ABSTRACT

MALHEIROS, DIMITRI MORAES. Influence of Laying Hen Stress Due to Differing Cage Densities and Saponin Phytobiotic Impacts on Performance and Egg Quality (Under the direction of Dr. Kenneth E. Anderson).

The effects of cage density documented on laying hens have been in multiple peer reviewed articles in the past where the majority of research points to a decrease in egg production, egg weight, feed intake, and an increase in mortality due to higher stocking densities. However, studies have contradicting findings when it comes to production parameters and higher stocking densities. The experimental design of this trial follows a 3X2 factorial arrangement, where each of the three rearing densities were then subsequently divided between two lay densities. During the pullet phase the highest stocking density pullets (310 cm^2) achieved higher body weights while consuming less feed. These better performance parameters had a fearfulness score trade off, where tonic immobility (TI) times were significantly increased in 310 cm^2 reared pullets. In the lay period, some differences in body composition, jejunum histomorphology, and blood biochemistry were observed, with a possible explanation is that these differences are due to resource competition. It was observed that the highest pullet stocking density of 310 cm^2 resulted in the highest values for hen day %, hen housed %, feed consumed, and egg production, which all point to better production as a whole. When looking at the performance interactions, it is apparent that higher stocking densities during the pullet phase resulted in the carryover effects of increased production parameters compared to hens reared in the lowest stocking densities.

Some minor differences were found in vitelline strength and egg weights attributed to lay period stocking density. When looking at the proportion of XL and S eggs, it is apparent that 445 cm^2 stocking density hens had significantly higher proportion of eggs in these categories

compared to hens housed at 889 cm^2 stocking density. Interestingly, 889 cm^2 hens had the highest proportion of eggs in the L and M size categories compared to 445 cm^2 hens. When looking at the size distribution it can be concluded that the more intense stocking density during the lay phase produced more eggs in the extreme sizes of XL and S, while hens in the lower stocking density produced eggs in the middle sizes categories of L and M.

One of the most prevalent poultry diseases is caused by *Eimeria* protozoan parasite resulting in an enteric disease infection called coccidiosis. In this current experiment, saponins were sourced from Quillaja Saponaria, to reduce coccidia oocyst production. The grow period consisted of two feeding programs, a Control (C) and Magni-Phi (MP) treatments. In the lay cycle, the grow treatments were divided to compose the four lay treatments. The hens remained unchallenged in this trial, with only exposure to the research station's facilities natural biologicals which did not include coccidia. Overall, this study explores the potential supplementation of Magni-Phi in the poultry industry as it offers a possible reduction of rearing feed costs with comparable pullet development, while in the laying phase maintaining comparable egg yields compared to the control, with an improvement in physical quality indicating an economically viable utilization even in healthy laying hens during production.

Influence of Laying Hen Stress Due to Differing Cage Densities and Saponin Phytobiotic Impacts on Performance and Egg Quality

by
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DEDICATION

I would like to dedicate this dissertation to my wife Paige, parents, and all of my friends who have helped me along the way.

BIOGRAPHY

Dimitri Moraes Malheiros, born on December 26th, 1996, in Ribeirão Preto SP, Brazil, is a dedicated researcher in the field of Poultry Science. He is the son of Dr. Ramon Malheiros and Dr. Vera Moraes, who are both figures in poultry research and academia, and who instilled in him the principles of hard work, kindness, and good nature from an early age.

From early in life, Dimitri was immersed in diverse cultural experiences, living abroad with his family. Dimitri spent much of his early childhood in Brazil, with some time in Belgium, before eventually settling in the United States at the age of 12. These international transitions not only broadened his worldview but also laid the foundation for his adaptable and multicultural perspective, which would later enrich his academic pursuits.

At North Carolina State University, Dimitri embarked on a trajectory of academic excellence, undertaking both a Bachelor's and Master's degree in Poultry Science. Throughout this period, he immersed himself in various facets of poultry management, health, nutrition, and physiology, honing his practical skills and theoretical knowledge under the mentorship of esteemed faculty.

Pulling from his experience and understanding of the poultry industry garnered from his preceding degrees, Dimitri pursued a PhD in Poultry Science, focusing his research on laying hen performance and egg quality. Under the guidance of Dr. Kenneth E. Anderson, his research endeavors have contributed to advancing the understanding of critical factors influencing poultry productivity and welfare.

Beyond his scholarly pursuits, Dimitri has demonstrated a commitment to academic leadership and community engagement. His active involvement in the Graduate Student

Association (GSA), where he served on the board for two years and held the esteemed position of GSA president for the poultry science department, underscores his dedication to fostering a supportive and inclusive academic and work environment.

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I would like to thank Dr. Kenneth E. Anderson for his great mentorship during my whole PhD program, I would not have been able to do it without him. Dr. Anderson has taught me a lot about laying hens, but more importantly he has taught me what a good leader is. For that I thank you!

Secondly, I would like to thank my wife, Paige. We got married halfway through my PhD program in the most hectic semester where I had live animal trials, was TA'ing a class, and had my prelims… I would not have had it any other way! Paige, you are my muse and role model, I love you more than you will ever know.

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CHAPTER 1

Literature Review

WHAT IS STRESS?

Vertebrate animals have developed sophisticated pathways for endocrinological mechanisms, which plays a pivotal role in the survivability of all animals. Prey animals have specifically fine-tuned these stress responses in order to face survival obstacles one of which is predation. This evolutionary adaptation cascades for the release of glucocorticoids, which can lead to elevation in blood glucose, increased heart rate, and increase in respiration rates (Vinterstare et al., 2021). All of these physiological changes happen in preparation of the "fight or flight" response within the animal, which is essential for predator evasion in the wild (Romero, 2004; Scanes, 2015; Vinterstare et al., 2021).

Conventional definition of stress in society associates the word with a negative event, which has detrimental effects on health. Whereas, in animals, even small and benign changes to the environment can trigger a stress response (Romero, 2004). This evolutionary stress mechanism has played a crucial role in adaptation to changing environments, as it aids in keeping the animals alert in new environment with potential new and unknown predators.

History of stress

Hans Selye is regarded as the father of stress, as his definition of stress has been used and expanded upon over the years. Although he was not the first to use this word in a biological context, in 1936 Selye was the first to define and introduce the word "stress" to the general public, which caused the definition to be known around the world (Bienertova-Vasku et al., 2020; Selye, 1936).

In 1976 Selye expanded upon his definition of stress, as not all stress elicits the same responses by the organism. In this body of work, Selye coins the terms distress and eustress, denoting negative and positive resistances of these responses (Selye, 1976). This dichotomy between distress and eustress can be contrasted by differentiating the responses by the body. For example, any pathogen or agent that elicits a response by the immune system can be categorized as distress, while the body's reaction to activities that leads to improvements upon itself, like physical labor, can be categorized as eustress (Bienertova-Vasku et al., 2020; Selye, 1976). However, this definition has been challenged by some researchers, as they think that the term eustress should be simply replaced by the term "stress" (Bienertova-Vasku et al., 2020). These researchers have concluded that eustress can be categorized as an adaptation reaction, where the organism faces a challenge and is then adapting to that demand by biological means. Furthermore, the characterization of distress having negative connotations and eustress as having positive connotations do not agree with the views of some professionals (Bienertova-Vasku et al., 2020); as the adaptation reaction is neither positive or negative, just an evolutionary means by which animals are able to adapt and change their livability in new environment challenges.

Selye himself acknowledged the important role that stress has on our lives, and details that "Total elimination of stress is impossible, even during short periods, because that would involve cessation of demands made upon any part of the body, including the cardiovascular, respiratory, and nervous systems. This would be equivalent to death" (Selye, 1976). The thought that no stress equals death is an interesting point to ponder upon, as doing so would negate all of our evolutionary advancements and forsake millions of years of evolution, as the body is always in a state of alertness to keep the organism in a living state. Furthermore, the stark idea

connecting the intrinsic connection between stress and life itself puts stress as an integral part of the animals' physiological functions and response to its environment.

Acute vs Chronic Stress

Stress in animals can manifest in two distinct and differing modes: acute and chronic. Acute stress can be characterized as a brief perturbation which lasts seconds or minutes, triggering rapid physiological responses by the body in order to aid in immediate action (Romero, 2004; Scanes, 2015). These rapid responses to an acute stressor usually result in no detrimental effects to the animals, and normal animal behavior can resume within minutes to hours after the acute stressor has ended (Scanes, 2015). In contrast, chronic stressors occur over extended periods, which can last hours or days. These chronic stressors can impose high energy demands and can disrupt the normal life cycle of the animal (Scanes, 2015). In the rest of this section, the key differences between acute and chronic stressors will be discussed, specifically on how laying hens experience and physiologically interpret these events. It is important to understand that, as mentioned above, even benign changes can be seen as a stressor event by the animal (Romero, 2004).

In the acute stress phase, the prey primal "fight or flight" instinct is triggered in order to aid in the animal's survival thorough rapid action (Romero, 2004; Scanes, 2015; Vinterstare et al., 2021). Acute stressors include attempts in predation, agonistic interactions, sudden loss of nest or offspring due to environmental factors, and brief human disruptions (Scanes, 2015). Through these events a cascade of responses happen, where acetylcholine is released, which triggers epinephrine release into the blood (Scanes, 2015). The release of epinephrine impacts the immediate response of the animal to the acute stressor, whereas corticosterone will be

released shortly after the initial “fight or flight” state. In this acute stress state, there are many rapid changes that take place. Some of these changes include increased heart rate, vasodilation of arterioles in skeletal muscle, vasoconstriction throughout the body, dilation of the pupil, piloerection, and mobilization of liver glycogen and free fatty acids (Scanes, 2015). Acute stressors are a common problem in commercial laying hen operations, which can affect their physiology and laying performance (Wang et al., 2017). During the life span of a layer, they are exposed to many stressors that are part of normal management practices. These practices include weighing, vaccination, moving to a different production house, sampling in research settings, etc. (Wang et al., 2017).

Chronic stressors can last hours or days and carry a high energy cost (Scanes, 2015). When an animal is experiencing a chronic stressor their normal life cycle is disrupted and many negative impacts to the body can be observed (Jalal et al., 2006; Romero, 2004; Scanes, 2015). Examples of chronic stressors include but are not restricted to: exposure to extreme weather, continued resource competition, increased predation potential, reduction in food resources, and prolonged human disturbances (Romero, 2004; Scanes, 2015). As an animal is exposed to this chronic stressor, their plasma corticosterone levels are elevated, in comparison to their base levels, and are maintained at those higher levels until the animal is no longer in that emergency state (Romero, 2004; Scanes, 2015). In the chronic stressed stage, the animals experience many physiological changes, like increased protein metabolism and gluconeogenesis, increase use of lipid energy from adipose tissues, decrease in body weights, and suppression of reproductive behaviors (Scanes, 2015).

Changes mediated by corticosterone show many improvements in the adaptiveness of an animal to a stressor, as the animal can adapt and improve its fitness at the cost of momentary

changes to its current life cycle (Scanes, 2015). As an animal becomes acclimated to the stressor, they don't respond to the stressor in the same manner. This acclimation to the stressor changes how it is perceived by the animal and the physiological responses are lessened, as now the animal doesn't perceive the stressor as severe (Romero, 2004). This acclimation to similar stressors can have many applications in animal production. One application that is widely used in the poultry industry is the acclimation process of heat stress in early life in order to improve livability and stress responses of subsequent heat stress events later in life (Madkour et al., 2022).

Measurements of stress

As animals are not able to communicate their stressed state in a reliable way, many assays have been extensively used in poultry research in order to quantify stress responses. One of these ways is to collect blood plasma and measure the levels of corticosterone as an indicator of acute stress in poultry (J. V. Craig & Craig, 1985; Davis et al., 2000; Gross & Siegel, 1983). As explained in the above section, as an animal encounters an acute stressor, the "fight or flight" response is activated. In this state, epinephrine is released first in order for the animal to immediately act and is shortly followed by the release of corticosterone (Scanes, 2015). It is at this stage that corticosterone levels can be collected as a means to measure the stress response. However, for this current body of work it is important to note that the cage density of birds has been shown to change the circulating corticosterone levels (Davis et al., 2000; Koelkebeck et al., 1987; Mashaly et al., 1984). It has also been elucidated that more intense stocking densities have been shown to increase corticosterone levels compared to lower stocking densities (Davis et al., 2000; Mashaly et al., 1984). In a study conducted with laying hens it was observed that caged

birds with five hens per 240 in^2 cage had higher serum corticosterone compared to hens with three or four birds per cage (Mashaly et al., 1984).

The heterophil to lymphocyte ratio (H:L) has been used as a valuable measure of chronic stress responses in poultry (Davis et al., 2000; Gross & Siegel, 1983; Siegel, 1980). Previous research has concluded that the decrease of lymphocytes and an increase in heterophil counts occurs in response to stress (Lentfer et al., 2015). (Meaning that a higher H:L ratio can lead to conclusions that the bird is more stressed compared to lower H:L ratios). Researchers (Gross & Siegel, 1983) have also stated that H:L ratios are a more reliable measure of chronic stress in poultry, compared to corticosterone measurements, as it detects physiological changes in the body whereas corticosterone in the blood measures before that physiological change can happen.

STOCKING DENSITY

Caged laying hen environments have drastically evolved since its start in the 1920's, where a drastic movement towards large scale production were emphasized in order to meet increased demands. Caged production system for laying hens has established itself as the standard commercial practice due to its cost effectiveness and high yielding hen production. Caged environments for poultry kept for longer periods of time, like in the case of laying hens, were thought to not be suitable as researchers saw many problems with rickets (Kidd & Anderson, 2019). This condition was caused by vitamin D deficiency, where calcium and phosphorus formation into bones is dependent upon. The discovery that vitamin D from cod liver could be fed to hens in the feed allowed for hens to be kept for longer periods in caged environments (Kidd & Anderson, 2019). By the 1940's about 20% of hens in the United States were kept in single hen cages, which was made possible through adoption of newer technologies

in cage building and feed lines (Hartman, 1950; Kidd & Anderson, 2019). The caged environment served as a great advancement for the industry, as there were many advantages of caged systems over floor rearing. These advantages included less labor for the caretakers, complete control of feeding (resulting in more uniform egg production), separation of feed and feces, no cannibalism (as at this point hens were still one bird per cage), ease of removal of low producing hens, and overall cleaner eggs (Kidd & Anderson, 2019). In contrast, there were some disadvantages of these new caged environments, as there was a high initial investment into these systems, fly breeding issues under caged if not kept dry, and occasional dirty eggs due to high humidity and feces sticking to the cages (Kidd & Anderson, 2019).

Research conducted in the 1960's-1970's related to caging systems allowed for the development of tiered systems in a stair stepped fashion (Kidd & Anderson, 2019). Furthermore, development of automation of egg collection and excreta pits allowed the tiered systems to evolve from a few tiers to eight or more (Kidd & Anderson, 2019). In the decades that followed, research and development into cage dimensions, hens per cage, ventilation, and manure belts have allowed for further production in the layer industry (Kidd & Anderson, 2019).

It is apparent that vast improvements in hen development, production, and mortality can be observed in just 50 years of advancement. In the 1950's, the age that a hen would have to reach in order to attain 50% egg production would be 182.9 days, where in the 2000's that age would be 138.8 days (Kidd & Anderson, 2019). This decreased maturation age of almost 45 days allowed for faster egg production from a flock and reduction of their feed costs. Hen day egg production was also greatly impacted, as there was about a 25% increase in egg production between the 1950's and the 2000's (56.9% & 82% respectively) (D. R. Jones et al., 2001; Kidd & Anderson, 2019). During the fifty years of advancement there was also a significant 6.8%

decrease on total mortality in the flocks (D. R. Jones et al., 2001; Kidd & Anderson, 2019). In the following decades and into the present there has been further developments that have allowed for better hen efficiency, industry advancement, and product diversification, which have allowed for better production for a growing world with continued increase in demands for nutrition.

Laying hen Cage Density Research

The effects of cage density in laying hens have been documented in numerous peer reviewed research articles in the past (K. E. Anderson & Adams, 1992; M. G. Anderson et al., 2021; J. Craig & Milliken, 1989; Davami et al., 1987; Hester & Wilson, 1986; Jahanian & Mirfendereski, 2015; Jalal et al., 2006; Saki et al., 2012), where the majority of research indicated that a decrease in egg production, egg weight, feed intake, and an increase in mortality occurs due to higher stocking densities. Cage density studies are usually manipulated by changing the number of hens per fixed cage space, not by manipulation of cage dimensions. Furthermore, studies referring to high stocking densities mean that more hens are housed per cage, as to say, less space per hen. The United Egg Producers (UEP) published guidelines for caged layers in 2002, that delineated space requirements per hen. With the newest update of these guidelines published in 2017, they have become a standard practice in laying hen production in the United States. The UEP has put forth that conventional cage requirements for hens is 67-86in^2 (432 cm^2-555 cm^2) per hen (*United Egg Producers*, n.d.). Literature prior to the UEP guidelines (2002) have explored stocking densities higher than 432 cm^2 (67in^2), where it has been determined that less than 432 cm^2 per hen can result in detrimental impacts on laying hen performance.

In a study conducted by Saki et al. (2012), it was concluded that feed intake was affected due to cage densities between 1 bird per cage (2000 cm^2 per bird) and 4 birds per cage (500 cm^2 per bird). It was determined that these differences were due to the higher cage density related to higher competition for drinker and feeder space and due to lower activity with less space per bird (Saki et al., 2012). Furthermore, Saki et al. (2012) found that hens in cages of 667 cm^2 and 500 cm^2 per hen had higher FCR (g/doz eggs) compared to hens in 1000 cm^2 and 2000 cm^2 per hen. They found that hens reared in the two lower densities (1000 cm^2 and 2000 cm^2) had a higher hen day egg production compared to the higher density treatments (667 cm^2 and 500 cm^2). In other studies it was observed that hens in cage densities of 348 cm^2, 464 cm^2, and 580 cm^2 (high, medium, and low densities; respectively) had impacts on production parameters of laying hens, where high density birds showed adverse effects in hen day production, egg weights, and mortality compared to medium and low densities (J. Craig & Milliken, 1989). Moreover, Jalal et al. (2006) found that feed intake was higher in hens in lower densities (690 cm^2 per bird) compared to higher densities (342 cm^2 per bird), and lower egg production was observed in the higher density compared to the low density by 10.1%.

In contrast, some studies have contradicting findings when it comes to production parameters and higher stocking densities. In a study conducted by Brake & Peebles (1992), no density effects were observed in hens housed in 344 cm^2, 516 cm^2, and 1032 cm^2 on egg weight differences. Additionally consistent effects on feed consumption were not attained in the two trials conducted, where in trial 1, 344 cm^2 reared hens had lower feed consumption than 516 cm^2 hens and in trial 2, 1032 cm^2 hens had higher feed consumption compared to 344 cm^2 hens, with 516 cm^2 hens being intermediate (Brake & Peebles, 1992). However, some studies have found that pullet density does not have a great impact on 18 week performance parameters. In this

instance, researchers have found that pullets reared in 221 cm^2, 249 cm^2, 277 cm^2, and 304 cm^2 showed no statistical differences in body weight uniformity, feed consumption, mortality, and fearfulness (K. E. Anderson & Adams, 1992). With some research pointing to detrimental impacts of higher stocking densities in the lay phase, while some research points to no impacts of cage densities in the pullet phase, it is clear that more research on the effects on rearing densities and its impacts on laying density needs to be conducted to elucidate on this carry over effect.

Animal welfare and consumer perceptions

In the past few decades, the poultry industry has received more attention from consumers as an increase in scrutiny regarding animal welfare has been more prevalent. While laying hen caged production is still the most popular housing system in use, the industry has faced pressure to reevaluate housing conditions primarily from a welfare standpoint. The interplay between production demands and welfare concerns is a hard issue to tackle, as these two sides seem to be inverses of each other. More research into these systems and into welfare needs to be done in order for the middle ground to be achieved. Furthermore, consumer awareness of how agriculture as a whole, and more specifically how animal production occurs, need to be elucidated. Special interest groups geared towards animal rights have taken an active role in tarnishing the reputation of animal production, and some of the population have taken that point of view, which in turn has had great impacts in the trajectory of how animal production in the modern world occurs.

Fearfulness Scoring

Studies have concluded that as cage density increases, the base corticosterone levels in the blood also increases (Davami et al., 1987; Mashaly et al., 1984; Onbaşılar & Aksoy, 2005). A previous section details the reasoning behind why the sampling of corticosterone alone is not a useful tool to measure chronic stress in laying hens, while a better parameter can be collected in conjunction. The use of heterophil to lymphocyte ratio (H:L) has been used as a valuable measure of chronic stress responses in poultry, as it detects physiological changes in the body whereas corticosterone in the blood measures before that physiological change can happen.

In a section above, it was elucidated that prey animals have specifically fine-tuned stress responses in order to face survival obstacles like predation. Tonic Immobility (TI) is characterized by motor inhibition, low responsiveness to stimuli, and suppression of the righting response (R. B. Jones, 1986). TI is generally regarded as a fear response as procedures to induce fear responses prolong the TI period, while fear reducing tactics tend to reduce TI periods (R. B. Jones, 1986). Tonic immobility responses have long been regarded as an anti-predator response, as cessation of movement or crouching can reduce detection by a predator, where flight can lead to escape measures if a predator approaches (R. B. Jones, 1986). Furthermore, TI may also take part after the predator has made contact with its prey, as a lack of movement may reduce stimulation for further attacks by the predator and ultimately can lead to loss of interest in its prey (R. B. Jones, 1986).

