An Assessment of the Molecular Basis of Toxin-induced Dilated Cardiomyopathy in an Avian Animal Model

By

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ABSTRACT

Dilated cardiomyopathy (DCM), a disease of the myocardium, causes morbidity and premature death in humans and other domestic animals including turkeys. Though DCM results from many different factors including those that are unknown or idiopathic, genetic factor is a major cause of idiopathic DCM. In this study, I assessed the molecular basis of toxin-induced DCM in turkeys by evaluating the association and effect of mutations in candidate genes in the nucleus and mitochondria on the incidence and severity of this disease. Echocardiographic measurements at 3 weeks of age showed that birds on furazolidone-containing diet exhibited a significant DCM phenotype (increased left ventricular end diastolic dimension and left ventricular end systolic dimension) with a marked decrease in the left ventricular shortening fraction. Pathological phenotype confirmed the dilated heart with extended cell necrosis. Two mutations, both in NADH dehydrogenase genes, were found to be associated with DCM. Real-time RT-PCR quantification indicated that mRNA expression of alpha cardiac actin gene (*ACTC*) were significantly different between control and treatment birds. While *ACTC* expression increased, though moderately, in control birds from week 1 to 3, it decreased significantly in treatment birds. These findings suggest that the mitochondrial DNA variation and *ACTC* expression may be associated with

the turkey's response to toxin. Therefore, further research is needed to investigate the molecular mechanism of toxin-induced DCM in the turkey.

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Chapter 1

INTRODUCTION

Native to North America, turkeys were first domesticated more than 2,000 years ago in Mexico and South America. Today, the domestic turkey, *Meleagris gallopavo*, has become the fourth most valuable agriculturally important animal commodity in the United States of America (USA). Annually, about 250-300 million turkeys are raised for meat in the USA, mostly for Thanksgiving meals. Since 1970, turkey consumption in the USA increased by about 108 percent with the average consumed per capita at 16. 9 pounds in 2007 (A farm sanctuary research report, 2007).

As demand for turkey meat continues to grow, concerns about infectious and genetic diseases also rise. In addition to their effect on efficiency of meat production, annual costs for disease prevention through vaccination and treatment in the USA involve billions of dollars (Mendoza et al., 2008). One disease which poses a major problem to the turkey industry, especially in the USA, is dilated cardiomyopathy (DCM). DCM is a prevalent syndrome in some commercial turkeys which leads to depressed growth rates and sudden death. Clinically, DCM is characterized by dilated ventricles and cardiac muscle hypertrophy. Affected birds present significant weight loss by 2 to 4 weeks of age. It has been estimated that deaths due to DCM in turkeys could occur as late as 14 weeks of age though the highest incidence, at a rate of 2-4%, occurs between 2 and 3. In some turkey populations in central Utah, the morbidity rate from DCM has been reported to be as high as 40-50% (Reed et al., 2007). Thus DCM can have a marked economic impact on turkey production.

DCM also affect other species including humans (Zifa et al., 2008), dogs (Oyama and Chittur, 2005), mice (Du et al., 2007), and cattle (Nart et al., 2004). Economic losses due to DCM therefore extend to many different livestock and poultry species, making it a major concern in animal agriculture. In humans, deaths due to DCM exceed 10,000 annually (Bowles, 2002).

The myopathic turkey heart has many features similar to that in human DCM including pathophysiological and biochemical characteristics such as gross morphology, myocardial energetics, Calcium metabolism, and the beta-receptor-adenylyl cyclase signaling system (Hajjar et al., 1993; Genao et al., 1996). The turkey can thus be used as an avian model to understand DCM in humans.

Factors responsible for the incidence and severity of DCM are variable. In some cases of DCM known as idiopathic, the cause is unclear or without a consensus. However, factors that are known to cause or contribute to DCM include genetic/familial (Zifa et al., 2008), viral infection (Knowlton, 2008), auto-immune disease (Portig et al., 2006), or environmental (Mayosi and Somers, 2007). It has been estimated that almost 20 to 30% of idiopathic DCM cases have a genetic/familial component (Kelly and Strauss, 1994; Grunig et al., 1998). Feeding a high concentration of furazolidone (700 ppm) leads to DCM similar to spontaneous DCM (Genao et al., 1996).

Genetic factors known to cause DCM include mutations in the mitochondrion DNA (mtDNA). Variations in the mtDNA have been implicated in DCM in which there are changes in myocardial protein and cardiac energy metabolism (Marin-Garcia et al., 2000). In the heart, mitochondria play an important role in energy generation and thus in contractile

function, and disturbances in these functions may lead to DCM. The metabolic pathways affected include fatty acid oxidation, mitochondrial respiratory chain and oxidative phosphorylation (OXPHOS). Diverse studies have indicated that some mitochondrial DNA (mtDNA) mutations may cause myocardial dysfunction (Kelly and Strauss, 1994; Marin-Garcia and Coldenthal, 1998). Also, there is increasing evidence from mouse transgenic models that disruptions in mitochondrial bioenergy due to mutations at specific loci can cause cardiomyopathy and cardiac failure (Zhang et al., 2000).

Like those in the mitochondria, genes in the nucleus have been shown to influence the incidence and severity of DCM. A significant fraction of these nuclear genes code for sarcomeric proteins. For example, α-cardiac actin gene (*ACTC*) has been reported to be linked to the development of DCM in both humans and other animals. As a component of thin filament, actin is vital to cell motility, transport, and cytoskeletal integrity (Rutkevich et al., 2006). It can polymerize into long helical filaments which form parts of the supporting cytoskeleton of the cell cortex in the heart. Given actin's important functions, mutations in the gene have been shown to influence physiological activities (Spudich, 2001). Olson et al (1998) reported that two mutations (G867A and A1014G) in the human *ACTC* were associated with the development of DCM.

In this thesis research, I hypothesize that *ACTC* and mitochondrial tRNA genes affect the incidence and severity of DCM in turkeys. To test this, I will assess the molecular basis of toxin-induced DCM in turkeys by evaluating the association and effect of mutations in candidate genes in the nucleus and mitochondria on the incidence and severity of this disease. The research will involve two specific objectives:

1. To evaluate the associations, if any, between mutations in the mitochondrial tRNA genes and DCM.
2. Use real-time RT-PCR to examine the differences between normal and furazolidone-induced affected turkey poults in mRNA level of α-cardiac *actin*.

Defining the expression and function of disease genes that underlie DCM would facilitate the development of new diagnostic and therapeutic tools for this severe disorder.

CHAPTER 2

LITERATURE REVIEW

Introduction

Dilated cardiomyopathy (DCM) is a heart muscle disease characterized by impaired myocardial contractility and ventricular dilatation. In some commercial turkeys, DCM is a prevalent syndrome (Roberson, 2005). Turkey DCM has many features similar to those observed in human DCM (Biesiadecki and Jin, 2002). In many cases of DCM, the cause is little understood. It has been shown that point mutations in the mitochondrial DNA (mtDNA) transfer ribonucleic acid (tRNA) genes have been associated with DCM (Marin-Garcia et al., 2000; Grasso et al., 2001; Mahjoub et al., 2007). Also, more than 10 candidate genes that encode sarcomeric proteins have been implicated including alpha cardiac actin gene (*ACTC*) (Morimoto, 2008). This thesis will involve an assessment of the role in toxin-induced DCM in turkeys of tRNA genes of the mitochondrial genome and a sarcomeric protein gene, actin.

2.1 Turkeys

Turkey, *Meleagris gallopavo,* native to North America, is a large poultry bird raised for food. The modern turkey, a descendant of the wild turkey, was domesticated in southern Mexico and brought to Europe in the 16th century. They can be found in most of the eastern United States (US) and in pockets throughout the western US. Generally, eight subspecies of *Meleagris gallopavo* are recognized: Broad-breasted White, Broad-breasted Bronze, Standard Bronze, Bourbon Red, Blue slate, Spanish Black, Narragansett Turkey, Chocolate Turkey,

Beltsville Small White, and Midget White Turkey (Eaton, 1992). The Broad-breasted White is believed to be the progenitor of commercial turkeys used primarily for large scale production. The choice of this variety is because it has more breast meat and are traditionally consumed as the main course at large feasts including Christmas and Thanksgiving in the USA and Canada.

Turkey consumption has increased by about 108% since 1970. In 2006, United States turkey grower raised 261.9 million turkeys and the average American ate 16.9 pounds of turkey. According to a survey by the National Turkey Federation, about 97% of Americans eat turkey at Thanksgiving Day. The increased population of turkey as a lunch meal has also contributed to the increased production. With the increased production has emerged concerns about diseases and other health problems. Both infectious and genetic diseases significantly affect the efficiency of poultry production. In commercial turkeys, dilated cardiomyopathy is prevalent in poults and causes growth retardation, poor blood circulation and death due to heart deformation (Roberson, 2005). The turkey has been reported as an effective animal model for human heart failure (Wu et al., 2004).

2.2 Types of Cardiomyopathy

In cardiomyopathy, the heart muscle becomes enlarged or abnormally thick or rigid, which causes cardiac dysfunction and ultimately heart failure, arrhythmia, and sudden death. It is a significant cause of morbidity and mortality in both children and adults and is reported to be the most common reason for cardiac transplantation. In the USA, the average annual cost and mortality rate due to cardiomyopathy are estimated to be about $200 million and

over 10, 000 deaths, respectively (Bowles and Bowles, 2004). In terms of etiology and pathophysiology, there are four main types of cardiomyopathy: hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), restrictive cardiomyopathy (RCM), and arrhythmogenic right ventricular dysplasia/ cardiomyopathy (ARVD/C) (Richardson et al., 1996).

Hypertrophic cardiomyopathy is a common autosomal dominant genetic disease of the cardiac sarcomere which, in humans, may afflict as many as 1 in 500 individuals (Maron et al., 1995). HCM occurs when the heart muscle thickens abnormally. It is clinically characterized by the presence of unexplained left ventricular hypertrophy (Mogensen et al., 1999; Lombardi and Betocchi, 2002; Ramirez and Padron, 2004). Pathological examination of tissues in HCM-affected individuals reveals characteristic myocyte hypertrophy, disarray, and replacement fibrosis. This type of cardiomyopathy can affect people of any age and is the most common cause of sudden cardiac death in the young and a major cause of morbidity and mortality in the elderly (Marian and Roberts, 2001). It is thought that more than 90% of HCM cases are inherited in an autosomal dominant pattern. Since HCM is a disease of the cardiac sarcomere, it can be caused by mutations in many genes including those that encode for β-myosin heavy chain, cardiac regulatory and essential myosin light chain, myosin binding protein C, α-cardiac actin, cardiac troponin T, cardiac troponin I, troponin C, titin and α-tropomyosin (Marian and Roberts, 2001). To date, HCM has been associated with over 200 mutations in sarcomeric protein genes. Among these, mutations in the genes for β-myosin heavy chain and myosin binding protein C are reported to account for 70% of HCM (Murphy and Starling, 2005).

Dilated cardiomyopathy, which affects both humans and livestock, is the most frequent among cardiomyopathies. It accounts for about 87% of all cases and is the leading cause of heart failure and sudden death (Gregori et al., 2008). The age of onset of this progressive disease in humans varies between 3 and 7 years of age (Broschk and Distl, 2005). Also called congestive cardiomyopathy, DCM can affect the chambers of the heart by weakening the walls. The phenotype can be characterized by an isolated cardiac dysfunction (isolated DCM) or include conduction defects (atrioventricular block or sinus node dysfunction) and/or skeletal muscular disorders (Villard et al., 2005). Despite its importance to both biomedicine and agriculture, its aetiology is still poorly understood so far.

DCM can be spontaneous or inducible. Spontaneously occurring DCM in dogs, like HCM in cats, is a common disease that is underutilized as models of human cardiac disease (Freeman and Rush, 2007). In turkeys, early deaths of toms and hens due to spontaneous DCM is about 1% and 4%, respectively (Zepeda and Kooyman, 2002). In order to provide further insights into the pathophysiology and progression of DCM, furazolidone is widely used to induce DCM (Wu et al., 2004; Lin et al., 2007). After receiving furazolidone for 2-3 weeks, turkeys develop cardiomyopathy and the myopathic heart is similar to that in humans in terms of gross morphology, myocardial energetics, Calcium metabolism, and the beta-receptor-adenylyl cyclase signaling system (Hajjar et al., 1993; Genao et al., 1996). Turkey is thus a good model for us to evaluate the pathogenesis of human DCM.

Restrictive cardiomyopathy is a rare heart muscle disorder that is characterized by impaired ventricular filling and reduced diastolic volume in the presence of normal systolic function and/or near normal myocardial thickness (Richardson et al., 1996). While RCM

shares many features with HCM, in RCM hearts, the walls of ventricles become stiff but not necessarily thickened. It can be classified as either primary or secondary (Bruce, 2005). Primary RCM includes endomyocardial fibrosis, Loeffler's endocarditis, and idiopathic RCM. Idiopathic primary RCM is often characterized by skeletal myopathy with an autosomal dominant mode of transmission (Hughes and McKenna, 2005). Secondary RCM is more common and is usually associated with other diseases including amyloid disease, scleroderma, Churg-Strauss syndrome, Gaucher's disease, Noonan's syndrome, reactive arthritis, Fabry's disease, and endomyocardial pathology with or without hypereosinophilia (Langlard, 1998; Stollberger and Finsterer, 2007). RCM can also occur as a result of radiation treatments, infections, or scarring after surgery. This type of cardiomyopathy leads to heart failure with shortness of breath and fluid accumulation in tissues (edema). Chest pain and fainting are less likely than in HCM, but abnormal heart rhythms (arrhythmias) and increased awareness of a change in the heartbeat are common.

