Effect of compensatory growth on forms of glycogen, postmortem proteolysis, and meat quality in pigs

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ABSTRACT: The current experiment was designed to examine if a compensatory feed regimen influenced storage of glycogen forms, activity of proteolytic systems, and meat quality. Female pigs (Large White × Landrace × Duroc cross) with an initial age of 74 d were allocated to 6 feeding treatment groups (n = 8 for each group). Groups then consumed feed ad libitum for 40 (A40), 42 (A42), or 82 d (A82). The compensatory growth groups were fed 0.70 of ad libitum intake for 40 d (R40) followed by refeeding for ad libitum intake for 2 (R40A2) or 42 d (R40A42). Pigs were slaughtered at the end of the restriction period (SL1), then after refeeding for 2 (SL2) and 42 d (SL3). The feeding regimen caused restricted animals at SL2 to have a decreased BW ($P = 0.039$), with the refeed animals undergoing compensatory growth by SL3 so BW was not different ($P = 0.829$). At SL1 there was a trend for the R40 pigs to have less intramuscular fat than A40 ($P = 0.084$). There was a trend for macroglycogen (MG; $P = 0.051$) and a significant effect for proglycogen (ProG; $P = 0.014$) to be greater at slaughter in R40 than A40, along with a greater postmortem decline in both MG ($P = 0.033$) and ProG ($P = 0.022$) over the first 2 h in R40, which was associated with the R40 having a lower pH at 24 h postmortem ($P = 0.043$). After refeeding for 2 d (SL2), only MG of R40A2 was greater ($P = 0.030$) than A42 and had a trend for a greater difference of decline at 24 h postmortem ($P = 0.091$), which was associated with lower pH at 24 h ($P = 0.012$). The data suggest that the concentrations of ProG are more labile and recovered to the concentrations of pigs fed for ad libitum intake sooner than MG. After full compensation in SL3, there was no difference for MG content (at 0 h, $P = 0.721$; at 2 h, $P = 0.987$; at 24 h, $P = 0.343$), ProG content (at 0 h, $P = 0.879$; at 2 h, $P = 0.946$; at 24 h, $P = 0.459$), and muscle pH (at 45 min, $P = 0.373$; at 24 h, $P = 0.226$). At all slaughter points, there was no difference in shear force (at SL1, $P = 0.101$; at SL2, $P = 0.420$; at SL3, $P = 0.167$). There were no significant effects of the feeding regimen on micro- and milli-calpain large subunit gene expression (for micro-calpain at SL1, $P = 0.450$; at SL2, $P = 0.171$; at SL3, $P = 0.281$; for milli-calpain at SL1, $P = 0.666$; at SL2, $P = 0.123$; at SL3, $P = 0.617$) or the activity of the 2 proteolytic enzymes at any of the slaughter dates (for micro-calpain at SL1, $P = 0.238$; at SL2, $P = 0.238$; at SL3, $P = 0.222$; for milli-calpain at SL1, $P = 0.296$; at SL2, $P = 0.230$; at SL3, $P = 0.615$). In R40 there was a trend ($P = 0.070$) for greater gene expression of caspase 3, whereas in R40A2 the increase was significant ($P = 0.009$) relative to pigs consuming feed ad libitum. However, gene expression of the E3 ligase, MuRF1, at SL3 was less in R40A42 ($P = 0.019$). Although compensatory growth does appear to influence the expression of various proteolytic systems, the changes do not appear to be associated with meat quality as measured by shear force.

Key words: compensatory growth, glycogen, meat quality, pig, proteolytic system


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INTRODUCTION

Compensatory growth is the process by which animals show a period of enhanced growth when they are refed on a greater plane of nutrition after a period of feed restriction. This compensatory process is believed to be mediated through an overall increase in protein turnover in the animals undergoing compensation (Jones et al., 1990). Both protein synthesis and degradation are increased, with the latter being suggested to have a subsequent positive effect on postmortem proteolysis, which could potentially influence meat tenderness (Therkildsen et al., 2004). Therefore, it appears that compensatory growth regimens lead to a range of metabolic adjustments that could potentially affect meat quality.

Muscle glycogen concentration at slaughter, the rate of its decline, and the associated formation of lactate along with a consequential drop in pH influence meat quality (Bendall and Swatland, 1988). There are 2 forms of muscle glycogen, acid-soluble macroglycogen (MG) and acid-insoluble proglycogen (ProG), which appear to have different turnover rates in vivo (Graham et al., 2001). The nutrition of pigs influences the concentration of the different forms of glycogen (Rosenvold et al., 2003; Bee et al., 2006) and, in other species, both glycogen forms degrade at different rates depending on the initial glycogen concentration (Shearer et al., 2001). The hypothesis for the current study was that restricted feeding during the feed restriction phase of compensatory growth would lead to an altered metabolic status compared with pigs fed for ad libitum intake and these alterations would, after allowing ad libitum access to feed, subsequently influence meat quality. Therefore, the objectives were to determine if, at different stages of a compensatory growth regimen, the concentrations of differing forms of muscle glycogen as well as expression of proteolytic systems were altered and whether this was associated with changes to meat quality.

MATERIALS AND METHODS

Because the live animal work was based on performance assessment only, Codes of Practice as laid down by the Department for Environment, Food and Rural Affairs (United Kingdom), which considered welfare and space allowances for commercial animals, were accordingly applied.

Animals and Sampling

Forty-eight female pigs (Large White × Landrace × Duroc cross, 24.6 ± 0.68 kg; 60 d of age) were allowed ad libitum feed intake for 14 d at 74 d of age. Animals were placed on a control (ad libitum access to feed throughout) or compensatory growth regimen; the latter consisted of a 40-d period of feed restriction at 0.70 (by weight) of ad libitum feed intake followed by 42 d of ad libitum access to feed. For the trial, pigs were allocated (based on equal average BW for every group) to 3 slaughter groups (SL1, SL2, and SL3 with slaughter at 114, 116, and 156 d, respectively). In SL1, 8 animals were allowed ad libitum feed intake (A40), whereas 8 others were feed restricted (R40) at 0.70 (by weight) of ad libitum intake for 40 d. For SL2, 8 animals were provided ad libitum access to feed (A42), whereas 8 others were restricted at 0.70 for 40 d, then refed for ad libitum intake for 2 d (R40A2). For SL3, 8 pigs were provided ad libitum access to feed for 82 d (A82) and 8 others were feed-restricted at 0.70 (by weight) of ad libitum intake for 40 d, then provided ad libitum access to feed for 42 d (R40A42) as presented in Table 1. (1 pig in this group died at the end of the experiment 1 d before slaughter).