In addition to collection of physiological changes of poultry like H:L and corticosterone due to stress, observations on the birds fearfulness scoring can also expand our understanding of the animal's condition and deepen the understanding between the interplay of stress and fearfulness. Differing cage densities and housing systems effect on TI scoring has been

researched in the past few decades, where floor reared leghorn hens had longer TI times when compared to cage reared hens (K. E. Anderson & Adams, 1994).

Egg Quality Impacts

Hen stocking density impacts on egg quality is of great importance to the egg industry. Past research has looked at key egg quality factors and how they are affected by laying hen stocking densities. Egg quality traits usually encompass shell strength, shell color, Haugh unit, yolk color, egg component percentage, vitelline membrane strength, and dry egg matter percentage. A study comparing W-36 laying hen caged stocking densities of 360 cm^2 and 257 cm^2 per hen concluded no differences in egg quality parameters, like shell thickness, Haugh unit, and yolk color were observed at egg quality sampling points (Jahanian & Mirfendereski, 2015). Similarly, a study looking at egg quality traits of caged Lohmann-Brown hens in high, medium and low densities (552.3 cm^2, 736.3 cm^2, and 1104.5 cm^2 per hen, respectively) found no statistical differences between the egg quality parameters observed: Haugh unit, yolk height, yolk color, shell thickness, and egg component percentage (Erensoy et al., 2021). Whereas a study conducted with W-36 laying hens caged in four stocking densities (2000 cm^2, 1000 cm^2, 667 cm^2, and 500 cm^2 per hen) found significant differences in yolk color and higher egg surface area attributed to the lower densities compared to the highest stocking density (Saki et al., 2012). These results point to some variation of stocking density effects on egg quality parameters, as some research points no density impacts while some other studies point to differences being observed.

ENTERIC STRESS

Chronic stress in laying hens is known to induce general metabolic effects that can compromise hen fitness, but a direct link between stress and disease immunity has also been researched (Shini et al., 2010; Siegel, 1980). As an animal is stressed, corticosteroids are released, which are known to possess lymphocytic and anti-inflammatory suppression effects, which leads to a direct link between stress and immune responses in some disease challenges (Siegel, 1980). It is offered however, that as a hen is faced with an acute stressor the production of antibodies is increased but continuation of that stressor has been shown to decrease antibody responsiveness to that same challenge (Shini et al., 2010). Furthermore, corticosterone has been shown to mediate strong anti-inflammatory agents, where the production of proinflammatory cytokines and prostaglandins are suppressed (Shini et al., 2010), leading to less inflammation. The anti-inflammatory effect can potentially be a double-edged sword, as it may compromise the immune system's ability to react to diseases after long periods of stress. This difference in inflammation responses to stress can be due to the evolutionary role that takes place when an animal is in the fight or flight response stage. With an acute stressor, the animal may be attempting an escape where inflammation of tissues would not aid in that swift escape.

During a chronic stress period, the production of heterophils is increased, which has been shown to increase resistance to bacterial diseases (Campo et al., 2008; Davis et al., 2000; Gross & Siegel, 1983; Lentfer et al., 2015; Shini et al., 2010; Siegel, 1980). Whereas, reduction of lymphocytes in the chronic stress stage has been shown to increase the susceptivity to viral diseases (Shini et al., 2010; Siegel, 1980). This difference in responses of differing immune cells emphasizes the intricate relationship between chronic stress and disease responses in laying hens.

Understanding this interplay between stress and bird immunity had innovative impacts in the poultry industry. Previous research has expanded on this point, where it is explained that prior stressful experiences in an animal's life, especially in the neonatal stage, can have lasting benefits later in life, which leads to better survivability. An example of this is the use of fasting birds during the neonatal stage in order to modulate better responses later in life due to high ambient temperatures (Zulkifli et al., 1994). Furthermore, it has been demonstrated that stress may play a role in alleviating effects on parasitic infections through the increase of heterophilic white blood cell in the blood (Gross, 1976) which are then in turn used to fight off parasitic infections.

Eimeria & Coccidiosis

Eimeria is a protozoan parasite that can be the causing agent of the enteric disease called coccidiosis. Coccidiosis can affect all species of livestock animals, but it is of special interest in poultry production. When a bird is infected by Eimeria causing coccidiosis, they can experience malabsorption, enteritis, and in severe cases, mortality can occur (Blake et al., 2020). A study concluded that chickens are the most economically important hosts of coccidia infection, as in 2018 alone, 68 billion chickens were farmed and 1.38 trillion eggs were produced globally (Blake et al., 2020). The sheer number of poultry farmed around the world highlights the importance of diseases affecting this large section of food production in the world, which can then have effects on global food security.

Coccidiosis infections are of great importance due to their damage to the intestinal tract (Bafundo, Duerr, et al., 2021; Bafundo, Männer, et al., 2021; Blake et al., 2020; Martins et al., 2022). Eimeria species have a four to seven day life cycle, which takes place outside and inside

of the chicken's body (Martins et al., 2022). Outside of the host, the parasitic eggs (oocysts) which are shed in the feces, undergo a process of sporulation where oxygen and warmth are required (Martins et al., 2022). Through a hens natural behavior of pecking, the oocysts are ingested orally by the bird where the thick wall protecting the parasitic eggs is broken down by the action of the gizzard (Martins et al., 2022). After the oocysts are broken in the gizzard, the sporozoites (infective parasites) are released to the anterior part of the intestine and into the duodenum (Martins et al., 2022). The protozoa multiply exponentially in the host's intestinal tract, causing damage to the intestinal cells which hinders nutrient absorption (Martins et al., 2022). Intestinal lesions caused by the parasite proliferation in the epithelial cells can facilitate other infections (Martins et al., 2022), like necrotic enteritis (Blake et al., 2020).

Coccidiosis infections in poultry has been deemed as one of the top diseases having a detrimental economic impact in poultry production globally. In 2019 poultry veterinarians were surveyed, and coccidia was ranked the top disease to impact poultry production, especially in the broiler industry (Blake et al., 2020). In a recent study where the total loss due to coccidia infection was analyzed, it was determined that the global loss in 2016 was between 9.8 and 16.8 billion dollars (Blake et al., 2020). This great loss of revenue was even broken down on a per bird basis, where in general it equaled around a twenty cent loss per chicken produced (Blake et al., 2020). Although most of the costs accrued were in the broiler industry, it is also important to note that coccidia impacts laying hen production as well. In fact, a survey of the US Association of Veterinarians in Egg Production (AVEP) showed that coccidiosis was deemed the most impactful disease in replacement flocks reared in cage free environments, and the second most impactful disease in hens reared in caged environments (Blake et al., 2020).

Saponins as a means of coccidia control

Saponins are a natural product found in plants, which are known to impact many biological activities (Augustin et al., 2011; Bafundo, Duerr, et al., 2021; Fleck et al., 2019). Saponins were first described in 1782 due to the foaming property of the bark when mixed with water (Fleck et al., 2019). The word saponin is of Latin origin, where "sapo" means soap, and this is due to their foaming action in water (Fleck et al., 2019). Available saponins that are most commonly used commercially are derived from *Quillaja saponaria*, the Chilean soap bark tree, and *Yucca schidigera* plants which are native to north and south America (Bafundo, Duerr, et al., 2021). These plants are raised due to their high content of saponins and their tolerance to arid conditions (Bafundo, Duerr, et al., 2021).

Saponins contain chemical structures that are both water and fat soluble, which has the ability to influence many biological processes and affect an array of disease causing organisms (Augustin et al., 2011; Bafundo, Duerr, et al., 2021; Fleck et al., 2019). It is believed that saponins take part in a plant's natural ability to prevent invasions by pathogens, which has led to the study of application of these molecules as having antimicrobial and antiparasitic activity (Bafundo, Duerr, et al., 2021). Saponins have been shown to cause instability in the parasitic cell membrane, which leads to uses in antiprotozoal proliferation and formation (Bafundo, Duerr, et al., 2021; Fleck et al., 2019). It has been deemed that the mode of action of saponins is mostly due to their detergent properties ultimately causing cell lysis (Fleck et al., 2019). Furthermore, saponins have been described as possessing lytic effects in bacteria, but that specific mode of action is not yet fully understood (Bafundo, Duerr, et al., 2021). One possible mode of action of saponins structures against bacteria, is the affinity of saponins structures to the bacterial cell wall which changes its composition leading to permeability and weakening of the cell (Fleck et al.,

2019). In past studies, the combination of quillaja and yucca saponins have been shown to play a role against Salmonella enterica, Escherichia coli, Staphylococcus aureus, and other Gram-positive organisms which can all have detrimental impacts to poultry health (Martins et al., 2022). Saponins have been used in poultry production to promote intestinal health and immunity (Bafundo, Duerr, et al., 2021; Bafundo, Männer, et al., 2021), with active modes of action against bacterial, fungal, parasitic, and viral infections (Augustin et al., 2011; Bafundo, Duerr, et al., 2021; Bafundo, Männer, et al., 2021; Blake et al., 2020; Fleck et al., 2019).

CONCLUDING THOUGHTS

In the intricate interplay of many factors which influence the performance and welfare of laying hens, stress emerges as a key factor in the evolutionary path of prey animals like laying hens. Stress plays a particular role in shaping the acute and chronic stress responses, where acute perturbations trigger the "fight or flight" response, while chronic stress can pose disruptions in the normal life cycle (Romero, 2004).

Research suggests that some negative impacts on performance observed at higher stocking densities, which plays a huge role in a laying hen's welfare and production. In contrast some research found that higher stocking densities in the rearing period showed no effects on performance. Furthermore, some studies point to differing laying hen stocking density having no statistical differences in egg quality traits observed, while other research found some impacts. It is important to further knowledge in the field of caged stocking densities for laying hens as there is no concluding takeaways that can be inferred from previous research on egg quality trait impacts. As industry moves away from caged production an increasing issue in laying hen production are parasitic infections. Parasitic infections from Eimeria, causing coccidiosis, has

been one of the top ranked poultry diseases in the last few years due to their impacts on industry. Saponins which contain antiparasitic properties, provide a potential solution for parasitic infection reduction and an overall bettering of the gut health of laying hens. As researchers delve deeper into an understanding of stress responses by optimizing stocking densities with changing consumer demands and exploring nutritional interventions to better parasitic infections, the intricate connection between hen health and stress responses are highlighted.

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CHAPTER II

Stocking density impacts on key pullet performance indicators.

ABSTRACT:

Cage production systems for laying hens has been established as the standard commercial practice for many decades worldwide. Scrutiny regarding animal welfare has been more prevalent in the media and in consumer buying preferences, resulting in changes in the poultry industry regarding housing environments. This study utilized conventional quad deck cages and a total of nine hundred and forty White Leghorns (Hy-Line W36) pullets. The rearing density treatments are as follows: 310 cm^2 (310 cm^2) with 13 pullets per cage, 361 cm^2 (361 cm^2) with 11 pullets per cage, and 445 cm^2 (445 cm^2) with 9 pullets per cage. Pullets were weighed bi-weekly, along with feed consumption parameters being collected. At 16 weeks of age body composition, tonic immobility, blood biochemistry, plasma corticosterone, heterophils and lymphocyte ratio, femur health, and gut histomorphology parameters were collected. Pullets reared in 310 cm^2 had the highest body weights compared to the other density treatments, while pullets reared in 445 cm^2 had the highest daily feed consumption and FCR (gF/gG). The highest stocking density of 310 cm^2 had the longest times while the shortest tonic immobility times were observed in 445 cm^2 pullets, which can point to a direct link between fearfulness scores and cage density. Blood corticosterone levels were significantly different between treatments, with the highest stocking density having the highest level of corticosterone and 361 cm^2 reared pullets having the lowest. In addition, blood chemistry results for higher partial pressure of CO_2 (pCO_2) in the blood of 310 cm^2 pullets had acidifying impacts on the pH of the blood. And lastly, 310 cm^2 reared pullets had the longest villi which influenced the total villi area. The highest stocking density pullets (310 cm^2) achieved higher body weights while consuming less feed. These better performance parameters had a fearfulness score trade off, where TI times were significantly increased in 310 cm^2 reared pullets.

INTRODUCTION:

Environments for caged laying hens have evolved substantially since the 1920's. Laying hen caged production systems have been established as the standard commercial practice due to their cost effectiveness and high yielding hen production. In the past few decades, the poultry industry has received more attention from consumers as scrutiny regarding animal welfare has been more prevalent in the media, and in consumer buying preferences. In response to this increased awareness by the consumer population, the United Egg Producers (UEP) published guidelines for caged layers in 2002, where space requirements per hen were delineated. With the newest update of these guidelines published in 2017, they have become a standard practice in laying hen production in the United States. The UEP has put forth that conventional cage requirements for hens is 67-86in^2 (432 cm^2-555 cm^2) per hen (*United Egg Producers*, n.d.).

Studies looking at caged pullet stocking densities have been conducted to assess the effects on live production parameters and welfare in differing spaces allotted per pullet. Previous studies exploring cage density effects on pullet performance, in large agree that higher stocking densities decrease feed intake (Carey, 1987; Leeson & Summers, 1984; Patterson & Siegel, 1998). Although these higher stocking density pullets consumed less feed compared to their low stocking density counterparts, they found no significant impacts on pullet body weights associated with cage density (Carey, 1987; Patterson & Siegel, 1998). In contrast some studies found that along with the decreased feed intake, the pullets in higher cage densities (222 cm^2) had reduced body weights compared to lower stocking density pullets (311 cm^2) (Carey, 1987). Furthermore, one study found no differences between differing densities (221, 249, 277, and 304 cm^2) pertaining to pullet body weights and feed intake (K. E. Anderson & Adams, 1992). These

differences between white laying hen pullet performance relating to stocking density needs to be explored further in order to determine the effects of cage density on pullet performance.

The heterophil to lymphocyte ratio (H:L) has been used as a valuable measure of chronic stress responses in poultry (Davis et al., 2000; Gross & Siegel, 1983; Siegel, 1980). Previous research has concluded that the decrease of lymphocytes and an increase in heterophil counts occurs in response to stress (Lentfer et al., 2015). Where, a higher H:L ratio leads to conclusions that the bird has higher stress compared to lower H:L ratios. Researchers (Gross & Siegel, 1983) have also stated that H:L ratios are a more reliable measure of chronic stress in poultry, compared to corticosterone measurements, as it detects chronic physiological changes in the body whereas corticosterone in the blood measures before that physiological change can happen.

Tonic Immobility (TI) is characterized by motor inhibition, low responsiveness to stimuli, and suppression of the righting response (Jones, 1986). TI is generally regarded as a fear response as procedures to induce fear responses prolong TI periods, while fear reducing tactics tend to reduce TI periods (Jones, 1986). Tonic immobility responses have long been regarded as an anti-predator response, as cessation of movement or crouching can reduce detection by a predator, where flight can lead to escape measures if a predator approaches (Jones, 1986).

The interplay between production demands and welfare concerns is a hard issue to tackle, as these two sides seem to be inverses of each other. Further research into cage density and into welfare needs to be conducted in order for middle ground to be achieved. Furthermore, consumer awareness of how agriculture as a whole, and more specifically how animal production occurs, need to be elucidated. The aim of this study is to assess if manipulation of pullet cage density, to be less dense than industry standards, will present better production parameters of the pullet phase.

MATERIALS AND METHODS:

This study was conducted at the North Carolina Department of Agriculture Piedmont Research Station in Salisbury, NC, USA and the animal study was approved by the North Carolina State University Institution for Care and Use of Animals Committee. This study utilized conventional quad deck cages measuring 61 cm (24 in) by 66 cm (26 in) per cage. Pullets were fed *ad libitum* though the experimental period, and feed formulation can be found in table 2.7. Analyzed nutritional feed components can be found in table 2.8. Feeder space and nipple drinkers were held constant for each cage regardless of cage density. Each cage consisted of 61 cm of feeder space along the front of the cage, and two nipple drinkers per cage. For this pullet phase experiment, a total of nine hundred and forty White Leghorn (Hy-Line W36) pullets were used. The pullets were randomly assigned between 24 total experimental replicates with three differing densities. The rearing density treatments are as follows: 310 cm^2 (48 in^2) with 13 pullets per cage, 361 cm^2 (56 in^2) with 11 pullets per cage, and 445 cm^2 (69 in^2) with 9 pullets per cage. Treatments of 361 cm^2 and 445 cm^2 per pullet had 7 replicates each with three cages per replicate, while pullets reared in 310 cm^2 consisted of 10 replicates with four cages per replicate. The experimental period was from day zero to 16 weeks of age.

Performance:

Pullets were weighed bi-weekly, and each bi-weekly period constituted of 14 days, totaling the 8 experimental pullet phase periods. Feed consumption per replicate data was transformed to feed consumed per pullet per day, where feed consumption per pullet in the cage can be observed. Lastly, Feed Conversion Ratio (FCR) of gram of feed consumed by gram of body weight gain achieved (gF/gG) was also analyzed in order to observe differences in feed

utilization for growth parameters. These performance parameters can serve as key indicators for future laying hen production, which is where egg production can be impacted once pullets reach sexual maturity.

Body Composition:

At this experimental phase completion of 16 weeks, 14 birds per treatment group were euthanized and processed to assess body composition. Seven replicates from each treatment were sampled, where one bird per cage was randomly selected from two cages per replicate for sample collection. Live body weights were collected as the first step in the body composition analysis. Pullets were euthanized by cervical dislocation and were subsequently exsanguinated for processing; each bird was completely exsanguinated and weighed before moving to the picker. Furthermore, to keep the exsanguination process consistent, the same procedures and times were followed for all birds. The carcasses were placed in a small scale scalder with a constant temperature kept between 130-150°F for a minute, the carcasses were subsequently placed in a tumble-one bird feather picker until the majority of feathers were removed. Following this process, the carcasses were weighed once more to determine the feather percentage weights. The featherless carcasses were separated into the following components and weighed individually: neck and head, fat pad, wings, breast with back and lungs, Proventriculus, empty gizzard, liver (without gall bladder), and small intestine without pancreas. From these weights the percentage of each body part was determined in relation to the animal's live body weight. The percentages of these parts were used for comparisons between the treatment groups. In order to keep variations to a minimum, multiple skilled researchers were trained to collect the same set of samples where partitioning of the body parts were kept consistent across all treatments.

Tonic Immobility:

For this experiment two observers were trained and subsequently performed the tonic immobility (TI) testing. TI was conducted in the pullet house on opposite sides of the house in order to avoid one TI bird startling other TI assessment. Methods described by Anderson et al. (2021) were slightly modified and used. A total of 72 pullets were observed for TI times at 16 weeks of age, where four pullets per replicate were randomly selected from the same cage for six replicates per treatment. Bird TI was induced by the observer gently placing the bird on its back in a V-shaped cradle, and one hand was placed on the sternum of the animal while the other hand was used to cup the head of the chicken in a downward position (Anderson et al., 2021). After these procedures, the birds were in a TI state and the observer immediately started a timer and slowly sat down. If the bird righted itself within 5 seconds of being in the TI state, the observer repeated the restraint steps, and the timer was restarted.

Blood:

Blood was collected at 16 weeks of age using a 3ml syringe with a 22-gauge hypodermic one inch needle. A total of 3 ml of blood was collected from the brachial vein, per pullet, where corticosterone, heterophil : lymphocyte ratio, and blood chemistry parameters were observed. For plasma corticosterone analysis, a total of 42 blood samples were collected. A total of 14 blood samples from each treatment were collected, where one bird per cage was randomly sampled from 7 replicates per treatment. Blood chemistry parameters were collected via an i-Stat machine in the pullet house as the birds were being bled. A total of twenty-four pullets were sampled for blood chemistry, where one hen per replicate was sampled. The blood cartridges were stored in a cooler to maintain optimum performance and were disposed properly after use.

Each blood chemistry test lasts 120 seconds, and during this time the next bird was bled. The parameters collected for blood chemistry were as follows: pH, partial pressure of carbon dioxide (PCO2), partial pressure of oxygen (PO2), The base excess (BEecf), Bicarbonate (HCO3), total carbon dioxide (CO2), Oxygen saturation (SO2), sodium (Na), Potassium (K), Glucose (GLU), Hematocrit (HCT), and Hemoglobin (Hb).

In order to determine the ratio between heterophils and lymphocytes methodologies from Lentfer et al. (2015) were followed with some modifications. Blood was collected in 5 ml heparinized tubes to prevent coagulation, and transported to a dust free laboratory where the smears occurred in accordance to previous methodologies (Lentfer et al., 2015). The slides were then stained using Wright's Giemsa stain (Fisher HealthCare, Pittsburg PA, USA) and were then allowed to dry. The blood smears were mounted with a cover slip to allow oil immersion magnification. Cells were enumerated under 100X magnification with a counter that allowed for separation between heterophil and lymphocyte counts. A sum cell enumeration counter was used for this analysis, and counting was stopped once the sum of heterophils plus lymphocytes totaled 100. The ratios between heterophils and lymphocytes were calculated post analysis.

After the blood analysis for blood chemistry and H:L smears, it was placed in a centrifuge at 3,000 rotations per minute for 20 minutes in order to obtain the plasma for corticosterone analysis. Plasma samples were then stored in labelled bullet tubes in -20℃ for further analysis. Plasma corticosterone levels were assessed using a corticosterone ELISA kit obtained from Cayman Chemical Company (Item: 501320, Ann Arbor MI, USA), following the manufacturer's guidelines. Concentrations were measured based on a standard curve in picograms of corticosterone per milliliter of plasma.

