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Characteristics and Effects of Muscular Dystrophy in Broiler Chickens

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BY

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Introduction

Muscular dystrophy (MD) is a degenerative muscle condition in animals, including humans and chickens. The condition can be described as a group of inherited diseases that leads to the weakening of the skeletal muscle over time (Matsumoto et al., 2007). While the disease was once thought to be inherited as an autosomal recessive trait (Julian 1973), it is now believed to be transmitted co-dominantly by a single gene (Matsumoto et al,. 2007). Therefore, if both parents were heterozygous for this trait, then only a fourth of their offspring would exhibit traits of the disease. However, they now have evidence to believe that co-dominance is occurring, which says that heterozygotes should exhibit traits of the disease, so over half of the offspring produced from heterozygous parents would also. The exact gene to cause muscular dystrophy is not yet known, but it is believed that the *WWP1* gene may be responsible (Hirokazu et al., 2008). Therefore if the WWP1 gene exhibits co-dominance, even if the parent chickens are heterozygous

The GGA2q region, found on the AM locus, may contain genes that cause abnormal muscle in chickens resulting in muscular dystrophy (Matsumoto et al., 2007). Although all of the genes found in the GGA2q region may, in some way, contribute to muscular dystrophy in chickens, there is evidence that points to the *WWP1* gene as the cause. *WWP1* is an E3 ubiquitin ligase found in the ubiquitin-proteasome pathway, which is used to target short-lived proteins for

degradation in eukaryotes. E3 ligases recognize and catalyze ubiquitin conjugation to specific protein substrates, like those found in muscle protein. *WWP1* is also responsible for other important cellular function like RNA splicing, transcription, and the cell division cycle (Hirokazu et al., 2008). One study found that a missense mutation, found only in dystrophic chicks, interfered with transcription, which led to an amino acid replacement in which arginine was switched to glutamine during translation. Because the ubiquitin-ligase pathway is used in important cellular functions like cell division, mutations in this region of the *WWP1* gene could lead to uncontrolled cellular growth, like in dystrophic chickens with enlarged pectoral muscles. Mutations in the *WWP1* gene could also lead to aberrant regulation of muscle protein by the ubiquitin-ligase pathway, resulting in an accumulation of muscle protein that should have been degraded. This could result in the muscle conditions found in dystrophic chicken's legs that prevent them from being able to stand (Hirokazu et al. 2008).

Many different phenotypic traits of muscular dystrophy have been found and used to characterize the disease in chickens. Birds with the disease cannot lift their wings and have a hard time raising themselves from flat surfaces when laid on their backs (Julian, 1973). This is caused either by myotonia, the inability to relax voluntary muscles after vigorous effort, or by the interference of the pectoralis, the major depressor muscle of the wing, with the supracoracoideus, the wing's major elevator muscle (Julian, 1973). Other common characteristics include a drooping neck and an enlarged pectoral muscle. The pectoral muscle is the thick, fan-shaped muscle in the center of the chest that may become enlarged due to excess fat in the pectoralis region. Researchers have found that dystrophic chickens have a large amount of lipids in the central zone of their pectoral muscles (Mitchell and Julian, 1971). By comparing surface membranes of pectoral muscles from normal and dystrophic chickens, scientists found

that cells of dystrophic chickens follow different maturation patterns due to elevated enzymatic activity in leucyl beta-naphthlamidase, adenyl cyclase, and guanylate cyclase, which results in proliferative cell changes in chickens with this condition (Malouf et al., 1981). These changes may include a honey comb shaped tubular network continuous with vesicular dilations, an increase in the density of invaginated caveolae, and disarray of the plasmalemma (Malouf et al., 1981). Elevated enzymatic activity, only shown in dystrophic chickens, indicates an abnormal developmental pattern in the dystrophic chicken muscle (Malouf et al., 1981).

