu producers due to the fact that emu are expensive to raise and maintain in comparison to current commercial poultry and have a lower rate of lay. The increased cost associated with
emu production is attributed to the fact that emu are such large birds, have a very slow
growth rate and feed conversion, a long incubation period, and have a very limited season of
lay. However, even with the very high cost associated with raising emu, little research has
been focused at many areas of emu production, especially with regards to egg storage,
incubation, and hatchability.

Egg storage is a very crucial factor to consider when setting up an incubation program. It has
been found in poultry that there is not a single set storage time, that there are many
contributing factors that must be considered. This time will vary depending on the age of the
bird, differences in strains and many other factors (Brake et al, 1997). Extended storage of
chicken and turkey hatching eggs has been shown to cause increased embryo mortality
(Brake et al, 1997; Elibol and Brake, 2008), decreased hatchability (Brake et al, 1997; Elibol
and Brake, 2008), variation in hatch time (Mayes and Takeballi, 1984), decreased egg quality
(Brake et al, 1997), increases in pH of the albumen to above optimal levels (Goodrum et al.,
1989). However, virtually no research has been conducted on emu egg storage in
comparison to the amount of literature available on current commercial poultry. There are
recommendations that vary greatly in regards to temperature, humidity, and duration many of
these practices come from emu producers and have very little if any scientific basis.

Moisture loss during storage and incubation is typically monitored. There are many factors
that can alter moisture loss. The age of the flock has been shown to have a significant impact
on shell thickness, pore length, and pore numbers (Brake et al, 1997). Females from young
flocks have been shown to produce thicker egg shells, and as the hen progresses in her lay
cycle the shells become thinner (Peebles and Brake, 1987; Roland, 1976). The porosity of
eggs is smaller in younger birds, but as production increases so does porosity of the egg (Peebles and Brake, 1987). Some researchers feel that humidity during storage is not a major factor to consider (Funk and Forward, 1960) and that moisture loss is not the cause of high mortality as a result of long term eggs storage (Kaufman, 1939). Since many emu producers have mixed aged flocks, and typically no set genetic lines as in commercial poultry production, most attention appears to be aimed at storage length and storage temperature.

The storage temperature for emu egg have a wide range, some sources suggest storage temperatures of 54.4° F - 64.4°F (Christensen, 1993), however Brake and Rosseland, 1995 suggest a storage temperature of 75°F if for only one day of storage and that eggs be stored at 55°F for longer periods of time. Delf and Roseland suggest initial storage at 69.8°F and gradually reducing the temperature down during storage to 59.9°F (Delfel and Rosseland, 1993). Some producers store eggs in normal refrigerators with temperatures ranging from 40°F to 60°F (Minnaar and Minnaar, 1998).

The length of storage is possibly the most disputed factor. Egg storage even if just over night is recommended on all emu eggs even freshly laid eggs. Brake and Rosseland (1995) suggest that fresh eggs be stored at 70-75F overnight to allow for the breakdown of the albumen (Brake and Rosseland, 1995). Some sources say that egg storage should be no longer than 7 days (Christensen, 1993) yet; there are other sources that suggest that eggs can be stored up to 21 days with minimal decreases in hatchability. The 21 day storage time is thought to be of similar duration that the eggs undergo in the wild (Brake and Rosseland, 1995). However, 28 day storage is said to be the standard storage time for most producers, but there have been reports of storage for up to 44 days before incubation (Minnaar and Minnaar, 1998).
The goal of this study was to analyze the effects that storage time has on hatchability of emu eggs over short and long term storage of eggs.

**MATERIALS AND METHODS**

Since emu are expensive to raise and to simulate real world application, this trial was conducted in collaboration with a local commercial emu producer in North Carolina. The trials consisted of 256 emu eggs, that were hatched in 7 different batches due to incubator and rearing constraints as well as the laying patterns of the birds. Eggs were picked up 3 times daily; the condition of the egg was noted. Each egg was dated and labeled and weighed. The eggs were then placed in an egg cooler that was maintained at 55°F and 60% relative humidity. Egg weights were then recorded again just prior to setting in the incubator, and then once more at 4 days of incubation. All eggs were incubated in Hatchrite Incubators at 97-97.5°F and relative humidity of 30%-33%. The eggs were then placed into a NatureForm hatcher (NatureForm Hatchery Systems, Jacksonville, FL) at 97°F and 50% relative humidity.

Hatchability and moisture loss were analyzed based upon storage times based upon weeks of storage ranging from 0-8 weeks of storage. JMP Pro 10 (SAS Institute, Cary, NC) was used to analyze data using the Chi Square and One-Way ANOVA features.

**RESULTS AND DISCUSSION**

Researchers have long studied the optimum storage time for eggs for commercially raised poultry (Brake et al, 1997; Elibol and Brake, 2008; Mayes and Takeballi, 1984). Extended storage of chicken and turkey hatching eggs has been shown to cause increased embryo
mortality (Brake et al, 1997; Elibol and Brake, 2008), decreased hatchability (Brake et al, 1997; Elibol and Brake, 2008). Emu eggs have been reported to have lower hatch rates than other types of commercial poultry (Szczerbinska, et al, 2004; Majewska, 2001). There has been very little research conducted on emu in comparison to other types of commercial poultry. In this trial there were significant differences (p<0.0104) in hatchability of emu eggs due to storage time (Table 1.). There were very high rates of hatch recorded from eggs that were stored less than one week with the mean hatchability being 90.81%. There was a slight decrease in hatchability (85.11%, 69.46 %) of eggs that were stored for 2 and 3 weeks of age respectively. The hatchability after storage for 1-3 weeks of age is surprisingly higher than expected when compared to hatch rates reported from previous (Majewska, 2001). These data are in agreement with Brake and Rosseland (1995) in that emu eggs can be store for up to 3 weeks with minimal loss in hatchability. Majewaska, 2001 reported a 1.68 % decrease in hatchability per day for emu eggs that are stored for more than 7 days. Chicken eggs have been said to decrease hatchability by up to 5% a day when stored longer than 7 days (Mayes and Takeballi, 1984). There were drastic decreases in hatchability on eggs stored 3 weeks (50.46%) all the way to 9.09% hatchability at 8 weeks of storage. This data is very similar to reports made on chicken and turkey egg storage for the first 2 weeks (Brake et al, 1997; Mayes and Takeballi, 1984) with the exception that it appears that emu eggs can be stored for 3 weeks with minimal losses. Storage for this length of time may be necessary in emu operations due to specific challenges associated with emu production. Not surprisingly there was a significant (p<0.0001) increase in moisture loss from eggs due to storage. These ranges ranged from a mean loss of 2.7% from the eggs stored less than one week, all the way
to the 16.20% in the eggs store for more than 6 weeks (Table 2.). What is surprising about these data is how high these moisture loss percentages are, chicken eggs stored for 34 days only lost about 1% (Kaufman, 1939). Mather and Laughlin reported weight loss of 0.5% for eggs stored for 7 days, and 1.1% loss in eggs stored 14 days (Mather and Laughlin, 1977). Several researchers state that humidity during storage is not a major factor to consider (Funk and Forward, 1960) and that moisture loss is not the cause of high mortality as a result of long term eggs storage in chicken eggs (Kaufman, 1939). However, from this trial it is evident that weight loss percentages appear to be much greater in emu eggs during storage than in chicken eggs. More research is required to determine the role that humidity during storage has on emu egg hatchability.

**CONCLUSIONS**

1. Optimal hatchability of emu eggs is achieved when eggs are stored for less than 7 days.
2. Emu eggs can be stored for up to 3 weeks with minimal decreases in hatchability.
3. Storage of emu eggs for more than 3 weeks causes a significant decrease in hatchability.
REFERENCES


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Majewska, D. 2001. The Influence of Emu (Dromaius Novaehollandiae) Egg storage time on hatchability and chick survival. EJPAU 4(2)


<table>
<thead>
<tr>
<th>Storage length</th>
<th>Hatchability %</th>
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<tbody>
<tr>
<td>0-6 days</td>
<td>90.82 %&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.71</td>
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<td>7-13 days</td>
<td>85.18 %&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>11.52</td>
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<td>14-20 days</td>
<td>69.5 %&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>11.52</td>
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<tr>
<td>21-27 days</td>
<td>50.46 %&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>13.31</td>
</tr>
<tr>
<td>28-34 days</td>
<td>41.00 %&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>16.29</td>
</tr>
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<td>35-41 days</td>
<td>64.29 %&lt;sup&gt;abcd&lt;/sup&gt;</td>
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<td>42-48 days</td>
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</tr>
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<td>56-63 days</td>
<td>9.09 %&lt;sup&gt;d&lt;/sup&gt;</td>
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Hatchability of Emu eggs for various storage lengths. <sup>abcd</sup> denotes significance at (p<0.05)
<table>
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<tr>
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<th>% Moisture Loss</th>
<th>SE ±</th>
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<tbody>
<tr>
<td>0-6 days</td>
<td>2.73 e</td>
<td>0.82</td>
</tr>
<tr>
<td>7-13 days</td>
<td>5.62 de</td>
<td>0.88</td>
</tr>
<tr>
<td>14-20 days</td>
<td>7.25 cd</td>
<td>0.79</td>
</tr>
<tr>
<td>21-27 days</td>
<td>9.10 bcd</td>
<td>1.00</td>
</tr>
<tr>
<td>28-34 days</td>
<td>12.00 ab</td>
<td>1.12</td>
</tr>
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<td>10.00 abcd</td>
<td>1.59</td>
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<tr>
<td>42-48 days</td>
<td>16.20 a</td>
<td>1.42</td>
</tr>
<tr>
<td>49-63 days</td>
<td>14.50 abc</td>
<td>2.24</td>
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Moisture loss of Emu eggs during storage for various storage lengths. abcd denotes significance at the (p<0.05)
Protein and Caloric Intake on the Reproductive Performance Parameters of Hy-Line W-36 Parent Stock males

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North Carolina State University, Raleigh, NC, 27606
Primary Audience: Researchers, Layer Companies
DESCRIPTION OF PROBLEM

Reproduction in poultry has been studied for numerous years, and is a crucial factor to consider in commercial parental lines. Considerable work has focused on broiler breeder males, which has led to the development of feed restriction practices, as well as sex separate rearing. Previously broiler breeders have been studied looking at the relationship between nutrition and reproductive performance. However, very little research as has been done on layer-type males. Decreases in semen production have been observed in broiler breeder males that have been placed on lower caloric diets; it has been shown the decrease in semen production becomes more evident when caloric intake drops below 330 kcal/day (Bramwell, et al, 1996). There are several studies that report decreases not only in semen volume, but decreases in sperm concentration per ejaculate (Sexton et al 1998a). In broiler breeders a negative correlation has been shown to exist between the body weight (BW) and sexual activity of the male (Burke and Mauldin, 1985) there have also been reports that there is no apparent relationship between testis weight and semen production in broiler breeder males (Brown and McCartney, 1983). Wilson et al. 1988 reported that that broiler breeder males fed diets containing crude protein (CP) levels from 9-15% showed no significant difference in semen volume or concentration when collected at 48 and 49 weeks of age. Hocking and Benard (1997) reported that feeding broiler breeder males on higher levels of CP(16%) did not change semen volume or concentration as compared to the low CP(12%) group, and that the average testis size was smaller in the birds fed the higher CP diet than the low CP diet. Simply losing weight does not solve the issue, in fact it is very well documented that decreases in male BW during production have detrimental effects on reproduction and result
in fertility issues (Bramwell et al., 1996; Harris et al., 1984; Duncan et al., 1990). Debates continue as to whether decreases in reproductive performance in the males may be attributed to a reduction in protein or due to a reduction in caloric intake. Parker et al. 1964 demonstrated that white leghorn males when placed on low energy diets had a decrease in semen volume, as well as a decrease in fertilizing capacity of the sperm. Sexton et al. 1989b proved similar findings in broiler breeder males on low caloric diets, these birds’ demonstrated reductions in BW, semen weight, and sperm concentrations. Broiler breeders on low calorie diets have been shown to have decreased sperm penetration ability (Bramwell et al., 1996) Decreases in protein intake have been shown by researchers to have no negative impact on fertility or semen volume in White leghorn males (Arscott, and Parker 1963) or in broiler breeders (Wilson et al 1987). However Romero-Sanchez et al. 2007 demonstrated that changes in protein levels in the latter part of rearing period do have some impact on fertility in broiler breeders. Fertility has been shown to decline when the nutrient intake is inadequate to support the current BW (Romero-Sanchez et al 2007b).