Bones:

The pullet's right femur was excised at 16 weeks of age during body composition determination. The excarnation of the legs occurred during the collection process, and the femur was placed in a labelled bag and frozen for further analysis. After the bones were allowed to thaw, their diaphysis diameter and length were measured using digital calipers (VWR International, Radnor PA, USA) and expressed in mm. Through the use of an ultrasound bone sonometer machine (Nanjing Kejin Industries Co. Ltd, Qixia District Jiangsu province, China) the bone quality index (BQI) of the femur bones were assessed. Comparison between BQI scores were used, where a standard curve for bone health is used. Femur bone breaking strength and bending moment was subsequently measured using a texture analyzer equipped with a 250 kg load cell measuring force in kilograms for peak force and kilograms per mm^2 for bending moment (TA-HDplus, Stable Micro Systems, Surrey, United Kingdom).

Gut Histomorphology:

At pullet phase termination of 16 weeks, a portion of jejunum measuring three centimeters was collected from each of the sampled birds and rinsed with saline to remove digestive contents (14 birds per treatment, totaling 42 total birds). After rinsing, the jejunum cuttings were fixed in 10 % neutral formalin for storage and transportation. These samples were then transported to the NC State veterinary histology department, where jejunum samples were dehydrated in a graded ethanol series and subsequently embedded in paraffin. For each pullet, a 4 µm thick section was stained with hematoxylin and eosin. Villus height (tip to bottom), villus bottom width, villus top width, muscularis mucosa, and crypt depth were examined using a 40x lens with a light microscope (AmScope T340B-DK-Led Siedentopf Trinocular Irvine, California,

USA) and camera (AmScope FMA050 Irvine, California USA). Ten villi were measured per hen and averaged as the morphological value. These villi were measured with the Amscope image software (AmScope 3.7 Irvine, California USA).

Statistical analysis:

Data was analyzed using JMP Pro 17 (SAS Institute). All data was analyzed using a one-way ANOVA. Analysis of variance and differences between the means were determined using Tukeys HSD. Multiple comparisons with a P value <0.05 were deemed significant.

RESULTS AND DISCUSSION:

Performance:

Table 2.1 contains data relevant to the pullet period performance. Average body weights were statistically different between treatments (p=0.0001), where 310 cm^2 pullets had higher body weights compared to other treatments. Additionally, feed consumption was also significant between treatments (p=0.0014), where 445 cm^2 pullets had a higher feed consumption per day compared to other treatments. These differences in feed consumption and bodyweights could be attributed to pullets in the 445 cm^2 treatment having more space to move and spend energy within the cage, and in order to do so, some of the energy would be spent on movement and not on accruing corporal mass. Conversely, 310 cm^2 pullets had the highest body weights while consuming less feed compared to 445 cm^2 pullets. Where due to possible movement limitations, energy was allowed to be stored and used for body weight gain. As a consequence of these differences in body weight and feed consumption differences, FCR of gram of feed per gram of body weight gain also showed significant (p=0.0002) differences between treatments. In this

experiment 445 cm^2 pullets had the highest FCR compared to the other treatments. The finding is in this study partially agree with previous findings in the literature, where higher feed intake was observed in lower stocking densities (Carey, 1987; Patterson & Siegel, 1998). Interestingly, little literature is available on the cage density effects during the pullet phase, but the available research agrees that as stocking densities increase, the feed intake decreases due to less energy requirements and more competition for resources (Jalal et al., 2006; Saki et al., 2012). However, other literature has found that pullet density did not have a significant impact on 18-week performance parameters. In this instance, researchers have found that pullets reared in 221 cm^2, 249 cm^2, 277 cm^2, and 304 cm^2 showed no statistical differences in body weight uniformity, feed consumption, mortality, and fearfulness (Anderson & Adams, 1992). Some periodic effects were observed, which are not at all surprising as they follow the pullets natural development and biological functions.

Body Composition:

Some differences in body composition parameters between the experimental treatments were observed and can be found in Table 2.2. Statistical ($p=0.0198$) differences between keel lengths were observed among treatments. Pullets reared in 310 cm^2 had longer keel lengths compared to pullets reared in 445 cm^2 (11.30 mm vs. 10.63 mm, respectively). This difference in keel length could be due to bigger frame size of these birds, as 310 cm^2 birds had limited movement space and more energy could be put towards growth. During body composition sampling there were no differences observed between treatments when looking at live body weights, but as the number of hens was smaller than the numbers of hens weighed in the pullet house, some of the differences could possibly not be detected with a smaller sample size.

Differences between the proventriculus weights could also be observed, as 310 cm^2 reared pullets had statistically significant (p=0.0062) higher values for proventriculus weight compared to body mass. No differences were observed in the shank length or in the percentages of blood, feather, neck and head, fat pad, wings, breast with back, crop, gizzard, liver, or small intestine. Past studies have determined that cage densities between 293 cm^2 and 586 cm^2 did not have a significant effect on body composition parameters (Leeson & Summers, 1984). These findings in the literature disagree with the current findings of this experiment, as there were some differences in keel length and proventriculus weights.

Tonic Immobility:

Tonic immobility scores provided some interesting insights into the fearfulness scores of these pullets. Results pertaining to TI can be found in table 2.3 along with other stress data. Tonic immobility times were statistically (p=0.0488) different among treatments, where the highest stocking density of 310 cm^2 had the longest times while the shortest TI times were observed in 445 cm^2 pullets (544.22 s vs. 320.68 s, respectively). Tonic immobility responses have long been regarded as an anti-predator response, as cessation of movement or crouching can reduce detection by a predator, where flight can lead to escape measures if a predator approaches (Jones, 1986). Furthermore, TI may also take part after the predator has made contact with its prey, as a lack of movement may reduce stimulation for further attacks by the predator and ultimately can lead to loss of interest in its prey (Jones, 1986). It can be inferred that pullets reared in the highest stocking density of 310 cm^2 per pullet have the higher fearfulness compared to 445 cm^2 per pullet treatments. These findings are in agreement with previous literature looking at broiler and laying hens tonic immobility, where birds in higher stocking densities

remain in the TI state for longer periods (Anderson et al., 2021; Jones, 1992). Although previous literature findings dealt with older laying hens and broilers, the current findings of this study have concluded that as cage density increases, higher TI times can be observed.

Blood:

Blood chemistry data is available in table 2.4 below. Pullets reared in 310 cm^2 had the lowest blood pH compared to 361 cm^2 reared pullets (7.235 vs 7.301, respectively). This statistical ($p=0.0197$) significance between treatments points to 310 cm^2 pullets having more acidic blood compared to 361 cm^2 pullets who in comparison have more basic blood. The difference in pH is further elucidated when looking at the partial pressure of CO_2 (pCO_2) in the blood. Pullets reared in 310 cm^2 had statistically ($p=0.0075$) higher values of pCO_2 compared to the other treatments. This difference in pH can be due to high pCO_2 concentrations, as pCO_2 presence in the blood forms carbonic acid which decreases overall blood pH (Yoon et al., 2012). No differences were found between the treatments between the following parameters: partial pressure of oxygen (PO2), base excess (BEecf), Bicarbonate (HCO3), total carbon dioxide (CO2), Oxygen saturation (SO2), sodium (Na), Potassium (K), Glucose (GLU), Hematocrit (HCT), and Hemoglobin (Hb).

Previous literature has concluded that a decrease of lymphocytes and an increase in heterophil counts happens in response to stress (Lentfer et al., 2015). Meaning that a higher heterophil and lymphocyte ratio (H:L) can lead to conclusions that the bird is more stressed compared to lower H:L ratios. In this current experiment, no differences between heterophil and lymphocyte ratios were observed between treatments. These findings agree with previous

research, where in pullet stocking density showed no significant differences in H:L ratios (Patterson & Siegel, 1998)

Lastly, when comparing corticosterone levels in the plasma between the rearing treatments; it is evident that a significant difference ($p=0.0094$) was observed between the cage densities. Pullets reared in 310 cm^2 had the highest levels of corticosterone, while pullets in 361 cm^2 had the lowest (0.2301 vs. 0.1538, respectively). These differences partially agree with previous literature, as the highest stocking density had the highest levels of corticosterone in the blood. Meanwhile, the lowest stocking density did not have the lowest amount of blood corticosterone, which does not follow the linear decrease of blood corticosterone in relation to stocking density, as seen in previous literature. Studies have concluded that as cage density increases, the base corticosterone levels in the blood also increases (Davami et al., 1987; Mashaly et al., 1984; Onbaşılar & Aksoy, 2005) which was partially observed in this study. It can be concluded that pullets reared in 310 cm had the highest TI times and corticosterone concentrations in the blood, which can lead to conclusions of overall higher stress due to the cage density environment.

Bones:

Femur bone data can be found in table 2.5. There were no statistical differences observed for this experiment when looking at any of the bone quality parameters. As there were no differences in the nutrition of the birds, this is expected. The only source of difference could possibly be from differences in movement with the cages, as pullets raised in lower stocking densities would have more room for movement and expression of natural behaviors.

Gut Histomorphology:

Jejunum histomorphology data is shown in table 2.6, where some differences among the treatments were observed. It was observed in this experiment that pullets raised in 310 cm^2 had significantly (p=.0027) higher values for villi height compared to other density treatments. These differences in villi height had an impact on the overall area of the villi, where 310 cm^2 reared pullets also showed significant (p=.0019) higher values compared to other treatments. This difference could be due to lower feed consumption, and the animal's body adapting to this change by increasing absorption area for nutrients. Interestingly, pullets reared in 361 cm^2 consumed statistically similar amounts of feed compared to 310 cm^2 pullets; therefore, this cannot offer a full explanation for these differences.

CONCLUSION:

In conclusion, there were some differences observed between treatments in performance, body composition, tonic immobility, blood chemistry, and jejunum histomorphology. Pullets reared in 310 cm^2 had the highest body weights compared to the other density treatments, while pullets reared in 445 cm^2 had the highest daily feed consumption and FCR (gF/gG). Although these differences in feed consumption can be explained by limited cage space for energy use, some differences in body composition like keel bone length and proventriculus weights cannot be as easily explained. Furthermore, the highest stocking density of 310 cm^2 had the longest TI times while the shortest TI times were observed in 445 cm^2 pullets, which can point to a direct link between fearfulness scores and cage density. In addition, blood chemistry results for higher partial pressure of CO_2 (pCO_2) in the blood of 310 cm^2 pullets had acidifying impacts on the pH of the blood. Lastly, 310 cm^2 reared pullets had the longest villi which influenced the total villi

area. All of these physiological changes point to better pullet production being achieved through higher stocking densities, as 310 cm^2 pullets achieved higher body weights while consuming less feed. These better performance parameters had a fearfulness score trade off, where TI times were significantly increased in 310 cm^2 reared pullets.

Table 2.1: Performance parameters observed in pullets reared in three differing densities. [1]

Rear Treatment	Average Body Weight (kg)	Feed per pullet day (g)	FCR[2] (gF/gG)
48	0.6766[a]	43.12[b]	4.359[b]
56	0.6636[b]	43.28[b]	4.436[b]
69	0.6624[b]	44.65[a]	4.784[a]
P Value	**0.0001**	**0.0014**	**0.0002**
SEM	0.1424	6.930	0.0662
Period			
1	0.1055[h]	11.42[g]	2.340[f]
2	0.2202[g]	21.65[f]	2.660[ef]
3	0.3934[f]	33.08[e]	2.677[ef]
4	0.5973[e]	44.77[d]	3.078[e]
5	0.8064[d]	56.84[c]	3.819[d]
6	0.9539[c]	56.33[c]	5.414[c]
7	1.084[b]	63.87[b]	6.917[b]
8	1.178[a]	61.47[a]	9.303[a]
P Value	**<0.0001**	**<0.0001**	**<0.0001**
SEM	0.0044	0.4949	0.1226
CV%	31.29	17.36	7.60

[1]All data was collected on a per replicate basis (n=24) and then transformed into a per bird basis, as cages differed in number of hens per cage

[2]Feed Conversion Ratio of grams of feed consumed per grams of body weight gain produced

Table 2.2: Pullet body composition at 16 weeks of age, comparing three differing rearing densities.[1]

Rear Treatment	Live BW (kg)	Keel length (mm)	Shank length[2] (mm)	Blood[2] (%)	Feather[2] (%)	Neck & Head[2] (%)	Fat Pad[2] (%)
48	1.179	11.30[a]	96.29	3.252	11.09	8.247	2.467
56	1.143	11.07[ab]	96.62	3.208	11.66	8.226	2.112
69	1.113	10.63[b]	95.62	3.033	10.62	8.568	1.820
P Value	0.2285	**0.0198**	0.7992	0.6291	0.1834	0.0842	0.1924
SEM	0.0263	0.1590	1.0351	0.1686	0.3900	0.1181	0.2470
CV%	11.63	18.50	24.83	5.015	7.622	18.88	2.307

Rear Treatment	Wings[2] (%)	Breast, back with lungs[2] (%)	Proventriculus[2] (%)	Crop[2] (%)	Gizzard empty[2] (%)	Liver-no gallbladder[2] (%)	Small Intestines-no pancreas[2] (%)
48	8.131	25.23	0.5608[a]	0.3653	2.309	1.789	3.136
56	8.295	25.88	0.4622[b]	0.3363	2.360	1.559	2.736
69	8.401	25.77	0.4724[b]	0.4310	2.415	1.436	2.817
P Value	0.3955	0.4487	**0.0062**	0.0647	0.6054	0.0603	0.4911
SEM	0.1394	0.3826	0.0225	0.0283	0.0738	0.1032	0.2488
CV%	15.86	17.90	5.923	3.566	8.551	4.131	3.111

[1]Body composition parameters were collected using one bird per cage in each replicate (n=24)
[2]Parameters were calculated in relation to the hen's initial live body weights

Table 2.3: Pullet Tonic Immobility & HL ratio at 16 weeks of age.

Rear Treatment	Tonic Immobility Time[1] (s)	H:L ratio[2]	Corticosterone[2]
48	544.22[a]	0.4024	0.2301[a]
56	388.54[ab]	0.3930	0.1538[b]
69	320.68[b]	0.2434	0.2025[ab]
P Value	**0.0488**	0.0845	**0.0094**
SEM	64.50	0.0507	0.0168
CV%	1.322	2.222	3.102

[1]A total of 72 pullets were sampled at 16 weeks of age, where four hens from six replicates per treatment were sampled from the same cage (n=18)
[2]Blood samples collected during 16 weeks of age were used to determine heterophil to lymphocyte ratios and corticosterone levels. A total of 42 samples were collected, where one hen from two cages from seven replicates per treatment were sampled (n=21)

Table 2.4: Pullet blood biochemistry data collected via i-Stat at 16 weeks of age. [1]

Rear Treatment	pH	PCO2 /mmHg	PO2 /mmHg	Beecf /mmol/L	HCO3 /mmol/L	Tco2 MMOL/l
48	7.235[b]	53.24[a]	78.29	-5.000	22.53	24.14
56	7.330[a]	41.93[b]	77.14	-6.857	21.89	23.00
69	7.301[ab]	44.86[b]	65.29	-4.428	22.00	23.29
P Value	**0.0197**	**0.0075**	0.4476	0.5685	0.7686	0.5023
SEM	0.0221	2.301	7.851	1.663	0.6636	0.7031
CV%	124.8	7.666	3.542	1.234	12.61	12.62

Rear Treatment	sO2 %	Na mmol/L	K mmol/L	GLU mg/dL	Hct %PCU	Hb g/dL
48	89.86	145.28571	5.329	224.57143	24.57	8.343
56	91.43	143	6.186	222.28571	23.14	7.871
69	89.57	143.42857	6.657	225	25.14	8.557
P Value	0.7874	0.346	0.0878	0.9081	0.1746	0.1648
SEM	2.032	1.145	0.4030	4.686	0.7423	0.2483
CV%	16.79	47.51	5.681	18.06	12.37	12.57

[1]Blood chemistry parameters were collected using one bird per cage in each replicate (n=24)

Table 2.5: Pullet femur bone health assessment at 16 weeks of age. [1]

Rear Treatment	Bending Moment (kg/mm^2)	Peak Force (kg)	Diameter (mm)	Diaphysis (mm)	Length (mm)	BQI
48	0.0646	18.82	38.25	7.064	80.33	88.72
56	0.0669	19.22	37.93	6.929	79.81	82.79
69	0.0675	18.76	37.65	7.031	79.39	85.77
P Value	0.8396	0.932	0.2008	0.5911	0.5462	0.4345
SEM	0.0035	0.9331	0.2280	0.0966	0.5879	3.213
CV%	5.012	5.425	44.48	19.38	36.30	7.133

[1]Blood chemistry parameters were collected using one bird per cage in each replicate (n=24)

Table 2.6: Pullet jejunum histomorphology assessment at 16 weeks of age.[1]

Rear Treatment	Height (μm)	Tip (μm)	Bottom (μm)	Crypt (μm)	Muscularis (μm)	Area (μm^2)	H:C
48	803.32[a]	109.44	150.74	122.06	196.36	103258.51[a]	7.003
56	586.62[b]	115.57	142.41	97.34	218.99	74968.86[b]	6.292
69	625.32[b]	102.69	129.61	105.17	203.10	71883.17[b]	6.285
P Value	**0.0027**	0.379	0.2246	0.1958	0.3149	**0.0019**	0.4571
SEM	43.75	6.332	8.361	9.672	10.63	6300.22	0.4583
CV%	4.111	4.617	4.513	2.992	5.183	3.549	3.810

[1]Blood chemistry parameters were collected using one bird per cage in each replicate (n=24)

Table 2.7: Nutritional Breakdown for Pullet Rearing Phase Basal Diets.

Ingredients (%)	Starter (0–8weeks)	Grower (8-12 weeks)	Developer (12-16 weeks)
Yellow Corn	62.330	61.425	62.942
Wheat Bran		9.652	10.362
SBM	33.280	24.665	22.187
Limestone	1.890	1.769	2.129
Dical. Phos.	1.740	1.742	1.593
Salt	0.250	0.250	0.250
L-Lys		0.060	0.018
DL-Met	0.154	0.137	0.069
Choline Chloride			0.150
Trace Mineral Premix[1]	0.200	0.200	0.200
Vitamin Premix[2]	0.050	0.050	0.050
Selenium Premix[3]	0.050	0.050	0.050
Amprolium[4]	0.050		
Nutrients			
AMEn (Kcal/kg)	2900	2800	2750
CP (%)	20.8	18.50	17.50
Lys (%)	1.10	1.00	0.90
TSAA (%)	0.85	0.73	0.65
Ca (%)	1.10	1.10	1.20
Av. P. (%)	0.45	0.45	0.42

[1]Mineral premix provides per kg of diet: manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; and cobalt.

[2]Vitamin premix provides per kg of diet: 13,200 IU vitamin A, 4000 IU vitamin D3, 33 IU vitamin E, 0.02 mg vitamin B12, 0.13 mg biotin, 2 mg menadione (K3), 2 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 4 mg vitamin B6, 55 mg niacin, and 1.1 mg folic acid.

[3]Selenium premix=1 mg Selenium premix provides 0.2 mg Se (as Na2 SeO3) per kg of diet. ME=metabolizable energy

[4]Used as a means of coccidia control, organic compound

Table 2.8: Analyzed feed values for pullet rearing phase basal diets.[1]

	Starter	Grower	Developer
Dry Matter (%)	86.34	87.11	88.69
Crude Protein (%)	22.75	21.11	18.49
Neutral Detergent Fiber (%)	10.33	10.32	10.05
Acid Detergent Fiber (%)	4.45	4.73	3.98
Non-fiber Carbohydrate (%)	60	61.99	62.11
Fat (%)	3.73	3.25	2.72
Calcium (%)	1.04	1.28	2.48
Phosphorus (%)	0.88	0.9	0.83
Sulfur (%)	0.29	0.27	0.28
Magnesium (%)	0.21	0.2	0.19
Sodium (%)	0.1	0.13	0.11
Potassium (%)	1.14	1.04	0.86
Copper (ppm)	16.91	20.23	28.24
Iron (ppm)	278.47	299.31	484.38
Manganese (ppm)	150.58	157.91	199.93
Zinc (ppm)	172.24	198.16	207.15
Ash (%)	6.32	6.54	9.69

[1]Values were determined in a dry matter state through the Steve Troxler Agriculture Science Center, North Carolina Department of Agriculture & Consumer Services (Food & Drug Protection Division, Animal Feed Program)

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CHAPTER III

Pullet stocking density carry over effects into the lay cycle with differing cage densities, using key physiological changes as determining factors for production.

ABSTRACT:

The effects of cage density on laying hens has been documented in numerous research papers in the past and the majority of research points to a decrease in egg production, egg weight, feed intake, and an increase in mortality due to higher stocking densities. However, studies have contradicting findings when it comes to production parameters and higher stocking densities. A total of 504 White Leghorns (Hy-Line W36) hens were used for the lay period portion of the trail. The experimental design of this trial follows a 3X2 factorial arrangement, where each of the three rearing densities were then subsequently divided between two lay densities. The treatments are as follows: 1) **310-445** (310 cm^2 pullet phase and 445 cm^2 lay phase, per bird) ,2) **310-889** (310 cm^2 pullet phase and 889 cm^2 lay phase, per bird), 3) **361-445** (361 cm^2 pullet phase and 445 cm^2 lay phase, per bird), 4) **361-889** (361 cm^2 pullet phase and 889 cm^2 lay phase, per bird), 5) **445-445** (445 cm^2 pullet phase and 445 cm^2 lay phase, per bird), 6) **445-889** (445 cm^2 pullet phase and 889 cm^2 lay phase, per bird). The pullets were transferred to the laying house for a production cycle from 17 to 69 weeks of age. Blood was collected at 31, 44, 57, and 69 weeks of age where corticosterone (CORT), heterophil: lymphocyte (HL) ratio, and blood chemistry parameters (i-Stat) were determined. At 69 weeks of age body composition, femur health, and gut histomorphology parameters were collected. Some differences in body composition, jejunum histomorphology, and blood biochemistry were observed, with a possible explanation being the differences are due to resource competition. It was observed that the highest pullet stocking density of 310 cm^2 resulted in the highest values for HD%, HH%, feed consumed, and egg production, which all point to better production as a whole. When looking at the performance interactions, it is apparent that higher stocking densities during the pullet phase resulted in the carryover effects of increased production parameters compared to hens reared in the lowest stocking densities.

