A Cellular and Molecular Investigation of Dilated Cardiomyopathy (DCM) in Dogs

by

Elizabeth Sinclair

A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Master of Science
in
Biomedical Sciences

Guelph, Ontario, Canada

© Elizabeth Sinclair, December 2012
We hypothesized that alterations in cardiac myofilaments are associated with hereditary canine DCM. DCM myofilaments demonstrated a reduction in EC$_{50}$ and a modest decrease in maximum activity compared to non-failing dog samples. Treatment of myofilaments with the calcium sensitizer, bepridil, showed a reduction in EC$_{50}$. Desmin and tropomyosin phosphorylation was increased in DCM. Desmin protein levels were increased in DCM. Total troponin I phosphorylation was unchanged, but S23/S24 phosphorylation was reduced in DCM. Myofilament-associated PKC-δ and -ζ were elevated in DCM, PKC-ε was modestly reduced, and PKC-α showed no change. These data are the first investigation of cardiac myofilaments in naturally occurring canine DCM, and support the hypothesis that alterations in cardiac myofilaments are associated with DCM.
Acknowledgements

This project would not have been possible without the support, input and commitment of many individuals.

Thank you to Michael O’Grady for coming in as a key collaborator and for making the first tissue donation happen. Thank you to Lynne O’Sullivan for continuing that collaboration, for Opie and Katz readings and for genuine support. Thank you also to the additional members of my graduate advisory committee: G.Pyle, G.Kirby and J.Macri.

Thank you to my colleague Maggie Schuckman for communicating with clients about the study, for coming in after hours to echo donors. Thank you to my long term friend Allison MacKay for his technical support, and for having a ruthless sense of humor.

I would like to thank my fellow graduate students and technical staff: Kelly, Nimmi, Faz, Tara, Faisal, Jordan, Peter, Elena, Feng Hua, Shaelyn.

I would like to thank the Ontario Veterinary College and the Ontario government for their financial support, and the department of Biomedical Sciences for their administrative and financial support.

Thank you to my family and friends for embracing change and taking this journey with me.

Thank you most of all to my partner Steve for helping me brainstorm and build the connections to bring this project into existence.
Declaration of Work Performed

I declare that all the reported work in this thesis was performed by me, with the exceptions of the items indicated below:

Echocardiography of canine donors was performed by either Dr. Lynne O'Sullivan or Dr. Maggie Schuckman.

Histopathology slides were prepared by the Animal Health Laboratory, and interpreted in conjunction with Dr. Jeff Caswell.

The Actinomyosin MgATPase assay comparing normal and DCM myofilaments were run in duplicate with Dr. Glen Pyle.

Phosphorylation and immunoblot SDS-PAGE was run in conjunction with Dr. Glen Pyle.
# Table of Contents

Introduction

Prevalence

Pathology

Genetics

Diagnosis

Model of Disease

Myofilaments

Myofilament regulation/dysregulation

Protein Kinase A

Protein Kinase C

Treatment

Calcium Sensitizers: Mechanism of Action

Rationale and Hypothesis

Materials and Methods

Results

Discussion

Future Directions and Limitations

References
List of Tables and Figures

Introduction

Table 1. Contractile Proteins of the Heart

Figure 1. Cross bridge formation

Figure 2. Simplified four step reaction mechanism for actomyosin ATPase

Figure 3. Myosin molecule

Figure 4. Cross section of a thin filament

Figure 5. PKA Activation

Figure 6. PKA Signal Transduction

Figure 7. Signalling pathways mediated through phospholipid hydrolysis

Results

Table 2. Animal Population Characteristics of Donor Dogs included in Myofilament and Molecular Analysis

Table 3. Echocardiographic parameters of hearts from normal and DCM dogs

Figure 8. Echocardiographic changes in naturally occurring DCM

Figure 9. Histological changes seen in naturally occurring canine DCM

Figure 10. Cardiac myofilament function in non-failing and end-stage DCM dogs

Figure 11. Cardiac myofilament function in response to treatment with calcium sensitizing drugs

Table 4. Comparison of actomyosin MgATPase Activity for normal and DCM hearts, with and without Bepridil or Levosimendan treatment

Figure 12. Cardiac myofilament protein phosphorylation is altered in end-stage canine DCM

Figure 13. Expression of cardiac myofilament protein desmin is increased in canine DCM

Figure 14. Phosphorylation of N-terminal cardiac troponin I is reduced in end-stage canine DCM

Figure 15. Myofilament associated protein kinase C levels are altered in end-stage canine DCM
Introduction

Originating from the Greek words 'kardia' (heart), 'myo' (muscle), and 'pathiae' (disease), we find the most basic meaning of cardiomyopathy. With the boldness of modern medicine, this literal definition of cardiomyopathy has morphed into “heart muscle disease of unknown cause”, as was adopted by the World Health Organization (WHO) Expert Report on Cardiomyopathies (27). Four categories of cardiomyopathy are currently recognized by the WHO: (i) dilation with atrial and ventricular incompetence, also known as dilated cardiomyopathy (DCM); (ii) cardiac hypertrophy with or without obstruction, also known as hypertrophic cardiomyopathy; (iii) cardiac constriction or restriction, also known as restrictive cardiomyopathy; and (iv) arrhythmogenic right ventricular cardiomyopathy characterized by fibro-fatty replacement of the right ventricular myocardium (26, 27). This same classification system is recognized in veterinary medicine. Given that there is a lack of knowledge about the cause and underlying mechanism of disease in DCM, and because DCM is prevalent in the canine population, allowing them to act as a large animal model of disease, this research project focuses on studying DCM as a naturally occurring disease in dogs. The term idiopathic or primary dilated cardiomyopathy is sometimes used to differentiate DCM of unknown cause from cardiac dilatation as sequelae to other disease processes, such as tachycardia. Throughout this paper, unless otherwise specified, the terms idiopathic DCM, primary DCM, familial DCM and DCM are used interchangeably.

Prevalence of Canine DCM

Dilated cardiomyopathy was first described in the veterinary medical literature in 1970 (29). On a population basis the prevalence of DCM in dogs is estimated at 0.5-1.4% (6). DCM is more commonly
found in large and giant breed dogs, and less common in small or medium sized dogs (with the exception of English and American Cocker Spaniels). The majority of the published veterinary literature with respect to DCM is focused on Doberman Pinschers. Taking that into account, and that half of the clinical DCM donors in this project are Doberman Pinschers, much of the following introduction will reflect a reporting bias as such. The breed prevalence of DCM in Dobermans ranges from 45-63% based on studies from both North America and European dog populations (2,3,9,14). By comparison, Irish Wolfhounds are also frequently affected with DCM and have a reported breed prevalence of 24% (87); Boxers and Great Danes are also recognized as commonly affected breeds (14). Overt DCM typically manifests in middle aged dogs (9,12), however a case report exists describing idiopathic DCM in two litters of Doberman Pinscher puppies (7).

There is a recognized gender preference for Doberman DCM, with males being affected more often than females (2,3,16). More recent work demonstrates that, although males are still thought to be more often affected with DCM, female dogs are affected more prevalently than was once thought; male Dobermans have been found to have an approximately 50% prevalence of DCM and females are around a 33% prevalence (28). One study from Europe found an equal gender distribution for dilated cardiomyopathy, however there was a significant gender difference in the manifestation of disease: females were more likely to have ventricular premature contractions as their only sign and males were more likely to have echocardiographic changes and overt congestive heart failure (CHF) (9). It has been found that female Doberman Pinschers manifest overt DCM at a slightly older age than male Doberman Pinschers, with a median age for female dogs of 8.6-9.5 years and 7.3-7.5 years for male (1, 12); another study found median ages of 8.6 and 7.3 years, respectively (1). These studies may explain why male Dobermans were apparently over-represented in earlier clinical studies whose inclusion criteria were dogs in CHF. The mode of inheritance of Doberman DCM is not completely known; one study was unable to elucidate an inheritance pattern (31), however a more recent study found an autosomal dominant
inheritance with incomplete penetrance (30). There is no evidence that the genetic cause of DCM is X-linked in Doberman Pinschers. This is in contrast to Great Danes where evidence suggests an X-linked mode of inheritance, and Irish Wolfhounds who show an autosomal recessive mode of inheritance with sex-specific allele (88). Human familial DCM, of which approximately 20-50% is inherited, and autosomal dominance is the most common mode of transmission (although autosomal recessive, X-linked and mitochondrial modes are also reported)(32). These modes of inheritance in humans are most similar to Doberman Pinschers.

Pathology

Idiopathic dilated cardiomyopathy of the dog and other species, including human, is characterized by progressive systolic dysfunction with no known cause, leading to dilatation of predominantly the left side of the heart and eventual congestive heart failure (59).

Most Doberman's with DCM have a prolonged occult or subclinical stage of disease (5, 12). Little is known about subclinical disease in other breeds. The occult phase is characterized by normal activity and well being, as witnessed by the owner, normal physical examination and cardio-respiratory examination, but abnormal morphologic and/or electrical changes of the heart. In affected individuals, the occult phase of disease can persist for years, or alternatively may progress to overt disease rapidly. Since the etiology of DCM has not yet been elucidated, the factors involved with transition to the overt stage are poorly understood. Once in overt failure, disease progression is rapid: dogs being treated in overt disease have a median survival time of 130.5-329 days, while untreated dogs in CHF live 14-50 days (19, 20). In Doberman Pinschers, it is also important to note that sudden death accounts for 25-30% of deaths from DCM, usually occurring in the occult phase of the disease (21, 22); this is thought to be significantly higher than the 10% rate of sudden death in other breeds (14). These sudden death events are presumed to be caused by rhythm disturbances such as ventricular tachycardia, however definitive
evidence is lacking.

Histopathology is the only definitive diagnosis of idiopathic DCM in dogs. Due to the lower sensitivity, risks and expertise associated with ventricular biopsy, particularly of the left ventricle where DCM lesions are often localized, histopathology is often only performed post-mortem. Microscopic changes in the ventricular myocardium of Doberman Pinschers were first described in 1983 (90). Currently, there are two histologically distinct forms of canine DCM that have been reported: (i) the attenuated wavy type characterized by attenuated, wavy collagen bundles and (ii) the fatty infiltrative-degenerative type (FID) characterized by fibro-fatty infiltration of the myocardium (15,92). Although Doberman Pinschers have been documented as having both types, they are primarily associated with having FID (2,15,16). Boxers are also over-represented as having FID DCM, and most other breeds have predominantly the attenuated wavy type (15). Since a safe, sensitive screening tool is not currently available, it is therefore important to caution against pre-emptively classifying any breed as having exclusively type (i) or type (ii) DCM. It is unclear whether the different histopathological characterization represents two different disease processes leading to morphological DCM, or two different manifestation of the same disease process. Although perhaps not of great clinical importance, this should be taken into account when interpreting studies involving different breeds of dogs, and could have importance in elucidating the pathology or etiology of disease.