Pigs were individually penned in a controlled-environment, experimental growth building. Animals were weighed weekly allowing calculation of growth rate through regression of BW over time. Feed intakes

<table>
<thead>
<tr>
<th>Age, d</th>
<th>SL1</th>
<th>SL2</th>
<th>SL3</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 to 73</td>
<td>A40</td>
<td>Adjusted to A</td>
<td>A42</td>
</tr>
<tr>
<td>74 to 113</td>
<td>R4</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>114 to 116</td>
<td>A</td>
<td>R</td>
<td>A</td>
</tr>
<tr>
<td>117 to 156</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
</tbody>
</table>

1Treatments: A40 = access to ad libitum feed intake for 40 d before slaughter; R40 = restricted feed intake at 0.70 (by weight) of ad libitum for 40 d before slaughter; A42 = access to ad libitum feed intake for 42 d before slaughter; R40A2 = restricted feed intake at 0.70 (by weight) of ad libitum for 42 d, then ad libitum feed intake for 2 d before slaughter; A82 = access to ad libitum feed intake for 82 d before slaughter; R40A42 restricted feed intake at 0.70 (by weight) of ad libitum for 40 d, then access to ad libitum feed intake for 42 d before slaughter.

2Slaughter groups: SL1, slaughter at age 114 d; SL2, slaughter at age 116 d; SL3, slaughter at age 156 d.

3A = access to ad libitum feed intake.

4R = restricted feed intake at 0.70 of ad libitum feed consumption.
were recorded weekly for both groups. Individual feeders were used in all pens and checked twice daily to ensure pigs had ad libitum access to feed during the period of ad libitum intake. Feed was weighed weekly into an external bin, and weekly feed intake was calculated from the final weight of the bin together with any collected refusals. Feeding levels of restricted-fed pigs were determined weekly on the basis of BW and ADFI of those that had ad libitum access to feed. The level of restriction imposed was 0.70 of measured ad libitum feed intake; this was within the accepted commercial range. For treatments, a standard grower/finisher meal-based diet was fed (DE, 3,322 Mcal/kg; CP, 165 g/kg; lysine, 10.4 g/kg; methionine + cysteine, 5.9 g/kg; and threonine, 6.3 g/kg as-fed basis). On the morning of slaughter, pigs received their morning feed at the usual time and were then left for 2 h. Subsequently, the first 4 animals (2 restriction and 2 control) were weighed and transported to the abattoir (distance of 500 m) to be slaughtered immediately. The order of slaughter alternated between restricted-fed and ad libitum-fed animals, with treatment-paired animals being slaughtered in succession. After the first 4 animals were slaughtered, the second group of 4 animals was transported to the abattoir. This strategy was designed to minimize the stress of mixing and isolation. Animals were stunned with low voltage electricity followed by bleeding and evisceration without scalding. Muscle samples (approximately 10 to 20 g) were taken immediately after slaughter (0 h) from LM on the left side of the carcass at the position between the 12th and 13th rib, were snap-frozen in liquid nitrogen, and stored at −80°C until analysis. Samples taken immediately after slaughter were used for measuring calpain activity, caspase activity, and analysis of gene expression. Carcasses were dressed and split longitudinally then hung from the Achilles tendon during the conditioning period and stored at 4°C for 48 h. Muscle pH was measured in the LM at 45 min after slaughter (pH45) and 24 h after slaughter (pH24h) on the right side of the carcass in the region of the last rib using a combination pH puncture electrode (Mettler-Toledo, Leicester, UK), with the measurement undertaken in the chiller. When stored at this temperature, 10 to 20 g of LM was taken from the left side of the carcass at 2 and 24 h postmortem and snap frozen in liquid nitrogen for assessing postmortem glycogen decline. Muscle was taken by using a core sampler, which ensured that muscle was never exposed to the air to avoid the effect from oxygen on metabolism of meat during conditioning. After 48 h at 4°C, the whole LM was dissected from the right side of the carcass and trimmed of visible fat. Three-centimeter-wide chops were then cut perpendicular to muscle fiber orientation, vacuum packed, and conditioned at 4°C until 8 d. They were frozen at −20°C overnight, then stored at −80°C until assessed for Warner-Bratzler shear force (WBSF).

**Glycogen Analysis**

The content of MG and ProG was determined in LM samples by measuring glucose concentrations produced from glycogen using an adapted protocol from Adamo and Graham (1998). Tissue samples were crushed in liquid N, and 0.3 g was homogenized (Kinematica, Lucerne, Switzerland) in 1.2 mL of 8% (wt/vol) HClO4, then centrifuged at 15,000 × g for 15 min at 4°C. One milliliter of 1 M HCl was added to 200 μL of supernatant to determine ProG concentrations, whereas the pellet was resuspended in the same volume of the acid to determine MG concentrations. Both solutions were incubated at 100°C for 2 h and centrifuged at 15,000 × g for 15 min at room temperature. To 1 vol of supernatant, 0.6 vol of saturated NaHCO3 solution followed by 0.6 vol of 0.2 M NaC2H3O2 buffer (pH 4.8) were added. After mixing, 200 μL of this neutralized solution was removed and 5 μL of amyloglucosidase (Thermo Electron, Scoresby, Australia), in which 4 μL of the amyloglucosidase-incubated, neutralized solution was added to 150 μL of glucose oxidase solution and incubated for 10 min at room temperature. Absorbance was measured at 550 nm, and the glucose concentration was calculated from a standard curve using bovine liver glycogen (Sigma) as described by Sensky et al. (2006). Duplicate assays were run for both samples and glycogen standards, which had a CV of 20%.