INTRODUCTION:

The effects of cage density in laying hens have been documented in numerous research papers in the past (K. E. Anderson & Adams, 1992; M. G. Anderson et al., 2021; Craig & Milliken, 1989; Davami et al., 1987; Hester & Wilson, 1986; Jahanian & Mirfendereski, 2015; Jalal et al., 2006; Saki et al., 2012), where the majority of research points to a decrease in egg production, egg weight, feed intake, and increase in mortality due to higher stocking densities. In contrast, some studies have contradicting findings when it comes to production parameters and higher stocking densities. In a study conducted by Brake & Peebles (1992), no density effects were observed in hens housed in 344 cm^2, 516 cm^2, and 1032 cm^2 on egg weight differences. Additionally, consistent effects on feed consumption were not attained in either of the two trials conducted, where in trial 1, 344 cm^2 reared hens had lower feed consumption than 516 cm^2 hens. In the second trial, 1032 cm^2 hens had higher feed consumption compared to 344 cm^2 hens, with 516 cm^2 hens being intermediate (Brake & Peebles, 1992).

For this current body of work, it is important to note that the cage density of birds has been shown to change the circulating corticosterone levels (Davis et al., 2000; Koelkebeck et al., 1987; Mashaly et al., 1984), where it has been determined that birds in higher stocking densities have higher baseline plasma corticosterone levels. Furthermore, heterophil to lymphocyte ratios (H:L) has been established as a valuable measurement of chronic stress responses in poultry (Davis et al., 2000; Gross & Siegel, 1983; Siegel, 1980), where previous literature has determined that a decrease of lymphocytes and an increase in heterophil counts happen in responses to chronic stress (Lentfer et al., 2015). In addition to collection of physiological changes of poultry, like H:L and corticosterone due to stress, observations on the birds fearfulness scoring can also elucidate our understanding of the animal's condition and deepen the

understanding between the interplay of stress and fearfulness. Tonic Immobility (TI) is characterized by motor inhibition, low responsiveness to stimuli, and suppression of the righting response (Jones, 1986). Differing cage densities and housing systems effect on TI scoring has been researched over the past few decades, where floor reared leghorn hens had longer TI times when compared to cage reared hens (K. E. Anderson & Adams, 1994).

Conventional definition of stress in society associates the word with a negative event, which has detrimental effects on health. Whereas, in animals, even small and benign changes to the environment can trigger stress responses (Romero, 2004). This evolution of defining the stress mechanism has played a crucial role in understanding an animal's adaptation to changing environments, as it aids in keeping the animals alert in a new environment and unknown environment. Selye, deemed as the "Father of stress", has acknowledged the important role that stress has on our lives, and details that "total elimination of stress is impossible, even during short periods, because that would involve cessation of demands made upon any part of the body, including the cardiovascular, respiratory, and nervous systems. This would be equivalent to death" (Selye, 1976).

Stress in animals can manifest in two distinct modes: acute and chronic. Acute stress can be characterized as a brief perturbation, which lasts seconds or minutes, triggering rapid physiological responses by the body in order to aid in immediate action (Romero, 2004; Scanes, 2015). In contrast, chronic stressors occur over extended periods, which can last hours or days. As an animal is exposed to chronic stressors, their plasma corticosterone levels are elevated, in comparison to their base levels, and are maintained at those higher levels until the animal is no longer in that emergency state (Romero, 2004; Scanes, 2015). Changes mediated by corticosterone show many improvements in the adaptiveness of an animal to a stressor, as the

animal can adapt and improve its fitness at the cost of momentary changes to its current life cycle (Scanes, 2015). As an animal becomes acclimated to the stressor, they don't respond to the stressor in the same manner. Therefore, animals like laying hens can adapt to stress and even outperform their non stressed counterparts. In a caged production system, the stressor is the cage density, as they are competing for resources like feed, water and space. In this system, the hens can acclimate to this stressor, especially if exposed to it in early stages of life, and cope better with that stress as they age.

The aim of this study is to evaluate the carry over effects of pullet rearing densities and determine if lay densities have an impact on hen productivity. With supporting physiological changes as a guide to elucidate why these changes occur.

MATERIALS AND METHODS:

The facilities at the North Carolina Department of Agriculture Piedmont Research Station in Salisbury, NC, USA were used for this experiment, and approval by the North Carolina State University Institution for Care and Use of Animals Committee was attained in order to conduct live animal trial procedures. For the pullet phase, a total of nine hundred and forty White Leghorn (Hy-Line W36) pullets were used. Feed formulation for the pullet phase is contained in table 3.7. The experimental pullets were randomly assigned between 24 total experimental replicates with three differing densities. The treatments were as follows: 310 cm^2 (48in^2) per hen with 13 pullets per cage, 361 cm^2 (56 in^2) per hen with 11 pullets per cage, and 445 cm^2 (69 in^2) per hen with 9 pullets per cage.

In the lay period the three pullet phase treatments were further divided into two lay period densities of 445 cm^2 (69 in^2) and 889 cm^2 (137 in^2) per bird. A total of 504 hens were used

for the lay period portion of the trail. The experimental design of this trial follows a 3 x 2 factorial arrangement, where each of the rearing densities were subsequently divided into two lay densities. Each cage in the lay house measured 20 in (51 cm) x 48 in (122 cm), and a total of 24 replicates were used during the lay period with four replicates per treatment. Additionally, each replicate consisted of two bird cages. Feed formulation for the lay phase can be found in table 3.8, while analyzed nutritional feed composition of experimental pullet and lay phases can be found in table 3.9. The treatments are as follows: 1) **310-445** (310 cm^2 pullet phase and 445 cm^2 lay phase, per bird) ,2) **310-889** (310 cm^2 pullet phase and 889 cm^2 lay phase, per bird), 3) **361-445** (361 cm^2 pullet phase and 445 cm^2 lay phase, per bird), 4) **361-889** (361 cm^2 pullet phase and 889 cm^2 lay phase, per bird), 5) **445-445** (445 cm^2 pullet phase and 445 cm^2 lay phase, per bird), 6) **445-889** (445 cm^2 pullet phase and 889 cm^2 lay phase, per bird). Feed and water were provided *ad libitum* and lighting followed Hy-Line management guidelines.

Performance:

Hens were weighed every period, where each period constituted 28 days, totaling 13 experimental lay periods. Feed consumption per replicate was also collected, but as the replicates differed in number of birds per cage, the total feed consumed data would not be suitable for analysis. The feed consumption per cage was transformed to feed consumed per hen per day, where feed consumption per hen in each cage can be observed. Furthermore, egg production also was analyzed on a per hen basis as there were differing number of hens per cage. FCR1 was calculated to show kg feed consumed per dozen of eggs produced, while FCR2 was calculated to show gram of egg produced per gram of feed consumed. Whereas a lower number for FCR1 points to better performance, a higher number for FCR2 points to better production. Overall

performance data looking at hen day egg production (HD%), hen housed (HH%), feed consumed, egg production, FCR1, and FCR2 were pooled for all periods in order to assess overall impacts during the whole lay experimental period between 17-69 weeks of age.

Body Composition:

At termination of the lay period (69 weeks of age), a total of 48 hens were euthanized and processed to assess body composition. All replicates from the lay phase were sampled, totaling eight birds per treatment being sampled with one hen per cage in each replicate randomly selected. Live body weights along with post bled body weights were collected to determine subsequent measurements and feather weights. Hens were euthanized by cervical dislocation and were subsequently exsanguinated for processing. The carcasses were placed in a scalder bath and for a minute, and subsequently placed in the feather picker. Following this process, the carcasses were separated into the following components and weighed individually: Neck and head, Fat pad, wings, Breast with back and lungs, rear quarter, proventriculus, crop, empty gizzard, liver (without gall bladder), and small intestine without pancreas, ovary, and oviduct. From these weights, the percentage of each body part was able to be determined in relation to the animal's body weight. The percentage of these parts were then used for comparison between the treatment groups. Trained individuals were used to separate each specific part as a means of reduction of person to person variability.

Tonic Immobility:

Two trained observers performed the tonic immobility (TI) testing. TI was conducted in the lay house on opposing sides of the hen house, so that one TI bird would not startle the other

through visual movement. One bird catcher was used to supply birds to the two observers. Modified methods described by Anderson et al. (2021) were used.

A total of 72 hens were used for tonic immobility at 69 weeks of age. Three birds per replicate were randomly selected from the same cage within each replicate, bird tag numbers were checked in order to ensure that the same animal was not sampled more than once. The observer gently positioned the bird on its back in a V-shaped cradle, with one hand on its sternum and the other cupping its head in a downward motion. This induced a TI state in the bird, after which the observer initiated a timer and slowly sat down. If the bird managed to right itself within 5 seconds, the observer repeated the restraint process, restarting the timer. Slight modifications on methodologies by Anderson et al. (2021) included having two designated locations for TI which was not adjacent to the bird's original cage.

Gut Histomorphology:

At the lay phase termination of 69 weeks, a portion of the jejunum measuring around three centimeters was collected from each of the sampled birds and rinsed with saline to remove contents (8 birds per treatment, totaling 310 total birds). After rinsing, the jejunum samples were fixed in 10 % neutral formalin for storage and transportation. The jejunum samples were sent to the NC State Veterinary School histology department where the samples were dehydrated in a graded ethanol series and embedded in paraffin. For each hen, one 4 µm thick section was stained with hematoxylin and eosin. Villus height (tip to bottom), villus bottom width, villus top width, muscularis mucosa, and crypt depth were examined using a 40x lens with a light microscope (AmScope T340B-DK-Led Siedentopf Trinocular Irvine, California, USA) and camera (AmScope FMA050 Irvine, California USA). Ten villi were measured per hen and

averaged as the morphological value. These villi were measured with the Amscope image software (AmScope 3.7 Irvine, California USA).

Blood:

Blood was collected at 31, 44, 57, and 69 weeks of age using a 5 ml hypodermic needle and 3 ml was taken from the brachial vein where corticosterone (CORT), heterophil: lymphocyte (HL) ratio, and blood chemistry parameters (i-Stat) were determined. For the subsequent CORT, HL, and i-Stat analyses a total of 48 blood samples were collected per sampling time point. A total of 8 blood samples from each treatment were collected from each time point, where one hen in both cages in each replicate was sampled from each of the 4 treatment groups. Plasma corticosterone levels were assessed using a corticosterone ELISA kit obtained from Cayman Chemical Company (Item: 501320, Ann Arbor MI, USA), following the manufacturer's guidelines. Concentrations were measured based on a standard curve in picograms of corticosterone per milliliter of plasma.

To enumerate the ratio of heterophils to lymphocytes, the methodologies outlined by Lentfer et al. (2015) were used with slight adaptations. Blood samples were collected in heparinized tubes to prevent coagulation and were transferred to a dust-free laboratory. Smears were prepared following established procedures (Lentfer et al., 2015) and subsequently stained using Wright's Giemsa stain (Fisher HealthCare, Pittsburg PA, USA) before allowing to dry. Finally, the blood smears were mounted with a cover slip to facilitate oil immersion magnification. Cells were enumerated under 100X magnification with a counter that allowed for separation between heterophils and lymphocytes counts with an addition tool that stops counting

once the total is achieved. For this enumeration 100 total heterophils and lymphocytes were counted per slide to achieve the ratio.

Blood chemistry parameters were collected via an i-Stat machine in the pullet house as the birds were being bled. The blood cartridges were stored in a cooler in order to maintain optimum performance and were disposed properly after use. Each blood chemistry test lasted 120 seconds, and during this time the next bird was being bled. The parameters collected for blood chemistry were as follows: pH, partial pressure of carbon dioxide (PCO2), partial pressure of oxygen (PO2), The base excess (BEecf), Bicarbonate (HCO3), total carbon dioxide (CO2), Oxygen saturation (SO2), sodium (Na), Potassium (K), Glucose (GLU), Hematocrit (HCT), and Hemoglobin (Hb).

Bones:

The hen's whole right leg was excised at 69 weeks of age after body composition steps were concluded. The whole leg was placed in a labelled bag and frozen in -20℃ for further analysis. The legs were thawed and deboned at a later date. The resulting femur was subsequently placed in the bag and labelled, and again frozen for further analysis. After the bones were thawed, measurements of the diaphysis diameter and length were taken using digital calipers (VWR International, Radnor PA, USA) and expressed in mm. The bone quality index (BQI) were assessed through the use of an ultrasound bone sonometer machine (Nanjing Kejin Industries Co. Ltd, Qixia District Jiangsu province, China), where BQI scores were compared between treatments in comparison to their scores in a standard curve. Bone breaking strength and bending moment were subsequently measured using a texture analyzer equipped with a 250 kg

load cell measuring force in kilograms for peak force and kilograms per mm^2 for bending moment (TA-HDplus, Stable Micro Systems, Surrey, United Kingdom).

Statistical analysis:

This experiment was arranged in a 3×2 factorial arrangement, with three pullet rearing densities and two rearing densities in the lay period. All data was analyzed using JMP Pro 17 (SAS Institute, Cary, NC), all data was analyzed using a split plot in time with period considered as a random effect. Analysis of variance and differences between the means were determined using Tukeys HSD. Multiple comparisons with a P value ≤ 0.05 were deemed significant.

RESULTS AND DISCUSSION:

Performance:

All data pertaining to laying hen performance is contained in table 3.1 below. For this experiment, it was observed that rearing density treatment had an impact on laying hen performance. Hens reared in 310 cm^2 during the pullet phase had higher production parameters of hen day percentage (HD%) and hen housed percentage (HH%), which was statistically ($p=<0.0001$) higher than the other pullet densities. Interestingly, it is apparent that lay density did not have significant differences in HD% and HH% production parameters. Interactions were observed in in HD% and HH%, where a trend can be observed that hens reared in higher stocking densities during the pullet phase show better production parameters compared to pullets reared in lower densities. It was observed that 310-889 and 361-445 hens had better HD% performance than 445-889 treatment. Interestingly, 445-889 treatment had the lowest stocking

density through the pullet and lay phases. Whereas 361-445 had the highest HH% compared to 445-445 treatment.

Feed consumption was the lowest for hens reared in 445 cm^2 during the pullet phase, with significant differences (p=0.0005) compared to the other treatments. Additionally, hens in 889 cm^2 treatment during the lay phase also consumed the most feed compared to 445 cm^2 hens. These differences in feed consumption could be due to possible movement allowances for 889 cm^2 hens, which allowed for movement and use of energy to do so, requiring more feed. An interaction was observed where 310-889 and 361-889 hens consumed more feed compared to the other treatments, which could be explained by the movement allowance statement above. Furthermore, differences in feed consumption agree with past studies where Saki et al. (2012) concluded that feed intake was higher in cage densities of 1 bird per cage (2000 cm^2 per bird) compared to 4 birds per cage (500 cm^2 per bird). It was determined that these differences were due to the higher cage density relating to increased competition for drinker and feeder space and due to lower activity with less space per bird (Saki et al., 2012).

Egg production was also affected by pullet rearing density where 310 cm^2 treatment had the highest egg production compared to the other two treatments. An interaction was also observed, where 310-889 and 361-445 had statistically (p=0.0043) higher egg production parameters compared to 361-889, 445-445, and 445-889 treatments. Lastly, FCR (g egg/g feed) also had significant (p=<0.0001) differences between the lay treatments, where 445 cm^2 had a higher FCR compared to the 889 cm^2 hens. These current findings disagree with research conducted by Saki et al. (2012), who found that white leghorn hens in cages of 667 cm^2 and 500 cm^2 per hen had higher FCR (g/doz eggs) compared to hens in 1000 cm^2 and 2000 cm^2 per hen. Interestingly, Saki et al. (2012) also found that hens reared in the two lower densities (1000 cm^2

and 2000 cm^2) had a higher hen day egg production compared to the higher density treatments (667 cm^2 and 500 cm^2), which disagree with finding for this experiment. Previous findings in the literature disagree with this experiment's results, where it was observed that high, medium, and low densities (310 cm^2, 464 cm^2, and 580 cm^2 respectively) had impacts on production parameters of white leghorn laying hens, where high density birds showed adverse effects in hen day production, egg weights, and mortality compared to medium and low densities (Craig & Milliken, 1989).

Body Composition:

Data pertaining to body composition can be found in tables 3.2a and 3.2b. Statistical ($p=<0.0001$) differences in feather percentage were observed, where lay density of 889 cm^2 had a higher percentage compared to 445 cm^2 hens (7.051% vs. 5.543%, respectively). Although this parameter was not observed, this could be due to bird feathering damage due to the higher density treatment birds damaging feathers in the cages, that could account for this 1.5% difference is feather percentage. Neck and head composition were also significantly ($p=0.0034$) impacted by the lay treatment, where 445 cm^2 hens had a higher proportion of their body weights attributed to neck and head compared to 889 cm^2 hens (8.473% vs. 7.577%, respectively). Neck and head weights are related to comb development, as comb size is related to sexual maturity and production. This may be further supported when observing HD% production parameter as it seems like treatment groups of 310-445 and 361-445 show a slight trend in higher production. Pullet rearing density affected fat pad percentages, as 445 cm^2 hens had bigger fat pads compared to 310 cm^2 hens. This finding is interesting as these hens reared in 445 cm^2 during the pullet phase consumed the least amount of feed but had a higher body percentage attributed to the fat

pad. Furthermore, pullet phase density also affected breast with back percentage, as 310 cm^2 hens had the lowest percentage compared to the other two rearing treatments. These differences could be due to higher space allowances, which allowed for more development of the breast muscle as hens had more opportunities to flap their wings.

Crop differences in percentages can be observed due to pullet and lay treatment, where the highest stocking densities for each had the highest values compared to the other treatments. Additionally, small intestine and ovary percentages followed the same pattern of statistical differences ($p=0.0273$ & $p=0.0184$, respectively) as pullet rearing treatment had an impact on these percentages, where 310 cm^2 hens had the highest values compared to 445 cm^2 hens. Furthermore, ovary weights could have played a role in egg production, as 310 cm^2 birds had the highest number of eggs produced compared to 445 cm^2 hens. There were no differences observed in live body weights, shank length, keel length. Additionally, no differences in the body composition percentages in the following were different amongst treatments: blood, wings, rear quarter, proventriculus, empty gizzard, liver, and oviduct.

Stress Parameters:

As observed in table 3.3, no statistical differences were observed in tonic immobility, heterophil to lymphocyte ratios, and plasma corticosterone levels in the blood among any of the pullet or lay treatments imposed. Furthermore, no differences in corticosterone levels were observed in the lay period, and no differences were observed between pullet rearing treatment, lay stocking density, or interaction. The findings relating to plasma corticosterone disagree with finding in the literature as it is deemed that as cage density increases, the base corticosterone levels in the blood also increase (Davami et al., 1987; Mashaly et al., 1984; Onbaşılar & Aksoy,

2005). In an experiment by Wan et al., 2023, it was observed that as cage density increases, so does the plasma corticosterone when comparing between Hy-line brown and Jinghong chickens in stocking densities of 423, 564, and 846 cm^2 per hen.

Jejunum Histomorphology:

Table 3.4 contains all data pertaining to Jejunum Histomorphology of hens at termination of the experimental period. Pullet rearing density showed impacts on villi tip width, where 310 cm^2 hens statistically ($p=0.0008$) had the widest villi, width compared to the other pullet rear treatments. Hens reared in 310 cm^2 during the pullet period also had the highest villi bottom width compared to 445 cm^2 pullet period density hens. Crypt depth also showed significance ($p=<0.0001$) between pullet period densities, as 310 cm^2 hens had the least muscularis thickness compared to other treatments. When observing for muscularis thickness parameter, it is evident that there is an interaction event, where 361-445 treatment hens have the highest muscularis thickness compared to 310-445 hens. Lastly, when observing villus height to crypt depth ratio the pullet density had an impact, where 310 cm^2 hens had the lowest ratio compared to the other treatments. It is unclear why these differences between pullet rearing treatments were observed, but a possible explanation could be related to resource competition. As the highest stocking densities compete for resources in the caged environment, it is possible that effects on the jejunum histomorphology can be observed. The findings in this experiment disagree with findings in the literature, where differing stocking densities of 423, 564, and 846 cm^2 per hen observed impacts in duodenal villus height and height to crypt depth ratios in Jinghong chickens, where hens in the lower stocking densities presented with significantly higher values than hens in the high and medium stocking densities (Wan et al., 2023).

