Molt and the Immune System of Laying Hens

By

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ABSTRACT

Feathers are complex structures essential to a bird's survival which must be periodically replaced by molting. The process of molt in birds is characterized by multiple physiological changes and is known to be expensive in terms of the nutrients required to produce quality feathers quickly. However, little is understood about the relationship between the costly processes of molt and immune function. The overall objective of this dissertation was to determine how molt affects the immune system in regard to both systemic shifts and a potential trade-off between molt and mounting an innate immune response. In the first experiment, we investigated how the spleen, thymus, and inflammatory state of the domestic chicken (*Gallus domesticus*) shifts over the course of molt. Half of the adult laying hens (N = 36) were induced to molt using a combination of oral thyroxine (T4), a reduced calorie diet and a shift from a long day to a short-day schedule. Blood, liver, spleen, and thymus samples were collected weekly to assess changes over the course of molt. To determine if molt induced either a pro- or anti-inflammatory state, the level of key pro- and anti-inflammatory cytokines were evaluated via qPCR and ELISA. The spleens of molting birds were 63% heavier than the spleens of non-molting birds, and the thymus weight of molting birds increased 6.8-fold ($P < 0.01$). Upon histological examination, the thymus of molting birds had clearly defined lobules and an increase in cortex and medulla tissue ($P < 0.01$). Molting birds had a significantly higher proportion of double-positive T cells in the thymus than non-molting birds ($P < 0.01$). Overall, the increase in mass of both the thymus and the spleen resulted in molting birds having a greater number of T cells. Both IL-8 and TNF-α expression increased in the liver during molt, but overall, there was no clear indication that molt leads to an increase in inflammation during feather growth. During molt, there are marked changes in the weight and lymphocyte populations of the spleen and

thymus which may be driven by molt or triggered by the hormonal shifts that occur with molt. In the second experiment, molting birds were subjected to an innate immune challenge to clarify how the nutritional requirements of molt may alter investment in an immune response. Adult hens (N = 16) were induced to molt using the same combination of cues described for experiment one. During week four of molt, birds were challenged with an intra-abdominal injection of lipopolysaccharide (1.5 mg/kg) to induce an innate immune response. Four hours post injection, liver, spleen, and blood samples were collected to evaluate changes in the expression levels and plasma concentration of key pro- and anti-inflammatory cytokines. The weight of the spleen and thymus were measured to assess changes in lymphoid tissue mass during molt. The spleen and thymus were both significantly heavier in molting birds than non-molting birds ($P < 0.01$). Based on inflammatory cytokine expression levels in the liver and spleen, both molting and non-molting birds mounted an innate immune response to LPS, but the response was dampened in molting birds. The expression levels of IL-6 and IL-8 were significantly reduced in molting birds. The concentration of IL-1β and IL-6 in the plasma of molting birds was also significantly reduced compared non-molting birds indicating that production of protein as part of the inflammatory response may be attenuated due to competing demands for protein deposition in feathers. The third experiment used a unique genetic line, the Scaleless High (ScHi) line, to further examine the trade-off between molt and an innate immune response by mitigating the cost of feather production. ScHi birds have reduced feathering due to a genetic mutation and were used to determine if the trade-off between molt and the immune system is driven mainly by the mass of feathers grown during molt or by the broader process of molt and the accompanying hormonal and metabolic changes. Adult hens from both genetic lines (N = 32) were induced to molt using the combination of hormonal, light, and dietary signals.

During week four of molt, half of the birds in each genetic line were challenged with an intra-abdominal injection of lipopolysaccharide (1.5 mg/kg) to induce an innate immune response. Five hours after the LPS injection, samples were collected from the liver and spleen to evaluate the expression of pro- and anti-inflammatory cytokines. The weights of the spleen and thymus were measured to determine if systemic changes in these lymphoid tissues during molt were dependent on the mass of feathers growing during molt. The spleen and thymus significantly increased in weight during molt in both genetic lines ($P < 0.01$). The thymus weight increased 7.8-fold while the spleen weight increased an average of 48% across molting birds from both genetic lines. The area of cortex in the thymus unexpectedly increased more in molting reduced feathering ScHi birds than in the fully feathered UCD-003 birds. The inflammatory response to LPS was dampened in both genetic lines, especially the expression levels of IL-6 and IL-8. The overall process of molt, rather than the mass of feathers grown, appears to drive the trade-off between molt and the immune system when both processes must occur concurrently. This research helps clarify how a nutritionally expensive component of immunity, the inflammatory response, is affected by molt when birds must balance the costs of the immune system with the costs of molting. When molt and an innate immune response must occur concurrently, the inflammatory response is dampened, likely as a mechanism to allow both molt and an innate immune response to progress during an energetic or nutritional bottleneck.

DEDICATION

This dissertation is dedicated to all the chickens that made the research possible.

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Figure A.2 The effect of oral thyroxine dose on body weight. Body weight was measured twice a week during each week of molt. There was an overall effect of week ($P < 0.01$) and a significant interaction between week and treatment ($P < 0.01$). All birds lost weight during the experimental period, even the control birds fed a commercial diet.

Figure A.3 The effect of oral thyroxine dose on body weight change. The change in body weight shown is relative to the pre-molt period. Birds from each treatment group experienced peak weight loss during week five.

Figure A.4 The effect of thyroxine supplementation and molt cues on laying rate. Adult laying hens were induced to molt with a change in photoperiod and a molt diet. Hens in the thyroxine treatment group also received oral thyroxine in their feed. Thyroxine led to a faster cessation of lay in the thyroxine treatment group compared to the hens that only received light and dietary cues.

Figure A.5 The effect of thyroxine supplementation and molt cues on body weight. Body weight and the change in body weight were measured in hens induced to molt with photoperiod and diet alone or photoperiod, diet, and oral thyroxine. A. There was a significant interaction between molt week and thyroxine due to thyroxine treated birds having the lowest body weight during the first week of molt. B. Both birds receiving thyroxine and those not receiving thyroxine had a significant decrease in body weight relative to the pre-molt period.

Figure A.6 The effect of thyroxine supplementation and molt cues on the spleen and thymus. At the end of two weeks of molting the organ and spleen were weighed. There was no difference in the spleens of birds supplemented with thyroxine and those without. Thyroxine supplementation led to a significant increase in the weight of the thymus ($P < 0.01$).

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
°C	Degrees Celsius
cDNA	Complementary deoxyribonucleic acid
Ct	Threshold value
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IL-1β	Interleukin-1-Beta
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
LPS	Lipopolysaccharide
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcription PCR
RNA	Ribonucleic acid
RPL13	Ribosomal protein L13
RT-PCR	Reverse-transcription polymerase chain reaction
ScHi	Scaleless high genetic line
TNF-α	Tumor necrosis factor alpha
TGF-β	Transforming growth factor beta
UCD-003	UCD-003 Genetic Line

Chapter 1

Review of the Literature

Introduction

Feathers are a defining characteristic of all avian species and essential for survival. Not only do feathers allow for flight, but they also serve additional important functions such as attracting mates, thermoregulation and serve as a protective barrier for the skin. The structure of feathers is complex to allow for flight while remaining incredibly light weight. Over time, the integrity of feathers begins to degrade due to ultraviolet exposure or they are damaged making it necessary for birds to periodically replace their feathers via the molting process. Birds will undergo multiple molts to produce different plumages throughout their lives. Molt involves replacing not only feathers, but also associated integumentary structures such as the feather sheaths (Alfred M. Lucas, 1972). Molt is considered to be both nutritionally and energetically costly as birds must produce high quality feathers that are mostly protein. The process of molt is characterized by both the regeneration of the feathers but also by broader physiological changes. One of the systems influenced by molt is the immune system, although how the immune system changes during molt and if molt affects the ability of a bird to mount an immune response remains difficult to define. To understand the interactions between molt and the immune system, it is necessary to first discuss the complexities of feather growth and the physiological shifts associated with molt.

Regulation of Molt

The generation of new feathers during molt occurs at the feather follicle through a series of complex and carefully controlled steps. The mesenchymal and epithelia components of the

feather follicle collectively produce a new feather during molt. The major mesenchymal structures of the feather follicle are the dermal papilla and dermal pulp, which will form the new feather (Lucas and Stettenheim, 1972). The stem cells needed for regeneration of a new feather are located in the collar bulge, one of the epithelial components of the feather follicle (Yue et al., 2005). Another important epithelial component of the feather follicle is the ramogenic zone where the structural components of the feather, like the rachis, will differentiate (Lin et al., 2006). The activity of the feather follicle is divided into three stages: initiation, growth, and rest (Lucas and Stettenheim, 1972). The initiation phase of molt occurs when crosstalk between the mesenchymal and epithelial components of the feather follicle initiates the growth of a new feather. As the new feather starts to grow, the old feather will be pushed out of the feather follicle and dropped (Watson, 1963; Yue et al., 2005). As the feather follicle progresses into the growth phase, the dermal papilla grows upwards into the feather follicle to form dermal pulp also known as the feather pulp. Although there are many physiological similarities between hair growth and feather growth, the development of the dermal pulp is unique to the process of feather growth (Schneider et al., 2009). As growth continues, the dermal pulp becomes heavily vascularized as invading endothelial cells form the capillaries needed to transport nutrients into the growing feather. When the end of molt is reached, the feather pulp, blood vessels, and all innervating nerves break down as the feather becomes fully keratinized and the follicle enters the resting phase (Lucas and Stettenheim, 1972). The feather growth across a bird's body follows a sequential pattern during molt. The pressure for birds to molt feathers in a sequential pattern so that they can retain maximum functionality during molt is so evolutionarily conserved that even feathered dinosaurs may have molted in sequential patterns (Kiat et al., 2020).

At the level of the whole organism, the induction of molt is controlled by a combination of environmental and physiological cues. A change in photoperiod is one of the major cues known to induce molt in many avian species. Most species of temperate zone birds will molt in the spring as daylength begins to increase from short daylight hours or in the fall as daylength shortens (Kendeigh and Lesher, 1941; Brake and Thaxton, 1982). Once sexual maturity is reached, chickens will usually molt once a year in the fall as daylight hours reduce from long days in the summer to shorter days in the fall (Lucas and Stettenheim, 1972). Although photoperiod is a fundamental cue for the initiation of molt, there are other important cues that work in conjunction with shifts in photoperiod to trigger molt. The diet of a bird also has a strong modulatory effect on molt patterns. Calorie reduction or altered amounts of specific nutrients can reliably induce molt in chickens. Forced molt programs that rely on diet to induce molt in laying hens include feed withdrawal or low calcium, low sodium, or high zinc diets (Douglas et al., 1972; Brake and Thaxton, 1979b; Shippee et al., 1979; Herrick and Ernest, 1981; Webster 2003). Reproductive status is another important controller for the timing of molt. For many species, peak reproduction and molt do not tend to overlap. Species of birds that do overlap molt and reproduction usually live in environments where food may become very abundant for part of the year or have a molting strategy that involves a very slow molt over the course of many months (Payne, 1972). Changes in photoperiod, diet and reproduction all produce the changes in hormone concentrations that lead to molt.

Concentrations of thyroid, stress and reproductive hormones are all essential to ensuring molt progresses properly and structurally sound feathers are generated. The effect of reproductive hormones on molt varies based on the specific hormone. Treatment with high levels of exogenous progesterone or GnRH can induce molt in laying hens and lead to a cessation of

lay (Spearman, 1971). Interestingly, blocking thyroid activity while also administering high concentrations of progesterone prevents molt, indicating endogenous progesterone induces molt indirectly through the thyroid. Exogenous estrogens or androgens can override cues from changes in photoperiod to slow and even arrest molt (Payne, 1972; Schleussner et al., 1985). The thyroid hormones, triiodothyronine (T3) and thyroxine (T4), are necessary for both molt and proper feather growth. Oral thyroxine can be used to induce laying hens to molt (Sekimoto et al., 1987; Bass et al., 2007). Thyroidectomy prevents a complete molt and leads to very slow and abnormal feather growth (Spearman, 1971). Thyroxine acts not only at a systemic level, but also at the level of the feather follicle. An application of thyroxine to the skin around a feather follicle will induce feather growth (Spearman, 1971). The levels of glucocorticoids during molt appears to vary across species. Passerines experience a drop in glucocorticoid concentration during their molt whereas chickens may have an increase in glucocorticoid levels at the onset of molt (Brake et al., 1979; Romero, 2002; Cornelius et al., 2011). The fluctuations in hormone levels during molt help instigate a range of metabolic and anatomical shifts characteristic of the process of molt.

Energetics of Molt

One of the characteristics of molt observed across avian species is an increase in metabolic rate during molt. The process of molt involves replacing a range of protein rich integumentary tissues including not only feathers but also feather sheaths and other keratinized structures. When birds molt the regeneration ratio by mass for feathers, feather sheaths and corneous structures is estimated to be 20:4:1 in White-crowned Sparrows with the ratio expected to hold true for many avian species (King and Murphy, 1990). Feathers and the related integumentary tissues replaced during molt are extremely protein dense tissues. The dry mass of

a feather is 90% protein and combined the plumage of a bird makes up nearly 25% of the total protein in a bird's body (Chilgren, 1977; Murphy and King, 1984a, c, b). Feathers have a unique amino acid profile compared to other protein dense tissues such as muscle. The most abundant amino acids in feathers are cysteine, glycine, and proline (Murphy and King 1986a). The main structural protein found in feathers is B-keratin, which is cysteine dense and results in the high proportion of cysteine found in feathers (Busch and Brush, 1979; Brush and Wyld, 1982; Stettenheim, 2000). To generate sufficient protein for feather growth, whole-body protein synthesis increases during the course of molt. During peak feather production, the whole-body protein synthesis of White-crowned Sparrows increases 33% above baseline levels (Murphy and Taruscio, 1995). Despite the increased protein accretion required to form feathers, the estimated dietary protein levels needed to support molt are calculated to be 10% of dietary protein from White-crowned Sparrows and between 8.6 – 12.4 % dietary protein for chickens recovering from a forced molt (Harms, 1983; Hoyle and Garlich, 1987; Murphy and King, 1991).

Although a low percentage of dietary protein is needed to support feather generation, the absolute amount of protein required increases during molt. Molting birds may increase daily food intake to ensure increased absolute protein consumption or utilize endogenous proteins sources. Increased levels of 3-methylhistidine indicate increased muscle degradation during molt which may be essential to supporting continual feather growth overnight (Taruscio and Murphy, 1995). Feather production requires about 75% the dietary maintenance amount of cysteine and 45% the maintenance amount of valine (Murphy, 1993). Beyond the specific nutritional requirements of molt, there are also increased energetic cost associated with molt. In White-crowned Sparrows, the energy needed to support peak feather growth is equivalent to 58% of the basal metabolic rate (Murphy and King, 1992). During molt, there are physiological and behavioral changes that

span beyond feather growth that are likely important contributors to the increase in metabolic rates seen across species during molt (Buttemer et al., 2019).

When birds undergo molt, there are physiological changes that occur that span beyond the integumentary system. For many bird species, molt occurs after a reproductive period when body mass and fat stores will be at their lowest point of the year (Chilgren, 1977; Bryant and Tatner, 1988). The reproductive organs, such as the oviduct and testes, are catabolized during molt and could represent a useful source of additional amino acids for feather growth when muscle mass is already low. Domestic chickens undergoing an induced molt will cease laying for the duration of molt. The period following induced molt in domestic chickens is characterized by a rejuvenation of laying in older hens, which gave rise to the practice of inducing molt in laying hens in the poultry industry (Webster, 2003). Molt in the domestic chicken is also accompanied by a decrease in adrenal weight and liver weight. The decrease in liver weight can, at least in part, be attributed to the decreased egg production (Brake and Thaxton, 1979b). The remaining anatomical shifts documented during molt are related to the lymphoid tissues of birds.

Molt and the Immune System

During molt there are noted shifts in the lymphoid tissues of birds, but it is not well understood why the shifts occur or how molt may affect immune function. The fundamental concept of ecoimmunology is that immunity is an essential component of an organism's biology influenced by life history and ecological trade-offs (Brock et al., 2014). By studying animals in the context of their ecological environments, rather than isolated from them, it has been shown that immune function can vary due to multiple factors including habitat, time of year, age, and natural history (Ots et al., 2001; Tieleman et al., 2005; Martin et al., 2008). For birds, there are a variety of nutritional costly life stages that must be balanced with the energetic costs of the

immune system. Of these different processes, molt is one of the most significant and essential to survival. Molt is expensive in terms of nutrient requirements due to metabolic costs associated with producing high quality feathers quickly, because their absence has very negative impacts on thermoregulation and flight needed for finding food, avoiding predators, and migrating. The trade-offs between investments in self-maintenance (e.g. immunity, antioxidant defenses) have been shown to be important during growth, migration, and reproduction, but the relationship between molt and the immune system remains unclear (Murphy and King, 1991; Lindstrom et al., 1993; Swaddle and Witter, 1997; Hemborg et al., 2001; Hegemann et al., 2012a). The energy expenditure during molt cannot be explained by the known costs of synthesizing new feathers, based on known costs of protein synthesis for tissue growth or egg production (Murphy and King, 1991, 1992). There are a variety of theories to justify the high metabolic cost of molt and apparently poor efficiency of feather growth, including differences in life histories and body size. Basal metabolic rate seems to be the most strongly correlated with increased molt costs, but even when body size is considered additional metabolic costs are still unaccounted for (Lindstrom et al., 1993; Hoye and Buttemer, 2011).

The majority of research aimed at defining the effects of molt on the immune system has been focused on the forced molt used in commercial egg production to reproductively rejuvenate laying hens. A forced molt induced through feed withdrawal leads to gross changes in the spleen and thymus of hens. In the spleens of molting hens, the number of apoptotic cells decreases while the number of agranulocytic cells like lymphocytes and macrophages increase (Brake et al., 1985). The cellular changes in the spleens of hens undergoing a forced molt do not clearly correlate with weight changes of the spleen (Brake and Thaxton, 1979b). The spleen weight of Willow Tits increases during molt, indicating that molt may be accompanied by changes in

spleen mass for some species (Silverin et al., 1999). The liver, which has an essential role in the acute phase response, decreases in weight when hens molt (Brake and Thaxton, 1979b). Finally, there is a recrudescence of the thymus during molt. Molting hens have an increased number of lymphocytes in the thymus (Brake et al., 1981). The thymus of tropical Yellow-vented Bulbuls increases in size during molt (Ward and D'Cruz, 1968). The Bursa of Fabricius appears to remain involuted during molt; there is no mention of changes in cellularity or size of the Bursa of Fabricius during molt in any avian species. Molt results in not only gross anatomical shifts in the spleen and thymus, but also shifts in the populations of different immune cells.

The populations of immune cells important for innate and adaptive immunity are altered during molt. In birds undergoing a forced molt, the circulating number of leukocytes decreases during the first two weeks of molt. After the second week of molt, the leukocyte numbers increase for the remainder of molt into the post molt period (Alodan and Mashaly, 1999). Monocyte numbers are elevated in birds undergoing an induced molt from two weeks into molt through the end of molt. The effect of molt on heterophils, eosinophils and basophils are more variable. In one trial, heterophils and eosinophils were increased the first two weeks of molt. In a replication of the trial, the number of eosinophils and basophils increased instead (Brake et al., 1982). There is also an increase in the heterophil to lymphocyte ratio throughout the entire molt period in domestic hens (Brake and Thaxton, 1979a; Brake et al., 1982; Alodan and Mashaly, 1999). The numbers of different lymphocyte populations also increase during molt. The percentage of $CD4^+$ T cells in the blood increases during day three to ten of molt while the percentage of $CD8^+$ T cells in the spleen of molting birds are elevated. Molting and non-molting birds have equivalent percentages of B cells in the spleen and blood (Holt, 1992a). These shifts in immune cell populations during molt imply that immune function may be shifting during molt,

although whether the shifts indicate increased activation or suppression of the immune system remains unclear.

In addition to the gross anatomical changes and changes in circulating cells numbers, the functional capacity of the immune system is also be affected by molt. Both the phagocytic and oxidative burst ability of macrophages appears to increase during molt, however the equivalent activity of heterophils decreases (Kogut et al., 1999; Sandhu et al., 2007b). Interestingly, the functionality of cell-mediated and humoral immunity seems to be differentially affected by molt. Molting birds have a decreased delayed-type hypersensitive response, but both molting and non-molting birds have equivalent antibody responses to sheep red blood cells (Holt, 1992b; Alodan and Mashaly, 1999). Birds that have undergone a zinc induced forced molt have higher antibody production in response to sheep red blood cells after molt (Sandhu et al., 2007a). The clearest indication that molt influences the immune system comes from studies looking at the effect of molt on *Salmonella enteriditis* infection. During the forced molt period, there is an increase in *Salmonella enteriditis* susceptibility and resulting pathology (Holt and Porter, 1992, 1993). The majority of the cited studies used laying hens at the end of their production cycles (60+ weeks of age) and induced molt by withdrawing feed, which led to body weight losses close to or above 30%. The protocols associated with extreme feed withdrawal used in older studies increase corticosterone levels and lead to metabolic changes (Brake, 1992; Webster, 2003). Therefore, although the trends observed may be used to describe the general effects that molt may have on the immune system, it is difficult to separate the effects of stress from food and water deprivation from the effects of molt.

Beyond the effects of molt on the immune system of domestic poultry, the trade-off between molt and the immune system has been investigated by ecoimmunologists in wild

species. In both juvenile and adult sparrows, the number of basophils and monocytes increased during molt while the number of lymphocytes and eosinophils stayed constant (Nava et al., 2001). An increase in basophil and monocyte number during molt has also been observed in Mallard Ducks (Driver, 1981). In Savi's Warblers, molt reduced the heterophil to lymphocyte ratio and increased the number of circulating leukocytes, heterophils and lymphocytes (Kulaszewicz et al., 2015). Hematocrit measurements, reflecting percentages of red blood cells, were reduced in a variety of species during molt (Ellis et al., 2012).

Similar to domestic chickens, alterations in the functional capacity of the immune system have been observed during molt in wild birds. The bactericidal capacity of the blood, which is indicative of the strength of an organism's constitutive innate immunity, has been shown to both increase and decrease during molt. Bactericidal activity of the blood was increased during molt in House Sparrows and Rufous-collared Sparrows (Pap et al., 2010; Merrill et al., 2015). In Brown-headed Cowbirds, the bactericidal ability of the blood decreased during molt (Ellis et al., 2012). The impact of molt on the immune system is likely to vary based on the stage of molt, so disagreements between studies could be due to sampling blood at different time points during molt. There is little understanding about the potential influences of molt on the adaptive immune response of avian species beyond domestic chickens. Sparrows showed the strongest cell mediated PHA immune response during pre-molt and heavy molt and the lowest response immediately post-molt (Martin et al., 2006). In King Penguins, plasma immunoglobulin levels increased by almost 40% during molt (Bourgeon et al., 2007). These data indicate that the process of molt seems to differentially influence both the different components of the immune system as well as the immune function in species with different molt strategies. Due to a lack of definitively comparable data across wild species, it is unclear if all components of immunity are

equally altered or if many of the observed changes could be the result of increased immunosurveillance while molt occurs (Hegemann et al., 2012b).

When evaluating the trade-off between molt and immunity, it is important to consider the bidirectionality of the trade-off where the immune system rather than molt has higher prioritization for energy and nutrients. Mounting an immune response concurrently with molt reduces the ability of birds to molt properly. When House Sparrows are injected with lipopolysaccharide (LPS), they fail to regrow healthy feathers (Ben-Hamo et al., 2017). An injection of phytohemagglutinin (PHA) also decreases the ability of both sparrows and Greenfinches to successfully molt and regrow feathers (Martin et al., 2006; Manniste and Horak, 2011). A similar decrease in molt success is observed when House Sparrows are infected with avian malaria, which is more analogous to a natural infection (Coon et al., 2016). Male Pied Flycatchers will delay the onset of their post-nuptial molt when challenged with sheep red blood cells (Sanz et al., 2004). These data support the concept that there is an energetic trade-off between supporting molt and supporting an immune response. The observed changes in immunity could be due to a trade-off in the nutritional requirement of growing feathers while maintaining immunity or changing requirements in immunity due to shifts in the integument that occur during molt.

Conclusion

The overarching goal of this thesis is to help define the effects of molt on the immune system of the domestic chicken. The studies performed to this end had two separate but closely related objectives: defining how the immune system shifts across molt and evaluating the potential trade-off between the energetic and nutritional requirements of molt and an inflammatory response. Although it may be more applicable to evaluate a trade-off in a wild

species instead of domestic species, the domestic chicken was chosen as the study organism to allow for increased control of experimental design and to utilize a broader range of immunological tools. The first study (Chapter 2) was conducted to understand, on a week-by-week basis, how the thymus and spleen change during molt. One of the major challenges in interpreting the existing literature on molt and the immune system is the high variability in the results for even the same metrics of immune function. Examining the changes in the immune system on a week-by-week basis will help provide a stronger framework to understand how the immune system is altered through the course of molt.

The main focus of the second (chapter 3) and third (chapter 4) experiments was to examine if the energetic and nutritional demands of molt lead to a trade-off in immune function when molting birds must mount an innate immune response. In the second study, chickens were challenged with an injection of lipopolysaccharide (LPS) to determine if molting birds had a decrease in inflammatory markers during molt. An innate immune response induces quick and nutritionally costly metabolic shifts that are more likely to induce a trade-off between molt and immunity compared to the lower long-term costs of an adaptive immune response. The third study build on the second to determine if the trade-off between molt and an innate immune response was driven by the cost of feather production or the broader physiological shifts that occur during molt to support feather production. Molting birds undergo broad immunological and metabolic changes during the process of molt beyond those changes directly associated with synthesizing new feather protein. Scaleless High chickens were used to mitigate the nutritional costs of molt directly associated with synthesizing feathers. The feathering of the Scaleless High genetic line is reduced; the birds have only a very small number of downy feathers remaining. Scaleless High birds maintain an annual molt without the expense of replacing most of their

feathers. The third study also helped to clarify if the changes observed in the immune system over the course of molt in study one were dependent on mass of feathers produced or the overall process of molt.

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Chapter 2

Physiological shifts in immune parameters during molt in the domestic chicken (*Gallus domesticus*)

Abstract

Feathers are complex structures that are essential to a bird's survival. Overtime, feathers are worn down and must be replaced through the process of molt. Molt is induced by a combination of environmental and physiological cues that lead to a range of hormonal and physiological shifts. The involution of the reproductive tract during molt is well documented, but it isn't well understood how the lymphoid organs are affected by molt. We investigated the impact of molt on selected immune parameters over the course of molt to clarify how the lymphoid organs and inflammatory state of a bird shifts as molt progresses. Molt was induced in domestic chickens (*Gallus domesticus*) using a combination of oral thyroxine (T4), a reduced calorie diet and a shift from a long day to a short-day schedule. During each week of molt, birds were sacrificed, and samples of blood, liver, spleen, and thymus tissue were collected. Changes in the weight of the spleen and thymus were assessed weekly. Histological examination of thymus tissue was also performed weekly. Shifts in the number of leukocytes were monitored using flow cytometry. To assess if molt induced either a pro- or anti-inflammatory state, the expression level and concentration of key pro- and anti-inflammatory cytokines were quantified using qPCR and ELISAs. The spleens of molting birds were 63% heavier than the spleens of non-molting birds ($P < 0.01$). The increase in thymus weight was even greater, with the thymus of molting birds increasing to be 6.8-fold heavier ($P < 0.01$). Unlike non-molting birds, the thymus of molting birds had clear demarcations between the cortex and medulla. The proportion

of the cortex tissue in the thymus increased as molt progressed ($P < 0.01$). The population of lymphocytes in the thymus also shifted during molt to closely resemble that of a young growing chick. Molting birds had a significantly higher proportion of double-positive T cells in the thymus than non-molting birds ($P < 0.01$). Overall, the increase in the weight of both the thymus and the spleen resulted in molting birds having a greater number of T and B cells. Both IL-8 and TNF-α expression increased in the liver during molt, but overall, there was no clear indication that molt leads to an increase in inflammation during feather growth. There are major shifts in the spleen and thymus during molt which may be essential to supporting feather regeneration or represent a concurrent but independent fluctuation in immune function during a bird's lifecycle.

Introduction

Feathers are a defining characteristic of all avian species and one essential to survival. Not only do feathers allow for flight, but they also serve additional important functions like thermoregulation or attracting mates. Feathers have a uniquely complex structure that allows for flight while remaining lightweight. Over time, the feather structure begins to wear down, or is damaged, making it necessary for birds to periodically replace their feathers by molting. The process of molt involves replacing feathers and other integumentary structures such as feather sheaths (Alfred M. Lucas, 1972). Molt is regulated by multiple abiotic and biotic factors that ensure feather growth occurs in specific patterns and while resources are available to support growing new feathers (Payne, 1972).

Induction of molt is controlled by a combination of environmental and physiological cues. Photoperiod is one of the well-defined cues known to induce molt across many species. The majority of temperate zone species that have been studied will initiate molt towards the end of summer after exposure to long daylight hours and when breeding has ended (Kendeigh, 1949; Farner, 1964). Birds in the order Galliformes are an exception, molting in the fall following a transition from long daylight hours to short daylight hours (Larionov, 1957). Shifts in hormone levels also play an essential role in regulating molt. The onset of the fall molt coincides with the involution of the reproductive organs when many species finish breeding for the season. Fluctuations in multiple reproductive hormones are important for regulating the initiation and progression of molt. The concentration of both estrogens and androgens are also important modulators of molt. High concentrations of exogenous estrogens or androgens can override cues from changes in photoperiod to slow or even arrest molt (Kobayashi, 1954; Tanabe et al., 1957). The thyroid hormones, triiodothyronine (T3) and thyroxine (T4), are important regulators of

feather growth. Supplementation of thyroxine in feed induces molt in domestic chickens (Sekimoto et al., 1987; Bass et al., 2007). The combination of environmental cues and shifting hormones produce a broad range of physiological changes that are necessary to cease reproduction and produce feathers.

The process of molt is characterized by not only the complex process of feather growth and related integumentary changes but also by the broad scale shifts that occur throughout the body. For many bird species, molt occurs following reproduction when their body mass is lowest. As birds enter molt, the reproductive organs involute. Domestic chickens will cease laying for the duration of their molt. Muscles, adipose, adrenals and liver of domestic chickens all decrease in mass during molt (Brake and Thaxton, 1979). Although reproductive organs shift to an inactive state during molt, the lymphoid tissues may become more active. During a forced molt in laying hens, the spleen fluctuates in weight and contains fewer apoptotic cells and more lymphocytes (Brake and Thaxton, 1979; Brake et al., 1985). The spleen size of Willow Tits increases during molt, indicating that spleen of wild species may also shift during molt (Silverin et al., 1999). The thymus undergoes a recrudescence when laying hens undergo a forced molt and increases in size in molting Yellow-vented Bulbuls (Ward and D'Cruz, 1968; Brake et al., 1981). Although shifts in the spleen and thymus have been observed during molt, it is not well understood why the changes are occurring.

Generally, the lymphoid organs of birds are considered to be the most active in the production of the lymphocyte repertoire in young birds. The primary lymphoid organs of birds, the Bursa of Fabricius and thymus, are essential for generating a bird's B-cell and T-cell repertoires respectively. The maximum Bursa of Fabricius size is reached from 8 – 10 weeks of age in the domestic chicken after which the organ begins to involute. The thymus is also

considered to be more active in growing birds compared to adult birds. The thymus reaches maximum size later than the Bursa of Fabricius, when chicks are 3 – 4 months old (Ciriaco et al., 2003). The spleen, although a secondary lymphoid organ, is also an important site for embryonic lymphopoiesis in growing birds. In chickens, B cell progenitors undergo some immunoglobulin gene rearrangement in the spleen before migrating to colonize the Bursa of Fabricius (Masteller and Thompson, 1994). The thymus and spleen are both large in growing birds when the immune system is being established. Little is known about why the spleen and thymus increase in size in adult birds during molt or even how the shifts in the organs progress over the course of molt.

The process of regrowing feathers is nutritionally and energetically costly. The preservation and growth of the spleen and thymus while other organs involute indicate that the immune system remains a priority during molt. The underlying causes for the shift in lymphoid organ size is not known and could be driven by aspects of feather replacement or it may be an unrelated coincidence. When birds molt, skin that is normally protected by feathers experiences more environmental exposure. Additionally, the growing feathers themselves can be vulnerable to damage and infection. One theory proposes that as birds molt, there is an increased need for lymphocytes which leads to the increase in thymus size (Ward and D'Cruz, 1968). An increase in lymphocytes could be necessary to support higher levels of immunosurveillance and immune defense at the skin and feathers during molt. To determine how the spleen and thymus are changing during molt, we tracked both gross histological and molecular changes in the spleen and thymus. To understand if there is increased immune defense occurring during molt, we looked at systemic markers of inflammation to determine if there might be a level of immune activation during molt that could necessitate an increase in lymphocyte number.

Materials and Methods

Animal Care

Seventy-two seven-month-old Hy-Line W-36 laying hens obtained from JS West (Atwater, CA) were housed in the Cole A research facility at the University of California, Davis. Prior to the start of the experiment, hens were given a two-week acclimation period. To allow for the maintenance of different lighting schedules, the hens were housed in two rooms directly across from each other. Hens were individually housed in sloped floor cages to facilitate egg collection and were provided ad libitum access to feed and water. For the duration of the experiment, control birds (N = 36) were maintained on a long day schedule (16L:8D) and a 16% protein high energy layer diet (Bar-Ale cat. no. WP16CC). Following the acclimation period, half of the birds (N = 36) were induced to molt using a combination of physiological signals. Over the course of a week, the day length of the molting birds was reduced from 16 hours to 8 hours. Birds were also administered a daily oral dose of thyroxine (T4) at 1.25 mg/kg for 7 days by sprinkling it on top of the feed in the morning. Finally, molting birds were fed a specially formulated molt diet that had a reduced content of metabolizable energy (2431.60 kcal/kg), protein (9.6%), fat (3.12%) and calcium (0.5%). The dietary levels of all other nutrients met the bird's daily nutritional requirements (NRC, 1994; diet formulation Appendix 3). Body weight and laying rate were monitored for the duration of the experiment. All husbandry and experimental practices were approved by the University of California, Davis IACUC #21304.

Experimental Design

This experiment was conducted to examine systemic shifts in immunity over the course of molt in laying hens. The start of molt was defined as the day that hens began to drop feathers

following seven days of thyroxine supplementation. The progression of molt was then monitored for the next six weeks. Each week, thymus, spleen, and blood were collected from six birds in the control and molt treatment to evaluate changes in systemic immunity (Figure 2.1). The weight of each organ was taken, in addition to samples being frozen at -80°C for further analysis or fixed for histology. The Bursa of Fabricius was checked during sample collection but remained involuted and grossly undetectable in both molting and non-molting birds.

Thymus Histology

A single thymic lobe was collected for histological examination from each bird. Thymic lobes were fixed for 48-hours in 10% buffered formalin before being transferred to 70% ethanol. Samples were trimmed and placed in tissue cassettes and sent to IDEXX BioAnalytics (Colombia, MO) for slide preparation and hematoxylin and eosin stain. To assess the changes in the cortex and medulla, ImageJ software was used to measure the area of cortex and medulla in three images per bird at 4x magnification. The cortex to medulla ratio was calculated as total cortex area divided by the total medulla area per image.