**Intramuscular Fat Composition Analysis**

The intramuscular fat (IMF) content was determined by petroleum ether extraction (Gerhardt, Königswinter, Germany) according to AOAC methods (AOAC, 1995) on crushed muscle sample produced from LM that had been conditioned for 5 d postmortem at 4°C. Duplicate assays were carried out for samples which had a CV of 30%.

**Shear Force Analysis**

The WBSF was measured as described previously (Kemp et al., 2006). Briefly, 8-d conditioned LM chops were cooked in vacuum-sealed bags in a water bath at 80°C until they reached an internal temperature of 70°C. Samples were cooled overnight at 4°C before being prepared into cores using a 1.27-cm diameter coring apparatus. From 3 successive chops, cores were cut in parallel to the alignment of the muscle fibers within the central region of the LM avoiding the edge of the chop. Eight randomly selected cores were subjected to shear using a TA-xT2i Texture Analyzer fitted with
Volodkevich-type jaws (Stable Micro System, Surrey, UK), typically generating a CV of 15% within an assay. Calibration of the apparatus was with a 25-kg weight set at a constant speed of 1.7 mm/s.

**Casein Zymography**

Micro- and milli-calpain LM activities at 0 h post-mortem were determined by casein zymography using an adaptation of the protocol described by Arther and Mykles (2000). Frozen tissue samples were crushed in liquid N, and 1 g of LM was homogenized in 3 mL of zymography extraction buffer [50 mM Tris/Cl (pH 7.5), 5 mM EDTA, 200 μg/mL of 2-(4-aminoethyl)-benzenesulphonyl fluoride, 1 μg/mL of leupeptin, 1 μg/mL of pepstatin] then centrifuged at 15,000 × g for 20 min at 4°C. The supernatant was removed and added to an equal volume of gel sample buffer [125 mM Tris-HCl pH 6.8, 0.1 M dithiothrietal (DTT), 20% (vol/vol) glycerol, 0.01% (wt/vol) Bromophenol blue]. This was then loaded on to a nondenaturing gel consisting of 2% (wt/vol) casein incorporated into 10% (wt/vol) acrylamide separating gel (75:1 ratio of acrylamide to bisacrylamide, 225 mM Tris-HCl, pH 8.8) with 5% (wt/vol) stacking gel (37.5:1 ratio of acrylamide to bisacrylamide, 160 mM Tris-HCl, pH 6.8) without casein. The gels were prerun for 30 min at 4°C in electrophoresis buffer (25 mM Tris, 125 mM glycerine, 1 mM EDTA, 1 mM DTT, pH 8.3) at 125 V. Samples were loaded with equal wet weight of tissue equivalent per well. To reduce the number of gels used, and thereby the intra-gel variations for this calpain activity assessment, single samples were assayed. To adjust for intra-gel variation, 3 replicated standards were run on each gel; using this approach, a CV of 8% was typical within an assay. Samples were run on gels under prerun conditions for approximately 4 h. After electrophoresis, gels were incubated for 1 h at room temperature with Ca2+ incubation buffer (50 mM Tris-HCl, pH 7.0, 5 mM CaCl2, 10 mM DTT) with 3 changes. After fixing for 20 min in 10% (vol/vol) acetic acid, gels were stained with a solution containing 0.2% (wt/vol) Coomassie blue, 0.2% (wt/vol) amido black, 10% (vol/vol) isopropanol, 10% (vol/vol) acetic acid for 30 min and destained with 10% (vol/vol) acetic acid. Band intensities of casein zymography were quantified using Quality-One Multi Analyst imaging software (Biorad, Hemel Hempstead, UK).

**Caspase Assay**

Caspase-3/7 activity was measured in LM samples using Apo-One Homogeneous Caspase-3/7 Assay (Promega, Southampton, UK), adapted for tissue samples (Wagner et al., 2003). Frozen tissue samples were crushed in liquid N, and 1 g was homogenized in 3 mL of extraction buffer [25 mM HEPES (pH 7.5), 0.1% (vol/vol) Triton X-100, 5 mM MgCl2, 2 mM 1,4-DTT, 74 μM antipain, 0.15 μM aprotinin, 1.3 mM EDTA, 20 μM leupeptin, and 15 μM pepstatin]. The homogenate was centrifuged at 15,000 × g for 20 min at 4°C, and the supernatant was removed for the assay. A 1:1 ratio of caspase reagent to sample supernatant was incubated at room temperature for 5 h (reaction end point), then the fluorescence measured at an excitation wavelength of 485 ± 20 nm and an emission wavelength of 530 ± 25 nm on a FluoStar Galaxy spectrometer (BMG Labtechnologies, Aylesbury, UK). Duplicate assays were carried out for samples with a CV of 30%.

**Gene Expression**

Total RNA was prepared from LM using Tripure reagent according to the manufacturer’s protocol (Roche Diagnostics, Rotkreuz, Switzerland). Total RNA was treated with deoxyribonuclease, and then first-strand cDNA was generated from 0.5 μg of total RNA by using random primers and Moloney murine leukemia virus reverse transcriptase as described by the manufacturer (Promega). The first-strand cDNA was subjected to quantitative PCR (Q-PCR) using the primers and dual-labeled fluorescent probe sets (Table 2). Quantitative PCR was carried out using LightCycler 480 Probes Master reaction mixture on a LightCycler 480 Q-PCR machine (Roche Diagnostics) as described previously (Hemmings et al., 2009). Quantitative PCR was performed using thermal cycling conditions of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Absolute quantification analysis was performed using standard curve method as described by the manufacturers (LightCycler 480 Basic Software version 1.2) using a serially diluted pool of LM first-strand cDNA as described previously (Sensky et al., 2006). The Q-PCR reactions were conducted in triplicate using 5 ng of total RNA equivalence, and cyclophilin mRNA expression was used as internal standard so that abundance of target gene expression was described as a ratio to cyclophilin mRNA expression. Duplicate assays were run for experimental samples and standards that had a CV of 20%.