Reductions in nutrient intake have also been shown to effect hormone production; fasted young male chickens have decreased levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Scanes et al, 1976). Birds that were fed diets that were deficient in essential fatty acids also exhibited decreased levels of LH (Engster, et al. 1978). Rearing birds on protein and caloric restricted diets have been shown to have decreased testicle growth, which was attributed to the decreased levels of LH (Buonomo et al. 1982).
Since there has been very little research aimed at the effects of crude protein levels and metabolizable energy in layer type birds a pilot study was conducted to compare the layer male response to similar research that has been conducted in broiler breeders.

MATERIALS AND METHODS

Day old Hy-line W-36 Parent Stock males were obtained from Hy-line international (Dallas Center, IA). The chicks were vaccinated with HVT/IBD, Rispens, SB1. All males were dubbed at day old. Then neck tagged and sorted by sex, and placed in a petersime brooder. At 5 weeks the cockerels were moved to alternative design grow out pens. Birds were assigned to one of the 3 diet groups Low, Control, and High. The Low group was fed a starter diet that contained 12% CP and 1000 kcal/lb, Control diet contained 18% CP and 1360 kcal/lb, and the High diet contained 24% CP and 1480 kcal/lb. The birds were fed the respective starter for 10 weeks, then were placed on a respective layer diet consisting of the Low containing 12% CP and 1000 kcal/lb, Control diet contained 18% CP and 1360 kcal/lb, and the High diet contained 24% CP and 1480 kcal/lb. The birds were allowed ad libitum feed and water for the entire trial. When the birds were 15 weeks old the extra males were euthanized, body weight was recorded and then the birds were necropsied. The right testicle was removed and weighed, and then the left was removed and weighed. A random sample of 5 testicles from each group were selected, cut in half horizontally and were placed in 10% buffered formalin for 24 hours, and were then placed in 70% ethanol at 4° F. The testicles were then trimmed and embedded in paraffin, cut to a thickness of 5µ, and affixed to a slide. The sample was dewaxed and stained with hematoxylin-eosin in order to see the
seminiferous tubules. The testicles were staged on a 1-6 scale based on maturation and development of seminiferous tubules (table 1).

Four males from each treatment were saved for semen collection using the abdominal massage method described by Burrows and Quinn, 1937. Birds were only stimulated 2 times during each collection to avoid damage and bleeding of phallus. Semen was collected in 1.5ml tubes immediately from the ejaculatory groves as soon as the phallus was erect. Great care was taken to avoid feces, urates, or blood contamination. The males were collected once a week from 16 weeks of age through 21 weeks of age. At each collection time point the semen volume was measured, and sperm content was determined by collecting semen in a capillary tube, sealing off one end with cryoseal, and then centrifuging the sample in a clinical centrifuge for 5 minutes. The hematocrit tube was then placed in a hematocrite reader and the percentage of packed sperm were recorded.

RESULTS AND DISCUSSIONS

The weights were analyzed using JMP Pro 10 One-Way ANOVA (SAS Institute Inc. Cary, NC). There were significant differences in body weights between the three different diets. The birds on the Low diet had a mean BW of 1214.87g which was significantly lower (P<0.0001) than the other treatment groups. The control group had a mean BW of 1435.41g which was 40g lighter than the High group which had a mean BW of 1475.96g, however these two groups did not differ significantly from each other (table 2). These results parallel those of Zang et al, 1999 as well as Romero-Sanchez et al. 2007, where crude protein levels resulted in significant changes in bodyweights.
There were significant differences in testicle weights between the treatments. The left and right testicles followed the same trends between the treatments with the Low group having a mean testicle weight of 1.1g and 1.39g respectively, which differed significantly (p<0.0001) from the other two treatment groups with the control having a left testicle weight of 4.9g, and a right testicle weight of 5.4g, and the high group having a left testicle weight of 6.2g and a right testicle weight of 7.09g. The weights for the control group even though several grams lower did not differ significantly from the High group (Figure 1, 2). There was no significant difference between the weights of the left and right testicles in any of the treatments. The total testicle weights were similar to the left and right break down weights. The Low group had a significantly lower (p<0.0001) mean weight from the other two treatments, and though slightly lighter in controls there was not a significant difference in total testicle weight between the Control and High diet (figure 3). These data similar to Vizcarra et al. 2010, where broiler breeder males were raised on full feed or restricted feed, the males on the full feed diet had significantly higher testicle weights upon necropsy than did the males on the restricted diet. Wilson et al. 1988 found there was a positive correlation between body weight and testicle weight. However there are reports that caged broiler breeders males fed 16% CP diets have smaller testicle size than the males fed the 12% diet (Hocking and Bernard, 1997).

Differences in semen volume were observed between the dietary treatment groups. There were significant differences in semen volumes seen at 4 of the 5 collection times. The males on the High diet always produced the largest volume of semen ranging from 310.9 ul on the initial collection, and increasing to 603.1 ul of semen on the last collection (table 3). The
Low group ranged from 93.8 ul on the initial collection to 385.9 ul on the fifth collection, this was significantly lower than the High groups at all time points except on wk 18 in which the volume was still lower, just not significantly different (table 3). The volume of the control group ranged from 207.8ul on the first collection and increased to 582.8 ul on the last collection. The only time point at which there were significant differences between the control group and either of the treatment groups was at 16 weeks, where the control group was significantly less than the high group, and higher than the low group (table 3). There were also differences in sperm concentrations with in the semen. The high group had significantly higher spermatocyte readings than the low group at weeks 18,19,20, with a range starting at 5.70% topping out at 11.90% on the fifth week. The control group was intermediate, and did not differ significantly from either treatment groups. The low group had a range from .95% sperm on week 16 and increased to 3.11% sperm on week 20 (table 4). These data similar to work done by Parker et al. (1943) in that broiler breeder birds on restricted diets produced lower levels of less concentrated semen. These trials however looked at changing the diets after rearing, where as our study examined the effects of CP and energy levels from brooding to production. Sexton et al (1998a) also showed that decreases in caloric intake resulted in decreased semen volume and sperm concentration. However, Wilson et al. (1988) found that growing broiler breeder males on CP levels ranging from 9%-15% resulted in no significant difference in semen volume or concentration from the males. Hocking and Bernard (1997) found that caged broiler breeders have the exact opposite response to increased levels of CP, Hocking showed that broiler breeder males in production that were fed 16 % CP diets had lower semen concentrations than males fed 12% CP. These
results different from the current study, probably due to the differences between broiler breeder males and layer type males.

There were significant differences observed from the histological examination and scoring of testicle development (table 1) of the testicles, the high group had a mean scoring with a mean value of 3.9 this score was significantly (p<0.0144) higher than that of the average score of the low group value of 1.2. There was no significant difference observed between the control group score and the scores of the high or low groups.

**CONCLUSIONS AND APPLICATIONS**

1. CP and ME play a very important role in the development of WL males
2. WL males seem to follow the same trends as broiler breeder males
3. Higher CP and ME result in faster maturing birds.
4. 24% CP diets resulted in increases in semen concentration and volume
5. Protein and caloric content of the diet significantly changed histological development of WL testicles.
REFERENCES AND NOTES


Romero-Sanchez, H., P.W. Plumstead, J. Brake. 2007. Feeding Broiler Breeder Males. 3. Effect of Feed Allocation Program From Sixteen to Twenty-Six Weeks and Subsequent Feed Increments During the Production Period on Body Weight and Fertility. Poult Sci 86:775-781


<table>
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<th>Score</th>
<th>Stage of Development</th>
<th>Description</th>
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<tr>
<td>0</td>
<td>Immature</td>
<td>Seminiferous epithelium is composed of spermatogonia and Sertoli cells. There is no differentiation.</td>
</tr>
<tr>
<td>1</td>
<td>V. early development</td>
<td>Most tubules have only spermatogonia and Sertoli cells, there are spermatocytes and possibly later stages in a few tubules.</td>
</tr>
<tr>
<td>2</td>
<td>Early development</td>
<td>Most tubules show early epithelial development.</td>
</tr>
<tr>
<td>3</td>
<td>Intermediate development</td>
<td>Tubules show early development with most having more advanced development to the early or, infrequently, the late spermatid stages.</td>
</tr>
<tr>
<td>4</td>
<td>Late development</td>
<td>Most tubules have late spermatids, but few, if any spermatozoa.</td>
</tr>
<tr>
<td>5</td>
<td>V. late development</td>
<td>Spermatozoa are present in some tubules and in epididymal ducts.</td>
</tr>
<tr>
<td>6</td>
<td>Mature</td>
<td>Spermatozoa are in almost all tubules and are abundant in the epididymis.</td>
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Table 2. Body weights for Hy-line w-36 males according to diet

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<td>Control</td>
<td>29</td>
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</tr>
<tr>
<td>High</td>
<td>34</td>
<td>18.7</td>
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Treatments with differing A,B,C with in column denote significant difference at P≤0.05 lack of A,B,C denotes no significant difference.
### Table 3. Mean Semen volume (ul) per treatment

<table>
<thead>
<tr>
<th></th>
<th>Low</th>
<th>Control</th>
<th>High</th>
<th>SEM</th>
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</thead>
<tbody>
<tr>
<td>16 weeks</td>
<td>93.8 C</td>
<td>207.8 B</td>
<td>310.9 A</td>
<td>25.3</td>
</tr>
<tr>
<td>17 weeks</td>
<td>262.5 B</td>
<td>393.8 AB</td>
<td>509.4 A</td>
<td>49.4</td>
</tr>
<tr>
<td>18 weeks</td>
<td>325.0 A</td>
<td>481.3 A</td>
<td>498.4 A</td>
<td>68.4</td>
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<tr>
<td>19 weeks</td>
<td>356.3 B</td>
<td>621.9 AB</td>
<td>670.3 A</td>
<td>79.2</td>
</tr>
<tr>
<td>20 weeks</td>
<td>385.9 B</td>
<td>582.8 AB</td>
<td>603.1 A</td>
<td>64.4</td>
</tr>
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Treatments with differing superscripts within column denote significant difference at \( P \leq 0.05 \) lack of superscript denotes no significant difference.
<table>
<thead>
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<td>16</td>
<td>.95 A</td>
<td>1.34 A</td>
<td>5.70 A</td>
<td>1.9</td>
</tr>
<tr>
<td>17</td>
<td>2.13 A</td>
<td>2.78 A</td>
<td>7.25 A</td>
<td>2.1</td>
</tr>
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<td>18</td>
<td>1.78 B</td>
<td>4.61 AB</td>
<td>10.95 A</td>
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<td>19</td>
<td>1.60 B</td>
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<td>2.1</td>
</tr>
<tr>
<td>20</td>
<td>3.11 B</td>
<td>8.71 AB</td>
<td>11.90 A</td>
<td>2.1</td>
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Treatments with differing A, B, C within column denote significant difference at P ≤ 0.05 lack of A, B, C denotes no significant difference.
**Figure 1.** Weight of Left Testicle by diet

Treatments with differing \(A, B\) with in column denote significant difference at \(P \leq 0.05\)
Figure 2. Weight of right testicle by diet

Treatments with differing \( A, B \) with in column denote significant difference at \( P \leq 0.05 \)
Figure 3. Total testicle weight by diet

Treatments with differing \( \text{A, B} \) with in column denote significant difference at \( P \leq 0.05 \)
Manuscript VI

Low Oxygen Incubation Effects on Performance

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ABSTRACT

There have been tremendous advances in genetic selection of broilers resulting in the fast-growing, efficient modern-day bird. As a consequence of this selection, broilers have difficulty obtaining the oxygen supply needed to meet the demands higher body weights (BW) require. Birds can adapt to this challenge over time by increasing oxygen carrying capacities through angiogenesis, polycythemia, modification of hemoglobin composition, or by increasing hematocrit levels. In this study, we investigated the effects of short-term hypoxic conditions on the embryo and long-term body composition. Broiler embryos were exposed to a 48 h period of low oxygen (LoOx =16%) from day 16 to 18 of incubation. Birds were hatched and grown under standard brooding conditions to 14 d of age, sampling at internal pip, external pip, day 7, and day 14 (n=12). Acute effects of LoOx were observed at the end of the hypoxic treatment including an impact on heart rate, yolk utilization, and blood gas parameters. LoOx treatment had a long-term effect on body composition and growth rate. At day 7 control birds had a significantly higher breast % and body weight, but by day 14 these differences were lost and LoOx significantly increased heart % and liver % determined by ANOVA (P<0.05). The observations made in this study may be useful to the broiler industry where birds are grown in varying environments. By manipulating incubation conditions including brief periods of hypoxia, the resulting birds may be better suited for specific environmental conditions or when specific modifications to body composition are desired.

