Proteomic profiling of bovine *M. longissimus lumborum* from Crossbred Aberdeen Angus and Belgian Blue sired steers varying in genetic merit for carcass weight[1]

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ABSTRACT: Bovine skeletal muscle is a tissue of significant value to the beef industry and global economy. Proteomic analyses offer the opportunity to detect molecular mechanisms regulating muscle growth and intramuscular fat accumulation. The current study aimed to investigate differences in protein abundance in skeletal muscle tissue of cattle from two breeds of contrasting maturity (early vs. late maturing), adiposity, and muscle growth potential, namely, Belgian Blue (BB) × Holstein Friesian and Aberdeen Angus (AA) × Holstein Friesian. Twenty AA (n = 10) and BB (n = 10) sired steers, the progeny of sires of either high or low genetic merit, expressed as expected progeny difference for carcass weight (EPD_{cwt}), and bred through AI, were evaluated as 4 genetic groups, BB-High, BB-Low, AA-High, and AA-Low (n = 5 per treatment). Chemical composition analysis of *M. longissimus lumborum* showed greater protein and moisture and decreased lipid concentrations for BB-sired compared with AA-sired steers. To investigate the effects of both sire breed and EPD_{cwt} on *M. longissimus lumborum*, proteomic analysis was performed using 2-dimensional difference gel electrophoresis followed by mass spectrometry. Proteins were identified from their peptide sequences, using the National Center for Biotechnology Information (NCBI) and Swiss-prot databases. Metabolic enzymes involved in glycolysis (glycogen phosphorylase, phosphoglycerate mutase) and the citric acid cycle (aconitase 2, oxoglutarate dehydrogenase) were increased in AA- vs. BB-sired steers. Expression of proteins involved in cell structure, such as myosin light chain isoforms and troponins I and T, were also altered due to sire breed. Furthermore, heat shock protein β-1 and peroxiredoxin 6, involved in cell defense, had increased abundance in muscle of AA-sired relative to BB-sired steers. Protein abundance of glucose-6-phosphate isomerase, enolase-3, and pyruvate kinase was greater in AA-sired animals of High compared with Low EPD_{cwt}. Changes in the expression of these proteins were supported by gene expression analysis using quantitative real-time PCR. This information will aid in our understanding of genetic influences controlling muscle growth and fat accumulation and could contribute to future breeding programs to increase lean tissue gain of beef cattle.

Key words: cattle, growth, proteomics, skeletal muscle, glycolysis

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INTRODUCTION

Bovine skeletal muscle is a tissue of significant economic importance worldwide. Genetic evaluations for a range of economically important performance traits across all of the main cattle breeds in Ireland are undertaken by the Irish Cattle Breeding Federation. Genetic merit for carcass growth rate, an important commercial trait reflecting lifetime growth, is estimated using a multitrait animal model and is

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expressed as the EPD for carcass weight (**EPD_{cwt}**; Campion et al., 2009a). Both sire breed type and EPD_{cwt} influence carcass characteristics, including yield and quality of saleable meat from cattle (Campion et al., 2009a,b; Keane and Moloney, 2010; Keane et al., 2011). Belgian Blue (**BB**) is a late-maturing breed noted for its exaggerated muscular growth and very lean meat deposition (Fiems et al., 2013)**,** whereas Aberdeen Angus (**AA**), an early-maturing breed, is renowned for greater levels of marbling fat in muscle, which is favorably associated with tenderness and flavor of beef (Kuber et al., 2004).

A number of studies to date have investigated the physical and physiological differences in animals varying in EPD_{cwt} (Crews et al., 2004; Keane et al., 2011). In addition, Bernard et al. (2009) examined the effects of genetic selection in favor of high muscle growth on global gene expression in muscle tissue of young Charolais bulls. Data from our own group show that both sire breed and genetic merit for carcass weight affect the expression of genes regulating the somatotropic axis in muscle of cattle (Keady et al., 2011), with greater gene expression of IGF-1 and reduced transcript abundance of IGFBP3 in muscle potentially having a role in increased muscle growth. Whereas transcriptomic studies have been fundamental in the establishment of the molecular mechanisms regulating important traits in cattle (McCabe et al., 2012; Zhao et al., 2012), mRNA expression does not always reflect corresponding proteomic abundance (Rogers et al., 2008). Consequently, proteomic studies examining large numbers of proteins simultaneously, including their posttranslational modifications (Rogers et al., 2008), offer a snapshot of the functional molecules of the cells under investigation.

Protein expression of skeletal muscle in both sheep (Hamelin et al., 2006) and cattle (Talamo et al., 2003; Bouley et al., 2004, 2005) has been examined. To the knowledge of the authors, few data exist on the effect of either breed or genetic merit for carcass growth on global protein abundance in bovine muscle. Therefore, the objective of this study was to determine the effect of 1) sire breed and 2) sire EPD_{cwt} on the expression of proteins in *M. longissimus lumborum* in AA- and BB-sired cattle using 2-dimensional difference gel electrophoresis (**2D-DIGE**) and mass spectrometry.

MATERIALS AND METHODS

All procedures were performed under license in accordance with European Community Directive 86-609-EC. Animals were slaughtered in a licensed abattoir (Meadow Meats, Rathdowney, Co. Laois, Ireland).

Experimental Design

This study utilized *M. longissimus lumborum* samples collected at slaughter from a larger study by Campion et al. (2009a). Briefly, in that study, steer progeny ($n = 114$) of Holstein-Friesian (**HF**) dairy cows sired by bulls of 2 contrasting beef breeds (AA and BB) were used. Within each sire breed, sires were selected on the basis of EPD for carcass weight (EPD_{cwt}) and classified as either **High** or **Low**. There were 32 steer progeny of High EPD_{cwt} sires and 24 of Low EPD_{cwt} sires. For BB there were 31 progeny of High and 27 of Low EPD_{cwt} sires.