**Statistical Analysis**

Effect of feeding regimen was analyzed by ANOVA using the General Procedure of Genstat (VSN International Ltd., Hemel Hempstead, UK). The statistical model was 2 feeding levels and 8 blocks (the positions of the pen in the pig house). Because of the strong effect of animal age between each slaughter group, statistical analyses were performed within each slaughter group. Results were presented as means and SED. Statistical significance was considered when P < 0.05, whereas a P > 0.05 to <0.1 was considered a trend. Correlation was used to estimate the relationship between meat quality attributes and glycogen forms as well as IMF. The death of a pig in an experimental group was accounted for in the statistical analysis through the capacity of
Table 2. Quantitative PCR primers and dual-labeled probes\(^1\) for quantifying gene expression

<table>
<thead>
<tr>
<th>Target transcript</th>
<th>Accession No.</th>
<th>Primer/probe</th>
<th>Nucleotide sequences (5′ to 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micro-calpain (\text{large subunit})</td>
<td>AF263610</td>
<td>Forward</td>
<td>GACACCCCTCTTGACCCGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>TCCACCACCTCCCCAAACT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probe</td>
<td>CCAACGCGCAAAACCTCTTCAGAAG</td>
</tr>
<tr>
<td>Milli-calpain (\text{large subunit})</td>
<td>U01181</td>
<td>Forward</td>
<td>ACATGCACACCACGCGCCTTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CGGCTCTGGCGCTCAGAAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probe</td>
<td>TGGCAGGAGATTGTTCGAG</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>AB029345</td>
<td>Forward</td>
<td>TTGAAGCCAGCTTCTTATGATCAT</td>
</tr>
<tr>
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<td></td>
<td>Reverse</td>
<td>CGCTGCAACAAATGGACTGGA</td>
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<tr>
<td></td>
<td></td>
<td>Probe</td>
<td>TTTCACGACCTTATTATGCTGAGC</td>
</tr>
<tr>
<td>MuRF1(^2)</td>
<td>EW363322</td>
<td>Forward</td>
<td>GGAGATGGTCTACCAAGCCCG</td>
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<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>TGCTTCAGATGGATTTTCGAG</td>
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<tr>
<td></td>
<td></td>
<td>Probe</td>
<td>CGGAATGTCACACGACATCTCCAG</td>
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<tr>
<td>MAFbx(^3)</td>
<td>EW308124</td>
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<td></td>
<td>Reverse</td>
<td>GGCTCTCGAGATGTCCTCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probe</td>
<td>AAAAAGTGGTACGGAATGCTGAGCAGC</td>
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<tr>
<td>Cyclophilin</td>
<td>F14780</td>
<td>Forward</td>
<td>ACCGCGAACCTTTCTGCGTCTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GCTTGGGCCGCGCTCCTTGAG</td>
</tr>
</tbody>
</table>

\(^1\)Probes: oligonucleotide probe labeled with reporter dye 5′FAM (5-carboxyfluorescein) and the quencher 3′TAMRA (3-carboxytetramethylrhodamine).

\(^2\)Muscle ring-finger protein 1.

\(^3\)Muscle atrophy F-box.

Generate to estimate the missing value; accordingly, this did not contribute to the residual sums of squares and the degrees of freedom were adjusted.

RESULTS

After the period of restricted feeding (0.7 of ad libitum intake) for SL1, there was no difference in BW \((P = 0.110)\) and carcass weight \((\text{CW}; P = 0.108)\) between R40 pigs and A40 pigs. Both BW and CW were less in R40A2 pigs than in A42 pigs \((P = 0.039; P = 0.019,\) respectively) at SL2. In SL3, there was no difference in BW \((P = 0.829)\) or CW \((P = 0.494)\) between the pigs that had been restricted, then refed (R40A42), and those that had been fed for ad libitum intake (A82), indicating that the former had undergone some compensatory growth (Table 3).

In LM at all the slaughter points, the content of MG was greater than ProG at slaughter \((P = 0.001)\). At SL1, there was a trend for the MG content of LM in R40 to be greater at 0 h \((P = 0.051)\) and greater at 24 h postmortem than in A40 pigs \((P = 0.048)\), whereas there was no difference \((P = 0.435)\) at 2 h postmortem (Table 3). As might be expected, MG declined more in R40 animals compared with A40 pigs from 0 to 2 h \((P = 0.033)\), and from 0 to 24 h, there was a trend for similar effect \((P = 0.087)\). For R40 group, ProG was greater than A40 at 0 h \((P = 0.014)\), whereas no difference was detected in others at other times postmortem (ProG 2 h, \(P = 0.550)\); ProG 24 h, \(P = 0.194)\). As with MG, the content of postmortem ProG of R40 pigs declined more than A40 pigs between 0 and 2 h \((P = 0.022)\), with a similar trend detected between 0 and 24 h \((P = 0.094)\).

At SL2, R40A2 pigs had a greater MG at 0 h than A42 \((P = 0.030)\), whereas there was no difference \((P = 0.730)\) in ProG content. In R40A2 pigs, there was a trend for postmortem MG to decline more than in A42 pigs between 0 and 24 h \((P = 0.091)\), whereas over the same time period there was no difference \((P = 0.726)\) in the decline of ProG between the 2 feeding groups. At SL3, there were no differences in the concentrations of MG \((P = 0.721)\) and ProG \((P = 0.879)\) at slaughter or decline of MG and ProG between 0 and 2 h \((\text{MG}, P = 0.482; \text{ProG}, P = 0.980)\) or between 0 and 24 h \((\text{MG}, P = 0.918; \text{ProG}, P = 0.553)\;\text{Table 3).}\

Although the concentrations of both MG and ProG at SL1 and SL2 were greater in pigs that had a restricted feed intake, there was no difference in pH\(_{24h}\) for any of the slaughter dates after 8 d of conditioning (SL1, \(P = 0.715\); SL2, \(P = 0.597\); SL3, \(P = 0.373\)). The pH\(_{24h}\) was generally less in those animals that had been feed restricted compared with the groups that had been fed for ad libitum intake throughout the trial, with pH\(_{24h}\) being less at SL1 \((P = 0.043)\) and SL2 \((P = 0.012)\) in the group that had been restricted-fed (R40 and R40A2) compared with the ad libitum feed intake group (A40 and A42). There was a trend for less IMF at SL1 in the LM of pigs \((P = 0.084)\) that were feed restricted (R40) compared with those with ad libitum access to feed (A40), whereas there was no difference at the other slaughter dates once the feed restriction had been removed, even when animals were allowed ad libitum feed intake for 2 d after restriction (SL2, \(P = 0.159\); SL3, \(P = 0.352\)). There was no difference in the LM shear force between treatments at any of the slaughter dates after 8 d of conditioning (SL1, \(P = 0.101\); SL2, \(P = 0.420\); SL3, \(P = 0.167\)). There were positive correlations between shear force samples conditioned for 8 d and both MG \((P = 0.002)\) and ProG \((P = 0.033)\) concentrations at slaughter (Table 4), and there was also a negative correlation between IMF with
Table 3. The effect of feeding treatments\(^1\) on pig BW, carcass weight, and LM characteristics for each slaughter group\(^2\)