A complex of findings has been proposed for the histopathological classification of Dobermans with the FID form of DCM (16,92). These findings are located primarily in the central portion of the left ventricular free wall and include: (i) vacuolar and hyaline changes and atrophy of myofibers; (ii) replacement of myocardium by dense bands of collagen; and (iii) clusters of adipocytes, which occur particularly at the central portion of the lesion complex (16). It is potentially important, when elucidating the underlying pathology and/or etiology of DCM, to note the distinct absence of inflammatory cells in the FID lesion complex; this has led to postulation that apoptosis could be a major contributor to myocyte
loss (16). Other pathological studies have found collagen and proteolytic enzyme abnormalities in the myocardium of Dobermans with DCM, although it is unclear if these changes are etiological or secondary to disease progression (17,18).

An analogy has been drawn between FID forms of DCM and the pathologic findings in Boxers and humans suffering from arrhythmogenic right ventricular cardiomyopathy (ARVC) (15,23,24,25). Although the lesion location is usually in the right ventricle for ARVC, as opposed to the left ventricle in dogs with DCM, the histological findings of FID characterized by fibrosis and adipose deposition are remarkably similar. Histopathological lesions found in human idiopathic DCM vary according to the etiology, but might show myofiber loss, thinning and fibrosis (89). Attenuated wavy fibres are described as a major finding in human DCM, although infrequently (92).

**DCM Genetics**

There is no known prevention for the development of DCM in affected individuals. Due to the high prevalence in certain dog breeds, and along family lines, primary DCM is thought to be genetic. In humans, 20-50% of DCM cases are thought to be familial (32). Genetic inheritance and penetrance is tremendously variable in people, making investigations into these areas challenging. Several genetic mutations have been identified in familial DCM in humans. Of particular interest, work has recently identified mutations in the cytoskeletal protein actin, which is an integral part of the cardiac myofilament (contractile unit) (38). Also in the contractile apparatus, at least six genetic mutations for DCM in people have been identified in the myofilament complex: β-myosin heavy chain, cardiac myosin binding protein C, titin, cardiac actin, α-Tm, cTnT, and cTnC (38). Inspired by this research, several genes have been investigated in dogs with DCM, without success. PCR analysis of myocardial RNA did not find consistent mutations in the following 10 genes in Doberman DCM vs normal: cardiac actin, α-actinin, α-tropomyosin, β-myosin heavy chain, metavinculin, muscle LIM protein, myosin binding protein C,
tafazzin, titin-cap (telethonin), and troponin T (164). Myocardial protein expression evaluated using western immunoblots did not find detectable differences in dystrophin, α-sarcoglycan, or β-dystroglycan in dogs with DCM (42). Analysis of the phospholamban gene has also been unfruitful in detecting causative mutations (43). One study looked at 5 Dobermans with DCM and did not find causative mutations in the genes encoding for troponin C, lamin A/C, cysteine- and glycine-rich protein 3, cardiac troponin T and the β-myosin heavy chain (39). Another study looking at portions of the actin gene sequence in Dobermans with DCM did not find a causative mutation either (40).

Recently, a study found a genetic anomaly that correlates with DCM in Doberman Pinschers. Using genome wide association analysis on blood from 66 Dobermans with clinical DCM, they found a 16 base pair deletion in the gene for the mitochondrial glucose metabolism enzyme pyruvate dehydrogenase kinase 4 (PDK4) on canine chromosome 14 (41). A similar study in Europe could not replicate the findings of a PDK4 mutation on a larger study population (165), creating a current hot topic in the field of Doberman DCM and certainly the area of ongoing research investigation.

**Diagnosis**

Since DCM dogs in the occult phase do not exhibit clinical signs, DCM is often first diagnosed after sudden death occurs or when overt CHF develops. The onset of CHF is characterized by typical clinical signs such as respiratory distress, exercise intolerance and coughing; at this time a diagnosis is primarily made based on clinical signs, breed predisposition, thoracic radiographs, echocardiography and electrocardiography (ECG). Therefore, the detection of DCM at the occult stage requires screening high-risk individuals when they are otherwise healthy. Current recommendations for clinically normal individuals of high risk breeds, such as Doberman Pinschers and Irish Wolfhounds, is to have annual screening in the form of an echocardiogram or Holter examination (14).

**Electrocardiography:** Thorough screening for occult DCM includes ECG examination, the
current gold standard of which is a 24-48 hour portable electrocardiogram (Holter monitor)(10). ECG findings are thought to be particularly important for identifying dogs at risk for sudden death; as 25-30% of dogs with DCM will die suddenly of presumed rhythm disturbances such as ventricular tachycardia or ventricular fibrillations (21, 22, 33). Criteria for abnormal Holter findings in occult DCM has been proposed as >50 ventricular premature contractions (VPCs) over a 24 hour period, or any couplets or triplets (21).

In contrast to occult DCM, the most common electrical abnormality found with overt DCM is atrial fibrillation secondary to structural myocardial changes in the atrium (1,34,59). Although atrial fibrillation is often persistent and is not considered life threatening in itself (providing that ventricular rate and adequate cardiac output is maintained), nor is it a risk factor for sudden death, it is often evidence of disease progression. (1,59). In one study, atrial fibrillation was found to be a negative prognostic indicator for Dobermans and decreased CHF mean survival time from 11 to 4.1 weeks (1). In another study, atrial fibrillation was not found to be a significant prognostic indicator, but pleural and pulmonary edema was found to be significant negative prognostic indicators (13).

**Ultrasound modes:** Currently echocardiography is the gold standard for diagnosing and evaluating the progression of occult and overt DCM (59). Criteria for diagnosing idiopathic DCM on echocardiography include progressive myocardial hypokinesis (measured via decreased fractional shortening (FS)), and severe dilatation of the left ventricle and atria, without other detectable cardiac abnormalities (6,59). A FS of <15% has been proposed by one investigator/group for the diagnosis of DCM (1). Recent recommendations for estimating human left ventricular (LV) volume from the American Society of Echocardiography suggest that M-mode, which is a 1-dimensional method using geometrical assumptions that can contain inaccuracies to estimate volume, be replaced by the 2 dimensional method, ejection fraction (EF). Evaluation of EF by the Simpson's Method of Disc, which uses a series of ellipses to estimate LV volume, has been evaluated in Dobermans and shows an increased
efficacy compared to M-mode at detecting early chamber changes in occult DCM (8).

Biomarkers as diagnostic/therapeutic tools: Cardiac biomarkers have garnered keen attention in both the clinical and research realm over the past several years, and blood tests for several biomarkers are commercially available. With respect to DCM in Dobermans, there exist specific clinical challenges that could potentially be addressed with biomarkers. These include: (i) disease classification of ambiguous cases; (ii) predicting rate of disease progression, and time to onset of CHF in occult DCM dogs; (iii) objective assessment of therapeutic response; and (iv) prediction of survival times (4).

Currently in the literature, B-type natriuretic peptide (BNP), pro-atrial natriuretic peptide (pro-ANP), and endothelin-1 (ET1) have gained much interest and have been shown to be significantly increased in CHF due to DCM (35). ANP was found to be increased in Dobermans with advanced occult DCM (4); and BNP was shown to be elevated in dogs with occult DCM (37). Cardiac troponin I (TnI) was increased in Dobermans with arrhythmias, and/or echocardiographic changes, or CHF compared to normal Dobermans (11). TnI levels alone were moderately accurate for the diagnosis of DCM, however they were not more accurate than evaluation with echocardiography and a Holter monitor (11). TnI levels were found to be increased in dogs with renal and other non-cardiac disease, which must be taken into account when evaluating TnI as a potential diagnostic tool (36).

Many, if not all, of the biomarkers evaluated for veterinary use stem from human medicine. Significant species differences have been found in the levels and detection of cardiac biomarkers (35), making a single diagnostic test or set of criteria impossible. A definitive biomarker with a high level of clinical accuracy and utility, such as a rapid screening test, does not exist in veterinary medicine for Doberman DCM.

Model of Disease

DCM in dogs has been well characterized, and there are notable similarities in the pathophysiology
and clinical course of DCM between dogs with naturally occurring DCM and human familial DCM (5,92). Therefore, in addition to the primary focus of benefiting canine patients, there exists the added potential of transferring information from this research project to humans. Compared to other laboratory models, such as murine and dogs paced to tachycardia induced dilatation, a natural canine disease model provides many advantages. For example, (i) dogs with naturally occurring DCM are a closer analogy to naturally occurring human disease versus artificial, acutely induced models, and thus broadens the scope of application and strengthens the scientific validity of our research results; (ii) a naturally occurring disease phenotype serves as a more realistic model for investigating both cell signalling and therapeutic targets in the laboratory; (iii) a more realistic model increases the likelihood of bringing research results into the clinical setting, thereby helping future heart failure patients, both human and canine.

**Myofilaments and Heart Failure**

Myofilaments are the central contractile units of the heart. Myofilaments are composed of 6 main proteins, each belonging to either the thick or thin filament (see Table 1). These thick and thin filaments interact in the process of excitation-contraction coupling, which results in contraction (Fig. 1 and 2). The force of contraction is then transmitted to the cell via cytoskeletal proteins.

The thick filament's main contractile protein is myosin. Myosin, along with kinesins and dyneins, belongs to the group of proteins known of “motor proteins” (82). Myosin is a tadpole shaped protein, with two “heads” and a “tail” (Fig. 3) that is composed of 2 heavy and 4 light chains. The heads, comprised of heavy chains, interact with actin on the thin filament to form cross-bridges; these “heads” contain the ATPase site of myosin, and form cross-bridges with actin filaments (part of the thin filament) to release the chemical energy of ATP hydrolysis that powers the inward step of mechanical contraction (Fig. 2)(82).

