Dilated cardiomyopathy is a primary myocardial disease and the second most common, nonparasitic acquired heart disease (after chronic valvular disease) in dogs. The disease is inherited in several breeds, although potential causative mutations have been identified in only a few and the mutations that have been identified differ among breeds. Treatment is largely directed toward controlling clinical signs of congestive heart failure, but to date, pimobendan is the only drug that has been shown to prolong the time to onset of congestive heart failure in dogs with DCM.

Genome-wide expression analyses have proven helpful in elucidating disease mechanisms, identifying causative genes, and suggesting therapeutic targets for specific diseases. A newer method of genome-wide expression profiling known as RNA sequencing (referred to hereafter as RNA-seq) has been shown to be more sensitive in detecting low-abundance or novel transcripts and biologically relevant gene isoforms. The purpose of the study reported here, therefore, was to identify, through the use of RNA-seq, cardiac tissue genes and gene pathways differentially expressed between dogs with and without DCM. We hypothesized that analysis of RNA-seq data would provide new insights into biochemical pathways that are dysregulated in dogs with DCM.

Materials and Methods

Ethics statement

All procedures were performed in keeping with local and national regulations. The study protocol was approved by the institutional animal care and use committees at The Ohio State University and North Carolina State University, where samples were collected.

Power calculation

A power calculation for the RNA-seq portion of the study was performed as described by Hart et al. However, the number of breeds included was limited.

Use of RNA-seq to identify cardiac genes and gene pathways differentially expressed between dogs with and without dilated cardiomyopathy

**OBJECTIVE**

To identify cardiac tissue genes and gene pathways differentially expressed between dogs with and without dilated cardiomyopathy (DCM).

**ANIMALS**

8 dogs with and 5 dogs without DCM.

**PROCEDURES**

Following euthanasia, samples of left ventricular myocardium were collected from each dog. Total RNA was extracted from tissue samples, and RNA sequencing was performed on each sample. Samples from dogs with and without DCM were grouped to identify genes that were differentially regulated between the 2 populations. Overrepresentation analysis was performed on upregulated and downregulated gene sets to identify altered molecular pathways in dogs with DCM.

**RESULTS**

Genes involved in cellular energy metabolism, especially metabolism of carbohydrates and fats, were significantly downregulated in dogs with DCM. Expression of cardiac structural proteins was also altered in affected dogs.

**CONCLUSIONS AND CLINICAL RELEVANCE**

Results suggested that RNA sequencing may provide important insights into the pathogenesis of DCM in dogs and highlight pathways that should be explored to identify causative mutations and develop novel therapeutic interventions. (Am J Vet Res 2016; 77:693–699)
on the basis of a negative binomial distribution with the following assumptions: \( \alpha = 0.05, \beta = 0.8 \), mean coverage per gene = 20X, coefficient of variation (\( \sigma \)) = 0.3, and detectable fold change = 2. Coverage of 20X per gene and \( \sigma \) of 0.3 were selected because these values have typically been recommended for studies involving inbred animals.\(^{15}\) The power analysis indicated a minimum of 5 samples would be needed in each group.

**Dogs**

Eight client-owned dogs with DCM that had been euthanized at the request of their owners because of progression of various chronic diseases were included in the study. Affected dogs included 3 Doberman Pinschers (2 castrated males and 1 spayed female), 2 Schnauzers (1 castrated male and 1 spayed female), a Dalmatian (castrated male), a Boxer (sexually intact male), and a Saint Bernard (castrated male). Age ranged from 1.5 years (Boxer) to 9 years (Doberman Pinscher), and fractional shortening ranged from 12% to 18%.

Dogs with DCM had been examined at The Ohio State University or North Carolina State University. In all dogs, the diagnosis of DCM had been made on the basis of echocardiographic evidence of fractional shortening < 20% with left ventricular internal diameter during diastole that exceeded the accepted value for the dog’s body weight.\(^{16}\) Dogs had been receiving furosemide, pimobendan, an angiotensin-converting enzyme inhibitor, a \( \beta \)-adrenoceptor blocker, or an antiarrhythmic drug, alone or in combination, at the time of euthanasia, and none had evidence of any cardiac disease other than DCM (eg, endocarditis or congenital heart disease) that could have complicated the diagnosis.

Five dogs without DCM (unaffected dogs) were also included in the study. Unaffected dogs consisted of 2 Beagles, 2 Boxers (9 and 10 years of age), and a Coonhound that had been part of a research colony and had been euthanized as part of other studies. These dogs were determined to be free from DCM on the basis of normal physical examination and echocardiographic (fractional shortening > 25% and left ventricular internal diameter during diastole within the reference range for the dogs’ body weight) findings. In addition, 24-hour ambulatory electrocardiography had been performed in the 2 Boxers, both of which had < 50 ventricular premature contractions during the recording period.

