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Differential Cardiac Gene Expression in Turkeys Genetically Selected for Increased Body Weight

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Abstract

Selective pressure for rapid and efficient growth in poultry may have unintended negative effects such as an increased risk of cardiac disease. Association between rapid growth and cardiac disorders suggests that birds selected for increased body weight exhibit differences in gene expression during development that predisposes them to cardiac irregularities. This study contrasts the expression of cardiac genes at two developmental time points (1 day post hatch [1 dph] and 16 weeks[16wk]) between two genetic lines (Random Bred Control 2 and F-line). Two independent measures of gene expression, a turkey-muscle-specific microarray and a cardiac-specific CEQ gene multiplex were used to examine total RNA extracted from cardiac muscle. Comparison of gene expression between 1 dph and 16 wk stages found 829 cases of putative differential expression ($p < 0.05$) in the RBC2 and 1325 in the F-line birds. A total of 39 comparisons were highly significant (false discovery rate, FDR < 0.05). Over 300 genes showed putative differential expression ($P < 0.05$) between genetic lines, however only a single transcript met the FDR cutoff ($p = 0.000002$, FDR = 0.012). Cardiac gene expression measured with the CEQ assay found the observed changes to reflect growth and development of the heart. Both experimental approaches identified variable expression in *troponins* that was confirmed by qRT-PCR. Results of this study of two key developmental time points indicate that 45 generations of selection for increased body weight have not detrimentally influenced turkey cardiac gene expression.

Keywords: Turkey; Cardiac muscle; Microarray; Gene expression; qRT-PCR

Introduction

Rapid and efficient skeletal muscle growth is a necessity for the commercial poultry industry and selection for these traits has produced dramatic change. For example, the growth rate of domestic turkeys has essentially doubled in the past four decades [1]. High growth rates, however, are associated with larger muscle fiber diameter, increased glycolytic fiber proportion, and reduced proteolytic potential of muscle leading to reduced meat quality [2]. Effects of increased growth rate are also observed under physiological insults such as heat stress, where fast-growing birds are less thermo tolerant [3]. Recent genetic studies suggest that meat quality can be improved without reduced profitability as there may be little genetic conflict between muscle quantity and quality [4].

In addition to meat quality effects, selective pressure for rapid and efficient growth may have other unintended negative effects, such as cardiac disease. Specific examples include cardiohepatic syndrome (round heart disease, RH) in the turkey and pulmonary hypertension syndrome (PHS or ascites) in the chicken. Although initially thought to be a strictly genetic disorder, hypoxia and rapid growth are important factors in the etiology of RH [5]. Likewise in meat-type chickens, a sudden increase in PHS during the 1980's was associated with increases in growth rate and feed conversion [6]. Research suggested that one underlying cause was the increased metabolic oxygen requirement resulting from high food intake and rapid growth [7,8].

The association between rapid growth and cardiac disorders suggests that birds selected for increased body weight may exhibit differences in gene expression during development that predisposes them to cardiac irregularities. To test this hypothesis, cardiac gene expression was examined in birds from two genetically related turkey lines. The lines chosen for study consisted of genetically selected birds (OSU F line) and unimproved birds (Random Bred Control 2, RBC2). The F line, derived from the RBC2 line, has been selected for 45 generations solely for increased 16 wk body weight (BW) [9-11] and has significantly higher pectoralis muscle weight than the RBC2 line [12]. Birds of the F line have 60% higher total body weight and twice the pectoralis major muscle weight at 16 weeks as compared to RBC2 birds [12]. The RBC2 line has been maintained without selection for any trait and is representative of a 1967 turkey [13]. Therefore, comparisons between these lines are not confounded by selection for traits such as disease resistance, reproductive efficiency or behavioral traits as in commercial birds.

The F line has been used in previous studies of the genetic basis of cardiac growth and maturation [14]. This study measured BW, heart weight, heart rate, myocardial glycogen and lactate concentration, and activities of plasma creatine kinase (CK) and lactate dehydrogenase (LDH). One interesting conclusion was that long-term selection for increased 16 wk BW resulted in a significant decrease in embryonic heart rate. F-line embryos also showed a different energy metabolism profile that relied much more on gluconeogenesis. These results indicate that the F-line birds (and the RBC2 control) represent an

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excellent model for examining the effects of genetic selection for BW on cardiac gene expression.

Molecular approaches such as quantitative real time PCR allow for detailed experimental comparisons of gene transcription between individuals, tissues and or even time points. However, the development of microarrays and more recently RNA-seq approaches allow for simultaneous examination of the expression of thousands of genes. Sporer et al. [15] developed a 6K turkey skeletal muscle long oligonucleotide (TSKMLO) microarray for examining changes in gene expression during muscle growth and development. This array includes 5809 duplicate-spotted 70-mer oligonucleotides corresponding to gene sequences obtained from turkey cDNA libraries [16,17]. Although the genes on the array are primarily derived from skeletal muscle, several cardiac-specific genes and isoforms are also included, making this array a potential resource for examining gene expression in cardiac tissue. In addition to testing the TSKMLO microarray, a species-specific multigene expression assay and supporting quantitative real time PCR (qRT-PCR) approaches were used to examine cardiac gene expression. The results of this study contrast the expression of cardiac genes at two developmental time points between genetic lines, providing a necessary framework for future studies of cardiac gene expression in the context of conditions such as RH.

Materials and Methods

Materials

Age-specific differences in gene expression have been documented, and multiple isoforms generated through alternative mRNA splicing are common during development; therefore it was important to examine cardiac gene expression at different stages of cardiac development. Males from both lines (F and RBC2) were randomly selected at 1 day post hatch (1 dph) and at 16 wk (adult) for comparisons. Samples for each of the four groups (line x age combinations) were obtained from three individuals. Tissues used in this study were collected following an approved IACUC protocol. The two lines were compared at each developmental time point (eg.16 wk F-line compared to 16 wk RBC2) in a series of 6 arrays. Likewise, the developmental stage (1 dph vs. 16 wk) was compared within lines in a separate series of 6 arrays (Table 1). Since expression differences are expected between developmental time points, the more relevant stringent comparisons are made between lines at each time point.

Methods

Total RNA was extracted from ~25 mg of cardiac ventricle tissue with TRIzol reagent (Invitrogen) according to manufacturer's protocol; the TRIzol extraction was followed by chloroform extraction and organic separation. Briefly, flash-frozen cardiac tissues were ground to a fine powder in liquid nitrogen using a mortar and pestle, and samples were homogenized using a glass homogenizer and Teflon rod. RNA was precipitated by adding 0.5 ml isopropyl alcohol per initial 1 ml TRIzol reagent, followed by centrifugation, re-suspension of the pellet in molecular grade dH_2O and quantification using a Nanodrop spectrophotometer. RNA quality was considered acceptable when the $A_{260/280}$ ratio was 1.9-2.0 and $A_{260/230}$ ratio was 1.9-2.1. RNA quality was visualized using 2-4 µg RNA on a denaturing agarose gel and was considered acceptable when two bands corresponding to 28S and 18S ribosomal RNA were visible with little visible degradation. DNA contamination was removed from the RNA preparation using the Turbo DNAfree Kit (Ambion) according to manufacturer's protocol. DNase treated RNA was re-quantified by Nanodrop spectrophotometer.