Key words: Hypoxia, Incubation, Growth Rate, Broiler
INTRODUCTION

There has been significant progress in the genetic selection of fast-growing broiler chickens. This improved growth rate (GR) was shown by Havenstein et al. (1994, 2003) and compared 1957 broilers with 1991 and 2001 broilers. As a consequence of these dramatic changes, come dramatic increases in metabolic rate (MR) that is apparent even during embryonic development (Hulet and Meijerhof, 2001; Tona et al., 2004).

However, selection for fast growth has coincided with inferior development of visceral systems (Havenstein et al., 2003b) that has contributed to the difficulties of broiler chickens in regulating O2 supply to match O2 demands under suboptimal environmental conditions, especially at higher BW when the metabolic demands are high. In growing broilers, the inability of the cardiovascular system to efficiently maintain O2 supply leads to cardiovascular disorders and ultimately death, and results in significant economic losses (Julian, 1993, 2000; Balog, 2003). In light of continuing selection for high GR and MR a situation is likely to arise in which the broiler’s cardiovascular system will be unable to cope with increasing oxygen demands, even when birds are grown in expensive environmentally controlled facilities.

There are two means to cope with hypoxemia (insufficient oxygen supply to the tissues): adaptation to hypoxia (Herrera et al., 2007), and pharmacological intervention (Adair, 2005). These strategies either increase oxygen supply or reduce oxygen demand. One of the adaptive responses to oxygen shortage is elevation of blood oxygen-carrying capacity by means of angiogenesis, polycythemia, and modification of hemoglobin isoforms. Dusseau and Hutchins (1988) reported that exposing chick embryos to 15% oxygen from E7 to E14
induced an increase in vascularity, with the vascular density index increasing by 34 to 41%. On the other hand, increasing oxygen demand by exposing chicks to ascites-inducing conditions (AIC) caused an increase in their hematocrit levels (Druyan et al., 2007b) as a result of increased erythropoiesis (Luger et al., 2003). Increases in blood vessel density caused by angiogenesis, coupled with increases in red blood cell numbers could lead to delivery of sufficient oxygen to the tissue/cells. However, a finely tuned balance must be maintained between erythropoiesis (which affects blood viscosity) and elongation of blood vessels, both of which could negatively impact blood flow resistance and the effective functioning of the entire blood system.

We hypothesize that a better and more efficient adaptive response to oxygen shortage would be reduction of oxygen demand. Improving the oxygen balance between supply and demand can be achieved by reducing oxygen demand by effectively reducing the broiler’s resting metabolic rate (RMR). Hypoxic exposure (HyE) during the last phase of embryonic development might reduce RMR, and thereby make additional energy available for growth due to a reduced resting metabolic rate. This would shorten the period to achieve marketing body weight, improve feed efficiency (FE), and lead to more efficient use of farm resources, and primarily feed. The molecular/genetic mechanism that is involved in reducing RMR in response to hypoxia is unclear. In addition, it is still unknown whether the influence of this mechanism on RMR plasticity and the ability to re-partition energy allocation would persist in broilers kept under variable growth conditions.
MATERIALS AND METHODS

General procedures

300 Broiler breeder eggs were obtained from the NCSU Poultry education unit (Raleigh, NC). The eggs were incubated under standard conditions, 99.5 F 60% relative humidity. On day 16 of incubation 150 of the eggs were moved to a low oxygen (16% oxygen) incubator which was also at 99.5 F and 60 % Relative humidity. The eggs were incubated in the low oxygen incubator for 48 hours, and then on day 18 the eggs were moved to the hatcher which was kept at 99.5 F and 65% Relative humidity. Upon hatch chicks were tagged based upon their treatment. The chicks were brooded in a petersime brooder. The treatments were grown mixed together to decrease variability. The birds were all fed a standard broiler starter diet (3035kcal/kg, 22.855%CP) ad libitum.

Sampling

Birds were sampled at numerous time points, 16 days of incubation (E16) 18 days of incubation (E18) 20 days of incubation (E20) Hatch (D1) at 7 days (D7) and at 14 days of age (D14). At each time period 12 birds from each treatment low oxygen and control were selected. Heart rates were determined using the Buddy digital egg monitor (Avitronics, UK) and egg weights were recorded for embryos only. Blood samples were also pulled from the embryonic stages, and were analyzed using the ISTAT-6 Cartridge. Blood gas and nutrients were collected for Na, Cl, Total CO₂ (TCO₂), Glucose, Hematocrit, Partial Pressure of CO₂ (PCO₂), and HCO₃. The body weight, yolk weight, heart weight, liver weight, and breast weight were recorded at all samplings.
RESULTS

Blood gas analysis

There was no significant change in blood Na, pH, or K at E 16, E18 low oxy, or E18 control. The E16 embryos had significantly higher Cl levels than the E18 controls; however neither were statistically different than the E18 low oxy birds. There was a considerable increase (p<0.0001) in TCO₂ in E18 (38.60 mmol/L) Control birds when compared to the E16 (28.0 mmol/L), and E18 Low (30.09 mmol/L). Similar patterns were observed with PCO₂ as well as HCO₃, the E16 (32.69 mm/Hg, 27.13mmol/L), and E18 (31.28 mm/Hg, 29.26 mmol/L) low oxy birds having significantly lower levels and E 18 control having higher levels (49.01 mm/Hg, 37.06 mmol/L) respectively in the blood (p<0.005). Glucose (200.09 mg/dL) and Hematocrit (27.27 % PCV) levels were highest (p<0.0001) in the E18 low oxy embryos, followed by E18 control (172.60 mg/dL, 23.00 % PCV), and E16 embryos having the lowest levels (140.09 mg/dL, 15.36 % PCV) (Table 1).

Organ weights

There were no significant changes embryonically in heart, liver, or breast weights as a % of whole body weight, however there were differences (p<0.0001) in heart rate, with E18 low oxy having the highest heart rate (305.41 BPM) there were no other differences between the treatments at the different time points. There were significant differences between the time points but not between the treatments for yolk free embryo weight. As expected the yolk weight was highest in E16 birds, there were no differences between the E18 low and control birds, However the E20 control embryos had lower yolk weights (p<0.0001)(14.28g) than did the E20 low oxy (17.45g) (table 2).
Once the birds hatched, there were differences between the time points, however there were no differences between the low and control groups for heart weight as a percentage of body weight. The liver and breast followed similar trends, with exceptions being in the in D14 chicks, the livers made up a significantly larger percentage of the body weight (p<0.0001) in the low oxy group (4.05%) and the livers made up a smaller percentage of total body weight in the control group (3.44%). The breast percentage of total body weight in D7 control made up a significantly larger (p<0.0001) percentage (9.54%) than the low oxy group (7.81%)(Table 3).

**DISCUSSION**

This study demonstrates the physiological changes that occur in an embryo as a result of hypoxic incubation conditions. This stressor (hypoxia) has proven to play a significant role on the developing embryo, which manifest its self in numerous changes in the physiological response of the embryo. The E18 low oxy group had significantly higher levels of glucose than the controls. These results are indicative of the stress response of the embryo. When an organism is subjected to a stressor such as heat or hypoxic conditions, this elicits a stress response within the organism which leads to an increase in corticosterone (De Smith et al. 2008). Corticosterone has been shown to initiate numerous physiological changes with in birds, especially with metabolic function. One main function of corticosterone is that it causes the mobilization or production of glucose in order to help provide the energy for the organism to combat the stressor (Virden and Kidd, 2009). Glycogen levels have been shown to decline rapidly during hypoxic conditions leading to a dramatic increase in blood glucose.
levels (Beattie, 1964). Stress has also been shown to cause dramatic reductions in growth rates and development, which may be attributed to a decrease in RMR (Virden and Kidd 2009, Edens 1977). This decrease in growth and development is further supported by the blood gas data collected from this trial, in that the E 18 low embryos had levels of Cl, TCO$_2$, PCO$_2$, and HCO$_3$ that were very close to the E16 levels, and the E18 control levels were significantly higher further evidencing the decrease in GR. Tazawa et. al. 2012 reported decreased HCO$_3$ levels in embryos exposed to hypoxic conditions, and theorized the response was due to the metabolic down regulation of the embryos, and that anaerobic glycolysis was a key determining factor in this down regulation. Hypoxic conditions have been show to increase blood oxygen carrying capacity by changes in hemoglobin as well as polycythemia (Dusseau and Hutchins, 1988). The data from our study showed that there was a significant increase in hematocrit levels in the low oxy birds in comparison to control birds. These results are in agreement with previous studies by Druyan et al. 2007, and 2012. These data help support the claim that brief exposure to hypoxic conditions alters red blood cell concentration, and thus affects blood oxygen carrying capacity.

There were no significant differences in embryonic organs weighed. The organs were all analyzed as percentage of the whole body weight. These results are similar to findings by Druyan et al. 2012 in that there were no differences between the relative breast, liver, and heart weights due to hypoxic treatment. The embryo free yolk weight increased with age, however there were no differences between the control and low oxy group at each time point. Even though there were no differences between the heart weights, there were differences between heart rates due to hypoxic exposure. The E18 low oxy embryos had a significantly
higher heart rate of 305.41 BPM compared to the control with 274.91 BPM. This difference between heart rate diminished between the low and control groups in the E20 embryos (Table 2). This increase in heart rate is thought to be correlated with the increase in hematocrit. Increases in hematocrit levels of blood cause the blood to become thicker and more viscous which causes the heart to exert more force and an increased rate of beating is required to keep up with oxygen demand by the body (Whittow, 2000). E20 low oxy group had higher mean yolk weights 17.45g compared to the control group with a weight of 14.28g. This difference is attributed to the decrease in growth that has been discovered to result from hypoxic incubation and the possible decrease in RMR (Tazawa et al. 2012, Virden et al. 2009, and Edens 1977). The differences in breast weight at D7 that were observed again follow along with previous data and supports the claim that hypoxia causes a decline in growth; however by D14 the low group reached the same weights as the control group.

In summary, these data suggest that birds exposed to brief hypoxic incubation conditions are indeed capable of faster rates of growth and may perform better throughout the growing period. The observations made in this study may be useful to the broiler industry, where birds are grown in varying environments. By simply manipulating incubation conditions to including brief periods of hypoxia, the resulting birds may be better suited for specific environmental conditions or when specific modifications to body composition are desired. More research is required to determine exactly how to perfect this methodology so that it is applicable to the commercial industry. A better understanding of the molecular effects that hypoxia induces is require to further perfect this procedure.
REFERENCES


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<td></td>
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<td>5.6</td>
<td>97.5 (^A)</td>
<td>28.0 (^B)</td>
<td>140.1 (^C)</td>
<td>15.4 (^C)</td>
<td>7.5</td>
<td>32.7 (^B)</td>
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<td>49.0 (^A)</td>
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\(^{A,B,C}\) Treatments with differing \(^{A,B,C}\) with in column denote significant difference at P<0.001 Lack of \(^{A,B,C}\) denotes no significant difference
### Table 2. ANOVA analysis of mean organ weights

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<td>262.1</td>
<td>35.8</td>
<td>17.5</td>
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Treatments with differing \( A,B,C \) with in column denote significant difference at \( P<0.001 \) Lack of \( A,B,C \) denotes no significant difference
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<td>11.84</td>
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</table>

Mean values as percentage of body weight.

