Novel observations of peroxiredoxin-2 profile and protein oxidation in skeletal muscle from pigs of differing residual feed intake and health status

by

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The student author, whose presentation of the scholarship within was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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NOMENCLATURE

2D-DIGE 2-Dimensional Difference in Gel Electrophoresis
ADFI Average Daily Feed Intake
ADG Average Daily Gain
AMPER Ammonium Persulfate
Dpi Days Post Infection
DNPH 2, 4-dinitrophenylhydrazine
GAPDH Glyceraldehyde 3-Phosphate Dehydrogenase
G: F Gain per Feed
HC High RFI Control
HI High RFI Inoculated
H₂O₂ Hydrogen Peroxide
HRP Horseradish Peroxidase
INRA Institute National de la Recherche Agronomique
IS Infection Status
ISU Iowa State University
iTRAQ Isobaric Tag for Relative and Absolute Quantification
LC Low RFI Control
LC-MS Liquid Chromatography Mass Spectroscopy
Li *Lawsonia intracellularis*
LI Low RFI Inoculated
Mh *Mycoplasma hyopneumoniae*
MhLi *Mycoplasma hyopneumoniae and Lawsonia intracellularis*
NFDM  Non-fat Dry Milk
O$_2$  Oxygen
O$_2^-$  Superoxide Anion
OH$^-$  Hydroxyl Radical
PAGE  Polyacrylamide Gel Electrophoresis
PHS  Pulmonary Hypertension Syndrome
Prdx  Peroxiredoxin
Prdx-2  Peroxiredoxin-2
PRRS  Porcine Reproductive and Respiratory Syndrome
PVDF  Polyvinylidene Difluoride
REF  Reference
RFI  Residual Feed Intake
ROS  Reactive Oxygen Species
SDS  Sodium Dodecyl Sulfate
S$_p$  Peroxidatic Cysteine
S$_r$  Reducing or Resolving Cysteine
-SH  Cysteine
-SOH  Sulfenic Acid
-SO$_2$H  Sulfinic Acid
-SO$_3$H  Sulfonic Acid
TEMED  Tetramethylenediamine
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ABSTRACT

The objective of this thesis was to determine the impact of health challenge in the form of a dual respiratory and enteric bacterial health challenge on the antioxidant protein peroxiredoxin-2 (Prdx-2) and protein oxidation in the skeletal muscle of pigs selected for differing residual feed intake (RFI), a measure of feed efficiency. It was hypothesized that differences would exist in Prdx-2’s profile based on RFI line and infection status (IS) and that high RFI (less efficient) and health challenged pigs would present a higher degree of protein oxidation due to increased oxidative stress.

Barrows (50±7 kg, total n=24) divergently selected for RFI from the 11th generation of the ISU RFI Project were used for this study. To induce a respiratory and enteric health challenge, half of the pigs (n=6 / RFI line) were inoculated with Mycoplasma hyopneumoniae (Mh) and Lawsonia intracellularis (Li) on 0 days post infection (dpi). Uninoculated pigs in a separate room served as controls (n=6 / RFI line). Pig ADG, ADFI, and G: F were calculated during a 21 day acclimation period (dpi -21 to dpi 0) and from inoculation to projected peak infection (dpi 0 to dpi 21). At dpi 21 all pigs were necropsied, and longissimus skeletal muscle samples were immediately collected for analysis. Protein oxidation, various forms of Prdx-2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) content was measured on longissimus skeletal muscle sarcoplasmic protein.

No significant differences were seen in protein carbonylation, but low RFI pigs showed an increase in disulfide bound creatine kinase and GAPDH in the sarcoplasmic protein of the longissimus skeletal muscle. Further analysis showed MhLi pigs had a greater amount of oxidized GAPDH (P=0.017). Significant differences were seen based
on both RFI line and infection status for non-reducing gel Prdx-2 suggesting differences in some posttranslational modifications. There was an increase in total Prdx-2 (P=0.035) and Prdx-2 decamer (P=0.0007) in high RFI pigs while hyperoxidized peroxiredoxin relative to Prdx-2 was increased in low RFI pigs (P=0.028).