The thin filament is composed primarily of actin, tropomyosin and the troponin complex. Two
actin polymer chains are wound together to form the backbone of the thin filament. After calcium binding by troponin C removes troponin-tropomyosin inhibition, actin activates the ATPase enzyme in myosin, resulting in mechanical contraction (82). The troponin complex, containing troponin C, troponin I and troponin T (TnC, TnI and TnT respectively), periodically decorate the actin filament (Fig. 4). TnT anchors the troponin complex to tropomyosin, and troponin C contains the calcium binding sites that regulate excitation-contraction cycling and relaxation (82). TnI, in conjunction with tropomyosin, has an inhibitory effect on myosin binding. Tropomyosin is a biologically inert protein that in the resting state is located within the grooves of the two actin strands and physically blocks the myosin binding site. In advance of cross-bridge formation, via calcium binding to troponin C, tropomyosin shifts position to expose the myosin binding sites on actin (Fig. 4).

Cardiac myofilaments are anchored to Z-discs, which define the lateral borders of each sarcomere. Z-discs are complex regions wherein thin filaments from adjacent sarcomeres are interwoven with a number of cytoskeletal proteins that transmit tension developed by the contractile proteins to the sarcolemma. For example, sarcomeric actin in the thin filament is anchored in the Z-disc by CapZ, along with alpha-actinin and nebulette. Desmin links the Z-disc to intermediate filaments that connect to desmosomes in the intercalated discs, and thus participate in transmitting forces between adjacent cardiomyocytes (82,108,114). Z-discs are also surrounded, by rigid bands called costameres which are made primarily of the proteins desmin and alpha-actinin (82,108). Work in the last decade or so has identified novel z-disc proteins and functions, such as signal transduction, making the z-disc an area of increasing interest and importance (169).

Myofilament Regulation and Dysregulation

Myofilament regulation often occurs via signalling cascades by chemical mediators of the autonomic nervous system secreted into the bloodstream or extracellular fluid. Myofilament regulation
also occurs through physical signals from both molecular and physical components (82). Acutely, these signalling cascades can lead to covalent modifications such as phosphorylation of target proteins, as well as oxidation and glycosylation of contractile elements (170-72). Neurohormonal and physical stimulators of muscle contraction may increase energy and oxygen demands, contractility and heart rate in an adaptive way. However, chronic stimulation of the heart increases energy and oxygen cost excessively, or may drive inefficient cross-bridge cycling, leading to the detrimental/maladaptive function in failing hearts.

Protein Kinases – PKA

Protein kinases are signalling enzymes that are involved with most cell signal transduction pathways throughout the body. When activated, they catalyze the transfer of phosphate groups from ATP to hydroxyl groups of serine, threonine or tyrosine amino acid residues on target proteins (82,93). This phosphorylation involves breaking the high energy acyl bond found in ATP and the creation of a low energy phosphoester bond. When discussing biological relevancy, phosphorylation involves a change in the activity or function of the target protein (many proteins can accept phosphate groups without exhibiting any such change) (93).

Protein kinase regulation is necessary for appropriate signal transduction, making the activation and de-activation of protein kinases crucial for homeostasis. Protein kinases families are activated by a variety of functional signalling molecules such as cAMP (protein kinase A), cGMP (protein kinase G), phospholipids (protein kinase C) and calcium (CAM kinase). De-activation occurs when cytosolic levels of these substrates drop, and dissociation occurs, or when kinases are modified by other signalling molecules to affect their sensitivity to activation co-factors. Protein kinase dependent protein activation is mitigated by protein phosphatases that hydrolyze the phosphoester bond attaching phosphate groups to effector proteins (93).
Although protein kinases are involved with ion handling, fuel metabolism and growth in the heart, of particular interest in this project is their role in contractility. In the heart, the beta-adrenergic system is strongly associated with the contractile state. Cardiac beta-adrenergic receptor agonism is associated with strong increases in inotropy, lusitropy and chronotropy. Beta-adrenergic receptor agonism relies on protein kinase A (PKA) at two levels with respect to cardiac contractility. Firstly, PKA impacts calcium handling in the sarcolemma and sarcoplasmic reticulum. Secondly, PKA affects contractility at the level of the myofibrils, which is the area of investigation of this project.

PKA is a signal transducer that is dependent on intracellular cyclic adenosine 3’5’-monophosphate (cAMP) levels for activation (Fig.5). PKA is a significant mediator of beta-adrenergic effect on contractility and glycogen metabolism in the heart. In fact, PKA’s effect on glycogen metabolism was the first effect ascribed to protein kinases (94). Glycogen metabolism is much more prominent in the liver and skeletal muscle in comparison to the normal heart (where most substrates are brought in exogenously), and even in times of cardiac hypoxia or ischemia, the internal carbohydrate stores in the heart are finite and inefficiently utilized (95). Therefore, moderating the beta-adrenergic signal on contractility is thought of as the main effect of PKA in the heart.

Beta-adrenergic receptors are activated by epinephrine/norepinephrine neurotransmitters from the sympathetic nervous system. In the canine heart, beta1 receptors are the only beta receptors in the ventricles, whereas in humans there are approximately 20% beta2 receptors in the ventricles (and 80% beta1). Both Beta-adrenergic receptor subtypes are associated with positive inotropic responses, although their signalling pathways are slightly different (103). We will focus on the more relevant Beta1-adrenergic receptors, which are coupled with the enzyme adenylyl cyclase by the stimulatory G protein, Gs. Adenylyl cyclase is the only cellular enzyme system that produces cAMP. When activated by Gs, adenylyl cyclase produces cAMP from ATP, which binds to the regulatory sub-units of PKA, thereby releasing the catalytic subunits to phosphorylate calcium channels, myofibrils, beta-adrenergic receptors
(in a negative feedback loop), and sarcoplasmic reticulum proteins (Fig. 5 and 6).

As mentioned, beta-adrenergic dependent effects of PKA on contractility will be discussed as it relates to the myofibril (vs. other contractility pathways activated by PKA, such as calcium handling in the sarcolemma and sarcoplasmic reticulum). In normal hearts, PKA increases the phosphorylation of both TnI and myosin binding protein C, which acts to decrease calcium sensitivity (96-98). This is thought to happen by increasing the off-rate of calcium binding to TnC. The diminished myofilament calcium sensitivity likely occurs by disrupting the interaction between TnC and the N-terminus region of TnI, since PKA phosphorylation happens at serines 23/24 in the N-terminus of TnI(99). It is logical that a decrease in calcium sensitivity would lead to decrease in contractility. Paradoxically, it leads to an increase in contractility (173-176). Evidence suggests that PKA mediates TnI phosphorylation, while decreasing calcium sensitivity, also increases the rate of crossbridge cycling (175), and also increases length dependent activation (174). This negative inotropy counters the concomitant positive inotropy of increased calcium cycling caused by other beta-Adrenergic PKA pathways – part of the checks and balances in homeostasis. PKA also improves rates of cardiac relaxation through TnI phosphorylation, which contributes to the chronotropic effects of beta-adrenergic receptor stimulation (173).

It is well established in both people and dogs that the beta-adrenergic system is down-regulated in heart failure (4,100-102). However, as we will see shortly, our understanding of how PKA responds in heart failure is not well understood. When the beta-adrenergic system is over stimulated, as is seen in heart failure, it down-regulates itself using both short and long term mechanisms. In the short-term, overstimulation of the beta-adrenergic receptors leads to self-inactivation by feedback molecules. For example, the beta-adrenergic receptor kinase (BARK) enzyme uncouples the beta-adrenergic receptor from the G-stimulatory protein, rendering the receptor inactive while also increasing its affinity for the inhibitory molecule arrestin (103). Another example of short term down-regulation is when PKA is present in high enough concentrations it will phosphorylate and inactivate beta-adrenergic receptors.
Long term mechanisms of beta-adrenergic desensitization include receptor internalization, sequestration and/or degradation (103).

With beta-adrenergic receptor down-regulation in the failing heart, one would expect to find a concomitant decrease in its messenger molecule PKA. It is thought that, since PKA phosphorylation of the myofilament is associated with a decrease in calcium sensitivity (lower affinity of calcium for TnC), then the increase in calcium sensitivity often seen in heart failure is due to down-regulation of the beta-adrenergic system (and the subsequent decreases in PKA and PKA-dependent phosphorylation). Following this logic, one would also expect an increase in myofilament calcium sensitivity with lower levels of PKA. In reality, the effects on myofilament function are not clearly defined. Many studies report decreased levels of TnI phosphorylation levels in heart failure (104-107), but some studies find no change in PKA and/or cAMP (104,108,109). With respect to myofilament regulation, many studies report an increase in calcium sensitivity during heart failure (102,110,111), but others find a decrease (112, 113). Therefore, there is considerable contradiction within the literature as to how PKA levels, and myofilament calcium sensitivity are altered in heart failure, if at all. In addition, there is little known about these qualities in dogs, and within this project we are able to investigate PKA-dependent TnI phosphorylation and calcium sensitivity in dogs with naturally occurring dilated cardiomyopathy.

**Protein Kinases - PKC**

PKC was first recognized as a protein kinase signal transducer in the brain, however, along with PKA, it is also a major protein kinase that affects myofilament function in the heart. Similar to PKA, PKC phosphorylates serine and threonine residues on target proteins. PKC activation in the heart is coupled by several pathways, including g-protein agonism of alpha-adrenergic and endothelin-1 receptors, and follows an analogous cascade of messengers as seen with PKA (Fig. 7). The functional influences of
PKC are ubiquitous, ranging from regulation of pH, myocyte hypertrophy/remodelling, calcium fluxes, and myofilament activation (116). In cardiac myofilaments, PKC is known to phosphorylate TnI, TnT and myosin binding protein C with the cumulative effect of decreasing calcium sensitivity and diminishing maximal contractility (116,117). However, given the breadth of pathways influenced by PKC, many of which are thought to converge on the end-point of myofilament function, the actual outcome of PKC phosphorylation on myofilament function can be highly variable, and increases in calcium sensitivity and contractility have also been observed (118,119). There is also evidence that PKC phosphorylation overrides PKA mediated phosphorylation in the myofilament (116).

PKC families can be categorized into three subfamilies: (i) Group 1, “classic”, includes the cardiac isoforms -alpha (α), -beta (β1/β2), and -gamma (γ), and are activated by diacylglycerol (DAG, or it's analogues) in the presence of calcium; Group 2, “novel”, is composed of cardiac PKC-delta (δ), -theta (η), -epsilon (ε), -mu (μ), and -eta (θ), and these isoforms are activated by DAG but do not require calcium; and Group 3, “atypical”, which includes PKC-zeta (ζ), -lamda (λ), and -iota (ι), is not responsive to DAG or calcium, and is activated by other lipid derived second messengers (93,115,120). All three groups require a phosphlipid such as phosphatidylserine (PtdSer) for activation (93, 115).