Treatments with differing superscript letters within column denote significant difference at P<0.001. Lack of superscript letters denotes no significant difference.
Manuscript VII

Parental Diets Effect on Egg Component Weights and Protein levels

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Primary Audience: Researchers, Layer Companies
SUMMARY

The cost of feed is one of the largest expenses associated with commercial poultry production. Protein and energy are the two most costly components of the feed. There has been much research aimed at developing the least costly diet while still maintaining maximum production. This research demonstrates the changes associated with feeding diets with either high, low, or control levels of protein and energy to layer type birds from day of hatch to production. There were significant changes in albumen, yolk, and total egg weights between the groups as well as differences in protein content of the albumen. Significant differences due to diet were also observed in shell thicknesses and porosity.

INTRODUCTION

One of the largest costs associated with commercial poultry production is feed. In fact, it has been estimated that 65-75% of the cost associated with egg production comes from feed costs (Bell and Weaver, 2002). Protein and energy are the two most costly components of poultry feeds, and make up about 85% of the total feed cost (Gunawardana, et al. 2008). As a result of the high cost associated with feeding poultry there has been much research conducted exploring how to maximize the performance of the birds, while minimizing input costs. This can be a challenging feat to accomplish since changes in protein and energy levels in the diets of laying hens have been shown to change egg production (Khajali, et al. 2008; Perez-Bonilla et al, 2012) egg size, (Zimmerman, 1997; Shim et al. 2013) yolk weight, (Shim et al. 2013). In fact, Zimmerman (1997) has proposed a method to manage egg size by precise delivery of amino acids that has no impact on the production of the hen. Egg weights have
been shown to vary depending on CP and E available in the diets. Shim et al. (2013) reported that hens fed higher protein diets have larger egg weights when compared to eggs from hens on lower CP diets. Gunawardana et al. (2008) reported increasing dietary protein intake from 13.8 to 17.1g/hen per day resulted in a 2.38g increase in egg weight, but changing dietary energy was found to have no effect in this trial. Similar results were also reported by Sohail et al. (2003) and Shim et al. (2013) in that hens on higher protein diets produced eggs with significantly larger weights. However, there are data that demonstrated egg weight is not affected by feeding low CP diets (Khajali et al. 2008). The majority of the research suggests that birds fed diets containing low CP produce eggs that weigh less than birds fed higher CP diets. Leeson and Caston (1999) demonstrated that birds fed 14.4% CP had lower egg weights than birds fed 16.8% CP diets.

Shim et al. (2013) states that yolk percentage is indirectly proportional to CP in young hens. Hens exposed to increasing dietary protein demonstrated a significant increase in yolk weight when compared to the controls. Gunawardana et al. (2008) reported significant increases in both yolk and albumen weights in the birds on the high CP diets in comparison with birds on low CP diets.

Eggs possess a unique property that allows them to “breathe”. Eggs must allow for the exchange of gases produced and required by the developing embryo, as well as exchange of moisture so that the embryo can maintain ideal conditions (Rahn et al., 1981; Christensen, 1982; Peebles and Brake, 1985). This diffusion of gas has been termed conductance and is a measure of the gas exchange of an egg (Rahn et al. 1981). There are several factors that affect conductance including pore concentration. There has been much research focused on
egg shell pore concentration associated with the age of the female (Peebles and Brake, 1987; Rahn et al., 1981, Christensen, 1983), genetics or strain of the bird (Christensen et al, 1982); however, there has been very little research looking at nutritional effects on pore number. It is well documented that as poultry progress through their laying cycle the thickness of their egg shells decrease as the size of their eggs increase. As a result of this the pore concentration decreases (Peebles and Brake, 1987; Brake et al. 1997; Rahn et al. 1981). This change in pore concentration has drastic effects on conductance and ultimately the hatchability of the eggs (Rahn et al. 1981). Since changes in diet have been shown to cause significant changes in egg weights, research looking at the effect this has on pore concentration is justified.

There has been much research examining the effect that diets have on the yolk contents from color to lipid modification (Hargis, et al. 1991; Elkin and Lorenz, 2009; Fletcher and Papa, 1985). Yet there has been no research to date examining the effect that dietary protein levels have on the protein content of the albumen.

**MATERIALS AND METHODS**

Eggs analyzed for this trial were obtained from birds being raised for a separate experiment. A brief background of the rearing of the birds is provided. The experimental procedure used in this investigation was approved by the North Carolina State University Animal Care and Use Committee. Day old W-36 Parent stock was obtained from Hy-line International (Dallas Center, Iowa). Each chick was neck tagged and sorted into one of three dietary treatments: Low, Control, and High (table 1). The low group was fed a starter feed containing 12% CP
and 1000 kcal/lb, Control diet contained 18% CP and 1360 kcal/lb, and the High diet contained 24% CP and 1480 kcal/lb. All chicks were raised in the same room in a Petersime brooder. The chicks were brooded under standard brooding conditions. At 10 weeks of age the birds were switched to a layer diet. The low layer diet was 12% CP and 1000 kcal/lb, Control diet 18% CP and 1360 kcal/lb, and the High diet 24% CP and 1480 kcal/lb. The birds were allowed ad libitum access to feed and water for the entire trial. Eggs were collected; whole egg weight, albumin weight, yolk weight, pore count, and shell thickness were recorded.

**Egg Contents**

The total egg weight was determined. Each egg was gently cracked open, and the yolk and albumin were separated and weighed individually. The shells were rinsed with tap water and allowed to dry overnight. The shell thickness was measured using a caliper to the nearest .01 mm, in triplicate in similar location around the large end of each egg. Egg pore staining was conducted using the methods of Peebles and Brake (1985) briefly; each egg was stained with a dye solution containing .5g of methylene blue crystals dissolved in 70% ethanol. The solution was placed on the inside of the egg for 30 min then the excess dye solution was poured off and the egg was allowed to dry. Then four equally spaced .25cm² squares were drawn on the large end of the egg. The pores in each square were counted using a stereo microscope on low power. The four numbers were then averaged.
**Protein Content of Albumen**

Total protein content of the albumen was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) following manufacturer’s instructions. Briefly, the whole albumin was placed into a whirl-pak bag, and homogenized in a stomacher for 30s. Then a 100µl sample of homogenized albumin was diluted into 900µl 1XPBS +2% SDS, this dilution was not sufficient, so another 1:10 dilution was required, again this dilution was not sufficient to obtain measurable results so a final 1:5 serial dilution was performed resulting in a final dilution of 1:500. 25µL of diluted sample and 200µL of working stock reagent (supplied by kit) was placed into a 96-well microplate. Each sample was run in triplicate. The plate was sealed and incubated at 37°C for 30 minutes. Absorbencies of the solutions were read at 570nm.

**RESULTS AND DISCUSSION**

There were significant differences (p< 0.0001) observed in egg weights between the treatments. Eggs from hens fed the low diet were smaller (59.75g) by almost 5 grams than the eggs from the high group (64.26g). The eggs from the control group (63.07 g) were significantly heavier than the low group but not statistically different from the high group (table 2). This data is in agreement with work reported by Shim et al. (2013) that hens fed higher levels of protein had larger egg weights. These data are also in agreement with the work of Gunawardana et al (2008) as well as work by Zimmerman (1997) who reported that you can control egg size in layers with precise nutrient delivery.
Similar trends were observed in both albumen and yolk weights. There were significant differences found (p<0.0001) between treatments. There were no differences found between the high and control groups in regards to albumen and yolk weights. However, the low group had significantly lower weights for both the albumen (35.45g) and yolk (16.04g) (table 2). These results being expected and explained by the fact that increased egg weights are a result of an increase in contents. These findings are in agreement with similar work conducted by Gunawardana et al (2008) who also showed increased albumen and yolk weights in birds on higher CP diets compared to birds on lower CP diets. The albumen percentage followed the same trend as the aforementioned data, in that the low group had significantly (p<0.0001) lower percentage albumen than the control and high groups. This data is in agreement with Noavk et al (2008), as well as Penz and Jensen (1991). This data is suggestive that the hens on lower CP diets could not be receiving sufficient amino acids for optimal albumen formation. The interesting element of the egg component data is that the control group had significantly higher yolk percentage (31.59%) than did the high or low groups 30.45% and 26.87% respectively (table 2). Novak et al (2008) reported similar findings in that birds on lower CP diets had larger percentages of yolk than birds on high CP diets. This supports our results for the differences observed between the control and high groups; however, our low treatment still had lower percentage of yolk than control and high group. Our low group was fed a diet with a CP (12%) that was much lower than that used by Novak et al. (2008). Also, our low diet contained lower caloric levels, which is crucial for the formation of the yolk, thereby explaining the discrepancy.
Differences were also observed in shell thickness and pore concentration. The low and control groups had significantly thicker (p<0.0001) shells than the high group (table 3). Gunawardana et al (2008) reported that increasing dietary protein significantly decreased egg shell percentages. Nahashon et al (2007) found that guinea fowl on either high CP or ME diets had decreased shell thicknesses. The average pore concentrations of the low and control groups were significantly higher (p<0.0004) than pore concentrations of the high group (table 3). This data can be explained and supported by the simple fact that as egg size increases the pore concentration decreases.

There were significant differences observed in total protein content of the eggs (table 4). The control group had the highest albumen total protein content (188,742.69 µg/ml) followed closely by the high group (182,650.10µl/ml). The high group had a total protein intermediate of the low and control groups, approaching significant difference from the low group (p≤ 0.07). However, the control group had significantly higher (p ≤ 0.04) levels of total albumen protein than did the low group. The actual mechanism responsible for its difference is unknown. There have been no publications to date that have examined the protein content of eggs in relation to dietary protein levels. One simple explanation for this phenomenon could be that there are less amino acids available when the tubular gland cells of the magnum begin to deposit albumen. Further exploration of this occurrence must be conducted to fully understand the processes that are occurring.
REFERENCES


Elkin, R.G., E.S. Lorenz. 2008. Feeding laying hens a bioavailable soy sterol mixture fails to enrich their eggs with phytosterols or elicit egg yolk compositional changes. Poult. Sci. 88:152-158


Table 1. Ingredient Composition of Diets

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<tr>
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<td>Oats</td>
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Formulated Percentages

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Analysis (actual percentages)

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<td>-------------</td>
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</tr>
<tr>
<td>Low</td>
<td>59.76 (^b)</td>
<td>35.45 (^b)</td>
<td>16.04 (^b)</td>
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<tr>
<td>Control</td>
<td>63.08 (^a)</td>
<td>40.17 (^a)</td>
<td>19.91 (^a)</td>
</tr>
<tr>
<td>High</td>
<td>64.27 (^a)</td>
<td>40.49 (^a)</td>
<td>19.58 (^a)</td>
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Mean weight for total egg weight and components as percentages, \(^abc\) superscripts denote significant differences within columns at \((p\leq0.05)\)
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Thickness</th>
<th>SEM</th>
<th>Pore #</th>
<th>SEM</th>
<th>n</th>
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</thead>
<tbody>
<tr>
<td>Low</td>
<td>42.04</td>
<td>.5</td>
<td>29.51</td>
<td>.9</td>
<td>25</td>
</tr>
<tr>
<td>Control</td>
<td>41.17</td>
<td>.5</td>
<td>25.10</td>
<td>.9</td>
<td>25</td>
</tr>
<tr>
<td>High</td>
<td>37.967</td>
<td>.5</td>
<td>23.98</td>
<td>.9</td>
<td>25</td>
</tr>
</tbody>
</table>

Mean shell thickness and pore count, \(^{a,b}\) superscripts denote significant differences within columns at (p≤0.05)
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total Protein (ng/ml)</th>
<th>SEM</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>147.5 B</td>
<td>14.3</td>
<td>22</td>
</tr>
<tr>
<td>Control</td>
<td>188.7 A</td>
<td>13.6</td>
<td>24</td>
</tr>
<tr>
<td>High</td>
<td>182.7 AB</td>
<td>13.6</td>
<td>24</td>
</tr>
</tbody>
</table>