Microarray Analysis: Cardiac gene expression was examined using a muscle-specific 6K (5257 transcript) microarray [15]. A complete description of the TSKMLO array can be found in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database [GEO: GPL9788]. Total RNA was amplified and dye-coupled using the Amino Allyl MessageAmp™ II aRNA Amplification Kit (Ambion, Inc.) per manufacturer instructions. Dye-coupled RNA was purified, quantified, and fragmented to 60-200 nt segments using RNA Fragmentation Reagents (Ambion, Inc.) for microarray hybridization as described in Sporer et al. [15].

After crosslinking, arrays were pre-hybridized in 5X SSC, 1% SDS, 1% (w/v) BSA, washed in 0.1X SSC, rinsed with Nanopure H_2O, and dried by centrifugation. Fragmented, Cy3- and Cy5-coupled RNAs were mixed according to Table 1, and hybridized at 54°C to the microarrays for 18 h in a GeneTac Hybridization Station (Genomic Solutions, Ann Arbor, MI), followed by a medium-stringency wash (2X SSC, 0.1% SDS) at 42°C, a high-stringency wash (0.2X SSC, 0.1% SDS) at 22°C, and a buffer postwash (0.2X SSC) at 22°C. Arrays were rinsed in 2X SSC and Nanopure H_2O, dried by centrifugation, and immediately scanned with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA). Image analysis was performed using GenePix Pro 6.0 with spot intensities exported as GPR files for statistical analysis. Fluorescence intensity data were not background corrected in accordance with previous recommendations [18,19] but were instead normalized for dye intensity bias using the LOESS procedure of the "Normalize WithinArray" function of the Bioconductor R software LIMMA [20].

Statistical analysis: The software LIMMA was also used for formal statistical inference [21]. For the arrays directly comparing lines within age and microarrays (arrays fr1-fr6 in Table 1), the linear model included the effects of age specific comparisons of the two lines as well as their difference (i.e., the interaction between line and age). For the arrays directly comparing ages within lines and microarrays (arrays dw1-dw6 in Table 1), the linear model included the effects of line specific comparisons of the two ages as well as their difference (i.e., the interaction between line and age from a different perspective). In all cases, inferences were specifically based on the moderated t-tests provided by LIMMA. Adjustments for multiple testing were based on converting the P-values from these moderated t-tests to false discovery rates (FDR) using the method of Benjamani and Hochberg [22] as implemented in LIMMA. Statistical significance was based on these

Array	cy 3			cy 5		
	Line	Stage	Sample ID	Line	Stage	Sample ID
RBC2 vs F						
fr1	F	1 dph	1	RBC2	1 dph	2.1
fr2	RBC2	1 dph	2.2	F	1 dph	2
fr2	F	1 dph	4	RBC2	1 dph	2.3
fr4	RBC2	16 wk	3	F	16 wk	4
fr5	F	16 wk	5	RBC2	16 wk	9
fr6	RBC2	16 wk	14	F	16 wk	6
1 dph vs 16 wk						
dw1	F	16 wk	4	F	1 dph	1
dw2	F	1 dph	2	F	16 wk	5
dw3	F	16 wk	6	F	1 dph	4
dw4	RBC2	1 dph	2.1	RBC2	16 wk	3
dw5	RBC2	16 wk	9	RBC2	1 dph	2.2
dw6	RBC2	1 dph	2.3	RBC2	16 wk	14

Table 1: Experimental design of microarray experiments. For each array, dye (cy3 or cy5), line, stage, and sample are given.

estimated FDR. Raw Cy5 and Cy3 intensities, LOESS-normalized log2 Cy5:Cy3 ratios, LOESS-normalized log2 average intensities, and GPR files for all arrays, were submitted to the National Center for Biotechnology Information (NCBI)'s Gene Expression Omnibus (GEO GSE27147).

CEQ multiplex expression assay: In order to specifically examine a set of key cardiac genes, a custom species-specific cardiac gene multiplex was developed for the Genomelab GeXP CEQ system (Beckman Coulter). This platform is designed to simultaneously screen a panel of genes across a large number of RNA samples. Screenings of candidate genes and large family linkage analyses have identified a suite of 20 genes associated with human dilated cardiomyopathy (DCM) [23]. These genes (excluding *Tafazzin*) were initially targeted for inclusion in the multiplex.

Oligonucleotide primer pairs were designed to amplify the target genes with Beckman Coulter eXpress designer software. Sequences from turkey cardiac ESTs were used whenever possible; however, chicken homologs from Genbank were used when turkey sequence was not available. Amplicons were designed to be 150-400 bp in length with a minimum 5 bp separation in size between adjacent fragments. Each primer comprised a chimeric sequence including a 20 bp gene-specific region plus a 16 bp universal sequence. The final multiplex included 23 amplicons (genes plus controls, Supplemental Table 1). Specific gene products included are *alpha actin* (*ACTC*), *alpha tropomyosin 1* (*TPM1*), *alpha tropomyosin 4* (*TPM4*), *ATP binding cassette* (*ABCC9*), *beta actin* (*ACTB*), *delta sarcoglycan* (*SGCD*), *desmin* (*DES*), *dystrophin* (*DMD*), *GATA binding protein 4* (*GATA4*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *hypoxanthine guanine phosphoribosyl transferase 1* (*HPRT1*), *malate dehydrogenase I* (*MDH1*), *myosin binding protein C* (*MYBPC3*), *myosin heavy chain 6 beta* (*MYH6*), *myosin heavy chain 7 beta* (*MYH7*), *phospholamban* (*PLN*), *titin* (*TTN*), *troponin C* (*TNNC1*), *troponin I type 1* (*TNNI1*), *troponin T type2 (cardiac)* (*TNNT2*), *vinculin* (*VCL*), and *voltage-gated sodium channel type V alpha* (*SCN5A*).

Reverse transcription (RT) was performed with the GenomeLab GeXP Start kit according to manufacturer's protocol using the chimeric tailed reverse primers. The RT reaction included sample RNA, a control RNA, and each of the reverse gene-specific primers. Some reverse primers were titrated in the final reactions to attenuate peak height of highly expressed genes (see below). Reactions were performed in a thermal cycler with the following protocol: 48°C for 1 minute, 42°C for 60 min, and 95°C for 5 minutes. The resulting cDNA was amplified by PCR in a multiplex reaction comprised of equal amounts of each forward primer, 25mM Abgene $MgCl_2$, 5X PCR buffer (Beckman Coulter), and 3.5 units Abgene Taq polymerase. Reactions were performed in a thermal cycler with the following protocol: initial incubation at 95°C for 10 minutes, followed by 35 cycles of the following: 94°C for 30 seconds, 55°C for 30 seconds, and 70°C for 1 minute. Primer concentrations were optimized such that amplification of products from the original forward gene-specific primers was limited to the first three cycles with the universal primer driving amplification in cycles 4-35 to reduce primer bias.

RNA from each of the experimental samples was pooled at an equal concentration for all of the multiplex design, optimization and attenuation runs. Serial dilutions of pooled RNA were analyzed to generate a standard curve. All reactions (standard curve and experimental) were run in triplicate to verify repeatability. Reactions were visualized on the Beckman Coulter Genome Lab GeXP genetic analysis system. PCR products were prepared for capillary electrophoresis by diluting 1 µl PCR product into wells of 96-well plate containing 38.5 µl CEQ Sample Loading Solution (SLS) and 0.5 µl CEQ Size Standard 400 (SS400).