Quantitative RT-PCR of Liver and Spleen Cytokine Gene Expression

Liver and spleen samples were weighed then immediately placed into RNAlater® and minced. Samples in RNALater ® were kept on ice for up to four-hours post-removal, then stored at -80°C prior to further laboratory processing. Tissue samples were homogenized, and total RNA extracted using a modified version of the Trizol® protocol for phenol-chloroform extraction of RNA. Following RNA extraction and isolation, a Thermo Scientific™ NanoDrop™ spectrophotometer was used to determine the RNA quality of each sample. A 260/280 ratio of 1.8 or higher was used to define RNA as good quality. To ensure RNA purity, Ambion's DNA-

free™ kit (Cat. No. AM1907) was used to remove any residual genomic DNA from RNA samples. Following RNA purification, Reverse Transcription PCR was performed with 1 μg of RNA per tissue sample using Bio-Rad's iScript™ Reverse Transcription Supermix (Cat. No. BR 1708840). PCR procedures were completed in a Bio-Rad PTC-100 Programmable Thermal Cycler. All cDNA samples were stored at -20° C until quantitative RT-PCR analysis.

To assess shifts in either an anti- or pro-inflammatory state, the expression level of the cytokines interleukin-1-beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor alpha (TNF-α), interleukin-10 (IL-10) and transforming growth factor beta (TGF-β) were evaluated using qPCR. All qPCR reactions were performed using the following protocol: 3 minutes at 95°C, then 40 cycles of 10 seconds at 95°C, 30 seconds at 55° C and 30 seconds at 72°C on a MyIQ iCycler PCR machine (Bio-Rad, Hercules, CA). Bio-Rad's iTAQ ™ Universal SYBR ® green Supermix was used for each reaction with the following components: 1 uL of 10 ng/uL cDNA, 10 uL of SYBR Universal SYBR ® Green Supermix along with variable amounts of DEPC water, forward primer and reverse primer based on specific primer set (Table A.7). Each experimental sample was run in triplicate with DEPC water as a NTC control. The fold-change values (ΔΔCt) for each cytokine were calculated using the ratio of the gene of interest ΔCt to the housekeeping genes (RPL13 and GAPDH) ΔCt.

Cytokine Quantification

Prior to euthanasia, blood was collected from the brachial vein into EDTA treated tubes using 25G ⅝ needles on 3 mL syringes. Whole blood was stored at 4°C then centrifuged for 15 minutes at 2000g to isolate plasma (Sorvall® Legend MACH 1.6). Plasma was aliquoted and stored at -20°C until further processing. Commercial ELISA kits were used to quantify the

amount of IL-1β, IL-6 and IL-10 (ABclonal® Cat. No. RK00591, RK00594, RK00587) and thyroxine (Aviva Systems Biology Cat. No. OKCA00265) in plasma samples according to the manufacturer's instructions. Cytokine concentrations were reported as picograms per milliliter for IL-1β, IL-6 and IL-10 and as nanograms per milliliter for thyroxine.

Evaluation of Immune Cell Populations

Whole blood and spleen samples were collected during weeks two and four of molt for flow cytometry. Thymus samples were collected only during week four. To prepare single-cell suspension from whole blood, 2 mL of whole blood was layered over 2 mL of Histopaque-1077 then centrifuged for 30 minutes at 400g. The isolated buffy layer of cells was then resuspended in 2 mL of cold RPMI-10 and washed in fresh RPMI-10 by centrifugation for 5 minutes at 1500 RPM. For the spleen and thymus samples, single-cell suspensions were prepared by mechanically disaggregating the tissue through a 40 μm cell strainer into cold RPMI-10. Approximately 10 million cells were then diluted in 4 mL of RPMI-10 and layered onto 4 mL of Histopaque-1077 and spun for 30 minutes at 400g. The isolated buffy layer was suspended in 2 mL of RMPI-10, washed by centrifugation for 5 minutes at 1500 RPM and resuspended in 2 mL of fresh RPMI-10.

For each tissue, single-cell suspensions were stained using two antibody panels. Cells were pelleted in a 96-well plate then incubated for 30 minutes on ice in 50 μL of staining cocktail. To evaluate T cell populations, a panel consisting of the following mouse anti-chicken antibodies was used: CD3-PE (Southern Biotech, Birmingham, AL, cat # 8200-09), CD4-FITC (Southern Biotech, Birmingham, AL, cat # 8210-02), CD8A-A647 (Southern Biotech, Birmingham, AL, cat # 8220-31), and Invitrogen ™ LIVE/DEAD™ fixable violet dead cell stain

(Thermo Fisher Scientific, Cat. No. L34955). A second panel for monocytes, macrophages and B cells was prepared using Bu-1-PE (Southern Biotech, Birmingham, AL, cat # 8395-09), KUL01-A647 (Southern Biotech, Birmingham, AL, cat # 8420-31), and Invitrogen ™ LIVE/DEAD™ fixable violet dead cell stain (Thermo Fisher Scientific, Cat. No. L34955). The KUL01 antibody marks both monocytes and macrophages and throughout this manuscript the term macrophage will be used to describe cells in the monocyte to macrophage lineage marked by the KUL01 antibody. After staining, cells were washed twice in 150 uL of staining media by centrifugation for 5 minutes at 1500 RPM and incubated for 30 minutes in 50 uL of Invitrogen ™ LIVE/DEAD™ fixable violet dead cell stain (Thermo Fisher Scientific, Cat. No. L34955). After all staining steps, cells were fixed in 75 uL of BD Cytofix/Cytoperm ™ fixation and permeabilization solution (Fisher Scientific, Cat. No. BDB554722) for 15 minutes then resuspended in 200 uL of staining media and kept at 4°C. Cells were processed for flow cytometric analysis on the same day as staining and fixation using a BD™ LSR II (BD Biosciences, San Jose, CA). Compensation controls, including fluorescence minus one (FMO) and single fluorescence controls, were prepared from single-cell suspensions of the appropriate tissue.

Statistical Analysis

All of the statistical analyses were performed using Microsoft Excel® Version 16.58, R© version 4.1.1 and RStudio© version 2021.9.0.351 (R Core Team, 2021; RStudio Team, 2021). For each model, normality was assessed by examining a Q-Q plot of the standardized residuals versus the theoretical quantiles in combination with the Shapiro-Wilks test of normality. Homogeneity of variance was assessed by visually examining a plot of the model standardized residuals by the fitted model values as well as using Levene's test. In each data set, extreme

outliers were defined as data points above the third quartile plus three times the interquartile range or the first quartile minus three times the interquartile range. Extreme outliers were then evaluated for removal using both the Bonferroni outlier test and Cook's distance to evaluate the outlier leverage. When performing model comparisons, the best fitting model was selected based on log-likelihood, AIC, and BIC values (Bates et al., 2015).

Both body weight and change in body weight were analyzed as 2 x 6 factorials with two levels of molt by six different times (week) of molt. For all body weight data, a linear mixed-effects model was constructed with molt status, time and the interaction of time and molt status as fixed effects and bird identification as a random effect. When examining the difference in laying between molting and non-molting birds, a linear model with molt, time and the interaction of molt and time was used. Welch's T-test was used to compare the concentration of thyroxine in the plasma of molting and non-molting birds.

Organ weight, ELISA, and qPCR data were analyzed as 2 x 6 factorials with two levels of molt by six different time (time) levels. Both qPCR fold-change data and ELISA cytokine concentration were log transformed to fit a normal distribution. A linear model was used to assess the following data sets: organ weights, plasma IL-1β concentration, plasma IL-6 concentration, plasma IL-10 concentration, and spleen IL-6 expression. A generalized least squares model with unique variance assigned for each level of molt was used for liver TNF-α expression, liver IL-10 expression, spleen IL-8 expression, spleen IL-10 expression, spleen TGF-β expression. For all remaining data sets including liver IL-1β expression, liver IL-6 expression, liver IL-8 expression, liver TGF-β expression, spleen IL-1β expression and spleen TNF-α expression a generalized least squares model with unique variance for each level of time was used. Whole blood and spleen flow cytometry data were analyzed as 2 x 2 factorials with two

levels of molt by two different times (week) of molt using linear models. The effect of molt on thymus data and cell counts were determined using t-tests.

A two-way analysis of variance (ANOVA) was used to evaluate the main effect of molt, time and the interaction of molt and time on measured variables. For data such as body weight, where multiple measurements were collected from the same individual, a repeated measures ANOVA was used. For all ANOVA and post-hoc tests, significance was accepted at $P \leq 0.05$ with a p-value between $P > 0.05$ and $P < 0.10$ considered to indicate a non-significant trend. Tukey's honestly significant difference test (HSD) was used to determine differences between the least squares means for contrasts evaluating the effects of molt and time. When performing planned contrasts, no p-value corrections were performed. A Bonferroni p-value correction was used for any additional *Post hoc* analysis performed. Experimental results are reported as the coefficient estimates, standard errors and 95% CIs.

Results

Body Weight and Laying

The body weight, change in body weight and laying rate were monitored during six weeks of molt. There was an overall main effect of time on both body weight and the change in body weight ($P < 0.01$). The body weight of non-molting birds increased by an average of 7.4 ± 0.90 percent whereas the body weight of molting birds decreased by an average of 15.0 ± 1.00 percent (Figure 2.2). There was also a significant main effect of molt on bird body weight and the change in body weight ($P < 0.01$). The non-molting birds were an average of 221 ± 16.8 grams larger than the molting birds ($P < 0.01$). The average difference in the percent change of

body weight when comparing the non-molting and molting birds was 23% (Table 2.1). The interaction between time and molt was significant for both body weight and the change in body weight ($P < 0.01$). The largest difference in body weight between the non-molting and molting birds occurred during week three (estimate: 380.6 g ± 20.2, CI: 325.4 – 435.9) followed by week four (estimate: 282.9 g ± 21.4, CI: 224.5 – 341.3; Figure 2.2). The percentage of birds laying eggs in the non-molting group was significantly higher than the molting group for each week of the study ($P < 0.01$; Figure 2.3). The percentage of birds laying in the molting group neared zero during week one (estimate: 10.2% ± 1.6, CI: 7.1 – 13.3) and by week two no birds in the molting group were laying. The molting birds began to resume laying during week five (estimate: 6.7% ± 1.7, CI: 3.2 – 10.0) and six (estimate: 13.3% ± 4.1, CI: 5.1 – 21.6). The percentage of non-molting birds laying each week remained above 95% for the duration of the study.

Spleen and Thymus Weight

At the end of each time, the weight of the spleen from six non-molting and six molting birds was measured. Time did not have a significant effect on the spleen weight. There was a significant main effect of molt ($P < 0.01$) and a significant interaction between time and molt on spleen weight ($P < 0.01$). The spleens of molting birds were on average of 63% larger than the spleens of non-molting birds across the molt period ($P < 0.01$). Molt caused the greatest difference in spleen weight between non-molting and molting birds during week four (Figure 2.4; $P < 0.01$). By week six, the difference in spleen weight between molting and non-molting birds (estimate: 0.89 g ± 0.12, CI: 0.57 – 1.2) had decreased by half compared to week four (estimate: 1.7 ± 0.16, CI: 1.3 – 2.0) but was still greater than week one (estimate: 0.50 g ± 0.16, CI: 0.18 – 0.81). The spleens of molting birds were 2.2 times heavier than the spleens of the non-

molting birds during week four (Table 2.2). When comparing spleen weights as a percentage of body weight, there was a significant main effect of both time and molt ($P < 0.01$). The spleens of molting birds accounted for 1.9 times more body weight than the spleens of non-molting birds. There was also a significant effect of the interaction between time and molt on the proportion of body weight made up by the spleen. During the first week, the spleen accounted for a slightly higher, but statistically significant, percentage of body weight in the molting birds than the non-molting birds (estimate: 0.03% ± 0.01, CI: 0.0 – 0.05; $P = 0.04$). By week four, the component of body weight taken up by the spleen in molting birds increased six-fold (estimate: 0.16% ± 0.01, CI: 0.13 – 0.19; $P < 0.01$).

There was a significant main effect of time and molt, and a significant interaction between them on the weight of the thymus ($P < 0.01$). The thymus of molting birds was an average of 1.8 grams and 6.8 times heavier than the thymus of the non-molting birds (Figure 2.5; Table 2.2). During week one, there was no difference in thymus weight when comparing molting and non-molting birds. The largest difference in thymus weight occurred during week three of molt (estimate: 3.3 g ± 0.3, CI: 1.9 – 4.7) followed by week four (estimate: 3.0 g ± 0.3, CI: 2.3 – 3.7). By week six, the difference in thymus weight was still 1.5 times greater than during week one and had only decreased to half of the peak weight observed during week four ($P < 0.01$). There was also a significant effect of time, molt, and the interaction of time and molt on the thymus as a proportion of body weight ($P < 0.01$). The thymus of molting birds accounted for 8.6 times more body weight than the thymus of non-molting birds ($P < 0.01$). Compared to non-molting birds, the thymus of molting birds made up an additional 0.15% of body weight (CI: 0.13 – 0.18). The largest difference in the percentage of body weight made up by the thymus occurred during week three (estimate: 0.3% ± 0.06, CI: 0.12 – 0.49) followed by week four

(estimate: 0.25% ± 0.03, CI: 0.17 – 0.34). By week six, the difference in the proportion of body weight accounted for by thymus was 38% lower than during week four.

Thymus Histology

Thymus samples were collected weekly to better understand the recrudescence of the thymus during molt. The thymus of non-molting birds was regressed and lacked clearly defined thymic lobules (Figure 2.6). In non-molting birds, there was no defined cortex tissue and a greater incidence of adipose tissue. The structure of the thymus of molting birds more closely resembled the thymus of a young bird and contains clearly defined thymic lobules. Within each lobule, the cortex and medulla tissue are clearly visible. There was a main effect of time on the percent of cortex tissue ($P < 0.01$), medulla tissue ($P < 0.01$) and the ratio of cortex to medulla ($P = 0.01$) in the thymus of molting birds (Figure 2.7). As birds progressed through molt, the area of cortex tissue decreased as the thymus decreased in weight. During week six, there was a significant reduction in cortex tissue compared to week two ($P < 0.01$). As the cortex tissue decreased, the thymus consisted of a greater percentage of medulla. By week six, there was a significantly greater percentage of medulla tissue in the thymus compared to week two ($P < 0.01$). The ratio of the cortex to medulla tissue decreases over the course of molt as the cortex tissue in the thymus is reduced. The cortex to medulla ratio is significantly higher in week two compared to week six ($P = 0.03$).

Plasma Thyroxine

As birds progressed through molt, plasma samples were collected from non-molting and molting birds to assess shifts in the concentration of thyroxine in the blood (Figure 2.8). There was a significant effect of time, molt, and the interaction between time and molt on the

concentration of thyroxine in the blood. The concentration of thyroxine in the blood of molting birds was on average 2.5 times greater than that of the non-molting birds. During weeks one and three, molting birds had significantly higher levels of thyroxine in their blood compared to non-molting birds ($P < 0.01$). Over the course of molt, the concentration of thyroxine in the blood steadily decreased. By weeks four and six, there were no differences in the concentration of thyroxine in the blood of molting and non-molting birds.

Cytokine mRNA Expression in the Liver and Spleen

Liver and spleen samples were taken each week to determine the effect of molt on the mRNA expression of pro-inflammatory cytokines. Neither time, molt, nor the interaction between time and molt significantly affected IL-1β expression in the liver or spleen (Table 2.3; Table 2.4; Figure 2.9A). The expression of IL-6 in the liver also was not affected by time, molt or the interaction between time and molt. There was a significant main effect of time ($P = 0.04$) but not molt ($P = 0.16$) or the interaction between time and molt ($P = 0.17$) on IL-6 expression in the spleen (Table 2.3; Figure 2.9B). During week four of molt, molting birds trended towards higher expression of IL-6 in the spleen (estimate: 1.0 ± 0.6, CI: -0.1 – 2.2; $P = 0.08$). Expression of IL-8 in the liver was significantly affected by time ($P < 0.01$), molt ($P < 0.01$) and the interaction between time and molt ($P < 0.01$). Molting birds had an increased level of IL-8 expression in the liver (estimate: 0.8 ± 0.3, CI: 0.1 – 1.5; $P = 0.02$) compared to non-molting birds. There was a significant effect of time ($P = 0.02$) and the interaction between time and molt ($P = 0.05$) but not molt alone on the expression of IL-8 in the spleen (Figure 2.9C). The expression of TNF-α alpha in the liver was significantly affected by time ($P < 0.01$) and molt ($P < 0.01$) while the interaction between time and molt trended towards significance ($P = 0.10$).

TNF-α expression in the liver of molting birds was, on average, almost a fold-change higher (estimate: 0.9 ± 0.2, CI: 0.5 – 1.3) than the TNF-α expression of non-molting birds ($P < 0.01$). The largest difference in the expression of TNF-α in the liver between molting and non-molting birds occurred during week four of molt ($P < 0.01$). There was a significant main effect of time ($P < 0.01$), molt ($P = 0.04$) and the interaction of time and molt ($P < 0.01$) on the expression of TNF-α in the spleen (Figure 2.9D). For all pro-inflammatory cytokine expression in both the liver and the spleen, there was a high amount of individual variation.

The mRNA expression of anti-inflammatory cytokines was also monitored in the liver and spleen. There was a significant main effect of time ($P < 0.01$) but not molt or the interaction of time by molt on the expression level of IL-10 in the liver. The expression of IL-10 in the spleen was also significantly affected by time ($P < 0.01$) but not molt (Figure 2.10A). There was a significant effect of the interaction of time and molt on IL-10 expression in the spleen ($P = 0.02$). The expression of TGF-β in the liver was not significantly affected by time but there was a significant main effect of molt ($P < 0.01$; Figure 2.10B). The interaction between time and molt was also significant ($P = 0.05$). There was no effect of time, molt, or their interaction on the expression of TGF-β in the spleen.

Cytokine Protein Concentrations

The concentration of IL-1β, IL-6 and IL-10 in the plasma of non-molting and molting birds was assessed during each week of molt. Neither time, molt, nor their interaction had a significant effect on the concentration of IL-1β in the plasma (Table 2.5; Figure 2.11A). There also was not a significant effect of time or molt on the plasma concentrations of IL-6 (Figure 2.11B). The interaction of time and molt trended towards affecting IL-6 concentration in the

plasma (P = 0.07). During week four of molt, molting birds trended towards a higher concentration of IL-6 in the blood compared to non-molting birds however there was a high level of variance within the molting group (P = 0.08 by pairwise comparison). Neither time, molt, nor the interaction between the two had an effect on IL-10 concentration in the plasma (Table 2.5; Figure 2.11C). The concentration of cytokines in the plasma of individual birds were quite variable across the duration of the experiment.

Immune Cell Populations

Spleen, thymus, and whole blood samples were collected to assess shifts in immune cell populations during molt. There was a significant main effect of time on the percentage of $CD4^+$ (P = 0.04), $CD8^+$ (P < 0.01), $CD4^+$ $CD8^+$ (P = 0.04) and $CD4^-$ $CD8^-$ (P < 0.01) T cells in the blood (Table 2.6; Table 2.7). There was also a significant main effect of molt on the percentage of $CD8^+$ T cells in the blood (P < 0.01) but not on the other T cell populations. The percentage of $CD8^+$ T cells in the blood was significantly affected by the interaction of time and molt (P < 0.01). During week two, molting birds had a significantly lower percentage of $CD8^+$ T cells in their blood compared to non-molting birds (estimate -10.0 % ± 2.3, CI: -14.8 – 5.1; P < 0.01; Figure 2.12). There was no main effect of the interaction between time and molt on the percentage of $CD4^+$, $CD4^+CD8^+$ or $CD4^-$ $CD8^-$ T cells in the blood.

Time had a significant main effect on the percentage of both B cells and macrophages in the blood of molting birds. The percentage of B cells (P = 0.02) and macrophages (P < 0.01) in the blood of molting birds were higher during week two than week four (Figure 2.13). There was a significant main effect of molt on the percentage of B cells but not macrophages in the blood. The proportion of B cells in the blood of molting birds increased by 2.9% which represents an

81% increase in the percentage of B cells in the blood compared to the non-molting birds (P =0.02). Neither the percentage of B cells nor macrophages in the blood were affected by the time by molt interaction.

Molt resulted in a shift in the T cell populations observed in the spleens of molting birds (Figure 2.14). There was a significant main effect of time on the percentage of CD4-CD8- T cells in the spleen ($P < 0.01$) but not on other T cell populations. Molt significantly affected the percentage of $CD4^+$ T cells ($P = 0.02$) and had a positive trend on the percentage of $CD8^+$ ($P = 0.06$) and $CD4^+$ $CD8^+$ T cells ($P = 0.08$) in the spleen. Molt led to a 32% increase in $CD4^+$ T cells in the spleen of molting birds compared to non-molting birds ($P < 0.01$). There was a significant interaction between time and molt on the percentage of $CD4^+$ ($P = 0.03$) and $CD8^+$ T cells ($P = 0.02$) in the spleen. During week two, molt led to a 70% increase in the percentage of $CD4^+$ T cells in the spleen compared to non-molting birds (estimate 11.3% ± 3.2, CI: 4.5 – 18.2). The proportion of $CD8^+$ T cells in the spleens of molting birds was 28% lower than the proportion of $CD8^+$ T cells in the spleens of non-molting birds (Figure 2.14).

The percentage of both B cells and macrophages in the spleen were both affected by molt (Figure 2.15). There was a significant main effect of molt ($P < 0.01$) but not time on the percentage of B cells in the spleen. The interaction between time and molt trended towards having a significant effect on the percentage of B cells in the spleen ($P = 0.09$). Molting birds had an overall lower proportion of B cells in the spleen compared to non-molting birds (estimate -9.2 ± 2.8, CI: -15.0 – 3.3; $P < 0.01$). During week two, molting birds had a 52% decrease in splenic B cells compared to non-molting birds ($P < 0.01$; Figure 2.15). There was a significant main effect of time ($P = 0.01$) and molt ($P = 0.05$) on the percentage of macrophages in the spleen (Table 2.8). There was no effect of the interaction between time and molt on macrophage

level. Molting birds trended towards a lower percentage of macrophages in the spleen compared to non-molting birds (estimate -0.3 ± 0.1, CI: -0.59 – 0; P = 0.06).

The process of molt altered the percentages of different T cell populations in the thymus of molting birds compared to non-molting birds. There was a significant main effect of molt on the percentage of $CD4^+$ T cells in the thymus. There was also a trend of molt affecting the percentage of $CD8^+$ T cells (P = 0.06) and the percentage of $CD4^+CD8^+$ T cells (P = 0.08). Non-molting birds had a larger percentage of both $CD4^+$ (P < 0.01) and $CD8^+$ (P < 0.01) T cells in the thymus. Although molting bird had lower percentages of single positive T cells compared to non-molting birds, there was a large increase in the percentage of $CD4^+CD8^+$ T cells in the thymus of molting birds (Figure 2.16). Molting birds had a 25-fold increase in the proportion of double-positive T cells compared to non-molting birds during week four (estimate 37.8 ± 1.9, CI: 32.6 – 43.0; P < 0.01). There was no effect of molt on the percentage of $CD4^-$ $CD8^-$ T cells in the thymus. There was a significant main effect of molt on both the percentage of thymic B cells and macrophages (P < 0.01; Table 2.8). Non-molting birds had a significantly greater percentage of thymic B cells and macrophages (P < 0.01; Figure 2.17).

Molting birds had a much higher total number of T cells in the thymus (2.18). Compared to non-molting birds, molting birds had a significantly greater number of $CD4^+$ (P = 0.01), $CD8^+$ (P = 0.05), $CD4^+$ $CD8^+$ (P = 0.02) and $CD4^-$ $CD8^-$ T cells (P < 0.01). The largest difference in T cell numbers was observed for the $CD4^+$ $CD8^+$ T cells (Figure 18). The thymus of molting birds contained millions of double-positive T cells whereas the thymus of non-molting birds contained around half a million double-positive T cells (P = 0.02). Molting birds had more macrophages (P < 0.01) but not B cells in the thymus (Figure 2.19).

Molting birds had increased numbers of $CD8^+$, $CD4^+$ $CD8^+$ and $CD4^-$ $CD8^-$ T cells in the spleen compared to non-molting birds ($P < 0.01$). Interestingly, molting birds did not have an increased number of $CD4^+$ T cells in the spleen (Figure 18). Overall, there were very few double-positive or double-negative T cells in the spleens of molting and non-molting birds. Molting birds also trended towards a higher number of macrophages ($P = 0.10$) but not B cells in the spleen.

Discussion

Feathers are extremely complex integumentary structures that are replaced annually by most bird species. The induction of molt involves multiple environmental and physiological cues that lead to hormonal shifts and broad physiological changes throughout a bird's body. In poultry, molt is accompanied by a decrease in body weight, reduction in liver weight and involution of the reproductive track (Brake and Thaxton, 1979). Another aspect of molt that remains poorly understood are the physiological shifts in the lymphoid tissue. To better understand the changes in the lymphoid tissues that occur during molt, we examined shifts in the spleen and thymus on both a gross morphological and molecular level.

There were marked increases in the weight of both the spleen and the thymus as birds progressed through molt. By mid-molt, the spleens of molting birds were 2.2 times heavier than the spleens of non-molting birds. An increased spleen weight during molt was previously observed in hens (Brake and Thaxton, 1979) undergoing an induced molt and in molting Willow Tits (Silverin et al., 1999). Upon histological examination, the spleens of molting hens have increased numbers of lymphocytes (Brake et al., 1985). The increase in thymus weight during

molt was even more pronounced, increasing 6.8-fold. The increase in thymus size during molt is not unique to poultry and has also been observed in tropical Yellow-vented Bulbuls (Ward and D'Cruz, 1968). The recrudescence of the thymus was characterized by the development of clearly defined thymic lobules and distinct cortex and medulla tissue. At the beginning of molt, molting hens had a greater cortex to medulla ratio, which decreased over the course of molt. The increase in lymphocytes and development of clearly defined cortex and medulla is a consistent trait of thymic recrudescence during molt (Brake et al., 1981). The increase in thymus weight is likely driven by increased lymphocyte development. The cortex is the main site of T cell development (Bucy et al., 1990) and the molting birds have increased cortex tissue compared to non-molting birds. The process of molt leads to shifts in the spleen and thymus that are essential to supporting increased lymphocyte development.

The marked increase in the weight of the spleen and thymus was accompanied by shifts in the immune cell populations. The increase in thymus weight involves a notable increase in the cortex area which is essential for T cell development. Molting birds had a much higher number of T cells compared to non-molting birds. The most striking difference in T cell populations between the molting and non-molting birds was the 25-fold increase in the proportion of double-positive T cells in the thymus of molting birds. The presence of double-positive T cells in the thymus of molting birds indicates that not only is the thymus increasing in weight, but that lymphocyte production and receptor diversification are increasing as well. The proportions of populations of T cells in the thymus of molting birds resembles what would normally be seen in young birds (Erf et al., 1998) supporting that the thymus is actively producing lymphocytes again during molt after involuting during sexual maturation. The increase in spleen weight during molt is also accompanied by increased numbers of lymphocytes. The spleens of molting birds contain

more $CD8^+$ T cells, B cells and macrophages than non-molting birds. The increase in $CD8^+$ T cells in the spleen of molting birds has also been observed in hens that have undergone a fasting-induced forced molt (Holt, 1992).

The process of molt appears to necessitate this increase in lymphocyte production in thymus and spleen, though it is unknown what purpose the lymphocytes are serving during molt. After the peak of molt, the weight of the spleen and thymus decreased. In laying hens, once birds finish molting the spleen returns to the pre-molt size (Brake and Thaxton, 1979). The process of molt involves losing old feathers and replacing new feathers all across the body. It has been previously proposed that the increase in thymus size observed during molt is due to an increased requirement for lymphocytes (Ward and D'Cruz, 1968). The exposure of skin normally protected by feathers to the environment along with the generation of new feathers could lead to an increased need for immunosurveillance and defense while birds are molting. Birds undergoing molt may need to temporarily expand the population of lymphocytes in order properly monitor the expanded and vulnerable integumentary surfaces. New feathers are protected by the feather sheath, a single layer of epithelial cells (Lucas and Stettenheim, 1972). The thin feather sheath and increased exposure of skin to the environment increases the environmental interface during molt. The extent of the interaction between the skin and environment may vary between wild and captive birds. The captive birds in this experiment may have a lower level of interaction between the skin and the environment due to a more carefully controlled environment. However, captive birds also have a reduced ability to modify their behavior and environment and may potentially experience a greater level of insult to the skin during molt due to caging or handling. In humans, the skin is known to house a high density of immune cells involved in immunosurveillance of the skin. There are more than twice the number of T cells in human skin than the blood (Clark et al.,

2006). During molt an increase in lymphocytes may be essential to defending the skin and feather follicle when there is a potentially greater risk of both skin damage and infiltration of pathogens at the skin and feather follicle. Maintaining an expanded population of lymphocytes is energetically and nutritionally costly and likely explains the expansion of lymphoid tissue only persisting through molt (Iseri and Klasing, 2013, 2014).

To assess if there is an increase of immune activation during molt, we looked at markers of inflammation during each week of molt. For the marked increase in spleen and thymus weight to only be driven by an increased need for defense at the skin barrier and feather follicle, there would likely need to be a significant amount of either damage or bacterial infiltration past the skin barrier to necessitate the investment in cell proliferation in the thymus and spleen during molt when other organs are catabolized. We chose to track systemic, rather than local, inflammation to determine if there is an increase in inflammation reflective of a high enough level of pathogen infiltration or damage to drive a greater need for lymphocytes during molt. Throughout molt, there was no increase in the expression level of IL-1β or IL-6 in the liver or spleen. The levels of IL-1β and IL-6 protein in the plasma also did not increase over the course of molt. There was an increase in expression of TNF-α in the liver mid-molt, which corresponds to when there is both new feather that has emerged from the feather sheath and active dermal pulp. The sequence for TNF-α has only been recently identified in the chicken but it is an important mediator of inflammation with behavior analogous to TNF-α of mammalian species (Rohde et al., 2018). The expression of IL-8 was increased during the first three weeks of molt. Both TNF-α and IL-8 induce changes that could be beneficial during molt. TNF-α promotes the adhesion of leukocytes while IL-8 promotes angiogenesis (Kaiser et al., 1999; Li et al., 2005; Chandrasekharan et al., 2007). At the start of molt, there is increased levels of vascularization as

new blood vessels develop in the growing feather. The increase in vascularization may be in part promoted by increased IL-8 levels.

Although there was no clear pattern of inflammation in molting birds, there was a high amount of variation across individuals. Some birds may experience greater damage to the skin during molt but only to a level that induces local inflammation and not long-term systemic inflammation. An increase in occasional damage to the skin during molt seems likely, but the lack of stronger inflammatory signals doesn't lend strong support to the idea that an increase in immunosurveillance and defense occurs at the skin during molt which is significant enough to drive the large increase in spleen and thymus weight and increase in lymphocyte numbers. Alternatively, it could be that there is a large increase in defense but that the inflammation is dampened during molt. Feather growth requires extreme remodeling of the integument and increased inflammation during a period when there is dynamic tissue generation and degradation could produce harmful autoimmune reactions. We evaluated levels of anti-inflammatory cytokines to determine if molt may lean towards being a more anti-inflammatory state to damp down inflammation and prevent autoreactivity. There was no defined increase in either the expression level of IL-10 and TGF-β or the protein levels of either over the course of molt. Similar to the pro-inflammatory cytokines, there was a high amount of individual variation but no clear trend to indicate that there may be a greater number of anti-inflammatory signals during molt to either quickly tamp down inflammation during immune defense or reduce the chance of self-reactivity during a period of intense tissue remodeling.

During the energetically and nutritionally expensive period of molt, the preservation and expansion of lymphoid tissue while other tissues are catabolized indicates that increased lymphoid tissue must be essential for molt. The increase in lymphoid tissue may be due to a need

for more lymphocytes to perform immunosurveillance and defense of the skin during molt (Ward and D'Cruz, 1968). There may still be an increased need for both during molt, but there doesn't appear to a high enough level of insult at the skin to increase markers of systemic inflammation. As the knowledge of the immune system has expanded, the role of the immune system as an important mediator of not only defense but also tissue homeostasis is starting to be understood. In mammals, the numbers of T cells in different subsets fluctuates during the different phases of hair growth (Paus et al., 1999). The importance of a specific subset of T cells, T regulatory cells, in skin homeostasis has recently been demonstrated. Regulatory T cells in the skin preferentially localize around the hair follicle (Rodriguez et al., 2014). The regulatory T cells in the skin of mice promote hair follicle stem cell activation and are essential to hair growth. Knocking out regulatory T cells severely reduces the ability of mice to regrow their fur (Ali et al., 2017). The expansion of lymphocytes observed during molt may be due to the immune system modulating feather growth at the feather follicle. T cells, and maybe even B cells, may be essential to ensuring molt progresses smoothly in avian species. Similar to mammals, the T cells produced by the thymus during molt may be localizing to the feather follicles to provide signals that help induce molt. Like mammals, chickens have T regulatory T cells, although their functions are not as well defined (Shanmugasundaram and Selvaraj, 2011, 2012; Burkhardt et al., 2022). If part of the expansion in lymphoid tissue during molt is due to needing more cells to localize at the feather follicle, it could help explain why the lymphoid tissue expansion occurs very quickly at the onset of molt and then decreases over the course of molt. It is possible that the increase in lymphoid tissue and leukocytes numbers during molt may not be a secondary characteristic of molt due to an increased skin surface area requiring immunosurveillance but rather a

fundamental characteristic of molt and necessary for the regulation of growth at the feather or even some combination of both.

The regeneration of feathers during molt is both nutritionally and energetically expensive for birds. During molt, the reproductive organs and portions of other organs are catabolized while the spleen and thymus increase in weight. Not only do the spleen and thymus increase in weight, but both organs undergo shifts in immune cell populations during molt. The active tissue of the thymus, the cortex and medulla, increase significantly during molt. There is also a significant increase in the double-positive T cells found in the thymus of molting birds. The increase in thymic activity during molt could be essential to the process of molt or represent a concurrent, but independent process. The thymus is considered to be most active in growing birds when the immune system is being established. The period of molt is apparently another phase of life where the thymus is highly active in birds. Molt usually coincides with a resource rich period and birds may use the nutrients being generated to support feather growth during molt to also support fluctuations in immune system such as increased cell proliferation and diversification of the T cell repertoire as birds transition through different phases of life.

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Tables

Table 2.1 ANOVA P-values for the main effects of time, molt, and the interaction of time and molt on body weight measurements. P-values are shown for both the body weight and the percentage change in body weight relative to pre-molt period.

	Body Weight (Grams)	Body Weight (% Change)
Time	< 0.01	< 0.01
Molt	< 0.01	< 0.01
Time*Molt	< 0.01	< 0.01

Table 2.2 ANOVA P-values for the main effects of time, molt, and the interaction of time and molt on spleen and thymus mass. P-values are shown for both organ weight in grams and organ weight corrected for body weight.