Mean total protein by treatment (µg/ml), ab superscripts denote significant differences within columns at (p≤0.05)
Dietary Conditioning of Turkey poults with Calcium and Phosphorus
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Primary Audience: Researchers, Turkey Producers, Feed Companies

Keywords: Turkey, Dietary Conditioning, Calcium, Phosphorus
INTRODUCTION

Calcium and Phosphorus are two crucial nutrients in poultry feeds. It has long been known that there is some correlation between calcium and phosphorus in animals. The digestibility of phosphorus has been shown to be affected by not only the levels of phosphorus in the diet but also the level of calcium as well (Liu, et al, 2013; Driver et al, 2005). Researchers have been studying the delicate balance of calcium to phosphorus in the diet for many years (Sullivan, 1962). Still after 50 + years of research, there is still some debate as to the proper ratio. Much of the phosphorus present in feeds is bound to phytate molecules. The binding of Phosphorus to phytate results in the organism having a very difficult time absorbing and utilizing Phosphorus from feed stuffs (Eeckhout and Paepe, 1994; Liu et al, 2013). The typical solution for many producers is to simply increase the levels of Phosphorus in the feeds. This practice however causes higher levels of Phosphorus to be present in the feces of birds. This litter is then spread on to fields and pastures (Honeyman, 1993). Phosphorus has been shown to accumulate in higher quantities than other nutrients on land where poultry litter is applied (Chapman, 1996). These high levels of Phosphorus in the soil have been shown increase the amount of phosphorus that is present in run-off water, which eventually runs in to lakes and streams and other surface water sources and causes eutrophication (Chapman, 1996; Sharpley et al. 1993) Runoff rates of P has been shown to range from 2.2% up to 7.3% of the P from litter spread on the fields (Chapman, 1996). Some producers are exploring the use of phytases in poultry diets; these trials have shown some promising results. The addition of phytases to diets increase the availability of bound P in the diets, however there are many different phytases available and great variations in the reported
efficacies of each phytase (Powers and Angel, 2008). DeLaune et al. (2001) reported that the uses of phytases in poultry diets do in fact increase the availability of P, however it also increases the amount of water soluble P in the litter. So another alternative would be finding a way in which the animal better utilizes the P in the diet allowing for less P excretion. Ashwell and Angel, 2010 demonstrated that you can condition broilers early in life to better utilize phosphorus. Broiler chicks were exposed to 90 hours of Phosphorus deficient diet immediately after hatch. The conditioned broilers had increased expression levels of the Na/P co-transporter IIb, and increased P utilization later in life. The sodium phosphorus type IIb cotransporter is considered the major sodium phosphorus cotransporter and is primarily reported to be found in enterocyte brush-border membranes (Hilfiker et al. 1998) These data yield promising results for chicken producers. The purpose of this trial is to further test this method in turkeys.

**MATERIALS AND METHODS**

**General overview**

384 tom poult s were obtained from a commercial hatchery (Butterball, Goldsboro, NC) Upon arrival each of the poult s were tagged, weighed, mean weights were used to equilibrate the weights. This trial consisted of 3 different treatments: Control diet for the entire trial (CCC), Control diet for 29 days then Low diet (CCL), and then Low diet for 4 days, Control diet for 25 days, then Low diet for remainder of the trial (LCL). All poult s were brooded in the same room in Alternative Design Brooders. The rooms were kept at 98°F for the first week, the temperatures were gradually decreased each week until supplemental heaters were
no longer necessary. There were 36 pens total, yielding 12 replicates of each treatment, each pen contained 12 poult. There were two different diets utilized in this trial both of which contained chromium oxide as a marker. The feces were collected for 24h periods from each pen on days 4, 29, 42. One bird from each pen was sampled (12 per treatment) at days 4, 29, 42. Body weights were recorded, the right tibia was removed and stored at -20°C, the liver and ileum were also collected and placed in RNAlater solution for gene expression.

Fecal analysis

The Feces from each 24 hour sampling were stored at -20°C until the completion of the trial. Then all samples were placed in a drying oven at 60°C for 24 hours. Upon drying all samples were removed from the oven, ground, and weighed. The samples were sent to the NCSU Environmental and Agricultural Testing Service (EATS) (Raleigh, NC) for evaluation of Calcium and Phosphorus levels using Inductively Coupled Plasma-Optical Emission Spectrometry. In brief, samples were placed in Muffle Furnace and ashed overnight. The ashes were cooled and approximately 2ml of distilled H$_2$O was added along with 4 ml of 6N HCl. The solution was dried down on steam plate. The solution was then warmed slightly and transferred to a flask and equal amounts of distilled water were added. The sample was mixed, filtered and then ran through an ion coupled plasma spectrometer (ICP).

To measure Chromium, 20ml of 10% Sodium Carbonate was added to sample and the sample was thoroughly mixed. The solution was dried on a steam plate and placed in a Muffle furnace for 8 hours. Then 8 ml of HCL was added to ash The solution was then warmed slightly and transferred to a flask and equal amounts of distilled water was added.
The sample was mixed, filtered and then ran through an ion coupled plasma spectrometer (ICP).

**Tibia Calcium/ Phosphorus levels**

The right tibias of the poultts were collected at days 4, 29, 42 and stored at -20°C. The excess flesh was removed from the bone to avoid any potential interference. The bones were then sent to USDA (Beltsville, MD) for density readings using the dual energy x-ray absorptiometry (DXA) (Lunar, DPX-L, Lunar Corp., Madison, WI) following the procedures of Mitchell et al. (1997). Bone mineral density (BMD) (g/cm2), bone mineral content (BMC) (g/bone), and tibial area (cm²) were measured.

**Gene Expression**

**mRNA extraction:** Approximately 0.1g of each tissue sample was homogenized by bead beating using a mini bead-beater (Biospec, Bartlesville, OK) and RNA was isolated using the Qiagen RNeasy mini kit (Qiagen, Germantown, MA) according to manufacturer protocol. Briefly, tissue was homogenized in kit Buffer RLT and then centrifuged. The supernatant was mixed with 70% ethanol and placed on the RNeasy column. Column was washed with supplied buffers RW1 and RPE. RNA was eluted off the column in 50 µL of nuclease-free water. The sample was spectrophotometrically scanned then at 460 nm and 480 nm wavelengths (Nano-Drop 2000 Spectrophotometer, Thermo-Scientific) to determine the concentration and purity of the extracted mRNA.
The RNA Sample was diluted with nuclease-free water to a concentration of 500 ng/µL. Then 1 µL of the extracted mRNA samples was placed into a well in a 96-well plate, along with loading dye and nuclease-free water. The plate was then run on a denaturing cycle at 65 degrees for 15 minutes. The samples were then run on a 1% ethidium bromide gel. The gel was then visualized on a UV light box to evaluate RNA quality by the appearance of the intact bands of the 28S and 18S ribosomal subunits. Non-degraded RNA was then used to synthesize cDNA for use in real time PCR.

**cDNA:** 1ug of each RNA sample was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Foster city, CA). The 20 µL cDNA synthesis reaction contained in addition to the RNA template, 2 µL of 10x RT buffer, 0.8 µL of 25XdNTP mix, 2 µL 10x RT Random Primers, and 1 µL MulitScribe Reverse Transcriptase. Nuclease-free water was added bring the reaction up to final volume.

**Real Time PCR:** The cDNA samples from each treatment group were diluted 1:20 with nuclease free water for use as template in real time PCR reactions. All real time reactions consisted of 1 µL of cDNA, 1 µL of primer, 10 µL Power SYBR green mastermix (Applied Biosystems, Foster city, CA) and 8 µL nuclease-free water. The thermocycler parameters consisted of: Initial denaturation of 95°C for 7 minutes followed by 40 cycles of 95°C denature for 30 seconds, annealing temperature for 30 seconds, and 72°C extension for 30 seconds, a final 72°C extension for 5 minutes, and a melt curve consisting of 95°C for 1
minute followed by 55°C initially for 1 minute with the temperature increasing by 0.5 °C each cycle for 80 cycles.

**Statistics**

Gene expression values, reported as threshold cycle (CT), were subjected to multivariate analysis of variance (MANOVA) and analysis of variance (ANOVA), Statistical Analysis System- JMP® 10 program (2012; SAS, Cary, NC). Individual contrasts were made via comparison of means using the Tukey-Kramer procedure in JMP® 10 (2012; SAS, Cary, NC). Significance levels were set at \( P \leq 0.05 \).

**RESULTS**

From the analysis of the feces at day 4 significant differences in Total Tract Retention of Ca and P are immediately manifested between the turkeys exposed to low Ca and P. The low group (LCL) had significantly higher retention levels of both Ca (95.18) (\( p<0.0001 \)) and P (92.36) (\( p<0.0123 \)) in comparison to the control group (72.98) and (88.97) respectively. However the differences in both Ca and P disappeared on the day 29 sampling when both groups were placed on the control diet. On the day 42 sampling there were differences in Ca retention between the CCC group (76.0) and the LCL group (86.44) CCL group (87.18) yet there were no differences found in TTR for P levels (Table 1,2).

There were differences found in bone mineral density (BMD) and bone mineral content (BMC) however there were no significant differences found in tibial area. No data are reported for day 4 samples because they were too small to get an accurate reading from the
machine. There were no significant differences found in BMD at day 29 between the LCL and CCC, however differences in BMD were discovered at 42 days with the CCC group having significantly higher levels than the CCL and LCL legs (table 3.). There were no differences found between the LCL and CCC group at day 29, yet at day 42 the CCC group had significantly higher BMC levels than LCL poults. There were no significant differences observed between the CCL and LCL birds (table 3.)

Real Time PCR was performed on both Ileum and Liver tissues at 4, 29, and 42 days of age using the Type IIb Sodium Phosphate Cotransporter gene (NPT2b). Multiple significant differences in expression of the NPT2b gene were discovered. There was a significant up regulation of expression (p<0.0001) in the LDL poults at day 4 compared to the CCC day 4 poult's in the liver. Similar data was collected from the ileum, however the increase in expression was not significantly different (Figure 1.) Expression levels at day 29 while both groups were on the control diet were not significantly different between either group or tissue. On the day 42 sampling significant differences (p<0.0001) in expression of the NPT2b gene were found. The LCL and CCL had significantly higher levels of expression than did the control group (CCC) in the liver. Differences were also seen in the ileum, CCL had significantly higher expression levels than did LCL poults, however these levels were not significantly different than CCC birds. No differences in expression were found between LCL and CCC poults in ileal NPT2b expression levels at 42d (Figure 1.)
DISCUSSION

This experiment was designed to demonstrate the changes associated with exposing turkey poults to low levels of dietary calcium and phosphorus for the first 4 days of life, in hopes that the birds will be more conditioned to utilize calcium and phosphorus more efficiently. When you combine all of the data from each analysis, the results do in fact tell a story. The LCL poults at day 4 had significantly higher TTR of both calcium and phosphorus, and increased expression levels of the NPT2b gene, the major sodium phosphorus cotransporter (Yadgary et al. 2001). These results are in agreement with work done by Yan et al, (2007) where broiler chicks were fed diet low in Ca and P for the first 4 days of life, and also demonstrated increased expression of the NPT2b gene in chicks fed deficient diets.

At the day 29 sampling all groups of poults were on the control diet, thus the lack of significance in expression levels which is further supported by the lack of significant differences observed in the TTR, BMD, BMC as well as tibial area suggest that even though these poults were deficient, they were able to catch up and perform at the same level as the control counterpart, while receiving less calcium and phosphorus.