There is discrepancy as to which of the PKC isoforms are present in myocardial tissue, and in what species. For example, early work identified PKC- α, -δ, - ε, - η in cultured myocytes from rats (178). Other research has disputed this by identifying PKC-ζ, -β1 and -β2 in rat cardiomyocytes (181). Another study found no PKC-β in rats or human hearts, much higher levels of -ε in rats versus humans, and identified PKC-η in rats but not humans (179). Rabbits exhibit a yet different PKC cardiac profile (177). These PKC differences speak to the necessary caution when translating research findings from animal models, and the importance of consistent research protocols.

In heart failure, PKC isoforms have differing responses, suggesting that there are functional differences between the PKC isoforms. Of the cardiac PKC isoforms mentioned earlier (PKC-α, -β, -δ, -
η, -ε, -ζ), it has been reported that PKC-α, -β, and -δ are increased, while PKC-ε is unchanged in the failing heart (120,121). To the author’s knowledge, PKC-η and PKC-ζ have not been investigated in heart failure.

**Treatment**

There is no known cure for DCM. Treatment is palliative, and centred on improving quality of life and survival times. With respect to preventing the clinical manifestation of DCM in dogs, only one study exists which retrospectively evaluated the utility of using an angiotensin-converting enzyme inhibitor (ACEI; benazepril) in dogs with occult DCM; this study showed a beneficial effect of treating occult canine DCM with benazepril (45). Investigating potential therapeutic interventions during the occult phase of DCM is an area of ongoing research, and investigative trials are underway (see below).

Even with treatment, the prognosis for Doberman Pinschers with overt DCM is particularly poor compared to other breeds (59). Standard therapy for dogs in CHF due to DCM includes therapeutics that increases contractility, decrease preload and/or afterload. The only interventions for overt DCM that have undergone peer reviewed investigative clinical trials in improving survival and/or quality of life in DCM are an angiotensin converting enzyme inhibitor (ACEI; enalapril)(44) and a calcium sensitizer (pimobendan) (19,20). The enalapril study showed adequate safety and beneficial clinical attributes in dogs in CHF due to DCM or mitral regurgitation (44). Pimobendan is the newest addition to standard treatment regimes, and has revolutionized treatment of DCM in dogs. Pimobendan has been shown to dramatically improve survival times in Dobermans with CHF due to DCM, when used along with standard treatments such as ACEIs, furosemide +/- digoxin (19,20). A marked improvement in quality of life was reported in one study (19). It is unclear if the improved mortality and morbidity seen with pimobendan therapy in DCM Dobermans with CHF is due to correction of a primary myofilament abnormality or masking a non-myofilament dysfunction which will be the subject of further discussion.
Clinical studies of pimobendan in human heart failure show a decreased number of hospitalizations, increased activity, increased or stable quality of life, increased cardiac index, decreased pulmonary wedge pressures and decreased norepinephrine levels, and no increased risk of arrhythmia at 6 month follow up (47-55). One study, although not designed as survival study, found a non-significant risk of mortality during the 24 week clinical trial (53). There is concern about calcium sensitizers having an increased risk of inducing diastolic dysfunction, by sensitizing myocardium to low levels of calcium, however this is not typically seen with pimobendan, and in fact lusitropy may be improved with pimobendan therapy (62). As with other calcium sensitizing drugs, pimobendan has some level of phosphodiesterase III (PDE III) inhibition. The PDE inhibitory potential of pimobendan and other calcium sensitizers has sparked concern that this class of drug could cause arrhythmias as is seen with other PDE III inhibitors such as milrinone. However, increased arrhythmias have not been linked to calcium sensitizers in patients (see below). Pimobendan is not currently licensed for human use in any country other than Japan (58). Clinical trials with pimobendan in Doberman's with CHF secondary to DCM show a marked improvement in mortality; Dobermans treated in overt disease have a median survival ranging from 130.5-329 days, while untreated dogs in CHF live 14-50 days (19,20). The potential clinical utility of pimobendan in occult Doberman DCM is currently under investigation.

Levosimendan is the only other calcium sensitizer currently in medical use. Compared to pimobendan, it produces an increased level of calcium sensitization, has a lower level of PDE inhibition (46,62). Levosimendan is thought to stabilize calcium binding conformation, but not affinity for TnC, and is thought to have additional vasodilatory properties by potassium channel regulation in the vasculature (46,62). Similar to pimobendan, levosimendan has not shown evidence of inducing diastolic dysfunction, although its arrhythmogenic potential is an area of some controversy (62). Clinical trials with levosimendan in people are limited to acute hemodynamic studies (46); it is licensed in many countries
for intravenous injection to human patients in refractory CHF, but has also been suggested for use in acute heart failure as well (63). Levosimendan has not been approved in the U.S. or Canada, and recent announcements citing commercial unviability are halting Phase III trials in North America (57). Levosimendan has not yet been evaluated in a natural canine disease model, but an abstract proceeding from 2009 describes a preliminary clinical trial for dogs in CHF (66).

**Calcium Sensitizers: Mechanism of Action**

Pimobendan is a benzimidazole-pyridazinone inodilator. During normal physiological states, pimobendan is thought to exhibit its venodilatory, arteriodilatory and inotropic effects primarily through inhibition of phosphodiesterase (PDE) III, via prolongation of cAMP and cGMP mediated increased efflux of intracellular calcium from vascular smooth muscle (70). Systemically, this correlates to a decrease in preload, afterload, and an increase in myocardial contractility. Unlike traditional PDE inhibitors, such as milrinone and amrinone, which have been associated with pro-arrhythmogenic properties in humans (65), pimobendan does not increase the risk of arrhythmia in humans or dogs (with the exception of one clinical trial in people that found an increased risk of ventricular premature contractions (VPCs) on Holter examination at 14 days follow up but not at 6 month follow up, and not associated with any clinical events)(20,47,52). Additionally, pimobendan does not increase myocardial oxygen demand or energy consumption (69,70). In fact, one study suggested that pimobendan use in human heart failure may lower myocardial oxygen demand (70).

During heart failure, the effects of PDE inhibition may be blunted due to down regulation of cAMP pathways secondary to beta-adrenergic and G-protein complex down-regulation (6,68). It is therefore hypothesized that the calcium sensitizing properties of pimobendan predominate during heart failure.

Calcium sensitization describes an inotropic effect secondary to increased affinity of existing intracellular calcium for cardiac troponin C (cTnC) (71,72). Calcium sensitizing drugs, such as
levosimendan and pimobendan, trigger troponin-calcium sensitivity without a calcium overload that would disrupt other calcium based signalling pathways in cardiomyocytes, or induce diastolic dysfunction through reduced lusitropy. This effect is triggered by calcium sensitizers binding at the junction between cNTnC (N regulatory region of cTnC) and the switch region of cTnI (84,85). This junction is critical for activating key cNTnC hydrophobic residues. These residues bind to the hydrophobic residues on TnI in a hydrophobic groove (84,85). This interaction leads to the movement of the adjoining inhibitory and C-terminal regions of cTnI away from actin, and releases the inhibition of the actomyosin ATPase, leading to muscle contraction (81). Thus, these compounds exert their effects by altering the dynamic equilibrium between closed and open cNTnC conformations, enhancing its affinity for cTnI and thereby increasing the calcium sensitivity of cardiac troponin (75-81).

Calcium sensitizers have been found to have unique binding sites on TnC (86). Bepridil is a calcium channel blocker, calmodulin antagonist and calcium sensitizer for which three binding sites on TnC have been identified (86). Levosimendan binds directly to the N-domain of calcium saturated TnC (86). Anapoe, a detergent, binds the switch region of TnI to enhance TnC calcium binding (86). Several other drugs have also been mapped to unique regions of TnC, which could be used to create a functional map of the cardiac troponin complex. This project looks at levosimendan and bepridil and how each alters myofilament function in DCM.

**Rationale and Hypothesis**

As previously mentioned, genetic mutations of myofilament proteins can lead to DCM. Several of these mutations have been identified in human DCM (38). Thus far, investigations into these mutations in canine DCM have been unfruitful (39,40,42,43), but have been far from exhaustive. Interestingly, despite the lack thus far to identify a causative relationship between myofilament protein mutation and DCM, the standard of care for canine DCM includes pimobendan – a calcium sensitizer that acts directly on cardiac
myofilaments (see treatment section above). Given that myofilament mutations are a common cause of DCM, and drugs that act on the myofilament are standard treatment for DCM, we hypothesize that myofilament function and regulation is altered in canine DCM.

More specifically, we put forward the following hypotheses: (i) we hypothesize that myofilament function is altered in naturally occurring canine DCM; (ii) we hypothesize that calcium sensitizers bepridil and levosimendan will correct myofilament dysfunction seen in naturally occurring canine DCM; and (iii) we hypothesize that myofilament associated PKA/PKC, and myofilament protein phosphorylation and expression is altered in naturally occurring canine DCM.
**Introduction: Tables and Figures**