Controls and attenuation: Multiple reaction controls were used to ensure data quality. An optimized concentration of control RNA derived from a *Kan(r)* gene was included as a positive reaction control (no turkey RNA template). A second control was run without reverse transcriptase enzyme. Single primer reaction controls were run to verify amplification of the target fragment, target amplicon size on the instrument and to detect if primers produced un-designed peaks (UDPs). Primers that did not produce a quality amplicon or that produced unacceptable UDPs were re-designed. Unacceptable UDPs fell within the range of target gene fragments.

Attenuation of peak height was performed on pooled RNA to ensure all peaks fell into the same fluorescence range. This is accomplished so that peak heights, when differentially expressed, did not pass out of the dynamic range of instrument where they would not be accurately quantified (Figure 1). Attenuation was performed by serially diluting the reverse primer concentration for amplicons showing high peaks and repeating the multiplex reaction with primers for the other genes held constant. Attenuations on the pooled RNA were considered successful when peak height for that dilution ranged between 2,500-40,000 fluorescent units (cfu).

Data analysis: Multiplex data were analyzed post-electrophoresis using Beckman-Coulter eXpress Analysis and Quant Tool software. Expression was normalized to *KanR*, then to *PLN* and compared to the standard curve. Profiles for each well (peak height and area under peak, AUP) were overlaid and statistically compared by correlation and K-means plot analysis. Data were transformed via Quant Tool software using AUP as the dependent variable with the following equation $GEQ=0.5942X^3 + 2.6346X^2 + 19.357X - 2.2262$, where X = AUP. The resulting GEQ value, which represents the relative amount of RNA present in the sample, was used to calculate expression fold change (FC) between samples. Statistical analyses were performed using R statistical software (ANOVA 2-factor, paired Student's T-test). GEQ values were normalized to both *Kan(r)* RNA and *PLN*. ANOVA (analysis of variance) was performed for each gene across developmental groups and selection lines and interaction between the two factors was examined. In cases with significant interaction of variables, as determined by ANOVA, Student's T-tests were performed on normalized GEQ values to determine statistical significance ($p < 0.05$) of differences in expression.

Quantitative Real Time PCR

For quantitative real time PCR (qRT-PCR), synthesis of cDNA from 1 µg total RNA was performed using Invitrogen Super Script II kit according to manufacturer's protocol. Expression analysis of gene-specific amplicons was performed with the Quanta's PerfeCTa Sybr green SuperMix with low Rox on a MX3000P Real-Time PCR system (Stratagene, La Jolla, CA). Two microliters of diluted cDNA (1:10), 2 picomoles each gene-specific primer, and 10 µl 2X PerfeCTa Sybr green SuperMix were used in each 20 µl reaction. Primers were designed for a subset of genes examined with the CEQ system using the same sequences as the GeXP experiment without the universal CEQ tags (Supplemental Table 1).

Controls: Based on CEQ and qRT experiments, expression of *Phospholamban* (*PLN*) among the samples was found to be invariant. This gene was therefore chosen for reaction normalization. In addition, for each amplicon, clones were constructed by amplifying fragments

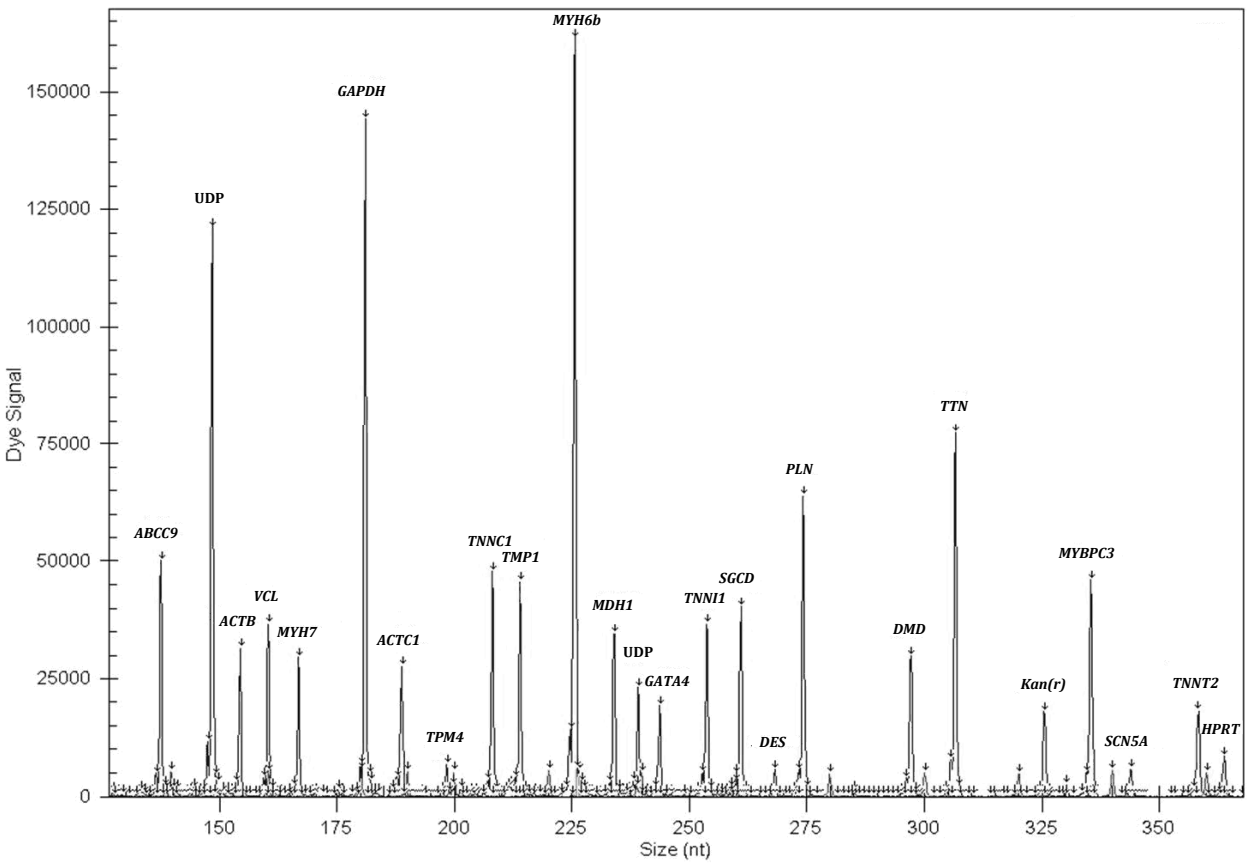

Figure 1: Example screen capture of CEQ chromatogram. X axis-fragment size (nt), Y axis-dye signal in relative fluorescent units.

from each gene via PCR and ligating these into pGEM-T easy T/A cloning vector system according to manufacturer's protocol (Promega). Ligations were transformed into DH5α subcloning efficiency cells according to manufacturer's protocol, plated onto selective media (100 μg/ml ampicillin plus Xgal), and grown overnight at 37°C. Single colonies were isolated in 3 ml Luria Bertani (LB) broth supplemented with 100 μg/ml ampicillin and grown overnight in a 37°C shaking incubator. Plasmid DNA was purified with a 96 well Qiaprep miniprep purification kit according to manufacturer's protocol (Qiagen). Isolated plasmid DNA was digested with *Eco*RI restriction enzyme to verify cloned insert size, and the identity was verified by DNA sequencing. Efficiency of primers was assessed via fit to standard curve. In addition to controls, a standard curve was generated by running multiple 20-μl reactions of cloned plasmid DNA containing gene-specific cDNA fragments with varying amounts of known starting template (0.005 pg - 10 ng).