<table>
<thead>
<tr>
<th>Trait</th>
<th>SL1</th>
<th>SL2</th>
<th>SL3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A40</td>
<td>R40</td>
<td>SED</td>
</tr>
<tr>
<td>n</td>
<td>8</td>
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</tr>
<tr>
<td>Initial BW, kg</td>
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<td>37</td>
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<tr>
<td>Final BW, kg</td>
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<td>64</td>
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<tr>
<td>Carcass wt, kg</td>
<td>55</td>
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<td>3.9</td>
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<tr>
<td>MG(^4) 0 h, (\mu)mol/g</td>
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<td>58</td>
<td>8.5</td>
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<tr>
<td>MG(^4) 2 h, (\mu)mol/g</td>
<td>33</td>
<td>37</td>
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<tr>
<td>MG(^4) 24 h, (\mu)mol/g</td>
<td>26</td>
<td>29</td>
<td>1.9</td>
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<td>∆ MG(^4) 2 h, (\mu)mol/g</td>
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<td>6</td>
<td>7</td>
<td>0.9</td>
</tr>
<tr>
<td>∆ ProG(^4) 2 h, (\mu)mol/g</td>
<td>4</td>
<td>7</td>
<td>1.5</td>
</tr>
<tr>
<td>∆ ProG(^4) 24 h, (\mu)mol/g</td>
<td>9</td>
<td>12</td>
<td>2.1</td>
</tr>
<tr>
<td>pH(_{45})</td>
<td>6.26</td>
<td>6.25</td>
<td>0.026</td>
</tr>
<tr>
<td>pH(_{24h})</td>
<td>5.56</td>
<td>5.50</td>
<td>0.023</td>
</tr>
<tr>
<td>IMF(^8), mg/g of dry wt</td>
<td>5.96</td>
<td>3.87</td>
<td>1.037</td>
</tr>
<tr>
<td>Shear force, kg</td>
<td>5.87</td>
<td>7.37</td>
<td>0.798</td>
</tr>
</tbody>
</table>

\(^1\)Treatments: A40 = access to ad libitum feed intake for 40 d before slaughter; R40 = restricted feed intake at 0.70 (by weight) of ad libitum for 40 d before slaughter; A42 = access to ad libitum feed intake for 42 d before slaughter; R40A2 = restricted feed intake at 0.70 (by weight) of ad libitum for 40 d, then access to ad libitum feed intake for 2 d before slaughter; A82 = access to ad libitum feed intake for 82 d before slaughter; R40A42 = restricted feed intake at 0.70 (by weight) of ad libitum for 40 d, then access to ad libitum feed intake for 42 d before slaughter.

\(^2\)Slaughter groups: SL1, slaughter at age 114 d; SL2, slaughter at age 116 d; SL3, slaughter at age 156 d.

\(^3\)One pig died, and the missing value was calculated.

\(^4\)LM glycogen forms at specific times postmortem: MG = macroglycogen; ProG = proglycogen.

\(^5\)Micromoles of glucosyl units/g of wet weight LM.

\(^6\)Difference in the concentration of LM glycogen forms postmortem between specific times postmortem: ∆ MG 2 h = difference in acid-soluble MG between 0 and 2 h; ∆ MG 24 h = difference in MG between 0 and 24 h; ∆ ProG 2 h = difference in acid-insoluble ProG between 0 and 2 h; ∆ ProG 24 h = difference in ProG between 0 and 24 h.

\(^7\)LM pH at specified times postmortem: pH\(_{45}\) = pH at 45 min postmortem; pH\(_{24h}\) = pH at 24 h postmortem.

\(^8\)IMF = LM intramuscular fat.
Table 4. Coefficient correlation of pig LM shear force 
vs. LM glycogen isoforms at slaughter or intramuscular fat (IMF)

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>r</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macroglycogen</td>
<td>48</td>
<td>0.44</td>
<td>0.002</td>
</tr>
<tr>
<td>Proglycogen</td>
<td>48</td>
<td>0.31</td>
<td>0.033</td>
</tr>
<tr>
<td>IMF</td>
<td>48</td>
<td>−0.37</td>
<td>0.013</td>
</tr>
</tbody>
</table>

1One pig died, and the missing value was calculated.

shear force ($P = 0.013$). The gene expression of selected targets involved in muscle proteolysis was examined. The MAFbx and MuRF1 are E3 ligases are part of the ubiquitin proteasome system, which is involved in protein degradation in skeletal muscle (Cao et al., 2005). However, at SL1 and SL2, the LM mRNA expression was not different for MAFbx (SL1, $P = 0.376$; SL2, $P = 0.472$) and MuRF1 (SL1, $P = 0.610$; SL2, $P = 0.801$). At SL3 MuRF1 gene expression was less in R40A42 than the A82 group ($P = 0.019$; Table 5), whereas for MAFbx it was not different ($P = 0.110$). There was no effect of feeding regimen on gene expression of micro- and milli-calpain large subunits (active calpain consists of a heterodimer of a large and small subunit; Table 5). At SL1 there was a trend for an increase in the abundance of caspase 3 mRNA in the restricted-feed intake group R40 ($P = 0.070$) compared with the control group fed for ad libitum intake, and at SL2 this increase was maintained in R40A2 ($P = 0.009$), whereas there was no difference between the groups at SL3 ($P = 0.551$).