**Table 1.** Contractile Proteins of the Heart (adapted from Katz, ref. 82)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Location</th>
<th>Approximate Molecular Weight (Daltons)</th>
<th>Number of Components</th>
<th>Salient Biochemical Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin</td>
<td>Thick Filament</td>
<td>500 000</td>
<td>2 heavy chains, 4 light chains</td>
<td>ATP hydrolysis, interacts with actin</td>
</tr>
<tr>
<td>Actin</td>
<td>Thin Filament</td>
<td>42 000</td>
<td>One</td>
<td>Activates myosin ATPase, interacts with myosin</td>
</tr>
<tr>
<td>Tropomyosin</td>
<td>Thin Filament</td>
<td>70 000</td>
<td>Two</td>
<td>Modulates actin-myosin interaction</td>
</tr>
<tr>
<td>Troponin C</td>
<td>Thin Filament</td>
<td>17 000</td>
<td>One (which contains four EF hands (alpha helical regions))</td>
<td>Calcium binding</td>
</tr>
<tr>
<td>Troponin I</td>
<td>Thin Filament</td>
<td>30 000</td>
<td>One</td>
<td>Inhibits actin-myosin interactions</td>
</tr>
<tr>
<td>Troponin T</td>
<td>Thin Filament</td>
<td>38 000</td>
<td>One</td>
<td>Binds troponin complex to the thin filament</td>
</tr>
</tbody>
</table>
**Figure 1.** Cross bridge formation. In the resting heart (left), the cross-bridges project almost at right angles to the longitudinal axis of the thick filaments. In the contracting heart (right) the cross-bridges draw the thin filaments toward the center (82).
Figure 2. Simplified four step reaction mechanisms for actomyosin ATPase. The process starts in diastole (lower right) where ATP binding to myosin has caused the muscle to relax by dissociating actin and myosin. Hydrolysis of myosin bound ATP transfers the energy of the nucleotide to the cross-bridge, but because the latter is not attached to actin, the muscle remains in a relaxed, energized state (lower left). Interaction of the cross-bridge with actin and release of Pi leads to the formation of a low energy rigor bond between the cross-bridge and thin filament; expenditure of chemical energy in this step allows the muscle to perform mechanical work (upper right). The cycle ends, and the muscle returns to its resting state, when ATP binding to the rigor complex dissociates the myosin cross-bridge from actin (82,83).
**Figure 3.** Myosin molecule that comprises the thick filament. Each myosin contains 2 heavy chains which wind together to constitute the “tail”, and continue into the paired “heads”. The hinges (circles) are the points of flexibility. (82).
**Figure 4.** Cross section of a thin filament at a region containing the troponin complex and tropomyosin in the resting (left) and active (right) state. At rest, the troponin complex holds the tropomyosin molecules toward the periphery of the groove between adjacent actin strands, which prevents actin from interacting with the myosin cross-bridges. In active muscle, calcium binding to troponin C weakens the bond linking troponin I to actin, which rearranges the regulatory proteins so as to shift tropomyosin deeper into the groove between the strands of actin, thereby exposing the active sites on actin for interaction with the myosin cross-bridges (82).
**Figure 5.** Protein kinase A (PKA) activation for signal transduction is dependent on cyclic AMP. When cyclic AMP levels are sufficiently high in the intracellular environment, they bind the regulatory subunits of PKA. When the regulatory sub-units are bound by cyclic AMP, the catalytic sub-units are activated and released.
Figure 6. PKA Signal Transduction in the Heart. Beta receptor agonism by epinephrine/norepinephrine leads to G-protein activation. G-protein activation increases cAMP levels via adenylyl cyclase. cAMP binds to the regulatory subunits of PKA, thereby releasing the active PKA subunits to phosphorylate contractile proteins (myofilaments).
Figure 7. Signalling pathways mediated through phospholipid hydrolysis. Binding of endothelin-I (ET-I) or α,-adrenergic agonists to their cell membrane receptors results in Gq dissociation and GTP activates the membrane-bound enzyme phospholipase C (PLCP) which stimulates hydrolysis of the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP2) to produce two second messengers: hydrophilic inositol-1,4,5-triphosphate (IP₃) and lipophilic diacylglycerol (DG). IP₃ regulates intracellular Ca²⁺ movements, but it is probably of limited importance in this regard in cardiac myocytes. DG activates classical and novel protein kinase C (PKC) isoforms in a process that involves translocation of soluble PKC to the membrane. PKC directly phosphorylates or indirectly brings about the phosphorylation of a number of proteins (93).
**Materials and Methods**

*Animal Use.* Canine tissue donors were treated according to standards of care in veterinary medicine.

*Canine Population:* Dogs were evaluated at the Health Sciences Centre, Ontario Veterinary College (Maggie Schuckman, DVM, ACVIM (cardiology); M. Lynne O'Sullivan ACVIM (cardiology), DVM; Elizabeth Sinclair, DVM) or at veterinary facilities in southern Ontario (Elizabeth Sinclair). Humane euthanasia elected by each owner was performed using intravenous injection of pentobarbital sodium (Schering-Plough) at a dose of 120 mg/kg. Immediately following euthanasia, tissue samples were obtained from the left ventricular free wall of dogs with end-stage DCM, or non-failing control dogs. Absence of cardiovascular disease in controls was based on normal physical examination and absence of histopathological changes in the myocardium. The diagnosis of DCM was made on the basis of echocardiography and confirmed post-mortem with gross pathology and histopathology. Written consent was obtained from clients for all procedures, including the post-mortem collection of myocardial samples.

*Echocardiography:* All measurements were performed using an echocardiographic system (Vivid 7 Dimension, GE Medical Systems, Milwaukee, Wisconsin) equipped with a 3 or 5 MHz transducer. Dogs were in left or right lateral recumbency with the imaging probe in the dependent position. A simultaneous, one-lead electrocardiogram was available throughout the study. Standard echocardiographic measures were performed to assess for chamber enlargements as well as systolic and diastolic dysfunction. Fractional shortening, left ventricular internal diameter in diastole (LVIDd), and systole (LVIDs) were measured using M-mode from the right parasternal long axis view of the left ventricle.
**Tissue Collection:** Following euthanasia, a standard thoracotomy was performed so as to exteriorize and incise the pericardial sac. Myocardium was sectioned from the left ventricular free wall and 1 cm$^3$ tissue samples were collected and placed into liquid nitrogen within 5 minutes of death, which has been shown to preserve protein biochemistry in human myocardium (122). Samples were then transferred to -80° C for long term storage.

**Pathology:** After tissue collection the remainder of the heart was placed in 10% formalin. Standard gross pathology was performed, including removal of the apex followed by sequential opening of the heart chambers along the flow of blood starting where the cranial and caudal vena cava enter the right atrium. Tissue was then trimmed for histopathology by taking longitudinal and transverse sections from the left and right ventricular free wall and septum. Tissue was embedded with paraffin, sectioned at a thickness of 7 μm, and stained with hematoxylin and eosin (HE). Slides were evaluated using light microscopy by a board-certified veterinary pathologist (Jeff Caswell, DVM, ACVP, PhD).

**Myofilament Isolation:** Cardiac myofilaments were prepared according to a modified protocol from Pyle et al (123). Briefly, hearts were homogenized using a tissue grinder in ice-cold Standard Buffer and centrifuged at 12,000g for 15 min at 4°C. The pellets were resuspended in ice-cold Standard Buffer containing 1% Triton X-100 and gently agitated at 4°C for 45 min. The suspension was washed three times (1,100g for 15 min at 4°C) in ice-cold Standard Buffer. Protein concentration was measured with a Bradford Protein Assay (Bio-Rad Laboratories Ltd., Mississauga, ON).

**Actomyosin MgATPase Assay:** Isolated cardiac myofilaments (50 μg) were incubated in reaction buffers containing variable free calcium concentrations made by mixing Activating and Relaxing buffers. Free
calcium was calculated using the program from Patton et al (125). Myofilaments were incubated in reaction buffers for 10 min at 32°C, and then quenched with ice-cold 10% trichloroacetic acid. Actomyosin MgATPase activity was determined using a modified Carter assay (124). Therefore, inorganic phosphate produced from ATP consumption was measured following the addition of an equal volume of 0.5% FeSO$_4$ and 0.5% ammonium molybdate in 0.5 M H$_2$SO$_4$, and after 10 minutes reading the absorbance at 630 nm on a spectrophotometer. Actomyosin MgATPase activity was plotted against free calcium and curves fit using the Hill equation (182).

**Calcium Sensitizers:** Calcium sensitizing drugs were added to the free calcium solutions used in the actomyosin MgATPase assay. Based on previously published reports, levosimendan (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the reaction buffer as a 10 µM treatment (166), and bepridil (Santa Cruz Biotechnology, Santa Cruz, CA) was added as a 100 µM treatment (167). Both drugs were handled as light sensitive and made fresh for each assay, using ethanol for reconstitution.

**Myofilament Protein Phosphorylation:** Myofilament proteins (10 µg) were separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gels were fixed overnight in 50% methanol-10% acetic acid at room temperature. Gels were incubated for 2 hours at room temperature with Pro-Q Diamond (Molecular Probes, Eugene, OR) to stain phosphorylated proteins. Gels were imaged with a Typhoon gel scanner (green laser 532 nm excitation, 560 nm longpass emission filter), GE Healthcare, Baie d’Urfé, PQ), and analyzed with ImageJ software (NIH, Bethesda, MD). Protein loading was assessed by Coomassie staining of gels after Pro-Q Diamond imaging.

**Immunoblotting:** Immunoblotting was done using a modified protocol from Pyle et al (126). Myofilament proteins (100 µg for PKC; 40 µg for myofilament proteins) were resolved by SDS-PAGE
using 10% (PKC) or 12% (myofilament proteins) separating gels. Proteins were transferred to nitrocellulose membranes and probed with primary antibodies overnight at 4°C. Antibodies for PKC-α, -δ, -ε, -ζ (BD Biosciences, Mississauga, ON, and Millipore, Billerica, MA), cardiac troponin T, cardiac troponin I, tropomyosin (Santa Cruz Biotechnology, Santa Cruz, CA), myosin light chain 2 (a gift from Dr. T.E. Gillis), or phosphorylated S23/S24 cardiac troponin I (BD Biosciences, Mississauga, ON, and Millipore, Billerica, MA) were diluted 1:1,000. Antibody for myosin binding protein-C (T.E. Gillis) was diluted 1:25,000. Protein loading for PKC immunoblots was assessed by stripping membranes with 0.1 M glycine (pH 2.9) and re-probing with an anti-cardiac troponin I antibody. Secondary antibodies (Sigma-Aldrich, Oakville, ON) conjugated to horseradish peroxidase were used at 1:5,000 dilution. Protein bands were detected using Western Lightning (PerkinElmer Life and Analytical Sciences, Woodbridge, ON), and analysis of band density was done using ImageJ software.

**Solutions:** Standard buffer contained 60 mM KCl, 30 mM imidazole (pH 7.0), 2 mM MgCl₂, 0.01 mM leupeptin, 0.1 mM PMSF, and 0.2 mM benzamidine. Activating buffer contained 23.5 mM KCl, 5 mM MgCl₂, 3.2 mM ATP, 2 mM EGTA, 20 mM imidazole, 2.2 mM CaCl₂, 0.1 mM PMSF, 0.01 mM leupeptin, and 0.2 mM benzamidine (pH 7.0). Relaxing buffer contained 26 mM KCl, 5.1 mM MgCl₂, 3.2 mM ATP, 2 mM EGTA, 20 mM imidazole, 4.9 µM CaCl₂, 0.1 mM PMSF, 0.01 mM leupeptin, and 0.2 mM benzamidine (pH 7.0).

