Metabolic adaptation of pigs to a *Mycoplasma hyopneumoniae* and *Lawsonia intracellularis* dual challenge¹

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ABSTRACT: Respiratory and enteric pathogens such as *Mycoplasma hyopneumoniae* (Mh) and *Lawsonia intracellularis* (LI) reduce lean accretion and feed efficiency (FE) in growing pigs. However, the metabolic mechanism by which this occurs is still unknown. Therefore, the primary aim of this study was to examine the metabolic adaptation of pigs presented with a dual Mh and LI challenge (MhLI). A secondary objective was to examine if selection for high FE, modeled by selection for low residual feed intake (RFI), alters molecular response to disease. Using a 2 × 2 factorial design, 6 littermate pairs from a high RFI (HRFI) and 6 littermate pairs from a low RFI (LRFI) line (barrows, 66 ± 2 kg BW) were selected, with 1 pig from each pair assigned to individual pens in either the challenge or the nonchallenge (control) rooms (*n* = 6 barrows per line/challenge). On days post inoculation (dpi) 0, MhLI pigs were inoculated intragastrically with LI and intratracheally with Mh. Pig and feeder weights were recorded at dpi 0, 7, 14, and 21. On dpi 21, pigs were euthanized and tissues and blood were collected. Markers of oxidative stress, skeletal muscle metabolism and proteolysis, and liver gluconeogenesis were evaluated to determine

the effects of MhLI, RFI line, and their interaction. The interaction of line and challenge was not significant (*P* > 0.05) for any measure. Overall, MhLI pigs had lower ADG (38%, *P* < 0.001), ADFI (25%, *P* < 0.001), and G:F (19%, *P* = 0.012) compared with controls. As expected, LRFI pigs had lower ADFI (*P* = 0.028) for the same ADG, giving them greater G:F (*P* = 0.021) than HRFI pigs. Challenged pigs had greater reactive oxygen species (ROS) production in the LM and liver (*P* < 0.10) but did not have greater skeletal muscle proteolysis. Liver gluconeogenesis was also not upregulated (*P* > 0.05) due to MhLI. These results provide further evidence that selection for LRFI does not negatively affect response to disease. In addition, these results suggest that postabsorptive metabolic functions are altered due to MhLI challenge. The MhLI challenge induced mitochondrial dysfunction, evident by greater ROS production, and caused pigs to favor glycolytic energy generation. However, skeletal muscle proteolysis and liver gluconeogenesis were not upregulated during MhLI challenge. These data suggest that during mild disease stress, pigs can meet energy demands without reliance on nutrient mobilization and gluconeogenesis.

Key words: *Lawsonia intracellularis*, metabolism, muscle, *Mycoplasma hyopneumoniae*, pig

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INTRODUCTION

Pathogen challenges and feed efficiency (FE) are 2 issues that affect pig production and productivity worldwide. In grow-finish pigs, *Mycoplasma hyopneumoniae* (Mh) and *Lawsonia intracellularis* (LI) are 2 pathogens that significantly contribute to respiratory and enteric disease, respectively, in the United States (USDA, 2012). These 2 pathogens, alone or in tandem with other infectious agents, reduce average daily gain, lean accretion, and FE (Ciprian et al., 2012; Paradis et al., 2012; Helm et al., 2018). However, the metabolic basis of these reductions in performance beyond reduced feed intake has been poorly characterized. It is widely accepted that during immune system stimulation, consumed nutrients are allocated away from lean growth to aid the pig in mounting an effective immune response (Reeds et al., 1994; Elsasser et al., 2000). During a severe stress response, feed intake is reduced enough that tissue reserves may be mobilized to provide energy and nutrients to fuel the immune response (Johnson, 2002; Klasing, 2007). Additionally, there is concern that intense selection for production traits such as high FE may alter the pig's ability to allocate resources during times of stress (Rauw, 2007). Previous research has demonstrated that pigs that differed in FE based on divergent selection for residual feed intake (RFI) performed similarly during pathogen challenge (Dunkelberger et al., 2015; Helm et al., 2018) or low RFI pigs performed better under unsanitary conditions (Chatelet et al., 2018).

Therefore, the aim of this study was to characterize the impact of a Mh and LI (MhLI) dual challenge on the metabolism of grow-finish pigs divergently selected for RFI. We hypothesized that a partial cause of the reduction in performance observed during this MhLI dual challenge (Helm et al., 2018) was a result of greater oxidative stress and skeletal muscle proteolysis, the latter of which would allow for mobilization and allocation of resources to the immune system over lean tissue accretion and growth.

MATERIALS AND METHODS

All animals were handled in accordance with the Iowa State University Institutional Animal Care and Use Committee (IACUC# 6-16-8298-S).