Pigs divergently selected for RFI may differ in antioxidant protein profile and oxidative stress response. High RFI pigs’ reduced efficiency appears to be the result of increased Prdx-2 production, resulting greater pool of reduced Prdx-2 to combat oxidative stress challenges.
CHAPTER 1. GENERAL INTRODUCTION

Oxidative stress in living tissues can be defined as a disturbance in prooxidant-antioxidant balance when the balance tips towards prooxidants (Sies et al., 2017). Oxidative stress and the reactive oxygen species (ROS) that contribute to it have a significant negative impact on livestock production efficiency by damaging cellular components including proteins, lipids, and DNA. Oxidized proteins may have reduced solubility, impaired function, and structural damage (Vansteenhouse, 1987). Nutrients that would otherwise be used for efficient growth are then allocated towards repairing damaged tissue. Increased ROS and the resulting protein oxidation have been implicated in multiple factors influencing pork production efficiency including feed efficiency and disease challenge response.

Pigs divergently selected for residual feed intake (RFI) represent a useful model for comparing animals of differing feed efficiency. Less efficient, high RFI pigs have demonstrated greater ROS production and increased electron leakage from the mitochondria of skeletal muscle (Grubbs et al., 2013). This may partially explain the difference in feed efficiency between high and low RFI pigs.

Health challenges can increase oxidative stress in pigs, possibly due to increased electron leakage and ROS production in the mitochondria. *Mycoplasma hyopneumoniae* and *Lawsonia intracellularis* are two bacterial pathogens commonly found in US commercial swine production (USDA, 2016). Though limited in terms of mortality, these pathogens can lead to a significant reduction in growth performance (Helm et al., 2018a).

Antioxidant proteins such as peroxiredoxin-2 (Prdx-2) can help to mitigate damage caused by oxidative stress by converting ROS to less reactive molecules. Peroxiredoxin-2 is an antioxidant enzyme which mitigates oxidative stress by converting hydrogen peroxide and
other hydroperoxides into more stable compounds such as water. Peroxiredoxin-2 can exist in multiple oxidation states and structural conformations which affect its antioxidant activity. In top down proteomics experiments, Prdx-2 profile has been seen to be altered in response to a wide range of oxidative stress challenges including heat stress (Cruzen et al., 2015), LPS immune challenge (Outhouse et al., 2015), physiological stress (Marco-Ramell et al., 2016), and health challenge (Genini et al., 2012). However, to our knowledge, no study has ever focused on comparing the various Prdx-2 oxidation states and structures in animals of differing feed efficiency or under health challenge. By better understanding the role of antioxidant proteins such as Prdx-2 in health challenge response and feed efficiency, we may be better able to manage livestock towards increased production efficiency, without a dramatic increase in inputs.

Thus, the objective of this thesis was to determine the impact of the health challenge resulting from dual infection with Mycoplasma hyopneumoniae and Lawsonia intracellularis on Prdx-2 protein profile and protein oxidation in the skeletal muscle of pigs divergently selected for RFI. It was hypothesized that differences would exist in the antioxidant protein Prdx-2’s profile based on RFI line and infection status and that greater protein oxidation would be seen in high RFI and health challenged pigs due to greater oxidative stress.

Literature Cited


CHAPTER 2. REVIEW OF LITERATURE

Introduction

The impact of disease challenges on the efficiency of meat animal production is an immensely important aspect of livestock production. Billions of dollars in productivity are lost annually due to livestock diseases in the United States (Holtkamp et al., 2013). Considering this, understanding the role of disease in production efficiency and how it influences feed consumption is invaluable in improving production efficiencies. By improving the overall efficiency of livestock production, we will be better able to meet future demand without negative impacts.

Demand for animal protein and fresh meat products is anticipated to increase dramatically in the coming years. An overall increase in the world population, increased wealth in the developing world, and the trend for consumers to consume greater quantities of protein as incomes increase are all expected lead to an increase in global consumption, especially in developing countries. Red meat is an invaluable, nutrient dense source of protein, iron, zinc, B-vitamins, folate, n-3 polyunsaturated fats, and conjugated linoleic acids in addition to preventing health issues such as nutrient deficiency and anemia (McAfee et al., 2010). Because of this, consumption of red meat will play a critical role in combating undernutrition and reducing the impact of diet related disease in the developing world in the future (McNeill and Van Elswyk 2012). The challenge of meeting this demand will be compounded by increasingly limited inputs including land, feed, and labor.

Producing pork, one of the most widely consumed meats around the globe, as efficiently as possible is imperative. By increasing our understanding of efficiency of muscle growth at the molecular level and leveraging this knowledge to the best of our abilities, we
will be able to increase production without a dramatic increase in inputs to feed a world hungry for animal protein.