There was no difference between the dams of the various genetic groups in estimated genetic merit for beef production and carcass weight (Campion et al., 2009b). The finishing diet consisted of a total mixed ration with a grass-silage:concentrate ratio of 30:70 on a DM basis (Campion et al., 2009a). For the current study, a representative subset of 20 steers was selected at slaughter in a 2 (sire breed) × 2 (sire EPD_{cwt}) factorial design ($n = 5$ per treatment), generating 4 genetic groups of AA-High, AA-Low, BB-High, and BB-Low based on the original blocking criteria of Campion et al. (2009a). The 20 animals represented the progeny of 12 sires (AA, $n = 6$, and BB, $n = 6$) with no over dominance of any particular sire within group. Mean values for the subgroups used in our study for the main outcome traits were similar to that of Campion et al. (2009a; i.e., mean animal slaughter weight for AA was 583 kg and for BB was 600 kg, whereas ADG for AA-High vs. Low were 0.64 kg and for BB were 0.71 kg). As crossbred BB animals were employed in this study, it must be noted that they were heterozygous for the myostatin null mutation.

Chemical Composition of M. longissimus lumborum

At 48 h after slaughter, steaks were cut from the *M. longissimus lumborum* between the 6th and 10th ribs, vacuum packaged immediately, frozen, and used for subsequent chemical analysis. Intramuscular fat and moisture concentrations were determined from thawed Longissimus thoracis et lumborum using the Smart System 5 microwave moisture drying oven and NMR Smart Trac Rapid Fat analyzer (CEM Microwave Technology Ltd., Buckingham, UK), using AOAC Official Methods 992.15 (1990). Protein concentration was determined using a LECO FP328 (LECO Corp., St. Joseph, MI) protein analyzer on the basis of the Dumas method and according to AOAC Official Method 992.15 (1990).

Tissue Collection, Extraction of Muscle Proteins, and 2D-DIGE

Campion et al. (2009a) reported that LM area and LM area per unit carcass weight were greater for Belgian Blue × Holstein Friesian (**BB-HF**) compared with Aberdeen Angus × Holstein Friesian (**AA-HF**) steers and also for AA animals sired by bulls with High compared with Low EPD_{cwt}. Specifically, *M. longissimus lumborum* is of high economic and commercial value. For these reasons *M. longissimus lumborum* was selected for proteomic analysis in the current study. Samples of *M. longissimus lumborum* were harvested from animals at slaughter and washed in sterile Dulbecco's PBS (Sigma-Aldrich Ireland Ltd., Dublin, Ireland). Samples were snap frozen at −80°C in liquid nitrogen within 20 min of slaughter. A sample was also stored at −80°C for quantitative real-time PCR (**qRT-PCR**) validation analysis. Because of differences in inter- and intramuscular fat in the muscle tissue from AA- vs. BB-sired animals, care was taken to collect lean muscle tissue only for protein and mRNA isolation while avoiding inter- and intramuscular fat.

Protein extraction and 2D-DIGE were performed as described by Mullen et al. (2011). Frozen muscle (100 mg) was weighed and crushed into a fine powder using a mortar and pestle and placed in 7 *M* urea, 2 *M* thiourea, 1% (wt/vol) dithiothreitol (**DTT**), 4% (wt/vol) 3-[(cholamidopropyl) dimethylammonio] propanesulfonic acid (**CHAPS**), and 8% (vol/vol) ampholytes (pH 3 to 10; Bio-Rad Laboratories Inc., Hercules, CA). To avoid protein degradation, protease inhibitors (Roche, Clarecastle, Co., Clare, Ireland) were added. Samples were shaken vigorously at 4°C for 150 min before centrifugation at 10,000 × *g* for 20 min at 4°C. Bradford colorimetric protein assays (Bio-Rad Laboratories Inc.) were performed to determine protein concentration using BSA as a standard. Immobiline DryStrips (GE Healthcare Biosciences, Buckinghamshire, UK), 24 cm, covering a pH range of 3 to 10 were rehydrated overnight in 500 μg of sample up to a volume of 450 μL of rehydration buffer [8 *M* urea, 0.5% (vol/vol) CHAPS, 0.2% (wt/vol) DTT, and 0.2% (vol/vol) ampholytes (pH 3 to 10; Bio-Rad Laboratories)]. DeStreak reagent (GE Healthcare Biosciences) was added to the sample solution to stabilize thiol groups and prevent nonspecific oxidation. Isoelectric focusing was performed in an Ettan IPGphor 3 Isoelectric Focusing Unit (GE Healthcare Biosciences), and the following voltage/time program was used: 100 V for 120 min, 500 V for 90 min, 1,000 V 60 min, 2,000 V for 60 min, 4,000 V for 60 min, 6,000 V for 120 min, 8,000 V for 150 min, 500 V for 240 min, and 8,000 V for 300 min. After focusing, strips were equilibrated for 15 min in 1% (wt/vol) DTT followed by 15 min in 2.5% (wt/vol) iodoacetamide. After equilibration, proteins were separated on a 12% polyacrylamide gel using the DALT*twelve* separation unit (GE Healthcare Biosciences).