On the d42 sampling the LCL and CCL birds had been on a low diet for 13d and the CCC birds remained on the control diet for the entire trial. The purpose of the switch back to low diets was to determine if the birds conditioned on the low diet could in fact utilize calcium and phosphorus more efficiently than the control birds. There were no significant differences between the CCL and LCL in TTR of calcium or phosphorus, nor was there significant differences in BMD and BMC, however there was a significant difference in ileal expression of the NPT2b gene. The poults from the LCL diet had significantly lower expression levels
than the CCL birds. In fact there was no difference in expression levels between the CCC and the LCL groups. This data in agreement with reports made by Ashwell and Angel (2010) that nutritionally “conditioned” (fed phosphorus deficient diets) broilers are able to utilize phosphorus later in life when phosphorus deficient diets were fed. The results from this trial are in no way conclusive regarding calcium and phosphorus condition in turkey poults. However, from the results it is evident that this procedure does in fact cause some changes in ca/p utilization as well as expression of the NTP2b gene. This trial was based upon previous research in broilers and other animals. It is well know that turkeys are much different than broilers. Since this trial is the first to date experiment looking at dietary condition with calcium and phosphorus in turkeys and was based off of boiler data, the lack of a definitive answer was not complete surprise. However, we now have background information with turkey specific data. The next step will be to customize this feeding procedure to turkeys in regards to the correct low calcium and phosphorus levels, duration of restriction, and the correct sampling time points. Another possible reason for the inconclusive answer from this trial could be the sample sizes used for this trial. More funding must be obtained and larger sample sizes could be collected and analyzed for future trial in hopes for a more definitive answer can be reached.
REFERENCES


Superscripts denote a significant difference within columns at $p \leq 0.05$, lack of superscripts denote no significant differences.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 4</th>
<th>Day 29</th>
<th>Day 42</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCL</td>
<td>95.18</td>
<td>80.09</td>
<td>86.44</td>
</tr>
<tr>
<td>CCC</td>
<td>72.98</td>
<td>79.1</td>
<td>76.00</td>
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<tr>
<td>CCL</td>
<td>-</td>
<td>-</td>
<td>87.18</td>
</tr>
</tbody>
</table>

abc superscripts denote a significant difference within columns at $p \leq 0.05$, lack of superscripts denote no significant differences.
Table 2. Total Tract Retention (TTR) of Phosphorus

<table>
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<tr>
<th>Treatment</th>
<th>Day 4</th>
<th>Day 29</th>
<th>Day 42</th>
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<tbody>
<tr>
<td>LCL</td>
<td>92.36 (^a)</td>
<td>90.2</td>
<td>88.76</td>
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<tr>
<td>CCC</td>
<td>88.97 (^b)</td>
<td>89.83</td>
<td>89.16</td>
</tr>
<tr>
<td>CCL</td>
<td>-</td>
<td>-</td>
<td>89.36</td>
</tr>
</tbody>
</table>

\(^{abc}\) superscripts denote a significant difference within columns at \(p \leq 0.05\), lack of superscripts denote no significant differences.
**Figure 1.** Real Time Ct values for mRNA expression of NPT2b in the Ileum and Liver of turkey poult at 4, 29, and 42 days of age. ABC values denote significant differences between treatments within each tissue at p≤0.05.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>BMD (g/cm²)</th>
<th>SEM</th>
<th>BMC (g)</th>
<th>SEM</th>
<th>Area (cm²)</th>
<th>SEM</th>
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<tr>
<td>CCC D42</td>
<td>0.126 A</td>
<td>0.00375</td>
<td>1.33 A</td>
<td>0.07506</td>
<td>10.7 A</td>
<td>0.47613</td>
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<td>CCL D42</td>
<td>0.103 B</td>
<td>0.00375</td>
<td>1.05 AB</td>
<td>0.07506</td>
<td>10.1 A</td>
<td>0.47613</td>
</tr>
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<td>LCL D42</td>
<td>0.097 B</td>
<td>0.00375</td>
<td>.94 BC</td>
<td>0.07506</td>
<td>9.7 A</td>
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</tr>
<tr>
<td>CCC D29</td>
<td>0.107 B</td>
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<td>.67 CD</td>
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<td>LCL D29</td>
<td>0.100 B</td>
<td>0.00419</td>
<td>.54 D</td>
<td>0.08392</td>
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<td>0.43465</td>
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Superscripts denote a significant difference within columns at p ≤ 0.0001, lack of superscripts denote no significant differences.
Table 4. Ingredient Composition of Diets

<table>
<thead>
<tr>
<th>Ingredient</th>
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</thead>
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<tr>
<td>Yellow Corn</td>
<td>666</td>
<td>666</td>
</tr>
<tr>
<td>Soybean Meal</td>
<td>600</td>
<td>600</td>
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<tr>
<td>Poultry Fat</td>
<td>26.6</td>
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<tr>
<td>Poultry Meal</td>
<td>120</td>
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<tr>
<td>Methionine</td>
<td>3.3</td>
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<tr>
<td>Lysine</td>
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<td>2.5</td>
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<tr>
<td>Salt</td>
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<td>3</td>
</tr>
<tr>
<td>Choline Chloride</td>
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</tr>
<tr>
<td>Vitamin premix</td>
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<td>1.5</td>
</tr>
<tr>
<td>Dicalcium Phosphate</td>
<td>28</td>
<td>0</td>
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<tr>
<td>Vermiculite</td>
<td>0</td>
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<td>Analysis (Actual)</td>
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<td>Calcium (ppm)</td>
<td>8352</td>
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<tr>
<td>Phosphorus (ppm)</td>
<td>7441</td>
<td>3359</td>
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</table>
Manuscript IX
Nutritional Sex Reprograming

Nutritional Effects on Parent Stock W-36 and its Influence on the Sex Ratio and Development of Offspring

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Primary Audience: Researchers, Layer Companies

Key Words: Layers, Sex Ratio, Nutrition
ABSTRACT

Past studies in birds have shown that there is a link between maternal condition, or resource availability, and the resultant sex ratio of offspring in avian species, both wild and domesticated. In the majority of cases, mothers will preferentially bias the sex ratio of their offspring to the sex that is most likely to survive in conditions in which nutritional resources are scarce or of poor quality; typically, this is the sex with the lower mature body weight, thus lower nutritional requirements, in sexually dimorphic species. This study analyzed how three diets of differing caloric and protein concentrations influenced the sex ratios of a commercially utilized avian species, the Hy-Line W-36 laying hen. As expected, development of both parent and filial generations was significantly affected by diet or egg composition, respectively. Sex ratio bias was significantly different only between CL offspring compared to the control, but there was a similar trend in the opposite direction for CH offspring. Results suggest that continual availability of either high- or low-nutrient density food resources does not predispose hens to bias the sex ratio of their offspring; however, a sudden change in diet, particularly to one of lower protein concentration, may influence such a change, suggesting linkage with the production of stress-related hormones. Further trials are needed to assess the efficacy of diet alteration prior to reproductive maturity as a means of sex allocation manipulation.
INTRODUCTION

There is evidence to suggest that environmental conditions, specifically the availability and quality of food resources, can bias the sex ratio of hatchlings in sexually dimorphic avian species (Howe, 1977; Kalmbach, et al., 2005; Love, et al., 2005; Nager, et al., 1999; Parker, 2002; Pike & Petrie, 2005; Pryke & Rollins, 2012; Trivers & Willard, 1973). Presumably, the smaller of the two sexes will be produced in greater numbers when there are scarcer resources because their rearing is less costly (Trivers & Willard, 1973). The effect appears to be maternally derived; based on her condition and the resources to which she has access, the female will skew the sex ratio of her offspring towards the sex that is more likely to survive on lower quality egg contents during incubation and in a poorer rearing environment (Nager, et al., 1999; Pryke & Rollins, 2012; Trivers & Willard, 1973). Typically, this is the sex that is capable of surviving with a lower total intake and quality of feedstuffs and, on average, has a lower mature body weight, normally the females (Nager, et al., 1999).

The mechanism by which this change occurs has not been definitively identified, but it has been proposed that increased maternal corticosterone production in response to stressful environmental conditions may play a major role in sex-biased maternal investment in offspring (Correa et al., 2005; Love, et al., 2005; Pike & Petrie, 2003; Pike & Petrie, 2005; Pike & Petrie, 2006; Pinson et al., 2001a; Pinson et al., 2001b). Increased corticosterone levels in the bloodstream of the mother result in the reciprocal rise in deposition in the yolk of the egg (Love, et al., 2005; Pike & Petrie, 2003). In one study of European starlings (Sturnus vulgaris), this finding was presumed to be the cause for the resultant skewed sex
ratio by three main effects: high corticosterone eggs had males with a lower hatch rate (higher embryonic mortality), decreased weight at hatch, and slower cell-mediated immune responses. Females matured from eggs with high corticosterone levels appeared to be unaffected (Love, et al., 2005). A similar study in which Japanese quail (Coturnix coturnix japonica) were subjected to chronic exogenous corticosterone administration via Silastic implants showed a significant negative linear relationship between fecal corticosterone concentrations and male sex ratio per clutch of eggs (Pike & Petrie, 2006). Conversely, Pinson et al. found that acute administration of corticosterone to laying hens (Gallus gallus domesticus) five hours prior to ovulation resulted in significantly more male (82.6%) than female embryos, suggesting a relationship with either species or the mode of hormone application (2011a).

It is still unknown whether or not a causal mechanism for biases in offspring sex ratio of birds can be confidently applied across species (Pike & Petrie, 2003). In a study with the blue-faced parrot finch (Erythrura trichroa), a species that has no discernible sexual dimorphism and is insensitive to changes in nutritional quality (in terms of body condition), yielded puzzling results. Birds on low-quality diets produced offspring that were highly male-biased (72.9%), but birds on high quality diets had an unbiased offspring sex ratio. In the young, females were found to be much more susceptible to the effects of a low quality diet than male birds, thus providing a possible explanation for the skewed sex ratio from parents on the low quality diet. This implies that maternal condition may not play as much of a role as in determining sex of offspring as much as the expected rearing environment (Pryke & Rollins, 2012). Similar results were seen in both the great skua (Stercorarius skua) and
the common grackle (*Quiscalus quiscula*, Icteridae), in which wild populations would progressively produce higher numbers of the less “expensive” sex with the deterioration of resource availability (Howe, 1977; Kalmbach *et al.*, 2005).

This study aims to identify whether environmental adjustments similar to those referred to in the included literature can create complementary biases in sex ratio of the domestic chicken (*Gallus gallus domesticus*), which may have been subject to the loss of such a trait due to many years of dependence on humans for survival. If present, positive results could prove to be of major benefit to both the commercial egg- and meat-production industries.

**MATERIALS AND METHODS**

200 male and 400 female day-old W-36 parent stock chicks were obtained from by Hy-Line International (Dallas Center, Iowa). The chicks were vaccinated with HVT/IBD, Rispens, and SB1. All males were dubbed at hatch. Birds were neck-tagged and evenly separated into three groups, each corresponding to a diet of specific total crude protein and energy composition: “Control” (C) - 18% CP, 1461.27 ME, “Low” (L) - 12% CP, 1000 ME, and “High” (H) - 24% CP, 1460 ME (Fig.1-2). Chicks were brooded in a Petersime Brood-Unit (Model 25D 24) to 4 weeks; then, birds were divided by treatment and moved to grow-out cages. At 17 weeks of age, 4 males and 24 females from each treatment group were selected for breeding. An additional 12 females from both the H and L groups were switched to the control diet at this time and were labeled as CH and CL, respectively. Body and testicle weights were recorded from unsaved males, histological slides of testicles were prepared, and semen production was monitored for a separate project, details presented in
Lowman et al., 2014. Beginning at 19 weeks, females were artificially inseminated twice weekly with 50 μL of pooled semen from all 4 males of the corresponding group—CH and CL females: H and L semen was used, respectively. Semen was collected from the males using the abdominal massage method described by Burrows and Quinn, 1937. Birds were stimulated 2 times at each collection; semen was immediately collected from the ejaculatory groves of the phallus.

Eggs were collected for two weeks after the second week of artificial insemination and stored at 60°F and 65% RH until incubation. Eggs were incubated in a NatureForm (Jacksonville, FL) at 99.5°F and 55 % RH for 18 days. For the last 3 days of incubation, eggs were placed in hatchers (G.Q.F. Model 1520 Circulating Air Incubators) at 99.5°F and 65 % RH. At hatch, chicks were weighed, tagged, and evenly mixed in each level of Petersime Brood-Units. All chicks were fed a standard layer starter (19.5% CP) and water ad libitum. The birds were weighed weekly to track rate of growth. Weights were recorded for three weeks before termination of the first hatch. All unhatched eggs were analyzed for fertility and, if applicable, the stage of embryonic death (i.e. early, middle, or late). After 3 weeks, birds were euthanized via cervical dislocation and sexes were determined visually by secondary sex characteristics. Birds called into question were necropsied to confirm sex.