Muscular dystrophy is very common among broilers, which are chickens raised specifically for meat production. When dystrophic chickens are mated, or when normal and dystrophic chickens are mated, it is very likely that at least fifty percent of the clutch will end up with muscular dystrophy since it is inherited as a co-dominant disorder (Fujiwara et al., 2009). For my study, eggs were obtained from a local farm, and allowed to hatch. Among these hatchings were chicks who exhibited some traits of muscular dystrophy. It is possible that the parents of the seemingly dystrophic chicks may carry the mutated *WWP1* allele which would cause the chicks to exhibit these traits. Because chickens are useful animal models of disease, it is important to try to understand the mutations in the *WWP1* gene so we can see exactly how it affects the ubiquitin-ligase pathway, and how we can work to reverse the mutations to eliminate the disease all together. Understanding the disease in chickens may help us to further understand muscular dystrophy in humans as well. By taking feathers from the potentially dystrophic chicks and their parents, phenotypic characterization of these chicks was done to confirm diagnosis of muscular dystrophy. Further DNA sequencing for the *WWP1* mutation associated with muscular dystrophy (Hirokazu et al., 2008) was performed to determine whether these chicks had the previously characterized mutation for muscular dystrophy in this gene.

Methods

Collection and phenotyping of chickens

To begin this experiment, chickens were bred at a small chicken farm in Aynor, SC and the eggs were allowed to hatch. After the eggs hatched, the chicks were observed by me, Dr. Lin, and Dr. Barthet, and phenotypic traits, such as color, neck position, leg development, proper eating, and overall health. Phenotypic abnormalities were recorded from the time of hatching until time of death.

A) 

B) 

Figure 1. Phenotype of Potentially Dystrophic Chicks
A) This photo depicts healthy chicks from the first clutch that were able to eat and walk on their own. B) This picture depicts a chick from the same clutch believed to have muscular dystrophy. Unlike the baby chick on the left, the potentially dystrophic chick has severely bent legs, drooped neck, and did not feed well.

Dissections

Chicks that displayed signs of muscular dystrophy based on phenotype died within one week of hatching (Figure 1), and were subsequently dissected to determine musculature. Muscle tissues were taken from the legs, pectoral region, and stomach, and the brains were removed to look for any evidence of other types of other conditions that may cause the same phenotypic traits as muscular dystrophy. For example, a connective tissue disorder or pathological changes can cause some of the same phenotypic traits, such as the drooping neck and inability to walk (Rigdon et al., 1962). The brains were removed to look for any signs of cancerous lesions that could produce similar phenotypic traits to those produced by muscular dystrophy. For further study, histological work was planned so microscopic analysis of the muscle samples could be done to look for other signs of muscular dystrophy. However, that was not done for this paper. Following the dissections, pictures of the dissected bodies were taken (Figure 2). A few days later, the live, healthy chicks were labeled based on color and observations of their behavior and health continues to ensure that no signs of muscular dystrophy develop.

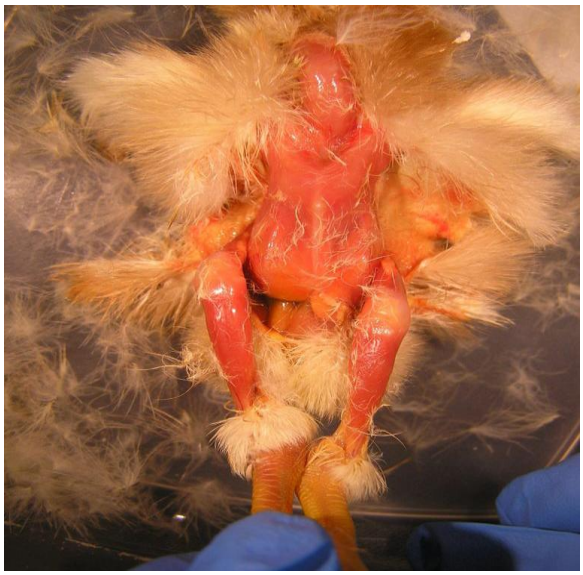

Figure 2. Dissection of Chick with Possible MD
Dissection of the potentially dystrophic chicks occurred post-mortem. The feathers were pulled off and then the skin was cut, so muscle and brain samples could be obtained. Nothing observed from the dissection showed any obvious signs of MD.

Phenotypic Characterization of Potential Parents

Photographs were taken of the healthy chicks and mature adult chickens (Table 1) to determine any phenotypic relationship between affected and unaffected individuals and parentage. Feather samples from the dystrophic and healthy chicks were collected for DNA and RNA analyses. These analyses would be used to look for alleles such as WWP1 (Hirakazu 2008) that are known to be present in muscular dystrophy. This would allow us to confirm that the dead chicks suffered from muscular dystrophy. Three other clutches were hatched, and the phenotypes of these chicks were recorded and used to compare to the chicks of the first clutch.