All reactions included a disassociation curve to ensure production of a single product and that the reaction was free from interfering primer/dimer. Results were interpreted using a standard curve (linearity is denoted by the R squared value (R^2) or Pearson Correlation Coefficient) and a quantitative comparison approach. Ct values were collected for each data set and expression in each RNA sample was normalized first to *PLN*, and then via comparison of Ct value to the standard curve. To determine statistical significance of expression differences, Student's T-tests were performed on normalized RNA quantity values, as determined by comparison to the standard curve. Differences between sample means were deemed significant if the p value was less than 0.05.

Results

Microarray

Comparison of gene expression between 1 dph and 16 wk stages found 829 cases of differential expression ($p < 0.05$) in the RBC2 and 1325 in the F-line birds (Supplemental Tables 2, 3). A total of 541 genes were differentially expressed in both lines. A total of 39 gene expression differences (Table 2) had significant adjusted P values (false discovery rate, FDR <0.05). One strength of the TSKMLO array is the inclusion of multiple, unique oligos for several genes. Thus, the 28 spotted oligos indicating significant expression differences in the RBC2 line correspond to 23 genes (*alpha-actin*, *TNNI1*, and *myotilin* are represented by multiple oligos), ten of which were also significant in the F line (Table 3). Similarly, the 23 significant expression differences in the F-line birds correspond to 20 genes. In each case where multiple oligos correspond to the same gene and may represent alternate exons, the direction and magnitude of the expression FC were similar. Likewise, genes showing significant differences within both lines also had similar FC parameters.

The two genes showing the largest fold changes were *myotilin* and *tropomyosin*. *Myotilin* was up-regulated in both lines, showing an average FC of 3.4 at 16 wk vs 1 dph. The largest negative FC was observed for *tropomyosin* in the F line where transcription was 3.8 fold

RBC2 unique	Gene	FC	P	FDR
	actin, alpha 1 , skeletal muscle (ACTA1) (array address 5, 13, 2)	-2.84	0.0002	0.0496
	actin, alpha 1 (ACTA1) (array address 5, 16, 8)	-3.12	0.0002	0.0496
	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1 (ATP2A1)	-2.23	0.0002	0.0496
	CDC28 protein kinase regulatory subunit 2 (CKS2)	-2.06	0.0001	0.0496
	collagen pro-alpha-1 (COL1AI)	-2.65	0.0002	0.0496
	glycosyltransferase 8 domain containing 1 (GLT8D1)	-1.52	0.0002	0.0496
	lamin B2 (LMNB2)	-1.63	0.0002	0.0496
	maternal embryonic leucine zipper kinase (MELK)	-2.00	0.0001	0.0496
	P311 POU, neuronal protein 3.1 (C5orf13)	-2.34	0.0000	0.0224
	proteoglycan (PRG)	-2.02	0.0000	0.0255
	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2 (SERPINE2)	-1.89	0.0001	0.0496
	*small nucleolar RNA host gene**	1.87	0.0002	0.0496
	tubulin, alpha (TUBA)	-1.81	0.0002	0.0496
	tubulin, beta 2B (TUBB2B)	-1.92	0.0001	0.0496
F Unique				
	actin, alpha 2, smooth muscle, aorta (ACTA2)	-1.92	0.0001	0.0354
	cardiac C-protein (MYBPC3)	1.75	0.0002	0.0498
	cysteine and glycine-rich protein 3 (cardiac LIM protein) (CSRP3)	2.49	0.0000	0.0183
	FRA10AC1 protein (*C10orf4*)	1.67	0.0001	0.0351
	Leber congenital amaurosis 5-like (LCA5L)	2.44	0.0001	0.0354
	microphthalmia-associated transcription factor (MITF)	1.76	0.0002	0.0470
	similar to *nexilin isoform s*	2.36	0.0000	0.0145
	unknown	1.58	0.0002	0.0470
	unknown protein	1.91	0.0002	0.0498
Shared	**Gene**	**Line**	**FC**	**P Value**	**FDR**
	actin, alpha	RBC2	-3.12	0.0000	0.0083
		F	-2.49	0.0000	0.0183
	CD74 molecule, major histocompatibility complex, class II invariant chain (CD74)	RBC2	2.43	0.0000	0.0195
		F	2.15	0.0000	0.0237
	family with sequence similarity 134, member B (FAM134B)	RBC2	1.72	0.0001	0.0496
		F	1.71	0.0001	0.0354
	Kazal-type serine peptidase inhibitor domain 1 (KAZALD1)	RBC2	-1.59	0.0001	0.0496
		F	-1.79	0.0000	0.0237
	Kelch domain containing 3 (KLHDC3)	RBC2	-1.62	0.0002	0.0496
		F	-1.68	0.0001	0.0354
	myosin regulatory light chain 2B, cardiac muscle isoform	RBC2	2.00	0.0001	0.0496
		F	2.72	0.0000	0.0183
	myotilin (MYOT) (array address 4, 7, 4)	RBC2	3.80	0.0000	0.0083
		F	4.15	0.0000	0.0061
	myotilin (MYOT) (array address 22, 9, 6)	RBC2	3.30	0.0000	0.0097
		F	2.50	0.0000	0.0207
	similar to *nebulin* (LOC771699)	RBC2	2.32	0.0001	0.0496
		F	2.68	0.0001	0.0331
	TNNI1 troponin I type 1 (skeletal) (array address 9,2,8)	RBC2	-2.70	0.0000	0.0223
		F	-2.23	0.0001	0.0351
	TNNI1 troponin I slow skeletal muscle isoform (array address 11, 1, 10)	RBC2	-2.13	0.0000	0.0195
		F	-1.97	0.0000	0.0237
	TNNI1 troponin I type 1 (skeletal, slow) (array address 18, 1, 14)	RBC2	-1.68	0.0001	0.0496
		F	-1.85	0.0001	0.0331
	tropomyosin, alpha (TPMI)	RBC2	-1.53	0.0002	0.0496
		F	-3.80	0.0001	0.0412
	unknown	RBC2	2.55	0.0002	0.0496
		F	3.08	0.0001	0.0354

*this gene contains four snoRNAs as introns

Table 2: Genes showing differential expression between developmental stages within genetic lines (RBC2 and F) as measured with the TSKMLO microarray. Genes in the Shared group were differentially expressed in both genetic lines. For each gene the directional fold change (FC; 16 wk versus 1 dph), P value, and false discovery rate (FDR) are given.

lower at 16 wk compared to 1 dph. Other prominent down-regulated genes in both lines were *alpha-actin* (avg. FC = -2.8) and *TNNI1* (avg. FC = -2.05). A single small nuclear RNA host gene was uniquely up regulated in the RBC2 line. This non-protein coding gene contains four snoRNAs (*SNORA16B/16A*, *SNORA44*, *SNORA61*, and *SNORD99*) within its introns. This transcript was included in the 1325 genes with significant P values in the F line, however did not meet the FDR cutoff. Presence of this transcript suggests a significant developmental up regulation of these four snoRNAs which are thought to function in modification of other snRNAs.