In humans, arrhythmogenic right ventricular cardiomyopathy or ARVD/C, also known as arrhythmogenic right ventricular dysplasia, is an inherited disease that affects approximately 0.002 to 0.004%. It is a major cause of sudden cardiac death and ventricular tachyarrhythmias in young and apparently healthy individuals including athletes (Wichter et al., 2005; Thiene et al., 2007; Awad et al., 2008). It is a clinical and pathological condition whose diagnosis relies on electrocardiographic and angiographic criteria (Moric-Janiszewska and Markiewicz-Loskot, 2007). ARVD/C is characterized by replacement of cardiomyocytes primarily in the right ventricle, and by fibro-fatty tissue (McKenna et al., 1994; Basso et al., 1996). The disruption of normal myocardial architecture in ARVD/C can result in severe

right ventricular (RV) dysfunction, life-threatening arrhythmias, and sudden death. Since ARVD/C has no cure, the primary treatment is to cure on prevention of secondary complications especially lethal ventricular tachyarrhythmias.

Unclassified cardiomyopathy is a category reserved for cardiomyopathies that do not fit the four types of cardiomyopathies discussed above. Major advances in diagnostic technology including echocardiography and Doppler ultrasound, magnetic resonance imaging, and radionuclear techniques have significantly helped in the clinical identification of the different forms of cardiomyopathies as well as discriminating among their etiologies.

2. 3 Dilated Cardiomyopathy in Animals

It has been reported that DCM can affect a variety of species including humans (Zifa et al., 2008), turkeys (Lin et al., 2006), dogs (Oyama and Chittur, 2005), mouse (Du et al., 2007), and cattle (Nart et al., 2004). The incidence of this disease in diverse animals implies a rich source of models to study both its pathophysiology and to evaluate new therapies. None of these can perfectly reproduce the human disease, but investigators can choose the model that best meet the specific aims and resources available for their research.

In the USA, the annual incidence of DCM in humans has been reported to be at least 5.5 cases per 100, 000 with a prevalence of 36 cases per 100, 000 (Cooper, 2005). DCM can develop at any age but is more common among adults 20 to 60 years of age, and about 10% in people older than 65. The disease occurs about 3 times more frequently in men than women and affects more African-Americans than Caucasians (Coughlin et al., 1997). The usual signs and symptoms of DCM include tiredness, shortness of breath, swollen ankles and

abdomen, heart palpitation, dizziness, and fainting during physical activity. However, some individuals with DCM may not have any clinical symptoms or signs, and can only be identified by diagnostic testing (Ku et al., 2003). Often, the best way to identify DCM–affected individuals is by echocardiography, which is an ultrasound test that produces images of the heart (Ku et al., 2003).

In addition to humans, there is also a high instance of spontaneous DCM in other species including turkeys and dogs. In turkeys, spontaneous DCM, also known as round heart (RH) disease, is characterized by dilated ventricles and cardiac muscle hypertrophy (Reed et al., 2007). Though deaths occur up to 14 weeks of age, the highest incidence occurs between 14 and 21 days of age. The morbidity rate for DCM in some turkey populations in central Utah has been reported to reach to 40–50% between hatch and four weeks of age (Reed et al., 2007). In commercial turkeys, DCM is a prevalent circulatory problem which can result in severe economic losses to producers (Fatkin and Graham, 2002). The naturally occurring turkey DCM can be mimicked by feeding a toxin, furazolidone [N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone] in a high concentration (700 ppm) for 2-3 weeks. Furazolidone was previously used extensively in commercial turkey starter diets to treat and reduce mortality from non-specific enteritis in poults. It is now known that high dietary levels of furazolidone is toxic to poults and is associated with cardiac dilation, ascites, and occasionally causes high mortality rate (Staley et al., 1978). However, the mechanism of the production of cardiomyopathy by furazolidone is still unknown. The myopathic turkey heart has many features similar to human DCM including pathophysiological and biochemical characterization such as gross morphology, myocardial energetics, muscle

physiology myofilament properties, Ca^{2+} metabolism, and the beta-receptor-adenylyl cyclase signaling system (Hajjar et al., 1993; Genao et al., 1996). These similarities make the turkey a promising model to provide new insights into the pathophysiology and progression of DCM.

In addition to turkeys, several large dog breeds including Doberman Pinschers, Newfoundlands, Boxers, Great Danes and Irish Wolfhounds show high prevalence of DCM. It has been reported that Doberman Pinschers are the most commonly affected of dog breed (Monnet et al., 1995; Hammer et al., 1996). The age of onset of this progressive disease in dogs varies between 3 and 7 years old. A juvenile form of DCM has been found in Portuguese Water Dogs and Doberman Pinscher Dogs (Broschk and Distl, 2005). There also seems to be a sex predisposition as male dogs are affected more often than female dogs. Dogs with DCM may have symptoms that include cough, depression, dyspnoea, weight loss, panting syncope, and polydipsia. The duration of the clinical signs before presentation is often very short, typically one to two weeks (Tidholm et al., 2001). Usually, dogs are used for breeding before the disease becomes apparent. The high prevalence of DCM in specific breeds of dogs suggests a genetic cause. Though no causal mutations have yet been identified, an autosomal dominant inheritance was found in a family of Doberman Pinschers and in English Cocker Spaniels (Hammer et al., 1996). Within each breed, DCM has unique characteristics and between breeds, it is probably a genetically heterogeneous disease.

Mice, compared to the other rodents, have been the most widely used in cardiovascular research because they are relatively less expensive and easier to handle. This preference for mice is due in part to the observation that 99% of human genes have murine orthologs (Recchia and Lionetti, 2007). Furthermore, mice have a high reproductive rate and

are suitable for selection of genetically modified individuals in a relatively short time. To date, a large number of mouse models genetically engineered for DCM has been developed. These transgenic models and knock-in mice have been used to analyze the functional consequences of DCM causing genes for sarcomeric protein mutations (Lombardi et al., 2008; Du et al., 2007).

In cattle, DCM with systemic circulatory failure due to cardiac insufficiency has been reported in many countries (Furuoka et al., 2001). There are at least three different types of bovine DCM reported: DCM in cattle of Canadian Holstein origin, cardiomyopathy in Japanese Black cattle and cardiomyopathy in Hereford cattle (Leifsson and Agerholm, 2003). The clinical signs and circulatory effects associated with DCM in cattle are reported to include oedema, hepatic and pulmonary lesions (Furuoka et al., 2001). Like mice and turkeys, hearts from bovine DCM display important clinical and biochemical similarities to human DCM. Thus, cattle could also be a possible animal model for understanding DCM in human.

2. 4 Causes of DCM

The etiology of DCM is multifactorial, and many different clinical conditions can lead to the phenotype. Sometimes, the cause of DCM is unknown, which is reported as idiopathic DCM. Approximately 20 to 30% of DCM seem to have a genetic component. Studies of hereditary DCM suggest that DCM is a heterogeneous disease (Grunig et al., 1998). Factors that have been known to cause or contribute to DCM in some specific circumstances include genetic/familial (Zifa et al., 2008), viral infection (Knowlton, 2008), auto-immune disease (Portig et al., 2006), or environmental (Mayosi and Somers, 2007). During recent years, it has

been evident that genetic factors play an important role in the etiology and pathogenesis of idiopathic DCM. In about 25% of relatives of patients with DCM, systematic family screening appear to show echocardiographic abnormalities, including DCM and isolated left ventricular enlargement. Moreover, 10-25% of the relatives with left ventricular enlargement will develop clinical DCM within 5 years (Baig et al., 1998). The genetics of DCM have been extensively investigated. According to an increased understanding of the genetic basis of DCM, two changes may occur in DCM at the cellular level: changes in myocardial protein and in cardiac energy metabolism (Marin-Garcia et al., 2000). The mitochondrial DNA and nuclear genes have been implicated in the incidence and severity of DCM. Better knowledge of the genetic basis as well as of other mechanisms that underlie DCM would facilitate the development of new treatment modalities and an increased likelihood of a better outcome in the affected animals.

2.4.1 Mitochondria DNA

The mitochondrion, an important organelle in cells, supplies a significant proportion of the usable energy needed for activities of the heart. The cellular energy is generated by oxidative phosphorylation (OXPHOS), a process associated with respiratory uptake of oxygen and the converting of dietary calories into heat and adenosine triphosphate (ATP). Unlike other intracellular organelles, the mitochondrion has its own DNA, as well as separate transcription and translation systems. In animal cells, the mitochondrial DNA (mtDNA) is a small double-stranded circular molecule that encodes 13 peptides involved in the respiratory chain. The mtDNA also has 22 transfer ribonucleic acids (tRNA) and 2 ribosomal RNAs

(rRNA) that are involved in the regulation of mitochondrial biogenesis (Marin-Garcia et al., 2000). Compared with nuclear DNA, mtDNA is maternally inherited. It has a higher spontaneous mutation rate due to the lack of an effective DNA repair system and the effect of toxic byproducts of OXPHOS--oxygen free radicals. Therefore, it is not surprising that point mutations in mtDNA arise more frequently in somatic cells, which may accumulate with age (Hayakawa et al., 1992).

At present, there is convincing evidence that proper mtDNA functioning is important for normal cellular metabolism. Disruptions of mtDNA function may lead to myocardial dysfunction, metabolic disorders, degenerative diseases, aging, and cancer (Wallace, 2005). Though the mechanism that underlies these disorders are still uncertain, it has been suggested that ATP deficiency may be responsible. Anomalies in mitochondrial structure affect the activity of the enzyme complexes (I, III, IV or V), causing an impaired OXPHOS that results in reduced ATP production. This is believed to explain the observation that the cells most affected by mitochondrial dysfunction are those found in energy-demanding tissues such as muscles, nerves, and pancreas (Krishnan et al., 2008).

Recently, mutations in mtDNA have been reported to be associated with inherited DCM in humans. There is also increasing evidence from mouse transgenic models that disruptions in mitochondrial bioenergy at specific loci or pathways can cause DCM and heart failure (Zhang et al., 2000). Mitochondrial tRNAs, which play a key role in mitochondrial protein synthesis, may undergo mutations that are intimately involved in the genesis of DCM. Point mutations have been directly associated with cardiomyopathies including $tRNA^{Leu}$ gene (Hsu et al., 2007), $tRNA^{Gly}$ gene (Merante et al., 1994), $tRNA^{Lys}$ gene (Akita et al., 2000),

$tRNA^{Ile}$ gene (Mahjoub et al., 2007), $tRNA^{Glu}$ gene (Van Hove et al., 2007). In addition, mutations in mtDNA tRNA genes of the mitochondrion have been associated with specific respiratory enzyme defects in DCM patients. Grasso et al (2001) reported a T12297C mutation in the mtDNA-$tRNA^{Leu}$ (CUN) of a 36-year-old male patient diagnosed with DCM. Akita et al (2000) reported that an 8-day-old baby girl with a fatal infantile form of hypertrophic obstructive cardiomyopathy also had an A8296G mutation in the mitochondrial $tRNA^{Lys}$ gene. Except mitochondrial tRNA genes, a number of mutations in genes coding for NADH dehydrogenase (ND) subunits and for cytochrome *c* oxidase subunits have been detected. Though Complex I is the largest of the membrane bound complexes and the first enzyme of the respiratory electron transport chain, it is currently the least understood. Mutations found in ND subunits replacing conserved amino acid residues may be responsible for the substrate binding activity of Complex I. Zifa et al (2008) found a novel homoplasmic mutation G3337A in the ND1 gene in a cardiomyopathy patient. Usually, the pathogenic mtDNA mutations are heteroplasmic, with an intracellular mixture of mutant and normal molecules (Wallace, 1994). mtDNA heteroplasmy has been recognized as a promising approach to assess a mutation's pathogenicity.

2.4.2 Nuclear Genes

A number of genes that encode proteins of the cytoskeleton of the cardiac myocyte or the sarcomere (the contractile unit of the cardiac myocyte and extrasarcomeric proteins), have also been identified in humans and turkeys as potential causative factors of DCM (Wiersma et al., 2007). This association implies that DCM is probably a disease of the heart sarcomere.

More than 10 candidate genes that encode sarcomeric proteins, those that form the thin and thick filaments, titin, and Z-discs, have been implicated. These include the genes for α-cardiac actin (*ACTC*), β-mysosin heavy chain, cardiac troponin T, cardiac troponin I, cardiac troponin C, α-tropomyosin, myosin-binding protein C, titin, titin cap, cysteine-rich protein 3, vinculin, and cypher (Morimoto, 2008). In thin filament proteins, a deletion mutation in cardiac troponin T (*cTnT*) residue 210, in two independent families is the first *cTnT* mutation found to be associated with familial primary DCM (Kamisago et al., 2000). Olson et al (1998) reported two missense mutations, R312H and E361G in *ACTC*, were responsible for DCM with apparently favorable prognosis. Both mutations affect universally conserved amino acids in domains of actin that attach to Z bands and the intercalated discs. In thick filament proteins, two mutations S532P and F746L in *MYH7*, were reported by Kamisago et al (2000) in families with early-onset phenotype of DCM. In the *titin* gene, mutation V154M, which has been associated with DCM, decreases the affinity of *titin* Z1-Z2 domains for T-cap/telethonin (Itoh-Satoh et al., 2002). T-cap binds to the N-terminus of *titin* at the Z-disc. Mohapatra et al (2003) reported that a patient with DCM had one missense mutation in muscle LIM protein CRSP3 that resulted in residue lysine 69 substitution for arginine (K69R). Transgenic mice expression cTnT-W141 protein known human DCM mutations in the mouse heart resulted in pathological and physiological defects associated with DCM and thus provided an excellent opportunity to understand the disease pathology.

Actins are one of the most highly conserved proteins, and form the major components of the thin filaments in the muscle sarcomere (Mogensen et al., 1999). They participate in many important cellular functions, including muscle contraction, cell motility, and shape.