Animals, Housing, and Experimental Design

The impact of a 42-d MhLI dual challenge on performance of Yorkshire pigs from lines that

were divergently selected for RFI has been previously reported (Helm et al., 2018). For the current study, a subset of 24 barrows (48.1 ± 6.7 kg BW), 6 littermate pairs from the low RFI (LRFI) line and 6 pairs from the high RFI (HRFI) line, were randomly selected and confirmed seronegative by ELISA for Mh and LI antibodies. These lines of pigs have been selected such that LRFI pigs consume less feed for the same amount of backfat and growth as their HRFI-selected counterparts, making LRFI pigs more feed efficient (Cai et al., 2008). Littermate pairs were randomly split across 2 rooms within 1 barn, placed into individual pens, and allowed to acclimate for 21 d prior to inoculation. The 2 rooms had identical heating, cooling, water supply, feeders, flooring, and pen space, but separate manure pits. Pigs were allowed free access to water and feed was not restricted at any point in the study. All pigs were fed a corn-soybean diet that was formulated to meet or exceed all NRC (2012) requirements for this size pig. The diet was a commercially available finisher diet that was formulated to provide 3,400 kcal/kg ME and contained 15.5% crude protein. Diet composition has been published elsewhere and no differences in performance were observed between the 2 rooms during the 21-d period prior to inoculation (Helm et al., 2018). Prior to inoculation, 1 room was designated as the control room and the other as the MhLI dual challenge room. The resulting experimental design was a 2×2 factorial consisting of 4 treatment groups ($n = 6$): 1) LRFI control, 2) HRFI control, 3) LRFI MhLI challenge, and 4) HRFI MhLI challenge.

On days post inoculation (dpi) 0, pigs (66.5 ± 7.9 kg BW) in the challenge room were dual inoculated with both Mh and LI, whereas control pigs were inoculated with a sham. While under snare restraint, pigs received 10-mL Mh inoculum (strain 232, containing 10^5 color-changing units/mL) via intratracheal inoculation and 40-mL LI inoculum (2 mL gut homogenate, containing 2×10^7 LI organisms) via intragastric gavage. Both inocula were prepared at the Iowa State University Veterinary Diagnostic Laboratory (ISUVDL, Ames, IA). The Mh inoculum was prepared from a crude lung homogenate and the LI from a crude gut homogenate of pigs confirmed positive for their respective organism. Individual pigs were weighed weekly and individual feed disappearance and G:F were calculated over the 21-d challenge period. At dpi 21, all pigs were euthanized via captive bolt followed by exsanguination. The dpi 21 time point was chosen as it was expected to have peak clinical symptoms such as intestinal lesions characteristic

of LI infections (McOrist and Gebhart, 2012; Guedes et al., 2017) as well as coughing and lung lesions characteristic of acute Mh infection (Pieters et al., 2009). However, it is important to note that bacterial pathogens have considerable variation with regards to time and extent of peak impact.

To confirm infection and pathogen colonization at dpi 21, lungs and intestines were evaluated for gross lesions characteristic of Mh and LI infection by a trained veterinary pathologist who was blinded to individual pig treatments. Lungs were scored based on the percentage of lung lesion coverage to confirm Mh infection (0%–100%) and intestines (ileum and colon) were evaluated for presence or absence of gross lesions of porcine proliferative enteritis caused by LI. Additionally, a section of the lung, ileum, and colon was formalin fixed and then trimmed, processed, and sectioned at the ISUVDL. Antigen presence was determined for Mh (lung) and LI (intestines) via immunohistochemistry (IHC) staining with antibodies specific to the respective antigen. A blinded veterinary pathologist at the ISUVDL recorded presence or absence of Mh antigen in lungs and LI antigen in ileum and colon. This is reported as the number of pigs that tested positive out of the total number of pigs in each treatment group.

At necropsy, a section of the LM and liver was snap frozen in liquid nitrogen and stored at -80°C . Additionally, LM and liver tissue samples were transported on ice to the laboratory for fresh tissue analyses. Liver samples were rinsed in Krebs–Henseleit buffer (25-mM NaHCO_3 , 120-mM NaCl, 1-mM MgSO_4 , 6.3-mM KCl, 2-mM CaCl_2 , and 0.32-mM NaH_2PO_4 , pH 7.4) to remove excess blood.

Blood Collection and Analysis

On dpi 0 and 21, blood samples (10 mL) were collected via jugular venipuncture into BD Vacutainer tubes (Becton, Dickinson and Company, Franklin Lakes, NJ). Samples were allowed to clot at room temperature, centrifuged ($2,000 \times g$ for 10 min at 4°C), and serum was collected, aliquoted, and stored at -80°C until analysis.

To confirm presence or absence of LI and Mh, serum samples were submitted to the ISUVDL and tested via ELISAs. To quantify LI antibody response in sera, SVANOIR Ileitis ELISA (Boehringer Ingelheim Svanova, Uppsala, Sweden) was used and the response was recorded as percent inhibition; samples with percent inhibition greater than 30% were considered positive. *Mycoplasma*

hyopneumoniae antibody response was assessed using the IDEXX Laboratories, Inc., Mh ELISA assay (Westbrook, ME) and serology results were reported as a sample to positive (S:P) ratio, in which an S:P ratio greater than 0.40 was considered positive for Mh. For analysis of the LI and Mh antibody ELISA results at dpi 21, pig serum results were summarized as being either positive or negative based on these thresholds.

In addition to antibody responses, dpi 21 serum was analyzed for metabolic markers such as glucagon, blood urea nitrogen (BUN), NEFA, glucose, and insulin. Glucagon and insulin concentrations were measured via ELISA Kits (R&D Systems, Minneapolis, MN) according to manufacturer's instructions. Serum BUN was quantified with a QuantiChrom Urea Assay Kit (BioAssay Systems, Hayward, CA) and NEFA concentrations were measured with the Wako Diagnostics kit (Wako Chemical Inc., Richmond, VA) according to the manufacturer's instructions. Serum glucose concentrations were measured utilizing Glucose Oxidase/Peroxidase Reagent (GO, Sigma-Aldrich, St. Louis, MO). Briefly, serum samples were plated onto a 96-well plate in triplicate and incubated with GO for 30 min at 37°C , after which the reaction was stopped with 12 N HCl and absorbances were read at 540 nm. All serum sample absorbance values were analyzed with a Cytation Hybrid Multi-Mode Reader using Gen 5 software (BioTek Instruments, Inc., Winooski, VT). All samples were run in triplicate and had CVs less than 7%.