**Tissue collection and RNA extraction**

Immediately after dogs were euthanized, a single sample of left ventricular tissue (approx 2 cm\(^3\)) was collected from each dog. Samples were immediately placed on dry ice and then stored at –80°C until analyzed. For each sample, RNA was isolated with a commercially available kit for fibrous tissue\(^{4}\) according to manufacturer-specified protocols. Quality of the isolated RNA was assessed by means of spectrophotometry\(^{9}\) and capillary electrophoresis.\(^{5}\) Samples were considered acceptable for further analysis if the ratio of absorbance at 260 nm to absorbance at 280 nm was > 2.0 or the RNA integrity number was > 7. A desktop fluorometer\(^{4}\) was used to perform RNA quantitation.

**Next-generation sequencing**

Sequencing was carried out at the Genomic Sciences Laboratory at North Carolina State University. Approximately 3 \( \mu \)g of RNA was depleted of rRNA with a commercially available kit.\(^{5}\) Library preparation was performed with standard reagents.\(^{1}\) Samples were barcoded and multiplexed into pools of 8 to 11 samples. Sequencing was performed with a desktop sequencer\(^{6}\) and commercially available chips.\(^{7}\) Each sample was sequenced 3 to 4 times, with 13.2 million to 30.5 million reads (mean, 20.2 million reads) generated for each sample, representing a total of 24.9 billion bases. Only bases with a Phred quality score > 20 were included in downstream analyses (81% of total). Mean read length was 93 bases (range, 79 to 114 bases).

**Differential expression analysis**

Sequencing reads were demultiplexed and adapters were trimmed from the sequences with standard software,\(^{1}\) with any sequences < 50 bases long discarded.\(^{17}\) Trimmed reads were subsequently aligned to the reference sequence of the canine genome\(^1\) with standard software,\(^{8}\) as described.\(^{18,19}\) Differential gene expression was assessed according to best-practice guidelines.\(^{20,1}\) Briefly, mapped reads were assembled into transcripts for each sample on the basis of the reference assembly,\(^1\) with fragment bias and mulitread correction.\(^{21}\) Assembled transcripts were then merged into a single assembly with the reference-guided option\(^{20}\) of the software.\(^{20}\)

Transcript assemblies from each dog were grouped into affected (8 dogs) and unaffected (5 dogs) categories, and assemblies from each dog were compared with the merged assembly,\(^9\) again with corrections for fragment bias and multiple reads.\(^{20}\) Differentially expressed genes were analyzed with standard software,\(^8\) as described.\(^{20}\) Given that the read depth was less than that generally recommended for alternative isoform interpretation,\(^15,22\) this analysis was not attempted.

Genes that were significantly differentially expressed between groups were identified as described.\(^{23,24}\) In short, a model for each group (affected vs unaffected) was created on the basis of number of reads mapped to each gene and variance among samples. Transcript abundances were normalized to the overall read depth expressed as fragments per kilobase of transcript per million mapped reads. Benjamini-Hochberg–corrected \( P \) values < 0.05 were considered significant.

**Validation of differentially expressed genes**

To validate differential gene expression results, qRT-PCR assays for 6 randomly selected genes (3 up-
regulated and 3 downregulated) were performed on samples from 3 affected dogs (Doberman Pinscher, Schnauzer, and Boxer) and 3 unaffected dogs (Coonhound, Beagle, and Boxer). The upregulated genes chosen for analysis were MYL6B, MMP28, and NPPB; the downregulated genes were PDCD4, TNNI1, and KCNIP2. Exon-spanning primers were designed for each gene with standard software, with the aim of amplifying an approximately 200 bp fragment of cDNA (Appendix). 24 Oligonucleotides were purchased from a commercial supplier. For these assays, RNA from the left ventricle of each dog was converted to cDNA with a commercially available reverse transcription kit. Relative quantification was performed with the ΔΔCt method, with canine glyceraldehyde-3-phosphate dehydrogenase used as an endogenous control and negative control wells used as the zero calibrator. Reaction mixtures for the qRT-PCR assays consisted of 50 ng of cDNA template, 0.5 µM of each primer, and a 1X master mix containing DNA polymerase, dNTPs, cyanine dye, and reaction buffers. Thermocycler conditions were 95°C for 10 minutes (activation), followed by 40 cycles of 95°C for 15 seconds (melting) and 58°C for 1 minute (annealing and extension). Melting curve analysis was performed between 55° and 95°C to rule out primer-dimer and spurious product formation. All reactions were performed in triplicate, and averaged values were used for analyses.