The activities of either micro- or milli-calpain were not different between treatment groups at any of the slaughter dates (Table 6). Caspase 3/7 activity was assessed using an assay that cannot distinguish between the activities of these isoforms. Both are effector caspases, being involved in the degradation of substrate cleaved during apoptosis such as cytoskeletal proteins. Caspase 7 has previously been reported as not being cleaved during apoptosis such as cytoskeletal proteins. The feeding regimen employed in the current study was able to induce a compensatory effect on pig growth; after a period of restricted intake, there was a reduction in the CW compared with a control group fed for ad libitum intake. After restriction, the pigs allowed to feed to ad libitum levels recovered their CW. In the current study both glycogen forms, MG and ProG, were principally identified based on their differing acid solubility, MG being soluble, whereas ProG is not, which is due to the greater protein content of the latter (Alonso et al., 1995). In addition, their molecular weights differ, MG being approximately $10^7$ Da and ProG being in the region of $4 \times 10^5$ Da (Lomako et al., 1991, 1993; Alonso et al., 1995). The protein glycogenin is the precursor of ProG, and this form of glycogen functions as intermediate for the synthesis and breakdown of MG (Lomako et al., 1993; Alonso et al., 1995). Studies have reported differing values for the relative ratios between the 2 forms of glycogen in pig muscle, with ProG being less than or greater than MG (Rosenvold et al., 2003; Bee et al., 2006; Ylä-Ajos et al., 2007; Young et al., 2009); the values described in the current study were similar to those reported in pigs by Bee et al. (2006). Exercise, genotype, and fiber type are known to influence the content of both MG and ProG. For example, in humans the resynthesis of muscle MG and ProG are at different rates after exercise (Graham et al., 2001). In the presence of high carbohydrate intake after exercise, the increase in total muscle glycogen content is due to a greater synthesis of the MG fraction than ProG (Adamo et al., 1998). Greater MG relative to ProG have been reported in Rendement Napole (RN”) pigs (Essén-Gustavsson et al., 2005), where the authors also found that the more oxidative fibers (type I and IIA) tended to have less glycogen after exercise than the fast type IIB fibers. Studies in rats have described that, as the quantity of muscle glycogen increases, the MG form becomes the greatest proportion of total glycogen, but when muscle glycogen concentrations are reduced the ProG form is retained and is at a greater amount than MG (Hansen et al., 2000). Therefore, the ratio of MG to ProG appears to be variable and influenced by several factors.

In the present study, at the end of the feed restriction period R40 pigs had greater MG and ProG than A40 animals. However, after the feed-restricted pigs were returned to have access to an ad libitum intake for 2 d (SL2), only MG content remained significantly larger, suggesting that the ProG pool is more labile to feed manipulation; ProG is the precursor of MG and MG must be converted to back to ProG, and it is this form of glycogen that is used to produce glucose (Lomako et al., 1991; Adamo and Graham, 1998). Therefore, the difference in MG content at SL2 could be due to ProG being used to synthesize MG when pigs were returned to ad libitum access to feed, resulting in MG content remaining significantly different (at greater concentrations than SL1) but ProG being the same. After the compensation period, glycogen content at SL3 was not different, indicating no lasting effects of feed restriction on glycogen pools. The greater amount of MG and ProG during the feed restriction period could be the result of metabolic adaptations affecting glycogen synthesis caused by feeding regimen. During restriction period, pigs were fed 0.7 of ad libitum intake at 2 fixed time points (morning and afternoon) per day, consuming their feed within less than 30 min, whereas the group fed for ad libitum access freely consumed feed throughout the day. Studies that have examined the effects of meal feeding, delivered by a single daily meal compared with nonrestricted ad libitum access,
### Table 5. The effect of feeding treatments\(^1\) on mRNA expression of proteolytic system components in pig LM at slaughter (0 h) for each slaughter group\(^2\)

<table>
<thead>
<tr>
<th>Gene mRNA target(^3)</th>
<th>SL1</th>
<th>SL2</th>
<th>SL3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A40</td>
<td>R40</td>
<td>SED</td>
</tr>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>MAFbx(^5)</td>
<td>0.60</td>
<td>0.41</td>
<td>0.204</td>
</tr>
<tr>
<td>MuRF1(^6)</td>
<td>0.87</td>
<td>1.07</td>
<td>0.371</td>
</tr>
<tr>
<td>Micro-calpain(^7)</td>
<td>0.69</td>
<td>0.82</td>
<td>0.160</td>
</tr>
<tr>
<td>Milli-calpain(^7)</td>
<td>0.85</td>
<td>1.01</td>
<td>0.368</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>0.45</td>
<td>1.00</td>
<td>0.258</td>
</tr>
</tbody>
</table>

\(^1\)Treatments: A40 = access to ad libitum feed intake for 40 d before slaughter; R40 = restricted feed intake at 0.70 (by weight) of ad libitum for 40 d before slaughter; A42 = access to ad libitum feed intake for 42 d before slaughter; R40A2 = restricted feed intake at 0.70 (by weight) of ad libitum for 40 d, then access to ad libitum feed intake for 2 d before slaughter; A82 = access to ad libitum feed intake for 82 d before slaughter; R40A42 = restricted feed intake at 0.70 (by weight) of ad libitum for 40 d, then access to ad libitum feed intake for 42 d before slaughter.

\(^2\)Slaughter groups: SL1, slaughter at age 114 d; SL2, slaughter at age 116 d; SL3, slaughter at age 156 d.

\(^3\)Values for gene targets are expressed as ratio of proteolytic gene mRNA:cyclophilin mRNA.

\(^4\)One pig died, and the missing value was calculated.

\(^5\)Muscle atrophy F-box.

\(^6\)Muscle ring-finger protein 1.

\(^7\)Gene expression of the large subunit was assessed.

