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Evidence of decreased muscle protein turnover in gilts selected for low residual feed intake

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Abstract
The objective of this study was to evaluate the contribution of muscle protein turnover (synthesis and degradation) to the biological basis for genetic differences in finisher pigs selected for residual feed intake (RFI). Residual feed intake is defined as the difference between expected feed intake (based on the achieved rate of BW gain and backfat depth of individual pigs) and the observed feed intake of the individual pig. We hypothesized that protein turnover would be reduced in pigs selected for low RFI. Twelve gilts from a line selected for 7 generations for low RFI and 12 from a contemporary line selected for 2 generations for high RFI were paired by age and BW and fed a standard corn–soybean diet for 6 wk. Pigs were euthanized, muscle and liver samples were collected, and insulin signaling, protein synthesis, and protein degradation proteins were analyzed for expression and activities. Muscle from low RFI pigs tended to have less μ- and m-calpain activities ($P = 0.10$ and 0.09, respectively) and had significantly greater calpastatin activity and a decreased μ-calpain:calpastatin activity ratio ($P < 0.05$). Muscle from low RFI pigs had less 20S proteasome activity compared with their high RFI counterparts ($P< 0.05$). No differences in insulin signaling intermediates and translation initiation signaling proteins [mammalian target of rapamycin (mTOR) pathway] were observed ($P > 0.05$). Postmortem proteolysis was determined in the LM from the eighth generation of the low RFI pigs versus their high RFI counterparts ($n = 9$ per line). Autolysis of μ-calpain was decreased in the low RFI pigs and less troponin-T degradation product was observed at 3 d postmortem ($P < 0.05$), indicating slowed postmortem proteolysis during aging in the low RFI pigs. These data provide significant evidence that less protein degradation occurs in pigs selected for reduced RFI, and this may account for a significant portion of the increased efficiency observed in these animals.

Keywords
Swine Feed Efficiency, calpain, calpastatin, proteasome, protein synthesis, residual feed intake, swine

Disciplines
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ABSTRACT: The objective of this study was to evaluate the contribution of muscle protein turnover (synthesis and degradation) to the biological basis for genetic differences in finisher pigs selected for residual feed intake (RFI). Residual feed intake is defined as the difference between expected feed intake (based on the achieved rate of BW gain and backfat depth of individual pigs) and the observed feed intake of the individual pig. We hypothesized that protein turnover would be reduced in pigs selected for low RFI. Twelve gilts from a line selected for 7 generations for low RFI and 12 from a contemporary line selected for 2 generations for high RFI were paired by age and BW and fed a standard corn–soybean diet for 6 wk. Pigs were euthanized, muscle and liver samples were collected, and insulin signaling, protein synthesis, and protein degradation proteins were analyzed for expression and activities. Muscle from low RFI pigs tended to have less μ- and m-calpain activities (P = 0.10 and 0.09, respectively) and had significantly greater calpastatin activity and a decreased μ-calpain:calpastatin activity ratio ($P < 0.05$). Muscle from low RFI pigs had less 20S proteasome activity compared with their high RFI counterparts ($P < 0.05$). No differences in insulin signaling intermediates and translation initiation signaling proteins [mammalian target of rapamycin (mTOR) pathway] were observed ($P > 0.05$). Postmortem proteolysis was determined in the LM from the eighth generation of the low RFI pigs versus their high RFI counterparts (n = 9 per line). Autolysis of μ-calpain was decreased in the low RFI pigs and less troponin-T degradation product was observed at 3 d postmortem ($P < 0.05$), indicating slowed postmortem proteolysis during aging in the low RFI pigs. These data provide significant evidence that less protein degradation occurs in pigs selected for reduced RFI, and this may account for a significant portion of the increased efficiency observed in these animals.

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INTRODUCTION

The physiology defining differences in feed efficiency (**FE**) and residual feed intake (**RFI**) in livestock is complex and multifaceted. Many of these factors have been quantified in poultry and beef and include physical activity, feed intake patterns and behavior, stress, digestion, and metabolism (Luiting, 1990; Herd and Arthur, 2009). In recent years, much attention has been given to feed efficiency in livestock production due to the rising costs of feed and other inputs. Therefore, selecting for pigs that more efficiently use feed enables our industry to remain competitive and sustainable. Pigs selected for reduced RFI, which is the difference between the expected intake and the actual feed intake of the individual pig necessary to reach a given performance, may help the industry achieve these goals.

In the Iowa State University RFI Yorkshire pig selection project, pigs in the low RFI line are more efficient and tend to have less carcass fat, greater carcass lean, and lesser ADG compared with control and high RFI lines (Cai et al., 2008; Smith et al., 2011; Young et al., 2011). Boddicker et al. (2011a,b) reported a trend

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for increased protein and decreased fat composition in carcasses from Yorkshire pigs selected for low RFI compared with the control line. In a similar swine RFI selection project, Barea et al. (2010) showed changes in metabolism and energy use, which may partially explain these divergences in FE.

Richardson and Herd (2004) estimated that 37% of the variation in cattle for RFI was due to protein turnover, tissue metabolism, and stress, and it is reasonable to suggest that this may be true for swine as well. The Iowa State swine selection lines provide a useful model to study the genetics and physiology defining FE. The objective of this study was to evaluate the contribution of protein turnover pathways to FE in growing pigs. We hypothesized that protein turnover is reduced in pigs selected for reduced RFI versus the high RFI line.