Protein Translational Efficiency

The ratio of protein to RNA was assessed in LM and liver tissue samples to determine how MhLI dual challenge and RFI may alter protein translational efficiency. Briefly, total RNA was isolated from frozen LM and liver samples as described previously (Schweer et al., 2016). Sarcoplasmic protein was extracted from frozen LM and liver samples (0.5 g) in 4 mL of HEPES cell lysis buffer (50-mM HEPES, 150-mM NaCl, 50-mM NaF, 2-mM EDTA, 1% Triton X-100, 0.1% protease inhibitor cocktail, and 5% glycerol), homogenized, and then centrifuged at $2,000 \times g$ for 10 min at 4°C . The supernatant was collected, protein concentrations were determined using a Pierce bicinchoninic acid (BCA) assay (ThermoFisher Scientific, Waltham, MA), and extracted protein was stored at -80°C until analyzed. Protein and RNA were corrected for start tissue weight, and then protein was expressed

relative to RNA to assess crude differences in protein translational efficiency (Pringle et al., 1993).

Muscle and Liver Mitochondrial Isolation, Reactive Oxygen Species Production, and Oxidative Stress

Longissimus muscle and liver tissue reactive oxygen species (ROS) production and oxidative stress markers were assessed at dpi 21. Briefly, mitochondrial isolation was performed on ice from fresh liver and LM samples using a procedure previously described (Iqbal et al., 2004; Grubbs et al., 2013). Final mitochondrial protein concentrations were determined using a BCA assay and then samples were diluted to 2-mg mitochondrial protein/mL for use in the ROS production assay.

Mitochondrial ROS production was determined by a 2',7'-Dichlorofluorescein diacetate (DCFH) assay as described previously (Iqbal et al., 2004; Grubbs et al., 2013). All assay reagents used were made daily from either raw chemicals or frozen stock. Fluorescence of DCFH was detected at an excitation/emission wavelength of 480/530 nm using a BioTek Synergy H4 microplate reader and Gen 5 Software (BioTek U.S., Winooski, VT). Mitochondrial hydrogen peroxide production was calculated from a hydrogen peroxide standard curve based on fluorescence values of DCFH. Samples were plated in triplicate using a black 96-well plate. Twenty units of superoxide dismutase (Sigma-Aldrich, St. Louis, MO) were added to each sample well to convert superoxide into hydrogen peroxide for quantification. Either hydrogen peroxide standards or 90 µg of mitochondrial protein were added to each well, as well as 45 µL of an assay buffer (145-mM KCl, 30-mM HEPES, 5-mM KH₂PO₄, 3-mM MgCl₂, and 0.1-mM EGTA) containing 51-µM DCFH and 8 µM of either glutamate or succinate. Glutamate and succinate were provided as an energy source for complexes of the electron transport chain (I for glutamate, II for succinate). Plates were incubated at 37 °C and read at 0, 5, 10, 15, and 20 min after the addition of the energy substrate. The 5 readings were used to calculate the rate of hydrogen peroxide production per minute, expressed as µmol hydrogen peroxide produced/mg mitochondrial protein/min.

To assess oxidative damage to LM proteins, protein carbonyl concentrations were evaluated. Briefly, sarcoplasmic protein was extracted in an EDTA/phosphate buffer (50 mM sodium phosphate, pH 6.7, 1 mM EDTA) from snap frozen LM tissue and the resulting solubilized protein extracts were assayed for protein carbonyl concentrations

using a commercially available kit (Cayman Chemical, Ann Arbor, MI). Carbonyl content was expressed as nmol carbonyls per mg protein.

Skeletal Muscle Proteolysis

Activities of calpain and calpastatin were measured in fresh tissue extracts from the LM as described previously (Cruzen et al., 2013). Activities of µ- or m-calpain or calpastatin-containing fractions were determined using casein as a substrate, using a modification of the Koohmaraie method (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 protein (nitrogen × 6.25) was estimated using an Automated LECO Nitrogen Analyzer (LECO-TruSpec N, LECO Corp., St. Joseph, MI) to calculate activity on a total protein basis.

Activity of the 20S proteasome substrate was determined in sarcoplasmic protein extracted from frozen LM tissue samples using the Chemicon 20S Proteasome Activity Assay Kit (MilliporeSigma, Billerica, MA) according to manufacturer's instructions. Activity was determined via fluorescence of fluorophore 7-amino-4-methylcoumarin after cleavage from the labeled substrate LLVY-AMC using a BioTek Synergy H4 microplate reader and Gen 5 Software (BioTek U.S., Winooski, VT) and was expressed as units of activity per gram of extracted skeletal muscle protein.

Easily releasable myofilaments (ERMs) were quantified from frozen LM tissue using a procedure detailed by Neti et al. (2009) and run in duplicate. Protein was determined in both crude myofibrillar extracts and final ERMs pellets via BCA. Quantity of ERMs was reported as percent of ERMs per mg crude myofibrillar protein.