**Statistical Analysis:** All data are shown as mean ± SEM. Statistical analysis was done using a one-way ANOVA and post-hoc t-test in Microsoft Excel. P<0.05 was considered statistically significant.
Results

Subject Selection

A total of six dogs in end stage DCM were included for molecular analysis in this study, as were an equal number of dogs identified as non-failing controls. However, because echocardiography was not available in all non-failing controls, the echocardiography analysis outlined in Figure 8 and Table 2 was from 6 DCM Doberman Pinschers and 6 normal Doberman Pinschers, all of whom were not donor dogs for the remaining of the investigation in this study. Also because echocardiography was not available for all control donors, there is a possibility that they had undetected cardiac disease. Together, this implies that there could be a disjunction between how we have characterized canine DCM in this study, our comparison to controls, and the myofilament function and protein analysis we draw conclusions from.

DCM donors included three Doberman Pinschers, one Newfoundland, one Labrador retriever and one Boxer. There were 5 males (neutered) and 1 female (spayed). Non-failing controls were weight matched mixed breed dogs, which were 4 female (3 spayed) and 2 male (1 neutered). Because of the high prevalence of DCM in large breed dogs including Doberman Pinschers, Newfoundlands and Boxers, it is generally recommended that these breeds not be used as non-failing controls. However, one non-failing Doberman Pinscher was included in the analysis, and results did not differ from other non-failing samples in any assay (data not shown). Donor dog characteristics are outlined in Table 2. From an original nine non-failing control dogs, three were excluded from all parts of the study on the basis of post-mortem diagnosis of heartworm infection.

Echocardiographic and Histological Analysis of Myocardium

DCM diagnosis was made on the basis of echocardiographic and physical examinations, and confirmed with post-mortem and histological staining. Echocardiography revealed a characteristic dilated
ventricular cavity and impaired systolic function in DCM dogs (Figure 8, Table 2). Non-failing control dogs were not in clinical heart failure and had structural histological values that were within the normal range, however as mentioned previously, most of them did not have echocardiography performed.

Histological assessment with H/E staining of myocardial samples revealed no pathological findings in the non-failing samples, but the fatty infiltration-degenerative findings in DCM samples. Also in DCM samples, hypereosinophilic areas consistent with early stages of myocardial necrosis were seen (Figure 9). These might represent acute hypoxic episodes experienced just prior to, or during death, and could have a confounding influence on our data interpretation.

**Table 2.** Animal Population Characteristics of Donor Dogs included in Myofilament and Molecular Analysis. *P<0.05

<table>
<thead>
<tr>
<th></th>
<th>Non-Failing Controls</th>
<th>DCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>5.6 +/- 0.8</td>
<td>6.8 +/- 1.3</td>
</tr>
<tr>
<td>Sex</td>
<td>2 males (1 neutered)</td>
<td>5 males (neutered)</td>
</tr>
<tr>
<td></td>
<td>4 females (3 spayed)</td>
<td>1 female (spayed)</td>
</tr>
<tr>
<td>Breed</td>
<td>Mixed breed (5), Doberman Pinscher</td>
<td>3 Doberman Pinscher, 1 Boxer, 1 Newfoundland, 1 Labrador Retriever</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>32.8 +/- 3.9</td>
<td>36.5 +/- 7.1</td>
</tr>
</tbody>
</table>
Figure 8. Echocardiographic changes in naturally occurring DCM. M-mode images from non-failing Doberman Pinscher dogs (left) and failing DCM Doberman Pinscher dogs. Summary data of echocardiographic assessments are presented in Table 2.

Table 3. Echocardiographic parameters from hearts of non-failing and dilated cardiomyopathic dogs. Data was collected from Non-failing and DCM Doberman Pinschers. *P<0.05

<table>
<thead>
<tr>
<th></th>
<th>Non-Failing (n=6)</th>
<th>DCM (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Weight (kg)</strong></td>
<td>36.1 ± 4.4</td>
<td>38.1 ± 6.2</td>
</tr>
<tr>
<td><strong>Left Ventricular End Diastolic Diameter (mm)</strong></td>
<td>38.4 ± 2.3</td>
<td>60.4 ± 7.1*</td>
</tr>
<tr>
<td><strong>Left Ventricular End Systolic Diameter (mm)</strong></td>
<td>29.2 ± 3.7</td>
<td>55 ± 6.2*</td>
</tr>
<tr>
<td><strong>Fractional Shortening (%)</strong></td>
<td>24.0 ± 7.2</td>
<td>7.7 ± 4.7*</td>
</tr>
<tr>
<td><strong>Heart Rate (bpm)</strong></td>
<td>113 ± 12</td>
<td>128 ± 27</td>
</tr>
</tbody>
</table>
Figure 9. Histological changes seen in naturally occurring canine DCM. Ventricular samples were taken from non-failing and DCM dogs and processed for histopathology with HE staining. Non-failing dogs (40x, top) exhibited no detectable abnormalities in myocardial histology. Myocardial samples from dogs diagnosed with DCM (10x centre and 40x bottom) demonstrated abnormal histologic changes suggestive of early stage necrosis (centre, region indicated by arrows), and/or fatty infiltration (bottom, arrowheads to representative areas).
Cardiac Myofilament Function in DCM and Non-failing Control Dogs, and in Response to Calcium Sensitizers

Actomyosin MgATPase activity was measured using myofilaments isolated from the left ventricles of DCM and non-failing control dogs. Actomyosin MgATPase activity at saturating levels of free calcium was 11% lower in DCM samples, as compared to non-failing controls (Figure 10). Normalized curves demonstrated a left-ward shift in the ATPase-calcium curve in DCM samples, illustrated by a significant reduction in the calcium concentration defined by mid-point ATPase activity (EC$_{50}$) from 0.31 ± 0.02 μM in non-failing controls to 0.23 ± 0.02 μM in DCM. Co-operativity as determined by the Hill coefficient was not different between groups.

Actomyosin MgATPase activity was also measured when the isolated myofilaments were incubated with one of the calcium sensitizing drugs bepridil or levosimendan. Treatment with levosimendan did not exhibit significant change. Myofilaments increased their level of sensitivity to calcium in response to bepridil treatment as measured by change in EC$_{50}$ (P<0.05) (Figure 11, Table 3). The DCM myofilaments had a significant but attenuated response to bepridil (P<0.05) (Table 3).
Figure 10. Cardiac myofilament function in non-failing and end-stage DCM dogs. A. Actomyosin MgATPase activity in isolated myofilaments from non-failing and end-stage DCM dogs, across a range of free calcium concentrations. Calcium activation was not significantly different between groups. B: Normalized actomyosin MgATPase-calcium curves demonstrate a left-ward shift in DCM myofilaments. C: Cardiac myofilaments from DCM hearts exhibited a significant decrease in EC_{50}, which is consistent with the left-ward shift seen in the normalized actomyosin MgATPase-calcium curves (B). D: Myofilament co-operativity as measured by the Hill coefficient was not different between non-failing and DCM samples. *P<0.05 vs Non-failing.
Figure 11. Cardiac myofilament function in response to treatment with calcium sensitizing drugs. Bepridil treatment of isolated myofilaments from non-failing controls (A) and from DCM dogs (B) showed a significant leftward shift in calcium sensitivity at the midpoint (EC$_{50}$). In comparison, levosimendan treatment of isolated myofilaments from non-failing controls (C) and from DCM dogs (D) did not show a significant change in response. Changes in EC50 are shown in Table 3.
Table 4. Comparison of actomyosin MgATPase Activity for normal and DCM hearts, with and without Bepridil or Levosimendan treatment. *P<0.05 vs untreated paired samples.

<table>
<thead>
<tr>
<th></th>
<th>Maximum Activity (nM Pi/min/mg protein)</th>
<th>EC\textsubscript{50} (μM Calcium)</th>
<th>Change in EC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>75.9 ± 4.7</td>
<td>0.31 ± 0.02</td>
<td>-</td>
</tr>
<tr>
<td>DCM</td>
<td>67.8 ± 5.0</td>
<td>0.23 ± 0.02</td>
<td>-</td>
</tr>
<tr>
<td>Normal + Levo</td>
<td>80.8 ± 18.2</td>
<td>0.23 ± 0.04</td>
<td>0.063 ± 0.03</td>
</tr>
<tr>
<td>DCM + Levo</td>
<td>64.8 ± 14.1</td>
<td>0.20 ± 0.02</td>
<td>0.026 ± 0.02</td>
</tr>
<tr>
<td>Normal + Bep</td>
<td>83.3 ± 7.6</td>
<td>0.13* ± 0.01</td>
<td>0.163 ± 0.03</td>
</tr>
<tr>
<td>DCM + Bep</td>
<td>89.1 ± 12.1</td>
<td>0.12* ± 0.02</td>
<td>0.082 ± 0.03</td>
</tr>
</tbody>
</table>
**Myofilament Protein Phosphorylation in DCM and Non-failing Control Dogs**

Changes in the phosphorylation status of cardiac myofilament proteins can mediate both acute and chronic changes in myofilament function. Cardiac myofilament proteins were separated by SDS-PAGE and stained with ProQ Diamond to quantify total protein phosphorylation levels. Densitometric analysis of Coomassie-stained gels was used to quantify total protein levels for phosphorylation. Protein phosphorylation levels normalized to protein load demonstrated significant increases in total desmin and tropomyosin phosphorylation in DCM hearts, as compared to non-failing controls. (*P<0.05 vs Non-failing). Staining of phosphoproteins revealed a 59 ± 14% increase in desmin phosphorylation and a 45 ± 7% increase in tropomyosin phosphorylation in DCM myofilament proteins, as compared to non-failing controls (P<0.05)(Figure 12). Troponin T, myosin light chain, myosin binding protein C and troponin I exhibited no detectable changes in total phosphorylation.