MATERIALS AND METHODS

All animal procedures were approved by the Institutional Animal Care and Use Committee of Iowa State University.

Experiment 1

Study Design and Animals. Twelve Yorkshire gilts from the seventh generation of the low RFI line and 12 high RFI Yorkshire gilts (second generation of divergent selection for high RFI after 5 generations of random selection) were randomly chosen from the Iowa State University RFI project population. The total 24 gilts used came from 24 different litters and 15 different sires.

Gilts were paired by line, based on age and BW, and each pair was assigned to an individual pen. Pigs had free access to water and were fed ad libitum a standard corn–soybean diet that was formulated to meet or exceed the nutrient requirements for pigs of this physiological stage (NRC, 1998). Weekly BW and feed intake were collected to calculate ADG. Ultrasound measurements of backfat (**BF**) and loin eye area were performed on d 0 and d 42 on feed. These data were used to calculate RFI for each pig. Residual feed intake indices were obtained as the residuals from analysis of ADFI using a model with BF and ADG included as covariates (Young et al., 2011). The difference in average RFI between the low and high RFI gilts was 0.19 ± 0.07 kg/d ($P = 0.06$), indicating that the low RFI pigs required 0.19 kg less feed per day to achieve the same rate of growth and backfat values as high RFI pigs. At the end of the test period, pigs were euthanized by captive bolt and subsequent exsanguination (68.4 ± 3.5 kg). The LM and red and white portions of semitendinosus (**RST** and **WST**, respectively) and liver samples were collected and either analyzed immediately or frozen in liquid nitrogen for later analysis.

Expression of Protein Synthesis Markers. Whole muscle extracts (10 m*M* phosphate and 2% SDS, pH 7.0) from the LM of 7 randomly chosen pigs from each line were prepared for immunoblotting. Protein concentration of the whole muscle extracts was determined using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Expression of total and phosphorylated Akt, insulin receptor, IGF receptor, total and phosphorylated mammalian target of rapamycin (**mTOR**, Ser2448), S6K1 (Thr389), and 4EBP1 (Thr70) were assessed using immunoblotting procedures (Kimball et al., 2003). In a 4 to 20% precast gradient gel (Lonza, Basel, Switzerland), 40 μg of protein was separated using standard techniques. After separation, the protein was transferred to a nitrocellulose membrane (GE Water and Process Technologies, Feasterfille-Trevose, PA). The membrane was blocked for 30 min with 5% milk in PBS-Tween (0.05% Tween 20). The membrane was subsequently incubated with primary antibody diluted in 1% milk with PBS-Tween at 4°C overnight (see Table 1 for antibody information). The next day, the membrane was washed 3 times for 10 min in PBS-Tween and incubated in secondary antibody for 1 h at room temperature and washed 3 more times. After the last wash, enhanced chemiluminescent substrate (Thermo Fisher, Rockford, IL) was added, the membrane was exposed to film, and the film of the membrane was developed in the darkroom. To analyze protein abundance, the film was scanned and band density was measured using Carestream molecular imaging software version 5.0 (Carestream Health Inc., Rochester, NY).

Protein Degradation Indicators. Calpain and calpastatin activity were determined in extracts of the LM, RST, and WST ($n = 11$ pigs from each line; 1 pig from each line was not used based on time constraints and equipment availability). Sarcoplasmic protein was extracted according to the method of Melody et al. (2004). Within 45 min postmortem, 10 g muscle samples were finely minced and 3 vol (wt/vol) of pre-rigor extraction buffer (100 m*M* Tris-HCl pH 8.3, 10 m*M* EDTA, 100 mg/L trypsin inhibitor, 2 μ*M* E-64, and 0.1% 2-mercaptoethanol) were added. Samples were homogenized in pre-rigor extraction buffer using a Polytron PT 3100 (Lucerne, Switzerland), in three 30 s bursts. The homogenate was centrifuged at 25,000 × *g* for 20 min at 4°C, and the supernatant was dialyzed against 40 vol of 40 m*M* Tris-HCl pH 7.4, 1 m*M* EDTA, and 0.1% 2-mercaptoethanol (**TEM**). The dialyzed sample was centrifuged at 25,000 × *g* for 20 min, and the supernatant was filtered through cheesecloth.

Supernatant protein samples were loaded onto a 20 mL Q-Sepharose Fast Flow (GE Healthcare Biosciences, Pittsburgh, PA) anion exchange column equilibrated with TEM (40 m*M* Tris, 1 m*M* EDTA, and 0.1% 2-mercaptoethanol). After washing, calpastatin,

Table 1. Antibodies and dilutions used in immunoblotting to determine expression of protein synthesis markers