Gel Staining and Image Analysis

For DIGE analysis, Cy3 and Cy5 dyes were reconstituted as a stock solution of 1 m*M* in fresh dimethylformamide. The stock solution was diluted to a working solution of 0.2 m*M* before protein labeling. Individual samples (50 μg protein) were minimally labeled with 200 pmol of Cy3 working solution (Karp and Lilley, 2005). A pooled sample consisting of equal quantities of protein from all replicates used in the experiment were labeled at a ratio of 200 pmol of Cy5 working solution to 50 μg of muscle protein. All samples were labeled with the appropriate amount of dye at pH 8.5 and then incubated on ice in the dark for 30 min (Mullen et al., 2011). Ruthenium (II) tris bathophenanthroline disulfonate (**RuBPS**), a well-established fluorescent dye for protein staining, was applied for 6 h, followed by destaining using 40% (vol/vol) ethanol and 10% (vol/vol) acetic acid for 15 h. Reference gels containing the pooled sample were generated for spot picking and stained with Coomassie Brilliant Blue (Doran et al., 2006). Imagemaster platinum analysis software v5.0 was used for imaging gels using a Typhoon variable mode image scanner (GE Healthcare Biosciences). Progenesis Samespots V3.2.3 software (NonLinear Dynamics, Newcastle upon Tyne, UK) was used for the detection of protein spots, background subtraction and detection of proteins with statistically significant differences across groups. Before analysis of individual gels, a single reference gel, representing electrophoretically separated proteins from muscle tissue from all animals in the study, was analyzed and further matched and normalized with non-gel spots manually filtered out on the basis of spot volume (Donoghue et al., 2010; Mullen et al., 2011). Differentially expressed protein spots ($P < 0.05$) between AA and BB were classified as biologically significant with a fold change greater than 1.9 and were selected for tryptic digestion from gels stained with Coomassie Brilliant Blue G-250. At a fold change of greater than 1.9, no difference in protein abundance was identified between High and Low steers for EPD_{cwt}. However, in complex biological systems physiological changes are often controlled by larger numbers of small fluctuations in signaling networks rather than a small number of large changes (Amaral et al., 2004). Therefore, a 1.5-fold induction in protein abundance was selected as a cutoff between High- and Low-sired steers for EPD_{cwt} (AA-High vs. AA-Low; BB-High vs. BB-Low), which is consistent with findings of previous research in relation to the High and Low for growth rate (Bernard et al., 2009).

Protein Spot Isolation and Identification Using Mass Spectrometry

Mass spectrometric identification of the muscle proteins of interest was performed as previously

described (O'Connell and Ohlendieck, 2009). In brief, protein spots were carefully excised using a sterile pipette tip and digested using trypsin (Promega, Southampton, UK) overnight at 37°C. The solution was removed, and 30% (vol/vol) acetonitrite (**ACN**)/0.2% (vol/vol) trifluoroacetic acid (**TFA**) was added to each gel plug and incubated for 10 min at 37°C, followed by 60% (vol/vol) ACN/0.2% (vol/vol) TFA for 10 min at 37°C. The solution was dried overnight, and 10 μL of 0.1% (vol/vol) formic acid were added to the protein pellet. Identification of proteins was performed with a Model 6340 Ion Trap LC/MS (Agilent Technologies, Dublin, Ireland). Proteins were identified from their peptide sequences, searching on the National Center for Biotechnology Information (**NCBI**; http://www.ncbi.nlm.nih.gov) database. This database was interrogated using the MASCOT search engine (http://www.matrixscience.com; Matrix Science, London, UK) with comparison with mammalian databases allowing up to one single trypsin missed cleavage.

Validation of Protein Expression using qRT-PCR

Ideally, validation of DIGE results of candidate proteins should have been conducted using Western blotting. However, in the current study, we experienced some technical issues with the Western blotting procedure. We did, however, validate the proteomic data generated using a qRT-PCR approach, and whereas some studies have shown a lack of correlation between the outcomes of these 2 techniques (Greenbaum et al., 2003), in the current study high positive correlation coefficients between gene transcript and protein data were observed.

For the purpose of validation of proteomic data, 6 proteins, 3 (HSPB1, PRDX6, and MYL1) found to be differentially expressed in the AA-sired compared with the BB-sired steers and 3 (ENO3, GPI, and PKM2) that were shown to be differentially expressed in High and Low EPD_{cwt} in muscle tissue of AA, were targeted using qRT-PCR analysis. Total RNA was isolated from approximately 40 mg of the same muscle tissue used for protein analysis using TRIzol reagent and chloroform (Sigma-Aldrich Ireland). Tissue samples were homogenized using a tissue lyser (Qiagen, Manchester, UK), after which the RNA was precipitated using isopropanol. Samples were then treated with RQ1 RNase-free DNase (Promega UK, Southhampton, UK), according to the manufacturer's instructions, to remove any contaminating genomic DNA. The quantity of the RNA isolated was determined by measuring the absorbance at 260 nm using a Nanodrop spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE). The RNA quality was assessed on the Agilent Bioanalyser 2100 using the RNA 6000 Nano Lab Chip kit (Agilent Technologies). The RNA quality was verified by ensuring all RNA samples had an absorbance ($A_{260/280}$) of between 1.8 and 2. The RNA samples with 28S/18S ratios ranging from 1.8 to 2.0 and an RNA integrity number (**RIN**) of between 8 and 10 were deemed high quality.

Total RNA (1 μg) was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) using the Multiscribe reverse transcriptase according to manufacturer's instructions. Samples were stored at −20°C for subsequent analyses. To select stable reference genes for the muscle tissue being investigated, analysis of putative reference genes was performed using the geNorm version 3.4 Excel software (Microsoft, Redmond, WA). The cycle threshold (**Ct**) values were transformed to relative quantities using the comparative delta Ct ($\mathbf{2^{-\Delta CT}}$) method to facilitate the calculation of the M value within the geNorm software. A gene was considered to be sufficiently stable if an M value of less than 1.5 was generated. Within this range of parameters, β-actin (***ACTB***), glyceraldehydes 3-phosphate dehydrogenase (***GAPDH***) and ribosomal protein SP (***RPS9***) were selected as suitable reference genes for this study. All primer sets (listed in Table 4; see Results section) to amplify reference and specific target genes (*ENO3*, *GPI*, and *PKM2*) were designed using the Web-based software program Primer 3 (http://frodo.wi.mit.edu/primer3/) and were obtained from a commercial supplier (Sigma-Aldrich Ireland).