This is because actin interacts with a large number of other proteins, including sarcomeric myosin heavy chains (Coumans et al., 1997). The cyclical interactions of actins with tropomyosin and that of the troponin complex with myosins of the thick filament, which are driven by the concomitant hydrolysis of ATP, are vital to the generation of contractile force and contraction in muscle (Boheler et al., 1991). It is possible that any changes in these interactions can alter the function of muscle. There are six isoforms known in the actin family: cardiac, skeletal, vascular, enteric muscle types and two non-muscle types (Takai et al., 1999). The α-cardiac actin is the major actin isoform found in muscle tissues and is a major constituent of the thin filaments of sarcomeres, the contractile apparatus of cardiac myocytes. In addition, actin can also transmit force by mechanically binding adjacent sarcomeres and myocytes in the heart (Gergorio, 1997). In this thesis, the cardiac *actin* gene will be potentially developed and characterized in turkeys.

Elucidation of the genetic basis of heritable DCM might provide new insights into the pathogenetic mechanisms of this disease in most animals.

CHAPTER 3

Mutation analysis of mitochondrial tRNA genes for toxin-induced dilated cardiomyopathy in the turkey (*Meleagris gallopavo*)

3.1 ABSTRACT

Dilated cardiomyopathy (DCM) is a leading cause of heart failure in humans and animals including the domestic turkey, *Meleagris gallopavo*, the fourth most important food animal species. Abnormalities in energy production and/or abnormal protein synthesis due to mutations in mitochondrial DNA may play an important role in the pathogenesis of DCM in the turkey. To evaluate the role of mitochondrial DNA (mtDNA) in DCM, I performed single nucleotide polymorphism (SNP) analysis in 11 tRNA genes including $tRNA^{Ile}$, $tRNA^{Gln}$, $tRNA^{Met}$, $tRNA^{Trp}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Ser}$, $tRNA^{Leu}$ and their flanking regions (2346 bp) of contiguous mtDNA sequences in control birds (n=15) on a normal turkey diet and treatment birds (n=35) on a diet containing toxic levels (700 ppm) of furazolidone. Echocardiographic measurements at 3 weeks of age showed that birds on furazolidone-containing diet exhibited a significant DCM phenotype including increased left ventricular end diastolic (LVEDD) and systolic dimensions (LVESD) with a marked decrease in the left ventricular shortening fraction (SF). Pathological phenotype confirmed the dilated heart with extended cell necrosis. Two SNPs, both in NADH dehydrogenase genes, were found to be associated with DCM. Birds with an AA genotype for the polymorphism at nt-3783 had 32.35% and 41.50% higher LVEDD and LVESD respectively than those with a

GG genotype. The results suggest that though the mitochondrial tRNA variation may not be important in response to toxin in the turkeys, NADH of the mtDNA may be. Further studies are needed to validate the relationship described here between mtDNA polymorphism and left ventricular dilation in response to toxin.

Keywords*:* Dilated cardiomyopathy, Mitochondrial DNA, Single nucleotide polymorphism, turkey

3.2 Introduction

Dilated cardiomyopathy (DCM) is widely accepted as a multifactorial disease with a strong genetic component. Because of the association between energy metabolism in the mitochondria and the heart muscle contraction, mitochondrial DNA (mtDNA) mutations have been reported to contribute to myocardial dysfunction.

Mitochondrion has its own DNA and carries out transcription and translation of its genes independent of the nucleus. Due to the lack of an efficient mtDNA repair system, there is a much higher rate of mutation in the mitochondria than in the nuclear genome (Marin-Garcia and Goldenthal, 1997). In mitochondrial protein synthesis, tRNA genes play an important role in the activity of the respiratory chain complexes, which may be involved in the genesis of DCM. Several point mutations in tRNA genes of the mtDNA have been reported to be associated with DCM in humans. For example, a T12297C mutation in the mtDNA-$tRNA^{Leu}$ (CUN) gene in a 36-year-old male patient was reported by Grasso et al (2001) to be associated with DCM. In another report, an A8296G mutation in the mitochondrial $tRNA^{Lys}$ was observed in an 8-day-old baby girl with a fatal infantile form of hypertrophic obstructive cardiomyopathy but not in normal individuals (Akita et al., 2000).

Although DCM has been shown to be a manifestation of molecular mitochondrial abnormalities, mtDNA's role in turkey DCM has not been previously investigated. To determine whether mtDNA may influence DCM incidence and severity in the turkey, I conducted a SNP analysis of 11 tRNA genes including $tRNA^{Ile}$, $tRNA^{Gln}$, $tRNA^{Met}$, $tRNA^{Trp}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Ser}$, $tRNA^{Leu}$, and their flanking regions (a total of 2346 bp) in poults fed toxic levels of furazolidone from hatch to 3 weeks of age.

As a reference, I also used *in silico* sequences to compare the level of polymorphism that was detected in my *in vitro* analyses to the targeted regions in both chicken (*Gallus gallus*) and human (*Homo sapiens*). Since SNPs are the most common type of genetic variation in the genome (Meyerson, 2003), they can provide a useful tool to perform linkage and association studies between these mitochondrial tRNA genes and DCM in the turkey.

3.3 Materials and Methods

3.3a Animals

Two hundred one-day-old commercial turkey poults (100 each of males and females) purchased from Ag LLC (Harrisonburg, VA) were randomly divided into control (n=100) and treatment (n=100) groups. The treatment group was fed normal diet with toxic levels (700 ppm) of furazolidone (Wu et al., 2004). Both groups were fed *ad libitum* and studied weekly using serial echocardiography in order to determine ventricular dimensions (Gyenai, 2005). Blood samples collected in week 3 from 15 control birds and 35 treatment birds were placed on Flinders Technology Associates (FTA) Classic Cards for mtDNA isolation and initial SNP detection. Heart samples from 10 randomly chosen birds from each of the control and treatment groups were collected in week 4 and fixed in 10% neutral-buffered formalin. In order to validate the echocardiography measurements, the hearts were used for histopathological diagnosis as described below.

3.3b Ultrasonic echocardiography examination

Starting at 2 weeks of age, transthoracic echocardiography was performed weekly using a portable Aloka echo machine (ProSound SSD-4000) with a 7.5-MHz liner interfaced array transducer to examine heart dimensions of both control and treatment birds according to Gyenai (2005). The following parameters were assessed using M-mode image obtained in the parasternal long- and short- axis views (mid-papillary level) of the left ventricle: left ventricular end-diastolic (LVEDD) and end-systolic dimensions (LVESD). Left ventricular shortening fraction (SF) was determined as follows:

%SF= [(LVEDD-LVESD)/LVEDD] x 100 (%), was an important mark of systolic function (Snider et al., 1997).

3.3c Histopathological diagnostics

Following euthanization of birds by electric shock, hearts were obtained and then fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections of 5 µm thickness were stained with haematoxylin and eosin (H&E) according to standard methods (Courtesy of Dr. G. K. Saunders, Department of Biomedical Science and Pathobiology, Virginia Tech). Tissue examination was performed and interpreted by Dr. G. K. Saunders lab according to standard histopathological morphology (Hughes and McKenna, 2005). Characteristic lesions were defined by light microscopy according to Hughes and McKenna (2005). Image acquisition was carried out using a digital camera (Nikon, Japan).

3.3d Molecular analysis

Genomic DNA was extracted from blood samples on FTA cards (Whatman Inc, Florham Park, NJ, USA) following manufacture's recommendation with modification as previously described (Guan et al., 2008). The regions of the sequences of the tRNA genes scanned are defined in Table 3.1. The sequences for these genes span a contiguous region of 2346 bp. Primers were designed to include these genes and their flanking regions using the Primer 3 computer program (http://frodo.wi.mit.edu) and to produce overlapping products used in the resequencing for SNP analyses. The turkey mitochondrial genome sequence used, Accession Number NC_010195, was recently described by Guan et al (2008). The optimal

conditions for PCR using each primer-pair were determined with the FailSafe™ PCR PreMix Selection Kit (Epicentre Inc., Madison, WI). The optimization PCR was carried out in a total volume of 25 µl containing 300 ng of genomic DNA as template, 12.5 µl FailSafe Master Mix, 0.25 µl FailSafe PCR Enzyme Mix, 0.25 µl 50 nmol/L each primer and 11 µl sterile water. Following optimization, PCR was carried out using melting temperatures as shown in Table 3.1 and standard cycling conditions of denaturation and extension. After PCR, 4 µl of the reaction product was analyzed by gel electrophoresis and stained with SYBR green. Each amplicon was purified using QIAquick 96 PCR Purification Kit (Qiagen, Valencia, CA, USA) and sequenced using the BigDye terminator sequencing protocol (ABI, Foster City, CA). The sequences were analyzed for SNPs using a combination of Phrap, Polyphred and Consed as previously described (Guan et al., 2007). According to Nickerson et al (1998), heterozygous sites can be identified through scanning the assembled sequence traces for the following: (i) the presence of a drop in normalized fluorescence peak height at a position when compared to the respective peak height for all individuals that are homozygous at the position; (ii) the presence of a second peak that accompanies the drop in fluorescence peak height.

3.3e In silico SNP analysis

A total of 10 unique sequences each from chicken (*Gallus gallus*) and human (*Homo sapiens*) were obtained from GenBank for the *in silico* SNP analysis in a region that corresponds to the targeted tRNA region defined above for the turkey. The Accession Numbers for the chicken sequences were AB086102 (Wada et al., 2004), AP003317

(Nishibori et al., 2003), AP003318 (Nishibori et al., 2003), AP003321 (Nishibori et al., 2005), AP003322 (Nishibori et al., 2005), AP003323 (Nishibori et al., 2005), AP003580 (Nishibori et al., 2003), AY235570 (Froman and Kirby, 2005), AY235571 (Froman and Kriby, 2005), and NC_001323 (Valverde et al., 1994) and those for human were AF346963 (Ingman et al., 2000), AF346972 (Ingman et al., 2000), AF346978 (Ingman et al., 2000), AF346988 (Ingman et al., 2000), AF346989 (Ingman et al., 2000), AF347013 (Ingman et al., 2000), AF382012 (Maca-Meyer et al., 2001), AY195787 (Mishmar et al., 2003), DQ112749 (Kivisild et al., 2006), and EF064330 (Olivieri et al., 2006).

3.3f Statistical analysis

Mortality for control and treatment birds was recorded from day 1 (week 0) and analyzed by Kaplan-Meier plot, and was compared using log-rank tests (Kaplan and Meier, 1958). Echocardiography data was analyzed with SAS software (SAS Institute Inc., Cary, NC, USA). Values of $p < 0.05$ were considered significant. Descriptive statistics of parametric variables are expressed as mean values and standard deviation. Comparisons between two groups were performed using independent groups two-tailed *t* tests.

3.4 Results and Discussion

As expected, mortality was significantly higher in the treatment than control group (Figure 3.2). At the end of week 1, 6% of birds in the treatment group had died while there was only 1% mortality in the control group. The Kaplan-Meier plot shows that survival probability in the treatment birds decreased 31.54% in week 3 than in week 1 while the decrease was only about 0.03% in the control birds.

Differences between control and treatment groups in LVEDD and LVESD were also significant (Figures 3.3-3.5). Echocardiography at 3 weeks of age showed larger increase in LVEDD and LVESD in treatment birds compared to control birds (1.77 ± 0.27 cm vs 0.69 ± 0.08 cm, $P<0.05$; 1.20 ± 0.31 cm vs 0.25 ± 0.06 cm, $P<0.05$). In treatment birds, echocardiographic examination from week 2 to 4 revealed progressive left ventricular dilatation. These observations support earlier reports of echocardiography as a useful tool for the evaluation of systolic and diastolic cardiac function and in the diagnosis of early DCM.

SF significantly decreased in treatment birds at weeks 3 and 4 (Figure 3.6). Reports suggest that a SF of 30% implies normal, while 26% to 30% is considered a mild decrease in function and below 25% considered abnormal (Keene and Oeffinger, 2000).

The histopathological analyses of hearts were in agreement with echocardiography results (Figure 3.7). Gross pathology of the heart of birds in treatment groups, based on visual inspection during necropsy, showed marked dilation of all four chambers, especially the left ventricle. Enlargement of the ventricular chamber is considered the defining morphological feature of DCM (Zifa et al., 2008). In humans, evaluating diastolic function is very important to monitor DCM patients for assessing prognosis and therapeutic effect. Hematoxylin and

eosin (H&E) stained left ventricle myocardium in treatment birds also revealed patchy areas of myocyte necrosis, increased fibrosis, vacuolar degeneration of myofibers (Figure 3.7a) and some with inflammatory cell infiltration (Figure 3.7b), compared with a cross-sectioned heart observed from a normal bird (Figure 3.7c). The loss of functional heart muscle and myocardial fibrosis can result in increased myocardial stiffness and reduced heart muscle compliance. This will further decrease cardiac diastolic and systolic function. Increased fibrosis has also been reported in human patients and in dogs with DCM (Vollmar et al., 2003; Ohtani et al., 1995). However, the histological changes associated with DCM are usually non-specific. The characteristic histologic findings presumably indicate DCM process.

The products of expected size were obtained from over 95% of samples used in the PCR (Figure 3.1). Two polymorphisms, both in NADH dehydrogenase (ND) genes, were found in the affected but not the unaffected birds (Table 3.2). The first was a G-A SNP found at position 3783 in the ND1 gene of the mtDNA sequence (Accession Number NC_010195) (Figure 3.8a). The A allele was found only in 5 treatment birds. The other SNP, also a G-A, was observed as a heterozygote, an unexpected phenomenon in the mitochondrion with its haploid genome (Figure 3.8b). The G12162A mutation, observed here only as heteroplasmic genotypes, was in the ND5 gene. The nucleotide variation results in an amino acid change from Alanine to Threonine (Figure 3.8b and Table 3.2).