Primary antibodies	Dilution	Species	Product no.
Phospho-Ser Akt	1:2,000	Rabbit monoclonal	Cell Signaling Technology, 4060
Phospho-Thr Akt	1:1,000	Rabbit polyclonal	Cell Signaling Technology, 9275
Total Akt	1:1,000	Rabbit polyclonal	Cell Signaling Technology, 9272
Insulin receptor	1:1,000	Rabbit polyclonal	Santa Cruz Biotechnology, sc-711
Insulin receptor substrate	1:1,000	Rabbit polyclonal	Santa Cruz Biotechnology, sc-7200
mTOR[1]	1:1,000	Rabbit polyclonal	Cell Signaling Technology, 2972
Phospho-mTOR (Ser2448)	1:1,000	Rabbit polyclonal	Cell Signaling Technology, 2971
P70 S6K1	1:1,000	Rabbit polyclonal	Cell Signaling Technology, 9202
Phospho-P70 S6K1 (Thr389)	1:1,000	Rabbit polyclonal	Cell Signaling Technology, 9205
4EBP1	1:1,000	Rabbit polyclonal	Cell Signaling Technology, 9452
Phospho-4EBP1 (Thr70)	1:1,000	Rabbit polyclonal	Cell Signaling Technology, 9455
Actin	1:4,000	Rabbit polyclonal	Abcam, ab15263
Secondary antibody	Dilution		Product no.
Donkey anti-rabbit IgG HRP	1:2,000		GE, NA934V

[1]mTOR = mammalian target of rapamycin.

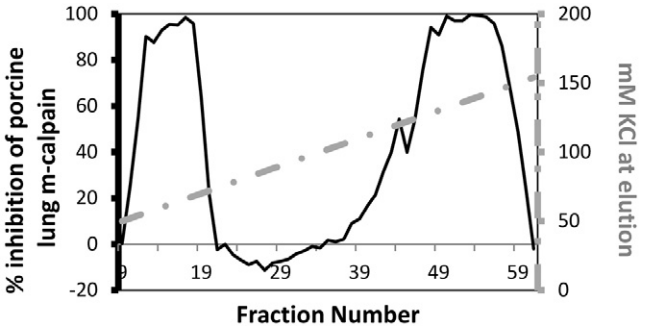

Figure 1. Representative example of calpastatin I and calpastatin II separation during elution using a Q-Sepharose ion exchange column. Dark line represents percent inhibition of porcine lung calpain and gray dashed line represents millimolar KCl at protein elution.

μ-calpain, and m-calpain were eluted using a linear gradient of 0 to 400 m*M* KCl in TEM. Calpastatin eluted in 2 separate and distinct peaks (calpastatin I and II, at 50 to 90 m*M* KCl and 120 to 190 m*M* KCl, respectively; Fig. 1) followed by μ-calpain (180 to 240 m*M* KCl) and m-calpain (300 to 400 m*M* KCl).

The activities of μ- or m-calpain or calpastatin-containing fractions were determined using casein as a substrate, using a modification of the method of Koohmaraie (1990). One unit of μ- or m-calpain activity was defined as the amount required to catalyze an increase of 1 absorbance unit at 278 nm in 1 h at 25°C. One unit of calpastatin activity was defined as the amount required to inhibit 1 unit of porcine lung m-calpain. Protein content of the original muscle sample was determined to calculate activity on a total protein basis. Crude protein (nitrogen × 6.25) was estimated using an Automated LECO Nitrogen Analyzer (LECO-TruSpec N, LECO Corp., St. Joseph, MI).

Proteasome activity of the 20S subunit and ubiquitination was measured in the LM and liver of the same pigs used to measure protein synthesis. Muscle samples were homogenized and diluted with PBS, and protein content was determined using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Proteasome activity of the 20S subunit was then determined using a commercial kit (Chemicon International, Billerica, MA). Additionally, samples were prepared for immunoblotting as described above and used to measure levels of ubiquitinated proteins (indicating protein tagging for proteasome degradation). Western blots were conducted as described above with the use of a polyclonal ubiquitin antibody (1:1,000, number 3936; Cell Signaling Technology, Inc., Danvers, MA).

Experiment 2

Study Design and Animals. To further assess protein degradation between the lines and its impact on meat quality, gilts (95.3 ± 6.9 kg) from the eighth generation of selection for low RFI and the contemporary third generation of divergent selection for high RFI were euthanized in pairs over 9 d ($n = 9$ per line). The total 18 gilts used came from 17 different litters and 4 different sires per line.

Postmortem Proteolysis. The entire LM from 1 side of each pig was removed immediately after death, placed on ice, and cut into thirds. Each portion of the LM was placed in a sealed plastic bag and stored at 4°C for 1, 3, or 7 d postmortem. Storage time for each LM portion was randomly assigned to minimize potential effect of muscle location. After storage, samples were removed from the bags and a 2.45 cm cube was removed from the center of each LM portion. This cube was minced, frozen in liquid nitrogen, powdered, and stored at –80°C, as described by Melody et al. (2004). Samples for d 0 were collected immediately postmortem and powdered the same day. The potential impact of cold shortening and oxidation, resulting from removal of the muscle immediately postmortem and storage in aerobic packaging, was minimized by storing large portions of the LM and using only the approximate center of each portion for analyses.

Immunoblotting for μ-calpain autolysis and troponin-T degradation was performed on whole muscle

extracts from the powdered samples, as previously described (Huff-Lonergan et al., 1996; Melody et al., 2004). For μ-calpain autolysis determination, an 8% polyacrylamide separating gel [acrylamide:N,N′-bis-methylene acrylamide = 100:1 (wt/wt), 0.1% (wt/vol) SDS, 0.05% (vol/vol) tetramethylethylenediamine, 0.05% (wt/vol) APS, and 0.5 *M* Tris HCl, pH 8.8] was used. To determine troponin-T degradation, 15% polyacrylamide gels were used. Hoefer 260 Mighty Small II units (Hoefer, Inc., Holliston, MA) were used to run all gels. Gels were loaded with 40 μg of protein and run at 20 V overnight. Gels were then transferred to polyvinylidene difluoride membrane, as described by Melody et al. (2004).