Table 1. Characteristics of the Possible Parents of the Dystrophic Chicks
The table lists possible parents of the first clutch of chicks that hatched and the phenotypes of each.

Chicken Label #	Sex-Breed	Coloration	Health	Overall Phenotype
K-1 (yellow, half circle shaped)	Rooter- Rhode Island Red Mix	Brownish feathers, some black	Mostly healthy, just blind in one eye	Triple cone; blind in one eye; most likely not the father
K-2 (green, key shaped)	Rooster- ?	Black and white feathers; yellow feathers on top	Seemingly healthy	Single cone, grey and white feet
K-3 (red, key shaped)	Rooster- mixed breed	Mostly black, some red	Healthy	Only black male--> may be father but he is not the alpha male
K-4 (blue, key shaped)	Rooster- ?	Brownish red	Healthy	Large cone; white legs and pink feet; alpha male
K-5 (pink, ring shaped)	Hen- lays white eggs so probably not Rhode Island red	Black, white spot on back	Healthy- but chunk taken out of head and some damage to feet (may be due to fighting?)	White feet; scarring on head; has laid a green egg but usually white eggs
K-6 (green, ring shaped)	Hen- Rhode Island Red	Dark brown, black, and a little red	Healthy	Small cone, white feet, not as much on feet as K5
K-7 (orange, ring)	Hen- Rhode Island Red	Mostly black with some brown and red feathers	Healthy	Small cone, white feet, younger
K-8 (yellow, ring)	Hen- Rhode Island red	Brown but mostly red	Missing a lot of feathers on her back	Golden/ yellow feet; may be main mom
K-9 (yellow, key cut in half)	Hen- ?	Mostly black but some brown and red	Healthy	White feet; fairly large
K-10 (red, key cut in half)	Hen- ?	Black- only one with solid black and no other colors	Healthy	Mostly black feet but some yellow
K-11 (tape placed on nail)	Hen- mixed breed	Black but brown towards bottom of hen and on its chest	Healthy	Younger, lays tiny eggs, golden legs, more brown on base of feet

DNA purification

The QIAamp DNA Mini Kit (Qiagen, Valencia, CA) was then used to purify the DNA. Twenty-five milligrams of tissue sample, whether feather or brains, was ground under liquid nitrogen. To obtain adequate feather samples, a feather was plucked from the chickens, and the piece at the end of the feather, closest to the root, was cut off and used for grinding. The tissue was then lysed using Buffer ATL (Qiagen, Valencia, CA), then placed in a QIAamp spin column (Qiagen, Valencia, CA) so the DNA could bind to the membrane. After the DNA was washed through the membrane and into a sterile microcentrifuge tube, a 1:50 dilution was placed in a spectrophotometer set at a wavelength of 260/280 to determine the concentration of each DNA sample.

PCR for sick chicks DNA

Three chicks who displayed the most severe signs of muscular dystrophy, as characterized by the mentioned phenotypic traits (chicks B, C, and D), were used for DNA analysis. PCR reactions were used to amplify the potentially dystrophic chick's DNA (Table 3). The first PCR reaction was done using WWP1s-F1 and WWP1s-R1 primers for the set of reactions and WWP1s-F2 and WWP1s-R2 primers for the second set of reactions. These reactions were used to amplify the WWP1 region (Figure 3) of the 3 sickest baby chicken's DNA. However, when the PCR reactions were run on a gel the product was too big. For the second PCR reaction the exact location of the mutation in WWP1 was found and this region was amplified. To do this the primers WWP1-mF and K2ReverseMD primers were used. PCR was done using a Bio Rad C100 Touch Thermal Cycler and conditions were set at 95ºC for 2 minutes, 94ºC for 30 seconds, 50ºC for 1 minute, 65ºC for 50 seconds, and the final was 65ºC for 10 minutes. This PCR reaction was

run for 50 cycles. A gel was run to make sure the PCR product was the right size, and then these products were then purified using the QIAgen PCR clean up kit.

Table 2. Primers Used on Potentially Dystrophic Chicks
In this table are the primers used for the PCR reactions done on the potentially dystrophic chicks.