Comparisons of the two genetic lines showed directional differences in gene expression change (Table 2). Within the RBC2 samples, a greater number of significantly different genes (20 of 28) were down-regulated at the 16-wk developmental stage. In the F line, 15 of 23 significantly different genes were up regulated. Differentially expressed genes shared between the lines were equally divided between those showing up (6) and down (5) regulation (Table 2, 3).

Over 300 genes showed putative differential expression ($P < 0.05$) in the direct comparisons between genetic lines, with 314 identified in the 1 dph comparison and 354 at 16 wk. However, only a single transcript fell under the FDR cutoff ($p = 0.000002$, FDR = 0.012) showing significant down regulation (-1.34 fold) in the F line. BLAST searches of the NCBI databases with the original EST sequence for this array oligo identified similarity to a predicted hypothetical protein (XP_430405.1; *BLOC1S3, biogenesis of lysosome-related organelles complex-1, subunit 3*). It is hypothesized that the BLOC-1 complex mediates the biogenesis of lysosome-related organelles through self-assembly and interaction with the actin cytoskeleton [24].

Genes with significant expression differences (FDR cutoff), were imported into Ingenuity Pathway Analysis (IPA, Ingenuity Systems, http://www.ingenuity.com) and mapped to their corresponding gene object in the IPA Knowledge Base. Pathway analysis identified three networks. A graphical representation of the molecular relationships between genes/gene products of the most significant network is presented in Figure 2. Top functions for this network are cell cycle, cell-to-cell signaling and skeletal and muscular system development and function.

CEQ Multi-gene expression

Gene expression measurements with the CEQ system showed consistency between experimental replicates. Differences among biological replicates as indicated by the standard deviations of GEQ values (Table 4) were uniformly low with few notable exceptions. Three genes (*ACTB*, *MYH7*, and *TPM1*) in the RBC2-line 1 dph samples had high standard deviations resulting from elevated expression in one individual. This difference was not consistent across all of the genes in the multiplex and thus is likely due to individual expression differences and not attributle to error in template quantification.

In comparisons between developmental stages, most genes in the CEQ multiplex were found to be down regulated (avg. - 1.45 x) at 16 wk compared to 1 dph (Table 4, Figure 3). Fold changes in the combined analysis ranged from 1.32 (*GAPDH*) to -16.67 (*TNNI1*). Three genes were slightly up-regulated. These included *GAPDH*, and the troponins *TNNC1* and *TNNT2*. The third troponin (*TNNI1*) was down regulated in both genetic lines, but FC was greater in the RBC2 line than in the F-line birds, -9.09 x and -50.00 x respectively. A similar trend of greater down regulation in the RBC2 birds as compared to the F line was seen for 10 of the 21 genes examined (Figure 3).

Direct comparisons between lines at the two developmental stages found minimal gene expression differences (Table 4, Figure 3). Combined fold changes in F versus RBC2 birds ranged from 1.31 (*ABCC9*) to -4.35 (*MYH7*). Greater line effects were seen at the earlier developmental time point. For example, three genes (*DES*, *DMD*, and *TNNT2*) showed lower expression in the F line at 1 dph than in the RBC2 line, but higher expression in the F line compared to RBC2 at 16 wk. Conversely, six genes (*ACTB*, *ACTC1*, *GATA4*, *TNNC1*, *TNNI1*, and *VCL*) showed higher expression in RBC2 at 1 dph than F and lower expression in RBC2 than the F line at 16 wk. Most notable was *TNNI1* where expression was 1.22x lower in the F-line birds at 1 dph but 4.52x higher than RBC2 birds at 16 wk. These changes may be indicative of altered troponin isoform switching during development.

Quantitative real time PCR

Quantitative real time PCR (qRT-PCR) was performed on a subset of genes to independently test the microarray and CEQ results. Members of the troponin family (*TNNT2*, *TNNI1* and *TNNC1)* that comprise the troponin protein complex and *HPRT1* were chosen for analysis. Expression of *HPRT1* in skeletal muscle is not affected by genetic line or developmental stage [15,25] and this gene was chosen as a secondary control for its consistency across samples in the CEQ experiment.

The CEQ and qRT-PCR methods showed similar trends in expression of the *troponin* genes. Results from qRT-PCR show all three genes (*TNNT2*, *TNNI1*, and *TNNC1*) with differential expression between the 1 dph and 16 wk groups (Table 5, Figure 4). The fourth gene *HPRT1* showed invariant gene expression between the developmental time points. *Troponin C* was down-regulated at 16wk compared to 1dph in the F line but showed the opposite trend in the RBC2 line. *Troponin T* showed a twofold up-regulation in 16-wk birds with qRT, whereas expression differences were only 1.2x in the CEQ experiment. The dramatic age effect for *TNNI1* expression as also observed by qRT-PCR with significant down-regulation in 16 wk birds from both lines. As seen in the CEQ experiment, the magnitude of this change was greater in the RBC2 line than the F-line samples.

Comparisons between genetic lines showed the qRT-PCR results to be consistent with those obtained in the CEQ experiments (Table 5). Two genes showed no major expression differences (*HPRT1 and TNNT2*). *Troponin C* was up regulated at 1 dph but down regulated at 16 wk in the F line compared to the RBC2 line. Likewise, *TNNI1* showed non-significant directional line effects at both time points with 1.85 x and 26 x fold increases in the F line over the RBC2 line at 1 dph and 16 wk, respectively. This is comparable to the microarray and CEQ experiments where the greatest line effects were also observed at 16 wk.

Discussion

Commercial turkeys are genetically selected for increased body weight and greater skeletal muscle for marketability. It is well documented that this intense selection can have detrimental effects on the health of the birds and the meat they produce. Pale, soft and

	RBC2	Unique to RBC2	F	Unique to F	Shared
Up regulated at 16 wk vs 1 dph	8	1	15	8	7
Down regulated at 16 wk vs 1 dph	20	15	8	3	5
Total	28	16	23	11	12

Table 3: Number and distribution of genes showing differential expression between developmental time points within genetic lines.