Membrane blocking was performed using 5% nonfat dry milk in a PBS solution containing 0.1% Tween-20 for 1 h at room temperature. Incubation in primary antibodies was at 4°C over night at 1:20,000 and 1:10,000 for anti-troponin-T (JLT-12; Sigma, St. Louis, MO) and anti-μ-calpain (MA3-940; Thermo Scientific), respectively. After washing with PBS-Tween, membranes were incubated in secondary antibody for 1 h at room temperature (Goat anti-mouse-HRP, number 2554; Sigma; diluted 1:30,000 and 1:20,000 for troponin-T and μ-calpain, respectively). Blots were developed using either ECL Plus (troponin-T) or ECL Prime (μ-calpain) Western Blotting Detection System (GE Healthcare, Piscataway, NJ). Images were captured using a ChemiImager 5500 (Alpha Innotech, San Leandro, CA) and Alpha Ease FC software (version 3.03 Alpha Innotech). Bands were quantified using densitometry; the 30 kDa troponin-T degradation product and the 80 (intact), 78, and 76 (autolysis products) kDa bands of μ-calpain were analyzed.

Statistical Analysis

Calpain and calpastatin activity data were analyzed using a split plot design with RFI line as the whole plot and muscle as the split plot. Analysis was done using the MIXED procedure (SAS Inst. Inc., Cary, NC). All other data were analyzed as a complete randomized design using the MIXED procedure in SAS. The model included the fixed effect of line, the random effect of day, and the covariate of BW at euthanasia. Phenotypic correlations were computed based on residuals derived using the CORR procedure of SAS.

RESULTS AND DISCUSSION

Experiment 1

Calpain System Activity. Protein degradation pathways, such as the calpain system and the ubiquitin-proteasome pathway, may play a role in FE (Bottje and Carstens, 2009) and are known to contribute to protein turnover through protein degradation in muscle (Smith and Dodd, 2007). Across muscles, μ- and m-calpain activities tended to be lesser ($P = 0.10$ and 0.09, respectively) in muscle from the more efficient low RFI pigs compared with high RFI pigs (Table 2). Furthermore, total calpastatin activity was greater ($P < 0.05$) in muscle from low RFI pigs, which is consistent with previous findings in younger pigs (Smith et al., 2011). The difference in total calpastatin activity can be attributed primarily to differences in calpastatin II activity ($P = 0.01$), given that activity of calpastatin I was not different between lines ($P = 0.79$). Additionally, in the low RFI animals the ratio of μ-calpain:calpastatin activity was less ($P = 0.04$) than in the high RFI line pigs (Table 2). This combination of reduced calpain activities and increased calpastatin activity in the low RFI pigs indicates the potential for decreased capacity for protein degradation in the muscles of these animals. Hence, gilts from the low RFI line are more efficient, at least in part, because of decreased protein degradation. These results are dissimilar to those of Le Naou et al. (2012), who documented no differences in total calpain activity in the LM or liver of either weaned or market weight Large White pigs selected for reduced or high RFI. This disparity may be due to differences in techniques used to measure activity; Le Naou et al. (2012) used a fluorometry technique that measured combined μ- and m-calpain activity of a sample extract whereas the current study used chromatography techniques to isolate the individual proteases (μ- and m-calpain) and their endogenous inhibitor (calpastatin) followed by determining their activities.

Table 2. Calpain and calpastatin activities (units of activity/g protein)[1] in muscle from gilts selected for low or high residual feed intake (RFI)[2]

Protease/Inhibitor	High RFI	Low RFI	*P*-value	SE
μ-Calpain	2.48	2.20	0.10	0.156
m-Calpain	9.00	8.25	0.09	0.422
Calpastatin I	4.55	4.49	0.79	0.207
Calpastatin II	8.11	9.57	0.01	0.473
Total calpastatin	12.67	13.99	0.04	0.563
μ-Calpain:calpastatin[3]	0.25	0.20	0.04	0.021

[1]One unit of μ- or m-calpain activity = amount required to catalyze an increase of 1 absorbance unit at 278 nm in 1 h at 25°C. One unit of calpastatin activity = amount required to inhibit 1 unit of porcine lung m-calpain.

[2]$n = 11$ gilts per RFI line.

[3]Ratio of μ-calpain activity to total calpastatin activity.

To our knowledge, the separation of the 2 isoforms of calpastatin using anion exchange chromatography has not been documented in porcine skeletal muscle although other groups have separated calpastatin from other species and tissues in this manner (Pontremoli et al., 1992; Averna et al., 2001; Samanta et al., 2010). Although there is general agreement that there are at least 2 isoforms of calpastatin, it is unclear if they result from

Table 3. Least square means for calpain and calpastatin activities (units of activity/g protein)[1] in LM, the red portion of the semitendinosus (RST), and the white portion of the semitendinosus (WST) muscles from gilts selected for low or high residual feed intake[2]

Protease/Inhibitor	LM	RST	WST	*P*-value	SE
μ-Calpain	3.53[a]	1.84[b]	1.66[b]	<0.0001	0.150
m-Calpain	7.00[c]	9.99[a]	8.89[b]	<0.0001	0.260
Calpastatin I	3.99[b]	5.16[a]	4.41[b]	0.0001	0.244
Calpastatin II	4.39[b]	11.42[a]	10.71[a]	<0.0001	0.562
Total calpastatin	8.30[c]	16.57[a]	15.11[b]	<0.0001	0.666
μ-Calpain:calpastatin[3]	0.46[a]	0.12[b]	0.11[b]	<0.0001	0.025

[a,b]Values in a row with different superscripts differ ($P < 0.05$).