The allele frequencies of G-A at positions 3783 and 12162 in 35 treatment birds were presented in Table 3.3. The frequency of allele G in treatment birds was 0.857 and 0.971 for positions 3783 and 12162 of the mtDNA, respectively (Table 3.3). The LVEDD, LVESD and

SF of the genotypes were different, with some genotypes appearing to be more susceptible to the toxic levels of furazolidone (Tables 3.5 and 3.6).

From *in silico* analyses, 9 SNPs in chicken (*Gallus gallus*) and 10 SNPs in human (*Homo sapiens*) in selected regions from 10 different GenBank samples with known geographic origin were detected (Table 3.4). It is also noteworthy that, comparison to similar regions of the mitochondrial genomes in chicken and human, the tRNA genes in the turkey were not polymorphic.

Several studies have reported the association between mtDNA mutations and DCM. These have included point mutations and deletions in tRNA and rRNA genes, cytochrome *b*, cytochrome *c* oxidase (*COX*) subunits I and II, NADH subunits 1 and 5, ATPase, and the D-loop (Ozawa et al., 1995; Li et al., 1997; Arbustini et al., 1998; Marin-Garcia et al., 2000; Marin-Garcia et al., 2002; Ruppert et al., 2004). For example, Moslemi et al (2000) found a mtDNA deletions in the *COX* gene in a 27-year-old man with severe DCM. Transgenic mice developed severe DCM and had increased cell death when point mutation levels had increased to on average two per mitochondrial genome (Zhang et al., 2003). However, few studies on mtDNA mutations are reported in turkeys with DCM. In this study, the genetic analysis was performed to identify mutations in tRNA genes and flanking regions of mtDNA in turkeys. It showed that the mitochondrial DNA variation may be associated with the turkey's response to the toxic levels of furazolidone. For ND complex genes, one of the mutations reported here leads to the substitution of an Alanine for a Threonine.

Since mtDNAs are present in hundreds or thousands of copies per cell (polyplasmy), mtDNA mutations are often heteroplasmic. Hereroplasmic mtDNA mutations are subject to

mitotic segregation and can accumulate to high levels in some cells, which create a mosaic pattern of respiratory chain deficiency in affected tissues (Rokas et al., 2003). In highly conserved regions of mtDNA complex I (including ND1 and ND5), enzymatic defects influence either single or multiple enzymes involved in the mitochondrial respiratory chain and oxidative phosphrylation. It has been reported that heteroplasmic mutations in mtDNA do occur in pathogenic mutations. Arbustini et al (1998) found heteroplasmic mtDNA mutations (9 tRNA, 5 rRNA, and 4 missense) only in DCM patients with ultrastructural mitochondrial abnormalities using mutation screening techniques. In another study, Ruppert et al (2004) determined that two of the mutations in the NADH subunits 1 and 5 and one in the *COX* subunits in patients with DCM were heteroplasmic.

In summary, mutations in mtDNA observed here appear to be associated with the turkeys response to furazolidone. They may contribute to a deficiency in complex I of a respiratory chain enzyme in the heart muscle and further result in disorders of energy production and depressed heart muscle contraction. Since commercial turkeys appear to be affected at a relatively young age (about week 3), mitochondrial dysfunction in the respiratory enzyme subunits may affect the heart's physiological activity and could be relevant to the pathogenesis of DCM in the turkeys.

Table 3.1. Sequences of primers used for SNP analysis in targeted tRNA sequences of the turkey mitochondrial genome

Region	Name	Primer sequences*	Size	Tm**
Ile-Gln-Met	6F	5'-TCCCCGCATGACCTTTAAC-3'	500	56.7
(3596-4307, 711bp)	6R	5'-AGGAAGCGAGAGGGTTGTTT-3'		57.3
	7F	5'-ACATGCCAGCATACCAATCA-3'	353	55.2
	7R	5'-CAGGCCAAGATTCAATGGTT-3'		55.2
	8F	5'-CAACCCTCTCGCTTCCTAAT-3'	451	57.3
	8R	5'-CAGACATGATGTGGGGTTGT-3'		57.3
Trp-Ala-Asn-Cys-Tyr	9F	5'-TAGTCGGAGGCTGAATAGGC-3'	536	59.4
(4763-5617, 854bp)	9R	5'-CAGAGGGTTATGGGCAGTA-3'		56.7
	10F	5'-CCCCCTAACTCGTCTAATCACA-3'	310	60.3
	10R	5'-ATATGGGATCAAGGCCCATC-3'		57.3
	11F	5'-TTCTGAGTGCAAACCAGACG-3'	352	57.3
	11R	5'-TGCTGTGCCGACTATACCTG-3'		59.4
His-Ser-Leu	14F	5'-CAGGAACTCCTCTATGCAAGC-3'	326	59.8
(11349-12148,799bp)	14R	5'-TGAGGGGAATTTTTGAGGTTT-3'		54.0
	15F	5'-CTCATCTTGGTGCAACTCCA-3'	501	57.3
	15R	5'-GCCGATGAGAGGAATGATA-3'		54.5

*Primer sequences were derived from *Meleagris gallopavo* mitochondrion complete genome (Accession Number NC_010195) in GenBank.

**Tm (^{o}C) represents the optimized annealing temperature at which a single amplicon of the expected size was obtained.

Table 3.2. Nucleotide variants in the DNA sequence of target region of the turkey mitochondrial genome

SNP Position*	Gene	Alleles	AA Change[a]
3783	ND1	G/A	NA
12162	ND5	G/A	Ala→Thr

*Position of the variant nucleotide in the turkey mtDNA sequence, GenBank Accession Number NC_010195.

[a]Where AA is amino acid.

Table 3.3. Sequence contexts and allele frequencies of single nucleotide polymorphisms (SNPs) observed for targeted tRNA regions of mtDNA in treatment turkeys (*Meleagris gallopavo*) on PCR-based resequencing experiments.

Turkey (*Meleagris gallopavo*)

SNP No.	SNP position*	Sequence context**	Frequency
1	3783	TGGCTT(G/A)CCTCCAAT	0.857(G), 0.143(A)
2	12162	AGAGTTC(G/A)CCACATG	0.971(G), 0.029(A)

* Position of the variant nucleotide within the consensus sequence in GenBank Accession Number NC_010195.
**Within each sequence context, alleles at the SNP locus appear in parentheses. The frequencies were based on resequencing data of 35 treatment birds, as described in the text.

Table 3.4. Sequence contexts and allele frequencies of single nucleotide polymorphisms (SNPs) identified by *in silico* analyses of tRNA regions of mtDNA in chicken (*Gallus gallus*) and human (*Homo sapiens)* that are similar to those targeted in the turkey for PCR-based variation analyses.

Chicken (*Gallus gallus*)

SNP No.	SNP position	Sequence context*	Frequency
1	5111	CCTTAA(T/C)CCTAGA	0.90(T), 0.10(C)
2	6769	TTCTAAT(T/C)CGCGCA	0.50(T), 0.50(C)
3	6811	GACGA(T/C)CAAATTTA	0.50(T), 0.50(C)
4	12694	CCGGCAC(C/T)GCAACA	0.80(C), 0.20(T)
5	12695	CCGGC(G/A)CCACAACA	0.90(G), 0.10(A)
6	12966	AAGCCA(G/A)CAAGAAG	0.90(G), 0.10(A)
7	13232	CAGCCT(A/G)ATCCCAAC	0.90(A), 0.10(G)
8	13234	GCCTAA(T/C)CCCAACAA	0.90(T), 0.10(C)
9	13262	TCAGG(G/A)GCAGAAA	0.90(G), 0.10(A)

Human (*Homo sapiens)*

SNP No.	SNP position	Sequence context*	Frequency
1	4073	GAACTCTA(C/T)ACAACAT	0.90(C), 0.10(T)
2	5444	CCACCCCA(T/C)TCCTCC	0.90(T), 0.10(C)
3	11895	GGGAGA(A/G)CTCTCTGT	0.90(A), 0.10(G)
4	11916	AACCAC(G/A)TTCTCCTG	0.90(G), 0.10(A)
5	11948	TACTTA(C/T)AGGACTCA	0.90(C), 0.10(T)
6	12093	CCCCCAT(T/C)CTCCTCC	0.90(T), 0.10(C)
7	12310	CCCCAA(A/G)AATTTTGG	0.80(A), 0.20(G)
8	12360	CTACTATA(A/G)CCACCCT	0.90(A), 0.10(G)
9	12374	AACCCT(G/A)ACTTCCC	0.80(G), 0.20(A)
10	12399	TCCTT(A/G)CCACCCTC	0.90(A), 0.10(G)

*Within each sequence context, alleles at the SNP locus appear in parentheses. All the SNP analyses were across a 2346 bp region. The frequencies were based on *in silico* analysis of a total of 10 unique sequences in GenBank from diverse labs (Accession Numbers were shown in the Materials and Methods).

Table 3.5. Mean±standard deviation of left ventricular dimensions of two mitochondrial genotypes based on a G-A SNP at nucleotide position 3783 in ND1 gene of mtDNA (Accession Number NC_010195) in 35 treatment birds and 15 control birds.

ECHO[a]	AA (n=5)[b]	GG (n=30)[b]	Control GG (n=15)[b]
LVEDD	2.04±0.29	1.72±0.24*	0.69±0.08
LVESD	1.47±0.43	1.16±0.27*	0.25±0.06
%SF	29.40±10.84	33.32±7.62	63.83±4.42

[a]Where ECHO, LVEDD, LVESD, and SF represent echocardiographic parameter, left ventricular end-diastolic dimension, left ventricular end-systolic dimension, and shortening fraction, respectively.
[b]Numbers of individuals with each genotype.
* Significant at $P<0.05$.

Table 3.6. Mean±standard deviation of left ventricular dimensions of two mitochondrial genotypes based on a G-A SNP at nucleotide position 12162 in ND5 gene of mtDNA (Accession Number NC_010195) in 35 treatment birds and 15 control birds.

ECHO[a]	GA (n=1)[b]	GG (n=34)[b]	Control GG (n=15)[b]
LVEDD	2.24	1.76±0.26*	0.69±0.08
LVESD	1.65	1.19±0.30*	0.25±0.06
%SF	26.34	32.95±8.13*	63.83±4.42

[a]Where ECHO, LVEDD, LVESD, and SF represent echocardiographic parameter, left ventricular end-diastolic dimension, left ventricular end-systolic dimension, and shortening fraction, respectively.
[b]Numbers of individuals with each genotype.
* Significant at $P<0.05$.

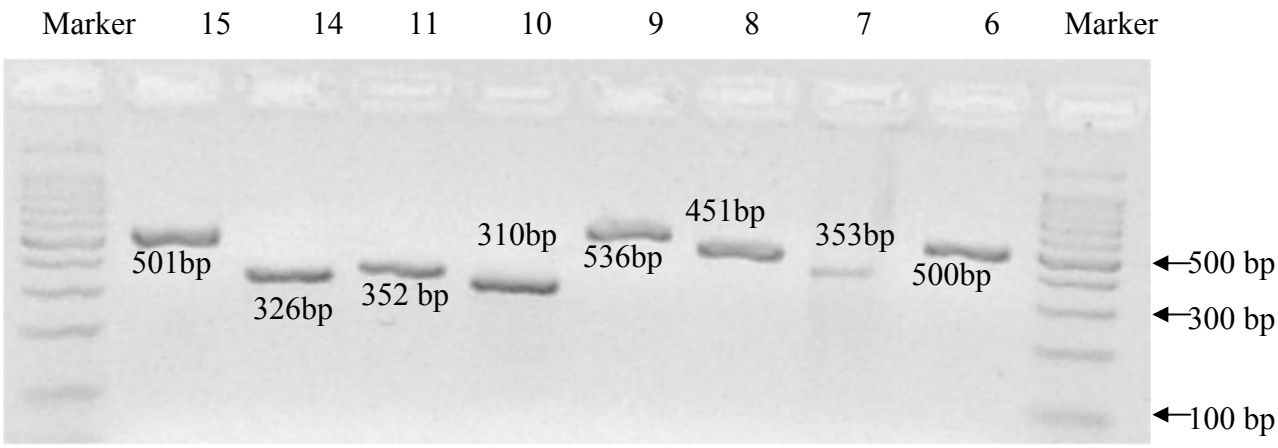
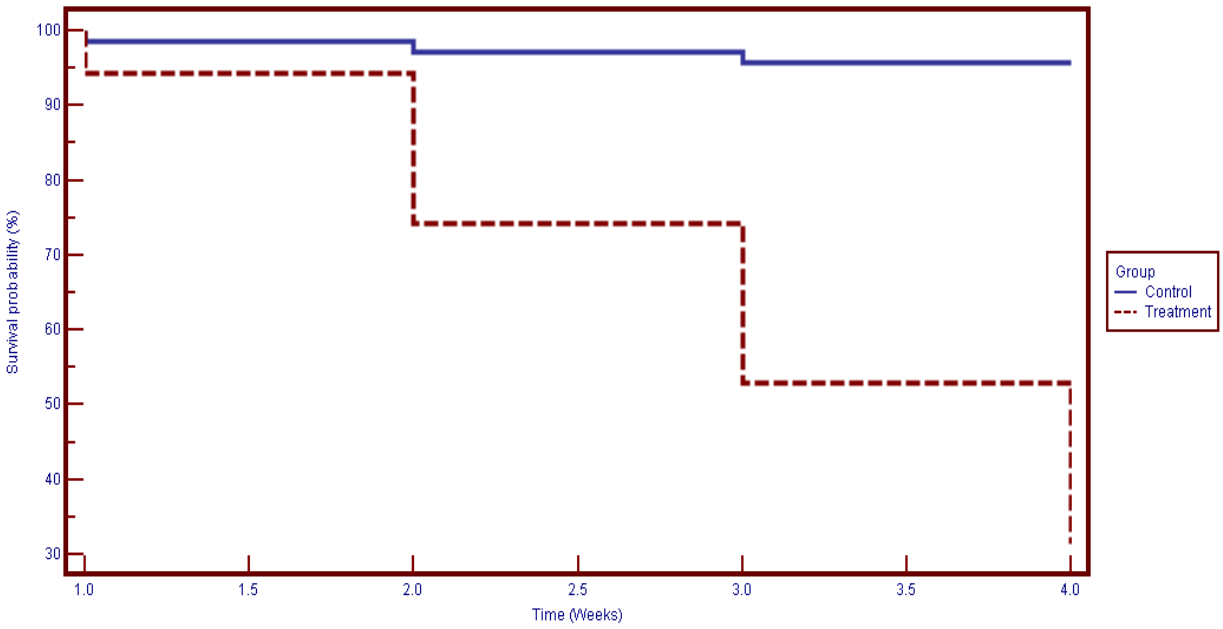