	Spleen (Grams)	Spleen (% BW)	Thymus (Grams)	Thymus (% BW)
Time	0.48	< 0.01	< 0.01	< 0.01
Molt	<0.01	< 0.01	< 0.01	< 0.01
Time*Molt	<0.01	< 0.01	< 0.01	< 0.01

Table 2.3 ANOVA P-values for the main effects of time, molt, and the interaction of time and molt on the expression level of pro-inflammatory cytokines in the liver and spleen.

Cytokine	IL-1β		IL-6		IL-8		TNF-α	
Tissue	*Liver*	*Spleen*	*Liver*	*Spleen*	*Liver*	*Spleen*	*Liver*	*Spleen*
Time	0.69	0.45	0.93	0.04	< 0.01	0.02	< 0.01	< 0.01
Molt	0.58	0.41	0.33	0.16	< 0.01	0.52	< 0.01	0.04
Time*Molt	0.48	0.34	0.21	0.17	< 0.01	0.05	0.10	0.01

Table 2.4 ANOVA P-values for the main effects of time, molt, and the interaction of time and molt on the expression level of anti-inflammatory cytokines in the liver and spleen.

Cytokine	IL-10		TGF-β	
Tissue	*Liver*	*Spleen*	*Liver*	*Spleen*
Time	< 0.01	< 0.01	0.64	0.73
Molt	0.42	0.59	< 0.01	0.67
Time*Molt	0.21	0.02	0.05	0.64

Table 2.5 ANOVA P-values for the main effects of time, molt, and the interaction of time and molt on the plasma concentration of pro- and anti-inflammatory cytokines.

Cytokine	IL-1β	IL-6	IL-10
Time	0.33	0.46	0.52
Molt	0.19	0.97	0.96
Time*Molt	0.37	0.08	0.77

Table 2.6 ANOVA P-values for the main effects of time, molt, and the interaction of time and molt on the percentage of single positive T cells.

Cell Type	**$CD4^+$ T Cell**			**$CD8^+$ T Cell**		
Tissue	***PBMC***	***Spleen***	***Thymus***	***PBMC***	***Spleen***	***Thymus***
Time	0.04	0.35	NA	< 0.01	0.37	NA
Molt	0.18	0.02	<0.01	< 0.01	0.06	<0.01
Time*Molt	0.92	0.03	NA	< 0.01	0.02	NA

Table 2.7 ANOVA P-values for the main effects of time, molt, and the interaction of time and molt on the percentage of double-positive and double-negative T cells.

Cell Type	**$CD4^+CD8^+$ T Cell**			**$CD4^-CD8^-$ T Cell**		
Tissue	***PBMC***	***Spleen***	***Thymus***	***PBMC***	***Spleen***	***Thymus***
Time	0.04	0.39	NA	< 0.01	< 0.01	NA
Molt	0.29	0.08	< 0.01	0.89	0.41	0.26
Time*Molt	0.19	0.54	NA	0.33	0.19	NA

Table 2.8 ANOVA P-values for the main effects of time, molt, and the interaction of time and molt on the percentage of B cells and macrophages.

Cell Type	**B Cell**			**Macrophage**		
Tissue	***PBMC***	***Spleen***	***Thymus***	***PBMC***	***Spleen***	***Thymus***
Time	0.03	0.51	NA	0.01	0.01	NA
Molt	0.02	<0.01	<0.01	0.69	0.05	<0.01
Time*Molt	0.23	0.09	NA	0.14	0.74	NA

Figures

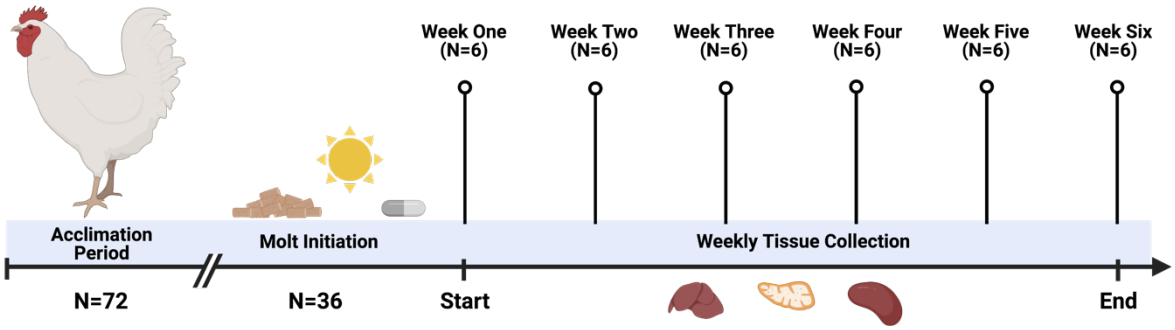

Figure 2.1 Experimental design of the time trial study to evaluate shifts in immunity during molt. Hy-Line W-36 laying hens were induced to molt (N = 36) using a combination of a shift from 12 to 8 hours of light, a molt diet, and a daily dose of oral thyroxine (1.25 mg/kg) for 7 days. During each week of the molt (N = 6), tissue samples were taken to assess systemic shifts in immunity.

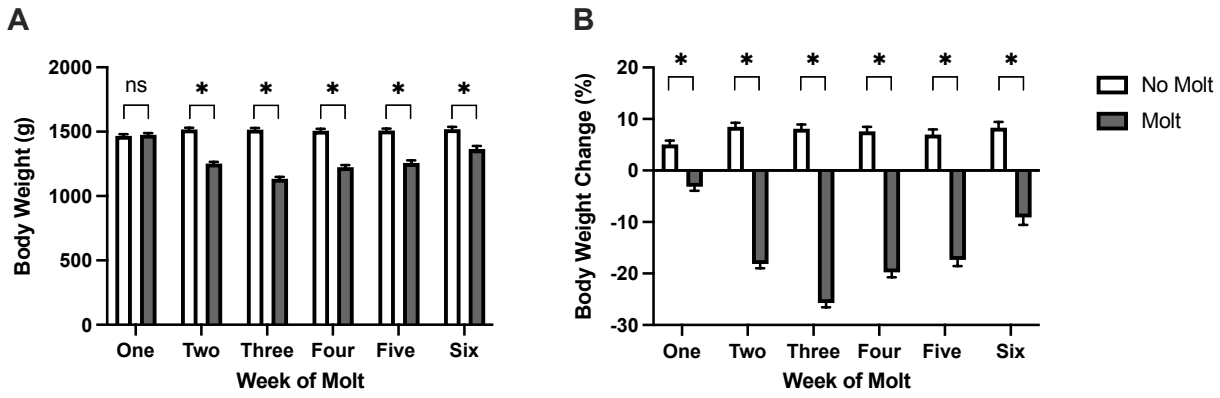

Figure 2.2 The effect of time on hen body weight A. Body weight measurements were taken weekly during the six-week molt period. On average, non-molting birds were 221 ± 16.8 grams heavier than molting birds ($P < 0.01$). Molting birds weighed significantly less than non-molting birds during week two through six of molt ($P < 0.01$). B. The body weight change is represented as the percent change in body weight relative to the pre-molt week. Non-molting birds had an average increase in body weight of 7.4 percent. Molting birds had an average decrease in body weight of 15 percent over the course of molt. Data are represented as mean + standard error with brackets and asterisks indicating significant differences ($P < 0.05$) between the non-molting and molt groups.

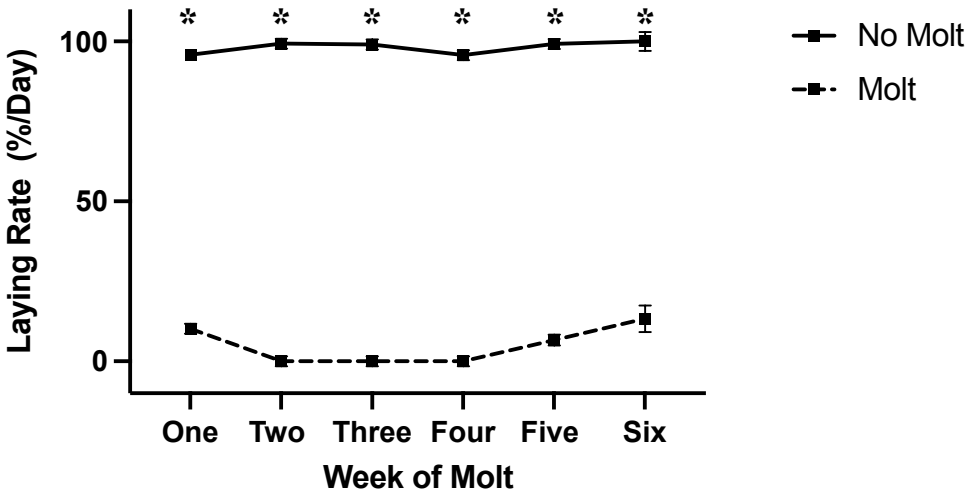

Figure 2.3 The effect of molt status on laying rate. During the six-week study period, the number of birds laying was assessed daily. The number of molting birds laying decreased drastically the first week (estimate: 10.2% ± 1.6, CI: 7.1 – 13.3) compared to non-molting birds (estimate: 95.9% ± 1.6, CI: 92.8 – 99.0). A significantly greater percentage of non-molting birds laid eggs compared to the molting birds during each of the six weeks ($P < 0.01$). Data are represented as mean + standard error with asterisks indicating significant differences ($P < 0.05$) between the non-molting and molt groups.

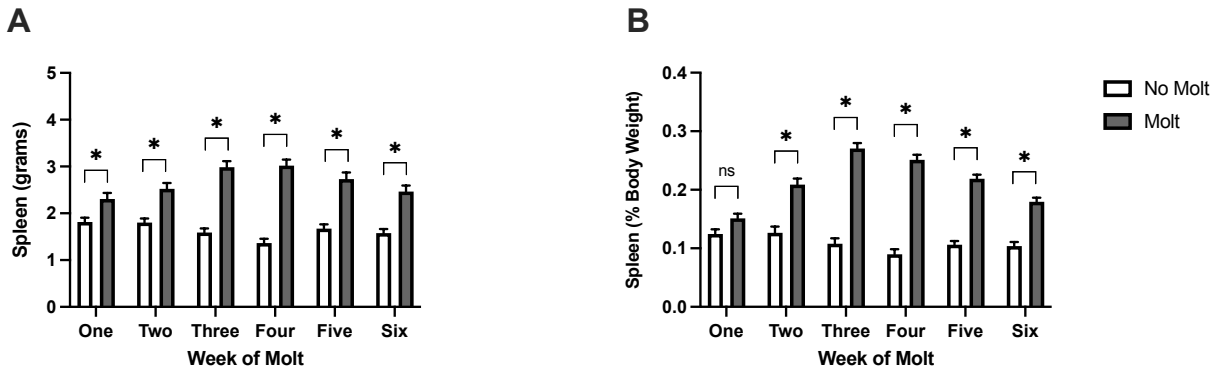

Figure 2.4 The effect of time on spleen weight. The spleen weight of non-molting (N = 6) and molting (N = 6) birds was measured each week for six weeks A. For the duration of the six-week molt, the molting birds had significantly heavier spleens than the non-molting birds ($P < 0.01$). The difference in spleen weight between non-molting and molting birds was greatest during week four (estimate: 1.6 g ± 0.2, CI: 1.2 – 2.1; $P < 0.01$) B. The spleen of molting birds accounted for a significantly greater proportion of the body weight during week two through six ($P < 0.01$). The largest difference between the non-molting and molting birds occurred during week three (estimate: 0.16% ± 0.01, CI: 0.12 – 0.21; $P < 0.01$).

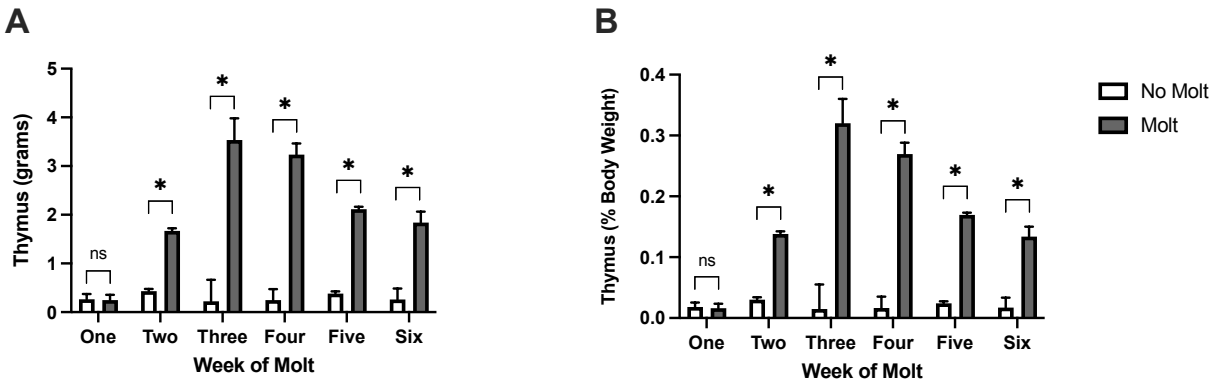

Figure 2.5 The effect of time on thymus weight. The thymus weight of non-molting (N = 6) and molting (N = 6) birds was measured each week for six weeks. A. The molting birds had significantly heavier thymuses compared to the non-molting birds from the second to sixth week ($P < 0.01$). The largest difference in thymus weight between molting and non-molting birds occurred during week three (estimate: 3.3 g ± 0.6, CI: 1.2 – 5.4; $P < 0.01$). The thymus of molting birds was an average of 1.8 grams heavier than the thymus of non-molting birds ($P < 0.01$). B. The thymus of molting birds made up a larger proportion of the body weight during weeks two through six of molt ($P < 0.01$). The largest difference occurred between the non-molting and molting birds during week three (estimate: 0.31% ± 0.06, CI: 0.12 – 0.49; $P < 0.01$).

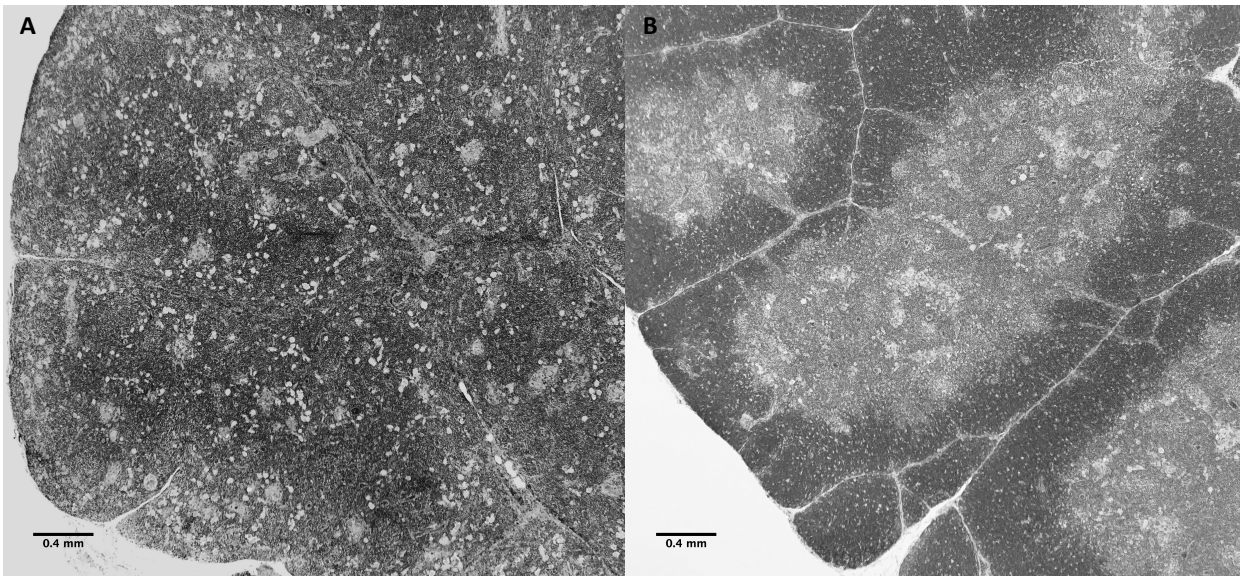

Figure 2.6 Anatomical shifts in the thymus during molt. Thymus sections were collected weekly from non-molting and molting birds and stained with hematoxylin and eosin. A. Thymus section from a non-molting bird (4x) had no well-defined thymic lobules or clearly defined areas of cortex or medulla. B. Thymus section from a bird four weeks into molt (4x). Thymic lobules are clearly visible as are distinct areas of cortex and medulla.

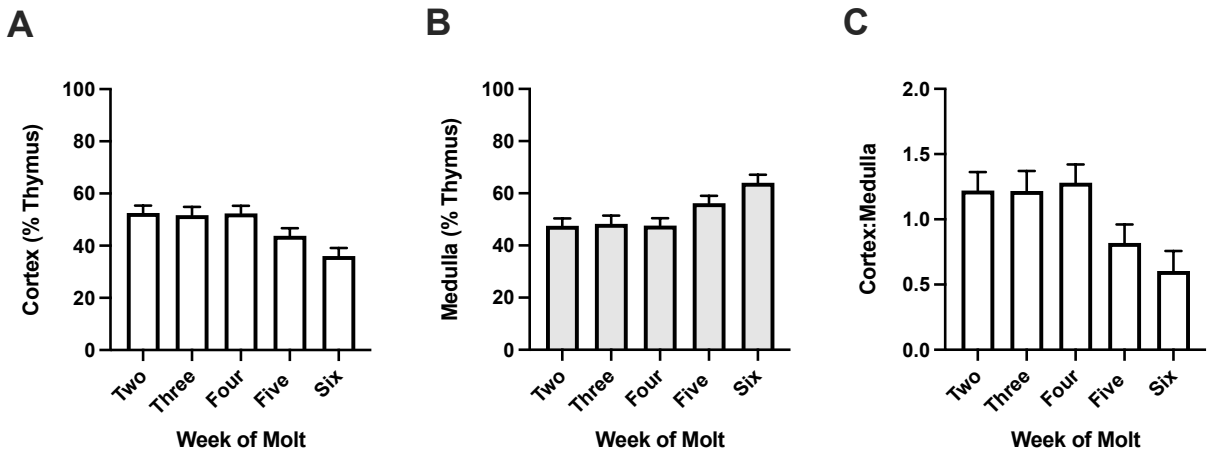

Figure 2.7 Changes in the cortex and medulla during molt. Thymus samples were collected weekly from molting birds (N = 6) to assess anatomical shifts during molt. A. There was a greater percentage of cortex tissue in the thymus at the beginning of molt compared to week six ($P < 0.01$). B. As birds progressed through molt, the percentage of cortex in the thymus increased. The thymus of birds from molt six had a greater percentage of medulla compared to week two ($P < 0.01$) C. The ratio of the cortex to medulla tissue decreased over the course of molt. The cortex to medulla ratio was significantly lower in week six compared to week two ($P = 0.03$). Error bars represent SEM.

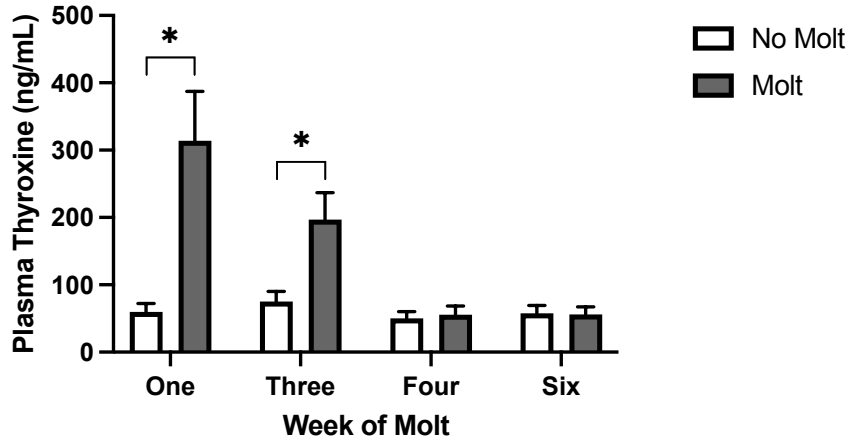

Figure 2.8 The effect of time on thyroxine concentration in the plasma. Plasma samples were collected from non-molting (N = 6) and molting (N = 6) birds to assess plasma thyroxine concentrations. Thyroxine concentrations are shown as average concentration with error bars representing the SEM. During the beginning of molt, molting birds had significantly higher concentrations of plasma thyroxine ($P < 0.01$). By week four, there was no difference in the plasma thyroxine concentration of non-molting and molting birds.

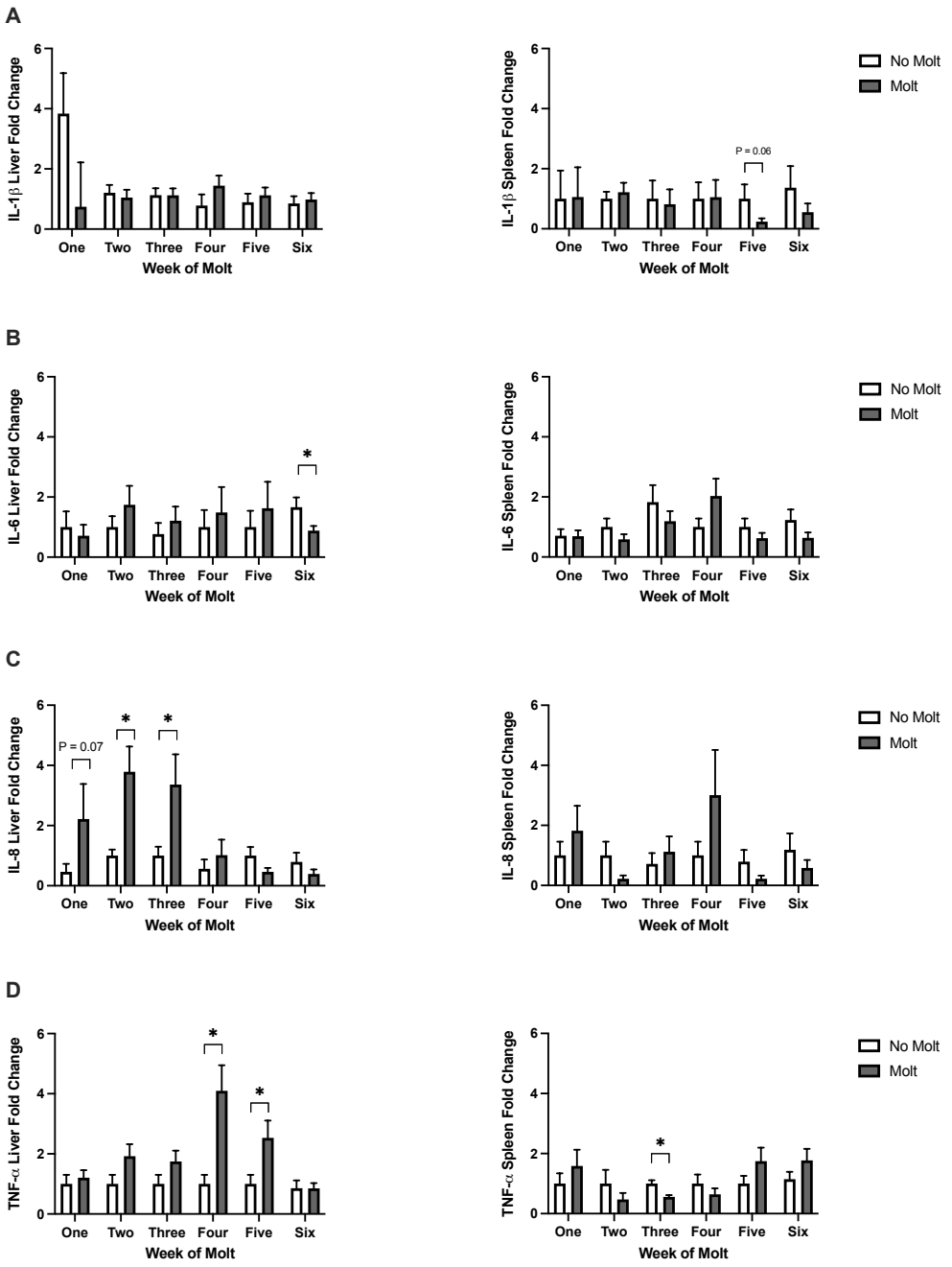

Figure 2.9 The impact of the time on the mRNA expression level of pro-inflammatory cytokines. Liver and spleen samples were taken from non-molting (N = 6) and molting (N = 6) birds at the end of each week. Gene expression levels are represented as the average fold-change relative to GAPDH and RPL13. Error bars are the SEM. A. The expression level of IL-1β in the liver and spleen was not affected by time. B. Time, but not molt or the interaction of time by molt, had an effect on IL-6 expression in the spleen (P = 0.04). C. IL-8 expression was increased in the liver of molting birds during the first three weeks of molt. D. The expression level of TNF-α in the liver and the spleen was significantly affected by both time and molt (P = 0.05).

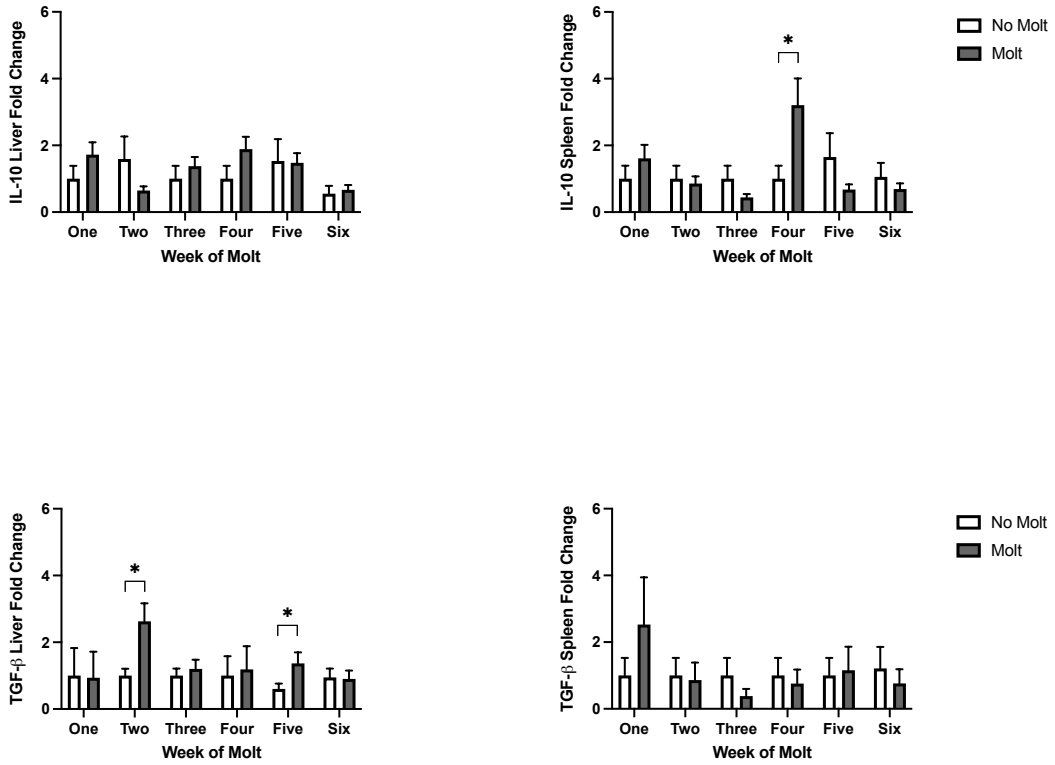

Figure 2.10 The effect of time on the mRNA expression level of anti-inflammatory cytokines. Liver and spleen samples were taken from non-molting (N = 6) and molting (N = 6) birds at the end of each week to assess anti-inflammatory cytokine level using qPCR. Gene expression levels are represented as the average fold-change relative to GAPDH and RPL13. Error bars are the SEM. A. The expression level of IL-10 in the liver was not affected by molt. IL-10 expression level in the spleen was significantly affected by the interaction between time and molt ($P = 0.02$). B. Molt significantly affected the expression level of TGF-β in the liver ($P < 0.01$) but not in the spleen.

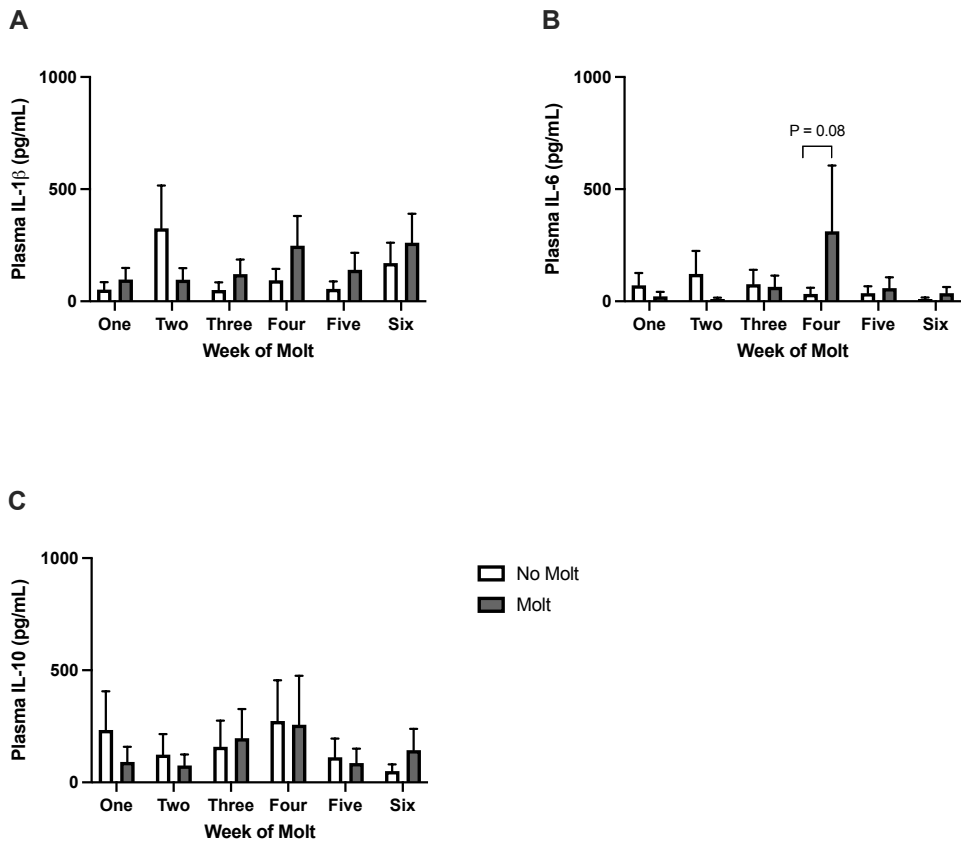

Figure 2.11 The effect of time on the concentration of cytokines in the plasma. Plasma samples were collected from non-molting (N = 6) and molting (N = 6) birds at the end of each week to assess weekly shifts in cytokine concentrations. Cytokine concentrations are shown as the average concentration with error bars representing the SEM. A. Neither time, molt or the interaction of time and molt had an effect on IL-1β concentration. B. The interaction between time and molt trended towards significance (P = 0.08) with molting birds having an increased concentration of IL-6 in the plasma during week four compared to non-molting birds (P = 0.08). C. There was no effect of time, molt, or their interaction on plasma concentrations of IL-10.

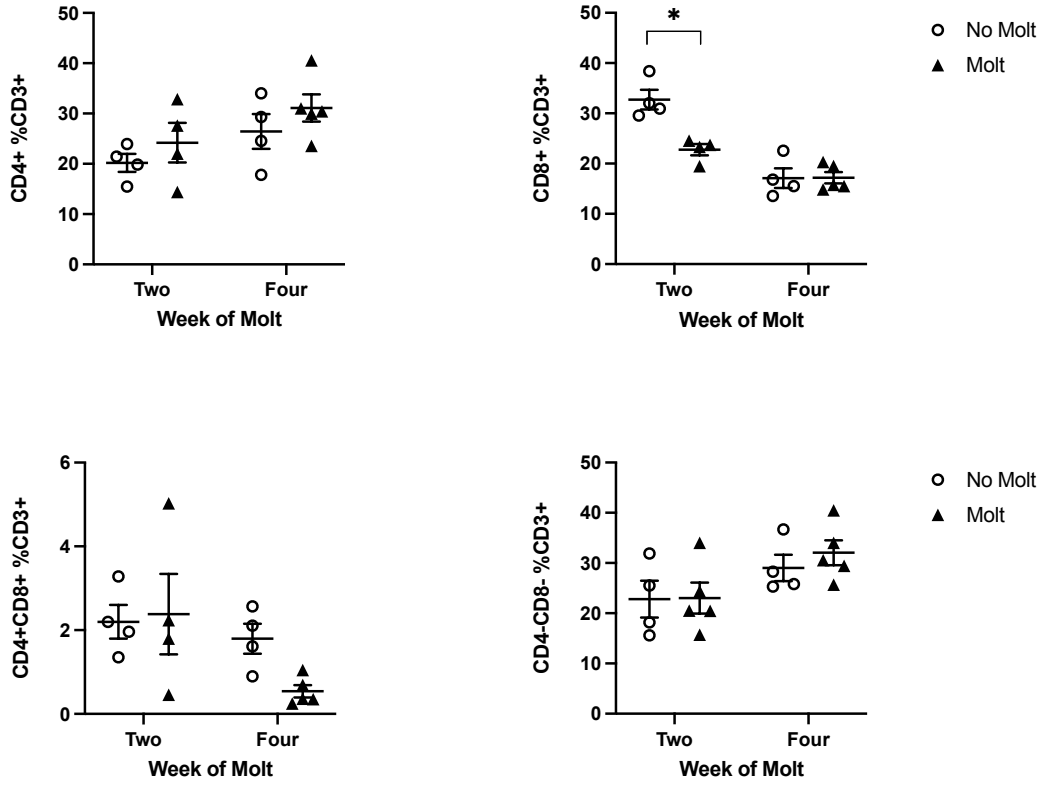

Figure 2.12 The effect of molt on T cell populations in the blood. The percentage of $CD4^+$, $CD8^+$, $CD4^+$ $CD8^+$ and $CD4^-$ $CD8^-$ T cells in the blood were examined during weeks two and four of molt. A. There was no difference in the percentage of $CD4^+$ T cells in the blood of non-molting and molting birds. Non-molting birds had a greater percentage of $CD8^+$ T cells in their blood during week two ($P < 0.01$).

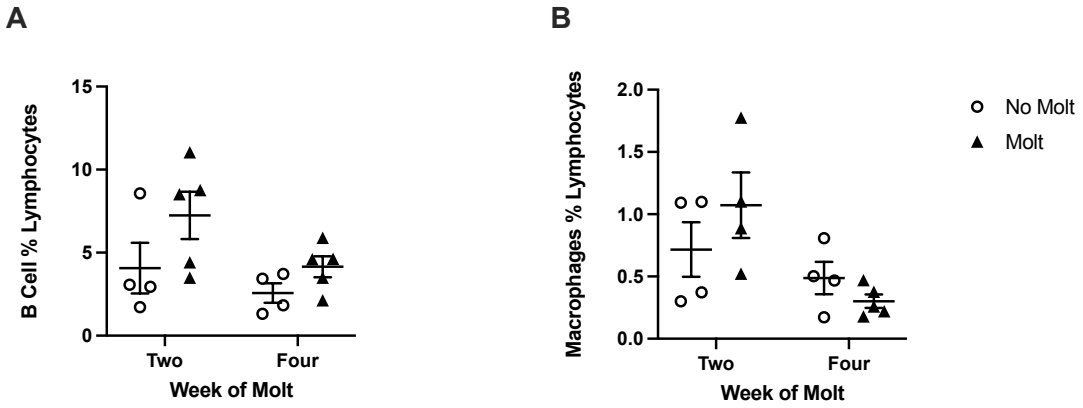

Figure 2.13 The effect of molt on B cells and macrophages in the blood. The percentage of B cells and macrophages in the blood were examined during weeks two and four of molt. Molting birds had a higher percentage of B cells ($P = 0.02$) and macrophages ($P < 0.01$) in their blood during week two of molt compared to week four.