	Mean GEQ values				P value			Directional fold change					
	1 dph		16 wk					16wk v 1dph (Age)			F v RBC2 (Line)		
Gene	F	RBC2	F	RBC2	Age	Line	Inter	F	RBC2	Combined	1 dph	16 wk	Combined
ABCC9	0.49 ± 0.16	0.44 ± 0.17	0.42 ± 0.24	0.25 ± 0.11	0.116	0.499	0.312	-1.18	-1.75	-1.41	1.11	1.67	1.31
ACTB	1.15 ± 0.16	1.77 ± 1.23	0.94 ± 0.49	0.83 ± 0.14	0.239	0.612	0.959	-1.23	-2.13	-1.67	-1.54	1.13	-1.25
ACTC1	1.24 ± 0.16	1.40 ± 0.46	0.73 ± 0.15	0.71 ± 0.13	0.001*	0.489	0.635	-1.69	-1.96	-1.85	-1.12	1.03	-1.06
DES	1.17 ± 0.29	0.97 ± 0.40	0.49 ± 0.17	0.57 ± 0.35	0.006*	0.786	0.830	-2.38	-1.69	-2.04	1.21	-1.16	1.08
DMD	0.82 ± 0.07	0.60 ± 0.10	0.40 ± 0.16	0.41 ± 0.15	0.003*	0.396	0.285	-2.04	-1.45	-1.75	1.37	-1.03	1.21
GAPDH	0.89 ± 0.15	1.05 ± 0.50	1.18 ± 0.33	1.37 ± 0.31	0.028*	0.753	0.367	1.33	1.30	1.32	-1.18	-1.16	-1.16
GATA4	1.10 ± 0.13	1.24 ± 0.35	0.89 ± 0.25	0.87 ± 0.35	0.212	0.773	0.873	-1.23	-1.43	-1.33	-1.12	1.03	-1.05
HPRT1	0.61 ± 0.12	0.68 ± 0.32	0.72 ± 0.12	0.76 ± 0.12	0.039*	0.699	0.299	1.18	1.11	1.14	-1.11	-1.05	-1.09
MDH1	0.89 ± 0.08	1.22 ± 0.57	0.74 ± 0.18	0.84 ± 0.08	0.198	0.510	0.527	-1.20	-1.47	-1.33	-1.37	-1.12	-1.25
MYBPC3	1.28 ± 0.07	1.16 ± 0.28	0.75 ± 0.12	0.74 ± 0.18	0.001*‡	0.136‡	0.013*	-1.72	-1.56	-1.64	1.11	1.01	1.07
MYH6	1.01 ± 0.10	0.97 ± 0.11	0.73 ± 0.13	0.65 ± 0.17	0.009*	0.423	0.974	-1.39	-1.47	-1.45	1.04	1.11	1.07
MYH7	0.95 ± 0.62	4.76 ± 7.38	0.48 ± 0.12	1.37 ± 0.37	0.205	0.081	0.123	-2.00	-3.45	-3.13	-5.00	-2.86	-4.35
SCGD	1.17 ± 0.06	1.03 ± 0.44	0.95 ± 0.25	0.62 ± 0.19	0.068	0.012*	0.832	-1.22	-1.67	-1.41	1.13	1.55	1.29
SCN5A	0.86 ± 0.13	0.77 ± 0.05	0.42 ± 0.17	0.46 ± 0.40	0.003*	0.55	0.573	-2.08	-1.89	-1.96	1.12	1.02	1.09
TPM1	0.78 ± 0.20	1.41 ± 1.08	0.71 ± 0.33	0.74 ± 0.09	0.677	0.872	0.985	-1.09	-1.92	-1.52	-1.82	-1.03	-1.45
TPM4	1.14 ± 0.07	1.29 ± 0.70	0.82 ± 0.25	0.94 ± 0.39	0.332	0.760	0.281	-1.39	-1.39	-1.39	-1.12	-1.14	-1.14
TNNC1	0.83 ± 0.31	0.92 ± 0.56	0.84 ± 0.50	1.18 ± 0.20	0.247	0.662	0.236	1.01	1.28	1.15	-1.11	-1.41	-1.27
TNNI1	1.06 ± 0.14	1.28 ± 0.74	0.12 ± 0.15	0.03 ± 0.01	0.000*	0.062	0.731	-9.09	-50.00	-16.67	-1.22	4.52	-1.11
TNNT2	0.79 ± 0.11	0.76 ± 0.30	0.85 ± 0.11	1.02 ± 0.13	0.014*‡	0.906‡	0.031*	1.08	1.33	1.20	1.03	-1.20	-1.09
TTN	1.03 ± 0.38	0.83 ± 0.42	0.54 ± 0.14	0.45 ± 0.06	0.013*	0.733	0.855	-1.92	-1.82	-1.89	1.25	1.19	1.23
VCL	1.13 ± 0.33	1.27 ± 0.28	0.72 ± 0.31	0.45 ± 0.22	0.013*	0.363	0.488	-1.59	-2.86	-2.04	-1.12	1.60	1.08

Table 4: Summary of CEQ multiplex analysis of cardac gene expression. For each gene the mean GEQ values (based on 3 individuals each with 3 experimental replicates) are normalized to *PLN*. Directional fold changes (calculated by dividing the GEQ values) and P values from 2 way ANOVA analysis of GEQ values are included for all comparisons. Significant P values (< 0.05) are indicated by asterisks and ‡ denotes statistical comparisons with significant interaction.

	RNA quantity (fg/ul) (mean/sd)				Directional fold change / p value					
	1 dph		16 wk		16 wk v 1 dph			F v RBC2		
Gene	F	RBC2	F	RBC2	F	RBC2	Combined	1 dph	16 wk	Combined
TNNC1	45.450	28.200	37.900	47.300	-1.20	1.67	1.16	1.61	-1.25	1.10
	23.350	6.000	7.000	5.050	0.65	0.02*	0.49	0.42	0.15	0.64
TNNI1	15.100	8.400	0.510	0.020	-29.71	-430.25	-44.54	1.80	26.01	1.85
	10.200	4.725	0.800	0.002	0.13	0.09	0.02*	0.41	0.40	0.47
TNNT2	6.450	7.250	12.900	11.800	2.00	1.64	1.82	-1.12	1.09	1.01
	2.695	1.060	1.905	6.500	0.04*	0.35	0.03*	0.67	0.81	0.96
HPRT1	0.487	0.491	0.685	0.695	1.41	1.41	1.41	-1.01	-1.01	-1.01
	0.169	0.053	0.088	0.157	0.17	0.17	0.01*	0.97	0.93	0.94

Table 5: Summary of qRT-PCR results. For each gene the mean quantity of RNA (fg/ul) in each qRT reaction is as determined through normalization to *PLN*. Directional fold changes and p values are given for each comparison. Significant P values (< 0.05; H_o = no difference) are indicated by asterisks.

exudative (PSE) is a meat quality defect with significant occurrence in poultry [26-28]. Meat characteristics associated with this disorder include lower water holding capacity, pale color, and higher shear force. Specific problems of the cardiovascular system in poultry include sudden death syndrome (flip over) and pulmonary hypertension in the chicken, ruptured aorta, and RH in the turkey [29]. Originally, it was hypothesized that rapid cardiac growth itself was the pathogenic cause for these abnormalities. However, metabolic imbalance caused by high nutrient and protein intake was also hypothesized as causative [7]. In addition, it is evident that egg incubation conditions such as temperature and hypoxia contribute to changes in cardiac development in the embryo [14]. The heart itself does not undergo rapid growth in growth-selected birds. In fact, the normal heart in heavy muscled birds is not proportionally larger but must support an increased body size of the fast growing birds [30].

This study utilized three separate methods to measure cardiac gene expression (microarray, CEQ, and qRT-PCR). Microarray analysis identified fewer than 30 genes with significant differential expression between developmental time points. Many of these have roles in cellular proliferation/differentiation or represent structural muscle components. Likewise, the single gene showing significant expression difference between genetic lines is also involved in biogenesis. In the CEQ experiments, small gene expression changes were observed between hearts of genetically selected birds (F line) versus the non-selected birds (RBC2). Only *MYH7* had greater than two fold expression difference between these groups (4.3 fold change). Four genes were up-regulated in the 16 wk birds (*GAPDH, HPRT1, TNNC1* and *TNNT2*) and the remainder showed down regulation. Four of the down-regulated genes showed a greater than two-fold difference (*TNNI1*, *DES*, *VCL* and *MYH7*). *Desmin* and *Vinculin* are both structural stabilizers of the sarcomere, whereas *TNNI1* and *MYH7* are contributors to force production in the muscle fiber. It is possible that subtle differences in gene expression may be responsible for significant phenotypic differences.