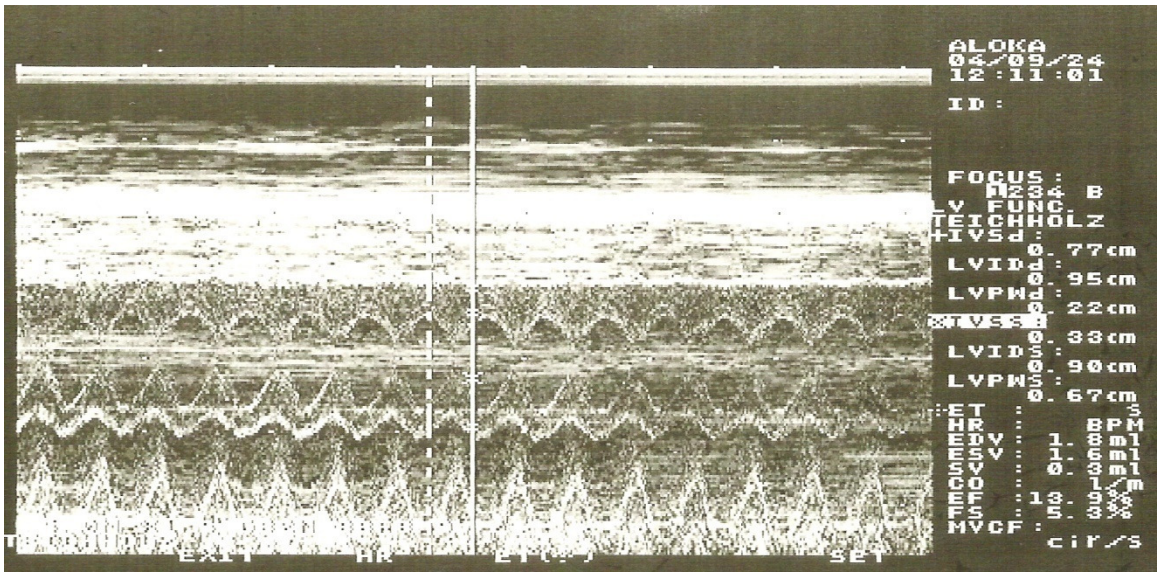
Figure 3.1. Agarose gel pattern of PCR products from amplification of turkey DNA using primers specific for turkey mtDNA tRNA genes. The number on a lane represents the identification of the primer used. The "Marker" is a 100 bp ladder. The positions of 100 bp, 300 bp and 500 bp bands are indicated.



	Comparison of survival curves (Logrank test)	
Endpoint: Observed n	3.0	48.0
Expected n	28.9	22.1
Chi-square	61.1071	
DF	1	
Significance	P < 0.0001	

Figure 3.2. Kaplan-Meier plot summary of turkey poults mortality. The plot depicts the time course of higher mortality rate for birds on furazolidone-containing diet. The dotted line is for birds in the treatment group, and the solid line is for birds in the control group. The logrank test shows significant difference between control and treatment groups (p<0.0001).

(A)


ALOKA
04/09/24
12:11:01
ID :
FOCUS :
M234 B
LV FUNC.
TEICHHOLZ
+IVSd :
0.77cm
LVIDd :
0.95cm
LVPWd :
0.22cm
IVSs :
0.33cm
LVIDs :
0.90cm
LVPWs :
0.67cm
ET : s
HR : BPM
EDV : 1.8ml
ESV : 1.6ml
SV : 0.3ml
CO : l/m
EF : 13.9%
FS : 5.3%
MVCF :
cir/s
EXIT
HR
CALC
SET

(B)

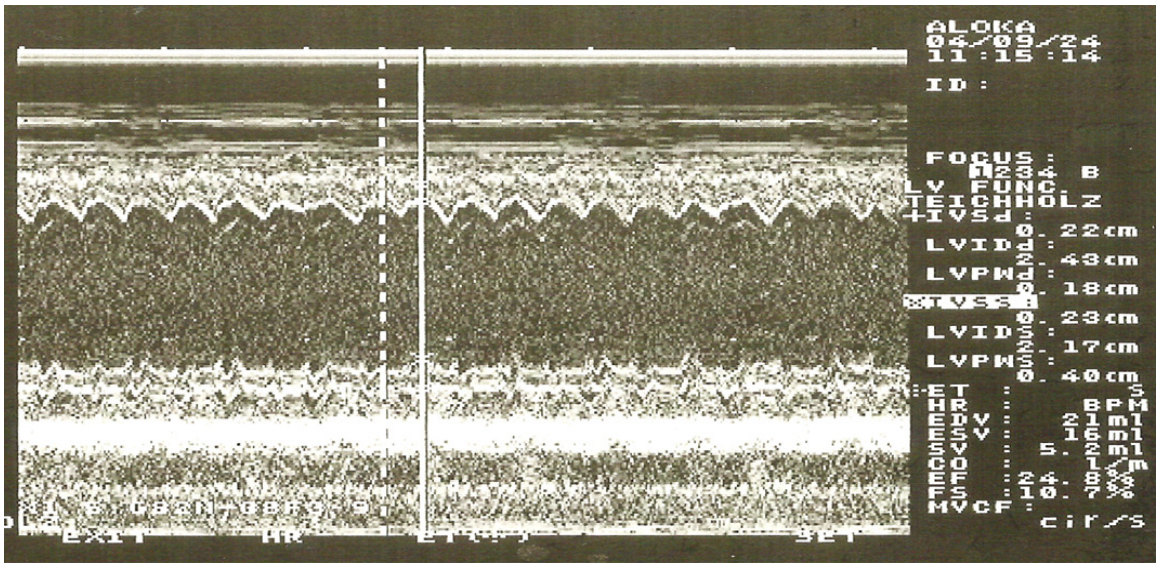


Figure 3.3. Representative M-mode echocardiography of the left ventricle from:

(A) a bird in the control group at 3 weeks of age. The numbers recorded (shown on the right hand side) for diastolic and systolic ventricular dimensions are shown as 0.95 and 0.90 cm, respectively.

(B) a bird in the treatment group at 3 weeks of age. The numbers recorded (shown on the right hand side) for diastolic and systolic ventricular dimensions are shown as 2.43 and 2.17 cm, respectively. Compared to Figure 3.3a, this echocardiography showed a significantly larger left ventricle.

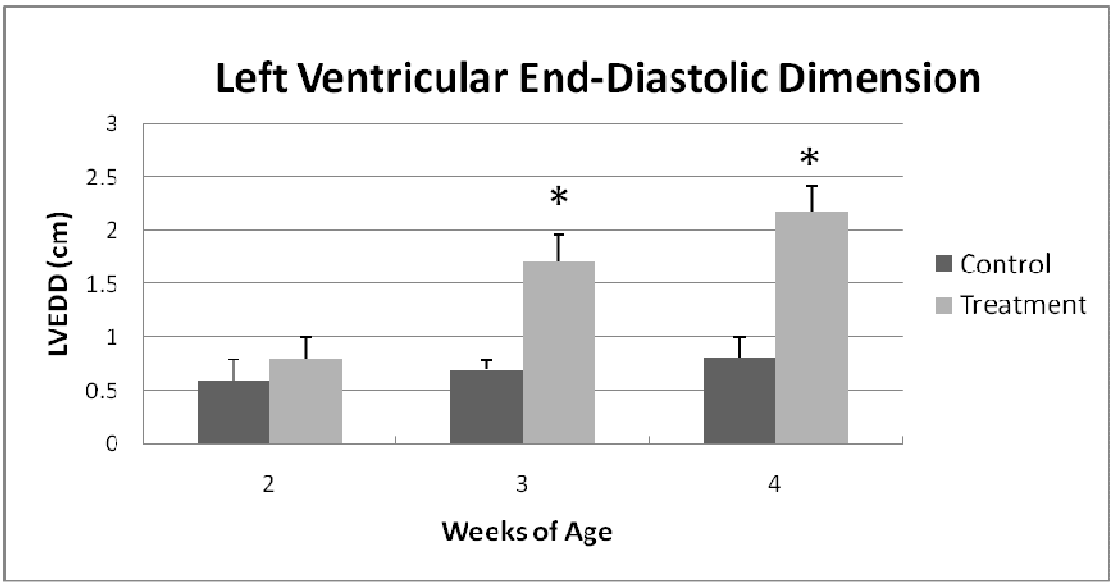


Figure 3.4. Average left ventricular end-diastolic dimension (LVEDD) in control and treatment birds at 2, 3, and 4 weeks of age.
* Significant at $P<0.05$.

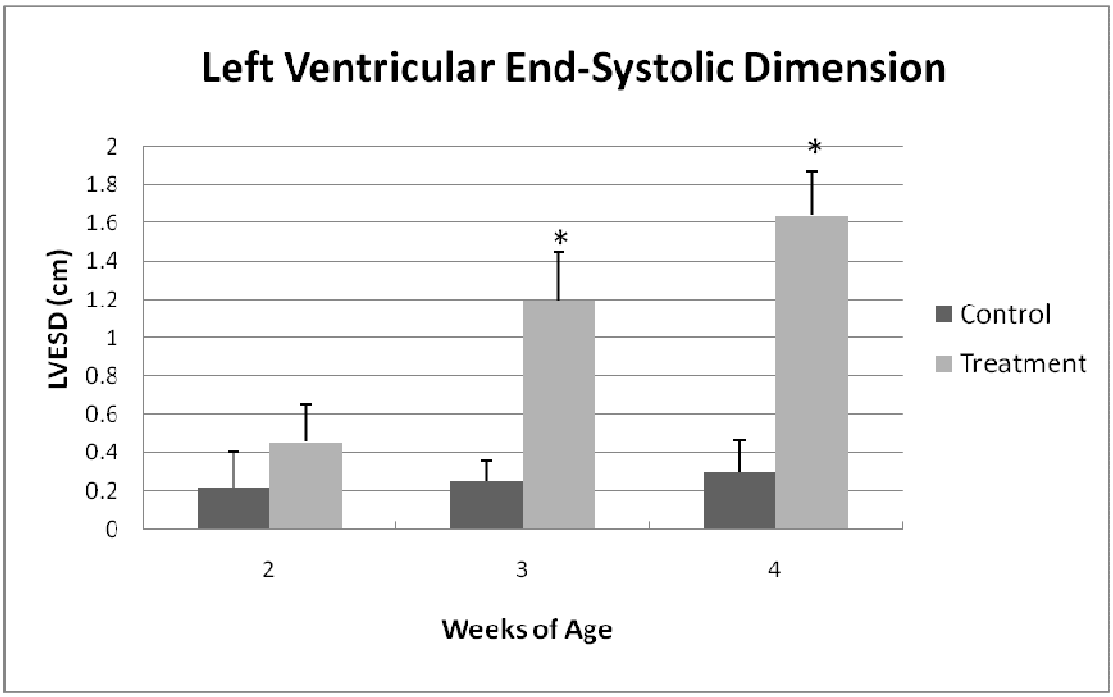


Figure 3.5. Average left ventricular end-systolic dimension (LVESD) in control and treatment birds at 2, 3, and 4 weeks of age.
* Significant at $P<0.05$.