[1]One unit of μ- or m-calpain activity = amount required to catalyze an increase of 1 absorbance unit at 278 nm in 1 h at 25°C. One unit of calpastatin activity = amount required to inhibit 1 unit of porcine lung m-calpain.

[2]Line by muscle interactions were not significant ($P > 0.05$); LM $n = 22$, RST $n = 19$, WST $n = 22$.

[3]Ratio of μ-calpain activity to total calpastatin activity.

posttranslational modification (Pontremoli et al., 1992; Averna et al., 2001) or alternative splicing (Geesink et al., 1998; Samanta et al., 2010).

Whereas the low RFI animals may be more efficient from a production perspective, decreased protein degradation has the potential to alter the normal tenderization process and impair meat quality. The protease μ-calpain and its inhibitor calpastatin play a large role in the proteolysis that occurs during meat aging; reduced postmortem proteolytic activity will result in less postmortem tenderization. Indeed, Smith et al. (2011) found positive correlations between RFI index value and pork tenderness ($P < 0.01$, $r = 0.24$) as well as negative correlations between RFI index value and star probe value ($P < 0.01$, $r = –0.26$) and chewiness ($P = 0.05$, $r = –0.15$) in the LM, which indicates that pigs with lower RFI have less tender meat.

Calpain and calpastatin activities for the LM, RST, and WST are reported in Table 3. Across RFI lines, the LM had the greatest ($P < 0.0001$) μ-calpain activity and the least ($P < 0.0001$) m-calpain activity across RFI lines. The RST and WST had similar μ-calpain activities, but the RST had greater ($P = 0.002$) m-calpain activity compared with the WST. Calpastatin I activity was less than calpastatin II activity in all muscles. The WST was similar in calpastatin I activity to the LM ($P = 0.08$); both the LM and WST had lower calpastatin I activity compared with the RST ($P < 0.05$). However, the WST and RST had similar calpastatin II activities, which were more than double that of the LM ($P < 0.0001$). These differences in "peaks" of calpastatin activity represent a previously undefined source of variation in calpastatin activity between muscles. Total calpastatin activity differed across muscles ($P < 0.0001$). The LM had the least total calpastatin activity followed by the WST and the RST. The LM and the semitendinosus sections had vastly different calpain/calpastatin profiles. The LM had a μ-calpain:total calpastatin ratio that was much more conducive to proteolysis ($P < 0.0001$). No significant ($P > 0.10$) line by muscle interactions were observed for calpain system activity.

Improved FE and low RFI could be partially explained be a reduction in the amount of electron leakage from mitochondria isolated from muscle, liver, and intestines, reactive oxygen species (**ROS**) production, and oxidative stress (Bottje and Carstens, 2009; Grubbs et al., 2013a). Decreasing ROS production could lead to a decrease in oxidative damage to DNA, lipids, and proteins, leading to a decrease in mitophagy and protein turnover (Bottje and Carstens, 2009). This would then contribute to a shift in energy use from cellular repair to improved lean growth of pigs, which is the ultimate goal for improving production efficiency. A greater amount of oxidized proteins appears to be a hallmark of low FE animals. Breast muscle mitochondria as well as gut, leg, heart, liver, and lymphocyte homogenates from low FE birds consistently exhibit a greater pervasive total protein carbonyl content compared with high FE birds (Bottje and Carstens, 2009). This supports previous observations of greater mitochondrial ROS production in low FE broilers (Bottje et al., 2002; Iqbal et al., 2004; Ojano-Dirain et al., 2005, 2007; Lassiter et al., 2006) and in our high RFI pig lines (Grubbs et al., 2013a). This is further supported by low FE steers having greater neck muscle mitochondria protein carbonyl content (Sandelin, 2005).

Interestingly, calpain activation appears to be associated with increased oxidative stress and ubiquitin-proteasome protein degradation (Barker and Traber, 2007). Oxidation of calpain can create a disulfide bond at the active site of the protease, resulting in inhibition of its activity, which is reversible in the presence of reducing conditions (Lametsch et al., 2008). However, oxidation also appears to decrease the efficacy of the inhibitor of calpain, calpastatin, at physiological pH (Carlin et al., 2006). Damaged proteins are also conveyed directly to the proteasome by molecular chaperones that include heat shock proteins such as heat shock protein 90 and α-crystallin B chain (Glickman and Ciechanover, 2002). This is supported by the fact that downregulation of heat shock proteins (LM) and acute phase proteins (serum) is observed in the Iowa State low RFI pig line (Grubbs et al., 2013b; Mani et al., 2013). As protein degradation is a very energetically expensive cellular process, both in terms of ATP required to operate the proteasome system and the loss of energy expended in synthesizing the proteins in the first place, protein turnover could represent a major contributing process in the phenotypic expression of poor feed efficiency in animals.