### Table 6. The effect of feeding treatments\(^1\) on proteolytic enzyme activity in pig LM at slaughter (0 h) for each slaughter group\(^2\)

<table>
<thead>
<tr>
<th>Trait</th>
<th>SL1</th>
<th>SL2</th>
<th>SL3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A40</td>
<td>R40</td>
<td>SED</td>
</tr>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Micro-calpain(^4)</td>
<td>5.19</td>
<td>4.98</td>
<td>0.168</td>
</tr>
<tr>
<td>Milli-calpain(^4)</td>
<td>3.68</td>
<td>3.51</td>
<td>0.151</td>
</tr>
<tr>
<td>Caspase 3/7(^5)</td>
<td>1,496</td>
<td>1,783</td>
<td>167.2</td>
</tr>
</tbody>
</table>

\(^1\)Treatments: A40 = access to ad libitum feed intake for 40 d before slaughter; R40 = restricted feed intake at 0.70 (by weight) of ad libitum for 40 d before slaughter; A42 = access to ad libitum feed intake for 42 d before slaughter; R40A2 = restricted feed intake at 0.70 (by weight) of ad libitum for 40 d, then access to ad libitum feed intake for 2 d before slaughter; A82 = access to ad libitum feed intake for 82 d before slaughter; R40A42 = restricted feed intake at 0.70 (by weight) of ad libitum for 40 d, then access to ad libitum feed intake for 42 d before slaughter.

\(^2\)Slaughter groups: SL1, slaughter at age 114 d; SL2, slaughter at age 116 d; SL3, slaughter at age 156 d.

\(^4\)One pig died, and the missing value was calculated.

\(^5\)Arbitrary densitometry units/0.3 g of wet weight muscle.

\(^6\)Combined caspase 3 and 7 activity, fluorescence/10 μg of protein.
have reported several metabolic adaptations in the for-
mer, such as increased intestinal absorption (Leveille,
1970), greater glycemia and increased glycogen syn-
thase activity, increased blood concentrations of insulin
(Ip et al., 1977; Batista et al., 2001), and enhanced
glycogen accumulation and resistance to its catabolism
during the intermeal period (Curi et al., 1984; Lima et
al., 1985). Indeed, feed restriction has been reported to
increase feed utilization by physiological adaptation,
increasing intestinal mucosa and gastric hypertrophy
as well as a decrease in gastric emptying rate (Lima
et al., 1981; Curi et al., 1984; Bazotte et al., 1989).
Although the effect of increasing intestinal mucosa and
 gastric hypertrophy due to meal feeding vs. ad libi-
tum access has predominantly been described in rats, it
is also known to occur in pigs (O’Hea and Leveille,
1969). The combined effects of such adaptations may,
in part, explain the increased concentrations glycogen
in restricted groups. It was clear the adaptations, which
were reflected in the differences in LM glycogen content
at the end of the restricted feeding period (R40) and
immediately afterward (R40A2), were lost at the end of
the compensation period. In contrast, Heyer and Lebret
(2007) reported that pigs subjected to a compensatory
growth feeding regimen had a reduction in their muscle
glycolytic potential and glucose content relative to ani-
mals with ad libitum access to feed; their regimen used
a restricted feeding phase that was 0.65 of ad libitum in-
take and was based on animals attaining a specific BW
(70 kg) before being switched to the refeeding phase.
Compared with the current trial, the restriction phase
was longer (65 d) and there was a shorter (34 d) refeed-
ing period in which restricted pigs caught up to the BW
of the access to ad libitum feed group at slaughter. The
differences in the indices of glucose metabolism may be
due to the distinctions between the level of feed intake
and time periods of exposure.

There was a significant difference in the decline of
both MG and ProG at SL1 between access to ad libi-
tum feeding (A40) and restricted-fed (R40) pigs that
was not seen at any other slaughter time points. Bee
et al. (2006) reported in pigs fed high or low dietary
carbohydrate that there was more MG in muscle than
ProG in animals fed high carbohydrate diets and their
MG content decreased more rapidly over the first 24
h postmortem, whereas there was no difference in the
amount or decrease of ProG between diets. In contrast,
Rosenvold et al. (2003) examined the effects of a car-
bohydrate-restricted diet fed for 21 d and found that a
restricted diet resulted in a decline in ProG being less
than control 45 min postmortem, whereas MG decline
was not affected. The significant decline in muscle ProG
but not MG postmortem was also observed by Ylä-Ajos
et al. (2007). The common feature of the latter 2 stud-
ies was that the ProG content at slaughter was greater
than MG, and therefore, as Bee et al. (2006) proposed,
it appears that when ProG is at greater concentrations
than MG, the ProG pool is preferentially catabolized.
At all the slaughter points in the current study, the pro-
portional decrease from 0 to 24 h in ProG was greater
than MG. This is indicated by the 0:24 h glycogen ra-
tio; for MG it was in the range 0.49 to 0.56, whereas
for ProG it was 0.26 to 0.50 across slaughter groups. As
indicated above, it has been suggested ProG is the pre-
cursor of MG and MG must be converted to ProG to
be utilized (converted to glucose); hence, its concentra-
tion decreased the most when glycogen was converted
to glucose (Lomako et al., 1991; Adamo and Graham,
1998). The reason that the decrease in ProG content
is greater than MG may be because the ProG is more
readily utilized in the animals in the current study.

At each slaughter point the lowest pH24h postmortem
was associated with the feed regimen group that had
the greatest quantity of either MG or ProG; however,
when considering all the slaughter groups, there was
no correlation between postmortem pH and slaughter
MG or ProG content or their decrease postmortem. In-
terestingly, whichever feeding group had significantly
increased MG concentrations, this was associated with
significantly lower pH24h, as was the case in the restrict-
ed groups at SL1 and SL2. This was an unexpected
observation because a reduced feed intake might be ex-
pected to reduce the quantity of glycogen in muscle,
thereby leading to high pH24h. When there was no dif-
fERENCE in glycogen concentrations between groups, for
example after the compensatory period (SL3), there
was no difference in postmortem pH, in agreement
with Heyer and Lebret (2007). However, across all the
slaughter groups, there was a significant positive cor-
relation between WBSF and MG or ProG stores at
slaughter; this may be a consequence of fat content of
the muscle because there was a significant negative cor-
rrelation between IMF and WBSF. These observations
agree with those of van Laack et al. (2001) who also
reported a significant negative correlation between IMF
and WBSF value of pork after aging for 7 d. However,
in the current study there was no significant difference
between IMF of the groups at each slaughter point.
Similarly, Therkildsen et al. (2002) and Mason et al.
(2005) did not find an effect on IMF after a compensa-
tory growth feeding regimen, although Kristensen et al.
(2004) did observe that IMF was reduced in castrated
male pigs after a compensatory growth feeding regi-
men. In the current study, at the end of the compensa-
tory feeding regimen, there was no significant difference
in WBSF values between compensatory and access to
ad libitum feeding strategies, which was similar to the
observations of others (Therkildsen et al., 2002; Heyer
and Lebret, 2007).