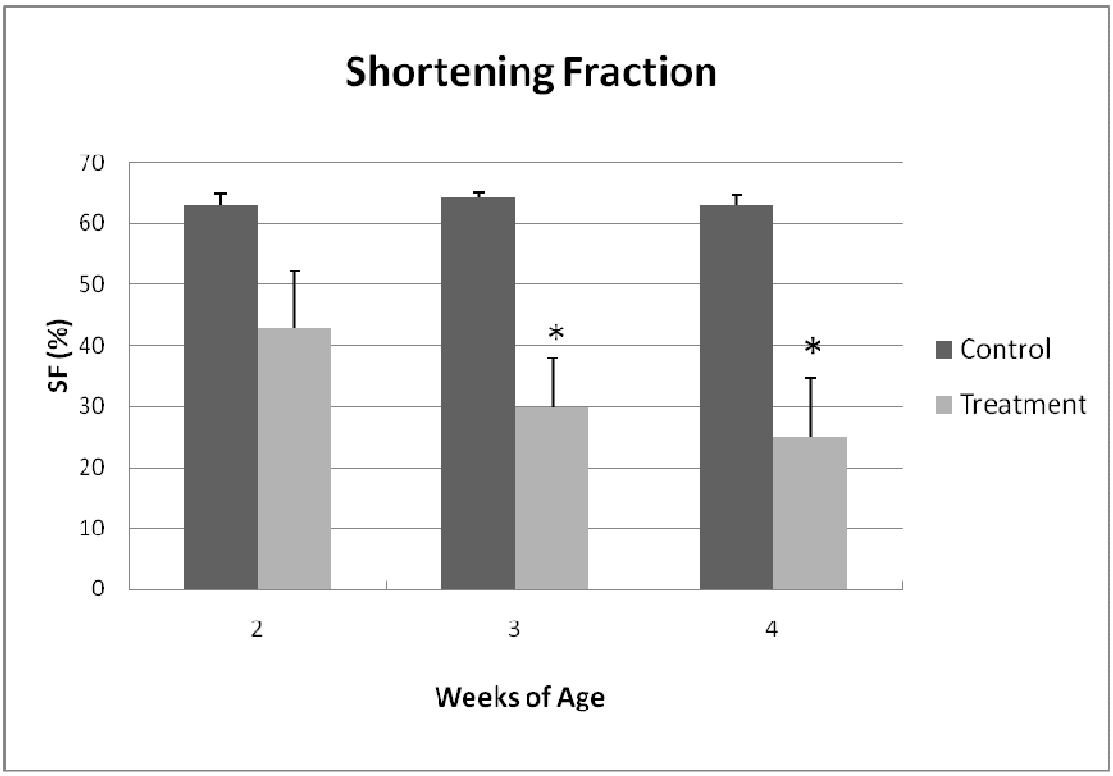
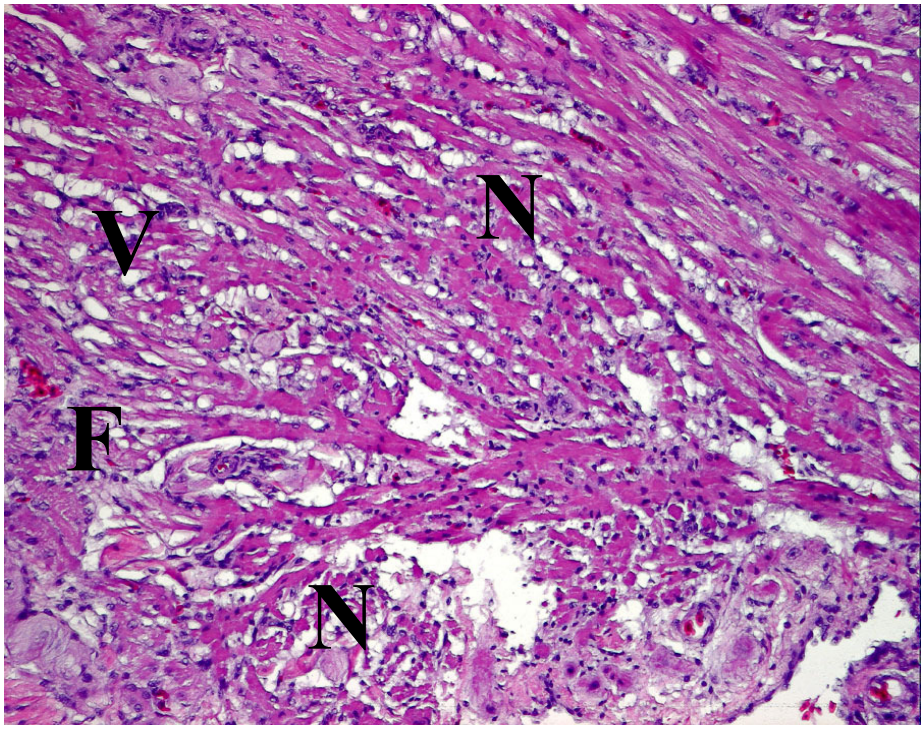
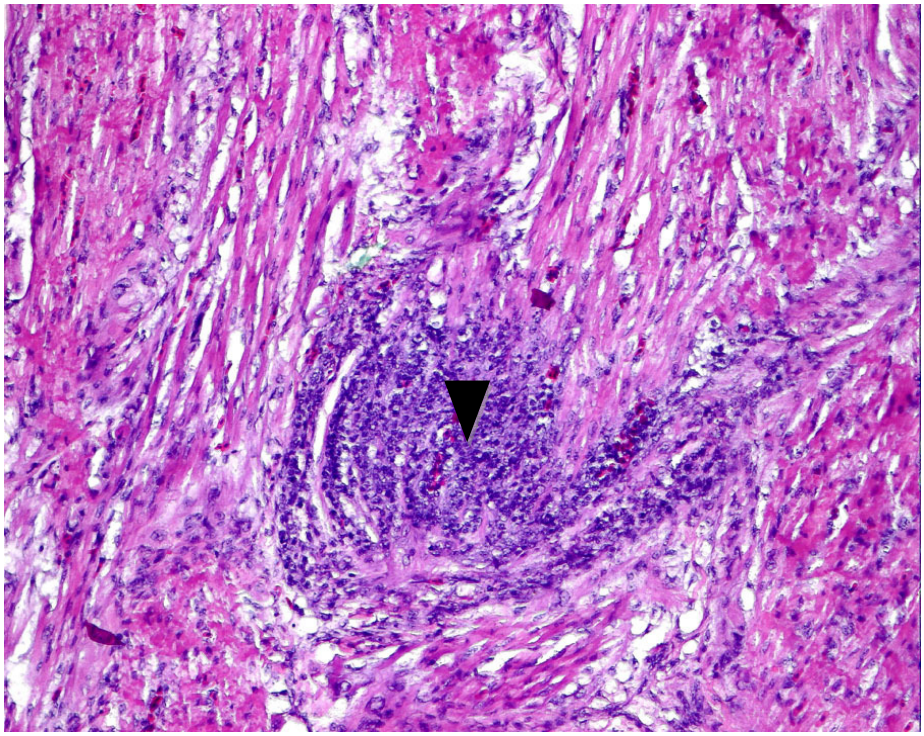


Figure 3.6. Average shortening fraction (SF) in control and treatment birds at 2, 3, and 4 weeks of age.
* Significant at $P<0.05$.

(A)



(B)



(C)

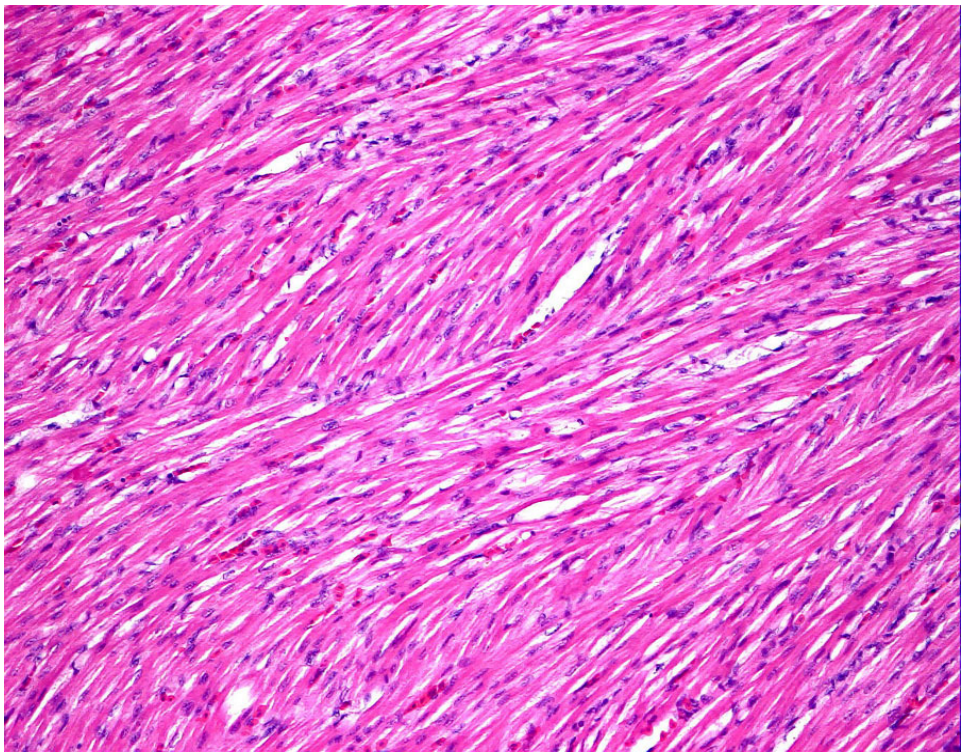
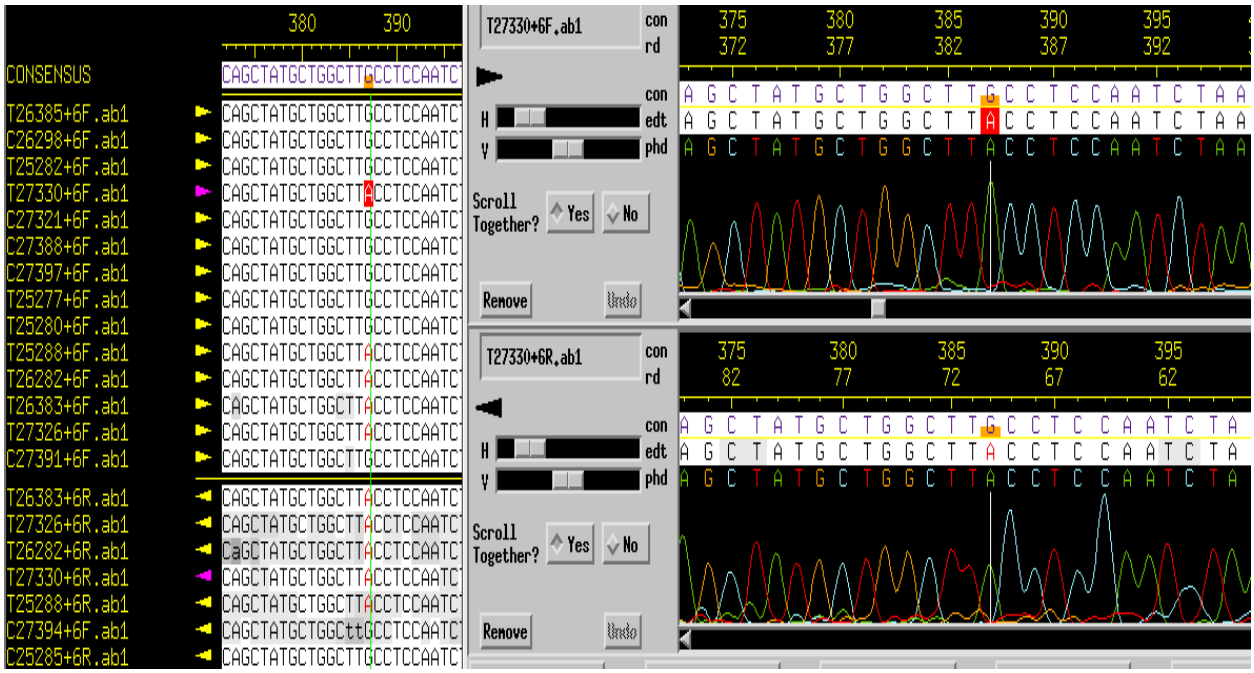


Figure 3.7. Hematoxylin and eosin (H&E) stained cross-sectioned heart from:
(A) a DCM-affected bird showing increased fibrosis (F), necrosis (N), and vacuolar degeneration (V) of myofibers. Original magnification was 200 X.
(B) a DCM-affected bird showing inflammatory cell infiltration (arrow head) including heterophil, lymphocyte, monocyte. Original magnification was 200 X.
(C) a normal bird showing the cardiac muscle cell has one central nucleus and is striated. Original magnification was 200 X.

(A)


380
390
CONSENSUS
CAGCTATGCTGGCTTGCCTCCAATC
T26385+6F.ab1
C26298+6F.ab1
T25282+6F.ab1
T27330+6F.ab1
C27321+6F.ab1
C27388+6F.ab1
C27397+6F.ab1
T25277+6F.ab1
T25280+6F.ab1
T25288+6F.ab1
T26282+6F.ab1
T26383+6F.ab1
T27326+6F.ab1
C27391+6F.ab1
T26383+6R.ab1
T27326+6R.ab1
T26282+6R.ab1
T27330+6R.ab1
T25288+6R.ab1
C27394+6F.ab1
C25285+6R.ab1
T27330+6F.ab1
T27330+6R.ab1
con
rd
edt
phd
H
V
Scroll Together?
Yes
No
Remove
Undo
375
380
385
390
395
372
377
382
387
392
82
77
72
67
62

(B)


Trace Window: Contig1
Dismiss
T27587+15F.ab1
T27587+15R.ab1
Scroll Together?
Yes
No
Remove
Undo
CONSENSUS

(C)

Figure 3.8. Consed aligned view and traces of a mtDNA polymorphism identified:

(A) at nucleotide position 387 of the alignment and 3783 in ND 1 gene in the turkey mtDNA genome sequence, Accession Number NC_010195.

(B) a heterozygous sequence based on a SNP at nucleotide position 348 of the alignment and 12162 in ND5 gene in the turkey mtDNA genome sequence, Accession Number NC_010195. The G and A peaks in the forward sequence were validated by reverse-primer sequencing of a second PCR product from the same bird T27587.

(C) a false positive heterozygous sequence based on a SNP at nucleotide position 348 of the alignment and 12162 in ND5 gene in the turkey mtDNA genome sequence, Accession Number NC_010195. The G and A peaks in the forward sequence were not observed or validated by reverse-primer sequencing of a second PCR product from the same bird T26280.

CHAPTER 4

Candidate gene expression for toxin-induced dilated cardiomyopathy in the turkey (*Meleagris gallopavo*)

4.1 ABSTRACT

Dilated cardiomyopathy (DCM), which affects diverse animals including turkeys, can be caused by both genetic and environment factors. Among factors responsible for the incidence and severity of DCM are genes that encode sarcomeric contractile proteins. Here, I evaluated the association, if any, between alpha cardiac actin (*ACTC*) gene expression and the incidence and severity of DCM in turkeys fed toxic levels of furazolidone (700 parts per million or ppm). A total of 200 birds obtained from commercial sources were evaluated from hatch to four weeks of age. Echocardiographic measurements were used to identify birds with dilated ventricles that are indicative of DCM. Real-time PCR quantification indicated that mRNA expression of *ACTC* was significantly different between control and treatment birds. The differences in expression suggest a role for *ACTC* in the turkeys response to toxin that induces DCM. Since results of the current work are strongly suggestive, additional evidence is needed to support the potential role of *ACTC* in the incidence of toxin-induced DCM.