After reverse transcription, cDNA quantity was determined and standardized to the required concentration for qPCR. Triplicate 20 μL reactions were performed in 96-well optical reaction plates (Applied Biosystems, Warrington, UK) containing 1 μL cDNA (10 to 50 ng of RNA equivalents), 10 μL Fast SYBR Green PCR Master Mix (Applied Biosystems), 8 μL nuclease-free H_2O, and 1 μL forward and reverse primers (250 to 1,000 n*M* per primer). Assays were performed using the ABI 7500 Fast qPCR System (Applied Biosystems) with the following cycling parameters: 95°C for 20 s and 40 cycles of 95°C for 3 s, 60°C for 30 s followed by amplicon dissociation (95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 15 s). Amplification efficiencies were determined for all target and reference genes using the formula $E = 10^{(-1/slope)}$, with the slope of the linear curve of Ct values plotted against the log dilution (Higuchi et al., 1993).

Only primers with PCR efficiencies between 90% and 110% were used. The software package GenEx 5.2.1.3 (MultiD Analyses AB, Gothenburg, Sweden) was used for efficiency correction of the raw Ct values, interplate calibration based on a calibrator sample included on all plates, averaging of replicates, normalization to the reference gene, and the calculation of quantities relative to the greatest Ct. Expression of each target

gene was normalized to the reference genes, and relative differences in gene expression were calculated using the $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

Data relating to the chemical composition of the muscle and key performance data from Campion et al. (2009a,b) were checked for adherence to normality using the UNIVARIATE procedure of statistical analysis software (SAS Inst. Inc., Cary, NC). Nonnormally distributed data were transformed, as appropriate, by raising to the power of λ (TransReg procedure). Data were analyzed using mixed models ANOVA (PROC MIXED). Sire breed and genetic merit for growth potential were included as fixed effects in the statistical model together with the interaction term, where appropriate. Sire was included as a random effect. The Tukey critical difference test was performed to determine the existence of statistical differences between treatment mean values.

Progenesis SameSpots analysis (**SSA**) was used to identify proteins of interest as described by Donoghue et al. (2010) and Mullen et al. (2011). The gel images were placed into groups (AA, BB, AA-High, and AA-Low), and analysis was carried as follows: AA vs. BB, AA-High vs. AA-Low, and BB-High vs. BB-Low. The Progenesis Samespots software uses a 1-way ANOVA test, and therefore, the interaction term could not be tested. For sire breed comparison, gel images from both breeds were matched to a single reference gel image. Power analysis was performed independently for each protein spot, with changes displaying a power of <0.8 being removed from the analysis. Statistical analysis of the relative abundance of each matched protein spot across the data sets was performed using Student's *t* test. Additionally, *t* tests between mean protein differences, with a *P*-value of >0.05, were removed from analysis.

For qRT-PCR analysis, a mixed model ANOVA (PROC MIXED, SAS) was conducted to determine the effect of High or Low EPD_{cwt} on the relative expression of each gene measured. The Tukey critical difference test was performed to determine the existence of statistical difference between the treatment groups. Fold changes in gene expression between the High and Low growth rates were determined. Spearman correlation coefficients were calculated to determine associations between the expression of proteins and genes found to be differentially expressed between High and Low for EPD_{cwt} in AA muscle tissue using the CORR procedure of SAS.

RESULTS

Chemical Analysis of LM

There was a difference in the chemical composition of the muscle between breeds (Table 1), with BB having greater ($P < 0.001$) protein and moisture content and a decreased ($P < 0.001$) lipid concentration compared with AA. There was no effect ($P > 0.05$) of EPD_{cwt} or sire breed × EPD_{cwt} interaction on LM composition after chemical analysis.

Effect of Sire Breed on Protein Abundance

After scanning with an Amersham Typhoon Trio variable imager and with the assistance of Progenesis 2D analysis software, a total of 612 two-dimensional spots were detectable on the reference gel. Twenty-one protein spots, relating to 16 protein products, were identified as different ($P < 0.005$) in their abundance across sire breed, with fold changes larger than 1.9 (Figure 1 and Table 2). To investigate the functional significance of the identified differentially expressed proteins, Ingenuity Pathway Analysis (**IPA**) software (IPA V9.0; Ingenuity Systems, Mountain View, CA, http://www.ingenuity.com) was used. Overall, the top canonical pathway identified was glycolysis/gluconeogenesis, with three proteins [glycogen phosphorylase (**PYGM**), phosphoglycerate mutase 2 (**PGAM2**), and aldolase A (**ALDOA**)] within the pathway activated. The second canonical pathway identified as activated was the citric cycle, with enzymes aconitase-2 (**ACO2**) and 2-oxoglutarate dehydrogenase (**OGDH**) identified as greater in abundance in AA compared with BB. The third canonical pathway was the protein kinase

Table 1. Effect of sire breed (B) and EPD for carcass weight (EPD_{cwt}) on the chemical composition (expressed as a percentage of 100%) of *M. longissimus lumborum* at slaughter

	B[1]			EPD_{cwt}[2]			*P*-value		
Variable	AA-HF	BB-HF	SED[3]	High	Low	SED[3]	B	EPD_{cwt}	B × EPD_{cwt}
Protein	22.22[a]	23.12[b]	0.16	22.76	22.57	0.16	***	0.25	0.88
Moisture	71.39[a]	73.29[b]	0.27	72.39	72.30	0.27	***	0.73	0.64
Fat	6.01[a]	2.99[b]	0.37	4.39	4.62	0.37	***	0.53	0.84

[a,b]Least squares means within a row with different superscript differ ($P < 0.05$); *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

[1]AA-HF = Aberdeen Angus × Holstein Friesian; BB-HF = Belgian Blue × Holstein Friesian.