Liver and Muscle Metabolic Enzyme Activity

Activity of 3 key gluconeogenic enzymes (phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphatase (F1,6BP), and glucose 6-phosphatase (G6P)) was determined in triplicate with liver protein extracted in HEPES cell lysis buffer as described above. All assays were read on a Cytation Hybrid Multi-Mode Reader using Gen 5 software (BioTek Instruments Inc., Winooski, VT) and expressed on a per protein basis.

Briefly, PEPCK activity was determined by a modified method as described previously (Wimmer, 1988; Jin et al., 2004; Curry et al., 2018) via measuring the 2-step transformation of oxaloacetate to phosphoenolpyruvate and then to ATP. Luminometric production of ATP was measured via reaction with 90- μ L luciferase reagent following the ATP Determination Kit (ThermoFisher Scientific, Waltham, MA). Blank reactions were performed using samples incubated without oxaloacetate in reaction buffer I and without inosine-5'-triphosphate. All samples were corrected for their respective blanks, and assay results were expressed as μ mol of ATP produced/min/ μ g protein.

Fructose-1,6-bisphosphatase activity was determined as previously described (Curry et al., 2018) using protocol EC 3.1.3.11 (Sigma-Aldrich, St. Louis, MO). All samples were blank corrected, and activity was expressed as μ mol NADPH produced per minute per mg hepatic protein. Activity of G6P was quantified by measuring the release of phosphate from glucose-6-phosphate as described previously (Curry et al., 2018). Activity was expressed as mM Pi released per minute per mg protein.

Skeletal muscle hexokinase (HK; glycolytic metabolism) and citrate synthase (CS; oxidative metabolism) activities were determined from LM protein extracted in HEPES cell lysis buffer as described above using commercially available kits (Sigma-Aldrich, St. Louis, MO) according to the manufacture instructions. One unit of HK activity is the amount of enzyme that is required to generate 1.0 μ mol of NADH per minute at pH 8.0 at room temperature, and activity is reported as μ mol NADH generated/min/mg protein. Activity of CS is reported as μ mol/min/mg protein. The ratio of CS:HK was then calculated to determine to potential shifts in oxidative to glycolytic metabolism.

Statistical Analysis

Statistical analysis of all performance data and laboratory assays was performed in SAS 9.4 (SAS Institute Inc., Cary, NC). Data were analyzed using the MIXED procedure with a 2×2 factorial design to examine the fixed effects of line, MhLI challenge, and their interaction. However, the interaction of line and MhLI dual challenge was not significant ($P > 0.05$), for any of the growth performance parameters, nor for any other assay performed in this experiment, thus only main effects of genetic line and MhLI dual challenge will be presented in the results and discussion, and however tables will contain interaction P -values. The model included

age and start BW as covariates and litter nested within line as a random effect. All data are reported as LSmeans with a pooled standard error of the mean (SEM). Differences were considered significant when $P \leq 0.05$ and a tendency when $0.05 < P \leq 0.10$.

RESULTS

Response to Challenge, Growth Performance, and Blood Metabolites

No antibiotic treatments were required and there were no mortalities during the MhLI 21-d challenge. Confirmation of challenge was assessed by daily visual assessment, serum samples collected at dpi 21, and lesion scoring at necropsy on dpi 21. At dpi 21, control pigs were confirmed negative for both Mh and LI by sera antibody response and pathogen lesion scoring (Table 1). One control pig did test positive via IHC for LI in the ileum; however, this was likely a false positive as the pig did not test positive for any other diagnostic parameters. As expected, all MhLI pigs were confirmed positive for both pathogens, as indicated by antibody presence in sera for both pathogens at dpi 21 (Table 1). To further confirm pathogen impact, MhLI pigs had compatible gross lesions and were positive for both pathogens by IHC at 21 dpi, indicating successful inoculation (Table 1). However, gross lesions were limited, and 5 pigs that responded immunologically to Mh and LI did not have grossly visible lesions. Additionally, outward symptoms of disease such as coughing and loose stools were limited, indicating a subclinical challenge model, which may be clinically undetected in commercial settings but still have performance impact.

In this cohort of 24 pigs, the dual challenge reduced 21-d ADG, ADFI, and G:F in both genetic lines of MhLI-challenged pigs compared with their naïve counterparts, similar to that of the larger group of pigs (Helm et al., 2018). Challenge pigs had a 38% reduction in ADG, a 25% reduction in ADFI, and a 19% reduction in G:F compared with control pigs (Table 2, $P < 0.05$). As a result, MhLI pigs were 7 kg lighter ($P < 0.05$) than their uninfected counterparts at dpi 21. As expected, genetic line affected performance measures, with LRFI pigs consuming 15% less feed than their HRFI counterparts for equivalent gain; thus, LRFI pigs were 21% more efficient over the duration of the study ($P < 0.05$) for both MhLI and control pigs.