The observed changes in cardiac gene expression reflect growth and development of the heart. Our experiments focused on contrasting gene expression in cardiac tissues at two important developmental time points, 1 dph and 16 wk. Age-specific gene expression has been

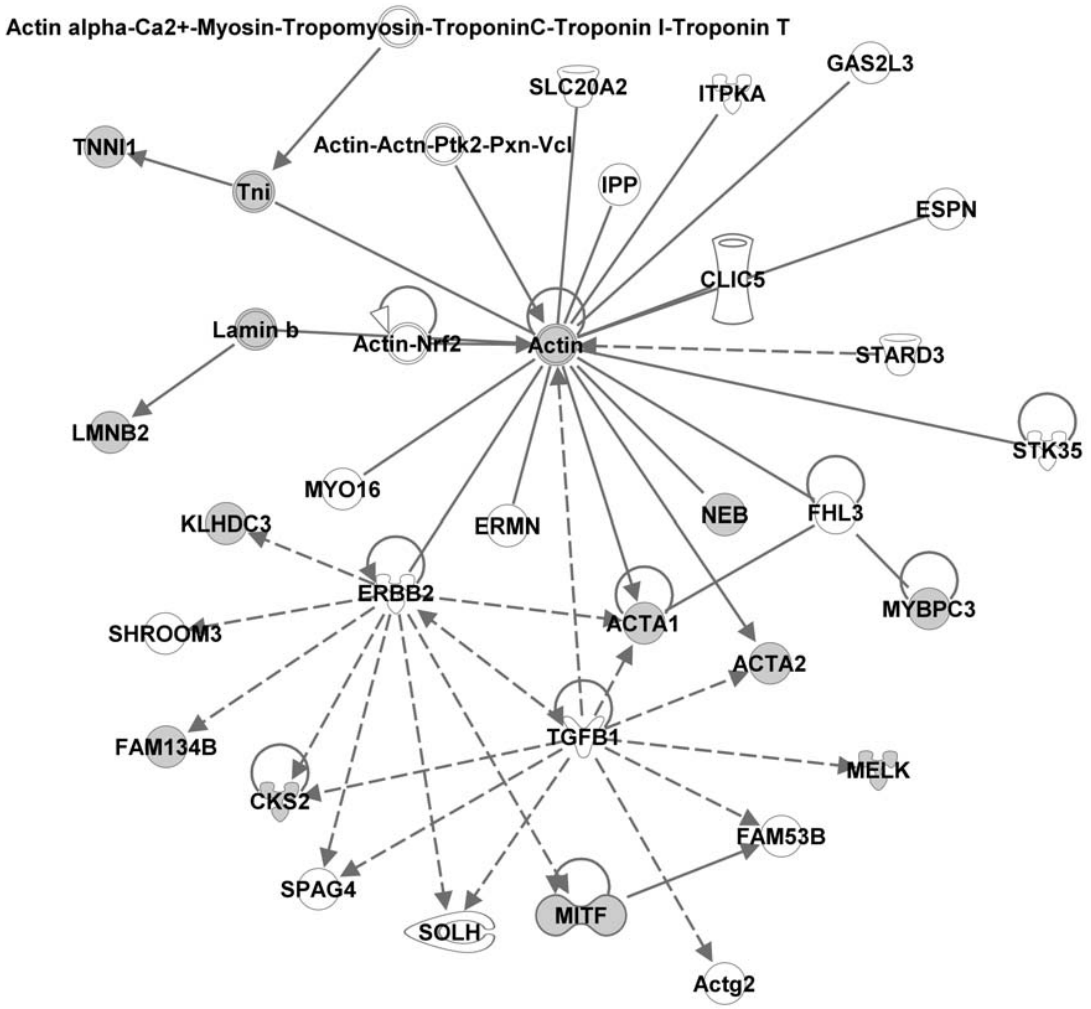

Figure 2: Graphical representation of network 1 from Ingenuity Pathway Analysis of genes showing significant expression differences (FDR cutoff) in the microarray analysis. Identifiers marked with an asterisk indicate the presence of multiple identifiers in the dataset that map to a single gene in the global molecular network. Solid lines denote direct interactions, whereas dashed lines are indirect interactions.

documented in a number of genes with multiple isoforms generated through alternative mRNA splicing during development. For example in turkey, the embryonic *myosin heavy chain* isoform appears by 22 days of incubation. Transition from the embryonic to neonatal isoform is observed after hatch and transition from neonatal to adult is initiated at 7 dph and is nearly complete by 28 dph [31,32].

Sporer et al. [25] examined both developmental and genetic line influences on gene expression in skeletal muscle with the TSKLMO array. Utilizing the same genetic lines used here, this study found significant differences between developmental stages at over 3000 genes. As observed for cardiac muscle, a much smaller number of genes (16) were significantly affected by genetic line (F vs RBC2).These genes were broadly categorized as being components of the extracellular matrix, involved in muscle differentiation and maintenance, or in cell signaling and muscle contraction. Included in the latter group were several *Troponins*. In skeletal muscle, *TNNI1* and *TNNT2* exhibited similar profiles with predominant expression in early development that decreased substantially at the other stages. Similar results were observed for *TNNI1* in cardiac muscle, but the reverse was seen for cardiac *troponinT (TNNT2)*.

Age-specific expression of *TNNT2* is to be expected. The gene is expressed during early stages of both cardiac and skeletal muscle development, but is repressed in skeletal muscle after early gestation [33]. Four major isoforms composed of higher and lower molecular weight proteins are found in cardiac *TNNT2*. Expression of *TNNT2* changes from high- to low-molecular-weight isoforms during cardiac muscle development. However, this transition is not found in skeletal muscle in the early stages of chicken development where only the high-molecular-weight isoforms were expressed [34]. *Troponin I* also undergoes switching in the heart of chickens with only one isoform detected up until two weeks post hatch, followed by a switch to the adult isoform [35]. Differences in the timing of isoform switching may represent a critical point where birds are most likely to develop cardiac irregularities such as RH. In mice, abnormal expression of skeletal muscle *troponin* isoforms in cardiac tissue significantly decreases cardiac function [36].

Mutations producing truncated *TNNT2* impair contractile performance resulting in diminished force output and cardiac hypertrophy [37]. In turkeys, Biesiadecki and Jin [38] reported an unusual low molecular weight (mw) isoform of *TNNT2* expressed at

a much higher level in the hearts of DCM birds as compared to birds with normal hearts. The low mw isoform was characterized by a 9 bp deletion in intron 7 that weakens splicing of downstream exon 8. They suggest this low mw isoform could be causative in DCM, however this same low mw isoform was also found in wild turkey hearts at a similar level as the DCM hearts.