Tissue samples were collected from all late dead embryos for sexing via PCR amplification. DNA was extracted using the Qiagen DNA extraction kit protocol. In 600 μL of cell lysis solution and 6 μL of proteinase K, ~ 10 ng samples were incubated in a rotor oven at 55°C overnight. 200 μL protein precipitation solution was added to each sample, then vortex, and centrifuged. Supernatant was added to 600 μL isopropanol, gently inverted and then
centrifuged a second time to from a DNA pellet. The supernatant was then discarded and samples were rinsed with 500 μL 70% ethanol and centrifuged a final time. The supernatant was discarded and DNA pellets were allowed to dry before being suspended in 50 μL of DNA rehydration solution. PCR reactions were composed of GoTAQ 10x buffer, TAQ polymerase, magnesium chloride, DNTPs, and Ribo (256 bp) and XhoI (415 bp) sexing primers, as described by Clinton et al. (2001) PCR was run for approximately 1.5 hours. Samples were then run on an 1.5 % agarose gel containing ethidium bromide for approximately 30 minutes.

Two more replicates were conducted using similar methodology. However, blood was taken from a random selection of C, H, and L chicks from the second hatch to measure corticosterone levels and all chicks were only tracked for growth for three weeks before termination. For the third hatch, blood samples were taken for PCR sexing at hatch, but chicks were not kept for tracking growth due to space limitations. Egg production records were kept for parental groups for the duration of the trial.

Experimental data collected during the trial were analyzed by ANOVA (JMP 10, SAS, Cary, NC) using Tukey-Kramer comparison of means. Weights and sexes were used as the experimental units and an alpha of 0.05 was used to establish significances.

RESULTS

Birds continually fed one diet, regardless of nutritional content (L, C, H), were not found to significantly skew the sex ratio of their offspring. This finding was consistent for hatched, late dead, and total offspring produced for all hatches (Tables 2-4).
There was a significant increase (p<0.042) in the percentage of male chicks/decrease in females that hatched from eggs produced by birds that had been switched from the L diet to the C diet at the onset of lay (Table 3). Birds that had been switched from the H diet to the C diet produced more female offspring at hatch at a level that approached significance (p<0.076) (Table 3). There were no significant differences observed between sexes from parents that had their diets changed for late dead or total offspring (Tables 2 & 4).

Blood corticosterone concentrations were significantly higher in offspring from parents on the L diet (p<0.01). Offspring from birds on the H diet had slightly higher blood corticosterone concentrations than did the C offspring, but not at a level of statistical significance (Table 5).

Between 21 and 26 weeks, percent lay was reduced in both the H and L groups compared to the control and was significantly lower (p <0.01) in the L group. The L group came into peak lay, approximately 80%, about 4 weeks later than the H and C groups. Birds that transitioned diets showed rapid changes in egg production between 21 and 26 weeks of age; although not statistically significant, production for the CH group was comparable to that of the C group at week 21, but had decreased by week 26. Conversely, production of the CL group was comparable to the L group at 21 weeks, but was the same as the C group by 26 weeks (Fig. 1).

At hatch, there were numerous significant differences in offspring weights between groups. The H offspring were significantly larger (p<0.0059) than the L offspring, when analyzed only by treatment (Table 6). When further broken down by treatment and sex, there were no significant differences between the males of each treatment, but the H females were
significantly larger than the L females, with the C females falling in the middle (Table 7). At the day 7 weighing, both the C and the H groups were significantly larger (p<0.0001) than the L birds (Table 6). The C and H males were both significantly larger than the L males, with the females following the same pattern (Table 7). 14- and 21-day weights revealed similar results; the H group weights were significantly larger than the L group with the C group being in the middle (Table 6). At day 21, there were no significant differences between the male weights of each group. However, the C females were significantly heavier than the L females (p<0.001) (Table 7).

**DISCUSSION**

A number of studies have demonstrated a positive causal relationship between a female’s physical condition and the sex ratio of her offspring in a number of avian species (Howe, 1977; Kalmbach, et al., 2005; Love, et al., 2005; Nager, et al., 1999; Parker, 2002; Pike & Petrie, 2005; Pryke & Rollins, 2012; Trivers & Willard, 1973). However, our results are not indicative of such a phenomenon. Hens raised from hatch on diets with either high or low metabolizable energy and crude protein concentrations did not skew the sex ratio of their offspring to any appreciable degree. Although, there were significant changes in the sex ratio of offspring from birds that had experienced a change in diet at the onset of lay. Our experimental design was novel in that we raised our parent stock on experimental diets differing in nutritional content; all of the studies previously mentioned had been conducted with birds subsisting in wild populations or raised under standardized nutritional protocols. As such, it may be that the hen’s physiological response to an abrupt change in resources, or
her perception of a change, rather than her actual body is the causative trigger that leads to a bias of the sex ratio of her offspring.

As expected, the offspring from parents raised on the low nutrient diet had the highest blood corticosterone levels at hatch, indicative of the higher deposition in the yolks of their eggs. This is in concordance with the findings of Schoech et al., 1997 and Pike & Petrie, (2006) who reported that birds will respond to stressful events, like changes in diet, via the production of hormones, particularly the stress hormone corticosterone (Pike & Petrie, 2003). There is evidence that corticosterone inhibits the production of testosterone (Wingfield et al., 1994). It was also shown that acute application of testosterone several hours prior to ovulation in domestic chickens can skew the resulting sex ratio of offspring toward males (Pinson et al., 2011b). As such, the inhibition of testosterone production via the action of chronically increased corticosterone levels in stressed hens may be a possible explanation for the skewing of the sex ratio toward females in poultry (Pike & Petrie, 2006). However, Pinson et al.(2011b), showed that acute application of corticosterone in domestic chickens, though far exceeding physiological capabilities, stimulates the production of more male offspring (2011a). Our results also indicate that high corticosterone levels, as seen in the offspring from the parents raised on the low-nutrient diet, may not necessarily cause any change in sex ratio. Again, this suggests that it is more likely a change in hens’ circulating hormone concentrations, perhaps in response to changing environmental conditions, that stimulates changes in offspring sex ratio, rather than the concentrations themselves. The delay in egg production in the hens on the low nutrient diet appears to be similar to research from broiler breeders (Joseph et al., 2000). Walsh and Brake (1997) reported that if
pullets do not meet the recommended threshold of energy and protein intake prior to photo stimulation, they will have a delayed onset of lay and can have decreased rates of lay once in production. Conversely, the hens that were over-conditioned did not lay as well as the control birds. However these birds did come into lay slightly earlier. This same trend has also been reported in the broiler breeder industry and is one of the reasons for the implementation of feed restriction programs in broiler breeders. More trials focused on examining how much of an increase in diet nutrient levels as well as how long those levels need to be sustained to stimulate a sex ratio bias will need to be conducted, and it must be determined if the sacrifice in egg production at the beginning of the laying period would justify the increase in female chicks produced.

In offspring of parents on the low diet, initial hatch weight was the lowest of all birds and weight gain was decreased, suggesting a link in total nutritional deposition in the egg and the resultant size and growth pattern of offspring. Both male and female offspring of parents on the low diet hatched smaller than all other chicks, but did maintain a comparable rate of growth. There did not appear to be a negative effect on growth rate associated with smaller egg size or lower nutrient contents, indicating that the poor nutritional status of parents does not adversely affect offspring in any way other than initial size. Chicks of parents on the high diet were the largest of all treatment groups for both sexes at hatch. However, by three weeks of age, high diet offspring had been overtaken in size by control offspring and were more comparable in weight to low diet offspring. Based on these data, it appears that the larger egg size and increased nutrient deposition within the yolk of eggs from high diet parents was initially beneficial to their offspring; however, their slower rate of growth
compared to control offspring on the same diet suggests a physiological disparity. There may be an epigenetic change occurring as a result of higher nutrient intake and obesity in the parental generation predisposing offspring to slower growth.

The presence of nutritionally-linked phenomenon for the manipulation of offspring sex ratio in domestic fowl may not facilitate the implementation of nutritional or managerial changes that would need to be made on farm production facilities in order to attain results as seen in the laboratory setting. Practical use of our findings is heavily dependent on the ease of integration of new practices into the current production system at the breeder level and economic feasibility. Further trials are needed to justify industry integration.

**CONCLUSION**

Continual access to either a high quality or low quality diet from hatch to sexual maturity does not seem to have an effect on the resultant sex ratio of offspring in W-36 layers. There may be a mechanism of sex ratio adjustment associated with the sudden change of diet type at the onset of sexual maturity, perhaps hormone-linked; further study is required to confirm this hypothesis. Parents on low-protein and energy diets have offspring with significantly smaller hatch weights, but the differences between these offspring and those of parents on high-protein and energy diets becomes indiscernible within a few weeks of hatching.
REFERENCES


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<th>Control</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
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<td>743.52</td>
<td>830.49</td>
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<td>Oats</td>
<td>250</td>
<td>500</td>
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<td>84.31</td>
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<td>529.27</td>
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**Formulated Percentages**

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<tr>
<td>Crude Protein</td>
<td>18.0%</td>
<td>12%</td>
<td>24%</td>
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<td>Crude Fat</td>
<td>10.0%</td>
<td>2.99%</td>
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<td>Crude Fiber</td>
<td>4.4%</td>
<td>10.46%</td>
<td>1.69%</td>
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<td>Calcium</td>
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<td>4%</td>
<td>4%</td>
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<tr>
<td>Metabolizable Energy</td>
<td>1360 kcal/lb</td>
<td>1000 kcal/lb</td>
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**Analysis (actual percentages)**

<p>| | | | |</p>
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<th></th>
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<td>Crude Protien</td>
<td>18.60%</td>
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<tr>
<td>Treatment</td>
<td>% Male</td>
<td>SE ±</td>
<td>% Female</td>
</tr>
<tr>
<td>-------------------</td>
<td>---------</td>
<td>------</td>
<td>----------</td>
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<tr>
<td>Low</td>
<td>60.81\textsuperscript{A}</td>
<td>9.34</td>
<td>39.19\textsuperscript{A}</td>
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<tr>
<td>Control</td>
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<td>48.90\textsuperscript{A}</td>
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<td>Control (Low)</td>
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<td>35.00\textsuperscript{A}</td>
<td>9.34</td>
<td>65.00\textsuperscript{A}</td>
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Percentages with differing \textsuperscript{A,B,C} with in column denote significant difference at p ≤ 0.05
Table 3. Percent Hatched Offspring by Treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Male</th>
<th>SE ±</th>
<th>% Female</th>
<th>SE ±</th>
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</thead>
<tbody>
<tr>
<td>Low</td>
<td>47.78&lt;sup&gt;B&lt;/sup&gt;</td>
<td>3.24</td>
<td>52.22&lt;sup&gt;B&lt;/sup&gt;</td>
<td>3.24</td>
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<tr>
<td>High</td>
<td>51.01&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>3.24</td>
<td>48.99&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>3.24</td>
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<tr>
<td>Control</td>
<td>47.25&lt;sup&gt;B&lt;/sup&gt;</td>
<td>3.24</td>
<td>52.75&lt;sup&gt;B&lt;/sup&gt;</td>
<td>3.24</td>
</tr>
<tr>
<td>Control (Low)</td>
<td>58.51&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.24</td>
<td>41.49&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.24</td>
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<tr>
<td>Control (High)</td>
<td>43.80&lt;sup&gt;B&lt;/sup&gt;</td>
<td>3.24</td>
<td>56.20&lt;sup&gt;B&lt;/sup&gt;</td>
<td>3.24</td>
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Percentages with differing <sup>A,B,C</sup> with in column denote significant difference at p≤0.05
Table 4. Percent Total Offspring by Treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Male</th>
<th>SE ±</th>
<th>% Female</th>
<th>SE ±</th>
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<tbody>
<tr>
<td>Low</td>
<td>54.30</td>
<td>5.21</td>
<td>45.70</td>
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<td>55.10</td>
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<td>Control (Low)</td>
<td>52.03</td>
<td>5.21</td>
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<td>Control (High)</td>
<td>42.27</td>
<td>5.21</td>
<td>57.73</td>
<td>5.21</td>
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Percentages with differing A, B, C with in column denote significant difference at p ≤ 0.05.
Table 5. Mean Offspring Blood Corticosterone Concentrations at Hatch