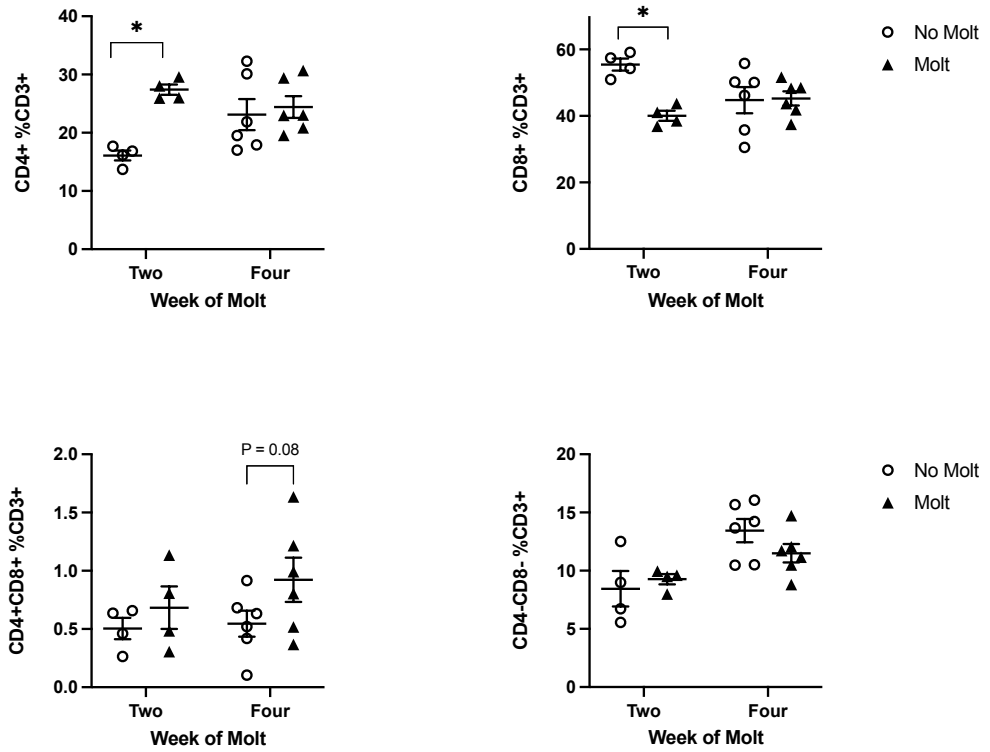

Figure 2.14 The effect of molt on T cell populations in the spleen. The percentage of $CD4^+$, $CD8^+$, $CD4^+CD8^+$ and $CD4^-CD8^-$ T cells in the spleen were examined during weeks two and four of molt. Molting birds had a higher percentage of $CD4^+$ T cells ($P = 0.03$) in the spleen during week two whereas non-molting birds had a higher percentage of $CD8^+$ T cells ($P = 0.02$).

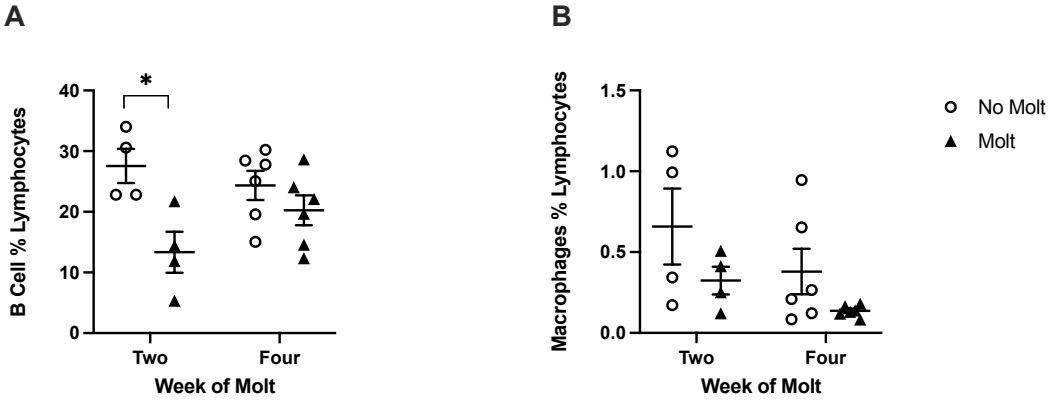

Figure 2.15 The effect of molt on B cells and macrophages in the spleen. The percentage of B cells and macrophages in the blood were examined during weeks two and four of molt. Molting birds had an overall lower percentage of B cells in the spleen compared to non-molting birds ($P < 0.01$). Molting birds trended towards a lower percentage of macrophages in the spleen ($P = 0.06$).

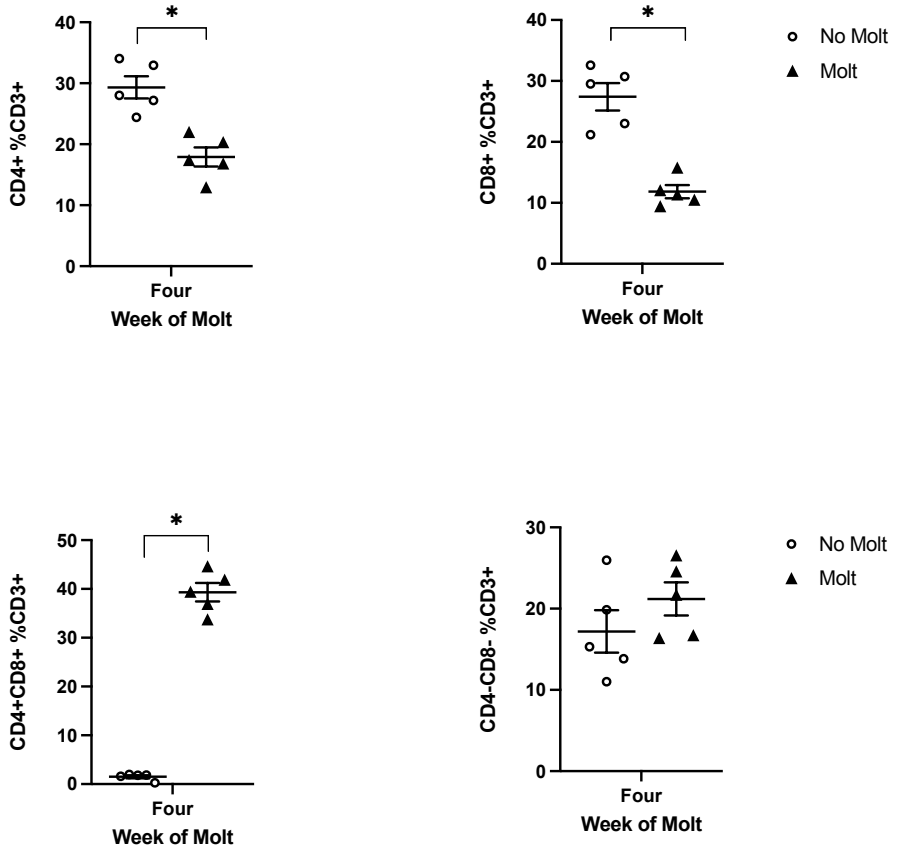

Figure 2.16 The effect of molt on T cell populations in the thymus. The percentage of $CD4^+$, $CD8^+$, $CD4^+CD8^+$ and $CD4^-CD8^-$ T cells in the thymus were evaluated during week four of molt. Molting birds had a lower percentage of $CD4^+$ ($P < 0.01$) and $CD8^+$ ($P < 0.01$) T cells but a much higher percentage of $CD4^+CD8^+$ T cells ($P < 0.01$) in the thymus.

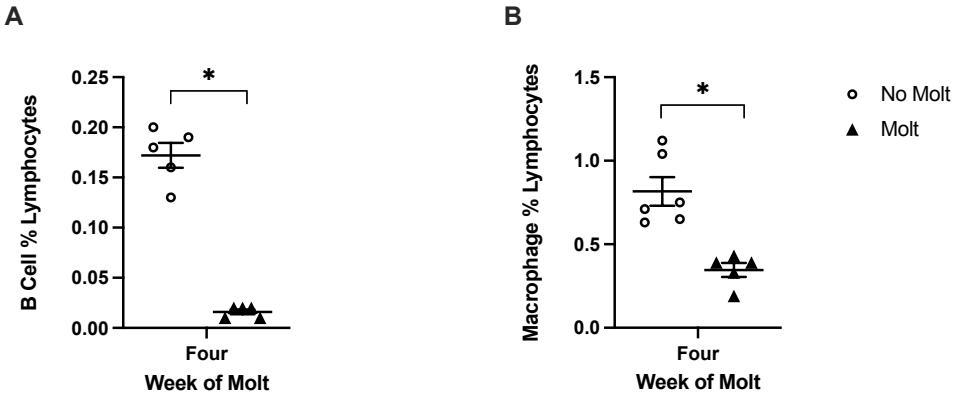

Figure 2.17 The effect of molt on B cells and macrophages in the thymus. The percentage of B cells and macrophages in the thymus were evaluated during week four of molt. Molting birds had an overall lower percentage of B cells and macrophage in the thymus compared to non-molting birds ($P < 0.01$).

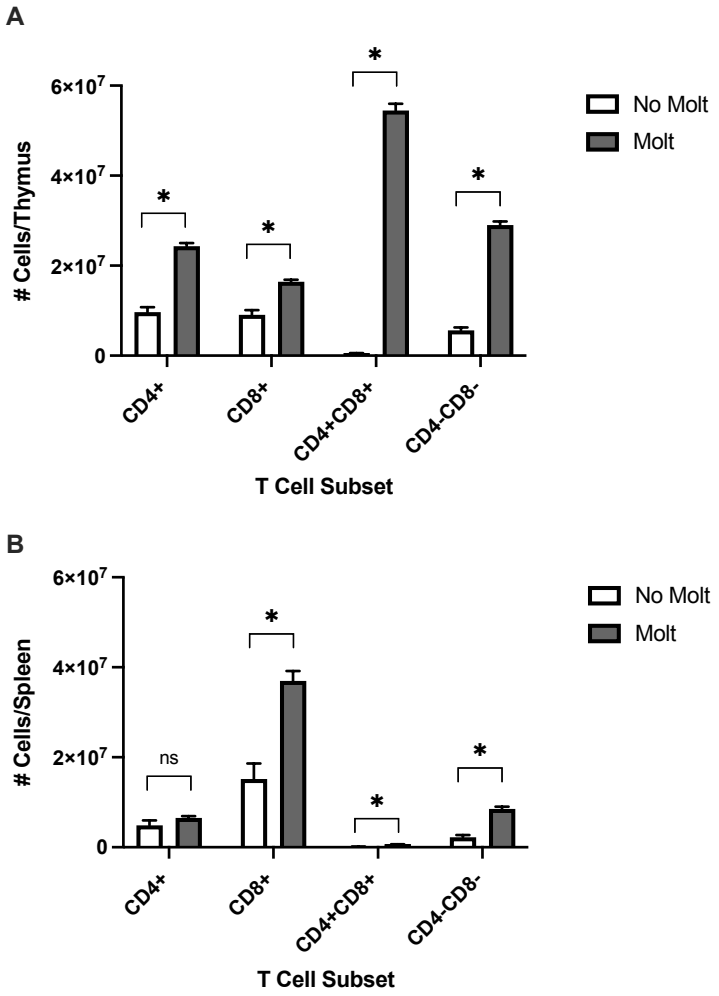

Figure 2.18 Number of T cells in lymphoid tissues during molt. The total number of single positive, double-positive, and double-negative T cells were estimated in the spleen and thymus of molting and non-molting birds A. The thymuses of molting birds had a significantly higher number of cells across all T cell subsets. The greatest difference in cell numbers was observed in the double-positive T cells. B. Molting birds had significantly more $CD8^+$, double-positive and double-negative T cells in their spleens. Molting and non-molting birds had similar numbers of $CD4^+$ T cells.

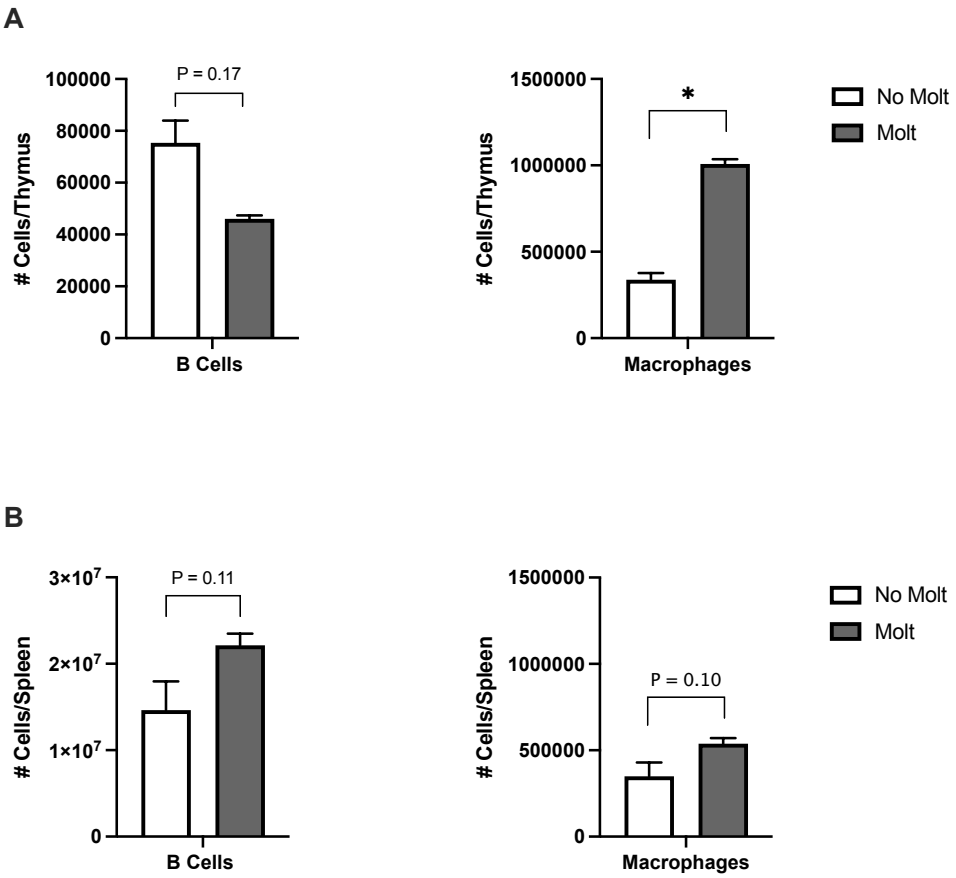

Figure 2.19 Number of B cells and macrophages in lymphoid tissues during molt. The total number of B cells and macrophages in the spleen and thymus of molting and non-molting birds were estimated. A. Molting bird had a significantly higher number of macrophages ($P < 0.01$) but not B cells in the thymus. B. There was a trend towards an increased number of macrophages in the spleen during molt but not B cells.

Chapter 3

Evaluation of the trade-off between molt and innate immunity in the domestic chicken (*Gallus domesticus*)

Abstract

There are a variety of nutritionally costly life stages and events for birds that must be balanced with immune system investments. The process of feather growth in birds is characterized by a range of physiological shifts and is known to be expensive in terms of the nutrients required to produce quality feathers quickly. However, little is understood about the relationship between the costly processes of molt and immunity. We investigated the impact of molt on innate immunity in order to clarify how the nutritional requirements of molt may alter investment in immunity during molt. Molt was induced in chickens (*Gallus domesticus*) using a combination of oral thyroxine (T4), a reduced calorie diet and a shift from a long day to a short-day schedule. During week four of molt, birds were challenged with an intra-abdominal injection of lipopolysaccharide (1.5 mg/kg) to induce an innate immune response. Four hours post injection, liver and spleen samples were collected to evaluate the expression levels of key pro- and anti-inflammatory cytokines. The plasma concentrations of IL-1β, IL-6 and IL-10 were also evaluated four hours post-injection. The weight of the spleen and all thymic lobes were measured to assess changes in lymphoid tissue mass due to molt. The spleen and thymus were both significantly heavier in molting birds than non-molting birds. The weights of the thymus and spleen of molting birds increased by an average of 1.6 and 1.0 grams, respectively. Based on inflammatory cytokine expression levels in the liver and spleen, both molting and non-molting birds mounted an innate immune response to LPS, but the response was dampened in molting

birds. The expression levels of IL-6 and IL-8 were significantly reduced in molting birds. Plasma concentrations of IL-1β and IL-6 from molting birds were also significantly reduced compared non-molting birds indicating that production of protein as part of the inflammatory response may be attenuated due to competing demands for protein allotment to developing feathers. Our data support the prediction that molting birds also undergo broad physiological changes associated with immunity that may be important for maintaining health while balancing nutritional resources during the complex process of molt. This research helps clarify how a nutritionally expensive component of immunity, the inflammatory response, is affected by molt when birds must balance both the costs of immunity with the costs of molting.

Introduction

For avian species, molt is a nutritionally costly life stage that is essential for survival. Worn and missing feathers negatively impact the ability of a bird to thermoregulate, forage, migrate, attract mates, defend territories, and avoid predators. Molt is an expensive process in terms of the nutrients required to produce high quality feathers. The energetic cost of producing feather protein during molt is 3.6 to 4.3 times higher than the cost of producing protein for growth or egg production, respectively (Murphy and King, 1991, 1992). The known energetic costs of producing feathers based on their chemical composition alone cannot account for this disparity. The timing of molt for the majority of avian species occurs so that molt does not overlap with other essential processes, such as reproduction, indicating that the nutritional and energetic costs of molt may be high enough to interfere with other expensive states. The ability to mount and maintain an immune response is another physiological process that is essential to a bird's survival. Due to the importance of the immune system, there are a variety of energetically and nutritionally costly life stages that must be balanced with the metabolic shifts induced by the immune system. The trade-offs between investments in self-maintenance (e.g., immunity, antioxidant defenses) have been shown to be important during growth, migration, and reproduction. However, little detail is known about the relationship between immunity and the process of molt (Sanz et al., 2004; Hegemann et al., 2012). Slowing down or halting molt in order to reserve resources for the immune system leaves a bird in an extended state of vulnerability while their feathers are growing and may even impact an individual's future fitness if the feathers don't grow properly. However, failing to mount a robust immune response has a potentially more immediate negative fitness impact if an individual succumbs to a disease or infection. Because both molt and the ability to mount an immune response are essential for long-

term survival, there are likely to be trade-offs between the competing energetic and nutritional costs of the two processes. Mounting an immune response represents a more immediate pressure for resources, however the process of molt involves complex whole body shifts that could alter the way resources are used by the different systems of the body.

During molt, there is an increase in daily energy use to support feather growth and shifts in protein synthesis. The process of molt involves replacing a range of integumentary tissues including not only feathers but also feather sheaths and other keratinized structures. The regeneration ratio by mass for feathers, feather sheaths and corneous structures is estimated to be 20:4:1 in White-crowned Sparrows with the ratio expected to hold true for many avian species (King and Murphy, 1990). Feathers and related integumentary tissues are extremely protein dense tissues with 90% of a feather's dry mass consisting of protein. Due to the high protein content of feathers, a bird's plumage accounts for up to 25% of the total protein in a bird's body (Chilgren, 1977; Murphy and King, 1984a, c, b). Feathers have a unique amino acid profile compared to other protein dense tissues such as muscle. The most abundant amino acids in feathers are cysteine, glycine, and proline (Murphy and King 1986a). The main structural protein found in feathers is B-keratin, which is cysteine dense and leads to the high proportion of cysteine found in feathers (Busch and Brush, 1979; Brush and Wyld, 1982; Stettenheim, 2000). To generate sufficient protein for feather growth, whole-body protein synthesis increases during the course of molt. At peak feather production, the whole-body protein synthesis in White-crowned Sparrows increased 33% above baseline (Murphy and Taruscio, 1995). Despite the increased protein accretion required to form feathers, the estimated dietary protein levels needed to support molt are calculated to be 10% of dietary protein from White-crowned Sparrows and from 8.6 – 12.4 % dietary protein for chickens recovering from a forced molt (Harms, 1983;

Hoyle and Garlich, 1987; Murphy and King, 1991). Although a low dietary percentage of protein is needed to support molt, the absolute amount of protein required increases. Molting birds may increase daily food intake to ensure increased absolute protein consumption or utilize endogenous protein sources. Increased levels of 3-methylhistidine indicate increased muscle degradation during molt which may be essential to supporting continual feather growth overnight (Taruscio and Murphy, 1995). The requirement for specific amino acids during molt is more likely to be limiting when considering the nutritional requirements of molt in the context of immunity. Feather production requires about 75% the dietary maintenance amount of cysteine and 45% the maintenance amount of valine (Murphy, 1993). Beyond the specific nutritional requirements of molt, there are also increased energetic costs associated with molt. In White-crowned Sparrows, the energy needed to support peak feather growth is equivalent to 58% of the basal metabolic rate (Murphy and King, 1992). The increased energetic and nutritional demands of molt are likely to limit the resources available to support other essential functions.

The process of molt and mounting an immune response are both characterized by shifts in metabolism and increased utilization of specific nutrients. The maintenance and activation of the immune system is commonly characterized as costly to an organism; however, the specific costs have historically been difficult to quantify due to the dispersed nature of the immune system and the dynamic phases of an immune response. The systemic immune system at maintenance, not including the mucosal immune system, is estimated to account for 0.15% of the BW of a domestic chicken and 0.38% of the total body lysine content (Iseri and Klasing, 2013b, a, 2014). Activation of an innate and adaptive immune response are both associated with increased nutrient utilization, but the energetic and nutritional costs of an innate immune response are higher due to a faster response initiation and reliance on acute phase proteins produced in the

liver. During the acute phase response due to a challenge with *E. coli* the components of the systemic immune system increase to 0.25% of body weight. When liver hypotrophy, a key marker of an acute phase response, is accounted for the estimate increases to 0.66% of body weight (Iseri and Klasing, 2013b). During an acute phase response, the use of specific nutrients by the immune system also rises with the production of protective proteins and leukocyte proliferation. The lysine content of effector proteins increased 114% above maintenance during the acute phase response and the overall lysine content in the immune system immune system rose 105% above the level of a healthy bird in response to an *E. coli* challenge (Iseri and Klasing, 2013a). Beyond elevated production of protective proteins and cells, a major modulator of energy use during an innate immune response is the febrile response. In Pekin ducks, an increase of 1°C in body temperature causes a 23% increase in metabolic rate (Gray et al., 2013). Both molt and an innate immune response place increased energetic and nutritional demands on birds.

An innate immune response induces quick and costly metabolic shifts that may lead to modulations in the progression of molt or immunity when both processes must proceed simultaneously. Currently, there is limited data indicating how an innate immune response may be affected by molt. One study examined an innate immune response induced by lipopolysaccharide (LPS) to determine the trade-off between molt and immunity. When House Sparrows were injected with LPS, they failed to fully regrow their feathers indicating that the process of mounting an innate immune response to LPS likely reduced the proportion of nutrients used for feather growth (Ben-Hamo et al., 2017). In hens undergoing a forced molt, the molting period was associated with an increased incidence of *Salmonella enteriditis* susceptibility and pathology (Holt, 1993; Holt and Porter, 1993).

The goal of this study was to determine the extent to which molt affects the innate immune response of chickens to a LPS injection during peak feather production. Compared to adaptive immunity, innate immunity has higher short-term metabolic costs which are more likely to drive a trade-off between molt and immunity. The costs associated with molt are not evenly distributed throughout the process, therefore a strong trade-off effect is most likely to be observed during peak feather synthesis. We predicted that molting hens would experience a trade-off between the nutritional costs of an innate immune response and molt and therefore mount a less robust innate response to an LPS injection compared to non-molting hens. An immune response is essential for survival in both the short term and long term and should therefore take precedence over molt. However, the speed at which molt progresses and the quality of feathers produced also affect the long-term fitness of a bird. Energetic and nutritional resources will be allocated to both processes to ensure survival, but because both molt and the immune system are resource intensive, we predict both should be reduced due to competing demands. This experiment examines the innate immune response component of the potential tradeoff between molt and the immune system.

Material and Methods

Animal Care

Thirty-two Hy-Line W-36 laying hens were obtained from JS West (Atwater, CA) and housed in the Cole A research facility at the University of California, Davis. Hens were housed individually in cages with sloped floors to facilitate egg collection. They were provided ad libitum access to feed and water. A week-long acclimation period was given prior to the

experiment start for the hens to adjust to their new caging. Following the acclimation period, half the birds (N =16) were induced to molt. A combination of light, hormone, and dietary cues were used to closely mimic the natural cues for molt. To allow for different light schedules, two separate rooms with sixteen birds per room were used. The non-molting birds were maintained on a light schedule of 16L:8D and fed 16% Bar-Ale high energy layer diet for the duration of the experiment. Over the course of seven days, the light schedule of the molting birds was reduced from 16 hours of daylight to eight hours of daylight. An oral dose of thyroxine (1.25 mg/kg) was added daily to the feed of molting birds. Finally, molting birds were fed a diet with a reduced content of metabolizable energy content of 2431.60 kcal/kg, 9.6% protein, 3.12% fat and 0.5% calcium. The dietary levels of all other nutrients met the bird's daily nutritional requirements (NRC, 1994). Throughout the experiment body weight and laying rate were monitored. All husbandry and experimental practices were approved by the University of California, Davis IACUC #21304.

Experimental Design

The experiment was performed to assess the effect of molt on an inflammatory response. The molting birds were considered to be molting when the hens ceased laying and began to drop their feathers, which occurred at the end of the seven-day molt induction period. At the end of week four of molt, half (N = 8) of the molting and non-molting birds received an intra-abdominal injection of LPS (1.5 mg/kg body weight). The goal of the experiment was to examine inflammation, rather than the specific effects of LPS. To avoid inducing inflammation due to an intra-abdominal injection of saline, control birds received no injection but were handled and had their abdomen area pinched to simulate the stress of an injection. Four hours post-injection or handling, 5 mL of blood were collected into EDTA tubes from the brachial vein and then all

birds were culled using CO2. The spleen and thymic lobes were collected immediately after death and then weighed (Figure 3.1).

Quantitative RT-PCR of Liver, Spleen, and Thymus Cytokine Gene Expression

Within five minutes of death, samples from the liver, spleen, and thymus were placed into ice cold RNAlater®, minced and stored at -80°C prior to RNA extraction. For RNA extraction, tissue samples were homogenized, and total RNA extracted using the Trizol® protocol for phenol-chloroform extraction of RNA. Following RNA extraction, a Thermo Scientific™ NanoDrop™ spectrophotometer was used to determine the RNA quality of each sample. RNA was defined as good quality if the sample had a 260/280 ratio of 1.8 to 2.0. To reduce DNA contamination, Ambion's DNA-free™ kit (Cat. No. AM1907) was used to remove any residual genomic DNA. Following RNA purification, Reverse Transcription PCR was performed with 1 μg of RNA per tissue sample using Bio-Rad's iScript™ Reverse Transcription Supermix (Cat. No. BR 1708840). All PCR procedures were completed using a Bio-Rad PTC-100 Programmable Thermal Cycler. The resulting cDNA samples were stored at -20°C until quantitative RT-PCR analysis.

To evaluate the strength and progression of the inflammatory response, the expression level of the cytokines IL-1β, IL-6, TNF-α, IL-8, IL-10 and TGF-β were monitored using qPCR. The genes GAPDH and RPL13 were used as reference genes for all cytokines. The following protocol was used for all qPCR reactions: 3 minutes at 95°C, then 40 cycles of 10 seconds at 95°C, 30 seconds at 55°C and 30 seconds at 72°C on a MyIQ iCycler PCR machine (Bio-Rad, Hercules, CA). Bio-Rad's iTAQ ™ Universal SYBR ® green Supermix was used for each reaction with the following components: 1 μL 10 ng/uL cDNA, 10 μL SYBR Universal SYBR ®

Green Supermix and variable amounts of DEPC water and variable forward and reverse primer concentrations based on the specific primer set. Each experimental sample was run in triplicate with DEPC water as a NTC control. The fold-change value (ΔΔCt) for each cytokine was calculated using the ratio of the gene of interest ΔCt to the housekeeping genes (RPL13 and GAPDH) ΔCt.

Cytokine Quantification

Whole blood was collected from the brachial vein into EDTA treated tubes prior to euthanasia using 25G ⅝ needles with 3 mL syringes. To separate the plasma, the whole blood was spun for 15 minutes at 2000g (Sorvall® Legend MACH 1.6**)**. Plasma samples were then aliquoted into 200 uL microcentrifuge tubes and stored at -20°C until further processing. Commercial ELISA kits from were used to quantify the amount of IL-1β, IL-6 and IL-10 (ABclonal® Cat. No. RK00591, RK00594, RK00587) and thyroxine (Aviva Systems Biology Cat. No. OKCA00265) in plasma samples according to the manufacturer's instructions. The concentration of IL-1β, IL-6 and IL-10 were reported as picograms (pg) per milliliter and as nanograms (ng) per milliliter for thyroxine.

Statistical Analysis

All statistical analyses were performed using Microsoft Excel® Version 16.58, R© version 4.1.1 and RStudio© version 2021.9.0.351 (R Core Team, 2021; RStudio Team, 2021). For all models, normality was assessed via visual examination of a Q-Q plot of the standardized residuals versus the theoretical quantiles in combination with Shapiro-Wilks test of normality. Extreme outliers were identified as values above the third quartile plus three times the interquartile range or the first quartile minus three times the interquartile range. Extreme outliers

were evaluated for removal using the Bonferroni outlier test and Cook's distance to evaluate the outlier leverage. Homogeneity of variance was assessed using both Levene's test and visual examination of a plot of the model standardized residuals by the fitted model values. When comparing models to determine the most appropriate model to select, log-likelihood, AIC and BIC values were used to select the best fitting model (Bates et al., 2015). Body weight was analyzed as a 2 x 5 factorial with two levels of molt by five different times (week) of molt. A generalized least squares model was constructed with molt status, the week of molt and the interaction of molt status and week of molt as fixed effects. To achieve homogeneity of variance, each level of molt was assigned a unique variance (Pinheiro & Bates, 2006). To assess the effect of molt on body weight loss over time, the same generalized least squares model was used with the key difference of four levels of week of molt rather than five as the pre-molt body weight data was used as a reference to calculate the change in body weight during molt. When examining the difference in laying between molting and non-molting birds, a linear model with molt, week of molt and the interaction between molt and week of molt was used. Welch's T-test was used to compare the plasma concentration of thyroxine for molting and non-molting birds.

Organ weight, ELISA and qPCR data were analyzed as a 2 x 2 factorial with two levels of molt (molt and no molt) and treatment (LPS and control) with molt, treatment, and the interaction of molt by treatment as fixed effects in all models. Both ELISA cytokine concentration and qPCR fold-change data were log transformed in order to fit a normal distribution. A linear model was used to assess the following data sets: organ weights, plasma IL-1β concentration, plasma IL-6 concentration, liver IL-1β expression, spleen IL-1β expression, thymus IL-1β expression, spleen IL-8 expression, thymus IL-8 expression, liver TGF-β expression, thymus TGF-β expression, and thymus IL-10 expression. A generalized least squares

model with unique variance assigned for each level of treatment was used for liver IL-6 expression, liver TNF-α expression, spleen TNF-α expression, liver IL-8 expression, liver IL-10 expression and TGF-β spleen expression. For all remaining data sets including plasma IL-6 concentration, spleen IL-6 expression, thymus IL-6 expression, spleen IL-10 expression and TNF-α thymus expression a generalized least squares model with unique variance for each level of molt was used.

A two-way analysis of variance (ANOVA) was used to evaluate the main effect of molt, treatment and the interaction of molt and treatment on measured variables with significant accepted at $P \leq 0.05$. When the p-value of a main affect was between $P > 0.05$ and $P < 0.10$, there was considered to be a non-significant trend of the effect. Tukey's honestly significant difference test (HSD) was used to evaluate differences between least squares means for contrasts evaluating the effect of molt and treatment. For planned contrasts, no p-value corrections were performed. For all additional *Post hoc* analysis, a Bonferroni p-value correction was used when multiple pairwise comparisons were performed. Results are reported as coefficient estimates, standard errors and 95% CIs.

Results

Body Weight and Laying

The body weight and laying rate of the birds were monitored weekly to observe the effects of molt. There were significant main effects of both time ($P < 0.01$) and mot ($P < 0.01$) on body weight (Figure 3.2A). The interaction between time and molt was also significant ($P < 0.01$) because the body weight of the body weight of the non-molting birds increased by an

average of 11.2% over the four-week period while the body weight of the molting birds decreased by an average of 13.4%. At the start of the experimental period, the average body weight of birds in the non-molting and molting groups did not differ. Non-molting birds were significantly heavier during week one (estimate: 317.5 g ± 31.1, CI: 236.9 – 398.1), week two (estimate: 361.7 g ± 31.1, CI: 281.1 – 442.3), week three (estimate: 347.0 g ± 36.2, CI: 253.0 – 441.1) and week four (estimate: 347.5 g ± 36.2, CI: 253.5 – 441.6) of the experiment.

The change in body weight relative to the pre-molt period was also tracked throughout the experiment. Time did not have a significant effect on the change in body weight ($P = 0.15$; Figure 3.2B). There was a significant main effect of molt ($P < 0.01$) on the change in body weight. Non-molting birds had an average increase in body weight of 11.2% compared to molting birds which had an average decrease in body weight of 13.4% (Figure 2). The interaction between week and molt was not significant ($P = 0.78$).

There was a significant main effect of week and molt and an interaction of week and molt on laying rate ($P < 0.01$). At the onset of molt, the cessation of lay for most birds occurred during week one. The majority of birds were laying at the end of the pre-molt week (estimate: 96.2% ± 3.9, CI: 88.3 – 104.1) and by the end of week one none of the molt birds were laying. The average laying rate for non-molting birds was 93% (Figure 3.3).

Plasma Thyroxine

The concentration of thyroxine was measured in the plasma of non-molting and molting hens following an LPS injection during week four of molt. There was no effect of an LPS injection on plasma thyroxine concentration (Figure 3.4). Molt had a significant main effect on the concentration of thyroxine in the plasma ($P = 0.03$). Birds that were not molting had a higher

average plasma thyroxine concentration (estimate: 65.8 ng/mL ± 4.1, CI: 55.7 – 75.8) than molting birds (estimate: 65.8 ng/mL ± 4.1, CI: 55.7 – 75.8). The interaction between LPS treatment and molt was not significant ($P = 0.35$).