Blood Biochemistry:

Blood biochemistry results can be found in table 3.5 below. For this experiment, the pullet phase rearing treatment had an influence on pH parameter. Hens reared in 310 cm^2 had more basic blood compared to 361 cm^2 hens. These differences in blood pH are hard to determine causation, as no other blood chemistry parameters that could impact pH were observed. Differences in blood pH can be due to high pCO_2 concentrations, as pCO_2 presence in the blood forms carbonic acid which decreases overall blood pH (Yoon et al., 2012). Furthermore, blood glucose levels were significant (p=0.0422) in the interaction, where 310-445 and 361-889 hens had the higher glucose concentrations in the blood compared to 310-889 hens. No differences were observed in partial pressure of carbon dioxide (PCO2), partial pressure of oxygen (PO2), The base excess (BEecf), Bicarbonate (HCO3), total carbon dioxide (CO2), Oxygen saturation (SO2), sodium (Na), Potassium (K), Hematocrit (HCT), and Hemoglobin (Hb).

Bone Health:

No differences between treatments were observed in any of the bone quality parameters tested. Results for this data can be found in table 3.6 below. These findings are not surprising, as diets did not differ among treatments, which could not in turn influence bone mineralization.

CONCLUSION:

The most consistent effects pertaining to cage densities were observed when looking at performance parameters. It was observed that the highest stocking density of 310 cm^2 during the pullet phase had the highest values for HD%, HH%, feed consumed, and egg production which

all point to better production as a whole. When looking at the performance interactions, it is apparent that higher stocking densities during the pullet phase have an increase in production parameters compared to hens reared in the lowest stocking densities. These findings contradict previous research indicating that higher stocking densities adversely affects production parameters.

Some differences in body composition, jejunum histomorphology, and blood biochemistry were observed, but no clear path to an explanation as to why is present. A possible explanation is that these differences are due to resource competition, and in order to adapt, the animals physiology changes. Lastly, no differences in tonic immobility, heterophil to lymphocyte ratios, and bone heath parameters were observed.

In conclusion, it is apparent that the findings in performance for this experiment disagree with previous research, but one conclusion stands out: higher stocking densities during the pullet phase have carry over effects that seem to enhance laying hen performance parameters.

Table 3.1: Laying hen performance in differing rear and lay cage density treatments.[1]

Rear Treatment	HD%[2]	HH%[3]	Feed Consumed (g/bird/day)	Egg production (egg/hen/day)	FCR[4] (kg feed/dozen)	FCR[4] (g egg/g feed)
310	86.73[a]	86.30[a]	99.86[a]	0.8673[a]	1.660	0.5318
361	86.02[b]	85.43[b]	100.61[a]	0.8602[b]	1.851	0.5259
445	85.22[c]	83.74[c]	98.28[b]	0.8522[c]	1.810	0.5282
P Value	**<0.0001**	**<0.0001**	**0.0005**	**<0.0001**	0.0746	0.2969
SEM	6.196	6.147	3.139	0.0620	0.4430	0.0372
Lay Treatment						
445	86.14	85.04	98.47[b]	0.8614	1.749	0.5359[a]
889	85.85	85.28	100.69[a]	0.8585	1.798	0.5214[b]
P Value	0.2408	0.4309	**<0.0001**	0.2408	0.4903	**<0.0001**
SEM	6.196	6.147	3.139	0.0620	0.4430	0.0372
Interaction						
310*445	86.40[ab]	86.12[ab]	98.22[b]	0.8640[ab]	1.700	0.5381
310*889	87.07[a]	86.49[ab]	101.50[a]	0.8707[a]	1.620	0.5255
361*445	86.69[a]	86.64[a]	98.74[b]	0.8669[a]	1.717	0.5376
361*889	85.35[bc]	84.22[c]	102.47[a]	0.8535[bc]	1.984	0.5141
445*445	85.33[bc]	82.36[d]	98.44[b]	0.8533[bc]	1.829	0.5318
445*889	85.12[c]	85.12[bc]	98.11[b]	0.8512[c]	1.791	0.5246
P Value	**0.0043**	**<0.0001**	**0.0012**	**0.0043**	0.0986	0.0966
SEM	6.196	6.147	3.139	0.0620	0.4430	0.0372
CV%	39.50	32.15	23.08	39.50	2.803	19.22

[1]All data was collected on a per replicate basis (n=24) and then transformed into a per bird basis, as cages differed in number of hens per cage. For this analysis period was considered as a random effect to account for variability across different time points, while the rearing and laying densities were considered fixed factors.
[2]Hen Day Percentage
[3] Hen Housed Percentage, which takes into account hen mortality
[4]Feed conversion Ratio

Table 3.2a: Body Composition of laying hens in differing rear and lay cage density treatments[1]

Rear Treatment	Live Body Weight (kg)	Shank length (mm)	Keel length (mm)	Blood[2] (%)	Feather[2] (%)	Neck & Head[2] (%)	Fat Pad[2] (%)	Wings[2] (%)	Breast with back[2] (%)
310	1.834	97.25	4.278	2.949	6.494	8.371	3.601[b]	6.335	20.54[b]
361	1.856	97.19	4.453	3.131	6.126	7.922	4.592[ab]	5.792	23.30[a]
445	1.880	97.75	4.531	3.156	6.270	7.783	4.994[a]	5.981	23.37[a]
P Value	0.7912	0.7821	0.0375	0.3849	0.6537	0.2327	**0.0239**	0.0968	**0.0004**
SEM	0.0476	0.6201	0.0687	0.1143	0.2826	0.2502	0.3549	0.1754	0.5259
Lay Treatment									
445	1.810	97.04	4.363	3.107	5.543[b]	8.473[a]	4.496	6.123	22.54
889	1.904	97.75	4.479	3.050	7.051[a]	7.577[b]	4.295	5.949	22.27
P Value	0.0942	0.3282	0.1491	0.6725	**<.0001**	**0.0034**	0.6266	0.3959	0.6684
SEM	0.0389	0.5063	0.0561	0.0933	0.2308	0.2043	0.2898	0.1432	0.4294
Interaction									
310*445	1.800	96.25	4.181	2.859	5.475	9.165	3.668	6.533	20.17
310*889	1.868	98.25	4.375	3.039	7.513	7.578	3.534	6.137	20.92
361*445	1.811	97.13	4.406	3.112	5.398	7.956	4.806	5.690	24.02
361*889	1.901	97.25	4.500	3.150	6.855	7.887	4.379	5.895	22.58
445*445	1.818	97.75	4.500	3.349	5.755	8.299	5.015	6.146	23.42
445*889	1.943	97.75	4.563	2.963	6.785	7.267	4.973	5.815	23.32
P Value	0.9114	0.4490	0.7810	0.2029	0.4556	0.1073	0.9235	0.4208	0.3417
SEM	0.0673	0.8769	0.0972	0.1617	0.3997	0.3539	0.5019	0.2481	0.7438
CV%	9.752	39.27	16.08	6.732	5.570	8.018	3.097	8.603	10.65

[1]Body composition parameters were collected using one bird per cage in each replicate (n=24), each replicate consisted of two cages

[2]Parameters were calculated in relation to the hen's initial live body weights. For this analysis period was considered as a random effect to account for variability across different time points, while the rearing and laying densities were considered fixed factors**.**

Table 3.2b: Body Composition of laying hens in differing rear and lay cage density treatments[1]

Rear Treatment	Rear Quarter[2]	Proventriculus[2]	Crop[2]	Gizzard empty[2]	Liver-no gall bladder[2]	Small Intestines[2]	Ovary[2]	Oviduct[2]
	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
310	28.45	0.7262	0.5482[a]	2.096	1.915	4.040[a]	2.853[a]	4.270
361	28.16	0.7792	0.3460[b]	1.923	1.820	3.718[ab]	2.763[ab]	4.666
445	29.14	0.7358	0.3711[b]	1.857	1.870	3.493[b]	2.315[b]	5.242
P Value	0.4554	0.5943	**0.0086**	0.2004	0.339	**0.0273**	**0.0184**	0.0825
SEM	0.5631	0.0389	0.0477	0.0956	0.0452	0.1386	0.1372	0.3003
Lay Treatment								
445	28.64	0.7653	0.4798[a]	2.024	1.853	3.608	2.682	4.708
889	28.52	0.7288	0.3638[b]	1.894	1.883	3.892	2.605	4.744
P Value	0.8567	0.4206	**0.0414**	0.2466	0.5698	0.0828	0.6313	0.9183
SEM	0.4598	0.0318	0.0390	0.0780	0.0369	0.1132	0.1120	0.2452
Interaction								
310*445	28.62	0.7494	0.6127	2.050	1.867	3.787	2.777	4.526
310*889	28.27	0.7030	0.4837	2.142	1.963	4.292	2.929	4.015
361*445	27.77	0.8107	0.3759	2.096	1.815	3.643	2.876	4.536
361*889	28.56	0.7477	0.3162	1.749	1.824	3.792	2.649	4.797
445*445	29.54	0.7359	0.4507	1.924	1.877	3.393	2.392	5.063
445*889	28.75	0.7357	0.2915	1.791	1.862	3.593	2.238	5.421
P Value	0.597	0.8395	0.7526	0.2774	0.6665	0.6218	0.5889	0.5384
SEM	0.7964	0.0550	0.0675	0.1352	0.0640	0.1960	0.1940	0.4248
CV%	12.69	4.801	2.209	5.123	10.33	6.765	4.817	3.934

[1]Body composition parameters were collected using one bird per cage in each replicate (n=24), each replicate consisted of two cages

[2]Parameters were calculated in relation to the hen's initial live body weights. For this analysis period was considered as a random effect to account for variability across different time points, while the rearing and laying densities were considered fixed factors.

Table 3.3: Stress parameters of laying hens in differing rear and lay cage density treatments

Rear Treatment	Tonic Immobility[1] (s)	H:L Ratio[2]	Corticosterone[2]
310	328.39	0.3257	0.1938
361	352.98	0.3547	0.1901
445	405.20	0.3336	0.1891
P Value	0.6262	0.7481	0.8345
SEM	57.13	0.0272	0.0176
Lay Treatment			
445	407.90	0.3488	0.1927
889	316.49	0.3272	0.1893
P Value	0.1705	0.5062	0.6173
SEM	46.64	0.0246	0.0173
Interaction			
310*445	351.38	0.3393	0.1978
310*889	305.40	0.3121	0.1898
361*445	446.60	0.3328	0.1936
361*889	259.37	0.3767	0.1866
445*445	425.72	0.3744	0.1867
445*889	384.69	0.2929	0.1915
P Value	0.5923	0.2951	0.6911
SEM	80.79	0.0400	0.0186
CV%	1.294	1.917	4.080

[1]A total of 72 hens were sampled at 69 weeks of age, where three hens from each replicate were sampled from the same cage (n=24). For this analysis period was considered as a random effect to account for variability across different time points, while the rearing and laying densities were considered fixed factors.
[2]Blood samples collected during 31, 44, 57, and 69 weeks of age were used to determine heterophil to lymphocyte ratios and corticosterone levels. A total of 48 samples per timepoint were collected, where one hen from both cages in each of the 24 replicates were sampled (n=24)

Table 3.4: Jejunum histomorphology of laying hens in differing rear and lay cage density treatments[1]

Rear Treatment	Villi Height (μm)	Villi Tip Width (μm)	Villi Bottom Width (μm)	Crypt Depth (μm)	Muscularis Thickness (μm)	Area (μm^2)	H:C
310	709.85	104.04^{a}	128.90^{a}	105.52^{a}	165.89^{b}	83217.89	6.882^{b}
361	833.88	89.86^{b}	115.75^{ab}	84.75^{b}	243.88^{a}	86253.14	10.044^{a}
445	828.70	87.55^{b}	110.57^{b}	94.89^{ab}	211.82^{a}	82997.97	8.699^{a}
P Value	0.1238	**0.0008**	**0.033**	**0.0132**	**<.0001**	0.9262	**0.0002**
SEM	47.01	3.745	4.899	4.650	11.11	6412.71	0.4868
Lay Treatment							
445	781.69	97.35	115.93	96.78	209.29	83423.62	8.230
889	799.93	90.27	120.88	93.32	205.11	84889.04	8.853
P Value	0.7415	0.1132	0.3918	0.5282	0.7492	0.8459	0.2799
SEM	38.38	3.058	4.000	3.797	9.075	5235.95	0.3975
Interaction							
310*445	625.26	110.69	121.19	103.03	136.80^{b}	72854.73	6.116
310*889	794.44	97.38	136.61	108.01	194.97^{abc}	93581.05	7.647
361*445	890.27	91.24	115.65	86.71	257.20^{a}	92603.20	10.403
361*889	777.50	88.47	115.85	82.79	230.57^{ab}	79903.07	9.684
445*445	829.54	90.13	110.95	100.60	233.86^{ab}	84812.93	8.170
445*889	827.86	84.96	110.19	89.17	189.77^{bc}	81183.01	9.227
P Value	0.1219	0.5911	0.4349	0.4652	**0.0051**	0.1823	0.2534
SEM	66.48	5.296	6.928	6.576	15.72	9068.94	0.6885
CV%	4.207	6.271	6.045	5.124	4.650	3.284	4.374

[1]Jejunum histomorphology parameters were collected using one bird per cage in each replicate (n=24), each replicate consisted of two cages

Table 3.5: Blood biochemistry parameters of laying hens in differing rear and lay cage density treatments[1]

Rear Treatment	pH	PCO2 /mmHg	PO2 /mmHg	Beecf /mmol/L	HCO3 /mmol/L	Tco2 MMOL/l	sO2% %	Na mmol/L	K mmol/L	iCa mmol/L	GLU mg/dL	Hct %PCU	Hb g/dL
310	7.370[a]	43.14	48.69	-0.7187	24.55	25.81	80.53	146.22	5.188	1.607	229.66	26.22	8.928
361	7.335[b]	46.52	52.47	-1.343	24.53	25.97	80.85	145.81	5.122	1.603	231.41	26.06	8.869
445	7.354[ab]	44.34	48.44	-1.218	24.39	25.72	79.78	146.22	4.988	1.605	228.97	26.50	9.019
P Value	**0.0236**	0.0767	0.1614	0.4684	0.9224	0.8581	0.7706	0.7764	0.1215	0.9835	0.4706	0.5772	0.5738
SEM	0.0306	3.429	3.769	0.5416	0.4376	0.4903	3.458	0.9344	0.1787	0.0341	2.5844389	0.5132	0.1775
Lay Treatment													
445	7.360	43.94	50.61	-1.000	24.48	25.79	81.25	146.02	5.065	1.617	230.19	26.56	9.046
889	7.346	45.39	49.11	-1.188	24.50	25.88	79.53	146.15	5.133	1.593	229.83	25.96	8.831
P Value	0.2004	0.2346	0.4286	0.6687	0.9586	0.8235	0.1685	0.8167	0.3931	0.1549	0.832	0.0828	0.0693
SEM	0.0301	3.375	3.657	0.4957	0.3989	0.4535	3.405	0.8949	0.1741	0.0330	2.447	0.4835	0.1676
Interaction													
310*445	7.366	43.18	49.25	-1.062	24.35	25.63	80.94	146.75	5.188	1.627	232.81[a]	26.19	8.925
310*889	7.374	43.11	48.13	-0.3750	24.76	26.00	80.13	145.69	5.188	1.588	226.50[b]	26.25	8.931
361*445	7.349	45.11	54.97	-1.063	24.53	25.94	83.25	145.31	5.050	1.610	229.69[ab]	26.81	9.131
361*889	7.321	47.93	49.97	-1.625	24.53	26.00	78.45	146.31	5.194	1.597	233.13[a]	25.31	8.606
445*445	7.364	43.54	47.63	-0.8750	24.56	25.81	79.56	146.00	4.956	1.615	228.06[ab]	26.69	9.081
445*889	7.344	45.14	49.25	-1.563	24.21	25.63	80.00	146.44	5.019	1.595	229.88[ab]	26.31	8.956
P Value	0.3418	0.624	0.3636	0.3681	0.6928	0.8266	0.2107	0.2748	0.7641	0.8047	**0.0422**	0.1669	0.1595
SEM	0.0318	3.587	4.101	0.6605	0.5373	0.5869	3.618	1.0439486	0.1917	0.0370	2.959	0.5935	0.2042
CV%	146.59	7.504	5.457	0.5114	13.89	14.16	13.37	55.47	12.99	19.46	28.21	15.57	15.64

[1]Blood chemistry parameters were collected using one bird per replicate (n=24), samples were collected during 31, 44, 57, and 69 weeks of age. For this analysis time was considered as a random effect to account for variability across different time points, while the rearing and laying densities were considered fixed factors.

Table 3.6: Bone quality parameters of laying hen femur in differing rear and lay cage density treatments[1]

Rear Treatment	Bending Moment (N/mm)	Peak Force (N)	Diameter (mm)	Diaphysis (mm)	Length (mm)	BQI
310	0.7492	206.53	37.58	7.300	79.53	76.01
361	0.8130	225.85	37.75	7.236	80.25	82.97
445	0.7551	201.43	37.43	7.236	80.75	83.19
P Value	0.6749	0.2799	0.9024	0.8609	0.2681	0.1987
SEM	0.0579	11.69	0.5126	0.0971	0.5364	3.186
Lay Treatment						
445	0.7375	205.94	37.69	7.248	80.62	82.83
889	0.8081	216.53	37.48	7.267	79.73	78.62
P Value	0.2886	0.4243	0.718	0.8622	0.1452	0.2461
SEM	0.0461	9.244	0.4055	0.0768	0.4243	2.520
Interaction						
310*445	0.7394	207.90	37.77	7.300	80.34	77.81
310*889	0.7581	205.16	37.39	7.300	78.72	74.22
361*445	0.7845	225.26	37.96	7.271	80.13	84.13
361*889	0.8424	226.44	37.53	7.200	80.36	81.81
445*445	0.6874	184.76	37.35	7.171	81.40	86.56
445*889	0.8228	218.10	37.52	7.300	80.10	79.83
P Value	0.7665	0.4701	0.8923	0.7452	0.4115	0.8715
SEM	0.0765	15.30	0.6712	0.1271	0.7023	4.172
CV%	3.587	4.895	19.80	20.19	40.38	6.849

[1]Femur bone health parameters were collected using one bird per cage in each replicate (n=24), each replicate consisted of two cages. For this analysis period was considered as a random effect to account for variability across different time points, while the rearing and laying densities were considered fixed factors.

Table 3.7: Nutritional Formulation Breakdown for Pullet Rearing Phase Experimental Diets

Ingredients (%)	Starter (0–8weeks)	Grower (8-12 weeks)	Developer (12-16 weeks)
Yellow Corn	62.330	61.425	62.942
Wheat Bran		9.652	10.362
SBM	33.280	24.665	22.187
Limestone	1.890	1.769	2.129
Dical. Phos.	1.740	1.742	1.593
Salt	0.250	0.250	0.250
L-Lys		0.060	0.018
DL-Met	0.154	0.137	0.069
Choline Chloride			0.150
Trace Mineral Premix[1]	0.200	0.200	0.200
Vitamin Premix[2]	0.050	0.050	0.050
Selenium Premix[3]	0.050	0.050	0.050
Amprolium 25% [4]	0.050		
Nutrients			
AMEn (Kcal/kg)	2900	2800	2750
CP (%)	20.8	18.50	17.50
Lys (%)	1.10	1.00	0.90
TSAA (%)	0.85	0.73	0.65
Ca (%)	1.10	1.10	1.20
Av. P. (%)	0.45	0.45	0.42

[1]Mineral premix provides per kg of diet: manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; and cobalt.

[2]Vitamin premix provides per kg of diet: 13,200 IU vitamin A, 4000 IU vitamin D3, 33 IU vitamin E, 0.02 mg vitamin B12, 0.13 mg biotin, 2 mg menadione (K3), 2 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 4 mg vitamin B6, 55 mg niacin, and 1.1 mg folic acid.

[3]Selenium premix=1 mg Selenium premix provides 0.2 mg Se (as Na2 SeO3) per kg of diet. ME=metabolizable energy

[4]Used as a means of coccidia control, organic compound

Table 3.8: Laying Hen Feed Formulation From 17-69 Weeks of Age

Ingredient	Control
	(%)
Corn	51.842
Soybean meal	32.241
Soybean Oil	3.729
D.L. Methionine	0.181
Ground Limestone	4.600
Coarse Limestone	5.000
DiCalcium Phosphate	1.809
Magni-Phi	0.000
Salt	0.250
Trace Mineral Premix[1]	0.200
Vitamin Premix[2]	0.050
Selenium Premix[3]	0.050
Choline Cl 60%	0.050
Calculated Analysis	
Protein %	19.5
Calcium %	4.14
A. Phos. %	0.45
Lysine %	1.1
TSAA %	0.83
ME kcal/kg	2922

[1]Mineral premix provides per kg of diet: manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; and cobalt.

[2]Vitamin premix provides per kg of diet: 13,200 IU vitamin A, 4000 IU vitamin D3, 33 IU vitamin E, 0.02 mg vitamin B12, 0.13 mg biotin, 2 mg menadione (K3), 2 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 4 mg vitamin B6, 55 mg niacin, and 1.1 mg folic acid.

[3]Selenium premix=1 mg Selenium premix provides 0.2 mg Se (as Na2 SeO3) per kg of diet. ME=metabolizable energy

Table 3.9: Analyzed feed values during the pullet and lay periods.