[2]High = high for EPD_{cwt}; Low = low for EPD_{cwt}.

[3]SED = SE of the difference.

A signaling (**PKA**) pathway, with proteins myosin light chain 1 (**MYL1**), myosin light chain, phosphorylatable (**MYLPF**), PYGM, and troponin I (**TNNI2**) differing in abundance across breed. Finally, the fourth canonical pathway identified was the pentose phosphate pathway, with ALDOA greater and phosphoglucomutase (**PGM1**) decreased in abundance in AA relative to BB sired steers, within the pathway. Other proteins identified as greater in abundance in AA compared with BB included AMP deaminase 1 (**AMPD1**), LIM domain binding 3 (**LDB3**), vinculin (**VCL**), capping protein α (**CAPZA2**), heat shock protein β-1 (**HSPB1**), and peroxiredoxin 6 (**PRDX6**).

Effect of EPD_{cwt} on Protein Abundance

No difference ($P > 0.05$) in protein spot abundance was detected between High and Low BB-sired animals. For AA, however, a difference ($P < 0.012$) in protein abundance of 3 glycolytic enzymes was observed between High and Low EPD_{cwt} groups. These 3 proteins, glucose-6-phosphate isomerase (**GPI**), enolase (**ENO1**), and pyruvate kinase (**PKM2**), were identified in the top canonical pathway, glycolysis/gluconeogenesis. These 3 proteins were greater in abundance in High compared with Low AA sired steers (Table 3).

qPCR Validation of Proteomics Data

To verify the DIGE results, a total of 3 proteins (glucose-6-phosphate isomerase, enolase-3, and pyruvate kinase) found to be differentially expressed in muscle of High vs. Low AA-sired steers were targeted to be analyzed by qPCR (Table 4). There was a statistically significant ($P < 0.05$) strong positive correlation between the protein and mRNA expression data for all 3 genes selected (Table 4).

DISCUSSION

Bovine skeletal muscle is a heterogeneous tissue comprised of several fiber types influenced by the genotype and breed of the animal (Bouley et al., 2005; Chaze et al., 2008). Additionally, its properties evolve during postnatal life and can be modified by environmental conditions (Therkildsen, 2005; Shibata et al., 2009). This study examined differences in protein abundance in *M. longissimus lumborum* in steers sired by 2 contrasting breeds of cattle (AA and BB) divergent for EPD_{cwt}. These breeds were selected on the basis of their well-documented differences in 1) maturation rates (early vs. late) and 2) intramuscular fat accumulation as well as perceived meat quality differences (Kuber et al., 2004; Sadkowski et al., 2009). We have shown that metabolic enzymes involved

Table 2. Proteins differentially expressed between Belgian Blue (BB) and Aberdeen Angus (AA) sired steers

No.	Identified protein	NCBI accession no. (Source)	Mascot score	Fold Change[1]	*P*-value	Matched peptides/sequence coverage, %	Theoretical pI/M_r, kDa[2]
Metabolic							
1	PYGM	gi|73983205 (canine)	224	5.7↑	<0.0001	6/10	6.6/97.5
2	PYGM	gi|154426116	1652	2.9↑	<0.0001	34/47	6.7/97.5
3	PYGM	gi|28461197	1374	2.8↑	<0.0001	31/37	6.7/97.6
6	PYGM	gi|28461197	609	2.0↑	0.002	18/24	
4	PGAM2	gi|84000195	643	2.9↑	0.004	13/49	9.0/28.8
5	PGM1	gi|116004023	1029	2.2↓	0.0001	21/52	6.4/61.8
7	ALDOA	gi|156120479	804	2.8↑	<0.0001	15/53	8.5/39.9
8	ACO2	gi|74268076	816	5.3↑	0.0006	17/30	8.0/85.9
9	OGDH	gi|115496742	491	3.9↑	0.0001	12/12	6.3/116.8
18	AMPD1	gi|154152079	279	4.0↑	0.0002	6/10	6.9/87.2
Contractile apparatus							
10	TNNT1	gi|21039010	219	3.9↑	<0.0001	5/23	6.2/30.1
11	TNNI2	gi|76658412	213	3.5↑	<0.0001	4/17	8.9/21.5
19	MYL1	gi|1181841	451	4.6↓	<0.0001	8/52	4.7/18.8
20	MYLPF	gi|115497166	206	2.8↓	0.003	5/31	4.9/19.1
21	MYLPF	gi|115497166	497	2.1↓	0.005	9/61	
Cell structure							
12	LDB3	gi|78369256	362	4.0↑	0.003	7/33	9.3/35.5
13	VCL	gi|194679459	440	2.3↑	0.006	10/13	5.9/11.7
17	VCL	gi|194679457	432	1.9↑	0.003	10/10	5.6/124.3
14	CAPZA2	gi|433308 (human)	382	2.2↑	<0.0001	8/44	5.6/32.9
Cell defense							
15	HSPB1	gi|85542053	750	2.2↑	0.0006	15/76	6.0/22.4
16	PRDX6	gi|27807167	626	1.9↑	0.0003	13/60	6.0/25.1

[1]An up arrow (↑) = increased in AA- vs. BB-sired steers; a down arrow (↓) = decreased in AA- vs. BB-sired steers.

[2]p_I = isoelectric point, M_r = molecular weight.

in glycolysis, and the citric acid cycle, cell structure, and cell defense were differentially expressed in AA- vs. BB-sired steers. In addition, protein abundance of glucose-6-phosphate isomerase, enolase-3, and pyruvate kinase was greater in High compared with Low AA animals. This information will assist in our understanding of genetic influences controlling muscle growth and fat accumulation and could contribute to future breeding programs to increase lean tissue gain of beef cattle.