To determine whether MhLI alters the circulating metabolic profile of pigs, blood glucose,

Table 1. Confirmation of dual challenge with *Mycoplasma hyopneumoniae* (Mh) and *Lawsonia intracellularis* (LI) as determined by lesion scores and serum antibody responses at days post inoculation (dpi) 21

Item	Treatment			
	LRFI-control	HRFI-control	LRFI-MhLI	HRFI-MhLI
Lung with gross lesions ¹ , %	0.0	0.0	15.0	12.5
IHC for Mh ²	0/6	0/6	5/6	6/6
Macroscopic ileal lesions ²	0/6	0/6	3/6	4/6
IHC for LI – ileum ²	1/6	0/6	4/6	5/6
IHC for LI – colon ²	0/6	0/6	4/6	5/6
Sera Mh antibody response ^{2,3}	0/6	0/6	6/6	6/6
Sera LI antibody response ^{2,4}	0/6	0/6	6/6	6/6

Infection was confirmed via macroscopic gross lesions and immunohistochemistry (IHC) staining for pathogen either in the lung (Mh) or ileum and colon (LI), or via ELISA tests for the presence of antibodies against Mh or LI antigen in the serum.

¹Scores are reported as percent (%) of lung involved with lesions (0%–100%).

²Number of positive pigs out of the total number of pigs in each treatment group.

³*Mycoplasma hyopneumoniae* antibody response is reported as a sample to positive (S:P) ratio in which an S:P ratio greater than 0.40 is considered positive.

⁴*Lawsonia intracellularis* antibody response is recorded as percent inhibition, in which percent inhibition greater than 30% is considered positive.

Table 2. Growth performance of low (LRFI) and high (HRFI) residual feed intake pigs in control and *Mycoplasma hyopneumoniae* + *Lawsonia intracellularis* dual-challenged (MhLI) groups over a 21 d challenge period

Item	Treatment				SEM	P-value		
	LRFI-control	HRFI-control	LRFI-MHLI	HRFI-MHLI		MhLI	Line	MhLI x Line
Start BW, kg	60.8 ^a	73.1 ^c	62.7 ^{ab}	69.2 ^{ab}	2.423	0.508	0.017	0.080
End BW, kg	86.9 ^a	84.8 ^{ab}	79.8 ^{bc}	77.2 ^c	1.669	<0.001	0.250	0.870
Overall performance (dpi 0–21)								
ADG, kg/d	0.88 ^a	0.84 ^a	0.57 ^b	0.50 ^b	0.076	<0.001	0.543	0.745
ADFI, kg/d	2.72 ^{ab}	3.11 ^a	1.97 ^c	2.41 ^b	0.147	<0.001	0.028	0.846
G:F	0.32 ^a	0.27 ^a	0.28 ^a	0.20 ^b	0.023	0.012	0.021	0.358

Estimates are represented by 6 pigs per line x challenge.

^{a,b,c}Means with differing superscripts indicate a significant ($P < 0.05$) difference.

insulin, glucagon, BUN, and NEFA were measured in serum at dpi 21. None of the serum metabolites differed ($P > 0.10$) as a result of the MhLI dual challenge, nor due to RFI line (Table 3).

Skeletal Muscle Proteolysis, Oxidative Stress, and Metabolism

The skeletal muscle metabolic profile was assessed in LM at 21 dpi (Table 4). Calpain and calpastatin activity, ERMs percent, and 20S proteasome activity were measured to assess skeletal muscle degradation. Surprisingly, MhLI pigs had 26% greater ($P = 0.016$) in calpastatin I activity compared with control pigs, suggesting an inhibition of skeletal muscle proteolysis. However, activities of calpain and 20S proteasome did not differ ($P > 0.05$) between challenge groups. Genetic lines did differ in skeletal muscle proteolysis regardless of challenge, with LRFI pigs having lower activity of μ -calpain ($P = 0.012$) and thus lower calpain

to calpastatin activity ratio ($P = 0.033$) compared with HRFI pigs. Additionally, LRFI pigs tended to have a lower protein:RNA ratio ($P = 0.091$) compared with HRFI pigs, regardless of challenge status. Challenge pigs tended to have 50% greater ($P = 0.088$) mitochondrial ROS production when provided with glutamate as a substrate. Due to these results, protein carbonyls were quantified in the LM to determine whether increased ROS production resulted in increased oxidation of skeletal muscle protein. However, protein carbonyls did not differ ($P > 0.05$) as a result of challenge or genetic line.

Activities of HK and CS were determined to examine relative levels of glycolytic (HK) and oxidative (CS) metabolism in the LM (Table 4). Additionally, the ratio of CS:HK was calculated to determine whether there was a shift from oxidative to glycolytic metabolism due to MhLI dual challenge or divergent selection of RFI. Although the specific activities of HK and CS did not differ due to

Table 3. Serum metabolite parameters of low (LRFI) and high (HRFI) residual feed intake pigs in control and *Mycoplasma hyopneumoniae* + *Lawsonia intracellularis* dual-challenged (MhLI) groups at days post inoculation (dpi) 21

Item	Treatment				SEM	P-value		
	LRFI-control	HRFI-control	LRFI-MhLI	HRFI-MhLI		MhLI	Line	MhLI x Line
BUN ¹ , mg/dL	34.6	28.1	37.7	31.0	6.499	0.546	0.408	0.981
NEFA, mmol/liter	0.11	0.06	0.10	0.13	0.034	0.232	0.846	0.137
Glucose, μ mol/liter	21.9	51.9	31.5	40.9	11.58	0.933	0.185	0.278
Glucagon, pmol/liter	0.91	1.01	0.70	0.79	0.443	0.541	0.853	0.981
Insulin, pmol/liter	56.0	81.3	68.3	103.0	22.51	0.237	0.334	0.756

Estimates are represented by 6 pigs per line x challenge.