Analyte	Pullet Phase (0-16 weeks)			Lay Phase (17-69 weeks)
	Starter	Grower	Developer	Layer Diet D
Dry Matter (%)	86.34	87.11	88.69	88.42
Crude Protein (%)	22.75	21.11	18.49	18.18
Neutral Detergent Fiber (%)	10.33	10.32	10.05	7.58
Acid Detergent Fiber (%)	4.45	4.73	3.98	3.3
Non-fiber Carbohydrate (%)	60	61.99	62.11	48.22
Fat (%)	3.73	3.25	2.72	5.63
Calcium (%)	1.04	1.28	2.48	3.48
Phosphorus (%)	0.88	0.9	0.83	0.67
Sulfur (%)	0.29	0.27	0.28	0.27
Magnesium (%)	0.21	0.2	0.19	0.22
Sodium (%)	0.1	0.13	0.11	0.14
Potassium (%)	1.14	1.04	0.86	0.85
Copper (ppm)	16.91	20.23	28.24	19
Iron (ppm)	278.47	299.31	484.38	317
Manganese (ppm)	150.58	157.91	199.93	124
Zinc (ppm)	172.24	198.16	207.15	146
Ash (%)	6.32	6.54	9.69	11.23

[1]Values were determined in a dry matter state through the Steve Troxler Agriculture Science Center, North Carolina Department of Agriculture & Consumer Services (Food & Drug Protection Division, Animal Feed Program)

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CHAPTER IV

Effects of differing cage densities during the pullet phase and lay phase on internal and external egg quality impacts due to carry over.

ABSTRACT:

The interplay between production demands and welfare concerns is a hard issue to tackle, as these two production characteristics tend to be inverse of each other. While laying hen caged production is still the most popular housing system in use worldwide, the US industry has faced pressure to reevaluate housing conditions primarily from a welfare standpoint. A total of 504 White Leghorns (Hy-Line W36) hens were used for the lay period portion of the trail. The experimental design of this trial follows a 3x2 factorial arrangement, where each of the rearing densities were subsequently divided into two lay densities. The treatments are as follows: 1) **310-445** (310 cm^2 pullet phase and 445 cm^2 lay phase, per bird), 2) **310-889** (310 cm^2 pullet phase and 889 cm^2 lay phase, per bird), 3) **361-445** (361 cm^2 pullet phase and 445 cm^2 lay phase, per bird), 4) **361-889** (361 cm^2 pullet phase and 889 cm^2 lay phase, per bird), 5) **445-445** (445 cm^2 pullet phase and 445 cm^2 lay phase, per bird), 6) **445-889** (445 cm^2 pullet phase and 889 cm^2 lay phase, per bird). Egg quality parameters were collected during experimental lay periods of: 1, 3, 5, 7, 9, 11, and 13. Where egg quality parameters of egg weights, shell color, Haugh unit, yolk color, vitelline and shell strength and elasticity, along with egg components and solids were analyzed. Furthermore, size and grade distribution were determined every period. Some minor differences were found in vitelline strength and egg weights attributed to lay period stocking density. When looking at the proportion of XL and S eggs, it is apparent that 445 cm^2 hens had significantly higher proportion of eggs in these categories compared to 889 cm^2 hens. Interestingly, 889 cm^2 hens had the highest proportion of eggs in the L and M size categories compared to 445 cm^2 hens. When looking at the size distribution it can be concluded that the more intense stocking density during the lay phase produced more eggs in the extreme sizes of XL and S, while hens in the lower stocking density produced eggs in the middle sizes categories of L and M.

INTRODUCTION:

In the past few decades, the poultry industry has received increased scrutiny from consumers regarding animal welfare. In response to this increased awareness by the consumer population, the United Egg Producers (UEP) published guidelines for caged layers in 2002, that delineated space requirements per hen. With the newest update of these guidelines published in 2017, they have become a standard practice in laying hen production in the United States. The UEP has put forth that conventional cage requirements for hens is 67-86in^2 (432 cm^2-555 cm^2) per hen (*United Egg Producers*, n.d.). While laying hen caged production is still the most popular housing system in use, the industry has faced pressure to reevaluate housing conditions primarily from a welfare standpoint.

The interplay between production demands and welfare concerns is a hard issue to tackle, as these two sides seem to be inverse of each other. More research into these systems and into welfare needs to be done in order for the middle ground to be achieved. Furthermore, consumer awareness of how agriculture as a whole, and more specifically how animal production occurs, needs to be elucidated. Special interest groups geared toward animal rights have taken an active role in tarnishing the reputation of animal production, and some of the population have taken that point of view, which in turn has had great impacts in the trajectory of how animal production in the modern world occurs.

The effects of cage density in laying hens have been documented in numerous research papers in the past (Anderson & Adams, 1992; M. G. Anderson et al., 2021; J. Craig & Milliken, 1989; Davami et al., 1987; Hester & Wilson, 1986; Jahanian & Mirfendereski, 2015; Jalal et al., 2006; Saki et al., 2012), where the majority of research points to a decrease in egg production, egg weight, feed intake, and an increase in mortality due to higher stocking densities. Cage

density studies are usually impacted by the number of hens per fixed cage space, not by manipulation of differing cage sizes.

The aim of this current study is to evaluate if pullet stocking density will have a carry-over effect onto the lay phase when looking at egg quality parameters, and to determine if the two stocking densities during the lay period will provide any further insights into egg quality changes.

MATERIALS AND METHODS:

Experimental Layout:

This study was conducted at the North Carolina Department of Agriculture Piedmont Research Station in Salisbury, NC, USA and the animal study was approved by the North Carolina State University IACUC. A total of 504 White Leghorns (Hy-Line W36) pullets were used. Pullet feed formulation data is contained in table 4.5. Pullets were randomly assigned between 24 experimental replicates with three differing densities. The treatments are as follows: 310 cm^2 (48 in^2) per hen with 13 pullets per cage, 361 cm^2 (56 in^2) per hen with 11 pullets per cage, and 445 cm^2 (69 in^2) per hen with 9 pullets per cage.

During the lay period, those three pullet phase treatments were further divided into two lay period densities of 445 cm^2 (69 in^2) and 889 cm^2 (137 in^2) per bird. A total of 504 hens were used for the lay period portion of the trail. Laying hen feed formulation can be found in table 4.6, while analyzed pullet and lay nutritional data can be found in table 4.7. The experimental design of this trial follows a 2X3 factorial arrangement, where each of the rearing densities were subsequently divided into two lay densities. Each cage in the lay house measured 20 in (51 cm) x 48 in (122 cm), a total of 24 replicates were used during the lay period, with four replicates per

treatment. Additionally, each replicate consisted of two bird cages. The treatments are as follows: 1) **310-445** (310 cm^2 during the pullet phase and 445 cm^2 during the lay phase, per bird), 2) **310-889** (310 cm^2 during the pullet phase and 889 cm^2 during the lay phase, per bird), 3) **361-445** (361 cm^2 during the pullet phase and 445 cm^2 during the lay phase, per bird), 4) **361-889** (361 cm^2 during the pullet phase and 889 cm^2 during the lay phase, per bird), 5) **445-445** (445 cm^2 during the pullet phase and 445 cm^2 during the lay phase, per bird), 6) **445-889** (445 cm^2 during the pullet phase and 889 cm^2 during the lay phase, per bird).

Egg quality:

All data pertaining to egg quality parameters were measured at 28-day intervals (periods), starting at 17 weeks and ending in week 69 of the hens' life. Egg quality parameters were collected during experimental lay periods 1, 3, 5, 7, 9, 11, and 13. Egg quality parameters were evaluated by assessing traits of 6 eggs per replicate for each measurement. All eggs were collected at the research station and immediately placed in a cooler for transport, the eggs were transported to egg lab cooler where they were stored at 45^{o}F for at least 24 hours prior to egg quality analyses. Shell strength and elasticity were determined using a texture analyzer (TAHDplus, Stable Micro Systems, Surrey, United Kingdom) using a 250 kg load cell, measuring the force in grams. Using a more sensitive texture analyzer (TA.XTplus, Stable Micro Systems, Surrey, United Kingdom) equipped with a 500g-load cell and a 1mm blunt probe, vitelline membrane strength and elasticity were analyzed. Shell color, albumen height, Haugh unit, and yolk color were determined utilizing the TSS QCD System (Technical Services and Supplies, Dunnington, York, UK). Eggshell color was determined based on light reflectivity scope with pure white representing 83.3% reflectivity, while 0% represents pure black

reflectivity. Egg solids were determined for whole egg (albumen plus yolk), yolk, and albumen by pooling 6 eggs per replicate, and homogenizing them. These homogenized egg mixtures were dispensed in metal tins where they were dried at 50 °C for 48 hours. These dry pans were weighed, and determination of solid percentages were then calculated. Egg components were determined by weighing the whole egg and separating the yolk from albumen. The yolks were weighed, and shells were washed and dried. After drying for a period of 48 hours, the eggshells were then weighed. With the weight of the dry shell, yolk, and whole egg; the weight of the albumen was determined. Lastly, shell thickness was measured utilizing calipers (iGaging speedmic, San Clemente, California, USA) where each shell was measured twice at the equator and averaged.

Egg grading and sizes:

Egg grades and size distributions were determined by collecting all eggs during a 24-hour period, during the third week of each period. During this time egg weights, along with USDA egg grades, and USDA egg sized were determined for all eggs. USDA grading for size and grading was conducted by a trained grader in accordance with the USDA grading handbook (Egg Grading Manual, 2000).

Statistical analysis:

The egg quality data was arranged as 3×2 factorial arrangement, with three pullet rearing densities and two laying densities. The data were analyzed using JMP Pro 17 (SAS Institute, Cary, NC) in a split-plot design in time, incorporating a mixed-effects model approach. Here, the period was treated as a random effect to account for variability across different time points, while

the rearing and laying densities were considered fixed factors. Analysis of variance (ANOVA) was conducted to evaluate the effects of cage dimensions (fixed factors) on various egg quality parameters, with differences between the means determined using Tukey's HSD test. Multiple comparisons with a P value ≤ 0.05 were deemed significant, indicating statistically significant differences between group means under the conditions tested.

RESULTS AND DISCUSSION:

Internal egg quality:

All data pertaining to internal egg quality parameters can be found in table 4.1 below. For this experiment, vitelline strength was impacted by laying hen cage density, where hens placed in the laying cage at 445 cm^2 had statistically ($p=0.0268$) higher values compared to hens reared in 889 cm^2 per hen. Furthermore, egg weights collected during egg quality analysis also differed significantly between hen's pullet rearing treatment and lay stocking density treatments. Hens reared in 361 cm^2 during the pullet phase had the heaviest eggs compared to 445 cm^2 hens. Additionally, hens in 445 cm^2 during the lay phase had significantly ($p=0.0156$) heavier eggs compared to 889 cm^2 hens (61.16g vs. 60.60, respectively). No statistical differences were found between pullet rearing treatment or lay stocking density for the following parameters: vitelline elasticity, shell strength, shell elasticity, shell reflectivity, albumen height, Haugh unit, and yolk color. A study comparing W-36 laying hen caged stocking densities of 360 cm^2 and 257 cm^2 per hen concluded that no differences in egg quality parameters, like shell thickness, Haugh unit, and yolk color were observed at egg quality sampling points (Jahanian & Mirfendereski, 2015). The lack of differences between stocking densities for these parameters were not surprising, as they are in agreement with previous findings in the literature pertaining to stocking density and egg

quality. In contrast, a study conducted with W-36 laying hens caged in four stocking densities (2000 cm^2, 1000 cm^2, 667 cm^2, and 500 cm^2 per hen) found significant differences in yolk color and higher egg surface area attributed to the lower densities compared to the highest stocking density (Saki et al., 2012). Although egg surface was not determined in this current experiment, yolk color in this previous research was statistically different among differing cage stocking densities.

Egg component & egg Solids:

Egg component percentages and egg solids data can be found in table 4.2. In agreeance with previous findings in the literature (Erensoy et al., 2021), no differences were observed for any of the egg components or egg solids data. There were no impacts of pullet rearing density or lay stocking density were found. Similarly, a study looking at egg quality traits of caged Lohmann-Brown hens in high, medium and low densities (552.3 cm^2, 736.3 cm^2, and 1104.5 cm^2 per hen, respectively) found no statistical differences between the egg quality parameters observed: Haugh unit, yolk height, yolk color, shell thickness, and egg component percentage (Erensoy et al., 2021).

Egg grade and size distribution:

Data pertaining to egg grade distribution can be found in table 4.3, while data pertaining to egg size distribution can be found in table 4.4 below. The proportion of A eggs was significantly affected ($p=0.0072$) by the lay stocking density treatment, where hens housed in 445 cm^2 had a higher proportion of grade A eggs compared to 889 cm^2 hens (94.44% vs. 92.91, respectively). Interestingly, that proportion was inverse when looking at check eggs. Hens

housed in 889 cm^2 had a significantly higher proportion of check eggs compared to hens housed in 445 cm^2. This disparity between A% eggs and check eggs could be attributed to the movement of A grade eggs shifting into the check category. These changes could also be due to more cage space in 889 cm^2 hens, which could allow for more possibility of checks to occur, as hens with more space can have more contact with the cage instead of other chickens when startled.

Furthermore, egg size distribution was impacted by the lay stocking density treatments. When looking at the proportion of XL and S eggs, it is apparent that 445 cm^2 hens had a significantly higher proportion of eggs in these categories compared to 889 cm^2 hens. Notably, 889 cm^2 hens had the highest proportion of eggs in the L and M size categories compared to 445 cm^2 hens. When looking at the size distribution, it can be concluded that the more intense stocking density during the lay phase produced more eggs in the extreme sizes of XL and S, while hens in the lower stocking density produced eggs in the middle sizes categories of L and M.

CONCLUSION:

In conclusion, some minor differences were found in vitelline strength and egg weights attributed to lay period stocking density. While these changes were significant, a practical significance cannot be determined, as the space requirements needed to house these hens are drastically different. Therefore, it can be concluded that rearing hens in higher stocking densities during the lay period can prove to be advantageous, as there seems to be no detrimental impacts on egg quality parameters observed. Furthermore, if a production farm aims to produce more eggs in the L and M categories, it may prove advantageous to house hens in lower stocking densities, as they produce a higher proportion of eggs in those categories.

Table 4.1: Internal egg Quality parameters of laying hens in differing rear and lay cage density treatments[1]

Rear Treatment	Vitelline Strength	Vitelline Elasticity	Shell Strength	Shell Elasticity	Shell Reflectivity	Egg Weight	Albumen Height	Haugh Unit	Yolk color
	(N)	(mm)	(N)	(mm)		(g)	(mm)		
310	0.0193	1.485	44.18	0.2446	81.28	61.04^{ab}	7.587	86.25	8.402
361	0.0199	1.571	43.20	0.2464	81.13	61.18^{a}	7.552	86.03	8.521
445	0.0194	1.492	44.27	0.2580	81.40	60.42^{b}	7.554	86.22	8.446
P Value	0.2449	0.1247	0.2011	0.2327	0.6613	**0.0175**	0.866	0.8651	0.0503
SEM	0.0008	0.0969	1.14	0.0080	1.336	2.606	0.1798	1.658	0.2120
Lay Treatment									
445	0.0198^{a}	1.544	44.31	0.2532	81.33	61.16^{a}	7.613	86.31	8.429
889	0.0192^{b}	1.488	43.45	0.2462	81.21	60.60^{b}	7.515	86.02	8.484
P Value	0.0268	0.1376	0.1092	0.3143	0.6043	**0.0156**	0.1049	0.4253	0.1663
SEM	0.0008	0.0950	1.11	0.0072	1.330	2.604	0.1773	1.648	0.2111
Interaction									
310*445	0.0195	1.477	45.14	0.2433	81.31	61.29	7.608	86.25	8.435
310*889	0.0191	1.493	43.22	0.2459	81.26	60.80	7.567	86.25	8.369
361*445	0.0198	1.573	43.38	0.2515	81.48	61.56	7.551	85.81	8.440
361*889	0.0199	1.568	43.01	0.2412	80.79	60.81	7.553	86.24	8.601
445*445	0.0202	1.582	44.43	0.2647	81.21	60.64	7.682	86.88	8.411
445*889	0.0186	1.402	44.11	0.2513	81.58	60.19	7.427	85.56	8.482
P Value	0.0532	0.0671	0.3919	0.6114	0.1963	0.8448	0.1763	0.129	0.0684
SEM	0.0008	0.1023	1.23	0.0100	1.352	2.614	0.1873	1.689	0.2148
CV%	4.327	2.518	5.108	2.258	21.23	16.45	7.907	14.70	13.28

[1] Egg quality parameters were collected during experimental lay periods 1, 3, 5, 7, 9, 11, and 13. Egg quality parameters were evaluated by assessing traits of 6 eggs per replicate for each measurement (n=24). For this analysis period was considered as a random effect to account for variability across different time points, while the rearing and laying densities were considered fixed factors.

Table 4.2: Egg Component & Egg Solids of laying hens in differing rear and lay cage density treatments[1]

Rear Treatment	Shell Thickness	Yolk (%)	Shell (%)	Albumen (%)	Whole Egg Solid (%)	Yolk Solid (%)	Albumen Solid (%)
310	0.3723	25.80	12.38	61.82	24.54	50.93	12.51
361	0.3686	32.92	9.64	57.43	24.79	49.99	12.66
445	0.3692	25.66	9.55	64.79	30.41	49.89	12.66
P Value	0.1036	0.3535	0.3478	0.4893	0.3419	0.2898	0.6241
SEM	0.0029	4.094	1.517	4.353	2.95	0.73	0.25
Lay Treatment							
445	0.3709	25.67	9.60	64.72	24.75	0.50.07	12.49
889	0.3692	30.58	11.45	57.98	28.40	0.50.46	12.74
P Value	0.2403	0.2964	0.3082	0.1816	0.3239	0.512	0.0936
SEM	0.0028	3.369	1.225	3.564	2.30	0.66	0.24
Interaction							
310*445	0.3734	25.34	9.45	65.21	24.54	50.32	12.28
310*889	0.3712	26.25	15.31	58.44	24.53	51.53	12.74
361*445	0.3684	26.15	9.82	64.02	24.76	49.84	12.61
361*889	0.3689	39.70	9.46	50.84	24.82	50.13	12.71
445*445	0.3710	25.54	9.54	64.92	24.96	50.05	12.57
445*889	0.3674	25.78	9.57	64.65	35.86	49.72	12.75
P Value	0.5465	0.4286	0.2883	0.5803	0.3831	0.5704	0.5779
SEM	0.0032	5.762	2.177	6.159	4.35	0.89	0.28
CV%	15.39	0.3782	0.3679	0.7683	1.112	13.06	13.34

[1] Egg quality parameters were collected during experimental lay periods 1, 3, 5, 7, 9, 11, and 13. Egg quality parameters were evaluated by assessing traits of 6 eggs per replicate for each measurement (n=24). For this analysis period was considered as a random effect to account for variability across different time points, while the rearing and laying densities were considered fixed factors.

Table 4.3: Egg Grade distribution of laying hens in differing rear and lay cage density treatments[1]

Rear Treatment	A%	B%	Check%	Loss%
310	93.87	0.1835	5.833	0.1105
361	93.67	0.1821	6.077	0.0687
445	93.80	0.2353	5.879	0.0874
P Value	0.9675	0.9194	0.9475	0.9331
SEM	0.9467	0.1113	0.9414	0.0763
Lay Treatment				
445	94.66[a]	0.2480	5.067[b]	0.0279
889	92.91[b]	0.1526	6.792[a]	0.1499
P Value	**0.0072**	0.4308	**0.0080**	0.1854
SEM	0.8897	0.0935	0.8842	0.0610
Interaction				
310*445	94.52	0.3671	5.030	0.0836
310*889	93.23	0.0000	6.635	0.1374
361*445	94.93	0.2039	4.867	0.0000
361*889	92.42	0.1603	7.287	0.1374
445*445	94.52	0.1731	5.304	0.0000
445*889	93.07	0.2976	6.453	0.1748
P Value	0.7038	0.2424	0.7191	0.8593
SEM	1.100	0.1528	1.095	0.1103
CV%	16.42	0.1876	1.039	0.1095

[1]During the first day of the third week of each period, all eggs produced in a span of 24 hours were collected per replicate (n=24) and used for egg grade distribution. For this analysis period was considered as a random effect to account for variability across different time points, while the rearing and laying densities were considered fixed factors.

Table 4.4: Egg size distribution of laying hens in differing rear and lay cage density treatments

Rear Treatment	XL%	L%	M%	S%	Peewee%
310	75.18	14.28	6.925	3.311	0
361	76.31	13.31	6.737	3.540	0
445	74.59	13.88	7.571	3.793	0.0876
P Value	0.2437	0.6892	0.5958	0.755	0.1267
SEM	9.149	5.062	4.391	3.528	0.0409
Lay Treatment					
445	76.59[a]	12.86[b]	6.233[b]	4.146[a]	0.0279
889	74.12[b]	14.78[a]	7.922[a]	2.950[b]	0.0305
P Value	**0.0038**	**0.0387**	**0.0167**	**0.0233**	0.9478
SEM	9.139	5.041	4.377	3.518	0.0355
Interaction					
310*445	76.46	12.74	6.842	3.626	0
310*889	73.89	15.82	7.008	2.996	0
361*445	76.60	13.02	5.371	4.939	0
361*889	76.02	13.60	8.102	2.141	0
445*445	76.72	12.84	6.485	3.874	0.0836
445*889	72.45	14.91	8.657	3.712	0.0916
P Value	0.2081	0.5408	0.2933	0.0931	0.9957
SEM	9.179	5.125	4.433	3.557	0.0539
CV%	10.07	1.697	1.142	0.7656	0.0817

[1]During the first day of the third week of each period, all eggs produced in a span of 24 hours were collected per replicate (n=24) and used for egg size distribution. For this analysis period was considered as a random effect to account for variability across different time points, while the rearing and laying densities were considered fixed factors.