**Myofilament Protein Changes in canine DCM**

Cardiac myofilament protein expression levels were individually quantified using immunoblotting. Of the myofilament proteins investigated, desmin from DCM samples showed a significant increase in total protein levels as compared to non-failing controls (Figure 13). Protein expression levels were measured and normalized to non-failing canine samples. Desmin protein levels were increased by 137 ± 19%. Interestingly, immunoblots showed a second, unidentified tropomyosin band that was present in more DCM samples than non-failing controls. Doublets were observed in 3 of 6 non-failing canine samples and 5 of 6 DCM samples. (Figure 13).
**Figure 12.** Cardiac myofilament protein phosphorylation is altered in end-stage canine DCM. A: total protein phosphorylation levels of cardiac myofilament proteins. B: total protein levels of myofilament proteins. C: Protein phosphorylation levels normalized to protein load demonstrated significant increases in total desmin and tropomyosin phosphorylation in DCM hearts, as compared to non-failing controls. *P<0.05 vs Non-failing.
Figure 13. Expression of cardiac myofilament protein desmin is increased in canine DCM. A: Levels of individual myofilament proteins were quantified by immunoblot analysis. Representative images of each immunoblot are shown. B: Immunoblots for tropomyosin revealed two distinct bands. Doublets were observed in 3 of 6 non-failing canine samples and 5 of 6 DCM samples. Bottom: Protein expression levels were measured and normalized to non-failing canine samples. Desmin protein levels were found to be significantly elevated in DCM samples. *P<0.05 vs Non-failing.

Key: NF, non-failing control; MyBP-C, myosin binding protein C; TnT, troponin T; TnI, troponin I; MLC2, myosin light chain 2.
Myofilament Regulation by PKA in DCM and Non-failing Control Dogs

Beta-adrenergic control of cardiac myocytes is often disrupted in DCM. Troponin I is a well known target of the beta-adrenergic signaling system through the phosphorylation of two N-terminal serines (S23/S24), and targeting of these residues can impact myofilament calcium sensitivity. Phosphorylation of N-terminal cardiac troponin I is reduced in end-stage canine DCM (Figure 14). Total troponin I phosphorylation was measured on an SDS-PAGE gel using ProQ Diamond staining, and found to be not different between non-failing and DCM samples. Total TnI was also found to not be different between groups. Antibodies for S23/S24 troponin I phosphorylation revealed a significant 25% decrease in N-terminal phosphorylation in DCM samples compared to non-failing controls. Immunoblotting for total troponin I showed no significant changes in troponin I protein levels between the two groups. *p<0.05 (Figure 14).

Myofilament-associated PKC is altered in DCM dogs

A reduction in troponin I phosphorylation by the beta-adrenergic system (PKA), coupled with no change in total troponin I phosphorylation suggests that there is a commensurate increase in troponin I phosphorylation at sites other than the N-terminus. The PKC family of signalling molecules can target several of the remaining sites in troponin I and are known to be upregulated in heart failure. Immunoblots of cardiac myofilament proteins revealed a 55 +- 29% and 257 +- 72% increase in PKC-δ and -ζ protein levels respectively, and a 25 +- 11% reduction in PKC-ε levels in DCM samples (Figure 15). There was no change in myofilament-associated PKC-α in dogs with DCM. These data are the first to determine myofilament-associated PKC levels in failing hearts.
Figure 14. Phosphorylation of N-terminal cardiac troponin I is reduced in end-stage canine DCM.  
A: Total troponin I phosphorylation was measured on an SDS-PAGE gel using ProQ Diamond staining (top), and found to be not different between non-failing and DCM samples. Total TnI (bottom) was also found to not be different between groups.  
B: Antibodies for S23/S24 troponin I phosphorylation revealed a significant decrease in N-terminal phosphorylation in DCM samples compared to non-failing controls (top). Immunoblotting for total troponin I showed no significant changes in troponin I protein levels between the two groups (bottom).  
C: Normalization of S23/S24 troponin I phosphorylation to total troponin I protein levels show a significant reduction in DCM samples. *p<0.05.  
Key: NF, non-failing control; TnI, troponin I.
**Figure 15.** Myofilament associated protein kinase C (PKC) levels are altered in end-stage canine DCM. A: Myofilament samples were probed on an SDS-PAGE gel from the presence of PKC-α, -δ, -ε, and -ζ. Protein loading was determined by probing for cardiac troponin I. Representative blots are shown. B: Myofilament associated PKC-α and PKC-ε protein levels exhibit no significant difference between non-failing and DCM samples. PKC-δ and PKC-ζ were both significantly increased in the myofilament compartment of DCM hearts. *P<0.05 vs Non-failing.
Discussion

This study is the first investigation of myofilament changes in naturally occurring canine DCM, and establishes a viable large animal model of disease. In support of our first hypothesis that myofilament function is altered in canine DCM, we report the novel finding that actomyosin MgATPase activity is altered in cardiac myofilaments from canine DCM hearts. In contradiction to our second hypothesis that calcium sensitizing drugs correct a myofilament dysfunction in DCM, we report that isolated myofilaments from DCM do not show a demonstrated deficiency with the actinomyosin MgATPase assay. In support of our third hypothesis that myofilament regulation is altered in DCM, we show evidence of the reduction in troponin I phosphorylation at the putative PKA sites. This suggests that beta-adrenergic receptor signalling is disrupted in DCM dogs. We also report increases in phosphorylated desmin and tropomyosin, and present the novel finding that several myofilament-associated PKC isoforms are affected in DCM, with increases in PKC-δ and -ζ, and a reduction in PKC-ε.

Naturally Occurring Canine DCM as a Model of Heart Failure

The rapid ventricular pacing model of heart failure in the dog produces similar changes in heart structure, myocardial function, as well as molecular and humoral alterations, as those of human heart failure patients (127,128). While there is no doubt that substantive advances have been made using rapid ventricular pacing as a pathological trigger, there are important differences between this experimental model of heart failure and the naturally occurring clinical entity. One of the most significant limitations of the rapid ventricular pacing model is the timeline of disease development. The pathological trigger (rapid ventricular pacing) is a sudden onset stressor that produces heart failure within 3-5 weeks in a (presumably) genetically normal heart. The abrupt onset of the cardiac stressor in an otherwise healthy animal does not necessarily recapitulate the phenotypical timeline of naturally occurring DCM conditions.
Our investigation of dogs in the end stages of DCM provides the advantage of yielding samples obtained following the normal disease progression of a naturally occurring cardiomyopathy in a manner that maximizes the core guidelines of reduction, refinement and replacement for animal use as set by the Canadian Council on Animal Care (CCAC), by capitalizing on a current and ongoing patient population.

It is worth noting the gender variation present within our sample population. Five of the six DCM donors were neutered males, with one spayed female. In comparison, the control population was composed of four females (three spayed, one intact) and two males (one neutered, one intact). In humans, it is well established that there are gender differences between men and women with respect to heart disease and failure, and worth acknowledging that the majority of large clinical trials under-represent women (184). For women, the risk of heart disease increases dramatically after menopause, and estrogen is thought to play an important role in this process (184). We also know that males and females respond differently to heart failure medications, such as digoxin (184). To the best knowledge of the author, there is no reported gender predisposition to the development of idiopathic DCM in people. As discussed in the introduction, there is thought to be a modest increased predisposition to developing DCM in male Doberman Pinschers compared to females, and there are differences seen in the clinical course of disease in female versus male DCM Dobermans (9, 28). However the role of sex hormones in these dogs is unknown. Given the gender and reproductive status variability in our study population, we must be cognisant of the potential role these factors play in our analysis and interpretation of data.

**Myofilament Function in DCM Hearts**

The data presented in this paper are the first investigation of myofilament function in naturally occurring canine DCM. Our data show that EC$_{50}$ is reduced in canine DCM myofilaments as compared to non-failing myofilaments. While the normalized actomyosin MgATPase-calcium data presented in Figure 10 clearly show a significant reduction in EC$_{50}$, raw data presented in the same figure indicate that
absolute changes at sub-maximum calcium levels are not significantly different between the two groups.

The lack of significant difference in sub-maximum calcium activation of non-failing and DCM actomyosin MgATPase activity does not necessarily mean that cardiac myofilaments are functioning properly. Normal myofilament function requires that ATP consumption be coupled with force generation. In canine DCM hearts an increased energy barrier between the weakly and strongly bound cross-bridge state causes the myofilaments to consume ATP without generating a power stroke (129). Such an uncoupling between actomyosin MgATPase activity and force production may contribute the systolic impairment that characterizes DCM hearts. It is also possible to speculate on other changes that could result in altered MgATPase activity, but not significantly different at sub-maximum calcium ranges. Perhaps the canine DCM myofilaments are unable to transfer force to the cellular cytoskeleton, due to dysfunction at the level of the anchoring proteins. This is seen with desmin mutations in people, and transgenic desmin deficiencies in mice, that develop DCM through a lack of mechanotransduction ability (32). As a canine example, variation of the genetic coding for the actin anchoring protein α-actinin has been identified in some Doberman Pinschers, suggesting altered myofilament anchoring as a component of canine DCM and warranting further investigation (164).

**Calcium Sensitizers in DCM Myofilaments**

Previous groups have established that both bepridil and levosimendan increase calcium sensitivity in isolated myofilaments from pig and guinea pigs, respectively (166-167). Our work is able to confirm this effect in normal canine cardiac myofilaments. To the best of our knowledge, bepridil has not been investigated in DCM myofilaments and we report that bepridil significantly increases calcium sensitivity in both normal and DCM canine myofilaments. Brixius et al (168) investigated the effects of levosimendan on human idiopathic DCM myocardium. They found that levosimendan increased myocardial calcium sensitivity in non-failing myocardium, but only increased calcium sensitivity in DCM
myocardium if it was first primed with increased intracellular calcium. This might explain why we did not see a response to levosimendan in isolated myofilaments. Bepridil increased calcium sensitivity, which may explain the clinical utility of calcium sensitizers in canine DCM as a supporter of contractility. The attenuated response of the DCM myofilaments could be due to alterations in the contractile apparatus, or changes in the calcium handling system during congestive heart failure, and are consistent with our hypothesis of altered myofilaments in canine DCM.

**Tropomyosin and DCM**

Tropomyosin is a steric myofilament dimer protein involved with thin filament regulation. The movement of tropomyosin in response to calcium binding to TnC partially exposes myosin binding sites on actin (131). The binding of a single myosin to the thin filament not only fully exposes nearby myosin binding sites, but is thought to move adjacent tropomyosin to expose neighbouring myosin binding sites and enable co-operative actin-myosin binding (130,131). Tropomyosins comprise a family of actin-binding proteins encoded by 4 different genes (*TPM1, TPM2, TPM3, and TPM4*). Each gene uses alternative splicing, alternative promoters, and differential processing to encode multiple striated muscle, smooth muscle, and cytoskeletal tropomyosin isoforms (132). In canine cardiac muscle, the αα dimer isoform is predominant, with little to no β component, whereas in humans, the β component constitutes 15-20% of the total, with preferential formation of heterodimers (αβ) (161-163).