Spleen and Thymus Weight

The weight of the spleen was measured at the end of week four to assess changes in response to an LPS injection and molt (Figure 3.5A). There was a significant main effect of an LPS injection on spleen weight ($P < 0.01$). LPS increased spleen weights in both non-molting (estimate: 0.26 g ± 0.12, CI: 0.02 – 0.50) and molting birds (estimate: 0.48 g ± 0.14, CI: 0.18 – 0.77). In both non-molting and molting birds, the increases in spleen weight following an injection of LPS were equivalent at a 19% and 21% percent increase, respectively. Molt also had a significant main effect on the weight of the spleen ($P < 0.01$). The spleens of molting birds were, on average, more than a gram larger than the spleens of non-molting birds (estimate: 1.0 g ± 0.1, CI: 0.8 – 1.2; $P < 0.01$), which is a 68% increase in weight. The interaction of an LPS injection and molt did not have a significant effect on spleen weight ($P = 0.24$).

To account for the difference in body weight between non-molting and molting birds, changes in spleen weight were also analyzed on a percent body weight basis and the effect of an LPS injection ($P < 0.01$) and molt ($P < 0.01$) remained significant after this correction (Figure 3.5B). When correcting for body weight, molt resulted in an 114% increase in the proportion of body weight made up by the spleen ($P < 0.01$). The effect of the interaction of LPS injection and molt on spleen weight as a proportion of body weight trended towards significance ($P = 0.08$)

because the response to LPS injection was 42% higher in the molting birds than the non-molting birds ($P < 0.01$ by pairwise comparisons).

The weight of the thymus was also monitored to track changes in the lymphoid tissue in response to an LPS injection and molt (Figure 3.6A). There was a significant main effect of both an LPS injection and molt on thymus weight ($P < 0.01$). The average thymus weight for molting birds (estimate: 2.1 g ± 0.1, CI: 1.8 – 2.3) was more than four times the weight of the thymus of non-molting birds (0.47 g ± 0.09, CI: 0.23 – 0.78; Figure 6). There was also a significant interaction between an LPS injection and molt ($P < 0.01$) because the injection of LPS decreased the weight of the thymus in molting (estimate: -0.94 g ± 0.21, CI: -1.37 – - 0.51) but not non-molting birds (estimate: 0.01 g ± 0.18, CI: -0.37 – 0.38).

When correcting for body weight, there was a significant main effect of treatment and molt and a treatment by molt interaction on the thymus ($P < 0.01$ respectively; Figure 3.6B). The thymus of molting birds accounted for a 45-fold greater increase in the proportion of body weight than the thymus of non-molting birds (estimate: 0.13% ± 0.01, CI: 0.11 – 0.16). The distinction between the weight of the thymic lobes of molting and non-molting birds upon visual inspection alone were noteworthy and striking. The thymuses of molting birds were significantly more responsive to an injection of LPS compared to non-molting birds. An injection of LPS led to a 38% decrease in the thymus as a percent of body weight ($P < 0.01$ by pairwise comparisons) in molting birds while the thymuses of non-molting birds were unaffected ($P = 0.96$ by pairwise comparisons).

Cytokine mRNA Expression in the Liver and Spleen

Four hours following an intra-abdominal injection of LPS samples were taken from the liver, spleen, and thymus to determine the effect of molt on the expression of immune related genes during an inflammatory response (Table 3.2). The IL-1β expression level increased to a similar extent in the spleen and liver of both non-molting and molting birds ($P < 0.01$ respectively) but was not increased in the thymus. Neither molt nor the interaction of LPS injection and molt significantly affected IL-1β expression in the liver, spleen, or thymus (Figure 3.7).

The expression level of IL-6 in the liver ($P < 0.01$), spleen ($P < 0.01$) and thymus ($P = 0.04$) increased significantly in response to an injection of LPS (Figure 3.7B). Molt significantly decreased IL-6 expression in the thymus ($P < 0.01$) and trended towards reducing IL-6 in the liver ($P = 0.07$) but did not lower expression in the spleen (Table 3.2). The interaction between the LPS injection and molt significantly affected IL-6 expression level in the liver ($P = 0.02$) but not in the spleen or thymus. The injection of LPS led to a significantly greater increase in IL-6 expression in the liver of non-molting birds compared to molting birds ($P = 0.02$ by pairwise comparison).

An injection of LPS significantly affected IL-8 expression level in the liver ($P < 0.01$) and trended towards affecting IL-8 expression in the spleen ($P = 0.08$; Figure 3.7C). An LPS injection did not affect the expression of IL-8 in the thymus ($P = 0.48$; Table 3.2). Molt trended towards significantly increasing IL-8 expression in the liver ($P = 0.09$) and significantly affected IL-8 expression in the thymus ($P = 0.01$). Compared to non-molting birds, molting birds trended towards a reduced increase in IL-8 expression in the liver in response to LPS ($P = 0.08$). There

was a significant interaction between LPS injection and molt significantly because non-molting birds had an increased expression level of IL-8 in response to LPS ($P = 0.01$) while molting birds did not. In the absence of LPS, the IL-8 expression level in the spleen of unchallenged molting birds was higher than the level of IL-8 in unchallenged non-molting birds ($P = 0.05$). The expression level of IL-8 in liver ($P = 0.41$) and the thymus ($P = 0.3$) were not affected by LPS.

The expression level of TNF-α in response to LPS trended towards increasing in the liver ($P = 0.07$). The expression of TNF-α in both the spleen ($P = 0.52$) and the thymus ($P = 0.66$) were not altered by an injection of LPS. Molt significantly altered the expression level of TNF-α in the thymus ($P < 0.01$) but not the liver or the spleen (Table 3.2). Molting birds had lower TNF-α expression levels, with the greatest reduction following an injection of LPS ($P < 0.01$ by pairwise comparison). The expression level of TNF-α was not affected by the interaction of LPS injection and molt in either the liver, spleen, or thymus (Figure 3.7D).

In the liver, spleen, and thymus ($P < 0.01$ respectively) an injection of LPS led to a significant increase in IL-10 expression (Figure 3.8A). Molt trended towards affecting the expression level of IL-10 in the thymus ($P = 0.07$) but did not lead to a change in IL-10 expression in the liver or spleen (Figure 3.8A). The interaction of an LPS injection and molt did not change IL-10 expression in the liver, spleen, or thymus.

In response to an LPS injection, there were significant change in the expression level of TGF-β in the liver ($P = 0.05$) and spleen ($P = 0.01$) but not the thymus (Figure 3.8B). The expression level of TGF-β increased in response to LPS, especially in the livers of molting birds ($P = 0.03$ by pairwise comparison). There was no effect of molt or its interaction with LPS on the expression of TGF-β in the liver, spleen, or thymus (Table 3.3).

Cytokine Protein Concentration

The concentration of pro-inflammatory cytokines IL-1β and IL-6 and the anti-inflammatory cytokine IL-10 were measured in plasma four hours after an intra-abdominal LPS injection. LPS led to a significant increase in the concentration of IL-1β in the plasma ($P = 0.02$) with the largest increase seen in non-molting birds ($P = 0.02$ by pairwise comparison). The IL-1β concentration in the plasma of molting birds injected with LPS did not increase significantly (Figure 3.9A). Neither molt nor its interaction with LPS influenced the plasma concentration of IL-1β.

An injection of LPS ($P = 0.02$), but not molt condition, altered the plasma concentration of IL-6 (Table 3.4; Figure 3.9B). There was a significant interaction between an LPS injection and molt because after an injection of LPS the plasma concentration of IL-6 increased significantly in the non-molting birds ($P < 0.01$) but not in the molting birds. Following an LPS injection, the concentration of IL-6 in the plasma of non-molting birds was 78% higher compared to the molting birds. Overall, molt led to a reduction in the concentration of both IL-1β and IL-6 in response to LPS in the plasma of molting birds compared to non-molting birds. Neither an injection of LPS or molt had an effect on the plasma concentration of IL-10 (Figure 3.9C). There was no effect of LPS injection on IL-10 concentration, but molt did trend towards increasing the plasma concentration of IL-10 ($P = 0.06$). When compared to non-molting birds, birds that were molting had a higher concentration of IL-10 in their plasma ($P = 0.05$). There was no significant interaction between an LPS injection and molt on IL-10 levels in the plasma.

Discussion

There are known trade-offs between the immune system and other essential processes such as growth and reproduction, however there is no clear understanding of how immunity may be affected by the process of molt. Molt and an innate immune response are both essential for survival and associated with large increases in energy and nutrient demands. To evaluate the potential trade-off between molt and immunity, we examined the effect of molt on the immune system in both the non-stimulated state and during an inflammatory response to an intra-abdominal injection of LPS.

Molting and non-molting birds started with equivalent body weights, but within the first week of molt the body mass of molting birds was reduced with the maximum average body weight reduction of 16% occurring during week two. When evaluating the trade-off between molt and immunity, it is important to consider the extent of the body weight loss that occurs with molt. Previous studies evaluating the effects of molt on immunity in domestic chickens have used an induced molt method that involves an extended period of nutrient restriction that leads to body weight losses of 20 – 30% (Brake and Thaxton, 1979; Webster, 2003). Extreme nutrient restriction and the resulting body weight loss can make it difficult to disentangle the effects of molt on immunity from the effects of starvation. The overlapping effects of and different methods of nutrient deprivation during molt may contribute to some of the contradictions in the literature about the effects of molt on immunity especially in studies of poultry. Due to the involution of the oviduct, feather loss, and muscle degradation to supply amino acids for feather synthesis there is an expected amount of body weight reduction as part of the molt process. The cues we used to induce molt were selected to keep body weight loss to a minimum in order to prevent confounding effects of molt and starvation on immunity. Although a hormonal stimulus

(thyroxine) was used to induce molt in this study, the difference in thyroxine levels were consistent with what has been previously observed during natural molt. Molting birds have a lower plasma thyroxine concentration compared to non-molting birds (Brake et al., 1979; Davis et al., 2000).

The weight of important lymphoid tissues, the spleen and thymus, increased as birds progressed through molt. The spleens of molting birds were, on average, 68% larger compared to the spleen of non-molting birds. The spleens of birds induced to molt by feed restriction have been shown to increase in weight, but to a lower magnitude than reported in this study (Brake and Thaxton, 1979; Brake et al., 1985). The recrudescence of the thymus in molting birds has been observed in hens undergoing an induced molt and is hypothesized to occur in wild avian species (Brake et al., 1981). However, no other studies of poultry have examined the absolute increase in thymus mass during molt. In non-molting birds the thymus weighs less than half a gram which increased to more than 2 grams during molt, which is a 336% percent increase. The comparative increase in the size of the thymus of molting birds compared to non-molting birds was much larger than the increase in spleen weight. The spleen and thymus of molting birds are not only heavier but also have more pronounced weight changes in response to LPS. The spleen of molting and non-molting birds both increased in weight in response to LPS by 18% and 21%, respectively. During an acute phase response, the blood flow to the spleen increases following elevated levels of IL-1β (Rogausch et al., 1997). Following an LPS injection, there are shifts in the populations of leukocytes in the spleen as the localization of cells in the spleen shifts in addition to varying efflux and influx of leukocytes over the course of an acute phase response (Bowen et al., 2009; Zhang et al., 2017). The approximately 20% increase in spleen weight seen in both molting and non-molting birds is likely to be driven more by an increase in blood flow

rather than an influx of leukocytes to the spleen given the four-hour sampling time point. In response to LPS, the thymus of molting birds decreased in weight but there was no change in weight of the thymus of non-molting birds. The recrudescence of the thymus is likely driven by increased lymphocyte proliferation (Brake et al., 1981). LPS is a potent inducer of thymocyte apoptosis in the thymus of young chickens (Huang et al., 2016). The thymuses of molting birds are expected to have an increased number of T cells compared to non-molting birds. The decrease in thymus weight following an LPS injection in the molting birds may be explained by LPS induced apoptosis of a greater number of T cells in the thymus of molting birds compared to non-molting birds.

When considering the energetic and nutritional costs associated with molt, the measured efficiency of protein deposition in feathers is much lower than other processes such as reproduction. The nutrient requirements of molt are attributed primarily to the production of feathers which may lead to an underestimate of the nutrients used by other tissues that undergo shifts during molt and an inflation of the nutritional costs of feather growth. Changes in organ mass of the spleen and thymus require protein and are difficult to properly estimate as the changes in organ physiology are dynamic over the course of molt. Therefore, the dynamic shifts in lymphoid tissue during molt could contribute to the unaccounted inefficiency of molt since even the most thorough studies do account for such changes in the final tally of protein production during molt (King and Murphy, 1990).

To examine the potential trade-off between molt and immunity, an innate immune response to LPS was induced during molt. The inflammatory response of molting birds was monitored using the gene expression of key pro-inflammatory cytokines in the liver, spleen, and thymus. The liver is an essential organ for the inflammatory response that undergoes significant

function shifts during an acute phase response. Shifts in the weight of the liver and protein production in the liver to support the inflammatory response are a major portion of the nutritional costs of an innate immune response (Iseri and Klasing, 2013b, 2014). Both feather growth during molt and an innate immune response requires the use of amino acids to produce essential proteins. If a bird does not have enough nutritional and energetic resources to support both simultaneously, one or both processes could be reduced or even paused. Both non-molting and molting birds experienced inflammation following and LPS injection, however molt appeared to dampen the inflammatory signal. Although both non-molting and molting birds had increased IL-6 and IL-8 expression levels in the liver, the increase in expression level of molting birds was significantly less compared to the increase observed in non-molting birds. A similar trend for IL-6 expression in the spleen of molting birds was also observed. Molting birds still demonstrated gene expression profiles consistent with an inflammatory response, but molt appeared to reduce the expression level of key inflammatory cytokines. When comparing feather growth and an inflammatory response, an inflammatory response is essential for immediate survival and is preserved during molt. It is likely that a combination of hormonal influences and increased nutrient demand for feather growth dampens the inflammatory response and its concomitant nutrient demands.

Plasma protein levels of inflammatory cytokines were measured to determine if they reflected mRNA expression levels in responding tissues. LPS increased plasma IL-1β levels to a similar extent in both molting and non-molting birds, indicating that the circulating concentration of the protein did not fully reflect the liver and splenic RNA expresion. The concentration of IL-1β in the plasma of molting birds did not increase significantly above the concentration of the unchallenged molting birds. In response to LPS, non-molting birds produced more IL-6 mRNA

and more IL-6 protein than molting birds. The dampening of both IL-1β and IL-6 expression levels in the liver and spleen and the dampened concentration of circulating IL-6 in response to LPS indicate that both the local and systemic responses to LPS are dampened during molt. During an acute phase response, the essential amino acid lysine used by the immune system is estimated to increase from equivalent to 332 feathers to as much as 667 feathers during an acute phase response (Iseri and Klasing, 2014). The level of abrupt increase in protein synthesis that is essential to producing the protective proteins needed for an innate immune response may be unattainable while birds are undergoing molt compared to during other life stages. An inflammatory response is maintained in molting birds but appears reduced during molt when important nutrients necessary for an inflammatory response are also being used for feather growth.

The trade-off between molt and an innate immune response is likely to be driven by an inability of the bird to allocate sufficient energy and protein to support both of these costly processes simultaneously. When exposed to salmonella, birds that underwent induced molt were more susceptible to a re-infection than non-molting birds (Holt, 1993; Holt and Porter, 1993). An injection of LPS slows the production of feathers in other species of birds (Houben et al., 2017). When birds must maintain both an immune response and the process of molt, it appears as though there is a redistribution of resources between the two processes. However, since both processes are essential to survival, rather than completely reducing or halting either process both appear to be attenuated. After an LPS injection during molt, the quality of feathers grown by house sparrows was reduced likely due to protein and essential amino acids being diverted away from feather growth to the immune system (Ben-Hamo et al., 2017). Failing to mount any immune response can be fatal but mounting a reduced immune response may still be sufficient to

control a pathogen while allowing a bird to finish the process of molt. Both the non-molting and molting birds had an inflammatory response to an injection of LPS; however, the inflammatory response was dampened in the molting birds. For key cytokines, the pro-inflammatory signal that is important for an acute phase response was reduced in the liver, which is an essential mediator of the acute phase response and the main site of acute phase protein production. IL-6, in particular, is important for helping mediate the febrile response and production of acute phase proteins. The reduction in IL-6 expression and plasma protein levels may be important for conserving energy and nutrients to balance molt and innate immunity. Both growing new feathers and sufficiently mounting an immune response are essential for the long-term fitness of birds. If a bird fails to quickly produce quality feathers, they risk remaining in a vulnerable state of molt for longer or having flight and thermoregulation impacted by structurally unsound feathers. The failure to mount an inflammatory response will have an immediate fitness cost if a bird succumbs to a pathogen making mounting an immune response arguably of higher priority than molt. The absolute essentiality of the immune system to survival even is further supported by the maintenance of lymphoid tissue during molt while other organs, such as those of the reproductive system, are involuted during molt (Brake and Thaxton, 1979). Overall, in a trade-off scenario between molt and an inflammatory response there appears to be a dampening of both molt and the inflammatory response which allows for the redistribution of resources in order to maintain both essential processes and ensure survival when nutrients may be limited.

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Tables

Table 3.1 ANOVA P-values for the main effects of treatment, molt, and the treatment by molt interaction on spleen and thymus mass. P-values are shown for both organ weight in grams and organ weight corrected for body weight.

	Spleen (Grams)	Spleen (% BW)	Thymus (Grams)	Thymus (% BW)
Treatment	< 0.01	< 0.01	< 0.01	< 0.01
Molt	< 0.01	< 0.01	< 0.01	< 0.01
Treatment*Molt	0.24	0.08	< 0.01	< 0.01

Table 3.2 ANOVA P-values for the main effects of treatment, molt, and the treatment by molt interaction on the expression level of pro-inflammatory cytokines in the liver, spleen, and thymus.

Cytokine	IL-1β			IL-6			IL-8			TNF-α		
Tissue	***Liver***	***Spleen***	***Thymus***	***Liver***	***Spleen***	***Thymus***	***Liver***	***Spleen***	***Thymus***	***Liver***	***Spleen***	***Thymus***
Treatment	< 0.01	< 0.01	0.11	< 0.01	< 0.01	0.04	< 0.01	0.08	0.48	0.07	0.52	0.66
Molt	0.89	0.45	0.26	0.07	0.16	< 0.01	0.09	0.39	0.01	0.61	0.74	< 0.01
Treatment*Molt	0.51	0.38	0.52	0.02	0.15	0.17	0.41	0.05	0.3	0.93	0.89	0.14

Table 3.3 ANOVA P-values for the main effects of treatment, molt, and the treatment by molt interaction on the expression level of anti-inflammatory cytokines in the liver, spleen, and thymus.

Cytokine	**IL-10**			**TGF-β**		
Tissue	***Liver***	***Spleen***	***Thymus***	***Liver***	***Spleen***	***Thymus***
Treatment	< 0.01	< 0.01	< 0.01	0.05	0.01	0.71
Molt	0.76	0.27	0.07	0.23	0.21	0.56
Treatment*Molt	0.25	0.5	0.45	0.24	0.46	0.99

Table 3.4 ANOVA P-values for the main effects of treatment, molt, and the treatment by molt interaction on the plasma concentration of pro- and anti-inflammatory cytokines.

Cytokine	**IL-1β**	**IL-6**	**IL-10**
Treatment	0.02	0.02	0.69
Molt	0.76	0.50	0.06
Treatment*Molt	0.32	< 0.01	0.33

Figures

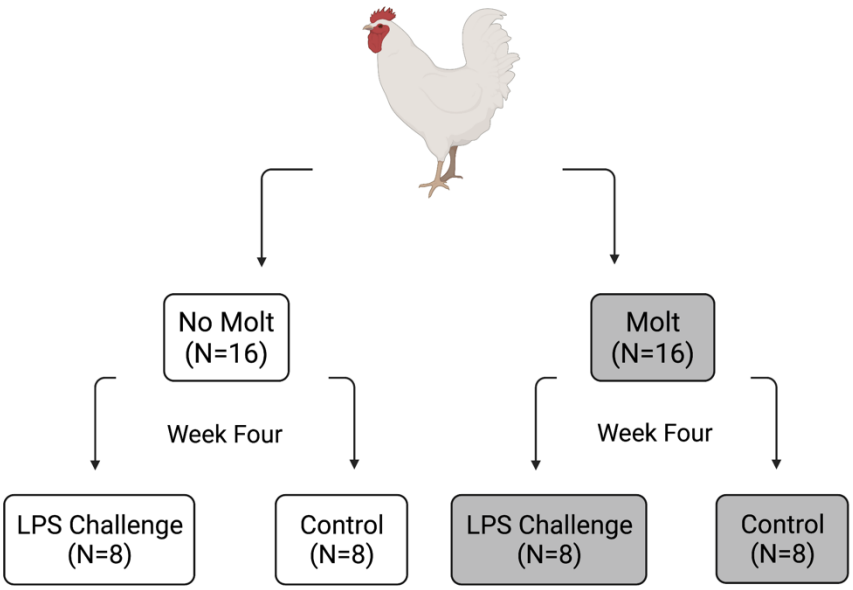

Figure 3.1 Experimental design for evaluating the trade-off between molt and innate immunity in the domestic chicken.

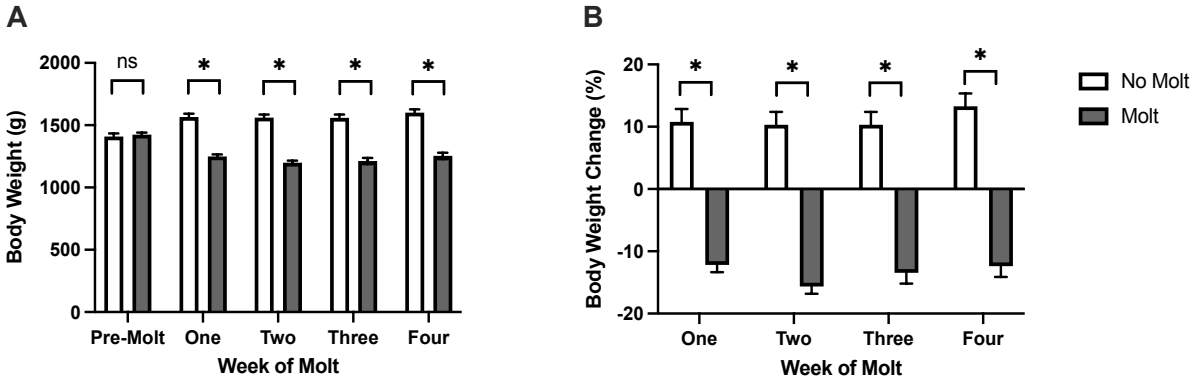

Figure 3.2 The effect of molt status on body weight A. For the duration of the five-week experiment, body weight was measured weekly. Over the course of the four-week molt period the non-molting birds were on average 343.45 grams heavier than the molting birds. B. Body weight change is represented as the percent change in body weight relative to the pre-molt week. Non-molting birds gained weight over the four-week molt period while molting birds lost weight. The greatest decrease in body weight occurred in the molting group during week two of molt (estimate: -15.7% ± 1.2, CI: -17.9 – -13.3). Data are mean + standard error with asterisks indicating significant differences ($P < 0.05$) between the non-molting and molt groups.

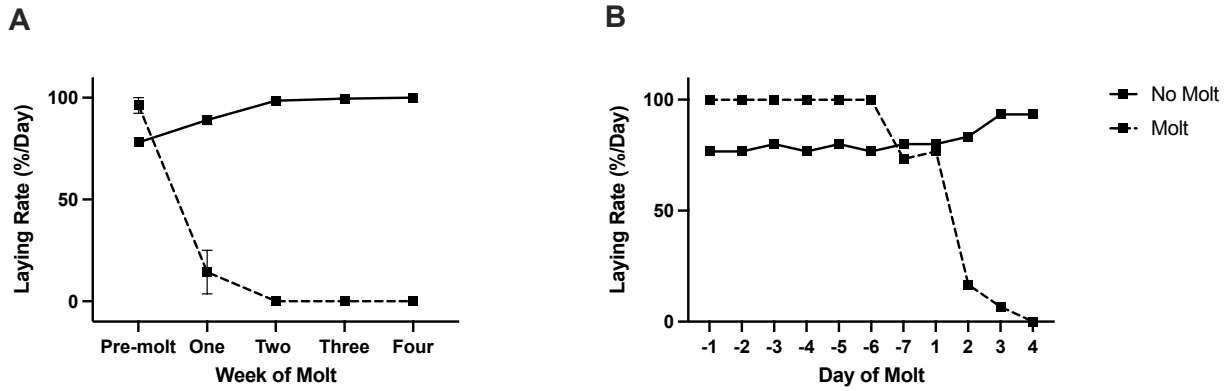

Figure 3.3 The effect of molt status on laying rate. During the course of the study, the number of birds laying was assessed daily. A. There was a steep decrease in the percent of birds laying between the pre-molt week (estimate: 96.2% ± 3.9, CI: 88.3 – 104.1) and week one (estimate: 14.3% ± 3.9, CI: 6.4 – 22.2). By the end of the first week of molt, none of the birds in the molting group were laying. B. The daily lay rate was reduced in the molt group at the end of the pre-molt week and by day four of the first week of molt no birds in the molting group were laying. Data are represented as mean + standard error.

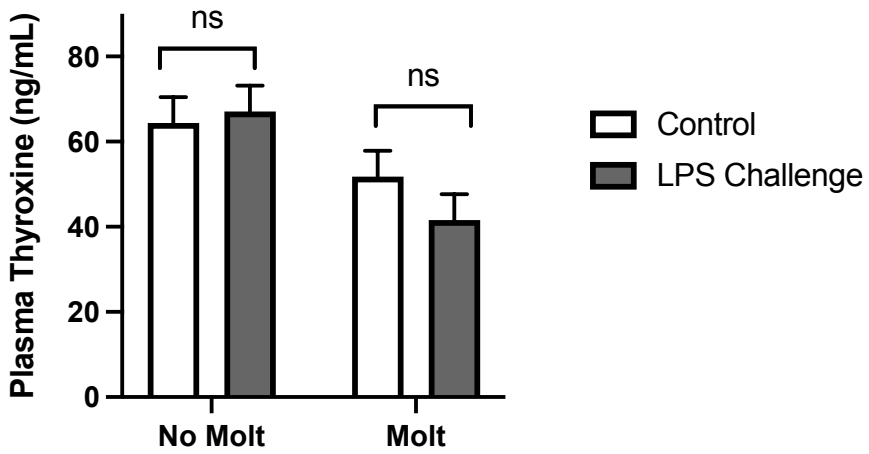

Figure 3.4 Plasma concentration of thyroxine in molting and non-molting birds. Plasma was collected at the end of the fourth week of molt and the concentration of thyroxine in the plasma was determined via ELISA. Molting birds had a lower concentration (P = 0.03) of plasma thyroxine (estimate: 65.8 ± 4.1, CI: 55.7 – 75.8) than non-molting birds (estimate: 65.8 ± 4.1, CI: 55.7 – 75.8). LPS treatment did not significantly affect the concentration of thyroxine in the plasma. The average concentration of thyroxine is shown with error bars representing the S.E.M.

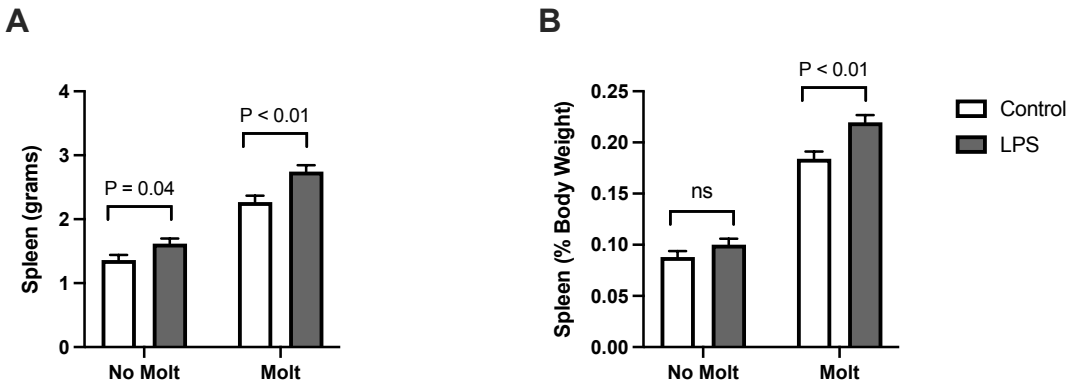

Figure 3.5 The effect of LPS injection and molt status on spleen weight. At the end of the fourth week of molt, the spleen weights of molting (N = 14) and non-molting (N = 18) birds were measured. A. Molting birds overall had significantly larger spleens (estimate: 2.5 g ± 0.07, CI: 2.4 – 2.7) than non-molting birds (estimate: 1.5 g ± 0.06, CI: 1.4 – 1.6). The spleen increased in weight in response to LPS in both the non-molting and molting birds. B. Spleen weight was divided by body weight in order to correct for differences in body weight between molting groups. The spleen accounted for a significantly greater percentage of body weight in molting birds (estimate: 0.20% ± 0.01, CI: 0.19 – 0.21) than non-molting birds (estimate: 0.09% ± 0.01, CI: 0.09 – 0.10). Following an LPS injection, the spleens of molting birds significantly increased in weight (estimate: 0.12% ± 0.01, CI: 0.1 – 0.14).

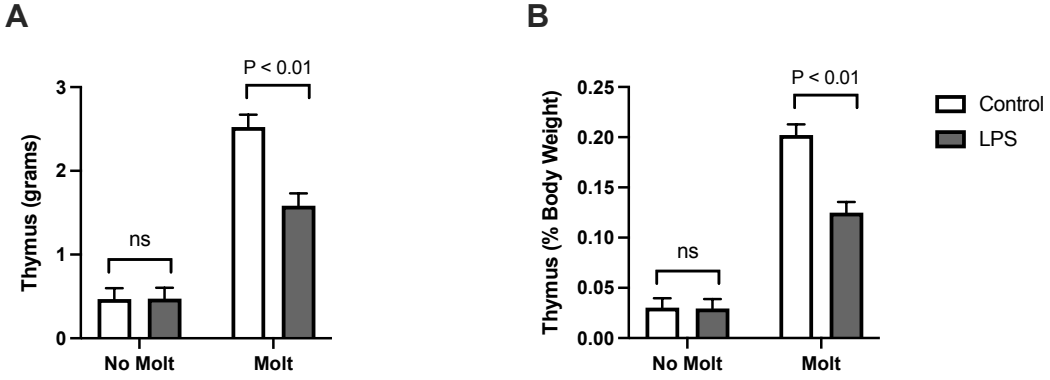

Figure 3.6 The effect of LPS injection and molt status on thymus weight. The thymic lobes of molting (N = 14) non-molting (N = 18) birds were collected and weighed at the end of the fourth week of molt. A. The thymus of molting birds was on average 1.58 ± 0.176 grams heavier than non-molting birds (CI: 1.22 – 1.94, P < 0.01). The thymus of molting birds significantly decreased in weight due to LPS (estimate: -0.94 g ± 0.21, CI: -1.34 – -0.51). B. When corrected for body weight, the average thymus weight was still significantly heavier in molting birds (estimate: 0.16% ± 0.01, CI: 0.15 – 0.18) than non-molting birds (estimate: 0.03% ± 0.01, CI: 0.02 – 0.04). An injection of LPS caused a significant reduction in thymus mass in molting birds (estimate: -0.08% ± 0.02, CI: -0.11 – 0.05).

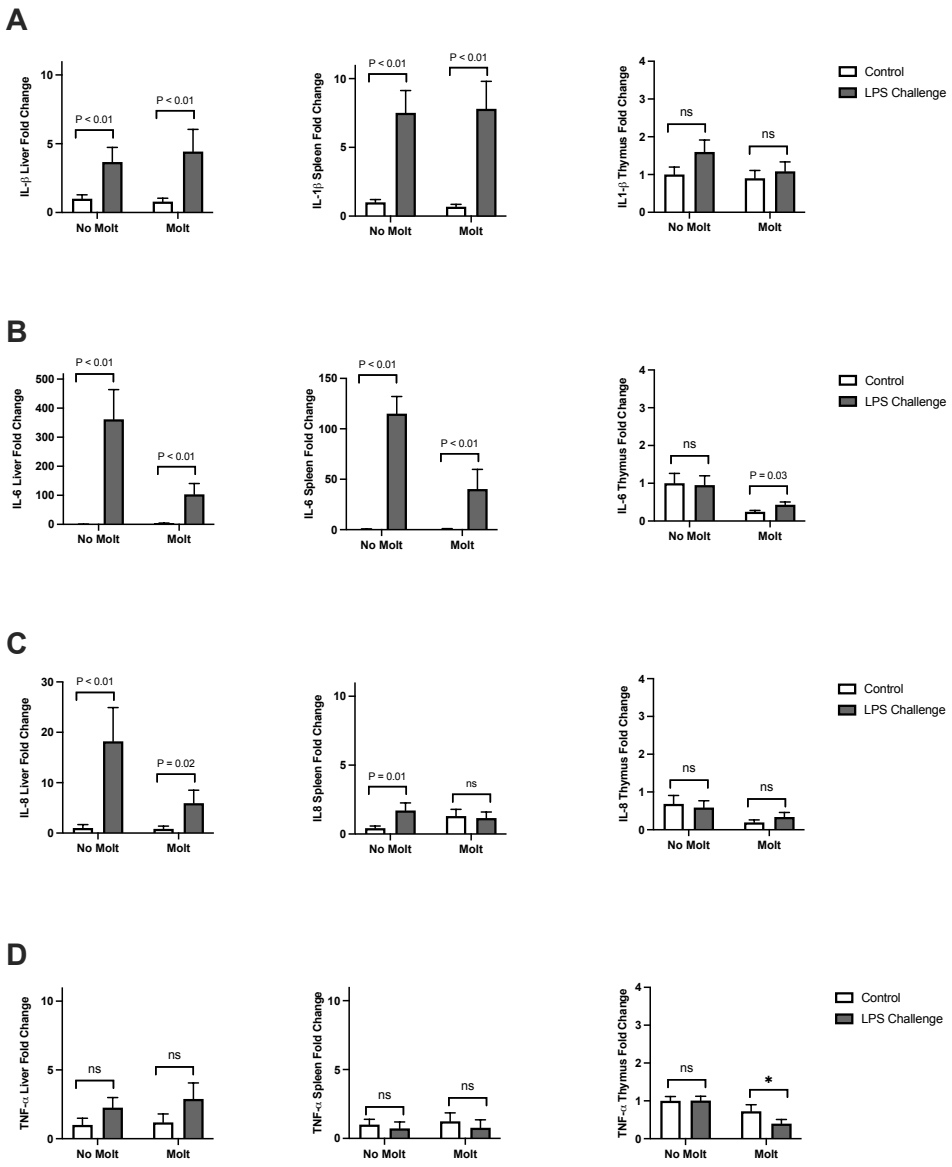

Figure 3.7 Impact of LPS injection on the expression level of pro-inflammatory cytokines in molting and non-molting birds. Birds were administered and intra-abdominal injection of LPS at 1.5 mg/kg. Four hours post injection, samples from the liver and spleen were collected to assess the expression level of selected pro-inflammatory cytokines. Gene expression levels are represented as the average fold-change relative to treatment control non-molting birds. Error bars are the SEM. A. The expression level of IL-1β in the liver and spleen, but not the thymus, increased significantly in response to LPS ($P < 0.01$). B. LPS injection also induced a significant increase in IL-6 expression in the liver and spleen of both molting and non-molting birds ($P < 0.01$) and in the thymus of molting birds ($P = 0.03$). C. IL-8 expression was significantly increased in the liver of molting ($P = 0.02$) and non-molting ($P < 0.01$) birds in response to LPS but only increased in the spleen of non-molting birds ($P = 0.01$). D. The expression level of TNF-α was not affected by either LPS injection or molt status.