**Mycoplasma hyopneumoniae and Lawsonia intracellularis**

Reduced live animal performance due to oxidative stress induced by health challenge can have a major impact on the efficiency and profitability of modern pork production by increasing the time and resources needed to produce a given amount of product. *Mycoplasma hyopneumoniae* and *Lawsonia intracellularis* are two pathogenic bacteria that negatively impact pork production by initiating subclinical disease challenges, resulting in decreased growth performance but limited observable symptoms. *Mycoplasma hyopneumoniae* is the primary pathogenic agent responsible for enzootic pneumonia leading to respiratory disease, coughing, and diminished productivity in pigs (Straw et al., 2006). *Lawsonia intracellularis* results in the thickening of the intestinal mucosa lining of the small intestine and colon leading to porcine proliferative enteropathy (also known as ileitis). Infected pigs may suffer lesions in addition to thickening in the intestines, diarrhea, reduced feed intake, and reduced gain (Straw et al., 2006). Both of these disease issues are prevalent throughout the United States with the United States Department of Agriculture estimating that *Mycoplasma hyopneumoniae* was present in 31.2% of breeding herds and 58.8% of grower/finisher operations, while *Lawsonia intracellularis* was found to be a problem in 18.7% of breeding herds and 28.7% of grower/finishers within the United States (USDA 2016). Both disease circumstances result in decreased productivity through reduced average daily feed intake and average daily gain (Straw et al., 2006).
Residual Feed Intake and Biological Efficiency

Residual Feed Intake (RFI) is a measure of feed efficiency. In the Iowa State University RFI Project pig model, RFI takes into account the difference between the amount of feed an animal consumes compared to its anticipated feed consumption based on average daily weight gain and backfat. Pigs with a higher residual feed intake must consume a greater amount of feed in order to gain the same amount of weight and attain the same depth of backfat as their low residual feed intake counterparts (Fig. 2.1) (Boddicker et al., 2011). The concept of RFI was first discussed by Koch et al. (1963) with the idea of measuring feed efficiency in beef cattle as a residual difference from an anticipated feed consumption when production traits are statistically fixed. Considering this independence from production traits, RFI can be seen as a measure of feed efficiency that represents true variation in metabolic efficiency or biological efficiency (Koch et al., 1963).

Various biological factors are thought to have an impact on variation in residual feed intake in beef according to Richardson and Herd (2004) including feeding patterns (2%), body composition (5%), the heat increment of fermentation (9%), activity (10%), digestibility (10%), and the metabolic factors of protein turnover, tissue metabolism, and stress (37%). The metabolic factors of protein turnover, tissue metabolism, and stress remain the most poorly understood of these attributes. Pigs divergently selected for RFI through the Iowa State University Residual Feed Intake Project provide a unique opportunity to examine the impact of these traits on animal efficiency. By examining differences at the molecular level between high and low RFI groups of pigs, progress may be made towards increasing the efficiency of swine and livestock production and in doing so increase production for a given quantity of resources. This information may also have a value in enabling us to better manage disease and oxidative stress challenges in pork production.
Models for RFI in commercial swine have been developed at two research institutions, with RFI calculations based on average daily gain and backfat composition for both models. The Institute National de la Recherche Agronomique (INRA) in France has continuously divergently selected pigs for high and low RFI (Lefaucheur et al., 2011). Pigs selected for RFI through the Iowa State University (ISU) RFI Project were selected for low RFI (improved feed efficiency) alongside a control group randomly selected for four generations, followed by divergent selection for high and low RFI for six generations, resulting in ten generations of selection. Following the tenth generation of selection more efficient low RFI were seen to consume an average of 201.4 g less of feed per day for the same amount of gain (Fig. 2.2) (Hsu et al., 2015).

Extensive research has been conducted on the meat quality, carcass composition, and live animal characteristics in pigs divergently selected for RFI over multiple generations. Quality and carcass composition traits that have been examined include ultimate pH, color (L values), instrumental tenderness (star probe), calpain activity, drip loss, protein degradation, fiber type, fat and loin depth, marbling, and moisture content (Lefaucheur et al., 2011; Faure et al., 2013; Smith et al., 2011; Arkfeld et al., 2015). Across studies, high RFI pigs have been seen to have significantly greater fat depth and higher ultimate pH values, while low RFI pigs have presented carcasses with significantly deeper loin eyes and lighter lean color based on Hunter L color values. Based on these results, selection for RFI is thought to play a role in quality attributes of fresh meat.