Table 4. Least squares means for 20S proteasome activity and ubiquitin protein expression in liver and LM samples from pigs selected for low or high residual feed intake (RFI)[1]

Tissue	High RFI	Low RFI	*P*-value	SE
Liver				
20S proteasome activity, RFU[2]	151.5	128.2	0.12	13.84
Ubiquitin, AU[3]	1.30	1.26	0.86	0.280
LM				
20S proteasome activity, RFU	63.8	48.3	0.03	5.90
Ubiquitin, AU	1.30	1.10	0.15	0.090

[1]*n* = 12 gilts per RFI line.

[2]RFU = relative fluorescence units.

[3]AU = arbitrary units based on densitometry intensity of lanes expressing ubiquitin tagged proteins.

Proteasome Activity and Ubiquitination. Calpain cleavage products often become substrates for the ubiquitin-proteasome system (Smith and Dodd, 2007). Proteins selected for degradation are polyubiquitinated in an ATP-dependent manner and then targeted to the proteasome for degradation (Nandi et al., 2006). The proteasome is a multicatalytic complex in the nucleus and cytosol of all eukaryotic cells. It is responsible for proteolysis of ubiquitin-tagged proteins. This system breaks down proteins into peptide fragments ranging from 7 to 25 residues in length. The 20S subunit is the catalytic core of the 26S proteasome, with 2 19S regulatory caps. The 20S core is where proteins are degraded and the 19S regulatory caps have multiple ATPase active sites and ubiquitin binding sites to recognize polyubiquinated proteins to be transferred to the catalytic core (Voges et al., 1999).

No differences in 20S proteasome activity or protein ubiquitination were observed in liver samples of low versus high RFI pigs (Table 4; P =0.10). However, liver 20S proteasome activity, although not significantly correlated with RFI index (r = 0.49, P = 0.10), was significantly correlated with backfat thickness (r = 0.56, P < 0.05) and ADFI (r = 0.59, P < 0.05). Proteasome activity in the LM was decreased by 24% in the low RFI pigs (Table 4) compared with high RFI pigs and was positively correlated with RFI index values (r = 0.60, P < 0.05; Table 5). These results are in contrast to those reported by Le Naou et al. (2012), who observed that proteasome activity tended to be increased (P = 0.08) in the liver of weaned (19 kg) Large White pigs selected for reduced RFI although no differences were found in the LM of these pigs. However, market weight pigs (115 kg) from the same study had no differences in proteasome activity in either tissue. The pigs in the current study were intermediate to the above study at 65 kg BW; it may be that the differences observed by Le Naou et al. (2012) are only evident during the early stages of growth. It is also possible that differing methods allowed more sensitive characterization of proteasome activity in the current study; again Le Naou et al. (2012) used fluorometry techniques that determined overall proteasome activity whereas the methods of the current study specifically measured 20S proteasome activity. Ubiquitination was similar between RFI lines in the LM. Decreased LM 20S proteasome activity in the current study is further evidence of reduced protein degradation in pigs selected for low RFI.

Table 5. Residual correlations between protein degradation markers, residual feed intake (RFI) index and performance traits in finisher pigs, including 20S proteasome activity, calpain and calpastatin activities, and protein ubiquitination, as determined by immunoblotting[1,2]

Item	RFI index	Backfat thickness	ADG	ADFI	FE[3]
RFI index	1.00				
Backfat thickness	0.66	1.00			
	0.021				
ADG	–0.20	–0.25	1.00		
	0.53	0.42			
ADFI	0.93	0.82	–0.02	1.00	
	<0.0001	0.001	0.96		
FE	–0.90	–0.80	0.53	–0.84	1.00
	<0.001	0.002	*0.07*	<0.001	
Liver 20S proteasome	0.49	0.56	–0.01	0.59	–0.45
	0.10	0.049	0.98	0.042	0.14
LM 20S proteasome	0.60	0.34	–0.21	0.48	–0.50
	0.038	0.28	0.52	0.11	*0.10*
LM ubiquitin	0.13	0.43	0.31	0.48	–0.22
	0.66	0.12	0.66	*0.07*	0.45
LM m-calpain	–0.07	–0.03	0.40	0.04	0.15
	0.82	0.94	0.22	0.90	0.67
LM μ-calpain	0.07	0.17	0.15	0.14	–0.09
	0.86	0.64	0.67	0.70	0.80
LM total calpastatin	–0.38	–0.25	0.10	–0.31	0.40
	0.25	0.45	0.77	0.35	0.23

[1]Upper row = residual correlations. Bottom row = *P*-values.

[2]n = 12 gilts per RFI line.

[3]FE = feed efficiency, defined as kilograms of gain per kilogram of feed.

Overall, the results of the current study are similar to those of Damon et al. (2012), in which gene expression of Large White and Basque pig breeds were compared. The Large White is a predominant breed known for greater lean, reduced fat, and a greater daily BW gain (similar to the low RFI pigs in this experiment) whereas the Basque breed is a breed local to France that grows slowly and has much less lean and greater fat production (similar to what would be an extremely high RFI pig in this study). Damon et al. (2012) observed that the Large White pigs had greater expression of the **CAST** (calpastatin) gene and decreased expression of genes that code for ligase enzymes involved in the ubiquitin-proteasome system (TRIM63 and

Table 6. Longissimus muscle expression of key insulin signaling and protein synthesis cascade proteins in pigs selected for low or high residual feed intake (RFI), as determined by immunoblotting[1]