Table 4.5: Nutritional Formulation Breakdown for Pullet Rearing Phase Experimental Diets

Ingredients (%)	Starter (0–8weeks)	Grower (8-12 weeks)	Developer (12-16 weeks)
Yellow Corn	62.330	61.425	62.942
Wheat Bran		9.652	10.362
SBM	33.280	24.665	22.187
Limestone	1.890	1.769	2.129
Dical. Phos.	1.740	1.742	1.593
Salt	0.250	0.250	0.250
L-Lys		0.060	0.018
DL-Met	0.154	0.137	0.069
Choline Chloride			0.150
Trace Mineral Premix[1]	0.200	0.200	0.200
Vitamin Premix[2]	0.050	0.050	0.050
Selenium Premix[3]	0.050	0.050	0.050
Amprolium[4]	0.050		
Nutrients			
AMEn (Kcal/kg)	2900	2800	2750
CP (%)	20.8	18.50	17.50
Lys (%)	1.10	1.00	0.90
TSAA (%)	0.85	0.73	0.65
Ca (%)	1.10	1.10	1.20
Av. P. (%)	0.45	0.45	0.42

[1]Mineral premix provides per kg of diet: manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; and cobalt.

[2]Vitamin premix provides per kg of diet: 13,200 IU vitamin A, 4000 IU vitamin D3, 33 IU vitamin E, 0.02 mg vitamin B12, 0.13 mg biotin, 2 mg menadione (K3), 2 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 4 mg vitamin B6, 55 mg niacin, and 1.1 mg folic acid.

[3]Selenium premix=1 mg Selenium premix provides 0.2 mg Se (as Na2 SeO3) per kg of diet. ME=metabolizable energy

[4]Used as a means of coccidia control, organic compound

Table 4.6: Laying Hen Feed Formulation From 17-69 Weeks of Age

Ingredient	Control
	(%)
Corn	51.842
Soybean meal	32.241
Soybean Oil	3.729
D.L. Methionine	0.181
Ground Limestone	4.600
Coarse Limestone	5.000
DiCalcium Phosphate	1.809
Magni-Phi	0.000
Salt	0.250
Trace Mineral Premix[1]	0.200
Vitamin Premix[2]	0.050
Selenium Premix[3]	0.050
Choline Cl 60%	0.050
Calculated Analysis	
Protein %	19.5
Calcium %	4.14
A. Phos. %	0.45
Lysine %	1.1
TSAA %	0.83
ME kcal/kg	2922

[1]Mineral premix provides per kg of diet: manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; and cobalt.

[2]Vitamin premix provides per kg of diet: 13,200 IU vitamin A, 4000 IU vitamin D3, 33 IU vitamin E, 0.02 mg vitamin B12, 0.13 mg biotin, 2 mg menadione (K3), 2 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 4 mg vitamin B6, 55 mg niacin, and 1.1 mg folic acid.

[3]Selenium premix=1 mg Selenium premix provides 0.2 mg Se (as Na2 SeO3) per kg of diet. ME=metabolizable energy

Table 4.7: Analyzed feed values during the pullet and lay periods.[1]

Analyte	Pullet Phase (0-16 weeks)			Lay Phase (17-69 weeks)
	Starter	Grower	Developer	Layer Diet D
Dry Matter (%)	86.34	87.11	88.69	88.42
Crude Protein (%)	22.75	21.11	18.49	18.18
Neutral Detergent Fiber (%)	10.33	10.32	10.05	7.58
Acid Detergent Fiber (%)	4.45	4.73	3.98	3.3
Non-fiber Carbohydrate (%)	60	61.99	62.11	48.22
Fat (%)	3.73	3.25	2.72	5.63
Calcium (%)	1.04	1.28	2.48	3.48
Phosphorus (%)	0.88	0.9	0.83	0.67
Sulfur (%)	0.29	0.27	0.28	0.27
Magnesium (%)	0.21	0.2	0.19	0.22
Sodium (%)	0.1	0.13	0.11	0.14
Potassium (%)	1.14	1.04	0.86	0.85
Copper (ppm)	16.91	20.23	28.24	19
Iron (ppm)	278.47	299.31	484.38	317
Manganese (ppm)	150.58	157.91	199.93	124
Zinc (ppm)	172.24	198.16	207.15	146
Ash (%)	6.32	6.54	9.69	11.23

[1]Values were determined in a dry matter state through the Steve Troxler Agriculture Science Center, North Carolina Department of Agriculture & Consumer Services (Food & Drug Protection Division, Animal Feed Program)

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CHAPTER V

The Effects of Magni-Phi® on the Performance Response of Commercial White Layers During Pullet Rearing and Through a Single Laying Cycle

ABSTRACT:

One of the most prevalent poultry diseases is caused by *Eimeria* protozoan parasite resulting in an enteric disease infection called coccidiosis. In this current experiment, saponins were sourced from Quillaja Saponaria. Saponins are used to reduce coccidia oocyst production, however this experiment aimed to observe the impact when hens are not challenged. In this grow/lay experiment, 988 W-36 pullets were used in a 2X2 factorial design. The grow period consisted of two feeding programs, a Control (C) and Magni-Phi (MP) treatments. In the lay cycle, the grow treatments were divided to compose the four lay treatments. The experimental lay treatments were allocated in the feed as C-C, C-MP, MP-C, and MP-MP. Water and feed were supplied *ad libitum,* while the vaccine schedule and lighting program followed W-36 management guide. The hens remained unchallenged in this trial, with only exposure to the research station's facilities natural biologicals which did not include coccidia. Feed consumption, FCR (g egg/g feed), egg production, Hen-Housed, Hen-Day, USDA Grading distribution, and egg incomes were also calculated on a per-period basis. Egg quality parameters like Haugh unit, shell color, yolk color, egg weights, shell and vitelline strength and elasticity were analyzed every other period from 17-69 weeks. Data was analyzed using JMP Pro 17 running a two-way ANOVA with $\alpha<0.05$ using Tukey's HSD to make comparisons and determine differences. During the grow period birds in the control group (C) consumed more feed than birds fed the Magni-Phi (MP) diet, but no differences in body weights were observed. Egg production, feed consumed, hen day, and hen housed production showed significant results in the lay periods, with C-C treatment groups showing statistically significant higher results when compared to MP-MP treatments. Albumen height and Haugh Unit showed significance improvement, with MP-MP treatment showing higher values when compared to C-C treatment. Overall, our study explores the potential supplementation of Magni-Phi in the poultry industry as it offers a possible reduction of rearing feed costs with comparable pullet development, while in the laying phase maintaining comparable egg yields when compared to the control, with an improvement in physical egg quality indicating an economically viable utilization even in healthy laying hens during production.

INTRODUCTION:

One of the most prevalent diseases in poultry around the world is caused by the *Eimeria* protozoan parasite. The enteric disease resulting from the *Eimeria* parasite infection is called coccidiosis, which has deep economic impacts and great detrimental effects on poultry production parameters. Coccidia infection in poultry can lead to enteritis, malabsorption, and in severe cases leads to death (Blake et al., 2020). Furthermore, intestinal lesions caused by the parasite proliferation in the epithelial cells can facilitate other infections (Martins et al., 2022), like necrotic enteritis (Blake et al., 2020). To lessen the effects and prevent coccidiosis infections many producers have started using naturally sourced additives that can prevent production losses due to this protozoan parasite. One of the products being used in the industry are saponins, which have been known to possess antimicrobial and antiparasitic activity (Augustin et al., 2011). Saponins are a natural product found in plants, which are known to impact many biological activities (Augustin et al., 2011; Fleck et al., 2019; Bafundo et al., 2021a). In this current experiment, saponins were sourced from Quillaja Saponaria, where in previous experiments it had been shown to reduce oocyst production of the three major species of chicken coccidia by 40 to 50 percent (Bafundo et al., 2021a). Furthermore, saponins have been used in poultry production to promote intestinal health and immunity (Bafundo et al., 2021b; a), with active modes of action against bacterial, fungal, parasitic, and viral infections (Augustin et al., 2011; Fleck et al., 2019; Blake et al., 2020; Bafundo et al., 2021b; a).

Saponins contain chemical structures that are both water and fat soluble, which has the ability to influence many biological processes and affect an array of disease causing organisms (Augustin et al., 2011; Fleck et al., 2019; Bafundo et al., 2021a). It is believed that saponins take part in a plant's natural ability to prevent invasions by pathogens, which has led to the study of

application of these molecules as having antimicrobial and antiparasitic activity (Bafundo et al., 2021a). Saponins have been researched to cause instability in the parasitic cell membrane, which leads to uses in antiprotozoal proliferation and formation (Fleck et al., 2019; Bafundo et al., 2021a).

This experiment aims to determine whether providing Magni-Phi to a pullet during its life may enhance bone growth, future egg production, and egg quality when the bird is raised in industry-standard conditions with no parasitic challenge. It is hypothesized that pullets fed Magni-Phi should reach their target weight at the conclusion of the rearing period with more uniformity, and throughout the laying cycle, they should exhibit improved production performance and livability according to the effects of the feed reported in other animals. The current study aims to validate Magni-Phi's anticoccidial properties in pullets and laying hens, as well as determine if supplementation throughout the pullet stage would result in additive reactions. To attain this objective, performance parameters will be analyzed for the growing and laying period, as well as evaluating egg quality parameters.

MATERIALS AND METHODS:

Pullet Phase:

This study was conducted at the North Carolina Department of Agriculture Piedmont Research Station in Salisbury, NC, USA and the animal study was approved by the North Carolina State University Institution for Care and Use of Animals Committee. In this experiment, 988 W-36 pullets were brooded and reared in Quad Deck cages through 16 weeks of age. Water and feed were supplied *ad libitum.* Vaccine schedule and lighting program followed W-36 management guide. Diet formulations can be found in table 5.7. There were 36 reps used

for this experiment, with each rep consisting of two cages with 13 pullets (48 in^2/bird) in each cage. All thirty-six pullet replicates were randomly assigned to one of the two experimental treatments, control (C) and Magni-Phi (MP), resulting in 18 reps per treatment. Bi-weekly body weights, feed consumption, mortality, flock uniformity CV, and New Castle and Bronchitis vaccine titer data were collected during the pullet phase.

Lay Phase:

The lay house consists of 4 rows of a Tri-Deck Stacked Cage System with Manure Belts and force ventilation. Throughout the whole experimental lay period, the hens received diet D formulation, which can be found in table 5.8. pullet and lay phase feed nutritional analysis can be found in table 5.9. To assess the carry-over effects of Magni-Phi activity into the lay period, the pullet phase reps were further divided into four treatments. A total of 36 replicates we used in the lay period of this experiment, with 9 replicates per treatment. The lay period treatments were divided in such a manner to attain a 2X2 factorial effect on performance between the two grow treatments on the four lay treatments. The experimental treatments all follow the same logic where the first letter related to the pullet phase treatment, and after the dash resulting in the lay period treatment. The four treatments are as follows: C-C, C-MP, MP-C, and MP-MP. Laying hen body weights were taken at 17 weeks and 69 weeks, at the beginning and end of the lay experimental cycle. The lay experimental cycle consisted of 13 periods, where each period lasted for 28 days. Feed consumption, FCR (g egg/g feed), and feed costs were calculated on a per-period basis. Egg characteristics like egg production, Hen-Housed, Hen-Day, USDA Grading and distribution, and egg incomes were also calculated on a per-period basis.

Egg Quality

Egg quality parameters like Haugh unit, shell color, yolk color, egg weights, shell strength and elasticity, and vitelline membrane strength and elasticity were analyzed every other period (1, 3, 5, 7, 9, 11, and 13), for a total of seven egg quality sampling time points being collected between 17-69 weeks of the hens' life. A total of six eggs per replicate were collected for egg quality analysis, eggs were collected in the third week of each period and stored at a constant 45^{o}F for at least 24 hours prior to egg quality analyses. Shell strength and elasticity and vitelline membrane elasticity were both collected using texture analyzers (TA.XTplus, Stable Micro Systems, Surrey, United Kingdom). Shell strength and elasticity were measured using a 250 kg load cell, while the vitelline membrane strength and elasticity used a 500 g load cell with a 1mm blunt probe; both measured the force in grams. The parameters of shell color, albumen height, Haugh unit, and yolk color were determined through the use of TSS QCD System (Technical Services and Supplies, Dunnington, York, UK) where Haugh unit is automatically calculated given the egg weight and albumen height. Eggshell color was determined using a light reflectivity scope with pure white color calibration representing 83.3% reflectivity, while 0% represents pure black color reflectivity.

Statistical Analysis:

Data was analyzed using JMP Pro 17 running a two-way ANOVA with $\alpha<0.05$ and if there were any differences Tukey's HSD was utilized to make comparisons and determine differences. Additionally, if Tukey's HSD did not detect any differences a Student's T test was used.

RESULTS AND DISCUSSION:

Pullet phase performance:

This study showed statistically significant differences between the treatment groups during the pullet phase in terms of feed consumption per bird, and all data relating to pullet phase performance can be found in table 5.1 below. It was evident that pullets in the control group (C) tended to consume more feed than those fed the Magni-Phi (MP) diet. Additionally, it is important to note that while feed consumption was statistically significant, BW between the two treatments were not. Meaning that the birds fed the MP diets were able to consume less feed while maintaining statistically similar body weights as the control pullets. This seems to indicate an improvement in the ability of the gut to absorb nutrients in the rearing diets. These findings are further supported by previous literature, where it was found that the supplementation of saponins to broilers showed a significant improvement in gut health of challenged and unchallenged birds (Bafundo et al., 2021b). It should also be noted that statistical differences naturally occur when comparing body weight (BW) and feed intake over the animals' life. These differences in feed consumption and body weight are due to the changing physiological needs and growth trajectories of chickens throughout the various stages of development.

Lay period performance:

Several key production parameters were found to be significant when comparing experimental treatments, and all data relating to lay period performance can be found in table 5.2 below. When assessing egg production, the C-C treatment showed the highest egg production, outperforming the MP-MP and C-MP treatment groups. These differences in egg production are not supported by previous research where laying hen and broiler breeder hen production was

assessed (Alagawany et al., 2016; Souza et al., 2023). In addition, feed intake showed differences according to treatment, with higher intakes observed in the C-C and C-MP groups when compared to the MP-C and MP-MP groups. The hen-day performance of the hens also showed significant differences, with C-C and MP-C treatments showing significantly higher values than MP-MP treatment. In addition, the hen housed production showed that the C-C treatment group performed better than the C-MP and MP-MP groups. Interestingly, when the feed conversion rate (kilograms of feed per dozen eggs, FCR-1) was examined, no statistical differences were found between the treatment groups. However, when evaluating the FCR-2 (grams of eggs per gram of feed), there was a significant difference, as MP-C treatment produced a higher egg weight per feed consumed than C-MP treatment. Importantly, mortality showed no apparent differences by treatment or period, suggesting that the product does not have a detrimental impact on hen livability. These variations in production parameters are consistent with the physiological aspects of the hens and have therefore been shown to be significant when assessed on a per period basis, due to the nature of chicken biology.

Lastly, although saponins were fed to these hens, no direct disease challenge was imposed. Therefore, the full mode of action of Magni-Phi as a means of parasitic challenge reducer could not be observed. It is proposed that these differences observed are attributed to saponins promotion of intestinal health and immunity as proposed by previous research (Bafundo et al., 2021b; a). Furthermore, although gut histomorphology was not assessed in this trial, previous research has found that saponins had positive effects on gut histomorphology through an increase in villus height in broiler studies (Alghirani et al., 2021; Dai et al., 2023), which could serve as a possible explanation as to why these changes in performance were observed.

Egg size and grade distribution:

Egg size distribution data can be found in table 5.3, where egg income and net income can also be found. Interestingly, it was determined that the different treatment regimens had no significant effect on the egg size distribution when looking at extra-large eggs, large, medium, small, and pee wee eggs. Rather, the observed differences were mainly due to periodic effects. As chickens get older and go through their reproductive cycles, a natural development occurs where hens produce larger eggs. This inherent physiological aspect resulted in statistical differences in egg size distribution on a per period basis. Regarding the analysis of total egg income per rep, no significant differences between the treatment groups were observed. This suggests that total egg revenue remained relatively constant regardless of dietary treatment. However, a key conclusion came from looking at net income, an indicator that takes into account both the egg income and the cost of feed. This was an unchallenged flock at all points in the pullet rearing and lay production periods, therefore improvements in profitability due to recuperation of losses due to enteric challenges were not observed. The C-C treatment group had the highest net income compared to the C-MP group. This difference could be attributed to the additional cost of incorporating the Magni-Phi ingredient into the feed. The C-C treatment possibly had higher net income due to the additional costs associated with Magni-Phi in the other treatment groups. Lastly, when looking at the egg grading distribution (Table 5.4) it is evident that significance on a per-period basis was present, which is normal due to the hen's natural laying progression. Additionally, it can be observed that there were no differences between the treatment groups and the egg grading distribution.

Egg quality:

When exploring the analysis of shell color, egg weight, and yolk color (Table 5.5), no statistically significant differences between the treatment groups were observed. These traits remained remarkably consistent across all dietary treatments, suggesting that treatment had no significant impact on these specific aspects of egg quality. However, by evaluating the level of albumen height, a compelling pattern emerged, with the MP-C and MP-MP treated groups showing the highest levels compared to the C-C treatment group. This observation is consistent with the subsequent Haugh Unit (HU) analysis, which indeed revealed significant differences between treatment groups. As expected, the importance of HU reflected albumen height scores, with the MP-MP and MP-C treatments showing higher scores than the CC treatment group. A plausible explanation for this is Magni-Phi's potential ability to improve HU by allowing chickens to use their energy resources more efficiently for optimal egg formation during the pullet phase, and not on fighting off enteric infections like coccidia. Saponin effect on Haugh unit scores is not supported by some literature (Guclu, 2003; Alagawany et al., 2016; Souza et al., 2023); however, more research needs to be conducted to determine these differences. Furthermore, due to the proposed bettering gut health benefits of saponins, pullets could have better utilized energy and nutrients for development of a better reproductive tract compared to control fed birds. One possible explanation for the improved Haugh unit scores and albumen height, could be that Magni-Phi fed hens are able to better uptake amino acids from the feed, which in turn contributed to better deposition into the albumen. In addition, it should be noted that the observed variations per period inherently reflect the natural progression of hens' performance and size distribution, thus illustrating the different dynamics of bird biology and egg quality.

By further evaluating egg quality traits such as shell elasticity and vitelline strength and elasticity found in table 5.6, the analysis revealed that there were no statistical differences between the treatment groups. These specific parameters remained consistent across the different treatment groups, suggesting that treatment had no significant impact on these aspects of egg quality. Interestingly, there was significance when looking at shell strength, where MP-C treatment group showed higher values for shell strength when compared to C-C treatment group. These findings are partially supported by previous research, where it was determined that feeding saponins to broiler breeders improved the shell strength (Alagawany et al., 2016; Souza et al., 2023) and thickness (Souza et al., 2023) compared to control fed birds. However, it must be taken into account that when comparing shell quality attributes by period, significant differences were found. This observed difference can easily be attributed to inherent changes in chicken performance and the natural evolution of chicken biology over time. As hens age and undergo various physiological changes, these traits can be expected to change during a hen's life cycle.

CONCLUSION:

In conclusion, the effects of Magni-Phi supplementation on poultry production provided valuable insights into various aspects of hen performance, egg production, and egg quality. During the pullet phase, the birds fed Magni-Phi showed a significant reduction in feed consumption while still maintaining comparable body weights to the control birds. However, the full potential of Magni-Phi gut health impacts could only become apparent during lay, as the MP-C and MP-MP groups had lower feed consumption than the C-C and C-MP groups. Surprisingly, C-C hens outperformed other treatment groups in egg production. This trend was further highlighted by the observed importance of the daily production parameters of Hen-day

and Hen-housed parameters, with the MP-MP treatment showing the lowest production values though egg quality parameters like albumen height and Haugh unit being enhanced in the MP treatments.

Analysis of feed conversion rate (FCR-2, grams of egg per gram of feed) also showed that MP-C treatment was superior to the C-MP treatment. Furthermore, the results showed that mortality rates remained constant across all lay treatments with no statistical differences observed across the treatment groups. The economic impact was significant, with C-C treatment generating the highest net income, which could be a consequence of the lack of additional costs associated with the Magni-Phi inclusion into the other treatments. Furthermore, this current study of egg quality traits found that Magni-Phi supplementation during the pullet phase had a positive effect on albumen height and Haugh unit parameters, which could potentially be due to nutrient and energy allocation in favor of reproductive potential in the pullet phase. This improvement of albumen height and HU could be advantageous in the egg breaker market, as it appears to have an influence on quality parameters which may contribute to the amount of egg solids produced. Overall, this study explores the potential supplementation of Magni-Phi in the poultry industry as it offers a reduction of feed consumption while maintaining comparable yields from eggs compared to control treatments, and thus an economically viable solution for the production of sustainable laying hen production.