Primer	Primer Direction	Sequence
WWP1s_F1 (Hirakazu 2008)	Forward	aggctccacatgggcagaactttgtc
WWP1s-R1(Hirakazu 2008)	Reverse	tcaaataggcagtacatagggttcag
WWP1s-F2	Forward	acttgctcatttccgttacttgtgtc
WWP1s-R2	Reverse	ttgaagattacctaacatcctcgtgg
WWP1-mF (Hirakazu 2008)	Forward	agagaaaatgagctatgcagtattac
Kay2RevMD	Reverse	taccacctgtact

Sequencing for sick chick DNA

Fifteen microliters of the purified PCR sample was sent to the University of South Carolina to be sequenced. Once the sequences came back, the chick's DNA were aligned to the WWP1 gene using Discovery Studios 3.5 Visualizer.

Results

In the first clutch, 6 out of 12 chicks displayed signs of muscular dystrophy. All six died within a week of hatching. The healthy chicks were livelier and had to be held so they wouldn't run away, while the sick chicks exhibited severe signs of muscular dystrophy. These individuals were unable to walk, had limp necks, and a poor appetite. By phenotyping chicks in other clutches we also found that over half the amount of chicks of each clutch displayed signs of muscular dystrophy as characterized in the chicks of the first clutch. Looking at the potential parents (Table 1) we found that the K3 rooster was the one that was most phenotypically similar to the chicks that were born, but he did not display any obvious signs of muscular dystrophy. While some of the hens displayed scarring and others were not of good health, none of them

displayed any significant signs of muscular dystrophy to lead us to believe they may be the mother of the chicks.

Products of about 500 bases were produced after PCR amplification of the WWP1 region was done using the WWP1-mF and Kay2Reverse primer pairs. Products were resolved using a 1% agarose gel (Figure 3).

In the alignments of the potentially dystrophic chick's DNA we found a mutation in the intron region of the DNA sequence at position 24,541 in the alignment (Figure 4).

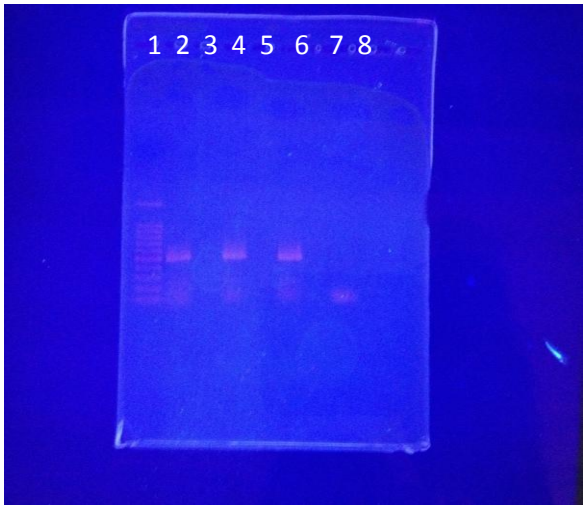

Figure 3.PCR product of the amplified *WWP1* region
The picture depicts the gel for the PCR product for the DNA of the chick's that had the most severe symptoms. In lane 1 is the Promega 100bp ladder, lane 2, 4, and 6 contains the DNA of the chicks, and in lane 8 is the control. Primers WWP1-mF and K2ReverseMD were used to produce a product of about 500 bases, which is what is shown here.