The results of the present study found only subtle differences in the expression of *TNNI1* and *TNNT2* between the F and RBC2 lines. This was not the case for *TNNC1* where qRT-PCR found the F line to display higher initial *TNNC1* expression, but lower levels at 16 wk. In mammals, cardiac expression of *TNNC1* increases through gestation, plateaus and then remains steady through adulthood despite protein turnover every 3-10 days [39]. During gestation and myocyte formation there is a progressive increase in the expression of *TNNC1*; however no change is seen in expression of either *TNNT2* or *TNNI* during this same period. This increase was positively correlated with both gestational age and fetal weight [40]. Our study included only two post-hatch developmental time points, multiple additional time points starting early in incubation would be valuable in future expression studies.

Cardiac troponin C is an important part of the troponin complex acting to bind calcium ions to alter interactions between actin, tropomyosin and myosin. The troponin complex is the first step in the sarcomere mechanics of muscle contraction. Troponin C is the first in the cascade. *TNNC1* has two domains; Ca^{2+} binds to the N lobe of *TNNC1* which induces a conformational shift where TNNI3 can bind. The troponin complex is tethered to the thin filament by *TNNT2*, which induces a movement of tropomyosin upon Ca^{2+} binding to *TNNC1,* allowing the formation of myosin crossbridges with actin [41-42].Therefore if the expression level of *TNNC1* is altered, contractility and cardiac function can also be altered. Mutations in *TNNC1* have been linked to certain forms of cardiomyopathy [43] and a loss of function mutation in the zebrafish homolog (*tnnc1a*) is characterized by a dysmorphic non-contractile ventricle [44]. Conditional knockdown in zebrafish with antisense RNA results in decrease heart rate and ventricular ejection fraction, asynchonized contractions and increased mortality [45]. Altered kinetics of either Ca^{2+} binding affinity or disassociation from *TNNC1* can impair contractility and/or relaxation [42,46]. It is therefore conceivable that reduced expression of *TNNC1* in the heavier muscled birds could increase susceptibility to cardiomyopathy. Christensen et al. [14] found F-line embryos displayed a different energy metabolism profile than their random bred counterparts, relying more on gluconeogenesis. The F-line also

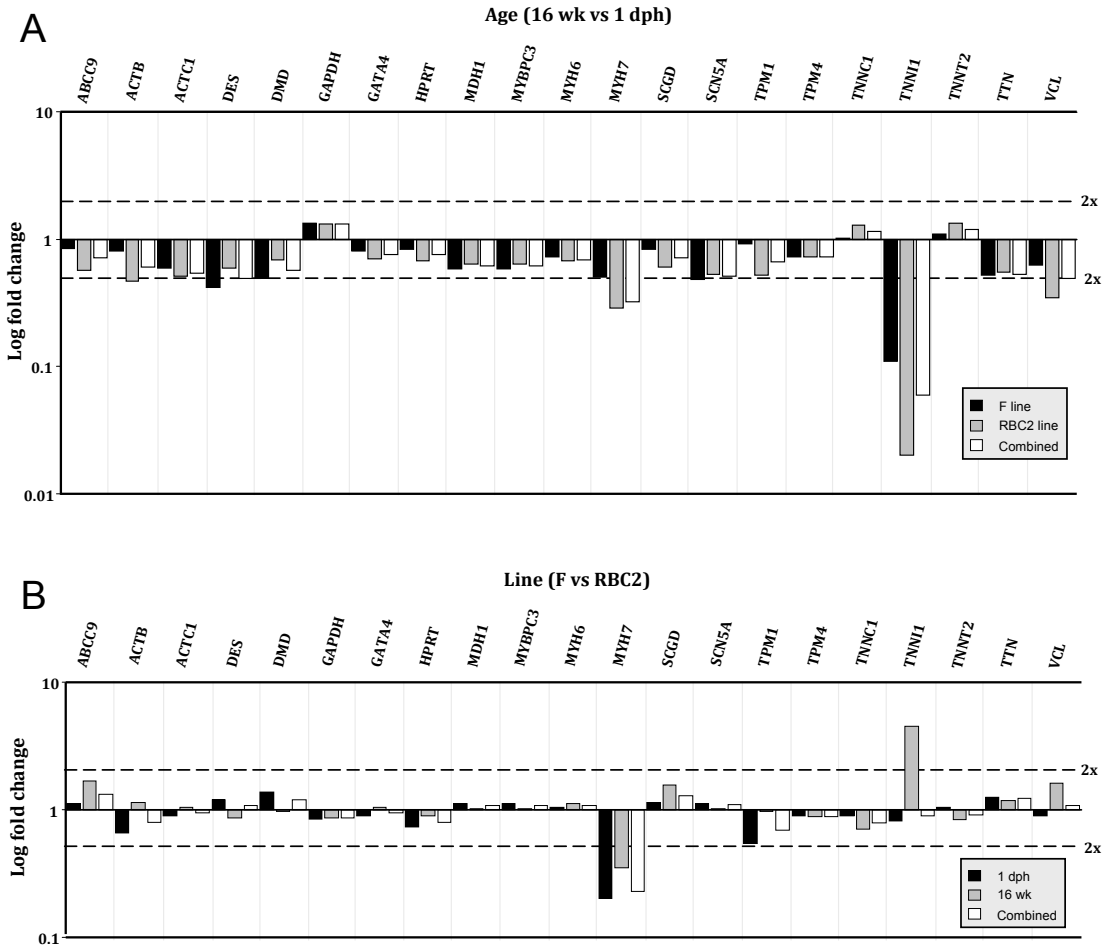

Figure 3: Log fold change observed at 21 genes in the multiplex CEQ analysis. A) Comparison between developmental time points (Age; 16 wk versus 1 dph) within genetic lines and for all individuals combined. B) Comparison between genetic lines (F and RBC2) within developmental time points and for all individuals combined. Dashed lines indicate 2x fold change.

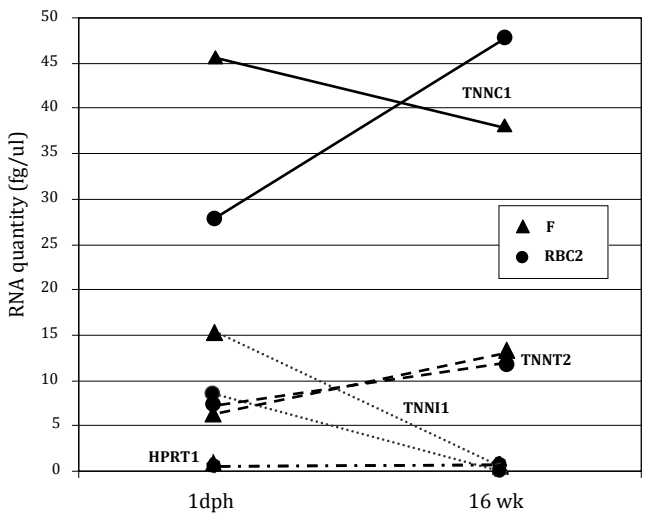

Figure 4: Mean RNA quantities determined by qRT-PCR at two developmental time points for each of four genes in F-line and RBC2 birds.

displayed elevated glycogen to lactate ratios in the myocardium, a condition that leads to increased frequency of death.

This study provides the necessary framework for expanded studies of cardiac gene expression in the turkey and identified a suite of candidate genes for future experimental investigations. Despite the limitation of relatively small sample size (and reduced statistical power), complementary supporting results were obtained from each of the experimental approaches. Importantly, this study demonstrates broader application of TSKMLO microarray in studying turkey cardiac muscle.

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