Pathway analysis
Differentially expressed genes were analyzed as described.26,27 Gene symbols were mapped to the Entrez Gene database, and enrichment analysis was performed with databases from GO, KEGG, and Wikipathways.28–31 A set of known human and canine protein-coding genes was used as a reference. Upregulated and downregulated genes were analyzed as separate lists. The hypergeometric test was used to determine enrichment, with Benjamini-Hochberg-corrected values of P < 0.05 considered significant. A minimum of 2 genes was allowed per pathway.

Results
Ribonucleic acid sequencing
During quality assurance testing of RNA-seq data, expression level distributions, dispersion plots, and cross-replicate variability were compared among individual samples and between affected and unaffected dogs and found to be highly similar (data not shown). A total of 34,726 transcripts were detected and evaluated for differential expression. One hundred forty-five transcripts were differentially expressed between affected and unaffected dogs; 85 were upregulated in affected dogs and 60 were downregulated (Figure 1; Supplemental Table S1, available at http://avmajournals.avma.org/doi/suppl/10.2460/ajvr.77.7.693). Of these 145 transcripts, 86 mapped to protein-coding genes (Figure 2) and 13 mapped to predicted genes or RNA sequences. Of the 86 protein-coding genes, 52 were downregulated and 34 were upregulated in dogs with DCM, compared with unaffected dogs.

Validation of RNA sequencing data
Results of qRT-PCR assays performed on 3 upregulated and 3 downregulated genes in 6 dogs confirmed that the directionality of the expression changes were identical to the RNA-seq findings (Figure 3).

Pathway analysis
An overrepresentation analysis for upregulated and downregulated genes was performed to group differentially expressed protein-coding genes into meaningful clusters (Supplemental Table S2, available at http://avmajournals.avma.org/doi/suppl/10.2460/ajvr.77.7.693). Use of the GO database to analyze downregulated genes in dogs with DCM revealed pathways associated with myocardial failure and with

Figure 1—Volcano plot of cardiac tissue RNA transcripts obtained from 8 dogs with and 5 dogs without DCM. Black dots represent 145 transcripts for which expression was significantly (P < 0.05) different (85 upregulated and 60 downregulated) between affected and unaffected dogs; gray squares represent transcripts for which expression did not differ significantly between groups.
medications used to treat dogs with DCM such as phosphodiesterase inhibitors (Figure 4). Additionally, analysis of clustering of downregulated genes with the KEGG and Wikipathways databases identified numerous pathways involved in cellular energy production in dogs with DCM. This included genes required for carbohydrate metabolism (eg, \( \text{PIK3R1} \), \( \text{PPARGC1A} \), and \( \text{ATP1A3} \)) and fat metabolism (eg, \( \text{ACSL1} \), \( \text{DECR1} \), and \( \text{NCOA1} \)).

**Discussion**

To our knowledge, the present study represented the first application of RNA-seq to dogs with DCM in an effort to elucidate novel pathways and mechanisms that may be responsible for this disease across a range of dog breeds. We identified 86 protein-coding genes that were differentially regulated in dogs with DCM, compared with unaffected control dogs. Of these 86 genes, 52 were downregulated in affected dogs, and many of these downregulated genes were significantly overrepresented in pathways associated with energy metabolism and cardiac function.

Downregulated genes involved in energy metabolism that were identified in the present study included genes for phosphoinositide-3-kinase regulatory subunit 1 (\( \text{PIK3R1} \)); peroxisome proliferator-activated receptor \( \gamma \)-coactivator 1 \( \alpha \) (\( \text{PPARGC1A} \)); acyl-CoA synthetase long-chain family member 1 (\( \text{ACSL1} \)); 2,4-dienoyl CoA reductase 1, mitochondrial (\( \text{DECR1} \)); ATPase, Na\(^+\)/K\(^+\) transporting, \( \alpha \) 3 polypeptide (\( \text{ATP1A3} \)); and nuclear receptor coactivator 1 (\( \text{NCOA1} \)). Phosphoinositide-3-kinase regulatory subunit 1 plays a key role in insulin signaling through production of second messengers such as phosphatidylinositol 3,4,5-trisphosphate and activation of serine-threonine kinases, and mutations in this gene have been associated with marked insulin resistance.\(^{32,33}\) Peroxisome proliferator-activated
receptor γ, coactivator 1 α, the protein product of the PPARGC1A gene, is a critical protein involved in activating mitochondrial oxidative metabolism and is known to be highly expressed in healthy cardiac tissue. The acyl-CoA synthetase long-chain family member 1 gene encodes for long-chain acyl-CoA synthetase 1, which plays a key role in energy production by catalyzing the first step in the intracellular metabolism of fatty acids, and DECR1 is similarly important in the metabolism of fatty acids. Mutations in ATP1A3 have recently been associated with alternating hemiplegia of childhood in humans, but the ATPase pump itself has been shown to be important in maintaining cardiac contractility and facilitating glucose uptake into cells. Downregulation of this gene has also been implicated in diabetic cardiomyopathy in mice. Steroid receptor coactivator 1, the protein generated by NCOA1, is also important in glucose homeostasis and cellular energy production. Taken together, downregulation of these genes points to severe cellular energy dysregulation in dogs with DCM.