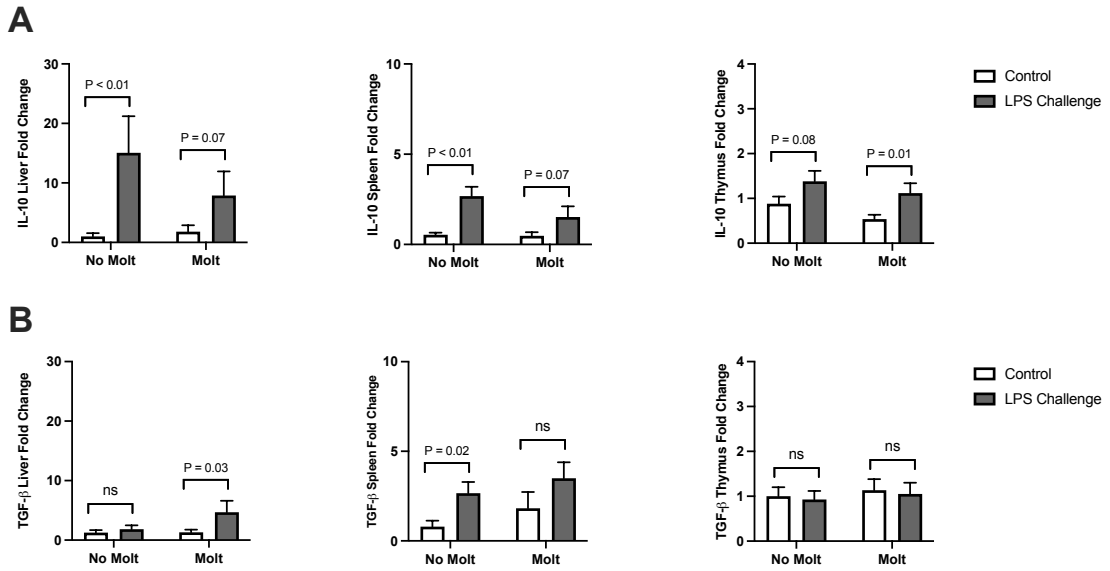

Figure 3.8 Impact of LPS injection on the expression level of anti-inflammatory cytokines in molting and non-molting birds. Liver and spleen samples were collected for gene expression assessment via qPCR four hours after an intra-abdominal injection of LPS (1.5 mg/kg). The gene expression levels are the average fold-change relative to treatment control non-molting birds with error bars representing S.E.M. A. The expression level of IL-10 significantly increased in the liver and spleen of non-molting birds ($P<0.01$) and trended towards a significant increase in molting birds ($P = 0.07$). LPS injection led to a significant increase in IL-10 in the thymus of molting birds and trended towards increasing IL-10 expression in non-molting birds. B. Non-molting birds had a significant increase in TGF-β expression in the spleen in response to LPS whereas molting birds had a significant increase in the level of TGF-β expression in the liver due to LPS injection.

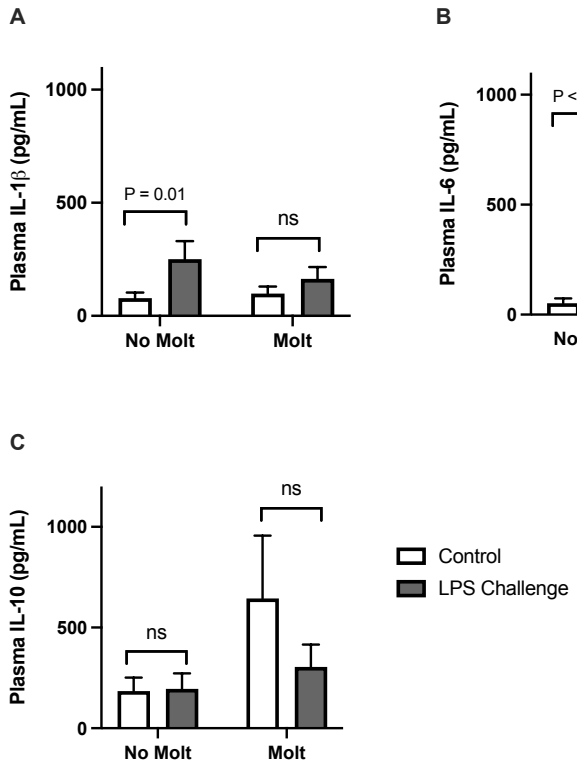

Figure 3.9 The effect of LPS injection on plasma cytokine concentration in molting and non-molting birds. Plasma samples were collected four hours after an intra-abdominal injection of LPS (1.5 mg/kg). Cytokine concentration was determined using commercial ELISAs. Data is expressed as average concentration in pg/mL with error bars representing the S.E.M. A. LPS injection led to a significant increase in the plasma concentration of IL-1β in non-molting birds ($P = 0.01$) but not in molting birds. B. In response to LPS, the concentration of IL-6 in the plasma of non-molting increased significantly more than in the plasma of the molting birds ($P < 0.01$). C. There was no strong effect of LPS on plasma concentration of IL-10 for either non-molting or molting birds. Molt trended towards increasing the concentration of IL-10 in molting birds ($P = 0.06$).

Chapter 4

Evaluation of the contribution of feather growth to the trade-off between molt and innate immunity in two genetic lines

Abstract

Feathers are essential for a bird's survival, but over time feathers become worn down and must be replaced. Birds replace feathers through the process of molt which is characterized by feather growth and also broader physiological changes. Feathers are extremely protein dense and require an increased energy and nutrient supply for their replacement. Due to the nutritional demands of molt, there is likely to be a trade-off between molt and other costly processes like mounting an immune response. However, the extent of the trade-off between molt and the immune system remains ill-defined. To better understand the trade-off between molt and a costly innate immune response, we used two genetic lines of birds: the UCD-003 line and Scaleless High (ScHi) line. The ScHi line of chickens have a genetic mutation that leads to a reduction in feather number and smaller feathers. If a trade-off between molt and the immune system is driven mainly by the mass of feathers grown, then the ScHi birds should have a reduced trade-off between an immune response and molt compared to a fully feathered bird. Chickens from both genetic lines (N = 32) were induced to molt using a combination of oral thyroxine (T4), a reduced calorie diet and a shift from a long day to a short-day schedule. During week four of molt, half of the birds in each genetic line were challenged with an intra-abdominal injection of lipopolysaccharide (1.5 mg/kg) to induce an innate immune response. Five hours after the LPS injection, samples were collected from the liver and spleen to evaluate the expression of pro- and anti-inflammatory cytokines. The spleen and thymus weights were measured to determine if

systemic changes in these lymphoid tissues during molt were also dependent on the mass of growing feathers during molt. The spleen and thymus both increased in weight significantly during molt in both genetic lines. The thymus of molting birds increased 7.8-fold while the spleen increased an average of 48% across genetic lines. The area of cortex in the thymus unexpectedly increased more in molting reduced feathering ScHi birds than in the fully feathered UCD-003 birds. The inflammatory response to LPS was dampened in both genetic lines, especially the expression levels of IL-6 and IL-8. The overall process of molt, rather than the extent of feather growth alone, appears to drive the trade-off between molt and an innate immune response when both processes must occur concurrently.

Introduction

Molt is the process by which birds periodically replace essential integumentary structures. The main part of the integument replaced during molt are the feathers, but other structures such as the feather sheaths are also replaced. Feathers are extremely protein dense structures with protein making up 90% of the dry mass of a feather (Chilgren, 1977). The production of new feathers requires a significant amount of protein. To support feather growth, the whole-body protein synthesis rate increases when birds are molting. In White-crowned Sparrows, the whole-body protein synthesis rate has been shown to increase by 33% during peak feather production (Murphy and Taruscio, 1995). The energetic cost of producing feather protein during molt is 3.6 to 4.3 times higher than the cost of producing protein for growth or egg production, respectively (Murphy and King, 1991, 1992). The energy used by White-crowned Sparrows to support peak feather growth is equivalent to 58% of their basal metabolic rate (Murphy and King, 1992). The disparity between the energy required to produce feather protein compared to protein for other essential tissues suggests that molt is highly inefficient or, alternately, considerable expenses are incurred concomitant with molt beyond growing feathers. There is some indication that energetic costs of feather production are highest in birds with smaller body weights and lower in larger birds (Lindstrom et al., 1993).

The energetically costly nature of molt is highlighted by the timing of molt relative to other essential life processes. Although there are exceptions, across many bird species there is minimal overlap between molt and reproduction. Birds that do regularly overlap reproductive cycles and molt tend to have potentially lower cost molts that are slower and longer lasting or they live in environments with abundant resources for a portion of the year (Payne, 1972). The

lack of overlap between molt and other necessary processes highlights the essential and seemingly energetically costly nature of molt.

When birds undergo molt, there are a range of other concurrent physiological changes that may contribute to the energy and nutritional demands molt places on a bird. As birds molt, there can be increased thermoregulatory costs when temperatures are cooler (Blackmore, 1969). Birds may reduce certain behaviors such as hunting or foraging during molt while increasing other behaviors like preening (Morton and Morton, 1990; Murphy, 1996). There are also shifts in different organ systems when birds undergo molt. In male and female birds, the reproductive organs are catabolized. Both the liver and the adrenals also decrease in size during molt (Brake and Thaxton, 1979). Interestingly, the thymus and spleen increase in size when other organs decrease in size (Ward and D'Cruz, 1968; Brake et al., 1981; Silverin et al., 1999). When re-growing feathers that have been plucked birds do not have an increased metabolic rate. Molting House Sparrows had a 28% increase in their resting metabolic rate but a similar increase in metabolic rate was not observed in birds regrowing an equivalent amount of plucked feathers (Buttemer et al., 2019). It is possible that the broader physiological changes that occur beyond molt may contribute un-accounted for energetic costs leading to the apparent inefficiency of molt. Specifically, shifts in other processes that require periodic renewal, such as activity of the immune system, may be occurring coincidently with molt. These processes could account for a portion of the unidentified energetic cost of molt that cannot be explained by the protein synthesis required for new feathers alone.

Due to the energetically and nutritionally costly nature of molt, there is likely to be an inability of birds to maintain molt and other expensive processes concurrently. One of the most important systems for an organism's survival is the immune system and there are a variety of life

stages that must be balanced with the metabolic demands induced by an immune response to a pathogen. Trade-offs between growth, migration and reproduction and investments in self-maintenance (e.g., immunity, antioxidant defenses) have been well documented. However, the potential trade-offs between molt and immune function remain poorly defined (Sanz et al., 2004; Hegemann et al., 2012). Both activation of the immune system and molt are characterized by increased production of specific proteins and a requirement for specific amino acids like cysteine (Busch and Brush, 1979; Brush and Wyld, 1982; Stettenheim, 2000; Iseri and Klasing, 2013b, a, 2014). Both processes also place increased energetic demands on the birds. In ducks, a 1°C increase in body temperature during the febrile response leads to a 23% increase in metabolic rate (Gray et al., 2013). Molt alone may be sufficient to induce a trade-off between molt and the immune system or there may be a more nuanced trade-off where the feather mass produced during molt modulates the magnitude of the trade-off.

The Scaleless lines of chickens, sometimes called naked chickens, have few to no feathers and can be used to try and disentangle the systemic effects of molt from feather growth during molt. Scaleless birds have a nonsense mutation in gene FGF20 on chromosome 4 that leads to improper signaling during feather placode development. Birds with this mutation fail to grow both scales and feathers (Houghton et al., 2007; Wells et al., 2012). Temperature selection was used to differentiate the original Scaleless line into the Scaleless Low line and the Scaleless High Line (Abbott, 1965b). Scaleless Low line birds have no feathers or scales whereas the Scaleless High line has about 30% feathering and mainly grow downy feathers, which are very small relative to the contour and flight feathers. The decreased number of feathers lowers both the methionine and cysteine requirement of the Scaleless chickens (Abbott et al., 1960). Despite the reduction in feather mass, Scaleless birds still maintain normal feather related behavior like

preening and wing flapping (Provine, 1981). In our experience, including the results described below, the Scaleless High line can also be induced into a molt state using traditional molt cues like decreased photoperiod. The Scaleless High line was selected as a reduced feather model because the decreased amount of feathering confirmed the birds were undergoing molt and allowed the progression of their molt to be tracked. It would be difficult to track the molt stage in the completely featherless Scaleless Low line. If a trade-off between molt and the immune system is driven mainly by feather growth, then the ScHi birds should have a reduced trade-off between an immune response and molt compared to a fully-feathered bird. If, on the other hand, the trade-off is driven by the broader physiological changes that happen during molt then both the ScHi birds and fully feathered birds should have a similar dampening of immunity during molt.

Materials and Methods

Animal Care

Sixty-four eight-month-old laying hens were housed in the Hopkins research facility at the University of California, Davis. Thirty-two of the hens from the University of California Davis UCD-003 colony and thirty-two of the hens were bred from the Scaleless High (ScHi) colony. The chicks used in the study were hatched on April 23, 2020, and sexed via the W gene to select for female chicks. Once the birds reached six months of age, they were housed in two rooms directly next to each other to allow for the maintenance of different light schedules. Each room contained thirty-two birds consisting of sixteen UCD-003 hens and 16 ScHi hens. The different genetic lines were evenly distributed across cage levels and cage racks. Birds were

individually housed in sloped floor cages to facilitate egg collection and were provided ad libitum access to feed and water. For the duration of the experiment, control birds (N = 36) were maintained on a long day schedule (16L:8D) and a commercial layer diet (Bar-Ale, Cat. No. WP16CC) containing 16% protein. A combination of light, dietary and hormonal cues were used to induce molt starting December 17th, 2020. Over the course of a week, the day length of the molting birds was reduced from 16 hours to 8 hours. Birds were also administered a daily oral dose of thyroxine (T4) at 1.25 mg/kg for 10 days by sprinkling the hormone on top of the feed in the morning. Finally, molting birds were fed a specially formulated molt diet that had a reduced content of metabolizable energy (2431.60 kcal/kg), protein (9.6%), fat (3.12%) and calcium (0.5%). The dietary levels of all other nutrients met the bird's daily nutritional requirements (NRC, 1994; diet formulation Appendix 3). Body weight and laying rate were monitored for the duration of the experiment. All husbandry and experimental practices were approved by the University of California, Davis IACUC #21304.

Experimental Design

This experiment was designed to determine if the mass of feathers produced during molt affects the shifts in lymphoid tissue mass or the ability of a bird to mount an inflammatory response during molt. Birds were considered to be molting when they began to drop feathers at the end of the ten-day molt induction period. Half of the birds (N = 8) from each molt group and genetic line received an intra-abdominal injection of LPS (1.5 mg/kg body weight) at the end of the fourth week of molt (Figure 4.1). The study is focused on general inflammation, rather than the specific effects of LPS, so the control birds received no injection but were handled and had their abdomen area pinched to simulate the stress of an injection but avoid inducing inflammation via a PBS injection. Five hours after the LPS injection or sham injection, 5 mL of

blood were collected into EDTA tubes from the brachial vein and then all birds were culled using CO2. Spleen and thymic lobes were then collected immediately after death and weighed.

Thymus Histology

From each bird, a single thymic lobe was collected for histological examination. The thymic lobes were fixed for 48-hours in 10% buffered formalin before being transferred to 70% ethanol. Samples were trimmed and placed in tissue cassettes and sent to IDEXX BioAnalytics (Colombia, MO) for slide preparation and hematoxylin and eosin stain. To assess the changes in the cortex and medulla, ImageJ software was used to measure the area of cortex and medulla in three images per bird at 4x magnification. The cortex to medulla ratio was calculated as total cortex area divided by the total medulla area per image. When medulla and cortex tissue were not discernable, the thymus tissue was classified as parenchyma.

Quantitative RT-PCR of Liver and Spleen Cytokine Gene Expression

After the spleen and thymus were weighed, the tissues were immediately minced in cold RNAlater®. The tissue samples in RNALater® were kept on ice for up to four-hours post-removal, then stored at -80°C prior to further laboratory processing. Tissue samples were homogenized, and total RNA extracted using a modified version of the Trizol® protocol for phenol-chloroform extraction of RNA. Following RNA extraction and isolation, a Thermo Scientific™ NanoDrop™ spectrophotometer was used to determine the RNA quality of each sample. A 260/280 ratio of 1.8 or higher was used to define RNA as good quality. To ensure RNA purity, Ambion's DNA-free™ kit (Cat. No. AM1907) was used to remove any residual genomic DNA from RNA samples. Following RNA purification, Reverse Transcription PCR was performed with 1 μg of RNA per tissue sample using Bio-Rad's iScript™ Reverse

Transcription Supermix (Cat. No. BR 1708840). PCR procedures were completed in a Bio-Rad PTC-100 Programmable Thermal Cycler. All cDNA samples were stored at -20° C until quantitative RT-PCR analysis.

To assess shifts in either an anti- or pro-inflammatory state, the expression levels of the cytokines IL-1β, IL-6, TNF-α, IL-10 and TGF-β were monitored using qPCR. All qPCR reactions were performed using the following protocol: 3 minutes at 95°C, then 40 cycles of 10 seconds at 95°C, 30 seconds at 55° C and 30 seconds at 72°C on a MyIQ iCycler PCR machine (Bio-Rad, Hercules, CA). Bio-Rad's iTAQ ™ Universal SYBR ® green Supermix was used for each reaction with the following components: 1 uL of 10 ng/uL cDNA, 10 uL of SYBR Universal SYBR ® Green Supermix along with variable amounts of DEPC water, forward primer and reverse primer based on the specific primer set (Table A.7). Each experimental sample was run in triplicate with DEPC water as a NTC control. The fold-change value (ΔΔCt) for each cytokine were calculated using the ratio of the gene of interest ΔCt to the housekeeping genes (RPL13 and GAPDH) ΔCt.

Plasma Thyroxine Concentration

Prior to euthanasia, whole blood was collected from the brachial vein into EDTA treated tubes using 25G ⅝ needles with 3 mL syringes. To isolate the plasma, the whole blood was centrifuged for 15 minutes at 2000g (Sorvall® Legend MACH 1.6**)**. Plasma samples were then aliquoted into 200 uL microcentrifuge tubes and stored at -20°C until further processing. A commercial ELISA was used to determine the amount of thyroxine (Aviva Systems Biology Cat. No. OKCA00265) according to the manufacturer's instructions. The concentration of thyroxine was quantified in nanograms (ng) per milliliter in the plasma.

Evaluation of Immune Cell Populations

To evaluate the effect of an inflammatory response on immune cell populations, single cell suspensions were prepared from the five birds in each of the UCD-003 treatment groups. Spleen samples were collected from the UCD-003 hens and stored in ice-cold RPMI until processing to single cell suspensions. To prepare single-cell suspensions, spleens were mechanically disaggregated through a 40 μm cell strainer into cold RPMI-10. Approximately 10 million cells were then diluted in 4 mL of RPMI-10 and layered onto 4 mL of Histopaque-1077 and spun for 30 minutes at 400 g. The isolated buffy layer was suspended in 2 mL of RMPI-10, washed by centrifugation for 5 minutes at 1500 RPM and resuspended in 2 mL of fresh RPMI-10. Each sample was stained with a single panel containing the following antibodies: CD3-PE (Southern Biotech, Birmingham, AL, cat # 8200-09), KUL01-A647 (Southern Biotech, Birmingham, AL, cat # 8420-31), and Invitrogen ™ LIVE/DEAD™ fixable violet dead cell stain (Thermo Fisher Scientific, Cat. No. L34955). The KUL01 antibody marks cells in the monocyte and macrophage lineage. Throughout this manuscript, the term "macrophage" will be used to refer to cells marked by KUL01 in the monocyte to macrophage lineage. After staining, cells were washed twice in 150 uL of staining media by centrifugation for 5 minutes at 1500 RPM and incubated for 30 minutes in 50 uL of Invitrogen ™ LIVE/DEAD™ fixable violet dead cell stain (Thermo Fisher Scientific, Cat. No. L34955). After all staining steps, cells were fixed in 75 uL of BD Cytofix/Cytoperm ™ fixation and permeabilization solution (Fisher Scientific, Cat. No. BDB554722) for 15 minutes then resuspended in 200 uL of staining media and kept at 4°C. Cells were processed for flow cytometric analysis on the same day as staining and fixation using a BD™ LSR II (BD Biosciences, San Jose, CA). Compensation controls, including fluorescence minus one (FMO) and single fluorescence controls, were prepared from splenic single-cell

suspensions. Data for the immune cell populations can be found in the appendix following this chapter.

Statistical Analysis

All of statistical analyses were performed using Microsoft Excel® Version 16.58, R© version 4.1.1 and RStudio© version 2021.9.0.351 (R Core Team, 2021; RStudio Team, 2021). For each model, normality was assessed by examining a Q-Q plot of the standardized residuals versus the theoretical quantiles in combination with the Shapiro-Wilks test of normality. Homogeneity of variance was assessed by visually examining a plot of the model standardized residuals by the fitted model values as well as using Levene's test. In each data set, extreme outliers were defined as data points above the third quartile plus three times the interquartile range or the first quartile minus three times the interquartile range. Extreme outliers were then evaluated for removal using both the Bonferroni outlier test and Cook's distance to evaluate the outlier leverage. When performing model comparisons, the best fitting model was selected based on log-likelihood, AIC, and BIC values (Bates et al., 2015).

Both body weight and change in body weight were analyzed as 2 x 2 x 5 factorials with two levels of molt and two levels of genetic line by five different times (week) of the study. For all body weight and laying data, a mixed effects model was constructed with time, genetic line, molt status and the interactions between all three effects as fixed effects and bird identification number as a random effect.

Organ weight and qPCR data were analyzed as 2 x 2 x 2 factorials with two levels of treatment (control and LPS challenge), molt (no molt and molt), and genetic line (UCD-003 and ScHi). To fit a normal distribution, qPCR fold-change data was transformed on a log scale. A

linear model was used to assess the following data sets: spleen IL-1β expression, spleen IL-6 expression, spleen IL-8 expression, liver TNF-α expression, spleen TNF-α expression, spleen IL-10 expression, spleen TGF-β expression. A generalized least squares model with unique variance assigned for each level of molt was used for thymus weight, liver IL-8 expression, liver IL-10 expression and liver TGF-β expression. For all remaining data sets including spleen weight, liver IL-1β expression and liver IL-6 expression a generalized least squares model with unique variance assigned for each genetic line was used. Flow cytometry data were analyzed as 2 x 2 factorials with two levels of treatment (control and LPS challenge) and two levels of molt (no molt and molt) using a linear model.

Analysis of variance (ANOVA) was used to evaluate the effect of immune challenge, molt, and genetic line, and on the measured variables. For all ANOVA and post-hoc tests, significance was accepted at $P \leq 0.05$ and a p-value between $P > 0.05$ and $P < 0.10$ was considered to indicate a non-significant trend. Tukey's honestly significant difference test (HSD) was used to determine differences between the least squares means for contrasts evaluating the effects of treatment, molt, and genetic line. Bonferroni p-value corrections were used for *Post hoc* analysis when appropriate. Experimental results are reported as the coefficient estimates, standard errors and 95% CIs.

Results

Body Weight and Laying

The change in body weight and laying rate were monitored throughout the study to evaluate if the extent of feather growth during molt affects the innate immune response and

spleen and thymus weight. There were significant main effects of time, molt, and genetics on body weight ($P < 0.01$; Table 4.1). Birds in the molt group averaged 272 grams lighter than non-molting birds. ScHi birds averaged 194 grams heavier than UCD-003 birds. The interaction between time and molt was significant ($P < 0.01$) because there was a decrease over time in body weight of molting birds but not non-molting birds (Figure 4.2). The interaction between time and genetic line was also significant ($P < 0.01$) because the UCD-003 birds lost more weight early in molt than the ScHi birds. There was no significant interaction between the main effects of molt and genetic line or the three-way interaction between time, molt, and genetic line. The relative change in body weight followed very similar patterns to those described for the absolute changes in body weight described above (Figure 4.3; Table 1).

The laying rate of the birds was significantly affected by the main effects of time ($P < 0.01$), molt ($P < 0.01$), and genetic line ($P < 0.01$; Figure 4.4). UCD-003 birds were more consistent layers and had a significantly higher lay rate than the ScHi birds (estimate: 11.6% ± 2.4, CI: -16.2 – - 6.9; $P < 0.01$). The three different two-way interactions between the main effects of time, molt and genetic line were all significant ($P < 0.01$). The laying rate of molting UCD-003 birds was higher than the laying rate of molting ScHi birds during the pre-molt period and molt induction period ($P < 0.01$ pairwise comparison). During week one of molt, the laying rate of molting birds from both the UCD-003 line and ScHi line fell to zero. The decrease in laying rate as UCD-003 birds entered molt was greater the decrease in laying rate observed for the ScHi birds. The UCD-003 birds decreased from a laying rate of 78% to 0%. The ScHi birds laying rate decreased from 51% to 0% during the same period (Figure 4.4).

Plasma Thyroxine

An injection of LPS, molt status and genetic line all affected the plasma concentration of thyroxine ($P < 0.01$, respectively). There was a significant interaction between LPS and molt because molting birds had a greater increase in thyroxine following an injection of LPS ($P < 0.01$; Figure 4.5). There was also significant interactions between LPS and genetic line ($P = 0.02$) and molt and genetic line ($P = 0.01$). Molting UCD birds had a higher plasma concentration of thyroxine than molting ScHi birds ($P < 0.01$ by pairwise comparison). The three-way interaction between LPS, molt and genetic line had a significant influence on plasma thyroxine ($P = 0.02$).

Spleen and Thymus Weight

Spleen weight was measured at the end of week four to evaluate changes in spleen weight due to an LPS challenge, molt, and genetic line (Figure 4.6A). Both an LPS challenge and molt had a significant effect on spleen weight ($P < 0.01$; Table 2). On average, the spleens of the molting birds were 0.6 grams (47%) larger compared to the non-molting birds ($P < 0.01$). The genetic line of the bird had a significant effect on the spleen weight ($P < 0.01$). The spleens of the ScHi birds were, on average, 0.5 grams (42%) larger than the spleens of the UCD-003 birds ($P < 0.01$). None of the two-way nor the three-way interaction of the main effects were significant. The relative spleen weight data also followed very similar trends to those described above for the absolute spleen weight (Figure 4.6; Table 4.2).

The thymus weight was also measured at the end of week four to examine changes in response to an LPS challenge, molt, and genetic lines (Figure 4.7A). Molt, but not LPS challenge, had a significant effect on thymus weight ($P < 0.01$). The thymuses of molting birds

were an average of 1.1 grams larger which equated to a 7.8-fold increase in thymus weight compared to non-molting birds ($P < 0.01$). The interaction between LPS and molt was significant ($P = 0.05$). The thymus weight of molting birds decreased by 31% in response to LPS, but there was little change in non-molting birds. There was no significant interaction between LPS challenge and the genetic line, but there was a significant interaction between molt status and genetic line ($P = 0.02$). The thymus weight increased more in response to molt in the ScHi birds than in the UCD-003 birds. There was a 12.8-fold increase in thymus weight during molt in the ScHi birds compared to a 4.8-fold increase in the UCD-003 ($P < 0.01$, respectively). The three-way interaction between treatment, molt and genetic line was not significant (Table 4.2). Correcting the thymus weight by body weight gave similar results to those described above with the exception that the molt by genetic line interaction was no longer significant ($P = 0.13$; Figure 4.7B; Table 4.2).

Thymus Histology

Thymus samples were collected at the end of week four to determine if there were differences in the recrudescence of the thymus between the UCD-003 and ScHi genetic lines. In both genetic lines, the thymus of non-molting birds was regressed with smaller thymic lobules and a greater incidence of adipose tissue (Figure 4.8A; Figure 4.8B). Neither genetic line had a notable presence of cortex (Figure 4.9C) or medulla tissue when not molting. In comparison, the thymus of molting birds from both genetic lines had well-defined thymic lobules containing cortex and medulla tissue (Figure 4.8C; Figure 4.8D). Molting UCD-003 birds had less cortex tissue ($P = 0.01$) and more medulla tissue ($P = 0.01$) in their thymus compared to molting ScHi birds (Figure 4.8A). Molting ScHi birds had a significantly higher ($P = 0.02$) cortex to medulla ratio compared to molting UCD-003 birds (Figure 4.9B). Molt led to a significant increase in

cortex tissue in the thymuses of both UCD-003 birds and ScHi birds ($P < 0.01$; Figure 4.9C). Medulla tissue was not distinguishable in the parenchyma of the thymus of non-molting birds from either genetic line.

Cytokine mRNA Expression in the Liver and Spleen

Five hours following an intra-abdominal injection of LPS samples were taken from the liver and spleen to determine the effect of molt on the expression of mRNA for selected inflammatory cytokines (Table 4.3). There was a significant main effect of LPS on IL-1β expression ($P < 0.01$) and also a significant LPS by molt interaction ($P = 0.02$). LPS greatly increased IL-1β expression in non-molting birds ($P < 0.01$ by pairwise comparison) but not in molting birds ($P = 0.10$ by pairwise comparison). There was also a significant main effect of molt on IL-1β expression ($P = 0.03$) and also a significant molt by genetic line interaction ($P = 0.02$). Molt decreased IL-1β expression to a larger extent in ScHi than in UCD-003 birds (Figure 4.10A). IL-1β The three-way interaction between LPS, molt and genetic line trended towards significance ($P = 0.07$).

In response to an injection of LPS, the expression level of IL-1β in the spleen increased significantly ($P < 0.01$). Neither molt status nor genetic line had a significant effect on the expression level of IL-1β. There was a significant interaction between LPS and molt on IL-1β expression in the spleen ($P = 0.01$; Figure 4.10A). The expression of IL-1β in the spleen was substantially higher in non-molting birds than those that were molting. No other interactions had a significant effect on IL-1β expression in the spleen (Table 4.3).

The expression of IL-6 in the liver was significantly increased in response to the LPS injection ($P < 0.01$: Figure 4.10A). The molt status of a bird also had a significant impact on the

expression level of IL-6 in the liver, with molting birds producing less IL-6 mRNA ($P < 0.01$). The interaction between molt and LPS was not significant, but there was a significant interaction between LPS and genetic line ($P < 0.01$). ScHi birds had lower levels of IL-6 expression in the liver compared to UCD birds in response to LPS ($P < 0.01$ by pairwise comparison). The three-way interaction between LPS, molt and genetic line was also significant ($P = 0.02$: Table 4.3).

The expression level of IL-6 increased significantly in the spleen following an injection of LPS ($P < 0.01$; Figure 4.10B). Molt led to a significant decrease in IL-6 expression in the liver ($P = 0.05$). There was also a significant impact of genetic line on IL-6 in the liver ($P < 0.01$) with UCD-003 birds having higher levels of IL-6. The interaction between LPS and molt was significant ($P < 0.01$) with molting birds having a dampened IL-6 expression level in response to LPS compared to non-molting birds ($P < 0.01$ by pairwise comparison). The expression level of IL-6 in the spleen was also significantly affected by an interaction between LPS injection and genetic line ($P = 0.03$). ScHi birds had a less robust response to an LPS injection that UCD-003 birds ($P < 0.01$ by pairwise comparison). No other interactions had a significant effect on IL-6 expression in the spleen (Table 4.3).

Both the LPS challenge ($P < 0.01$) and molt ($P < 0.01$) had a significant effect on the expression of IL-8 in the liver (Figure 10C) and there was a strong interaction between these two factors with increased IL-8 expression being much more robust in non-molting birds. Genetic line also significantly affected IL-8 expression, and UCD-003 birds had an elevated level of liver IL-8 compared to ScHi birds ($P = 0.01$). There was an interaction between molt and genetic line ($P < 0.01$) because molting UCD-003 had a much higher level of liver IL-8 mRNA compared to molting ScHi birds ($P < 0.01$ by pairwise comparison). The interaction between LPS, molt and genetic line had a significant effect on IL-8 expression level in the liver ($P = 0.03$).

An injection of LPS caused a significant increase in IL-8 expression in the spleen ($P < 0.01$). There was no main effect of either molt or genetic line on IL-8 expression in the spleen. Molt and an LPS challenge significantly interacted ($P = 0.01$) because there was a significant increase in spleen IL-8 expression in response to LPS in the non-molting group ($P < 0.01$ by pairwise comparison) but not in the molting group. The three-way interaction between LPS injection, molt and genetic line trended towards significant ($P = 0.07$; Table 4.3).

The LPS challenge led to a significant increase in TNF-α expression in the liver ($P < 0.01$; Figure 4.10D) and there was a strong LPS by molt effect because non-molting birds had a significant increase in TNF-α expression in the liver following the LPS challenge ($P < 0.01$ by pairwise comparison) but molting birds did not due to higher overall TNF-α levels. Molt status alone did not have a major effect on liver TNF-α expression. There was a significant interaction between molt and genetic line ($P = 0.01$). Molting ScHi birds had a lower f TNF-α expression in the liver during molt ($P < 0.01$ by pairwise comparison) but not in non-molting birds. No other interactions were significant (Table 4.3).

TNF-α expression in the spleen increased significantly in response both an injection of LPS ($P < 0.01$) and molt ($P < 0.01$). There was a strong LPS by molt interaction due to the significant increase in the TNF-α expression in response to LPS of non-molting birds ($P < 0.01$ by pairwise comparison) whereas molting did not have a significant increase. The expression level of TNF-α did not vary by genetic line. No other interactions had a significant effect on TNF-α expression level in the spleen (Table 4.3).

There was a significant increase in liver IL-10 expression due to LPS ($P < 0.01$) and the interaction between LPS and molt trended towards significance because non-molting birds had

slightly higher IL-10 expression in the spleen following an LPS injection ($P = 0.06$; Figure 11A). Molt did not have a main effect on liver IL-10 expression. The level of IL-10 expressed in the liver was higher in UCD-003 birds than in ScHi birds ($P < 0.01$). There was also a significant interaction between molt and genetic line ($P < 0.01$). Molting UCD-003 birds had higher levels of IL-10 liver expression compared to molting ScHi birds ($P < 0.01$ by pairwise comparison). The three-way interaction between LPS, molt and genetic line was significant ($P = 0.01$).

An LPS injection led to a significant increase in IL-10 expression in the spleen ($P < 0.01$). Molt, on the other hand, led to a significant decrease in IL-10 expression in the spleen ($P < 0.01$) and there was a significant interaction between LPS and molt, because non-molting birds had a higher expression level of IL-10 in response to LPS than molting birds ($P < 0.01$ by pairwise comparison). The bird genetic line did not have an effect on spleen IL-10 expression levels. No other interactions between LPS, molt or genetic line were significant (Table 4.4).