Proteomic technologies have been used to characterize protein expression of muscle (Talamo et al., 2003; Bouley et al., 2004) and to assess changes in muscle proteins postmortem (Lametsch and Bendixen, 2001; Lametsch et al., 2003). Two-dimensional gel electrophoresis (**2D-GE**) is one of the most commonly used techniques in proteomics (Ohlendieck, 2011). It has been shown that up to 10,000 protein spots can be separated in 1 gel, allowing high-resolution proteomic analysis (Klose and Kobalz, 1995). Two-dimensional difference gel electrophoresis (Unlü et al., 1997) addresses some of the major problems, including lack of reproducibility and quantitation, associated with traditional 2D-GE. Thus, comparative protein expression and post-translational protein modification profiling are tasks that are best performed using 2D-DIGE. This technique has recently been employed to examine quantitative and qualitative differences between fast- and slow-type muscles in Holstein cows (Oe et al., 2011).

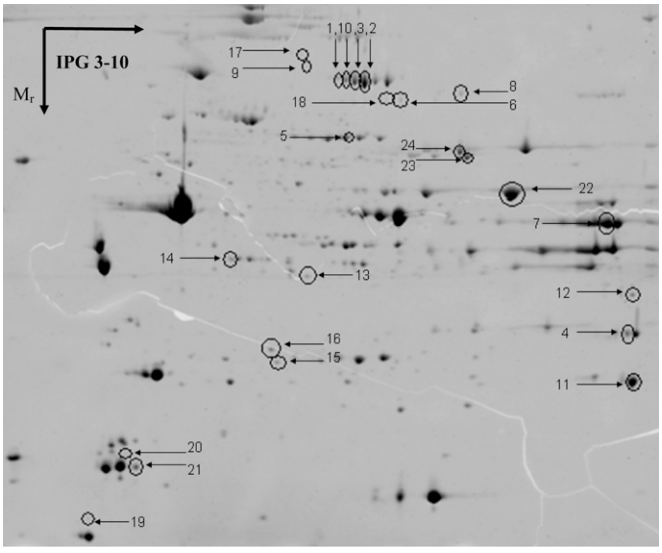

Figure 1. Representative 2-dimensional gel image of M. longissimus lumborum of crossbred steers. The proteins are indicated by spot number, which corresponds to those identified as differentially expressed between the different groups (see Tables 2 and 3).

Effect of Sire Breed on Protein Abundance

The top canonical pathway identified was glycolysis/gluconeogenesis with 3 proteins, PYGM, PGAM2, and ALDOA, found to be in greater abundance in AA compared with BB. Bernard et al. (2009) found that glycolytic enzymes were increased in bulls divergently selected for high compared with low muscle growth. However, in the current study, AA had greater abundance of proteins involved in glycolysis and the citric cycle compared with BB, despite the greater muscularity in the latter. Results from our study and those of Bernard et al. (2009) possibly differ mainly in the intramuscular fat relative to muscle in AA arising because of differences in sire breed genetics and age of the animals.

In fatty acid synthesis, the reduced form of nicotinamide adenine dinucleotide phosphate **(NADPH)** is required to support lipogenesis. In ruminants, 50% to 80% of NADPH required for fatty acid synthesis in adipose tissue is produced by glucose oxidation via the pentose phosphate pathway (Vernon, 1981). Up to 50% of NADPH used in fatty acid synthesis is also generated via decarboxylation of isocitrate to α-ketoglutarate (isocitrate dehydrogenase pathway; Vernon, 1981; Nafikov and Beitz, 2007). This action is preceded by the activity of the enzyme ACO2 and followed by a reaction involving the enzyme OGDH. These enzymes, which were increased in AA compared with BB steers in the current study, are involved in the citric acid cycle, the third canonical pathway identified to be activated. This suggests greater protein abundance of enzymes relating to oxidative metabolism in AA-sired steers. As there are also greater quantities of mitochondria, where the citric acid cycle takes place, in slow-twitch compared with fast-twitch muscle fibers as evident in AA, this finding is not surprising and warrants

Table 3. Proteins differentially expressed in muscle tissue between Aberdeen Angus–sired steers of either High or Low for sire expected progeny difference for carcass weight (EPD_{cwt})

No.	Identified protein	NCBI accession no. (Source)	Mascot score	Fold change[1]	*P*-value	Matched peptides/sequence coverage, %	Theoretical pI/ M_r kDa[2]
				Metabolic			
22	ENO3	gi|87196501 (Canis)	1117	1.5↑	<0.0001	21/50	7.6/47.5
23	GPI	gi|94966765	646	1.8↑	0.003	13/24	7.3/63.0
24	PKM2	gi|73587283	894	1.7↑	0.012	14/34	8.6/62.0

[1]An up arrow (↑) = increased in High compared with Low for EPD_{cwt}.

[2]p_I = isoelectric point, M_r = molecular weight.

Table 4. Validation of proteins found to be differentially expressed in *M. longissimus lumborum* using difference gel electrophoresis and mass spectroscopy by quantitative real time polymerase chain reaction