Item	High RFI	Low RFI	*P*-value	SE
Insulin signaling cascade				
Phospho-Ser Akt, AU[2]	1.00	0.76	0.20	0.125
Phospho-Thr Akt, AU	1.00	0.32	*0.09*	0.256
Total Akt, AU	1.00	1.03	0.90	0.147
Ser:total Akt, AU	1.10	0.73	*0.07*	0.134
Thr:total Akt, AU	1.16	0.41	*0.10*	0.293
Insulin receptor, AU	1.00	0.82	0.46	0.172
Insulin receptor substrate, AU	1.00	0.88	0.64	0.178
Protein synthesis pathway				
mTOR,[3] AU	0.95	0.95	0.99	0.027
Phospho-Ser2448 mTOR, AU	0.97	0.97	0.99	0.012
S6K1, AU	0.95	0.95	0.99	0.027
Phospho-Thr389 S6K1, AU	0.97	1.04	*0.09*	0.036
4EBP1, AU	0.97	0.97	0.99	0.013
Phospho-Thr70 4EBP1, AU	0.98	1.02	0.14	0.025
Ser:total mTOR, AU	1.01	1.01	0.99	0.027
Thr:total S6K1, AU	1.03	1.09	0.28	0.059
Thr:total 4EBP1, AU	1.01	1.05	0.29	0.034

[1]n = 7 gilts per RFI line.

[2]AU = arbitrary units based on densitometry intensity of lanes expressing ubiquitin tagged proteins.

[3]mTOR = mammalian target of rapamycin.

FBX032). Although compelling, it is important to note that the results of Damon et al. (2012) are based on transcript expression whereas the current data are based on protein activity, which may or may not mirror gene expression.

Protein Synthesis Pathway. Overall, the insulin signaling and protein synthesis markers measured were not significantly affected by RFI line (Table 6; $P > 0.05$). However, Akt phosphorylation on Thr308 and the ratio of Akt phosphorylation on either Thr308 or Ser473 to total Akt protein expression tended to decrease by approximately 30 to 60% in pigs selected for low RFI compared with high RFI pigs ($P < 0.10$). As Akt is upstream of mTOR and tended to have decreased activation (as evaluated by reduced phosphorylation level), the expression and phosphorylation of several mTOR pathway components were also measured. Akt is upstream of several pathways involved in protein synthesis and prevention of protein degradation, including activation of mTOR (Otto and Patel, 2010; Braun and Gautel, 2011). Reduced Akt phosphorylation should therefore result in decreased protein synthesis; however, no protein markers in the mTOR pathway were significantly different between the 2 lines ($P < 0.05$). Le Naou et al. (2012) found that the total protein synthesis rate tended to be increased ($P = 0.06$) in livers of sixth and seventh generation weaned Large White pigs selected for reduced RFI; however, no differences were detected within the LM of these animals, supporting the data in the current study. Additionally, hepatic protein synthesis may have been upregulated due greater insulin concentrations in the high RFI pigs at the time of measurement (Le Naou et al., 2012). Therefore, changes in protein synthesis do not appear to have a large impact on increased growth efficiency in low RFI pigs.

In aggregate, decreased protein degradation coupled with similar protein synthesis would be predicted to result in a net decrease in protein turnover and progressive protein accretion in low RFI pigs compared with high RFI pigs. Indeed, previous findings using pigs from these lines have measured greater nitrogen retention and greater protein accretion (Harris, 2012). Muscle from low RFI pigs has been shown to be composed of a greater proportion of type IIB fibers (Lefaucheur et al., 2011), which are known to exhibit decreased protein turnover when compared with oxidative fibers (Lewis et al., 1984; van Wessel et al., 2010). These data may explain a portion of the increased efficiency observed in low RFI pigs due to decreased energy required for protein degradation. In addition to the ubiquitin-proteasome pathway, which is ATP dependent, the production of urea to dispose of excess nitrogen after AA breakdown, known as ureagenesis, also requires energy in the form of ATP. Slowing the degradation processes will therefore ultimately result in less energy expenditure by the animal from multiple sources.

Experiment 2

Postmortem Muscle Proteolysis. When exposed to calcium concentrations required for activation, μ-calpain will undergo progressive autolysis from 80 to 78 kDa and eventually to 76 kDa. This autolysis reduces the calcium requirement for activity from approximately 1 to 50 μ*M* to 0.5 to 2.0 μ*M* (Edmunds et al., 1991; Goll et al., 2003). In pork from generation 8 of the low RFI line at d 3 postmortem, the 80 and the 78 kDa bands were more abundant and the 76 kDa band was less abundant, compared with pork from high RFI pigs (Table 7; Fig. 2). This indicates that the rate of calpain autolysis was slower in the low RFI lines compared with the high RFI line, which would lead to a slower rate of postmortem proteolysis and tenderization. These results are consistent with the calpastatin data because greater calpastatin activity inhibits calpain activity and therefore slows the rate of autolysis (Koohmaraie, 1992). At d 0 and 1 postmortem, μ-calpain autolysis was similar between the 2 lines (Table 7; Fig. 2).