Keywords: Turkey, Dilated cardiomyopathy, Alpha cardiac actin gene, Real-time PCR

4.2 Introduction

Dilated cardiomyopathy (DCM), a primary cardiac disorder, is characterized by a progressive course of cardiac dilation and reduced systolic function in humans (Zifa et al., 2008), turkeys (Lin et al., 2006), dogs (Oyama and Chittur, 2005), and mice (Du et al., 2007). Although DCM is the most frequent form of cardiomyopathy (accounting for 87% of all cases) and one of the leading causes of heart failure, sudden death, and heart transplantation, its aetiology is still largely unknown. In about 20-30% of cases, DCM appears to be genetic (Grunig et al., 1998).

In commercial turkeys, DCM, also known as round heart disease, is a prevalent circulatory problem that can result in severe economic losses to producers (Fatkin and Graham, 2002). Feeding furazolidone [N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone] at a level considered toxic (700 ppm) for 2-3 weeks, causes turkey poults to develop cardiomyopathy (Wu et al., 2004). Since furazolidone has been extensively used in commercial turkey starter diets to help treat and reduce mortality from non-specific enteritis, it is not surprising, therefore, to see high incidence of cardiac dilation, ascites, and mortality (Staley et al., 1978).

To date, the candidate gene approach is the most commonly used research method in genetic DCM. Mutations in sarcomeric protein genes have been reported to cause DCM (Wiersma et al., 2007; Morimoto, 2008). The alpha cardiac actin gene (*ACTC*) is the first sarcomeric protein gene reported to be associated with DCM. In two unrelated families affected by DCM, Olson et al (1998) found two missense mutations in *ACTC*: Arg312His and Glu361Gly. Actins, one of the most highly conserved proteins, are also the major components

of the thin filaments in the muscle sarcomere (Mogensen et al., 1999). In almost all eukaryotic cells, actin takes part in a broad range of functions including cell motility, cell shape, and muscle contraction (Bertola et al., 2008). Actins interact directly with other sarcomeric proteins like β-myosin heavy chain, the light chains, troponin I, α-tropomyosin, actinin and dystrophin (Gregorio 1997). The cyclical interactions of actins with tropomyosin and that of the troponin complex with myosins of the thick filament are vital to the generation of contractile force and muscle contraction (Boheler et al., 1991).

Primary actins that have been identified in birds and mammals include alpha-skeletal (ACTA1), alpha-cardiac (ACTC), alpha-smooth muscle (ACTA2), gamma-smooth muscle (ACTG2), beta-cytoplasmic (ACTB) and gamma-cytoplasmic isoactin (ACTG1) (Bertola et al., 2008). In addition to being a major constituent of the thin filaments of sarcomeres, ACTC also forces part of the contractile apparatus of cardiac myocytes. It has also been shown to transmit force by mechanically binding adjacent sarcomeres and myocytes in the heart (Gergorio, 1997). Though previous studies have shown that mutations in the *ACTC* gene are associated with DCM in humans (Takai et al., 1999; Mayosi et al., 1999; Tesson et al., 2000; Karkkainen et al., 2002; Shimizu et al., 2005), little is understood about its role in the incidence and severity of DCM in other animals including turkeys. Here I used real-time PCR to examine differences in *ACTC* expression between turkeys fed normal diet and those fed diet containing toxic levels of furazolidone.

4.3 Materials and Methods

4.3a Animals

Two hundred one-day-old turkeys used in this study were obtained from a commercial vendor, divided into control and treatment groups, and fed diet containing 700 ppm furazolidone. Weekly echocardiography (ECHO) was used to determine left ventricular dimensions for both control and treatment birds. In weeks 1 and 3, 0.5 ml blood was collected from birds in both groups. At the time of blood collection, heart samples were also collected from 15 birds each in the control and treatment groups. Left ventricular end-diastolic (LVEDD) and end-systolic dimensions (LVESD) measured by ECHO were the basis on which birds were selected for heart sample collection. Although *ACTC* is primarily expressed in the heart muscle, it was reasoned that change of *ACTC* expression with cumulative feeding of furazolidone could be observed if *ACTC* mRNA can be determined from blood. Therefore, in a pilot study, 0.5 ml blood was collected by brachial venipuncture into 2 ml tubes containing RNA*later* (Applied Biosystems, Foster City, CA, USA). The samples were thoroughly mixed by inverting the tube several times and then stored at -20^{o}C if not used immediately. Hearts were harvested immediately after humane euthanization according to the VT Institution Animal Care and Use Guidelines. Approximately 170 mg of each sample was put into 4 ml RNeasy midi columns (Qiagen Inc., Valencia, CA, USA) pre-loaded with RNA*later*.

4.3b Total RNA isolation

Total RNA from whole blood and heart tissue were isolated using the Mouse RiboPure-Blood Kit (Applied Biosystems, Foster City, CA, USA) and the RNeasy Midi Kit (Qiagen Inc., Valencia, CA, USA), respectively, according to the manufacturers' protocols. The integrity was estimated using 1% agarose-formalin gel and the concentration of RNA was determined using a Bioanalyzer (Agilent Technologies, Foster City, CA, USA).

4.3c Primer design and optimization

Since there is no turkey *ACTC* gene sequence in GenBank, the chicken sequence (Accession Number NM_001079481) was used as reference to design primers for RT-PCR. BLAST analysis of expressed sequences tags database identified a turkey sequence (Accession Number EX719246) (Reed et al., 2007) that showed significant sequence similarity (95%-99%) to the chicken *ACTC* gene (Accession Number X02212). Since alpha and beta actin sequences in chicken are similar (79%-87%), it was assumed that they are also similar in the turkey. To reduce the chances of analyzing beta actin, primer design focused on the 3' end of the chicken *ACTC* gene that appeared to be unique. Primers for amplifying the turkey *ACTC* were designed using Primer-BLAST (NCBI). Primers were similarly designed for Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), which was chosen as a controller housekeeping gene. Turkey *GAPDH* primers were designed with Beacon Designer™ software (PREMIER Biosoft International, Palo Alto, CA, USA) using chicken *GAPDH* mRNA sequence (Accession Number NM_204305) as a reference. All primers used in this research were synthesized by MWG Biotech (Huntsville, AL).

4.3d Real-time polymerase chain reaction (RT-PCR)

Reverse transcription-PCR was performed using WT-Ovation^{TM} RNA Amplication System (NuGEN, San Carlos, CA, USA). The primer specificity was tested by running a regular PCR for 34 cycles at 92 ^{o}C for 10s, 60 ^{o}C for 30s and 72 ^{o}C for 20s, and followed by an agarose gel electrophoresis. Sample products from each primer-pair were sequenced and a BLAST analyses carried out to confirm that the targeted amplicon was either *ACTC* or *GAPDH*. The RT-PCR reaction was conducted by Virginia Bioinformatics Institute on the BioRad iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using a quantitative PCR assay for *ACTC* and *GAPDH*. The standard curve for the amplicon was generated for 6 points: 100pg/ μ l, 10 pg/ μ l, 1 pg/ μ l, 0.1 pg/ μ l, 0.01 pg/ μ l, 0.001 pg/ μ l. The iCycle PCR amplifications were performed by amplifying 4.5 μ l of 1:5 diluted cDNA with the iQ SYBR Green Supermix containing Taq DNA polymerase (iTaqTM polymerase), Mg^{2+}, SYBR Green I, dNTPs, and fluorescein. Each sample was amplified in triplicate. Cycling conditions were 95 ^{o}C for 3 min, followed by 40 cycles of 95 ^{o}C for 10 sec, 60 ^{o}C for 30 sec. The fluorescence data used for quantization were collected and analyzed using the iCycler iQ5 software (Bio-Rad).

4.3e Data analysis

Expression stabilities were evaluated via the comparative threshold (C_t) and standard deviation after determining the C_t value for the reference (*GAPDH*) and target genes (*ACTC*) in each sample. The relative abundance of *ACTC* mRNA expression level was calculated after normalization to the endogenous housekeeping gene, *GAPDH*.

The comparative C_t method, also known as the comparative $2^{-\Delta Ct}$ method, used involved: $\Delta C_t = C_{t,\ ACTC} - C_{t,\ GapDH}$. Values were expressed as mean $\pm$ standard deviation. A value of $P<0.05$ was considered as significant difference (Gilli et al., 2003).

4.4 Results and Discussion

As expected, furazolidone administration to turkey poults from 1 day of age led to marked dilation in left ventricular parameters. Comparison of the echocardiography measurements including LVEDD and LVESD demonstrated significant differences between control and treatment groups in weeks 2, 3, and 4 (data was shown in Chapter 3).

RNA isolated from the heart muscle is presented in Figure 4.1. The products from RT-PCR for *ACTC* and *GAPDH* were 198 and 140 bp, respectively (Figure 4.2). BLAST analyses of the sequences (Accession Number FJ529668) showed a 93% identity to Gallus gallus *ACTC1*, mRNA (Figure 4.3a). The sequences also showed 95% identity with an EST Accession Number EX719288, which is a Meleagris gallopavo cDNA, mRNA sequence from the heart of a 16 week old bird (Figure 4.3b).

The expression of *GAPDH* mRNA remained constant in control and treatment birds. In the control group, mRNA levels of *ACTC* in week 3 increased by 38.75% compared to week 1 (Figure 4.4). In contrast, *ACTC* expression level in the treatment birds in week 3 decreased by 44.78% relative to week 1 (Figure 4.4).

Change in expression levels of *ACTC* in turkey heart muscle of control and treatment birds at 3 weeks of age with their echocardiographic parameters (LVEDD and LVESD) are presented in Table 4.2. The mRNA expression of *ACTC* increased in treatment birds with a marked increase in LVEDD and LVESD. The altered expression of the *ACTC* observed here, may lead to disorders of actin-myosin interaction which could influence the normal structure and function of the cardiac myocyte. As a highly conserved protein, actin takes part in a broad range of functions in many eukaryotic cells including the maintenance of the cell shape

and motility and muscle contraction. Alpha-cardiac actin is the main actin isoform in the heart and the major component of thin filament of sarcomere. DCM has been considered to be a disease of the heart sarcomere and may result from the impaired contractile performance of the sarcormere. An impaired myosin binding to actin filament weakens the sarcomere contraction, contributing to a compensatory dilation of the heart.

Although *ACTC* has previously been described as a putative causative gene for DCM, it should also be noted that some previous reports have failed to detect any mutations in *ACTC* in human patients with DCM (Mayosi et al., 1999; Takai et al., 1999; Tesson et al., 2000). Few data have been reported on mRNA expression level of *ACTC* in DCM models. Here, our study is the first to investigate the changes in expression of *ACTC* in apparently normal turkeys and those fed toxic levels of furazolidone. The result of this study indicated that *ACTC* was upregulated in treatment birds compared with control birds ($p<0.05$), which was consistent with real-time RT-PCR quantification findings from the transgenic DCM mouse model (Glu54Lys in α-tropomyosin) described by Rajan et al (2007). Olson et al (1998) suggested that dysfunction of actin can reduce the transmission of the force generated in sarcomeres. Our observation of a decreased *ACTC* expression with progression of DCM was also previously observed by Kumar et al (1997). They suggested that the decreased alpha cardiac actin probably exacerbates the physiological changes that eventually lead to DCM.

In summary, mRNA expression of *ACTC* increased in the treatment birds compared to control birds. With the severity of DCM development, *ACTC* expression level decreased from week 1 to week 3. This finding illustrated that *ACTC* may be associated with toxin-induced DCM in the turkeys.

Table 4.1. Sequences of primers used for real-time PCR

Gene	Primer name	Sequences	Size (bp)	Tm (^{o}C)*
ACTC	ACTC-F	5'-ACCCTTGAACCCTAAAGCCAATC-3'	198	60.6
	ACTC-R	5'-CATGATAGCATGAGGCAAA-3'		52.4
GAPDH	GAPDH-F	5'-CACACGGACACTTCAAGG-3'	140	56.0
	GAPDH-R	5'-GGACTCCACAACATACTCAG-3'		57.3

***** The optimized annealing temperature at which a single amplicon of the expected size was obtained.

Table 4.2. Mean±standard deviation of left ventricular dimensions and *ACTC* expression in control and treatment birds at 3 weeks of age

Birds	**LVEDD[a] (cm)**	**LVESD[a] (cm)**	**mRNA expression[b]**
Control (15)	0.69±0.09	0.25±0.06	0.88±0.61
Treatment (15)	1.74±0.27*	1.15±0.29*	2.64±0.92*
***P*-value**	<0.05	<0.05	<0.05

[a]Where LVEDD and LVESD represent left ventricular end-diastolic and end-systolic dimensions, respectively.
[b]The relative levels of *ACTC* mRNA expression were calculated by comparing their readings (arbitrary units) to the readings of a housekeeping gene (*GAPDH* mRNA).
* Significant at $P<0.05$.

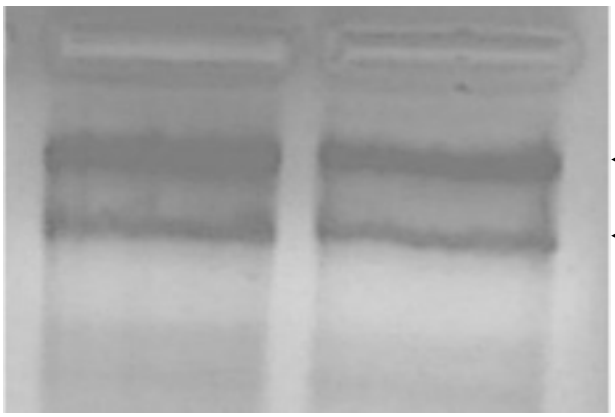


Figure 4.1. Formaldehyde agarose gel patterns of total RNA isolated from turkey heart muscle. The 28 S and 18 S RNA bands indicate RNA of good quality and high integrity.