The injection of LPS led to an increase in the expression of TGF-β in the liver ($P < 0.01$; Figure 4.11B). Genetic line, but not molt status has a significant effect on TGF-β expression ($P < 0.01$). UCD-003 birds had a higher level of TGF-β in the liver compared to ScHi birds. There was a significant interaction between molt status and genetic line because molting UCD-003 birds had higher levels of TGF-β in the liver than molting ScHi birds ($P < 0.01$). The three-way interaction between LPS, molt and genetic line trended towards significance ($P = 0.09$). There were no significant main effects or interactions of main effects that influenced TGF-β expression in the spleen (Table 4.4). ScHi birds trended towards a higher expression level of TGF-β in the spleen compared to UCD-003 birds ($P = 0.08$).

Discussion

As birds molt, the growth of new feathers is supported through increased energy and protein use, much of which is mobilized from body tissues. When birds must undergo expensive life stages, like molt, at the same time as similarly expensive processes such as reproduction or migration there is the potential for a trade-off to occur. A trade-off happens when the overlapping requirements of two processes cannot be met concurrently resulting in one or both processes being altered to reduce the cost to the animal. Molt is both costly and essential to birds' survival and is therefore likely to induce a trade-off with other important processes like mounting an immune response. During an active immune response, especially an innate immune response, energy usage increases during the febrile response. The immune system also increases amino acid usage to produce proteins and for energy (Gray et al., 2009; Iseri and Klasing, 2013a, 2014). The trade-off between molt and the immune system remains difficult to define, with many contradictory results showing both increased and decreased immune function ((Brake et al., 1981; Brake et al., 1982; Ben-Hamo et al., 2017).

To examine the trade-off between molt and the immune system, we used two unique chicken genetic lines that grow different numbers of feathers to try and determine if greatly reducing the feather mass produced during molt ameliorates the dampened inflammatory response observed in chapter 3. Markedly reducing the metabolic demands of feather growth would be expected to provide more resources for a robust inflammatory response. The Scaleless genetic line of birds have a developmental mutation that disrupts the formation of the feather placode and prevents the birds from developing full plumage (Wells et al., 2012). Additional temperature selection led to the development of the Scaleless Low line, which have no feathers, and the Scaleless High line. Birds from the Scaleless High line still grow feathers, but the

amount of feathering is extremely reduced and limited to mainly downy feathers (Abbott, 1965a). The Scaleless High line was specifically selected for this study because the presence of some feathering allowed the molt status of the birds to be monitored while still reducing the overall demands of feather growth during molt. Both the fully feathered UCD-003 line and the Scaleless High demonstrated defining features of molt during this study. In response to changes in photoperiod and oral thyroxine, the Scaleless High birds dropped their feathers on the same timeline as the UCD-003 birds. The molting Scaleless High birds also appear to undergo the metabolic shifts associated with molt including a decrease in body weight and cessation of lay indicating catabolism of the oviduct. Due to the broader metabolic and hormonal shifts that are associated with molt, it was important to ensure the ScHi birds underwent a full molt since there is a known difference in the energetic effects of molting versus regrowing feathers. Birds that are regrowing plucked feathers, but not molting, do not have an equivalent increase in metabolic rate that is observed in molting birds so plucking feathers does not represent an adequate equivalent for the process of molt (Buttemer et al., 2019). The Scaleless High birds underwent molt but with a reduced feather mass which allowed us to try and disentangle if the extent of feather growth or molt overall was a stronger driving force behind the trade-off between molt and the immune system.

The increase in the weight of the thymus and spleen during molt is a characteristic of molt overall and does not appear to be dependent on the mass of feathers growing during molt. The thymus of molting birds from the reduced feather Scaleless High birds and the fully feathered UCD birds increased 7.8-fold compared to birds that were not actively molting. Although the weight of the thymus increased in both genetic lines, the low feather Scaleless High birds had a higher cortex to medulla ratio driven by a greater increase in thymic cortex tissue.

The spleens of molting birds were 48% heavier than the spleens of the birds not molting. It is unclear if the increase in the weight of the spleen and thymus during molt is driven by molt or occurring in response to the environmental and hormonal cues that lead to the induction of molt. The increase in the spleen and thymus weight during molt may be driven by an increased need for additional lymphocytes to monitor and defend the skin barrier (Ward and D'Cruz, 1968) or even potentially to help regulate the tissue microenvironment around the feather follicle. The increase in the weight of the spleen and thymus in both genetic lines indicates that the increase in spleen and thymus weight may not depend on the mass of feathers being produced. Rather, the increased weight of the spleen and thymus appears to be due to molt and any amount of feather growth. It is also possible that the Scaleless High birds were still molting enough feathers, even though the number was greatly reduced, to necessitate the changes in the spleen and thymus.

From a systemic point of view, the process of molt involves major changes in the levels of multiple hormones including thyroid and reproductive hormones (Spearman, 1971; Brake et al., 1979). There are also broader metabolic changes associated with molt which are necessary to produce the nutrients and energy needed for generating new feathers. The combination of hormonal and metabolic changes during molt potentially creates an environment conducive to rejuvenating components of the immune system. The energetic and nutritional costs of molt are associated with increased mobilization of amino acids to support the growth of protein dense feathers. Proliferating cells also have increased amino acid requirements (Palm and Thompson, 2017). The increased number of lymphocytes seen in the thymuses of molting birds may also be supported by the increased mobilization of amino acids during molt as a result of catabolism of the reproductive track and other organ systems. Many birds molt following their breeding period, which may be a useful time to rejuvenate their immune system since resource usage will have

shifted away from supporting reproduction and caring for offspring back to the individual. The fact that that the weight of the spleen and thymus increased in both genetic lines, even the molting Scaleless High birds that have less feather mass, could also indicate that the regulatory environment needed for molt may result in concurrent changes in the immune system.

If the trade-off between molt and mounting an innate immune response was dependent upon the feather mass produced during molt, then the ScHi birds would be predicted to have a more robust inflammatory response during molt compared to the fully feathered birds which much use additional nutrients to produce a greater feather mass. The expression level of key inflammatory cytokines, especially IL-6 and IL-8, in response to LPS were reduced in in both genetic lines during molt. The Scaleless High birds should have reduced nutrient requirements during molt (Abbott et al., 1960) which was expected to reduce the extent of the trade-off between molt and an innate immune response. If the mass of feathers growing during molt was the driving force behind the trade-off, then the molting Scaleless High birds were expected to have a more robust inflammatory response to LPS than the molting UCD-003 birds. Contrary to this prediction, The Scaleless High birds had a similar dampening in the amplitude of their inflammatory response during molt compared to the UCD-003 birds, although overall the Scaleless High birds had a less robust response to LPS. The dampening of the inflammatory response in both genetic lines indicates that the feather mass generated during molt isn't the main driver behind the trade-off between molt and an innate immune response. Despite a reduction in feather costs, molting Scaleless High birds still had a significant reduction in their inflammatory response. The extent of the feather mass produced during molt does not appear to be the main driver behind a trade-off between molt and the immune system.

When examining the trade-off between molt and the immune system, it is important to consider the bidirectionality of the trade-off. This study focused on the trade-off from the perspective of the impacts of molt on the immune system. Molt, regardless of the mass of feathers being generated, led to a dampening of the inflammatory response. The broad physiological changes that accompany molt, rather than the extent of feather growth alone, resulted in a dampened inflammatory response. When considering the molt aspect of the trade-off, there are additional studies indicating that activation of the immune system also affects feather growth. Mounting an immune response during molt results in birds having slowed feather growth and reduced quality feathers. Generally, changing the rate of molt can result in lower quality feathers (Dawson et al., 2000). Sparrows challenged with LPS or PHA grew their feathers more slowly and the feathers were of lower quality after mounting an immune response while molting (Martin, 2005; Ben-Hamo et al., 2017). To ensure survival, birds appear to dampen both molt and inflammation during an innate immune response in order to maintain the two essential and costly processes simultaneously. The broad hormonal and metabolic changes that lead to the induction of molt and support feather growth, rather than the extent of feather growth alone, appear to be the stronger drivers of the trade-off between molt and the inflammatory response.

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Tables

Table 4.1 ANOVA P-values for the main effects of LPS challenge, molt status, genetic line, and all main effect interactions on body weight measurements. P-values are shown for both the body weight and the percentage change in body weight relative to pre-molt period.

	Body Weight (Grams)	Body Weight (% Change)
Time	< 0.01	< 0.01
Molt	< 0.01	< 0.01
Genetic Line	< 0.01	< 0.01
Time*Molt	< 0.01	< 0.01
Time*Genetic Line	< 0.01	0.06
Molt*Genetic Line	0.91	0.03
Time*Molt*Genetic Line	0.3	0.04

Table 4.2 ANOVA P-values for the main effects of LPS challenge, molt status, genetic line, and all main effect interactions on organ measurements. P-values are shown for both the organ weight and the organ weight as a proportion of body weight.

	Spleen (Grams)	Spleen (% BW)	Thymus (Grams)	Thymus (% BW)
LPS	< 0.01	< 0.01	0.18	0.19
Molt	< 0.01	< 0.01	< 0.01	< 0.01
Genetic Line	< 0.01	< 0.01	0.02	< 0.01
LPS*Molt	0.99	0.56	0.05	0.05
LPS*Genetic Line	0.49	0.28	0.18	0.25
Molt*Genetic Line	0.39	0.82	0.02	0.13
LPS*Molt*Genetic Line	0.75	0.56	0.56	0.73

Table 4.3 ANOVA P-values for the main effects of LPS challenge, molt status, genetic line, and all main effect interactions on the expression level of pro-inflammatory cytokines in the liver and spleen.

Cytokine	IL-1β		IL-6		IL-8		TNF-α	
Tissue	*Liver*	*Spleen*	*Liver*	*Spleen*	*Liver*	*Spleen*	*Liver*	*Spleen*
LPS	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Molt	0.03	0.73	<0.01	0.05	<0.01	0.20	0.41	<0.01
Genetic Line	0.14	0.88	0.22	<0.01	0.01	0.99	<0.01	0.43
LPS*Molt	0.02	0.01	0.85	<0.01	<0.01	0.01	<0.01	<0.01
LPS*Genetic Line	0.2	0.56	<0.01	0.03	0.25	0.19	0.34	0.65
Molt*Genetic Line	0.02	0.87	0.73	0.51	<0.01	<0.01	0.01	0.47
LPS*Molt*Genetic Line	0.07	0.8	0.02	0.87	0.03	0.07	0.77	0.73

Table 4.4 ANOVA P-values for the main effects of LPS challenge, molt status, genetic line, and all main effect interactions on the expression level of anti-inflammatory cytokines in the liver and spleen.

Cytokine	**IL10**		**TGF-β**	
Tissue	***Liver***	***Spleen***	***Liver***	***Spleen***
LPS	<0.01	<0.01	<0.01	0.67
Molt	0.47	0.01	0.21	0.30
Genetic Line	<0.01	0.87	<0.01	0.08
LPS*Molt	0.06	0.03	0.59	0.71
LPS*Genetic Line	0.41	0.40	0.7	0.82
Molt*Genetic Line	<0.01	0.58	0.02	0.39
LPS*Molt*Genetic Line	0.01	0.45	0.09	0.30

Figures

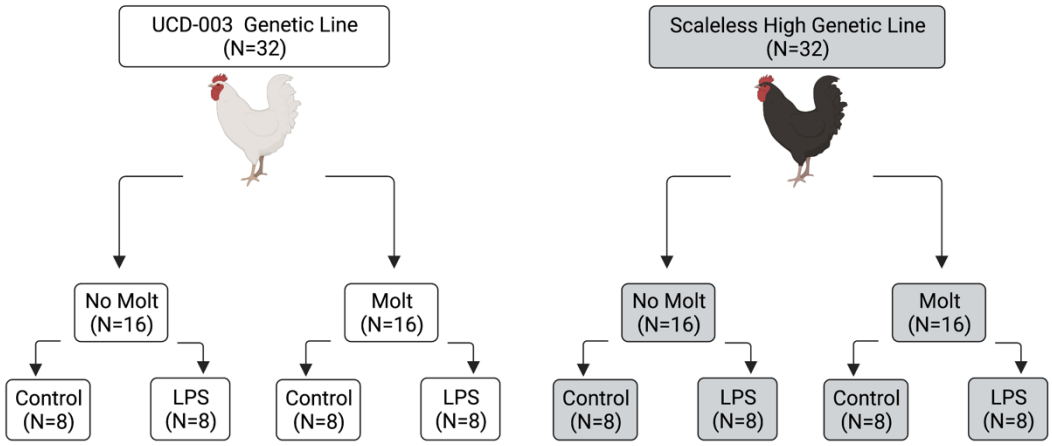

Figure 4.1 The 2x2x2 experimental design to evaluate the effect of feather amount on molt. UCD-003 and ScHi hens (N = 32) were induced to molt using a combination of a shift from 12 to 8 hours of light, a lower nutrient diet, and a daily dose of oral thyroxine (1.25 mg/kg) for 10 days. At the end of the fourth week of molt, half of the UCD hens (N = 16) and half of the ScHi hens (N = 16) received an intra-abdominal injection of LPS to assess the inflammatory response during molt.

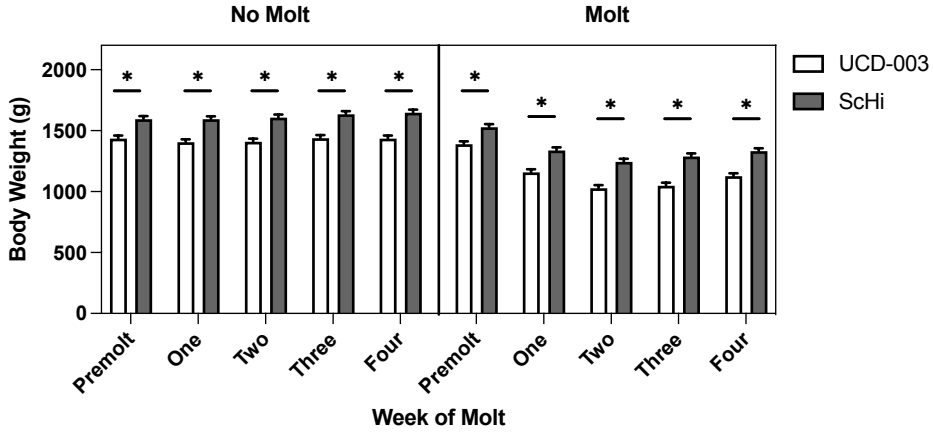

Figure 4.2 The effect of molt status on body weight among genetic lines A. Body weight measurements were taken weekly during the pre-molt period and each week of molt. ScHi birds weighed more than UCD-003 birds throughout the study ($P < 0.01$). Both molting UCD-003 and ScHi birds weighed significantly less than their non-molting counterparts ($P < 0.01$). Molting ScHi birds weighed more than molting UCD-003 birds ($P < 0.01$). Data are represented as mean $\pm$ standard error with asterisks indicating significant differences ($P < 0.05$) between groups.

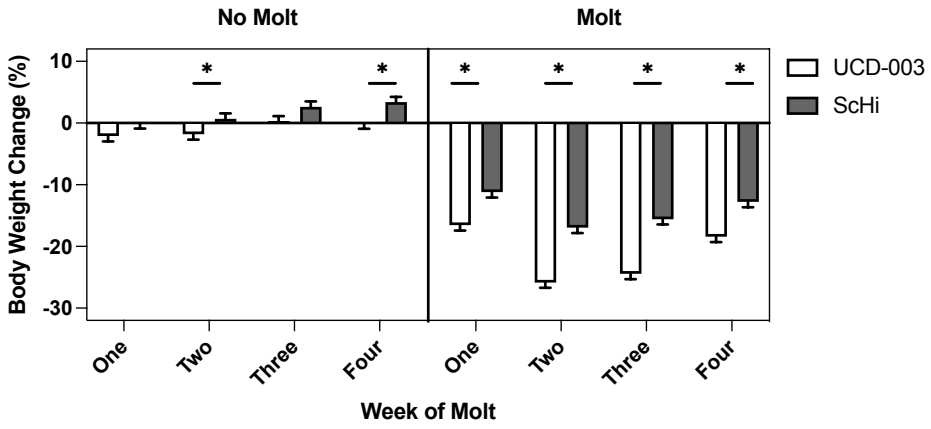

Figure 4.3 The effect of molt status on the change in body weight among genetic lines. The body weight change was calculated as the percent change in body weight relative to the pre-molt period. During each week of molt, the UCD-003 birds lost significantly more weight than the ScHi birds. Data are represented as mean ± standard error with asterisks indicating significant differences ($P < 0.05$) between groups.

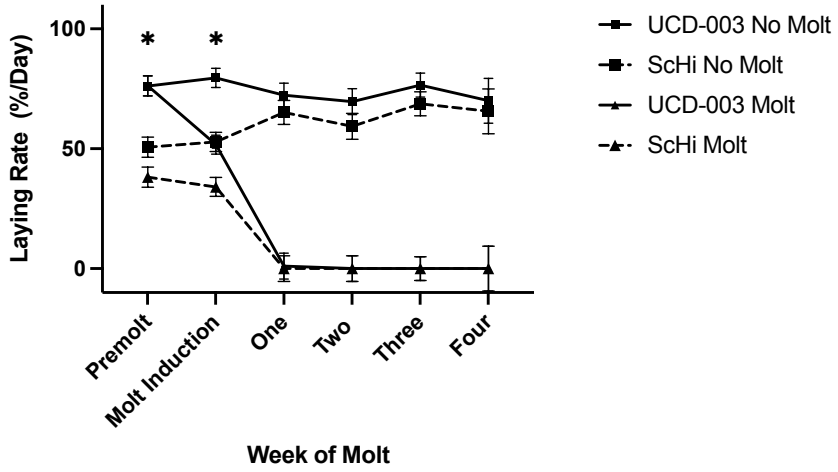

Figure 4.4 The effect of molt status on laying rate among genetic lines. The number of birds laying was assessed daily for the duration of the study. Both the molting UCD-003 birds and ScHi birds ceased laying during week one of molt and did not resume laying during the four weeks of molt. The non-molting birds had a significantly higher laying rate than the molting birds from week one through four ($P < 0.01$). ScHi birds had a laying rate that was on average 12% lower than the laying rate of the UCD-003 birds ($P < 0.01$). Data are represented as mean ± standard error with asterisks indicating significant differences among molting genetic lines.

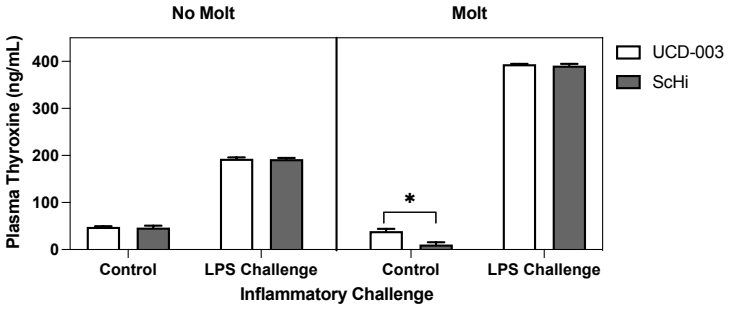

Figure 4.5 Effect of molt status and genetic line on plasma thyroxine concentration. Plasma was collected at the end of the fourth week of molt and the concentration of thyroxine in the plasma was determined via ELISA. An injection of LPS led to a significant increase in plasma thyroxine concentration across both genetic lines ($P = 0.02$). Molt led to an increased concentration of plasma thyroxine ($P < 0.01$). The average concentration of thyroxine is shown with error bars representing the S.E.M.

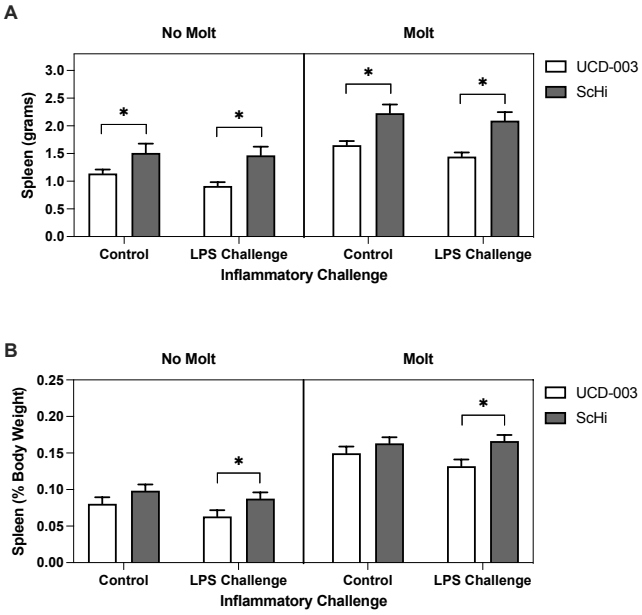

Figure 4.6 The effect of LPS challenge, molt status, and genetic line on spleen weight. Spleen weights from each group (N = 8) were measured at the end of the fourth week of molt. A. The spleens of non-molting and molting UCD-003, but not ScHi, birds decreased significantly in response to LPS. The spleens of the molting birds were significantly heavier than the spleens of the non-molting birds ($P < 0.01$). ScHi birds had, on average, heavier spleens than the UCD-003 birds ($P < 0.01$). B. Spleen weight was divided by body weight in order to correct for differences in body weight between molt and genetic groups. The spleen accounted for a significantly greater amount of body weight in the molting birds ($P < 0.01$). The increase in spleen weight in the molting birds compared to the non-molting birds was consistent across the two genetic lines. Data are represented as mean $\pm$ standard error with asterisks indicating significant differences ($P < 0.05$) between groups.

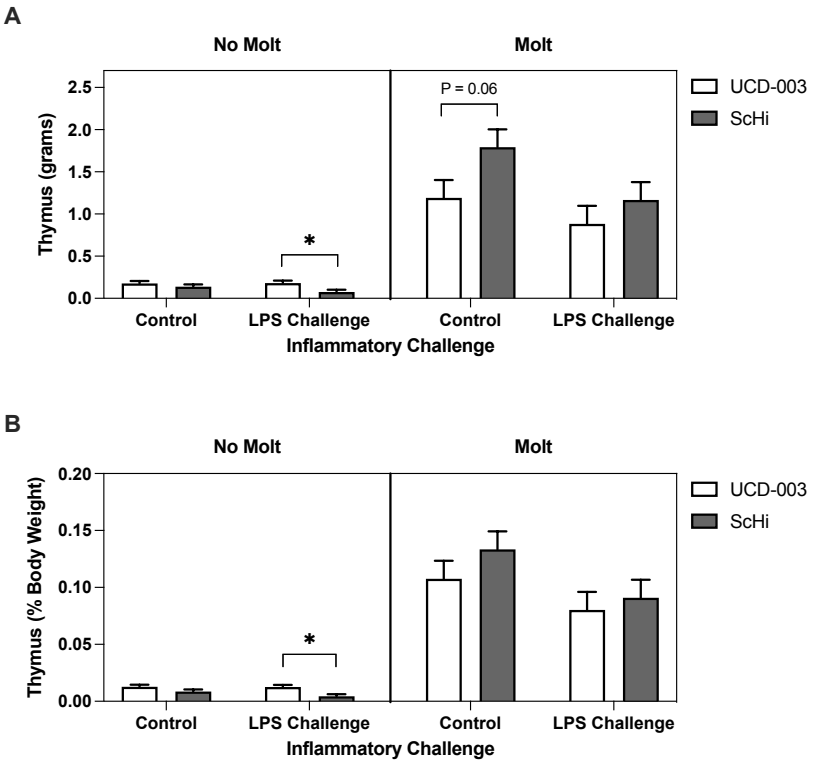

Figure 4.7 The effect of LPS challenge, molt status, and genetic line on thymus weight. The thymic lobes were weighed at the end of the fourth week of molt. A. Thymus weight increased significantly due to molt in both genetic lines ($P < 0.01$). In response to LPS, the thymus weight decreased significantly in the molting birds ($P = 0.04$) but not in the non-molting birds. B. When thymus weight was corrected for body weight, the average thymus weight of molting birds was 9.8-fold higher compared to the non-molting birds ($P < 0.01$). An injection of LPS only caused a reduction in the thymus mass of molting birds ($P = 0.04$). Data are represented as mean + standard error with asterisks indicating significant differences ($P < 0.05$) between groups.

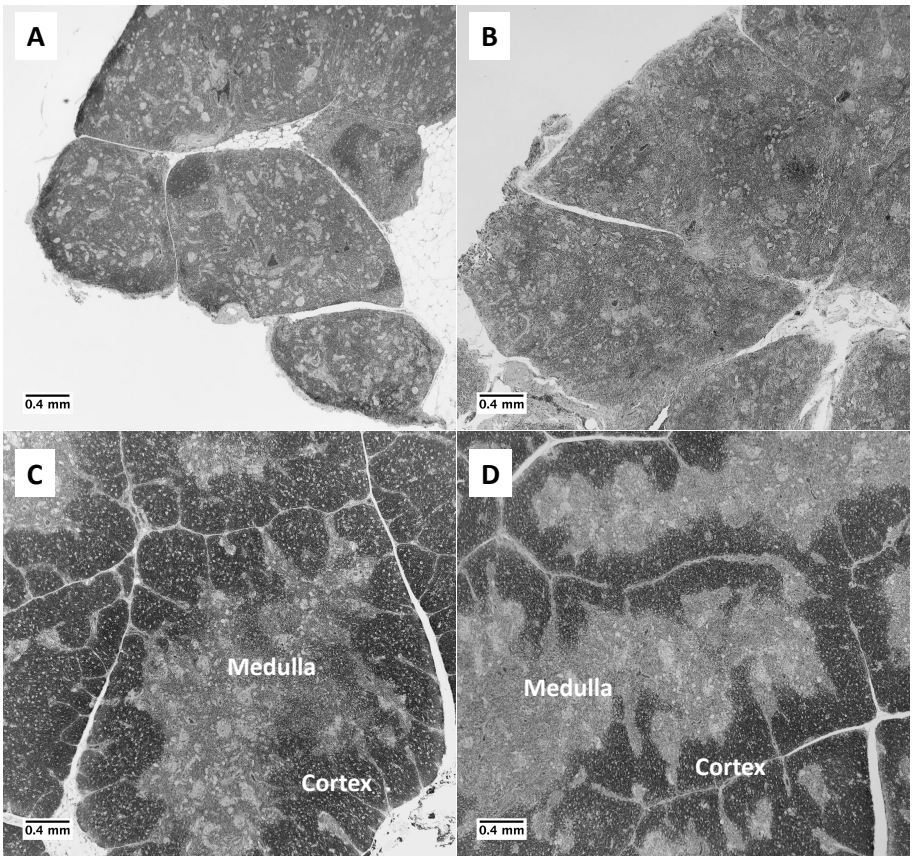

Figure 4.8 Histological changes in the thymus during molt. Thymus samples were collected at the end of week four from non-molting and molting birds in both genetic lines then stained with hematoxylin and eosin. All images were taken at 4x magnification. A. Thymus section from a non-molting UCD-003 bird showing no well-defined thymic lobules or clearly defined areas of cortex or medulla. B. Thymus section from a non-molting ScHi bird showing no clear cortex or medulla. C. Thymus section from a molting UCD-003 bird with clearly defined thymic lobules containing cortex and medulla tissue. D. Thymus section from a molting UCD Scaleless bird showing defined thymic lobules containing cortex and medulla tissue.

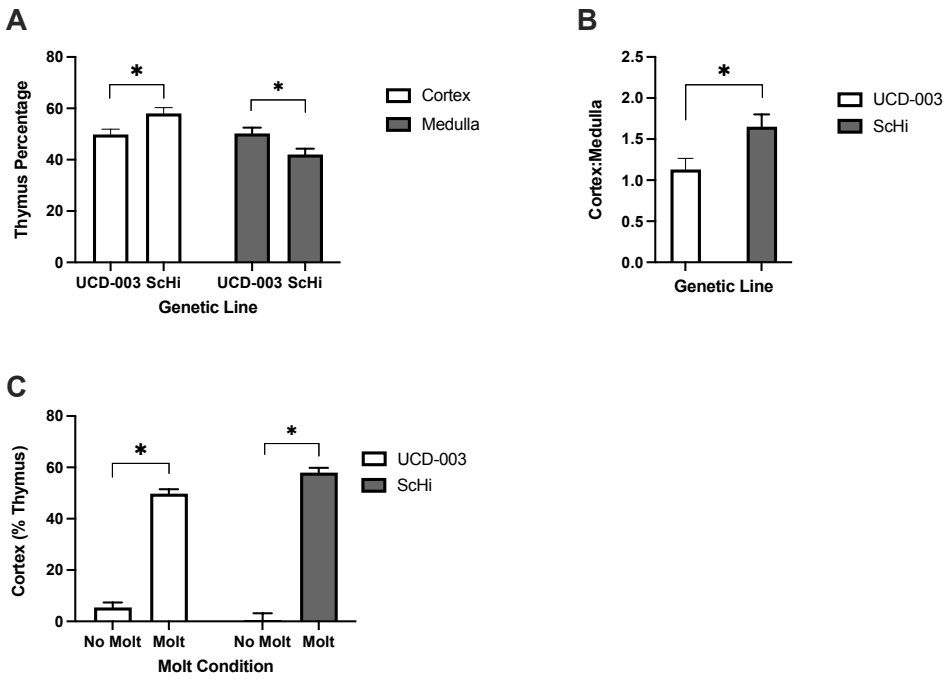

Figure 4.9 Changes in the relative area of the cortex and medulla during molt in two genetic lines. Thymus samples collected at the end of week for were analyzed for the area of cortex and medulla tissue using ImageJ at 4x magnification. A. UCD-003 birds had a higher percentage of cortex tissue ($P = 0.01$) but lower percentage of medulla tissue ($P = 0.01$) than ScHi birds during molt. B. Molting ScHi birds had a higher thymic cortex to medulla ratio than molting UCD-003 birds ($P = 0.02$). C. Birds from both genetic lines had a significantly greater proportion of cortex tissue in the thymus during molt ($P < 0.01$).

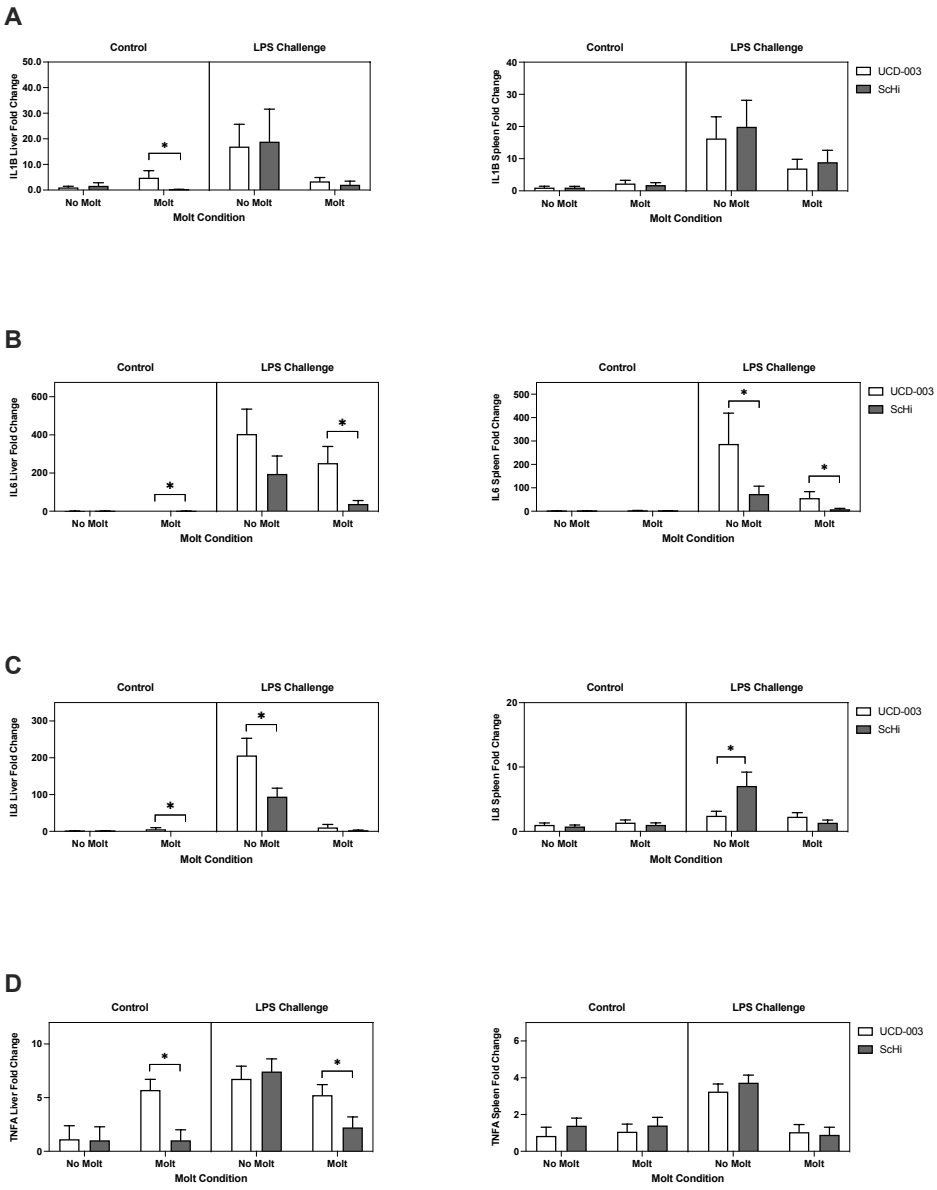

Figure 4.10 Impact of LPS injection on the expression level of pro-inflammatory cytokines in molting and non-molting birds of two genetic lines. Birds were administered and intra-abdominal injection of LPS at 1.5 mg/kg. Five hours post injection, samples from the liver and spleen were collected to assess the expression level of selected pro-inflammatory cytokines. Gene expression levels are represented as the average fold-change relative to treatment control non-molting birds. Error bars are the SEM. A. The expression level of IL-1β in the liver and spleen was dampened in molting birds ($P < 0.01$). B. ScHi birds had reduced levels of IL-6 expression in the liver and the spleen in response to LPS ($P < 0.01$; $P = 0.03$ respectively). C. IL-8 expression in response to LPS was reduced in the liver and spleen of molting birds from both genetic lines ($P < 0.01$; $P = 0.01$, respectively). D. Molt also led to a significant reduction in TNF-α expression in the liver and the spleen of molting birds following an injection of LPS ($P < 0.01$).