¹BUN = blood urea nitrogen.

Table 4. Characterization of skeletal muscle metabolic profile as determined in the LM from low (LRFI) and high (HRFI) residual feed intake pigs in control and *Mycoplasma hyopneumoniae* + *Lawsonia intracellularis* dual-challenged (MhLI) groups at days post inoculation (dpi) 21

Item	Treatment				SEM	P-value		
	LRFI-control	HRFI-control	LRFI-MhLI	HRFI-MhLI		MhLI	Line	MhLI x Line
μ -Calpain ¹	2.85 ^a	4.19 ^{bc}	3.17 ^{ab}	4.20 ^c	0.314	0.551	0.012	0.592
m-Calpain ¹	7.59	7.19	8.00	6.76	0.499	0.984	0.240	0.362
Calpastatin I ¹	3.34 ^a	2.50 ^a	4.21 ^b	3.63 ^b	0.530	0.016	0.387	0.739
Calpastatin II ¹	7.28	5.94	6.46	5.51	0.978	0.460	0.395	0.832
Total calpastatin ¹	10.69	8.36	10.72	9.10	1.256	0.722	0.260	0.757
μ -Calpain:calpastatin ²	0.27 ^a	0.51 ^{ab}	0.31 ^{ab}	0.48 ^b	0.064	0.849	0.033	0.600
20s proteasome ¹	2.32	12.80	2.45	7.24	4.796	0.368	0.309	0.394
ERMs ^{3,4}	0.199	0.222	0.234	0.251	0.064	0.461	0.813	0.948
ROS								
Glutamate ⁵	0.16	0.14	0.25	0.34	0.096	0.088	0.797	0.533
Succinate ⁶	0.11	0.12	0.14	0.13	0.022	0.392	0.945	0.664
Protein carbonyls ⁷	0.32	0.44	0.25	0.36	0.090	0.296	0.366	0.916
Hexokinase ⁸	468	963	683	523	253.2	0.558	0.603	0.129
Citrate Synthase ⁹	2003	2412	2029	1791	387.9	0.236	0.868	0.244
CS:HK, AU	4.84 ^a	6.06 ^{ab}	0.07 ^{bc}	-1.07 ^c	3.081	0.018	0.985	0.217
Protein:RNA	225	371	275	297	40.14	0.722	0.091	0.089

Estimates are represented by 6 pigs per line x challenge.

^{a,b,c}Means with differing superscripts indicate a significant ($P < 0.05$) difference.

¹Units of activity/g protein.

²Ratio of μ -calpain activity to total calpastatin activity.

³ERMs = easily releasable myofilaments.

⁴Percent ERMS/crude myofibrillar extract.

⁵Basal reactive oxygen species (ROS) production from isolated mitochondria as H₂O₂ produced, μ mol/min/mg mitochondrial protein, when glutamate is provided as a substrate.

⁶Basal ROS production from isolated mitochondria as H₂O₂ produced, μ mol/min/mg mitochondrial protein, when succinate is provided as a substrate.

⁷nmol protein carbonyls/mg sarcoplasmic protein.

⁸Hexokinase (HK) activity reported as μ mol/min/mg sarcoplasmic protein.

⁹Citrate Synthase (CS) activity reported as μ mol/min/mg sarcoplasmic protein.

MhLI or genetic line ($P > 0.10$), the ratio of CS:HK did differ ($P < 0.05$) due to MhLI dual challenge. Pigs inoculated with MhLI had a 25% lower ratio of CS:HK activity ($P = 0.018$) compared with control pigs, indicating a greater preference for glycolytic metabolism in the LM of MhLI pigs. There were no differences ($P > 0.05$) in LM metabolism due to RFI.

Liver Metabolic Profile

The MhLI dual challenge did not affect ($P > 0.05$) the activities of G6P or PEPCK; however, MhLI pigs demonstrated a 29% reduction ($P = 0.037$) in F16BP activity compared with control pigs (Table 5). Mitochondrial ROS production in

Table 5. Activity of key liver gluconeogenic enzymes and mitochondrial reactive oxygen species (ROS) production from low (LRFI) and high (HRFI) residual feed intake pigs in control and *Mycoplasma hyopneumoniae* + *Lawsonia intracellularis* dual-challenged (MhLI) groups at days post inoculation (dpi) 21

	Treatment					P-value		
Item	LRFI-control	HRFI-control	LRFI-MHLI	HRFI-MHLI	SEM	MHLI	Line	MHLI x Line
Gluconeogenesis								
G6P ¹	0.18	0.24	0.18	0.21	0.027	0.408	0.252	0.412
F16BP ²	1.91 ^a	2.05 ^a	1.21 ^b	1.62 ^{ab}	0.214	0.037	0.326	0.371
PEPCK ³	11.24	10.26	8.83	12.29	2.271	0.914	0.487	0.080
ROS								
Glutamate ⁴	0.46 ^a	0.59 ^{ab}	0.73 ^b	0.70 ^{ab}	0.125	0.034	0.800	0.339
Succinate ⁵	0.65 ^a	0.88 ^{ab}	1.09 ^b	0.99 ^b	0.087	0.002	0.552	0.053
Protein:RNA	58.0	64.4	50.9	61.5	5.974	0.321	0.239	0.684

Estimates are represented by 6 pigs per line x challenge.

^{a,b,c}Means with differing superscripts indicate a significant ($P < 0.05$) difference.