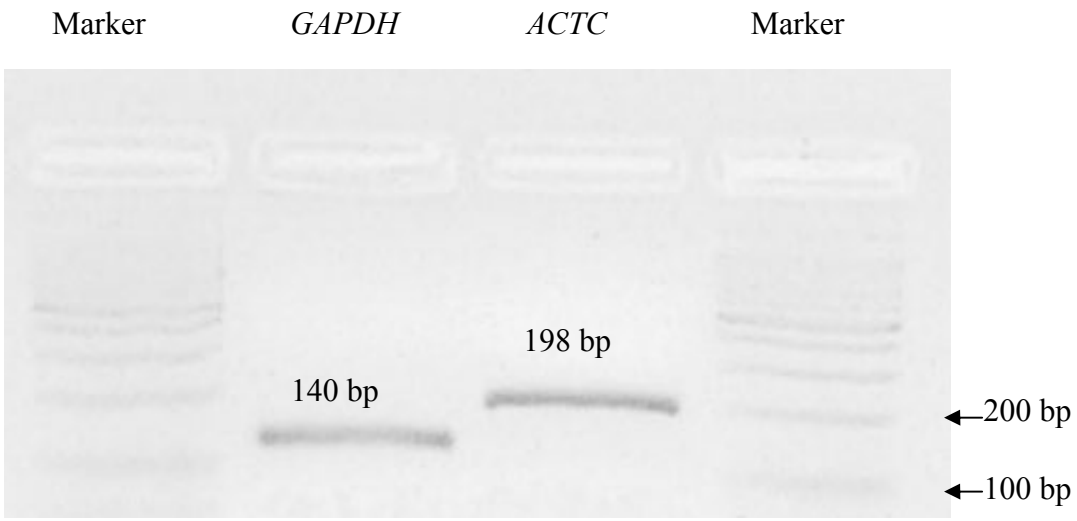


Figure 4.2. Agarose gel patterns of turkey amplicons produced by primers specific for turkey *GAPDH* and *ACTC* genes. The size of the amplicons (140 bp and 198 bp) are consistent with the expected RT-PCR products. The number on a lane represents the identification of the primer used. The "Marker" is a 100 bp DNA ladder, and the positions of 100 bp and 200 bp bands are indicated.

(A)

```
Query  13   ATGTTTGAGACCTTCAATGTACCTGCTATGTATGTGGCGATCCAGGCTGTCCTGTCCCTG  72
            ||||||||||||||||||||||| ||||||||||||| ||||||||||||||||||||||
Sbjct  433  ATGTTTGAGACCTTCAATGTACCCGCTATGTATGTGGCAATCCAGGCTGTCCTGTCCCTG  492

Query  73   TGTGCCTCTGGTCCTACCACAGGTATTGTTCTTGACTCTGGCGATGGAGTCACCCACAAT  132
            | ||||||||||| ||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  493  TATGCCTCTGGTCGTACCACAGGTATTGTTCTTGACTCTGGTGATGGAGTCACCCACAAC  552

Query  133  GTGCCCATTTATGATGGGTATGGTTTGCCTGGTGCTATCAT  173
            ||||||||||||||| || |||| |||||||  |||||||||
Sbjct  553  GTGCCCATTTATGAAGGTTATGCTTTGCCTCATGCTATCAT  593
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(B)

```
Query  13   ATGTTTGAGACCTTCAATGTACCTGCTATGTATGTGGCGATCCAGGCTGTCCTGTCCCTG  72
            ||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  423  ATGTTTGAGACCTTCAATGTACCTGCTATGTATGTGGCAATCCAGGCTGTCCTGTCCCTG  482

Query  73   TGTGCCTCTGGTCCTACCACAGGTATTGTTCTTGACTCTGGCGATGGAGTCACCCACAAT  132
            | ||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  483  TATGCCTCTGGTCGTACCACAGGTATTGTTCTTGACTCTGGCGATGGAGTCACCCACAAT  542

Query  133  GTGCCCATTTATGATGGGTATGGTTTGCCTGGTGCTATCAT  173
            ||||||||||||||| ||||||| |||||||  |||||||||
Sbjct  543  GTGCCCATTTATGAGGGGTATGCTTTGCCTCATGCTATCAT  583
```

Figure 4.3. BLAST-2-alignment of the sequence of the amplicon in turkey produced using primers specific for chicken alpha cardiac actin mRNA sequence:

(A) Query is the sequence of the turkey amplicon produced using primers specific for chicken *ACTC* described in Table 4.1. Sbjct is the matched GenBank sequence, Accession Number NM_001079481. The sequences share a 93% sequence identity.

(B) Query is the sequence of the turkey amplicon produced using primers specific for chicken *ACTC* described in Table 4.1. Sbjct is the matched GenBank sequence, Accession Number EX719288. The sequences share a 95% sequence identity.

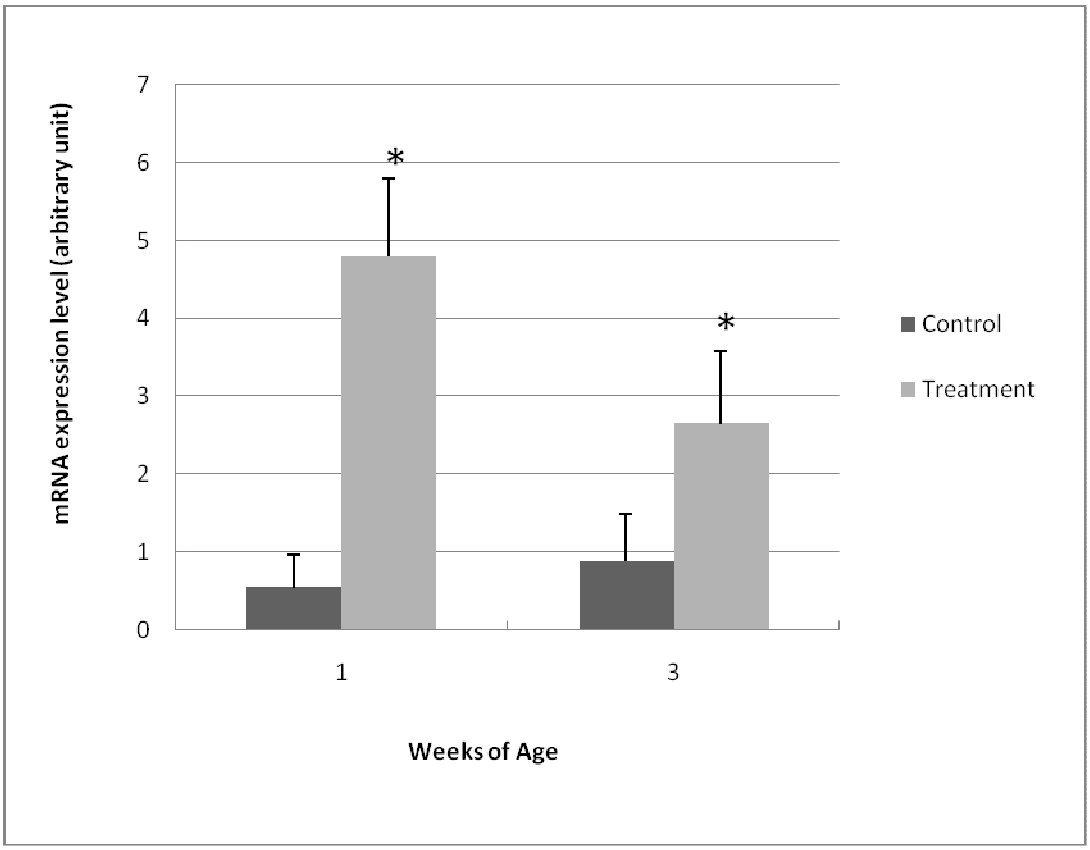

Figure 4.4. Alpha cardiac actin gene (*ACTC*) mRNA expression in control and treatment birds normalized to *GAPDH*. The mRNA expression of *ACTC* in treatment birds was significantly higher than that of control birds. The mRNA levels were presented as an arbitrary unit. Represented data: mean±standard deviation (n=15).
*Significant at $P<0.05$.

CHAPTER 5

SUMMARY OF THESIS

This thesis research investigated the hypothesis that alpha cardiac actin gene (*ACTC*) and mitochondrial genes affect the incidence and severity of toxin-induced DCM in the turkeys. Specific conclusions are:

1. Two mutations in NADH dehydrogenase (ND) genes appear to be associated with DCM.
2. The *ACTC* expression was significantly higher in birds on furazolidone-containing diet than those on normal diet at weeks 1 and 3.

Future work

The present study evaluated the role of *ACTC* and mitochondrial genes in the response to toxic levels of furazolidone that causes DCM in the turkey. Defining the mechanisms of this disorder will be important to both agriculture and biomedicine. Specific future research that could help increase our understanding of the molecular basis of DCM in turkeys include:

1. Identify and characterize the mutation in *ACTC*, if any, that affects expression in toxin-induced DCM.
2. Generate transgenic mice that express mutant *ACTC* (G867A) in the heart to investigate the functional consequences of *ACTC* mutations associated with DCM.
3. Use PCR-RFLP to confirm the SNPs found in the mitochondrial genes using a specific restriction enzyme for the more subjects.

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APPENDIX

Appendix Table 1. Left ventricular dimensions of 35 birds in the treatment group at 3 weeks of age

Birds ID	LVEDD (cm)	LVESD (cm)	Birds ID	LVEDD (cm)	LVESD (cm)
T27593	1.48	0.87	T27362	1.62	0.93
T26278	1.66	1.11	T27330	1.96	1.31
T27586	1.52	1.06	T26398	2.07	1.57
T27577	1.69	1.14	T25288	1.71	1.05
T27765	1.47	0.85	T26399	1.82	1.36
T26296	1.67	1.16	T27326	2.25	1.56
T27399	1.58	1.06	T25285	1.49	0.74
T27323	1.72	1.23	T26393	2.03	1.53
T27583	1.86	1.39	T26282	1.89	1.25
T27590	1.82	1.41	T25594	1.59	1.02
T25278	1.38	0.79	T27331	2.05	1.47
T26383	2.43	2.17	T26280	2.14	1.58
T26394	1.56	1.07	T27328	1.86	1.41
T25281	1.86	1.27	T27337	1.58	0.87
T26388	1.96	1.38	T25292	1.55	0.98
T26385	1.33	0.75	T27329	1.44	0.94
T25290	1.97	1.34			
T27366	1.68	0.92			
T27587	2.24	1.65			

Appendix Table 2. Left ventricular dimensions of 15 birds in the control group at 3 weeks of age

Birds ID	LVEDD (cm)	LVESD (cm)
C27391	0.72	0.28
C26279	0.59	0.19
C27395	0.66	0.20
C27321	0.55	0.18
C27324	0.67	0.22
C27379	0.82	0.35
C26293	0.68	0.25
C27302	0.65	0.23
C27303	0.76	0.24
C26392	0.63	0.21
C26300	0.86	0.36
C27350	0.79	0.31
C27334	0.73	0.24
C27358	0.77	0.29
C26299	0.61	0.27

Appendix Table 3. Comparative threshold cycle (ΔC_t) of *ACTC* expression measured by real-time PCR in control and treatment birds at 1 week of age

Control ID	ΔC_t	$2^{-\Delta Ct}$	Treatment ID	ΔC_t	$2^{-\Delta Ct}$
C1H1	1.33	0.40	T1H1	0.77	0.58
C1H2	2.55	0.17	T1H2	-2.37	5.17
C1H3	4.36	0.05	T1H3	-2.99	7.94
C1H4	2.20	0.21	T1H4	-2.35	5.09
C1H5	0.60	0.66	T1H5	-3.68	12.82
C1H6	1.12	0.46	T1H6	-3.72	13.17
C1H7	-0.31	1.24	T1H7	-2.02	4.05
C1H8	0.09	0.94	T1H8	-2.90	7.46
C1H9	-0.41	1.33	T1H9	0.26	0.83
C1H10	1.28	0.41	T1H10	-2.04	4.11
C1H11	0.65	0.63	T1H11	1.79	0.29
C1H12	2.27	0.21	T1H12	-1.52	2.86
C1H13	1.43	0.37	T1H13	-0.61	1.53
C1H14	0.27	0.83	T1H14	-1.55	2.93
C1H15	2.65	0.16	T1H15	-1.59	3.01

Appendix Table 4. Comparative threshold cycle (ΔC_t) of *ACTC* expression measured by real-time PCR in control and treatment birds at 3 weeks of age

Control ID	ΔC_t	$2^{-\Delta Ct}$	Treatment ID	ΔC_t	$2^{-\Delta Ct}$
C3H1	2.17	0.22	T3H1	-2.44	5.43
C3H2	0.10	0.93	T3H2	-0.91	1.87
C3H3	-0.12	1.09	T3H3	-2.02	4.05
C3H4	-0.65	1.02	T3H4	-1.58	2.98
C3H5	-0.13	1.09	T3H5	-0.21	1.16
C3H6	0.80	0.57	T3H6	-1.49	2.98
C3H7	1.48	0.36	T3H7	-1.90	1.16
C3H8	2.24	0.21	T3H8	0.09	3.73
C3H9	0.05	0.97	T3H9	-1.55	2.93
C3H10	-1.07	1.09	T3H10	-1.24	2.36
C3H11	-1.98	0.66	T3H11	1.00	0.50
C3H12	-0.64	1.56	T3H12	-0.92	1.89
C3H13	0.04	0.97	T3H13	-1.83	3.55
C3H14	-2.79	1.23	T3H14	-1.50	2.83
C3H15	-1.29	1.20	T3H15	-1.41	2.66