It is unknown, however, whether the downregulation of these genes represents a primary or secondary effect in the pathogenesis of DCM. It is possible that the onset of DCM stems from an underlying defect in energy metabolism, as has been described in several studies of people and dogs. However, it is also possible that the architecture of the cardiac myocytes in dogs with DCM has been altered, leading to secondary dysfunction of energy regulation. Two separate histologic characterizations of DCM have been proposed in dogs: the fatty infiltration-degenerative type seen primarily in Boxers and Doberman Pinschers and the attenuated wavy fiber type seen primarily in medium- and large-breed dogs. Thus, dysregulation of energy metabolism could potentially be primary in dogs with fatty-infiltration degenerative DCM, but secondary in dogs with attenuated wavy fiber DCM. Because of the diversity of breeds included in the present study, dogs with both phenotypes may have been included. Further studies are needed to determine the definitive causative mutations in dogs with DCM across multiple breeds.
In the present study, we also identified 34 protein-coding genes that were significantly upregulated in dogs with DCM. Some of these genes, such as natriuretic peptide B (ANP), were likely upregulated in response to increased effective circulating volume often seen in patients with late-stage heart disease. Other noteworthy upregulated genes include those associated with muscle contraction and calcium signaling such as myotilin (MYOT), purinergic receptor P2X 6 (P2RX6), myosin light chain 6B (MYL6B), death-associated protein kinase 3 (DAPK3), and calcium voltage-gated channel subunit α 1 H (CACNA1H). Alterations in expression of these genes may have been due to medications that affected dogs were receiving (such as pimobendan) or may have represented compensatory pathways triggered by a primary defect in cardiac contractility.

We compared our findings with those of a previously reported microarray study of 2 dogs with DCM. Broadly, our findings were similar, as both studies demonstrated severe dysregulation of cellular energy metabolism in affected dogs. Additionally, both studies showed differential expression of both structural and functional cardiac genes that encode for proteins such as myosin subunits, collagen isoforms, ATPases, and G-protein complexes. However, the specific genes implicated varied widely. We identified only 8 overlapping genes between the 2 studies (LG13, HSPA12A, MYL6B, UCHL1, APOE, FNDC3B, DPP6, and ATP1A3), all of which were downregulated. These discrepancies are expected when comparing studies with different sample sizes, breed mixtures, and gene expression detection technologies. More important is the overlap in gene pathways identified, which confirms many of the basic physiologic pathways that are affected in dogs with DCM.

There were several limitations to the present study. Notably, we included a mixture of dog breeds in the affected and unaffected groups. Because the causative mutations for DCM may differ by breed, the underlying pathogenesis and resulting differentially regulated genes may also differ by breed. Therefore, applying these findings to a particular dog breed might prove challenging. The objective of this study, however, was to identify common pathways or genes that were altered across the overall population, and the evaluation of a number of dogs of different breeds with DCM was informative for this objective. Additionally, although it would have been ideal to use age- and breed-matched controls, we believe that the controls we used provided information that was representative of the normal canine myocardium. Although we cannot be certain that the control dogs did not have an underlying genetic mutation and would not eventually develop DCM, this was unlikely given the breeds (Beagle and Coonhound) of 3 of the unaffected dogs. It was also unlikely that the 2 unaffected Boxers would go on to develop DCM, given that both were > 9 years old and had normal echocardiography and ambulatory ECG results. Finally, we did not control for stage of disease or medications that each dog was receiving, both of which could affect gene expression. Future studies that control for stage and treatment type could help clarify the effects of various treatments on gene expression in dogs with DCM.

Our findings confirmed that altered cellular energy metabolism is involved in the pathogenesis of DCM in dogs and suggested important pathways for future research. First, studies are needed to identify the causative mutations for DCM in multiple dog breeds. Second, studies are needed to evaluate whether treatments to help reverse the altered metabolic pathways we identified may help slow or reverse the course of the disease in affected dogs. Such research is pressing in canine cardiology given the limited therapeutic options and poor prognosis for dogs with DCM.

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Footnotes
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e. Ribominus, Life Technologies, Carlsbad, Calif.
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