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (ng/mL)</th>
<th>SE ±</th>
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<tr>
<td>Low</td>
<td>51.23^A</td>
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<tr>
<td>Control</td>
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<td>High</td>
<td>36.28^AB</td>
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Concentrations with differing superscripts within column denote significant difference at p ≤ 0.01.
Figure 1. Parent Stock Percent Egg Production 21-26 Weeks.
Table 6. Total Mean Body Weights for Offspring by Treatment

<table>
<thead>
<tr>
<th></th>
<th>Day 0 wt (g)</th>
<th>Day 7 wt (g)</th>
<th>Day 14 wt (g)</th>
<th>Day 21 wt (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>40.04 &lt;sup&gt;B&lt;/sup&gt;</td>
<td>63.97 &lt;sup&gt;B&lt;/sup&gt;</td>
<td>122.58 &lt;sup&gt;B&lt;/sup&gt;</td>
<td>176.20 &lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>40.54 &lt;sup&gt;AB&lt;/sup&gt;</td>
<td>68.39 &lt;sup&gt;A&lt;/sup&gt;</td>
<td>128.55 &lt;sup&gt;AB&lt;/sup&gt;</td>
<td>187.84 &lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>High</td>
<td>40.98 &lt;sup&gt;A&lt;/sup&gt;</td>
<td>67.78 &lt;sup&gt;A&lt;/sup&gt;</td>
<td>129.71 &lt;sup&gt;A&lt;/sup&gt;</td>
<td>183.13 &lt;sup&gt;AB&lt;/sup&gt;</td>
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Weights with differing <sup>A,B,C</sup> within column denote significant difference at p≤0.01.
<table>
<thead>
<tr>
<th>Treatment</th>
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<th>Day 0 wt (g)</th>
<th>Day 7 wt (g)</th>
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<th>Day 21 wt (g)</th>
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<tbody>
<tr>
<td>Low Female</td>
<td>Female</td>
<td>39.84&lt;sup&gt;C&lt;/sup&gt;</td>
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<tr>
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<td>Female</td>
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<td>66.33&lt;sup&gt;B&lt;/sup&gt;</td>
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<tr>
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<td>133.89&lt;sup&gt;A&lt;/sup&gt;</td>
<td>201.19&lt;sup&gt;A&lt;/sup&gt;</td>
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<td>Female</td>
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<tr>
<td>High Male</td>
<td>Male</td>
<td>40.93&lt;sup&gt;AB&lt;/sup&gt;C</td>
<td>69.69&lt;sup&gt;A&lt;/sup&gt;</td>
<td>133.39&lt;sup&gt;A&lt;/sup&gt;</td>
<td>195.2&lt;sup&gt;A&lt;/sup&gt;</td>
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</table>

Weights with differing <sup>A,B,C</sup> within column denote significant difference at p<0.01.
PROJECT CONCLUSIONS

This research has explored multiple different methods of early life manipulations (hypoxia, nutritional programming, immediate access to nutrition at hatch, dietary manipulation, and alterations in sanitation practices) as well including a very broad spectrum of avian species (Emu, Broilers, Layers, and Turkeys). The data presented from this research includes both molecular gene expression data from projects that have been more actively researched, as well as more applied production research from projects that are just beginning to be explored at the molecular level. At first glance this research seems fairly unrelated; however, it is very evident that the conditions an organism is exposed to during embryonic development and within the early portions of its life greatly impact the phenotypic development of the organism. There is very limited data available that provides a clear understanding of exactly how the environment and diet affect the organism at a molecular level. There have been many recent advances in molecular biology that has created the field of “nutrigenomics”. This new field of nutrigenomics examines how different diets or nutrients effects the up or down regulation of certain genes within the organism. There is growing evidence that suggests certain environmental factors can “turn on” expression of certain genes, that can either result in susceptibility or resistance to wide range of diseases such as heart disease or obesity. This concept is new to many people, the common assumption of genetics is that the gene is either present or absent, so it will either be expressed or not expressed. This assumption is true a portion of the time, however, this rule does not always hold true. There are many genes present in organisms that are regulated by a whole host of mechanism including environmental conditions, diet compositions etc. Finding a more complete
understanding of the pathways that are either turned on or off by certain factors or diets is required, in order to gain a further understanding of how the diets can be formulated/manipulated to cause the bird to perform at more optimal levels.

**Calcium and Phosphorus**

There are several benefits to the aforementioned research, with the primary advantage being that all manipulations performed are inexpensive to implement and can yield major gains in growth, performance, livability, and efficiency of the bird. Since production agriculture and especially the poultry industry are operated on such an unfathomably large scale, even minute changes in growth, performance, or feed efficiency has the potential of saving millions of dollars. Even though making money is the bottom line for the companies, this research demonstrates that by conditioning poultry with in the early portion of their life, it is possible to “program” the birds to utilize nutrients such as Ca and P more efficiently; similar results were also reported by Ashwell and Angel (2010). A potentially valuable tool for commercial agricultural production, since the recent increased concern from consumers in regards to the high amounts of phosphorus produced from these operations. There have been numerous reports of elevated levels of phosphorus in run-off water from application of animal waste on pastures. Many environmentalists express great concerns about the potential eutrophication that could ensue as a result of the high levels of phosphorus. If a detailed method of nutritional programming can be developed, the benefits, both environmentally and monetary would be immense.
Ensuring future production of safe, affordable, and healthy food is the goal of many commercial agricultural companies. In animal production agriculture there is a critical need for antimicrobial products that are safe, effective and affordable to the food industry as well as acceptable for food production use by the consumer. Many disinfectants and sanitizers do an adequate job at killing bacteria; unfortunately they can be dangerous to humans as carcinogens or residuals in the environment. Poultry and swine producers in the near term will likely have limited options for antimicrobial intervention due to societal concern regarding the use of certain disinfectants and pharmaceuticals. Currently there are very limited options for approved products that are safe, effective, affordable, versatile, and capable of being utilized throughout most of the poultry industry that meet societal criteria. Bac-D™ appears to be a very versatile sanitation product, with applications ranging from a human skin antiseptic, to a potentially efficient method of sanitizing hatching eggs. The data reported in this paper with regards to Bac-D are more applied currently. They served as the preliminary applied type data since the data is primarily production data. Now that Bac-D has shown promising results in regards to decreases in bacterial contamination, feed conversion ratios, and growth rates more trials are underway to determine the actual molecular mechanism that are responsible for the changes that are occurring in the birds. The trial that are being conducted are examining the effects that Bac-D™ has on gut microflora, intestinal histology, as well as the expression of numerous genes with in the gut.

**Bac-D**
Sex Ratio

The idea of skewing sex ratios of birds by maternal dietary manipulation is a very interesting phenomenon. Even though a slight skewing was observed in our trial, the actual mechanism responsible is still unknown. Based on our data and previous studies in various types of wild birds, the causative mechanism appears to involve stress hormones, much work has attributed this skewing to increases in corticosterone due to changes in nutrient availability. This research demonstrates that there is some skewing in the sex ratio of the offspring based upon different diets. The next step in this research needs to be aimed at determining what molecular changes are occurring as a result of changing the diets. If corticosterone is the responsible agent, a more in depth understanding of which genes are either up or down regulated when in the presence of increased levels of corticosterone will be required. Yet, much research is still required before a definitive mechanism is discovered. Finding a method to alter sex ratios could be of great benefit to the layer industry. Male layers from the commercial lines are of no benefit to the layer industry. In fact the extra male chicks have unfortunately drawn lots of unwelcomed attention to the poultry industry since many consumers do not like the idea of just killing the extra males at hatch. Not to mention that it is a huge burden to automatically kill half of your hatch at day 0. If an efficient method of corticosterone administration or an appropriate feeding regimen that maximizes the production of the desired sex, producers would not have to keep as many parent stock breeders to produce the amount of female chicks needed. This could result in unfathomable gains for the layer industry.
Dietary effects on male reproductive performance

Reproduction in poultry has been studied for many years, and is a crucial factor to consider when selecting and raising parental lines. Considerable work has focused on broiler breeder males, which has led to the development of feed restriction practices, as well as sex separate rearing, however very little research as has been done on layer-type males. In broiler breeders a negative correlation has been found to exist between the body weight (BW) and sexual activity of the male. To explore the role of diet during grow out on reproductive traits in leghorn type males. Nutrition has long been known to be a crucial factor in the development of any organism. Genetics are a major deciding factor in the developmental potential of an organism. However, regardless of the quality of genetics an animal possesses, if they do not receive sufficient nutrition throughout the growing period or during the reproductive period the animals will never be able to reach their full genetic potential.

Broiler breeders reared on low protein diets have been shown to exhibit decreased fertility as compared to broiler breeder females that were reared on higher levels of CP during grow-out. It was suggested that the low CP group did not receive an adequate amount of CP during growing, thus not allowing for optimum development of the oviduct nor the spermatozoal storage ducts yielding decreases in fertility. Low CP diets during rearing have been shown to greatly influence the growth rate of broiler breeders. Low CP diets produce males with lower body weights than males reared on diets that contain a higher CP content.

The data collected from the current trial suggests that that feeding high nutrient feeds to layer type males during rearing does not result in a decrease in reproductive performance as
observed in broiler breeder males, and that low nutrient feed are not as beneficial in layer males as in broiler breeder males. This data is valuable since there have been some issues with fertility in layer type males. Even though this data is suggestive that higher nutrient feeds may be of benefit to the reproductive performance of layer males, additional research is required to further validate the claim, an ideal test of this research would be to implement this feeding regimen under commercial settings and see if the results are similar.

**Hypoxia**

There have been tremendous advances in genetic selection in today’s current broiler lines, so much so that these birds can have difficulty providing enough oxygen to the tissues to keep up with such rapid growth. There is much evidence including this research, that suggest hypoxic incubation of eggs during certain points during incubation of the eggs causes significant changes in blood oxygen holding and carrying capacity. These simple embryonic changes, once perfected, could potentially allow for major increases in growth and performance of the birds. There are trials underway examining the molecular effects that hypoxic incubation has on the organism. The data collected from the trial described only looked at physical weights and physiological changes in blood gas parameters, however further research is needed to understand what genes are being differentially regulated that cause the changes that are being observed in the birds.

**Emu**

Emu are very expensive birds, and there has been very little research focused on how to efficiently produce them due to the lack of commercial production. However with the increase in sales of emu oil due to cosmetics application, the antibacterial, and the anti-
inflammatory properties associated with emu oil, there seems to be a growing demand for emu. This increase in demand requires more research be aimed at emu production in order for efficient production of emu to fulfill the new demand. The two trials reported in this manuscript address ways to increase early growth rates in emu, and decrease the typical weight loss associated within the first week of life, as well as provide an actual study conducted in the commercial sector that addresses the fictitious rumors of extremely long term storage of emu eggs. There were no molecular aspects to either of the emu trial, the emu industry is still very small in comparison to other poultry industries. Before molecular work is carried out on emu there needs to be much more research focusing on the more applied areas of emu production such as feed conversion, growth rates, optimal CP and ME diet formulation. Once the emu industry has a more scientific understanding of the basic aspects of emu production, then a more fine-tuned “tweaking” of the diets and environments can be implemented.

This research however, is only a demonstration of just how crucial the developmental conditions are for avians, and that making small alterations in them result in dramatic phenotypic changes. Before these principals becomes applicable to the commercial industry, there is still much research required at fine tuning and perfecting the alterations to maximize the specific species and production system of the company. But once the correct alterations have been made to the procedures, these methods could be of great importance and benefit for all aspects of poultry production.