Table 5.1: Pullet performance from 0-16 weeks of life (Pullet phase) when supplemented with saponin product.[1]

	BW[2] (kg)	Feed Consumed[3] (kg per bird)
Treatment (T)		
C	0.6820	0.587[a]
MP	0.6810	0.574[b]
P-Value	0.6377	**0.0004**
SEM	0.0018	0.0028
Weeks of life (W)		
2	0.108[h]	0.157[f]
4	0.235[g]	0.302[e]
6	0.406[f]	0.438[d]
8	0.614[e]	0.547[c]
10	0.826[d]	0.771[b]
12	0.962[c]	0.783[b]
14	1.106[b]	0.872[a]
16	1.196[a]	0.776[b]
P-Value	**<.0001**	**<.0001**
SEM	0.0035	0.0055
Interaction (T x W)		
P-Value	0.8742	**<.0001**
SEM	0.0050	0.0078
CV%	3.099	5.720

[1]Data during the pullet phase was collected for all of the experimental replicates (n=36)
[2]Body weight data is on a per pullet basis where data was collected every other week from 1-16 weeks
[3]feed consumed data was obtained on a per replicate basis (n=36) and further transformed into average per pullet consumption

Table 5.2: Performance parameters for laying hens from periods 1-13 of laying hens when supplemented with saponin product. [1]

	Egg Production (Number of eggs)	Feed Consumed (kg)	Hen Day (%)	Hen Housed (%)	FCR-1 (kg feed/Doz)	FCR-2 (g egg/ g feed)	Period Mortality (%)
Treatment (T)							
C-C	572.71^{a}	64.80^{a}	86.00^{a}	85.22^{a}	1.68	0.5403^{ab}	0.1426
C-MP	565.72^{b}	64.66^{a}	85.78^{ab}	84.18^{b}	1.72	0.5355^{b}	0.2882
MP-C	570.10^{ab}	63.93^{b}	86.34^{a}	84.84^{ab}	1.72	0.5440^{a}	0.3997
MP-MP	565.61^{b}	63.95^{b}	85.08^{b}	84.17^{b}	1.81	0.5396^{ab}	0.1441
P-Value	**0.0046**	**0.0144***	**0.0004**	**0.0046**	0.0672	**0.0408**	0.1336
SEM	1.66	0.2440	0.2126	0.2471	0.0349	0.0021	0.0898
Period (P)							
1	80.94^{i}	44.57^{g}	12.05^{h}	12.05	6.91^{a}	0.083^{f}	0.0001
2	579.06^{g}	56.50^{f}	86.27^{g}	86.17	1.17^{b}	0.582^{bc}	0.1158
3	649.17^{a}	64.59^{cd}	96.83^{a}	96.60	1.19^{b}	0.583^{bc}	0.1158
4	643.44^{ab}	66.33^{bc}	96.20^{ab}	95.75	1.24^{b}	0.577^{bcd}	0.2317
5	640.42^{abc}	67.70^{ab}	96.09^{abc}	95.30	1.27^{b}	0.577^{bcd}	0.3475
6	631.56^{bc}	69.76^{a}	94.87^{bc}	93.98	1.33^{b}	0.564^{de}	0.1580
7	626.33^{cd}	69.01^{a}	94.32^{cd}	93.21	1.32^{b}	0.576^{bcd}	0.2367
8	615.69^{de}	68.97^{a}	92.93^{d}	91.62	1.35^{b}	0.568^{cde}	0.2367
9	609.97^{e}	69.01^{a}	92.52^{d}	90.77	1.36^{b}	0.569^{cde}	0.4683
10	594.25^{f}	67.92^{ab}	90.22^{e}	88.43	1.37^{b}	0.568^{cde}	0.1158
11	585.64^{fg}	68.54^{a}	89.11^{ef}	87.15	1.41^{b}	0.556^{e}	0.2417
12	571.83^{gh}	63.19^{d}	87.55^{fg}	85.09	1.33^{b}	0.591^{b}	0.5842
13	562.64^{h}	60.26^{e}	86.47^{g}	83.73	1.29^{b}	0.623^{a}	0.3575
P-Value	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	0.4448
SEM	2.99	0.4398	0.3833	0.4454	0.0628	0.0038	0.1619
Interaction (T x P)							
P-Value	0.9911	0.7938	0.4495	0.9911	**<.0001**	0.1925	0.1087
SEM	5.99	0.8796	0.7667	0.8908	0.1258	0.0076	0.3238
CV%	3.1586	4.1012	2.6807	3.1588	21.7769	4.222	4.7715

***Students T test was used to find differences between the means*

[1]Experimental lay period data was collected on a per replicate basis (n=36) from 17-69 weeks of life (periods 1-13)

Table 5.3: Egg size distribution and revenue data from periods 1-13 of laying hens when supplemented with saponin product.

	Ex Large (%)	A large (%)	Medium (%)	Small (%)	PeeWee (%)	Egg Income per rep[2] ($)	Net Income[2] ($)
Treatment (T)							
C-C	75.08	16.17	5.14	3.40	0	153.08	116.19[a]
C-MP	75.55	14.81	5.24	4.16	0.04	150.98	113.90[b]
MP-C	73.16	17.68	6.44	2.45	0	152.37	115.99[ab]
MP-MP	73.84	16.93	5.64	3.36	0	151.42	114.75[ab]
P-Value	0.1451	0.0628	0.3341	0.1058	0.3927	0.0783	**0.0235**
SEM	0.7925	0.7810	0.5549	0.4891	0.0203	0.6268	0.6042
Period (P)							
1	2.14[g]	1.53[f]	53.72[a]	42.61[a]	0	13.54[g]	-9.59[f]
2	22.85[f]	63.31[a]	13.23[b]	0.61[b]	0	145.27[f]	115.94[e]
3	46.70[e]	51.88[b]	1.18[c]	0.12[b]	0	170.64[ab]	137.11[a]
4	61.33[d]	37.57[c]	1.10[c]	0[b]	0	171.31[a]	136.87[a]
5	75.48[c]	23.91[d]	0.24[c]	0[b]	0	168.65[ab]	132.76[ab]
6	88.99[b]	10.89[e]	0.13[c]	0[b]	0	170.87[ab]	133.89[a]
7	88.97[b]	10.56[e]	0.48[c]	0[b]	0	168.79[ab]	127.73[bc]
8	94.20[ab]	5.06[ef]	0.24[c]	0[b]	0	166.99[abc]	125.94[c]
9	96.57[a]	2.51[f]	0.79[c]	0[b]	0	165.62[bcd]	124.56[cd]
10	97.54[a]	1.69[f]	0.50[c]	0[b]	0	162.35[cd]	120.20[de]
11	97.59[a]	1.30[f]	0.98[c]	0[b]	0	161.64[d]	119.10[e]
12	97.36[a]	1.58[f]	0.13[c]	0.13[b]	0	154.92[e]	115.70[e]
13	97.91[a]	1.38[f]	0.28[c]	0[b]	0	164.94[e]	117.54[e]
P-Value	**<.0001**	**<.0001**	**<.0001**	**<.0001**	.4480	**<.0001**	**<.0001**
SEM	1.43	1.41	1.00	.8817	.0367	1.13	1.09
Interaction (T x P)							
P-Value	0.9992	0.9998	.1569	**.0039**	.4725	.7152	.7670
SEM	2.86	2.82	2.00	1.76	.0733	2.26	2.18
CV%	11.5173	51.5192	106.8852	158.2140	2163.3173	4.4618	5.6730

[1]During the first day of the third week of each period, all eggs produced in a span of 24 hours were collected per replicate (n=36) and used for egg size distribution

[2] Experimental lay period income data was collected on a per replicate basis (n=36) from 17-69 weeks of life (periods 1-13)

Table 5.4: Egg Grade distribution from periods 1-13 of laying hens when supplemented with saponin product.

	A (%)	B (%)	Check (%)	Loss (%)
Treatment (T)				
C-C	94.20	0.391	5.32	0.0856
C-MP	94.20	0.156	5.64	0
MP-C	94.09	0.268	5.56	0.0902
MP-MP	93.74	0.204	5.94	0.1204
P-Value	0.8558	0.3148	0.6323	0.5690
SEM	0.5299	0.0954	0.5066	0.0625
Period (P)				
1	98.92^{a}	0^{b}	1.08^{e}	0
2	97.88^{ab}	0^{b}	2.12^{de}	0
3	96.79^{abc}	0.116^{ab}	3.09^{cde}	0
4	95.34^{abcd}	0.132^{ab}	4.53^{bcde}	0
5	97.82^{ab}	0.116^{ab}	2.06^{de}	0
6	93.81^{bcde}	0.863^{a}	5.33^{bcde}	0
7	94.44^{abcd}	0^{b}	5.56^{bcd}	0
8	92.60^{cde}	0.374^{ab}	7.02^{abc}	0
9	92.70^{cde}	0^{b}	7.16^{abc}	0.139
10	91.40^{de}	0.917^{a}	7.34^{ab}	0.139
11	89.39^{e}	0.374^{ab}	10.10^{a}	0.132
12	92.08^{de}	0.139^{ab}	7.53^{ab}	0.253
13	89.55^{e}	0.278a^{b}	9.87^{a}	0.300
P-Value	**<.0001**	**.0002**	**<.0001**	0.5241
SEM	.9553	.172	.9132	0.1127
Interaction (T x P)				
P-Value	.5846	.7880	.6260	.6102
SEM	1.91	.3438	1.83	.2253
CV%	6.0938	405.1024	97.5862	912.5187

[1]During the first day of the third week of each period, all eggs produced in a span of 24 hours were collected per replicate (n=36) and used for egg grade distribution

Table 5.5: Egg quality characteristics from period 1-13 of laying hens when supplemented with saponin product.[1]

	Shell Color (%)*	Egg Weight (g)	Albumen Height (mm)	Haugh Unit (HU)	Yolk Color (Roche fan)
Treatment (T)					
C-C	79.91	61.05	7.53^{b}	85.70^{b}	7.47
C-MP	80.24	61.18	7.63^{ab}	86.46^{ab}	7.38
MP-C	79.56	60.96	7.83^{a}	87.61^{a}	7.41
MP-MP	80.37	60.78	7.76^{a}	87.06^{a}	7.41
P-Value	0.1658	.6115	**.0008**	**.0008**	.3589
SEM	0.277	.218	.056	.346	.034
Period (P)					
1	75.46^{e}	48.97^{f}	8.23^{ab}	93.25^{a}	8.01^{a}
3	75.63^{e}	57.77^{e}	7.95^{bc}	88.88^{bc}	6.52^{e}
5	80.89^{c}	60.47^{d}	7.76^{cd}	87.35^{c}	7.18^{d}
7	85.38^{a}	62.98^{c}	8.29^{a}	89.85^{b}	7.98^{a}
9	83.80^{b}	64.43^{b}	7.30^{e}	83.77^{d}	7.44^{c}
11	79.80^{cd}	66.04^{a}	7.54^{de}	84.37^{d}	7.66^{b}
13	79.17^{d}	66.27^{a}	6.75^{f}	79.47^{e}	7.13^{d}
P-Value	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**
SEM	0.366	0.289	0.074	0.457	0.045
Interaction (T x P)					
P-Value	0.0895	0.9743	0.2759	0.4339	0.8125
SEM	0.733	0.578	0.148	0.914	0.090
CV (%)	6.73	6.96	14.20	7.50	8.92

**Shell color is determined as the % light reflectance off of the egg shell.*

[1] Egg quality parameters were collected during experimental lay periods 1, 3, 5, 7, 9, 11, and 13. Egg quality parameters were evaluated by assessing traits of 6 eggs per replicate for each measurement (n=36).

Table 5.6: Shell and vitelline strength and elasticity through periods 1-13 of laying hens when supplemented with saponin product.[1]

Treatment (T)	Shell Strength (N)	Shell Elasticity (mm)	Vitelline Strength (N)	Vitelline Elasticity (mm)
C-C	42.99^{b}	0.248	0.0203	1.65
C-MP	43.84^{ab}	0.239	0.0197	1.54
MP-C	44.56^{a}	0.242	0.0203	1.61
MP-MP	43.35^{ab}	0.252	0.0197	1.61
P-Value	0.0415	0.206	0.0893	0.1481
SEM	0.411	0.0048	0.0002	0.0355
Period (P)				
1	45.51^{b}	0.238^{b}	0.0225^{a}	1.76^{ab}
3	43.40^{bc}	0.247^{ab}	0.0221^{ab}	1.87^{a}
5	43.24^{c}	0.248^{ab}	0.0201^{c}	1.64^{bc}
7	48.03^{a}	0.271^{a}	0.0181^{d}	1.46^{cd}
9	43.22^{c}	0.237^{b}	0.0209^{bc}	1.82^{ab}
11	45.45^{bc}	0.241^{b}	0.0181^{d}	1.38^{d}
13	36.9685^{d}	0.238^{b}	0.0183^{d}	1.30^{d}
P-Value	**<.0001**	**0.0013**	**<.0001**	**<.0001**
SEM	0.543	0.0063	0.0003	0.0470
Interaction (T x P)				
P-Value	0.5580	0.5166	0.1942	0.0618
SEM	1.09	0.0126	0.0001	0.0940
CV%	18.28	37.65	23.14	43.07

[1] Egg quality parameters were collected during experimental lay periods 1, 3, 5, 7, 9, 11, and 13. Egg quality parameters were evaluated by assessing traits of 6 eggs per replicate for each measurement (n=36).

Table 5.7: Nutritional Formulation Breakdown for Pullet Rearing Phase Experimental Diets

Ingredients (%)	Starter (0–8weeks) Control/ Magni-Phi	Grower (8-12 weeks) Control/ Magni-Phi	Developer (12-16 weeks) Control/ Magni-Phi
Yellow Corn	62.330/62.320	61.425/61.335	62.942/62.872
Wheat Bran		9.652/9.609	10.362/10.319
SBM	33.280/33.170	24.665/24.654	22.187/22.177
Limestone	1.890/1.890	1.769/1.767	2.129/2.127
Dical. Phos.	1.740/1.750	1.742/1.743	1.593/1.594
Salt	0.250/0.250	0.250/0.250	0.250/0.250
Magni-Phi	0.000/0.125	0.000/0.125	0.000/0.125
L-Lys		0.060/0.060	0.018/0.018
DL-Met	0.154/0.154	0.137/0.136	0.069/0.069
Choline Chloride			0.150/0.150
Mineral Premix[1]	0.200/0.200	0.200/0.200	0.200/0.200
Vitamin Premix[2]	0.050/0.050	0.050/0.050	0.050/0.050
Selenium Premix[3]	0.050/0.050	0.050/0.050	0.050/0.050
Amprolium[4]	0.050/0.050		
Nutrients			
AMEn (Kcal/kg)	2900	2800	2750
CP (%)	20.8	18.50	17.50
Lys (%)	1.10	1.00	0.90
TSAA (%)	0.85	0.73	0.65
Ca (%)	1.10	1.10	1.20
Av. P. (%)	0.45	0.45	0.42

[1]Mineral premix provides per kg of diet: manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; and cobalt.

[2]Vitamin premix provides per kg of diet: 13,200 IU vitamin A, 4000 IU vitamin D3, 33 IU vitamin E, 0.02 mg vitamin B12, 0.13 mg biotin, 2 mg menadione (K3), 2 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 4 mg vitamin B6, 55 mg niacin, and 1.1 mg folic acid.

[3]Selenium premix=1 mg Selenium premix provides 0.2 mg Se (as Na2 SeO3) per kg of diet. ME=metabolizable energy

[4]Used as a means of coccidia control, organic compound

Table 5.8: Laying Hen Feed Formulation From 17-69 Weeks of Age

Ingredient	Control	Magni-Phi
	(%)	(%)
Corn	51.842	51.799
Soybean meal	32.241	32.175
Soybean Oil	3.729	3.712
D.L. Methionine	0.181	0.181
Ground Limestone	4.600	4.600
Coarse Limestone	5.000	5.000
DiCalcium Phosphate	1.809	1.809
Magni-Phi	0.000	0.125
Salt	0.250	0.250
Vitamin Premix	0.200	0.200
Trace Mineral Premix	0.050	0.050
Selenium Premix	0.050	0.050
Choline Cl 60%	0.050	0.050
Calculated Analysis		
Protein %	19.5	19.5
Calcium %	4.14	4.14
A. Phos. %	0.45	0.45
Lysine %	1.1	1.1
TSAA %	0.83	0.83
ME kcal/kg	2922	2922

[1]Mineral premix provides per kg of diet: manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; and cobalt.

[2]Vitamin premix provides per kg of diet: 13,200 IU vitamin A, 4000 IU vitamin D3, 33 IU vitamin E, 0.02 mg vitamin B12, 0.13 mg biotin, 2 mg menadione (K3), 2 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 4 mg vitamin B6, 55 mg niacin, and 1.1 mg folic acid.

[3]Selenium premix=1 mg Selenium premix provides 0.2 mg Se (as Na2 SeO3) per kg of diet. ME=metabolizable energy

Table 5.9: Analyzed feed values during the pullet and lay periods.[1]

Analyte	Pullet Phase (0-16 weeks)						Lay Phase (17-69 weeks)	
	Starter	Starter MP	Grower	Grower MP	Developer	Developer MP	Layer Diet D	Layer Diet D MP
Dry Matter (%)	86.34	86.89	87.11	87.35	88.69	88.29	88.42	88.53
Crude Protein (%)	22.75	22.45	21.11	19.97	18.49	19.14	18.18	21.57
Neutral Detergent Fiber (%)	10.33	9.57	10.32	10.41	10.05	10.41	7.58	8.25
Acid Detergent Fiber (%)	4.45	4.53	4.73	4.58	3.98	4.01	3.30	4.22
Non-fiber Carbohydrate (%)	60.00	61.44	61.99	63.10	62.11	60.84	48.22	50.72
Fat (%)	3.73	3.09	3.25	3.44	2.72	2.93	5.63	7.42
Calcium (%)	1.04	1.16	1.28	1.15	2.48	2.49	3.48	4.49
Phosphorus (%)	0.88	0.85	0.90	0.80	0.83	0.90	0.67	0.83
Sulfur (%)	0.29	0.30	0.27	0.27	0.28	0.28	0.27	0.33
Magnesium (%)	0.21	0.20	0.20	0.20	0.19	0.19	0.22	0.24
Sodium (%)	0.10	0.13	0.13	0.11	0.11	0.11	0.14	0.16
Potassium (%)	1.14	1.12	1.04	0.96	0.86	0.90	0.85	1.02
Copper (ppm)	16.91	19.80	20.23	18.17	28.24	27.78	19.00	17.36
Iron (ppm)	278.47	265.06	299.31	260.12	484.38	533.12	317.00	423.15
Manganese (ppm)	150.58	136.14	157.91	131.09	199.93	230.99	124.00	177.17
Zinc (ppm)	172.24	176.36	198.16	173.42	207.15	219.51	146.00	190.01
Ash (%)	6.32	6.58	6.54	6.30	9.69	9.68	11.23	14.74

[1]Values were determined in a dry matter state through the Steve Troxler Agriculture Science Center, North Carolina Department of Agriculture & Consumer Services (Food & Drug Protection Division, Animal Feed Program)

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CHAPTER VI

Overall conclusions

In the beginning of modern laying hen production systems in the 1920's, hens were in free range settings where foraging and ample space was available for hens. Due to increased demands for protein and modernizations in management, laying hen production shifted to caged environments. For the past decades, laying hens have been selected and optimized to reside in caged environments. Where now, strong consumer demands have shifted laying hen production to housing environments where more space per hen is required. This consumer shift is due to perceived welfare concerns due to conventional cages and its space allowances. Laying hen egg production, in a way is moving backwards, as a great shift from conventional cages back to alternative designs like cage free and free range is occurring. Previous research in the literature has determined that caged hen management allows for complete control of feeding, ease of removal of low producing hens, and overall cleaner eggs due to greater separation from feces. Lastly, with a shift away from conventional cages, increased land use for hen production will be required and prices will increase. Leaving many protein nutritional requirements unmet by certain socioeconomic groups of people as egg prices and population protein requirements increase.

The rationale in the stocking density experiments contained in this dissertation is to explore greater space allowances in the caged environment, and its effects on hen production, stress responses, and fearfulness. Furthermore, exploration of stocking density in the pullet phase effects on laying hen egg production was also explored. One of the main findings from the pullet experiment was that pullets in 48 in^2 achieved higher body weights while consuming less feed compared to 69 in^2 pullets. Where a possible explanation could be that 69 in^2 pullets had higher

space allowances in the caged environment, which allowed for more movement and subsequent more energy expenditure into those movement actions. Furthermore, better performance parameters of 48 in^2 pullets had a fearfulness score trade off, where TI times and corticosterone levels were significantly increased compared to other treatments. In the lay phase, however, it was observed that the hens from the highest pullet stocking density of 48 in^2 resulted in carry over effects as they offered the highest values for HD%, feed consumed, and egg production, which can all point to better production as a whole regardless of lay stocking density. It was additionally observed that no statistical differences in heterophil to lymphocyte ratios, plasma corticosterone, and tonic immobility were observed in the lay phase. These findings point to early life pullet stocking densities playing a big impact on overall pullet performance and stress parameters and subsequent performance parameters in laying hens. It is hypothesized that as hens move into the laying phase, they adapt to cage density stress and for this reason no differences in heterophil to lymphocyte ratios, plasma corticosterone, and tonic immobility were observed during the lay phase. These findings offer great impacts to the laying hen industry, as this experiment determined that hens in higher stocking densities do not seem to have higher stress parameters, which is contradictory to consumer notions. In addition, as the world population increases the requirement for protein will also continue to increase, it is important that the industry moves forward in a path that is backed by research and not solely on consumer demands.

Lastly, the saponin phytobiotic trial offered interesting results where performance changes occurred although the animals remained in a n unchallenged state. Overall, the findings pointed to pullets fed Magni-Phi (MP) showing a significant reduction in feed consumption while still maintaining statistically similar body weights to the control birds. Which could serve

useful for the industry as the main costs in animal rearing are feeding costs. Additionally, hens fed MP during the lay phase also continued to consume less feed overall. Furthermore, MP fed hens showed improvements in egg quality parameters of albumen height, Haugh unit, and shell strength. As this was an unchallenged trial, it is deemed that the main differences in production and egg quality are due to changes in the hen's gut, where the saponin feed additive is promoting intestinal health and immunity.