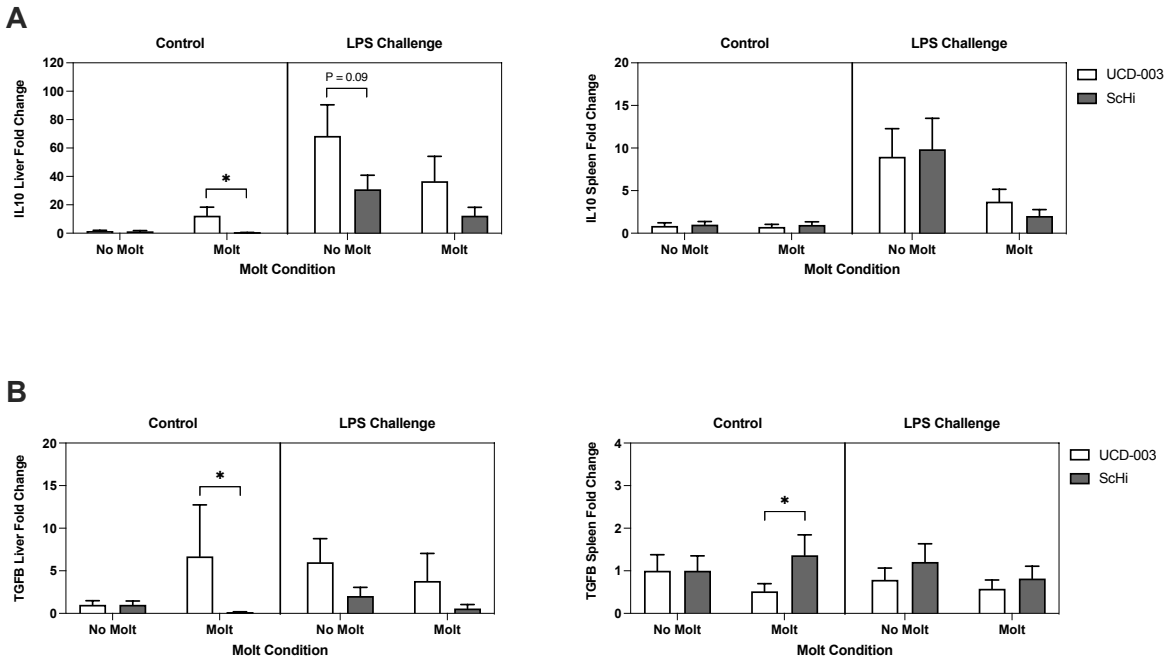

Figure 4.11 Impact of LPS injection on the expression level of anti-inflammatory cytokines in molting and non-molting birds of two genetic lines. Liver and spleen samples were collected for gene expression assessment via qPCR five hours after an intra-abdominal injection of LPS (1.5 mg/kg). The gene expression levels are the average fold-change relative to treatment control non-molting birds with error bars representing S.E.M. A. Molt led to a decrease in the expression level of IL-10 in response to LPS in the spleen ($P = 0.03$) but not the liver. B. ScHi birds had lower TGF-β expression in the liver ($P < 0.01$) and trended towards lower levels in the spleen ($P = 0.08$).

Appendix

Immune Cell Populations of the Spleen

An injection of LPS led to a significant reduction in the proportion of T cells in the spleen ($P < 0.01$; Table A.1). Molt, on the other hand, resulted in an increased proportion of T cells in the spleen ($P < 0.01$; Figure A.1). Molting birds had a 39% increase in the proportion of T cells in the spleen compared to non-molting birds ($P < 0.01$ by pairwise comparison). The interaction between LPS and molt trended towards significant ($P = 0.06$) with LPS causing a greater drop in the proportion of T cells in the spleens of molting birds (27% decrease) than non-molting birds (13% decrease). The proportion of macrophages in the spleen was not altered by an injection of LPS, molt status nor the interaction between the two (Figure A.1; Table A.1).

Table A.1 ANOVA P-values for the main effects of LPS challenge, molt status, and the interaction between LPS and molt status on the percentage of T cells and macrophages.

Cell Type	**CD3+ T Cell**	**Macrophage**
LPS	< 0.01	0.96
Molt	< 0.01	0.60
LPS*Molt	0.06	0.42

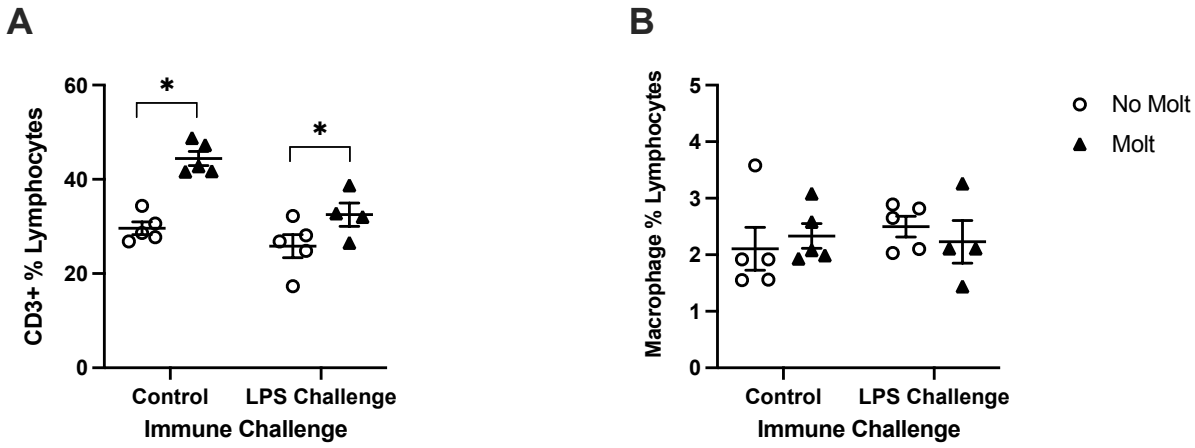

Figure A.1 The effect of molt on immune cell populations in the spleen. The percentage of T cells and macrophages in the spleen (N = 5) were examined in UCD-003 at the end of week four following an intra-abdominal injection of LPS. A. Molting birds has a significantly higher percentage of T cells compared to non-molting birds ($P < 0.01$). B. Molt did not influence the percentage of macrophages found in the spleen.

Chapter 5

Overall Dissertation Discussion

The period of molt is a truly impressive and fascinating phase of a bird's life. Despite the ubiquity of molt across avian species, many questions surrounding the process of molt still remain unanswered. The overall goal of this dissertation was to further the fundamental understanding of how molt affects the immune system. The research presented attempted to answer two separate, but closely related, questions about molt and the immune system. The first question, does the process of molt cause systemic shifts in the immune system, was the subject of chapter one of this dissertation. There is a fairly striking amount of conflict in the literature about the effects of molt on the immune system. Due to differences in molt strategies and species differences, it is expected that there would be at least some level of variance in the interactions between molt and the immune system. However, in many instances, there is directly contradictory evidence even when the same facet of immune function or the same species is studied. When considering the existing literature, one of the ways that the discrepancies in the data could make sense was if the immune system was altered while birds were molting. If the activity of the immune system varied over the course of molt, then the period of molt when samples were taken could contribute to the observed variance. There were indications that the immune system was altered during molt based on a few studies demonstrating shifts in cell populations and the weight of the spleen and thymus during molt. The time study described in chapter two was performed to track changes in the spleen and thymus weekly over the course of molt to better understand how the phase of molt may affect the immune system. The greatest change in the spleen and thymus occurred during mid-molt. Each phase of molt, whether birds

are just beginning to grow feathers or in peak feather production, may have slightly different effects on the immune system although additional studies are needed to clearly define the interaction between the phase of molt and the immune system.

When considering the changes in the immune system during molt, the spleen and thymus weight increased significantly during molt in each experiment presented in this dissertation. The increase in the mass of the spleen and thymus was consistently observed across three different genetic lines. The peak increase in the weight of both the spleen and the thymus corresponded to the period of peak feather growth mid-molt. Although the extent of the increase varied by genetic line, I feel confident defining an increase in the weight of the spleen and thymus as a main characteristic of molt. The changes in the thymus were examined more in depth than the spleen due to the striking recrudescence of the thymus during molt and data from other authors indicating potential shifts in T cell populations. There was also an increase in the weight of the spleen and shifts in the immune cell populations of the spleen during molt. I choose to focus on the thymus more than the spleen, but the changes in the spleen are also extremely interesting and worth following up on. The Bursa of Fabricius is another essential lymphoid tissue for birds and the site of B cell development in growing birds. Despite a concerted effort to locate the Bursa of Fabricius in adult molting birds, the organ remained involuted during molt and so only the spleen and thymus were focused on.

The thymus of a chicken is considered most active in young growing birds before mostly involuting in adult birds. Not only did the thymus increase in weight in molting birds but histological examination of the thymus during the peak weight increase revealed an increase in cortex and the medulla tissue. The thymus of molting birds across multiple genetic lines had clearly defined lobules as well as more cortex and medulla tissue. One of the most important

takeaways from this dissertation is that the thymus not only increased in weight but also appears to potentially be producing new T cells during molt. Due to the costly nature of molt, it is surprising that the thymus increases in size when many other organs are partially catabolized during molt. Examination of the T cell populations from the thymuses of molting birds during week four of the time trial study revealed a marked increase in the percentage of double-positive T cells. When the overall increase in thymus weight was considered, the molting birds had a much greater number of double-negative, double-positive, and single-positive T cells. The increase in the weight of the thymus during molt and the potential generation of a new T cell repertoire during molt implies that the thymus may have greater plasticity than has been traditionally assumed. Additional studies would need to be performed to confirm that new T cells, and not just clonal expansion, occurs in the thymus of molting birds. Flow cytometry can be used to examine in more detail the T cell receptors and T cell phenotypes in the thymus over the course of molt. Immunohistochemistry should also be used to track the location of specific T cell subsets in the thymus of molting birds over the course of molt. The flow cytometry data for the T cell phenotypes seen in the thymus during molt represent only one week of sampling from one study. It would be ideal to complete another time series experiment focused only on the thymus and to carry the experiment through an entire molt and into the post molt period. Although the data presented in this dissertation is striking, additional studies are needed to support the findings especially considering the important implications for the plasticity of thymus function in chickens and birds more generally.

For future experiments that involve the induction of molt in chickens using photoperiod, diet, and hormonal signals there are a couple experimental design considerations to be made. One of the most significant challenges was ensuring that the birds had a strong molt and molted

on a very similar timeline. A combination of cues were used to induce a robust molt in chickens including a switch from 16 hours to 8 hours of daylight, a molt diet, and an oral dose of Thyroxine. Preliminary studies showed that the combination of all three signals was the most effective method to induce a robust and consistent molt across all birds. For the studies in this dissertation, daylength was slowly reduced over multiple days to try and mimic a more natural reduction in light. To induce a faster transition into molt, it may be more efficient to drop the day length over a shorter period of time. The body weight of birds must also be closely monitored over the course of the experiment to ensure birds do not have too high of a reduction in body weight. A significant proportion of the body weight loss that occurs during molt is attributable to the regression of the oviduct as well as the loss of feathers. The change in daylength also reduces the amount of time for the birds to eat. Based on my personal observations the birds also reduced the time spent eating during molt in favor of preening. For anyone inducing molt, it would be recommended to use a lower calorie molt induction diet for the shortest possible period before switching to a molt support diet and post molt diet to encourage an increase in feed intake while the birds are molting and coming out of molt. Since molt affects the immune system, it is also essential to ensure preventative health measures are taken prior to molt such as treating for ectoparasites. All of the birds used in these studies were treated for mites during the acclimation period prior to the start of the experiments.

A preliminary trial was used to determine the oral dose of thyroxine needed to induce a consistent molt. The dose of thyroxine needed was on the higher end (1.25 mg/kg) likely due to the oral route of administration. The thyroxine dose induced a strong molt that more closely resembles a catastrophic molt compared to other molt strategies. To ensure that each bird received an appropriate dose of thyroxine, a concentrated thyroxine diet mixture was prepared

and weighed out for each bird individually based on body weight. During the molt induction period, the individual doses of thyroxine needed for each bird were prepared and added to the feed each morning. The process of properly dosing the thyroxine was very labor intensive and would be difficult to scale up for larger studies. The largest experiment carried out for this dissertation was just under seventy animals, which can be considered small for researchers who perform poultry experiments that involve hundreds of animals but was still very time intensive in-terms of animal care.

The number of birds used is the final important experimental consideration. The time trial study used six birds per treatment which represented the lowest N-value of all of the studies. Although there was no overall increase in inflammation, there was a higher-than-expected amount of individual variation in cytokine expression levels. There were some molting birds that did have higher levels of inflammatory markers. It would be interesting to run another study looking at systemic inflammation with increased numbers of birds to try and clarify some of the more nuanced aspects of inflammation during molt on an individual level. Birds molt multiple times over the course of their lifetimes and it could be that sometimes molt does induce a higher level of inflammation depending on the specific environmental factors of that molt. The number of birds used for the two trade-off studies were increased from six to eight to try and account for the variance seen in cytokine expression, but a high level of individual variance was still observed in the subsequent studies.

Molt presents a unique opportunity to examine the nutritional and energetic bottleneck that may occur as a result of mounting an immune response concurrently with other expensive processes. The third and fourth chapters of this dissertation were performed to determine if there is a trade-off between molt and mounting an innate immune response. Both molt and an innate

immune response are essential for a bird's survival and have high energetic and nutritional costs. The experiments in this dissertation demonstrated that when birds have to mount an innate immune response while simultaneously molting, the inflammatory response is dampened. The dampened signal apparent in the decrease in the expression of key pro-inflammatory cytokines and reduced plasma levels of pro-inflammatory cytokines.

The Scaleless High line of birds was used to determine if a decrease in the feather mass generated during molt would reduce the bottleneck between molt and the immune system. Despite the reduction in feather replacement costs when the Scaleless birds molted, there was still a significant decrease in the amount of inflammation observed during an innate immune response. As birds enter molt and maintain molt there are a range of metabolic and hormonal changes that occur. These hormonal and metabolic changes have broad affects throughout a bird's body and appear to be the main driver behind the trade-off between molt and an innate immune response rather than the mass of feathers being grown. The reduction in the inflammatory signal observed in both of the studies performed to examine the trade-off was consistent and significant. I feel confident stating that when chickens undergo a molt where they are re-growing most of their feathers there is dampening of the innate immune response. These studies only looked at general markers of inflammation during an innate immune response and it would be interesting to expand these studies to look at acute phase proteins or other aspects of innate immunity where there is also likely to be a bottleneck due to overlapping nutrient requirements. The reduction in the inflammatory response has important implications for the adaptive immune response as well. The signals of the innate immune response are essential modulators of the adaptive immune response. A pathogen challenge or even vaccine challenge

during molt would build on the small amount of existing literature on molt and the adaptive immune system to help further clarify the trade-off between molt and the immune system.

The chickens in this study were artificially induced into a strong molt that involved molting most of their feathers at once. When considering trade-offs, the type of molt used in this study is costlier and more likely to induce a trade-off between molt and an immune response than other types of molts. The molt strategies employed by different bird species are extremely diverse and therefore the extent to which there is a trade-off between molt and the immune system will most likely vary by species. That molt rarely overlaps with other expensive processes in most species of birds indicates that molt is likely to induce a trade-off across different species even with differences in molt strategies. The results of the research in this dissertation indicate that the molt strategy of a bird, the stage of molt and the metric of immune function must be carefully considered when designing experiments to examine interactions between molt and the immune system.

The research in this dissertation showed that the immune system of birds is altered during molt and that there is a trade-off between molt and an innate immune response. As any good data does, the results presented here raise many additional interesting and important questions about the process of molt and the avian immune system The very first paper documenting an increase in the number of thymic lymphocytes via histological examination was published by Brake et al. in 1981. Brake's original study, and my studies building on that research, have important implications for not only avian biology but also how the activity of the immune system is more broadly understood. It is my most sincere hope that other researchers build upon the data presented in this dissertation so that these fascinating lines of inquiry do not remain dormant for another 40 years.

Appendix 1

Thyroxine Dose Trial

Experiment Purpose

This experiment was conducted prior to the experiments in chapters 2, 3 and 4 of this dissertation in order to determine the ideal inclusion rate of thyroxine in the feed. The final thyroxine dose used was decided based upon which inclusion rate induced a consistent and strong molt across all birds. It was important that all hens, to the greatest extent possible, started molting at the same time and followed a similar molt timeline in order to keep the molt strength and rate consistent across birds.

Materials and Methods

Experimental Design

Twenty-two adult single-comb white leghorn hens from the UC Davis Hopkins Avian Research Facility colony were housed in the UC Davis Meyer Hall Avian Facility. Hens were housed individually in cages with sloped floors to facilitate egg collection. To induce molt, daylength was reduced from 16 hours of light to 8 hours of light. Thyroxine was tested at five different feed inclusion rates (N = 4): 3.5 mg/kg feed, 7 mg/kg feed, 10.5 mg/kg feed, 14 mg/kg feed and 17.5 mg/kg feed. Control birds were kept in the same room and underwent the change in daylength but were maintained on a commercial layer diet (Bar-Ale, Cat. No. WP16CC). The laying rate and feed intake were monitored daily for the duration of the study. The body weight of the birds and molt status were also monitored multiple times a week for the duration of the

study. The level of molt was classified as either low, medium, or high. Birds categorized as low molt dropped very few or no feathers during their molt. Birds classified as having a medium level of molt molted wing feathers, tail feathers and sometimes a few body feathers. Birds in the high molt category molted most of their wing, tail, and body feathers.

Statistical Analysis

All of the statistical analyses were performed using Microsoft Excel® Version 16.58, R© version 4.1.1 and RStudio© version 2021.9.0.351 (R Core Team, 2021; RStudio Team, 2021). For each model, normality was assessed by examining a Q-Q plot of the standardized residuals versus the theoretical quantiles in combination with the Shapiro-Wilks test of normality. Homogeneity of variance was assessed by visually examining a plot of the model standardized residuals by the fitted model values as well as using Levene's test. For body weight data, a linear mixed-effects model was constructed with week, thyroxine dose and the interaction between week and thyroxine dose as fixed effects and bird identification as a random effect.

Results

There was a significant main effect of experimental week on body weight ($P < 0.01$) and also a significant week by thyroxine dose interaction due to birds on higher thyroxine doses losing more weight mid-molt ($P < 0.01$). All birds lost weight during the molt period and there were no significant differences between groups by pairwise comparison. The percent change in body weight relative to the first week of molt followed the trends described above. There was a significant effect of week ($P < 0.01$) and a significant interaction between week and thyroxine dose ($P < 0.01$). The peak weight loss occurred during week five of the experiment (Figure A.3). Birds in the control group lost very few feathers and were some of the last birds to stop laying.

The birds given the lowest doses of thyroxine, 3.5 and 7.0 mg/kg, fell into the low molt category and continued to lay for longer. The highest dose of thyroxine resulted in the most consistent molt pattern and the fastest onset of molt characterized by the quick dropping of feathers and cessation of lay.

Conclusion

The highest dose of thyroxine was chosen for use in consequent experiments and was dosed based on body weight for all subsequent experiments (1.25 mg/kg body weight). Since the hormone is administered in the feed, the birds are unlikely to always ingest the full dose for a given day. A high dose is needed to ensure the birds ingest enough thyroxine to induce a robust and consistent molt.

Figures

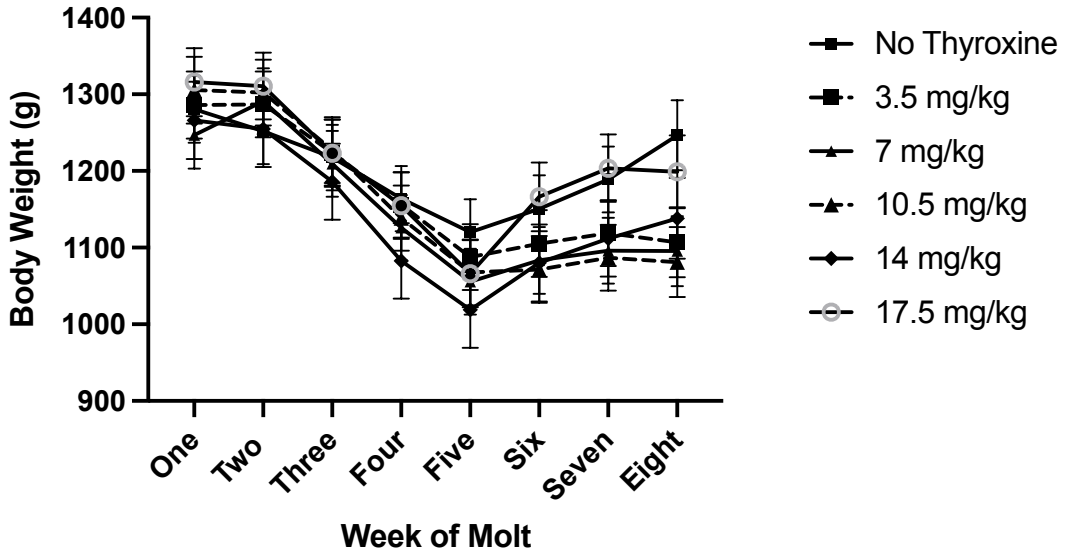

Figure A.2 The effect of oral thyroxine dose on body weight. Body weight was measured twice a week during each week of molt. There was an overall effect of week ($P < 0.01$) and a significant interaction between week and treatment ($P < 0.01$). All birds lost weight during the experimental period, even the control birds fed a commercial diet.

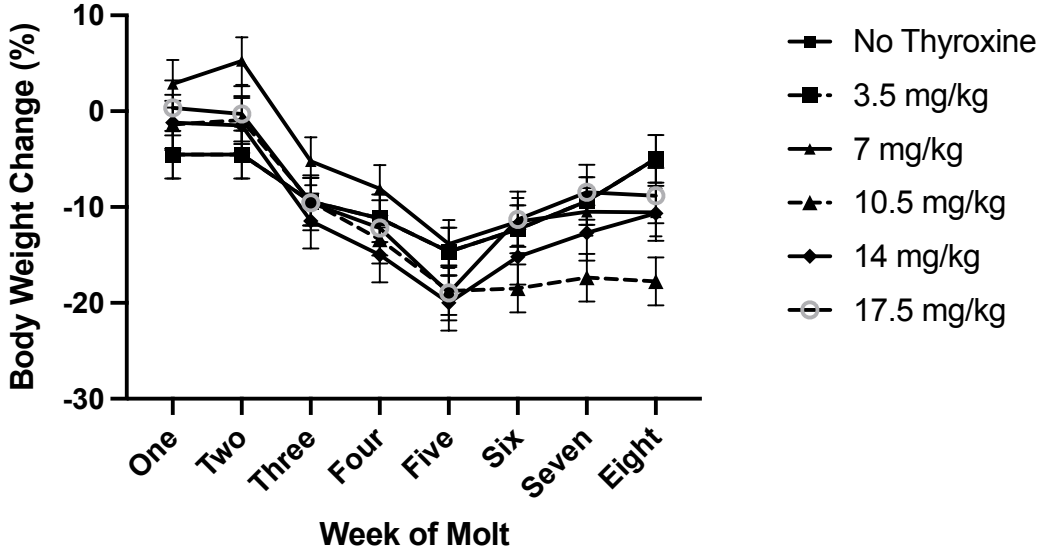

Figure A.3 The effect of oral thyroxine dose on body weight change. The change in body weight shown is relative to the pre-molt period. Birds from each treatment group experienced peak weight loss during week five.

Appendix 2

The effect of thyroxine on molt induction and spleen and thymus weight

Experiment Purpose

This experiment was conducted to determine if a robust molt could be induced without the use of thyroxine. Supplementation of a hormone adds another level of experimental complexity and, if possible, it would be ideal to induce molt without using exogenous hormone supplementation.

Materials and Methods

Experimental Design

Eleven Hy-Line W-36 laying hens obtained from JS West (Atwater, CA) were housed in the Cole A research facility at the University of California, Davis. Hens were housed individually in cages with sloped floors to facilitate egg collection. To induce molt in all birds, daylength was reduced from 16 hours of light to 8 hours of light and a molt diet was fed. To test the role of thyroxine in inducing molt, half of the birds (N = 6) received the molt diet mixed with thyroxine (1.25 mg/kg body weight). The lay rate was monitored daily until the cessation of lay occurred. Body weight was taken weekly to monitor the health of the birds. The birds were culled after two weeks, and the weight of the spleen and thymus were recorded.

Statistical Analysis

All of the statistical analyses were performed using Microsoft Excel® Version 16.58, R© version 4.1.1 and RStudio© version 2021.9.0.351 (R Core Team, 2021; RStudio Team, 2021). For each model, normality was assessed by examining a Q-Q plot of the standardized residuals versus the theoretical quantiles in combination with the Shapiro-Wilks test of normality. Homogeneity of variance was assessed by visually examining a plot of the model standardized residuals by the fitted model values as well as using Levene's test. For body weight and change in body weight, a linear mixed-effects model was constructed with week, thyroxine treatment and the interaction between week and thyroxine dose as fixed effects and bird identification as a random effect. A linear model and ANOVA were used to determine the effect of thyroxine supplementation to induce molt on the weight of the spleen and thymus. For all ANOVA and post-hoc tests, significance was accepted at $P \leq 0.05$ and a p-value between $P > 0.05$ and $P < 0.10$ was considered to indicate a non-significant trend.

Results

Hens induced to molt using thyroxine in addition to a molt diet plus the change in photoperiod ceased laying by day six. It took the control hens that did not receive thyroxine supplementation nearly twice as long to cease laying (Figure A.4). Thyroxine treatment trended towards affecting body weight ($P = 0.08$). There was a significant main effect of molt week ($P < 0.01$) and the interaction between molt week and thyroxine treatment ($P = 0.04$) because birds given thyroxine had lower body weight during molt. The week of molt ($P < 0.01$) and the interaction between molt week and thyroxine ($P < 0.01$) also had a significant effect on the decrease in body weight during molt (Figure A.5).

Thyroxine supplementation did not result in a difference in spleen weight between the molting birds given thyroxine and the molting birds without. There was a significant effect of thyroxine supplementation on the weight of the thymus ($P < 0.01$). The thymus of a bird supplemented with thyroxine was on average 5.8-fold larger than the thymus of a bird not given thyroxine. (Figure A.6)

The supplementation of thyroxine in addition to a change in photoperiod and dietary change quickly induced molt. Birds given all three cues were molting their primaries and tail feathers by the second week of the experiment. None of the birds induced to molt using only the molt diet and a change in photoperiod began to molt.

Conclusion

A change in photoperiod, molt diet and supplementation with thyroxine are all needed to induce molt. The birds that only experienced a change in photoperiod and the molt diet were not induced to molt. The decrease in body weight and cessation of lay observed in this group is due to decrease in feeding time due to the shorter day and molt diet which is not designed to support a laying hen. There was a major increase in the weight of the thymus in the molting thyroxine supplemented group but not the thyroxine control group. Thyroxine is needed to experimentally induce molt, birds do not enter molt without the combination of photoperiod, diet, and a hormonal cue.

Figures

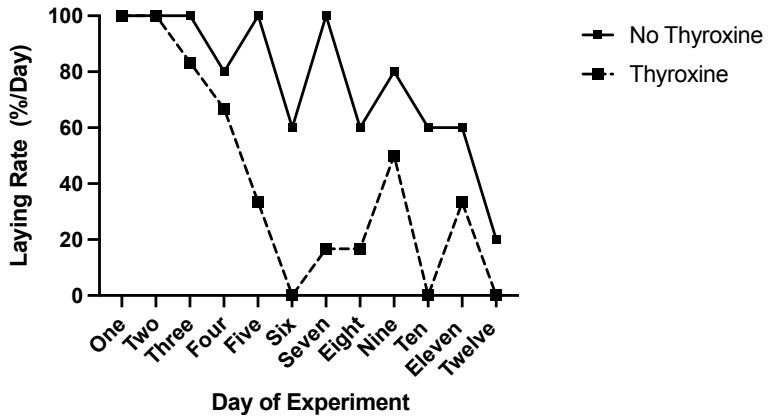

Figure A.4 The effect of thyroxine supplementation and molt cues on laying rate. Adult laying hens were induced to molt with a change in photoperiod and a molt diet. Hens in the thyroxine treatment group also received oral thyroxine in their feed. Thyroxine led to a faster cessation of lay in the thyroxine treatment group compared to the hens that only received light and dietary cues.

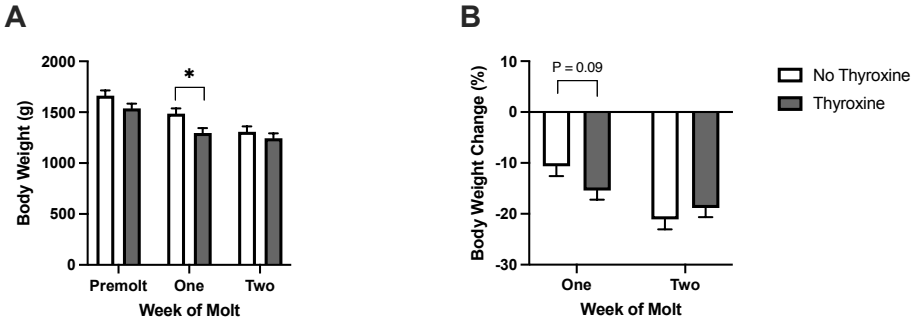

Figure A.5 The effect of thyroxine supplementation and molt cues on body weight. Body weight and the change in body weight were measured in hens induced to molt with photoperiod and diet alone or photoperiod, diet, and oral thyroxine. A. There was a significant interaction between molt week and thyroxine due to thyroxine treated birds having the lowest body weight during the first week of molt. B. Both birds receiving thyroxine and those not receiving thyroxine had a significant decrease in body weight relative to the pre-molt period.

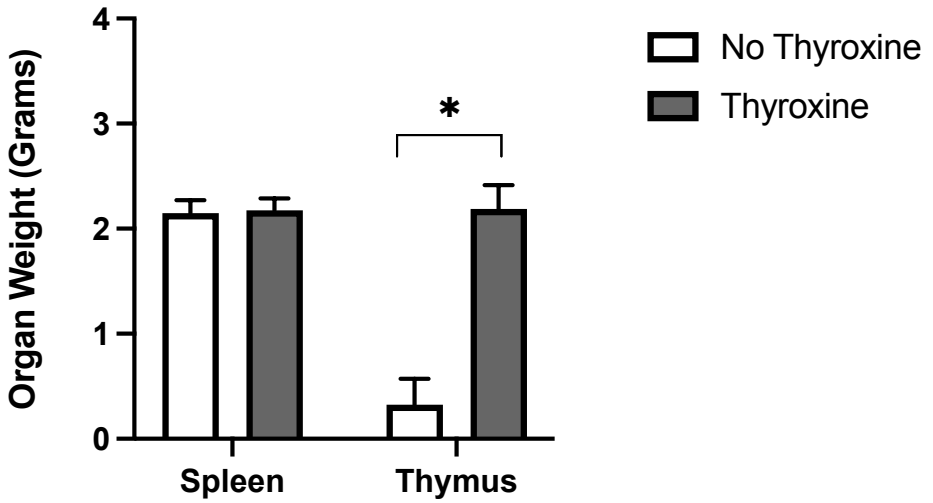

Figure A.6 The effect of thyroxine supplementation and molt cues on the spleen and thymus. At the end of two weeks of molting the organ and spleen were weighed. There was no difference in the spleens of birds supplemented with thyroxine and those without. Thyroxine supplementation led to a significant increase in the weight of the thymus ($P < 0.01$).

Appendix 3

Diet Compositions

Table A.2 Nutrient composition of experimental molt diet.

Nutrient	Unit	As fed
Metabolizable Energy	kcal/kg	2431.6
Protein	%	9.6
Fat	%	3.12
Calcium	%	0.5
Phos total	%	0.44
Phos avail	%	0.25
Potassium	%	0.78
Chloride	%	0.13
Sodium	%	0.03
Iron	%	208.08
Manganese	mg/kg	89.42
Copper	mg/kg	11.94
Zinc	mg/kg	59.83
Vit A	IU/kg	1500.06
Vitamin E	IU/kg	15
Choline	%	1050.01
Arginine	%	0.3
Isoleucine	%	0.24
Lysine	%	0.33
Methionine	%	0.23
Meth+Cyst	%	0.36
Threonine	%	0.24
Tryptophan	%	0.12
Valine	%	0.32

Table A.3 Ingredient composition of experimental molt diet.

Ingredient	Percentage of Diet
Corn, 2017	57.16
Soybean hulls	28.97
Alfalfa meal dehydrated 17% CP	9.55
Rice bran	2.53
Calcium phosphate dibasic	0.96
Mineral Mix - NRC -wo iron	0.25
Vitamin mix - NRC	0.25
L-lysine HCl 95%	0.137
DL-methionine 99%	0.108
Choline chloride	0.077
Salt, Iodized	0.012

Table A.4 Nutrient composition of experimental post-molt diet.

Nutrient	As fed
Metabolizable Energy	3000.00
Protein	16.06
Fat	9.41
Calcium	2.00
Phosphate total	0.70
Phosphate avail	0.50
Potassium	0.84
Chloride	0.38
Sodium	0.20
Iron	335.57
Manganese	108.08
Copper	10.09
Zinc	65.75
Vit A with b-carotene	2675.83
Vitamin E	19.68
Choline	1800.02
Arginine	0.97
Isoleucine	0.66
Lysine	0.81
Methionine	0.49
Meth+Cyst	0.75
Threonine	0.61
Tryptophan	0.28
Valine	0.76
Linolenic, alpha n-3	0.50

Table A.5 Ingredient composition of experimental post-molt diet.

Ingredient	As fed
Corn Dent Yel grain	54.58
Soybean meal, 46.5%	19.54
Alfalfa meal dehy 17% CP	12.34
Vegetable oil	6.78
Limestone, ground	3.35
Calcium phos. dibas	2.15
Salt, Iodized	0.43
Mineral Mix - NRC	0.25
Vitamin mix - NRC	0.25
DL-methionine 99%	0.23
Choline chloride	0.101

Table A.6 Nutrient analysis of experimental molt and post-molt diets. Analysis performed at the University of California, Davis analytical laboratory. Values are reported on a 100% dry matter basis. All values represent a percentage with the exception of sodium content which is reported in ppm.

Diet	Dry Matter[1]	ADF	Nitrogen (Total)	Protein	TDN	Crude Fat	Ash	Phosphorus (Total)	Calcium (Total)	Sodium[2]
Molt Diet	91.5	18.9	1.69	10.5	61.4	2.95	5.18	0.454	0.689	1387
Post-molt Diet	93.0	8.3	2.75	17.2	68.6	9.57	10.78	0.757	2.206	2291

[1] All results reported on a 100% dry matter basis.

[2] ppm reported

Appendix 4

qRT-PCR Primers

Table A.7 Primers used to study the expression levels of pro- and anti-inflammatory cytokines. The genes GAPDH and RPL13 were used as housekeeping genes for all studies.

Target Gene	Forward Primer Sequence (5' - 3')	Forward Primer Concentration (nM)	Reverse Primer Sequence (5' - 3')	Reverse Primer Concentration (nM)	Annealing Temperature
GAPDH	GGTGGTGCTAAGCGTGTTAT	400	ACCTCTGCCATCTCTCCACA	400	59
RPL13	GGAGGAGAAGAACTTCAAGGC	300	CCAAAGAGACGAGCGTTTG	300	58
IL-1β	CTACACCCGCTCACAGTCCT	400	GAGCTTGTAGCCCTTGATGC	500	59
IL-6	GCTTCGAC GAGGAGAAATGC	400	GCCAGGTGCTTTGTGCTGTA	500	60.5
IL-8	GGCTTGCTAGGGGAAATGA	400	AGCTGACTCTGACTAGGAAACTGT	400	60.5
TNF-α	CGCTCAGAACGACGTCAA	400	GTCGTCCACACCAACGAG	400	56
IL-10	CGGGAGCTGAGGGTGAA	500	GTGAAGAAGCGGTGACAGC	500	59
TGF-β	CGGGACGGATGAGAAGAAC	500	CGGCCCACGTAGTAAATGAT	400	58