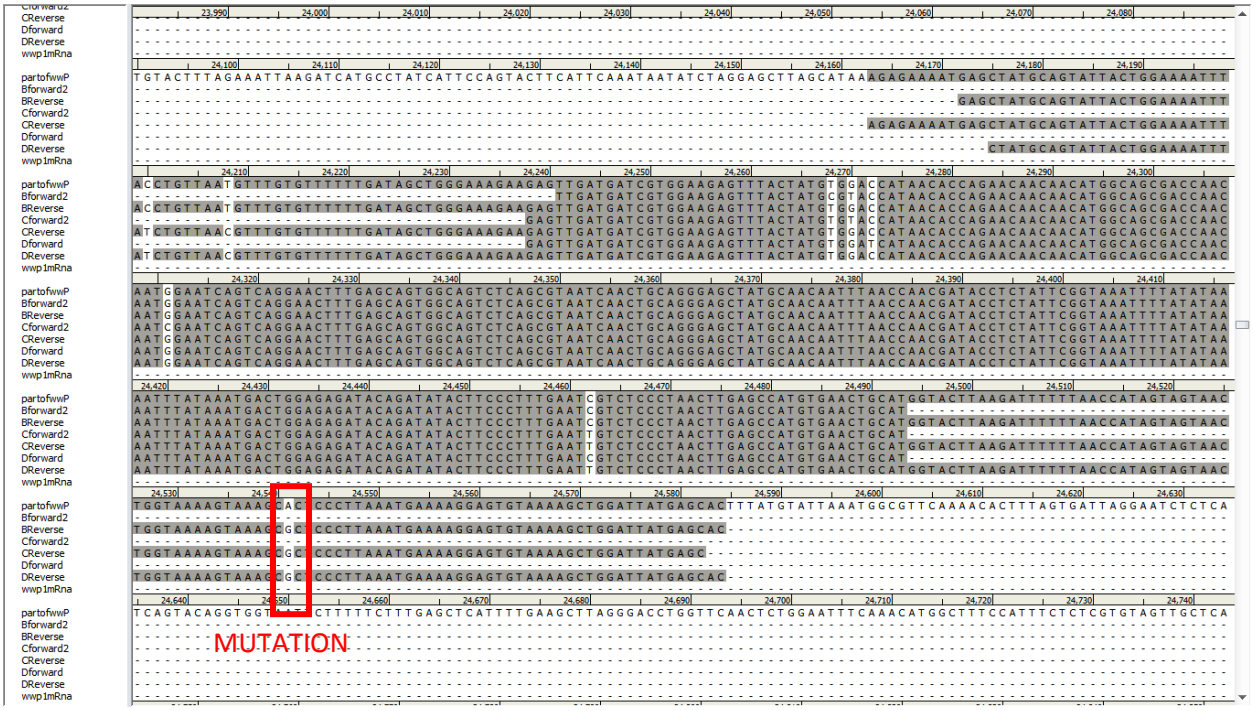

Figure 4. Alignment of Potentially dystrophic chick's DNA and the WWP1 gene
Each of the three potentially dystrophic chick's DNA was aligned to the wild type of the WWP1 gene from Pubmed (Pubmed, Bethesda, MD). The first line is the *WWP1* gene, the next six lines are the forward and reverse sequences of each chick. After aligning the *WWP1* mRNA we found that the mRNA aligned with the wild-type gene up until base 24,491 and did not align with the *WWP1* gene again until hundreds of bases later. This indicated that there was a mutation in the intron region of the gene. There were some other misalignments in the DNA; however, this was most likely due to misreading of the sequences.

Discussion

By phenotyping chicks of the first clutch, along with the three other clutches, we have found that at least half the chicks of each clutch exhibit signs of muscular dystrophy, like the drooping neck and bent feet. This suggests and supports the theory that muscular dystrophy found in chickens is inherited as a co-dominant disorder. At first we thought that the K3 rooster was the parent; however we found that when the K1 rooster was taken out of the barn, none of the other chicks hatched after he was removed have displayed any signs of muscular dystrophy.

For further study we plan to sequence the DNA of the all the rooster and hen sample we have and see if they carry the new mutation found in the potentially-dystrophic chicks.

Using primer pairs WWP1-mF and Kay2Reverse, we expected to get a product of about 500 bases, which was confirmed in the agarose gel (Figure 3), so we know that the correct portion of the DNA was amplified and sequenced. After aligning these sequences to the *WWP1* gene we found that there is a mutation in the intron region of the DNA found in at least three of these chicks, however it is not the same missense mutation found previously from other research (Hirokazu et. al., 2008). Because the mutation was found in an intron, we cannot say this mutation will affect the phenotypes of the chicks for certain; however, each chick with very severe cases of muscular dystrophy carry this same exact mutation, which suggests that we may have found a novel mutation that could cause the disease as well.

If this truly is a new mutation that causes muscular dystrophy, we could look and see how this mutation affects the ubiquitin-ligase pathway. This may explain why some of the other phenotypic traits associated with muscular dystrophy develop, such as the large hematomas found on the chicks leg. The causation of these traits was not explained in any of the studies I read about for this paper. We could also use this new discovery to begin looking at what happens in the WWP1 gene to cause these two very distinct mutations, and how these different mutations can cause the same phenotype. By furthering my research with the histological work and more sequencing, hopefully I can provide more evidence that this really is a novel mutation worth looking into.

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