Therkildsen et al. (2002) observed that pig compen-
satory growth regimens significantly decreased the ac-
tivity of the proteolytic enzyme micro-calpain, and this
was associated with the animals that had shorter peri-
ods of access to ad libitum feeding after the feed restric-
tion. These changes were also associated with changes
in indicators of protein synthesis, which led the investi-
gators to suggest that protein synthesis increased at a
faster rate after the transition to ad libitum feeding af-
ter restriction than protein degradation and that these changes could influence meat quality. Micro-calpain is the component of the calpain proteolytic system that is thought to be a key mediator of the proteolysis associated with meat tenderness (Koohmaraie and Geesink, 2006). In the current study neither calpain activity nor mRNA abundance of their corresponding large subunits were significantly different between restricted and ad libitum feeding in any of the slaughter groups. However, it is interesting to note that micro- and milli-calpain activities were less in the restricted feed group (R40) compared with the group having access to ad libitum feeding (A40), similar to the observations of Therkildsen et al. (2002) and Kristensen et al. (2002), which corresponded to a decreased WBSF. The changes in calpain activity that have been described as being associated with compensatory regimens, particularly the restriction and transition phases to access to ad libitum feeding, could be involved with the suggested role of muscle proteolysis, and particularly the calpain system, associated with the alterations in muscle growth (Goll et al., 2003).

Caspases are proteolytic enzymes associated with the process of programmed cell death (apoptosis). Herrera-Mendez et al. (2006) hypothesized that caspases could contribute to postmortem proteolysis, thereby influencing meat quality. Caspase activity has been associated with muscle differentiation (Fernando et al., 2002) so potentially may be involved in muscle protein turnover; activated caspase activity is associated with pathology-associated muscle atrophy, for example, that associated with burn injury (Yasuhara et al., 2000). Our laboratory has previously identified that caspases are expressed in pig muscle, that they degrade myofibril proteins, and that there is a potential association between caspase activity and tenderness (Kemp et al., 2006; Kemp and Parr, 2008). The initiator caspases (caspase 8, 9) cleave and activate the executor or effector caspases (caspases 3, 6, 7; Kemp et al., 2010). Caspase 3 is an effector caspase; the isoforms of the caspase system are involved in degradation of cytoskeletal proteins, many of which are associated with the tenderization process (Herrera-Mendez et al., 2006; Kemp et al., 2010). The present study found that, although caspase 3/7 activity was not significantly greater in R40 than in A40 pigs, at the mRNA level there was a trend for a difference in caspase 3 mRNA and this difference between groups was significant at SL2. The current study suggests that the caspase system may be involved in the proteolytic events associated with muscle growth and remodeling. This proteolytic system has been implicated in process of muscle atrophy associated with aging (sarcopenia; Dupont-Versteegden, 2005); however, the current study does not give indications of the potential role of caspases in meat quality.

As with the caspase system, the ubiquitin protein ligases, MAFbx and MuRF1, have been associated with muscle remodeling, particularly the processes associated with atrophy (Cao et al., 2005). Unlike the caspase and calpain systems, the quantity of both MAFbx and MuRF1 appeared not to be influenced at SL1 and SL2. However, after the full refeeding period (SL3), the group that had experienced restricted feeding (R40A42) showed less gene expression than the group with access to ad libitum feeding (A82) for MuRF1. Ubiquitin-protein ligase activity is associated with protein degradation (Cao et al., 2005); the decrease in expression of these at SL3 may be associated with decreased protein turnover via the proteasome system. Interestingly, in previous studies compensatory growth regimens have been shown to affect the concentration of IGF-I. Studies have described IGF-I concentrations are decreased during the restriction phase but during the compensatory phase IGF-I concentrations are the same, as was the case in the current study (data not shown), or are increased relative to animals fed with access to ad libitum feed throughout (Ellenberger et al., 1989; Renaville et al., 2000; Therkildsen et al., 2004). Both MAFbx and MuRF1 mRNA expression are known to be decreased by increased IGF-I concentrations (Sacheck et al., 2004), suggesting that growth regimens that alter IGF-I concentrations may decrease expression of these enzymes that have been strongly associated with catabolic states potentially enabling protein synthesis to be accentuated and there to be increased growth. More importantly, these observations suggest a difference in the regulation of the various proteolytic systems in skeletal muscle, which perhaps reflects their functions, with caspase and calpain having a role remodeling functions and the enzymes associated with the proteasome system being involved in general proteolysis.

In summary, the feeding regimen was able to induce compensatory growth as pigs recovered their BW at the end of the compensatory period. Associated with these changes was a recovery in concentrations of MG and ProG from the differences between access to ad libitum feed and restricted feeding groups at SL1 and SL2, so that at the end of the compensatory period the concentrations were the same and a lack of a lasting effect was also observed on meat quality as assessed by pH and WBSF. Surprisingly, in pigs fed a restricted amount of intake, muscle glycogen concentrations were greater than groups with access to ad libitum feeding at SL1 and SL2. This may have been, in part, caused by an adaptive effect of gastric and intestine hypertrophy that has been previous reported by others; however, further studies are required to confirm this effect. It is apparent that such significant differences in glycogen content due to restricted feeding influences postmortem decline of glycogen and ultimate pH (assessed at 24 h postmortem). It was apparent that, on introduction of access to ad libitum feeding after feed restriction, there was an adjustment of ProG concentrations to the same quantity as pigs with ad libitum access to feed throughout, that probably reflects the more labile nature of this glycogen form, ProG acting as an intermediate in the formation of MG. The only proteolytic system influ-
enced by the feeding regimen was the caspase system, but as was seen with glycogen concentrations, there appeared to be no significant lasting effects at the end of the compensatory period.

LITERATURE CITED


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