In support of the μ-calpain autolysis data, the presence of the 30 kDa degradation product of troponin-T was lesser in the d 3 postmortem samples of the low RFI line in generation 8 compared with the high RFI

Table 7. μ-Calpain autolysis at d 0, 1, and 3 postmortem, measured by immunoblotting. Values represent the percent of the 3 bands present[1]

μ-calpain band	High RFI	Low RFI	*P*-value	SE
		d 0		
80 kDa	28.24	35.57	0.50	7.45
78 kDa	29.97	27.52	0.55	2.75
76 kDa	41.64	37.06	0.63	6.64
		d 1		
80 kDa	12.74	19.06	0.30	4.12
78 kDa	30.24	31.70	0.75	3.13
76 kDa	57.99	48.27	0.25	5.73
		d 3		
80 kDa	5.89	14.98	0.046	2.91
78 kDa	19.47	27.19	0.078	4.08
76 kDa	74.97	57.51	0.029	7.20

[1]Comparison between pigs genetically selected for high or low residual feed intake (RFI). Values indicated are a ratio of the sample band density to an in-gel reference. $n = 9$ gilts per RFI line.

line (Fig. 3). No differences were observed at d 0 and 1 postmortem ($P = 0.80$). These data indicate a slower rate of postmortem protein degradation in the more efficient low RFI line (Fig. 3). However, a significant difference in the extent of proteolysis at d 7 was not detected between the lines ($P = 0.46$). From a meat quality perspective, these results raise the possibility of slowed or impaired tenderness development in pork from the low RFI line, as troponin-T is thought to be a key indicator protein in the development of meat tenderness (Huff Lonergan et al., 2010). Again, these data are supported by Smith et al. (2011), who, although observing no differences in sensory tenderness between the fifth generation low RFI and control lines, documented a significant positive correlations between RFI value and both desmin degradation and tenderness score, which suggested that continued selection for RFI could result in decreased postmortem proteolysis and therefore impaired pork tenderness. Additionally, selection for lean growth efficiency in Duroc pigs has been shown to result in greater calpastatin activity in the semitendinosus, decreased troponin-T degradation in the LM, and greater

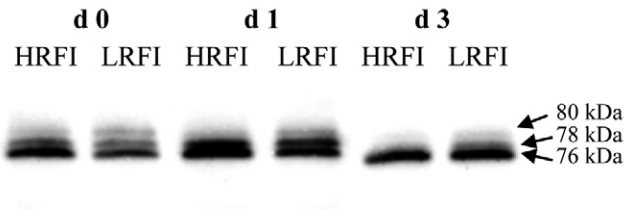

Figure 2. Representative Western blot of μ-calpain autolysis at d 0, 1, and 3 postmortem. Comparison between pigs genetically selected for high or low residual feed intake (RFI). Blot shows autolysis within the LM over time in 1 animal from each line (HRFI = high RFI and LRFI = low RFI). $n = 9$ gilts per RFI line.

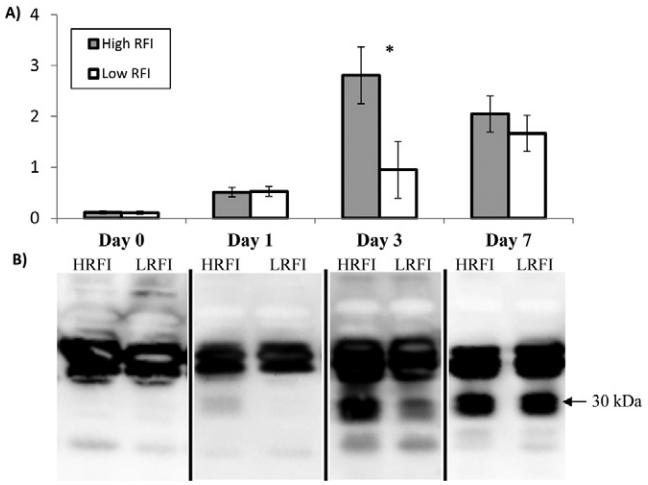

Figure 3. Troponin-T degradation (30 kDa) at d 0, 1, 3, and 7 postmortem. Comparison between pigs genetically selected for high or low residual feed intake (RFI). A) Values indicated are a ratio of the sample band density to an in-gel reference. Error bars reflect the SEM. *Within a day, values differ significantly ($P < 0.05$). B) Representative Western blots show the troponin-T degradation in LM from 1 animal from each line (HRFI = high RFI and LRFI = low RFI) over time. $n = 9$ gilts per RFI line.

Warner-Bratzler shear force values in both the LM and the semitendinosus after 5 d of postmortem storage (Lonergan et al., 2001). However, in another study conducted in France using French Large White pigs, no sensory tenderness differences have been observed between 2 lines of pigs after 6 generations of divergent selection for low or high RFI (Faure et al., 2013). Continued selection for low RFI merits monitoring of postmortem protein degradation and sensory attributes to identify whether tenderness has become a problem.

Conclusion

Overall, these results indicate that selection for low RFI in pigs may indirectly select for reduced protein degradation and turnover. Protein synthesis pathways in the context of RFI and FE have been poorly characterized in livestock and do not appear to be related to observed phenotype in these lines. Conversely, there is substantial evidence that protein degradation systems within muscle have decreased activity in low RFI versus high RFI line pigs. This overall reduced protein turnover could potentially be a cause of the greater efficiency in these pigs, resulting in decreased costs of production. However, due to the reduced levels of μ-calpain and increased calpastatin activity and given the evidence of slowed postmortem proteolysis in muscle from low RFI pigs, decreased tenderness and therefore reduced quality may be an unintended consequence of these changes. The benefits and potential adverse effects must be carefully considered as we strive for more efficient, sustainable production of meat animals.

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