Implications for live animal health have also been examined in pigs divergently selected for RFI. Grubbs et al. (2013) found that selecting for low RFI resulted in reduced reactive oxygen species in skeletal muscle mitochondria compared to that seen in high RFI
pigs. They also noted a positive correlation between RFI and hydrogen peroxide production for mitochondria isolated from both red and white muscle tissue. Research has also shown the mitochondrial protein profile from the muscle of pigs selected for low RFI had increases in some antioxidant proteins including catalase and heat shock proteins. There were also potential modifications of metabolic processes related to oxidative stress, metabolism, and tissue repair through alterations in the quantity of certain proteins within the mitochondrial protein profile, including those related to the citric acid cycle and heat stress response (Grubbs et al., 2013). This may help to explain some of the difference in efficiency between the two RFI lines. It is possible that high RFI pigs are required to expend more energy towards combatting oxidation, maintaining redox homeostasis, and repairing tissue damage caused by greater oxidant load, compared to low RFI counterparts.

**Residual Feed Intake and Reactive Oxygen Species**

Reactive oxygen species production has been seen to play a role in differences in biological efficiency between high and low RFI animals. Reactive oxygen species was first measured in a livestock species in relation to feed efficiency by Bottje et al. (2006). Since then multiple studies have been carried out to further elucidate the impact of ROS production from the mitochondria on RFI in chicken, beef, and swine livestock species.

Early experiments focused on ROS production in less efficient broiler chickens with pulmonary hypertension syndrome (PHS) (Iqbal et al., 2001b). Pulmonary Hypertension Syndrome is a respiratory condition in poultry caused by factors including cardiopulmonary insufficiency, mitochondrial dysfunction, and oxidative stress (Iqbal et al., 2001a). When compared with their contemporaries, PHS broilers are less efficient and have greater mitochondrial ROS generation and electron leakage in lung mitochondria (Iqbal et al., 2001a). In another experiment, Bottje et al. (2002) compared the most and least feed
efficient broiler chickens from the same genetic line and found that the less efficient birds exhibited more electron leakage and ROS production in the mitochondria of muscle, small intestine, and liver tissue. This increase in ROS production from a diverse set of tissues from less feed efficient broiler chickens suggests that animals with lower feed efficiency may be less efficient at the biological/molecular level due in part to increased oxidative stress.

The relationship between ROS generation and feed efficiency has received a lesser amount of study in beef cattle. Kolath et al. (2006) compared the mitochondrial function of high and low RFI Angus steers that had been sourced from the same farm and shared a sire in order to reduce variation. In this study, more efficient cattle were actually found to produce greater total quantities of hydrogen peroxide in longissimus skeletal muscle mitochondria. However, when hydrogen peroxide production was expressed as a ratio to mitochondrial respiration rate, no difference was seen, suggesting that mitochondrial respiration rate in addition to electron leakage and mitochondrial efficiency play a role in efficiency in cattle. This suggests that the relationship between feed efficiency and mitochondrial function varies between different livestock species.

Grubbs et al. (2013) examined differences in mitochondrial ROS production in pigs divergently selected for RFI following the eighth generation of selection for the Iowa State University Residual Feed Intake Project. Through this work, they reported that ROS production was greater in the mitochondria of both the red and white portions of semitendinosus muscle from less efficient, high RFI animals. A positive correlation was also seen between RFI and ROS production in the longissimus muscle of divergently selected pigs. Various proteins playing a role in mitochondrial function and efficiency were found to differ in quantity between high and low RFI pigs in muscle and liver samples (Grubbs et al.,
Proteins related to oxidative stress response including catalase, heat shock protein 60, and heat shock protein 70 were also found to differ in muscle and liver samples from divergently selected RFI pigs (Grubbs et al., 2013). These data as a whole support the hypothesis that selection for RFI impacts mitochondrial function and the response to oxidative stress in pigs. This difference in mitochondrial electron leakage may contribute to feed efficiency differences by shifting energy from lean growth towards cellular repair and overall upkeep or vice versa. By gaining greater insight into the role of ROS production and the response it triggers within the body, we may be better able to increase efficiency in swine production.