¹Pi released, mM/min/mg protein.

²NADPH produced, $\mu\text{mol/min/mg}$ protein.

³ATP produced, $\mu\text{M/min}/\mu\text{g}$ protein.

⁴Basal ROS production from isolated mitochondria as H_2O_2 produced, $\mu\text{mol/min/mg}$ mitochondrial protein when glutamate is provided as a substrate.

⁵Basal ROS production from isolated mitochondria as H_2O_2 produced, $\mu\text{mol/min/mg}$ mitochondrial protein when succinate is provided as a substrate.

the liver was also affected by MhLI dual challenge. ROS production was greater in MhLI pigs when glutamate (27%) or succinate (26%) was provided as energy substrates ($P < 0.05$). Genetic selection for LRFI did not alter gluconeogenic enzyme activity nor mitochondrial ROS production ($P > 0.05$).

DISCUSSION

Health challenges negatively affect pig performance, FE, and profitability of pork production year-round (Williams et al., 1997; Curry et al., 2017; Schweer et al., 2017). Based on the resource allocation theory (Beilharz et al., 1993; Rauw, 2007), animals have a finite set of resources that must be allocated to growth, maintenance, and immune function. Differences in how nutrient resources are allocated may be contributing factors to differences in FE and how animals resolve health challenges. We have previously reported that this MhLI dual challenge resulted in a 7% reduction in G:F and a 17% reduction in lean accretion and ADG compared with control pigs over a 42-d challenge period (Helm et al., 2018). Therefore, the objective of this study was to investigate mechanisms associated with the reductions in performance resulting from this MhLI challenge by evaluating changes in postabsorptive metabolic adaptation and resource allocation in pigs divergently selected for RFI. To evaluate this objective, a subset of pigs was necropsied at dpi 21.

In 4 separate experiments utilizing genetic lines of pigs that were divergently selected for high vs. low RFI, the more FE pigs (low RFI) did not demonstrate any disadvantage when presented with inflammatory (Rakhshandeh et al., 2012; Merlot et al., 2016) or pathogen challenges (Dunkelberger et al., 2015; Helm et al., 2018). In fact, when using poor hygiene as a model to stimulate the immune system, LRFI pigs had less affected growth rates than their HRFI counterparts (Chatelet et al., 2018). However, in the current study, there were no significant interactions between line and challenge for any of the assays performed.

Genetic selection for LRFI has resulted in pigs with 10%–20% greater FE in the grow-finish phase of production compared with HRFI-selected counterparts (Boddicker et al., 2011; Gilbert et al., 2017). In agreement with these findings, LRFI pigs in this study had approximately 20% greater FE compared with their HRFI counterparts. Previous experiments using RFI selection lines have also reported that LRFI pigs have more efficient protein accretion (Le Naou et al., 2012; Harris et al., 2013), coupled with a decrease in markers of skeletal muscle protein degradation (Smith et al., 2011; Cruzen et al., 2013) compared with their HRFI counterparts. The current work supports these findings, as LRFI pigs had a lower calpain to calpastatin ratio compared with their HRFI counterparts. Furthermore, LRFI pigs tended to have a lower protein to RNA ratio in their LM compared with HRFI pigs, which could

indicate a slower rate of protein turnover (Radley-Crabb et al., 2014). This notion of a greater rate of muscle protein turnover in HRFI pigs compared with LRFI pigs has been previously suggested by Cruzen et al. (2013) and may also contribute to the greater energy expenditure observed in HRFI pigs (Barea et al., 2010).

Greater energy expenditure and higher protein turnover may be partially explained by electron leakage and ROS production by the mitochondria. Oxidative damage from mitochondrial electron leakage and ROS production forces cells to repair or phagocytose damaged proteins (as well as lipids and DNA), resulting in further energy expenditure and increased proteolysis (Bottje and Carstens, 2009). Greater skeletal muscle mitochondrial ROS production has been associated with an increase in protein carbonylation in the same tissue of broilers of low FE (Bottje et al., 2006). In pigs, selection for LRFI has been demonstrated to reduce the amount of ROS leakage from the electron transport chain in skeletal muscle and liver (Grubbs et al., 2013). However, in the current study and irrespective of the pathogen challenge, we observed no significant differences in ROS production between the RFI lines from mitochondria isolated from LM and liver tissue. Although ROS production and mitochondrial efficiency have been explored with regards to FE differences (Bottje et al., 2006; Bottje and Carstens, 2009; Grubbs et al., 2013), its role in pathogenic infections is poorly defined.

Pathogen infections from swine influenza virus A (SVA) and Mh can increase oxidative stress in affected pigs, presumably because of excessive formation and leakage of ROS from the mitochondrial electron transport chain (Turrens, 2003; Deblanc et al., 2013). Thus, we hypothesized that the MhLI pigs would have greater mitochondrial ROS production in the LM and liver that would result in increased oxidative stress. In support of this, we reported the MhLI pigs to have greater mitochondrial ROS production in both the LM and liver, indicating a greater level of electron leakage and mitochondrial dysfunction. However, this did not translate into significant increases in LM concentrations of oxidized proteins (protein carbonyls) due to the MhLI dual challenge. These data suggest that the increase in ROS production was either not severe enough to induce oxidative damage or that antioxidant machinery was sufficient to clear excess ROS.