Differential expression in transgenic mice of a newly identified tropomyosin isoform, κ -Tm, has been associated with the DCM phenotype. (131,132,135,137). The κ -Tm isoform, which is similar to skeletal Tm-α, is expressed in the normal human heart and comprises 3-5% of Tm expression (132). Expression of κ -Tm in dog hearts is unknown. However, in human DCM and heart failure, κ -Tm expression is increased (132). Our results showing a tropomyosin doublet have not been previously reported, and might indicate isoform switching, mutation, or degradation correlated with the development
of the DCM phenotype, although it is important to note that the doublet was present in some normal myofilaments as well.

**Desmin and DCM**

The cytoskeletal protein desmin sits at the intracellular interface between cardiac Z-discs, myofilaments, and the sarcolemma. Through those sarcomeric attachments, desmin connects the contractile apparatus to the nuclear, mitochondrial and sarcolemma membranes – acting to maintain structural integrity of the cell during contraction, and also to aid in force transmission (133-134). Desmin also constitutes a key part of the desmosome which acts as a mechanical coupler between myocytes (133-134). In DCM hearts, desmin levels have been reported to both increase (139-141) and decrease (138), which confounds understanding of its role in heart failure.

Mackiewicz et al (139) reported that increased desmin in DCM hearts diminishes force transmission, while isolated cardiomyocytes showed no reduction in cell shortening. They suggested that calcium transient amplitudes were increased in DCM as compensation for impaired force transmission, and hypothesized that myofilament calcium sensitivity may also be increased. Results of our study are in agreement with Mackiewicz et al (139) and others (140-141) who reported upregulated desmin levels in failing hearts. However, our finding that myofilament calcium sensitivity is not altered in canine DCM hearts in conjunction with increased desmin levels contradicts the hypothesis advanced by Mackiewicz and colleagues (139). Moreover, previous work by Cory et al (142) found that sarcoplasmic reticulum calcium release and sequestration are impaired in end-stage canine DCM, also in opposition to Mackiewicz et al (139). It is possible that these discrepancies represent differences between the experimentally induced mouse model of failure used by Mackiewicz’s group, and the naturally occurring canine DCM investigated here.
Myofilament-Associated PKC in Canine DCM

Myofilament proteins exhibit altered phosphorylation states in failing hearts, possibly because of increased PKC activation (143-144). Given the inhibitory effects of PKC on cardiac myofilament function, it has been hypothesized that PKC-dependent inhibition of cardiac myofilaments plays a significant role in the contractile dysfunction that underlies heart failure progression. Despite extensive research into the role of PKC isoforms in heart failure (115), no previous studies have examined changes in myofilament-associated PKC in end-stage heart failure. Our data show that PKC-α, -δ, -ε, and -ζ are associated with cardiac myofilaments from non-failing dog hearts. In end-stage DCM, myofilament levels of PKC-δ and -ζ increase, whereas PKC-ε levels decline and PKC-α shows no change in total protein. Total PKC-α activity has been observed to increase in failing hearts (145,146), and inhibition of PKC-α can attenuate the functional decline (147). While our results show no change in myofilament-associated PKC-α, this does not necessarily contradict earlier studies as we have confined our investigation to PKC-α alterations in the myofilament compartment, whereas other studies have looked at whole cell levels of PKC-α. Cardiomyocyte PKC-δ has been reported to increase in hypertensive heart failure [51], a finding that agrees with our discovery of increased myofilament-associated PKC-δ in DCM. Changes in PKC-ζ in failing hearts have, to our knowledge, not been investigated before, making our report of increased myofilament PKC-ζ the first investigation of its kind.

The importance of identifying subcellular changes in PKC is supported by the diverse and paradoxical effects of the various PKC isoforms. It has been proposed that therapies targeting specific PKC isoforms in discrete subcellular compartments offer the greatest potential to treat heart failure, while minimizing unwanted side-effects (148). A key element in this approach is to identify the specific PKC isoforms that might be modulated. The results of our work advance this area by uncovering significant changes in select myofilament-associated PKC isoforms, thereby identifying PKC isotypes that may be
targeted in the management of chronic heart failure.

**Molecular Mechanism of Modified Myofilament Activation in Canine DCM**

The definitive mechanism by which myofilament activation is altered in canine DCM is unknown. However, based on our data we are able to advance several options. First, phosphorylation of the N-terminal serine residues in cardiac troponin I decreases myofilament calcium sensitivity. The decreased phosphorylation at these sites discovered here would permit an increase in myofilament calcium sensitivity. Second, increased phosphorylation of desmin increases myofilament calcium sensitivity (149). Finally, tropomyosin phosphorylation at S283 by an unknown kinase (150-151) increases myofilament calcium sensitivity (152). Together, these changes we observed would be expected to increase myofilament calcium sensitivity.

PKC is a prominent regulator of myofilament function, in large part through the phosphorylation of troponin I. Although some PKC isoforms can phosphorylate the N-terminal serines of troponin I typically targeted by PKA, the primary residues phosphorylated by PKC are serines 43 and 45 (S43/S45), and threonine 144 (T144). Phosphorylation of S43/S45 reduces myofilament activation (156-157), while T144 phosphorylation has been reported to both increase (158) and have no effect on myofilament calcium sensitivity (159). Interestingly, the effects of PKC on myofilament activation can be modified under conditions that occur in failing hearts. For example, with oxidative stress PKC-δ switches its substrate preferences from S23/S24 to T144, and depresses myofilament activation (153). Under acidic conditions PKC phosphorylates S43/S45 and T144 to also inhibit myofilament activation (154). The ability of PKC to reduce myofilament calcium sensitivity is also enhanced in failing myocardium where PKA phosphorylation of cardiac troponin I is reduced (155).

In results presented here we show that PKA regulation of cardiac myofilaments is depressed in end stage canine DCM, which occurs in conjunction with increased desmin and tropomyosin phosphorylation.
All of these changes are expected to increase myofilament calcium sensitivity. However, concomitantly we found an increase in myofilament-associated PKC-δ and PKC-ζ and no change in total troponin I phosphorylation. Together these results have lead us to propose a mechanism in which increased calcium sensitivity modifications are off-set by changes that decreased myofilament calcium sensitivity to yield no net change in myofilament calcium sensitivity. Although neutral in the functional assays used here, the changes in myofilament phosphorylation may drive hearts further into the cyclical decline of heart failure by disrupting cross-bridge cycling. PKC phosphorylation of cardiac myofilaments is associated with compromised cardiac function and poor long-term prognosis (154), and increased desmin phosphorylation is an accurate predictor of myocardial dysfunction (136).

**Future Directions**

Based on the work conducted in this study so far, there are several future directions that can be taken. Firstly, it is appropriate to further investigate myofilament cycling by conducting actomyosin MgATPase assays with simultaneous force measurements. This allows for a deeper understanding of how changes in myofilament cycling are likely to impact cardiac contraction in vivo.

An inquiry that is occurring as an offshoot of this work is identification of novel myofilament protein changes seen in 2D-DIGE. This work may inspire new directions of investigation such as novel pathways in the pathology of disease, compensatory mechanisms of disease, or biomarker identification.

Also, identifying a genetic cause of naturally occurring DCM in dogs could be possible by comparing genetic compositions between normal and DCM dogs. This has not been done before due to lack of myocardial sample quantity and quality. Since we have high quality cardiac tissue from a relatively large group of DCM dogs, we have sent isolated RNA for transcriptome analysis (for which we are awaiting results). This will provide a cardiac specific analysis of transcribed protein changes in canine DCM, and will likely allow us to contribute significantly to the current hot topic discussion on genetic
causes of DCM in Doberman dogs. The Meurs paper, which found a splice variant of the PDK4 gene associated with DCM formation in Dobermans, and absent in mixed breed dogs, is challenged by the Owkzarek-Lipska paper that did not find an association of the splice variant with DCM, and also found the variant present in mixed breed dogs (165,41). These differences could be due to geographical variations in population genetics, but could also be due to variations in techniques such as sample handling, laboratory methods, etc. We hope to be able to contribute to this ongoing discussion with the transcriptome results.

Limitations

Limitations of Clinical Study Design: Inherent in a clinical study design such as this is the dependence on clinical cases, which can result in small sample sizes as seen in this project. In order to reach our study goals, significant effort was put into collaboration and donor recruitment. These efforts included mail-outs to local veterinary clinics, information pamphlets for OVC clients, and collaborations with cardiologists from both OVC and the local referral hospital, Mississauga-Oakville Emergency Veterinary Hospital.

Also inherent to clinical study design is variation in sample population. End of life decisions are made primarily by the owners based on many complex variables such as perceived quality of life, prognosis, and finances (160). Consequently, veterinary patients are euthanized at different stages of heart failure which might result in variations between samples. In addition, although standard of care practices are similar, treatment regimes may vary somewhat, and co-morbidities will vary between individuals.

Technical Limitations: SDS-PAGE (1D and 2D), phosphorylation and immunoblot investigations are limited primarily by technical challenges inherent with proteomic studies. For example, the accuracy of protein concentration readings and assessing protein loading can be variable with these
semi-quantitative methods, making validation challenging.

Limitations with the actomyosin ATPase assay are that myofilament cycling is indicative of myofilament function, but not necessarily of myofilament force generation. Force studies also allow for 3D structures. In order to correlate myofilament functioning with force generation, either on the myofilament or cellular level, force studies would need to be conducted at the same time points as the ATPase assay. The main limitation of working with calcium sensitizing drugs are that they are light sensitive and unstable over time so must be prepared fresh for each experiment in a light restricted environment.
References

18. Gilbert SJ, Wotton PR, Tarlton JF, Duance VC, Bailey AJ. Increased expression of promatrix


36. Porciello F, Rishniw M, Herndon WE, Birettoni F, Antognoni S, Simpson KW. Cardiac troponin I is


90. Hazlett MJ, Maxie MG, Allen DG, Wilcock BP. A retrospective study of heart disease in Doberman
109. Unverferth DV, Schmidt WR, Fertel RH. Cyclic nucleotide analysis of myocardial biopsies in