**Figure 2.1** Residual feed intake (RFI) is calculated based on expected average daily gain and back fat deposition. More efficient pigs that eat less than expected have low residual feed intake, while their less efficient, high residual feed intake counterparts eat more than expected for the same amount of body weight gain.
Figure 2.2 Graphical depiction of the effect of selection for the Iowa State University model for residual feed intake (RFI). Low RFI selection was conducted over 10 generations, while selection for high RFI was conducted over 6 generations following 4 generations of random selection. At the 10th generation, the LRFI line had 241 g/d lower RFI (Hsu et al., 2015).

**Reactive Oxygen Species**

Reactive oxygen species are the primary causative agent of the oxidative process and oxidative stress in living tissues. Oxidative stress in living tissue can be defined as a disturbance in the prooxidant-antioxidant balance in favor of prooxidants (Sies, Berndt, and Jones 2017). Reactive oxygen species can be easily separated into two distinct groups, free radical molecules and nonradical molecules. Free radical molecules possess an odd number of electrons and include such compounds as the superoxide anion, nitric oxide, hydroxyl, peroxyl, and alkoxy radicals (Vansteenhouse 1987). Non-radical ROS comprise a diverse group of chemicals including hydrogen peroxide, organic peroxides, aldehydes, ozone, and molecular oxygen. As a group these oxidative molecules have distinct chemical properties in terms of reactivity and half-life. For example, a hydroxyl free radical would indiscriminately
react with oxidizable biological molecules, whereas hydrogen peroxide preferentially binds with free cysteine groups. A hydroxyl radical’s half-life is also much shorter than that of a more stable hydrogen peroxide (Bachi, Dalle-Donne, and Scaloni 2015).

Reactive oxygen species can be produced by a variety of processes within the cell. Reactive oxygen species generation involves the complete reduction of molecular oxygen (O₂) to two molecules of water (H₂O). This process requires the transfer of four electrons, however, oxygen is only able to accept one electron at a time, leading to the release of radicals (Bachi, Dalle-Donne, and Scaloni 2015). Though reactive oxygen can be produced within the cell in many ways, three cellular systems can be credited with the majority of ROS production within the cellular environment. These include: (1) electron leakage from cellular respiration within the mitochondrial membrane during oxidative phosphorylation, (2) hydrogen peroxide production by monoamine oxidase during oxidative deamination of biogenic amines within the outer mitochondrial membrane, and (3) oxygen metabolizing reactions carried out by multiple enzymes through metabolic processes such as the citric acid cycle including xanthine oxidase, nitric oxide synthase, and various other oxidase enzymes. Reactive oxygen species can also be introduced by external stimuli including osmotic stress, ultraviolet radiation, heavy metals, metabolic distress, and through disease challenges (Bachi, Dalle-Donne, and Scaloni, 2015). Reactive oxygen species have been implicated in a wide variety of disease instances in both humans and livestock species. Amongst the diseases ROS have been seen to play a role in are renal diseases (Liebert and López-Novoa, 2002), neurodegenerative diseases (Crichton et al., 2007), cancer (Kim et al., 2007), malaria (Kawazu et al., 2008), apoptosis regulation (Zhou et al., 2000), and many others (Martínez-Cayuela, 1995).
It is also important to remember that though we may be tempted to view ROS as a whole when considering their implication in living tissues, we must appreciate that all reactive oxygen species do not react in the same way as they have different reactivities with antioxidants, are produced by several processes, and may impact different proteins with a wide range of functionalities including glycolytic enzymes, structural proteins, regulatory proteins, heat shock proteins, and proteases. This is further complicated by the fact that the origin of an oxidant may also affect its impact due to membrane permeability. For instance, whether a cell generates oxidants extracellularly or in a compartmentalized area such as the mitochondria will affect the impact on a cell (Winterbourn and Hampton, 2008).