Gene name	Primer sequences	Accession no.	Efficiency[1]	Amplicon size, bp	Fold change[2]	Fold change *P*-value	Spearman partial correlation coefficient[3]	Spearman *P*-value
Target genes								
GE in AA- vs. BB-sired steers[4]								
HSPB1	F: TGGAGATCACTGGCAAGCAC R: ATTTGCGAGTGAAGCAACGG	NM_001025569	1.04	72	1.81	0.02	0.75	0.02
PRDX6	F: TGACTGCTCGTGTGGTGTTT R: CAGGGATGGTTGGAAGGACC	AF090194	1.01	198	1.83	0.01	0.82	0.03
MYL1	F: TGTTCTAGCCACACTAGGTGAA R: ACCTTGAGAGCTCCATTTAGTT	NM_001079578	0.97	140	-4.21	0.003	0.91	0.01
GE in high vs. low sire EPD_{cwt} in AA-sired steers[4]								
ENO3	F: GATTGGCTCGGTGACTGAAT R: GGGCACCAGTCTTGATCTGT	NM_001034702	0.98	155	1.56	0.02	0.68	0.04
GPI	F: CCCTGTTGTGACTGGTCCTT R: CAGACCAGCTTCCCTCAAAG	NM_001040471	0.92	117	1.81	0.03	0.87	0.02
PKM2	F: CACGCAGAGACCATCAAGAA R: GCATTGTCCAGGGTGATCTT	BT030503	1.02	191	1.78	0.04	0.72	0.03
Reference genes								
ACTB	F: AGCAAGCAGGAGTACGATGAGT R: ATCCAACCGATGCTGTCA	NM_173979.3	0.91	147				
RPS9	F: CCCTCCACGATGCCAAAGT R: CCTCCAGACCTCACGTTTGTTC	NM_001101152	1.08	64				
GAPDH	F: GGCGTGAACCACGAGAAGTATAA R: CCCTCCACGCTGCCAAAGT	NM_001034034	0.98	119				

[1] $E = 10^{(-1/slope)}$.

[2] Increased in High compared with Low for EPD_{cwt} or in Aberdeen Angus (AA) compared with Belgian Blue (BB) sired steers.

[3] Spearman partial correlation coefficient for the association between the muscle expression of proteins and genes.

[4] GE = gene expression, and EPD_{cwt} = expected progeny difference for carcass weight.

further investigation. This study reveals that different metabolic actions are taking place in the muscle tissue that directly relate to sire breed type in cattle. Intramuscular fat accumulation may be associated with increased abundance of enzymes relating to glycolysis, and the current research is consistent with these findings (Murgiano et al., 2010; Sieczkowska et al., 2010).

Glucose-1-phosphate is converted to glucose-6-phosphate during glycogen breakdown by phosphoglucomutase (**PGM1**), allowing it entry into the glycolytic pathway (McMurry and Begley, 2005). Alternatively, this enzyme can also function in reverse, facilitating glycogen synthesis. A 2.2-fold increase was observed in the expression of the PGM protein for BB compared with AA cattle. The activity of PGM can be attributed to that of at least 2 closely related isoforms (PGM1 and PGM2) encoded by different genes (Oh and Hopper, 1990). Consistent with our results, Hamelin et al. (2006) reported that muscle hypertrophy in the ovine is associated with an upregulation of enzymes involved in glycolytic metabolism together with oxidative metabolism in LM. They also showed that protein abundance of PGM1 and PGM2 was increased in rams with muscular hypertrophy compared with conventional genotypes (Hamelin et al., 2006). Muscular hypertrophy, or "double" muscling, occurs because of

mutations in the myostatin coding sequence, resulting in the production of a truncated protein (McPherron et al., 1997). Bouley et al. (2005) found that heterozygote and homozygote animals had a greater proportion of fast-twitch glycolytic fibers in muscle, resulting in differences in protein expression. Therefore, the myostatin deletion was consistent with an increased fast muscle phenotype (Bouley et al., 2005) and reduced slow-twitch oxidative fibers (Keane and Moloney, 2010). Fast-twitch glycolytic fibers, also referred to as white fibers, store high quantities of glycogen (Sherwood and Wagers, 2006). As it is known that PGM plays a role in glycogen catabolism as well as glycogen synthesis (Penha et al., 2005) and given that BB have a greater number of fast-twitch fibers, PGM may function in either glycogen synthesis or breakdown in muscle fibers of BB-sired progeny. However, further research is warranted to investigate the exact role of PGM in muscle of BB-sired compared with AA-sired animals.

The second canonical pathway identified in the current study was the PKA signaling pathway that incorporated proteins such as MYL1, MYLPF, and TNNI2. This pathway is central to many functions in the cell as well as playing a role in cytoskeleton regulation. Belgian Blue have large numbers of fast-twitch fibers because of the myostatin deletion (Bouley et al., 2005). One of the main molecular indicators of fast glycolytic fiber type is fast-twitch myosin (MYL1; Lehnert et al., 2006). Therefore, it is not surprising that in our study MYL1 protein expression was decreased in AA-sired steers relative to BB. In the study of Murgiano et al. (2010), LM from 2 distinct pig breeds, Casertana and Large White, were compared. Proteins related to MYL1 were increased in Large White, a breed that excels in growth of lean muscle tissue, consistent with our data for BB cattle. Troponin T (**TnT**) is an important regulatory and structural component of skeletal muscle thin filaments (Perry, 1998). Troponin T type 1 (**TNNT1**) and TNNI2, found in slow- and fast-twitch skeletal muscle, respectively, are involved in muscle contraction (Picard et al., 2010) and were increased in expression in AA-sired relative to BB-sired steers. Sadkowski et al. (2008) found that TNNT1 was a potential myostatin-dependent gene possibly explaining the differential expression in its protein product in AA-sired relative to BB-sired steers. In double-muscled animals, proteomics studies have demonstrated the underexpression of slow TnT isoforms and the overexpression of fast TnT isoforms in fast glycolytic muscles (Picard et al., 2010) and could potentially explain differences observed in the expression of these proteins in the current study due to sire breed. Furthermore, in postmortem bovine muscle, a 30- or 32-kDa peptide, which was immunologically identified as a TnT fragment, is the first degradation product of a skeletal muscle component that is associated with meat tenderness (Olson and Parrish, 1977). The degradation of TnT progresses simultaneously with the postmortem tenderization of beef, indicating a strong correlation between the 2 events (Penny and Dransfield, 1979). As AA is an early-maturing breed associated with beef tenderness (Kuber et al., 2004), it is not surprising that isoforms of TnT were present in greater abundance in muscle of the AA-sired compared with BB-sired steers.