In a larger cohort of pigs, we have reported that this MhLI challenge significantly reduced ADG, lean accretion, and FE over a 42-d period (Helm et al., 2018). During immune stimulation, which is often accompanied by reduced feed intake, resources are allocated to generate and fuel the immune response, decreasing capacity for tissue accretion (Johnson, 2012; Curry et al., 2017; Schweer et al., 2017; Helm et al., 2018). Appetite reductions during pathogen and inflammatory challenges have been recognized as an adaptive host response to facilitate disease resistance and recovery (Johnson, 2002). This host response is orchestrated by proinflammatory cytokine signaling that targets the brain and other peripheral tissues. The dual respiratory and enteric live pathogen challenge utilized in our study resulted in a significant reduction in feed intake, which may describe a significant proportion of the attenuated pig performance, particularly BW gain. However, we also reported a 19% reduction in G:F, suggesting not all can be described by feed intake reductions.

To provide AA and energy to produce and support immune response components such as cytokines and acute phase proteins during a time of attenuated feed intake, skeletal muscle proteolysis may be enhanced (Reeds et al., 1994). In rodent models of sepsis, skeletal muscle proteolysis, especially that of myofibrillar proteins, occurred at an accelerated rate (Hasselgren et al., 1989). In piglets, caspase-3 mediated increases in skeletal muscle protein degradation are induced by lipopolysaccharide (LPS) and its associated inflammation (Orellana et al., 2012). Additionally, our group has reported increases in specific activity of μ -calpain, indicative of increased proteolysis, in nursery pigs challenged with Porcine Reproductive and Respiratory Syndrome virus (PRRSV) and a PRRSV/porcine epidemic diarrhea virus (PEDV) co-infection (Lonergan et al., 2015). However, based on the results of the current study, there was minimal evidence (i.e., ERMs, 20S proteasome, and calpain activities) to suggest that skeletal muscle proteolysis was enhanced during MhLI dual challenge. In fact, MhLI pigs demonstrated enhanced activity of calpastatin I, the endogenous inhibitor of proteolytic calpains, suggesting that skeletal muscle proteolysis may be somewhat inhibited during MhLI dual challenge.

Although contrary to our original hypothesis, the results of the current study are supported by Curry et al. (2018) who observed similar levels of abundance of calpains and a tendency for greater calpastatin abundance in PEDV-inoculated nursery

pigs at dpi 20. One possible explanation for these observations is that during times of moderate and localized pathogen stress, protein turnover may slow in an attempt to conserve body resources and energy expenditure, as skeletal muscle proteolysis is an energetically costly endeavor (Herd and Arthur, 2009). Additionally, since acute phase proteins are initially released early in the immune response (Reeds et al., 1994), the timing of LM sample collection in the current experiment may have been too late to capture the initial amino acid nutrient mobilization for the acute phase protein response. Furthermore, fatty acids may be providing the majority of the energy needed to fuel the immune system or less glucose may be utilized for lipogenesis, as adipose accretion was 17% lower in MhLI pigs (Helm et al., 2018).

Metabolic adaptation to inflammatory and pathogenic stress and the metabolic switch from oxidative to glycolytic metabolism appears critical for appropriate immune cell activation and function (Calder et al., 2007; O'Neill et al., 2016). Glycolysis is a quick ATP generator and is preferred to maintain the immune response during times of stress (Tannahill et al., 2013; O'Neill and Pearce, 2016). However, metabolic flexibility in tissues such as skeletal muscle during immune stimulation has been poorly defined. In the LM, MhLI pigs had a lower CS to HK ratio, signifying a greater preference for glycolytic metabolism compared with their control counterparts. A shift towards glycolytic metabolism suggests a greater glucose demand, which is known to occur during immune system stimulation (Meszaros et al., 1991; Calder et al., 2007; Kvidera et al., 2017). To support this demand, the use of dietary and body tissue AA and fatty acids is repartitioned towards the production of glucose as a source of ATP (Klasing and Johnstone, 1991). As the liver is a major producer of glucose via gluconeogenesis, we examined the activity of key liver gluconeogenic enzymes. However, the current study indicated that the MhLI dual challenge had minimal effect on the activities of gluconeogenic enzymes in the liver; in fact, MhLI pigs had decreased activity of F16BP compared with the unchallenged control pigs. Although contrary to our hypothesis, these data are similar to what we have reported in pigs during PEDV challenge (Curry et al., 2018). The serological hormone and metabolite profile of the MhLI pigs reported herein also corroborate the lack of gluconeogenesis upregulation. Although not examined, this suggests that tissue glycogen or lipid stores may have been sufficient for energy generation during the current MhLI dual challenge.

In agreement with previous work (Helm et al., 2018), the data herein suggest that genetic selection for divergence in RFI did not alter pig performance during a MhLI dual challenge. Additionally, irrespective of RFI line, this dual enteric and respiratory subclinical challenge modulated postabsorptive metabolic functions in growing pigs, as evident by increased ROS production, and metabolic switching. However, contrary to our hypothesis, markers of skeletal muscle proteolysis and liver gluconeogenesis were not different at dpi 21 in the MhLI compared with the control pigs. Collectively, these data suggest that this subclinical MhLI dual pathogen challenge reduces feed intake, ADG, and FE irrespective of genetic selection for RFI. These pigs appear to meet the energy demands of this challenge without reliance on the up-regulation of skeletal muscle proteolysis and liver gluconeogenesis.

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