**Reactive Oxygen Species Impact on Tissue**

According to Xiong et al. (2000) many components of tissue including oxidizable lipids, iron-based heme molecules (such as myoglobin and hemoglobin), transitional metal ions, and various oxidative enzymes can serve as catalysts to increase production of ROS. These free radicals can have a positive impact within an organism, for instance by damaging or destroying microbial pathogens within the immune system. However, the presence of these reactive compounds is also likely to cause damage to host tissue components including lipids, proteins, carbohydrates, and RNA or DNA molecules if left unchecked. Membrane bound unsaturated lipids may have altered permeability following ROS exposure, resulting in loss of structure and function. Moreover, products of lipid oxidation including malondialdehyde and lipid peroxy radicals may be toxic and further damage tissue. In proteins, ROS may cause a variety of problems including reducing solubility, impairing function, and structure damaging crosslinking. DNA and RNA may undergo nucleic acid base modification and scission in response of ROS exposure, resulting in cell mutation and death (Vansteenhouse, 1987).
Reactive Oxygen Species as Signaling Molecules

It would be easy to look at ROS impact on living systems solely in terms of their negative impact and how their presence is combatted through redox systems. However, this may be oversimplified according to (Winterbourn and Hampton, 2008). In aerobic living systems, cells are constantly exposed to reactive oxygen species (ROS) through normal cellular processes. Cells use a combination of antioxidant defenses to combat the presence of ROS and maintain redox homeostasis. However, minute changes in ROS presence and redox homeostasis can act to initiate signaling responses and in turn trigger a response to oxidative stress. Furthermore, oxidants may serve other roles, for instance to activate or inactivate transcription factors, membrane channels, and metabolic enzymes and to alter calcium dependent and phosphorylation signaling pathways making their role more complex in living systems (Winterbourn and Hampton, 2008). The fact that ROS have both potential negative impacts on tissue as well as beneficial signaling roles highlights the complex role these molecules play in tissues. With this in mind it is worth pointing out that the most relevant ROS signaling molecules is one of the less reactive species: hydrogen peroxide (Sies 2017).

In recent years hydrogen peroxide has emerged as a signaling molecule in organisms with a significant role.

Hydrogen Peroxide

Hydrogen peroxide (H₂O₂) is a well-known ROS molecule that plays a role in oxidative stress as well as cell signaling in animal tissues. Its role is furthered by its ability to easily diffuse through cell membranes compared to other ROS impaired by their molecular charges (Sies 2017).

Numerous electron reduction reactions are known to produce H₂O₂ with systems in the cell. These include NADPH oxidases and the mitochondrias’ electron transport chain as
major contributors. In total, thirty-one H$_2$O$_2$ generating enzymes are known to be present in human tissues (Go, Chandler, and Jones, 2015). It also bears mentioning that highly reactive ROS species such as superoxide anion (O$_2^-$) are readily converted to less reactive H$_2$O$_2$ in the cellular environment by antioxidants such as superoxide dismutase (Lambeth et al., 2004). This process catalyzed by superoxide dismutase enables ROS to diffuse away from an origin site more easily due to greater H$_2$O$_2$ membrane permeability.

Hydrogen peroxides may be removed from a cellular system by a wide variety of enzyme-initiated reactions. The enzymes catalase, peroxiredoxin, and glutathione peroxidase are highly reactive with peroxides, with significantly greater rate constants for converting H$_2$O$_2$ to water, and carry out the majority of H$_2$O$_2$ removal. Catalase carries out a dismutation reaction, regenerating one oxygen molecule, while the two latter enzymes reduce H$_2$O$_2$ to a water molecule. Other water producing enzymes reduce H$_2$O$_2$ to a lesser extent including myeloperoxidase, eosinophil peroxidase, and lactoperoxidase (Sies 2017).

Hydrogen peroxide is best recognized as a ROS responsible for contributing to oxidative stress and tissue oxidation. Though H$_2$O$_2$ is lowly reactive on its own with a fairly limited set of targets including cysteine protein residues (Sies 2017), through reactions in the cell it can easily be converted to a much more readily reactive hydroxyl radical. In the Fenton reaction, H$_2$O$_2$ in the presence of an iron catalyst is converted a hydroxyl radical (OH$^-$), which may then exact oxidative damage on to surrounding cellular tissues (Koppenol 1993).

Fenton Reaction: Fe$^{2+}$ + H$_2$O$_2$ $\rightarrow$ Fe$^{3+}$ +OH$^-$ +OH

Through this process the more membrane permeable H$_2$O$_2$ is able to diffuse through cell
membranes, potentially leading to more widespread oxidative damage.

Hydrogen peroxide has been implicated in a wide array of cellular signaling functions due to its uniqueness among ROS (Forman, Maiorino, and Ursini 2010). A crucial feature of secondary messengers is the need to have specificity to signaling pathway targets. Hydrogen peroxide alone meets this requirement among its fellow ROS. Its specificity for swift thiol oxidation, its production points, and its reduction pattern add to its unique value.