In the current study, heat shock protein (**HSP**) β-1 and peroxiredoxin 6 (**PRDX6**), involved in cell defense, were increased in AA-sired relative to BB-sired steers. Heat shock proteins are essential for normal cellular stress responses. Heat shock protein β-1 is a relatively small (27 kDa) molecular chaperone protein associated with cellular development, differentiation, and signal transduction, and a microarray analysis revealed that mRNA expression of HSPβ1 increased during the intramuscular fat development stage in cattle (Wang et al., 2009). Zhang et al. (2010) found that HSPβ1 increased during fat accumulation in bovine skeletal muscle. This is consistent with the fact that AA-sired steers have greater intramuscular fat compared with their BB-sired contemporaries, which was confirmed by chemical analysis of muscle tissue in the current study. Recently, Zhang et al. (2012) found that protein expression and mRNA expression of HSPβ1 was upregulated in skeletal muscle tissue in response to differentiation, playing an important role in myogenesis, indicating that muscle tissue in AA-sired steers, an early-maturing breed relative to BB, was possibly at a more advanced stage of development. Peroxiredoxin 6, a member of antioxidant protein superfamily, plays an important role in oxidative stress, catabolism of lipids, and phospholipid liposomes (Fisher, 2011). There is little information in the literature on protein or mRNA expression of PRDX6 in cattle, and this is the first published study to show the protein expression of PRDX6 in bovine skeletal muscle. It may be hypothesized that as AA-sired steers contain greater proportions of intramuscular fat compared with BB-sired steers, an increase in PRDX6 is required for lipid catabolism. In addition, 2 SNP in *Prdx6* have been shown to be associated with meat quality traits (especially the muscle tenderness; Liu et al., 2011). This is not surprising, considering that meat from AA has been shown to be positively associated with having increased tenderness and flavor (Kuber et al., 2004).

Effect of EPD_{cwt} on Protein Abundance

The glycolytic pathway provides cells with metabolic precursors and a rapid source of energy (Murgiano et al., 2010). The results of the current study show that within AA, a statistically significant difference in enzymes relating to glycolysis and gluconeogenesis was observed between animals of High compared with Low EPD_{cwt}, with GPI, ENO3, and PKM2 exhibiting increased protein expression

in AA-High compared with AA-Low animals. Bernard et al. (2009) examined the effects of genetic selection in favor of increased muscle growth on gene expression in the muscle of young Charolais bulls using microarray technology. Consistent with our findings, the authors reported that gene expression of *GPI* and *ENO3* was increased in bulls of High compared with Low muscle growth. In addition, it has been established that animals undergoing accelerated muscle growth (e.g., compensatory growth) exhibit greater gene and protein abundance of GPI, ENO3, and PKM2 (Lametsch et al., 2006; Lehnert et al., 2006). Teltathum and Mekchay (2009) and Doherty et al. (2004) reported that during pectoralis muscle growth in chickens, enzymes relating to the glycolytic pathway (ENO3 and PKM2) were greater in abundance during a growth phase compared with at hatching. In ovine studies, Hamelin et al. (2006) reported that the protein abundance of ENO3 and PKM2 was greater in the LM of fast-growing compared with slow-growing rams. These findings indicate that in AA increased muscle growth potential may be associated with increased glycolysis and, in the case of Bernard et al. (2009), decreased oxidative metabolism.

In contrast to our findings for AA, no effect of EPD_{cwt} on protein abundance was detected in BB animals. This result is consistent with the performance data for these animals, reported by Campion et al. (2009a), where no difference in growth rate or carcass weight between BB-sired steers of High or Low EPD_{cwt} was observed. The authors of that study proposed that the absence of an effect of genetic merit on growth rate in BB steers may be associated with the fact that these animals are crossbred from HF cows and are heterozygous for the mutation in the myostatin gene, whereas EPD_{cwt} values are calculated on the basis of performance data from both crossbred and purebred (heterozygous and homozygous for the mutation in the myostatin gene, respectively) animals.

We have provided evidence for different metabolic processes taking place in muscle of crossbred AA- and BB-sired steers that are specific to breed type. Aberdeen Angus, an early-maturing breed, accumulates intramuscular fat at an earlier age, whereas late-maturing breeds like BB continue growing to a heavier mature weight. Proteins related to fiber type were increased in BB-sired steers, with proteins involved in glycolysis and the citric acid cycle in greater abundance in the muscle of AA-sired animals. In addition, this study facilitated greater insight into differential muscle proteome expression across bovine breeds divergently selected for muscle growth rate potential, with proteins involved in glycolysis increased in AA-sired steers of High compared with Low genetic merit for carcass growth.

Skeletal muscle is a very heterogeneous tissue consisting of diverse cell types. Intramuscular fat in bovine muscle is primarily accumulated and stored within adipocytes located in connective tissue between bundles of muscle fibers (Harper and Pethick, 2004). As differences exist in fat accumulation between BB- and AA-sired steers, the authors took care during tissue collection to harvest lean muscle tissue only, avoiding intramuscular fat. In addition the protocol used in this study was optimized for protein extraction from muscle tissue (Mullen et al., 2011; Donoghue et al., 2010). There is also no evidence of differential expression of markers of fat metabolism between the AA- and BB-sired steers in the current study. Although technologies such as laser microdissection (Albrecht et al., 2011) further enhance the ability to isolate myocytes, it is expensive and laborious and was beyond the scope of this study.

Data from this study will aid in our understanding of genetic influences controlling muscle growth and fat accumulation, and future work will continue to examine the potentiating or inhibitory effects of sire breed and EPD_{cwt} at more critical time points during the growth phase of the animal to elucidate key proteins regulating muscle growth. Glycolytic enzymes are potential candidates for high muscle growth for future investigation, including exploration of SNP. After appropriate validation, these markers could be incorporated into future cattle breeding programs to improve the accuracy of selection for muscle growth.

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