One of the earliest examples of H$_2$O$_2$ being recognized as a signaling molecule occurred in the 1970s when researchers noticed that ROS could mimic growth factor in cells (Czech 1976). However, the molecule was not well recognized for its role as a signaling molecule until late 1980s when research showed that H$_2$O$_2$ addition led to proliferation in cells (Burdon, Gill, and Rice-Evans 1989) as well as playing a role in immune system activation (Schreck, Rieber, and Baeuerle 1991). Since these initial observations, H$_2$O$_2$ has also been seen to trigger signal cascades through the production of prostaglandins (Hemler and Lands 1980), isoprostanes (Morrow et al., 1996) and aldehydes (Benedetti, Comporti, and Esterbauer 1980). Through these systems H$_2$O$_2$ is able to serve as a signal for a wide array of responses, highlighting its importance outside of its role as an oxidant molecule. The unique signaling role of H$_2$O$_2$ can be further seen through its interaction with the protein glyceraldehyde 3-phosphate dehydrogenase.

**Glyceraldehyde 3-Phosphate Dehydrogenase**

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is present in the tissues of all organisms and is best recognized for its role in glycolysis where it catalyzes the oxidation of glyceraldehyde 3-phosphate dehydrogenase to glycerate 1,3-bisphosphate (Seidler, 2013). The enzymes functional form exists as a homotetramer with two dimers coming together to form the structural unit.
Though GAPDH’s role in glycolysis has been recognized for decades, more recently the protein is gaining recognition for the role it plays in redox signaling. The protein GAPDH is known to be highly sensitive to oxidation to H$_2$O$_2$ compared to other proteins, with its reactive cysteine reacting with H$_2$O$_2$ at a greater rate than free cysteine, glutathione, and most redox regulated proteins (Hildebrandt et al., 2015). However, it bears mentioning that GAPDH’s reaction rate is slower than that of antioxidant proteins that directly react to reduce H$_2$O$_2$ to H$_2$O such as the peroxiredoxins (Winterbourn and Hampton, 2008). It is thought that the high reactivity of GAPDH with H$_2$O$_2$ is based on the enzymes structural conformation (Seidler, 2013).

The high reactivity of GAPDH’s cysteines with H$_2$O$_2$ enable it to play a dynamic role as a metabolic and redox signal transmitter (Barford, 2004). Potential modifications of GAPDH include S-glutathionylation, S-Nitrosylation, S-sulphydrlation, disulfide bond formation, and sulfenic, sulfonic, or sulfonic acid formation. These modifications can reduce GAPDH activity and trigger signal responses to a variety of stimuli including oxidative stress (Peralta et al., 2015).

Recent research suggests that under oxidative stress conditions NADPH production and concentration is increased by increased activation of the pentose phosphate pathway, decreasing glycolysis, and leading to increased concentration of the antioxidant protein glutathione. This diversion from glycolysis towards the pentose phosphate pathway was mediated by the oxidation of GAPDH active sites, with increased oxidized GAPDH leading to reduced glycolysis, increased activation of the pentose phosphate pathway, and increased reduction of glutathione (Kuehne et al., 2015). This role of GAPDH oxidation in increasing reduced glutathione concentrations has implications for the oxidative stress level in the
cellular environment as it may protect tissue from more extreme oxidative damage. In this way GAPDH may work with other better recognized antioxidant proteins to mitigate oxidative stress and maintain cellular homeostasis.

**Protein Oxidation**

Protein oxidation caused by ROS can have major implications for protein function and animal health. Reactive oxygen species can lead to multiple protein oxidation issues affecting function including decreases in protein solubility due to polymerization, enzyme activity loss, and reduced efficiency in carrying out functions (Stadtman and Oliver, 1991; Agarwal and Sohal, 1994; Shindoh et al., 1992).

ROS, such as hydrogen peroxide, can preferentially target sulfhydryl containing amino acids such as cysteine (Freeman and Crapo 1982). Cysteine, one of the amino acids that is most susceptible to oxidation, is among the first to be oxidized (Xiong 2000). The sulfhydryl group of cysteine is well known to play the role of an electron donating nucleophile in tissue. The redox state of cysteine’s active site plays a role in addition reactions in enzymes such as S-transferase, glyceraldehyde 3-phosphate dehydrogenase, and glutathione reductase as well as in peptidase activity in caspases, papain, and calpains. As such, the cysteine’s sulfhydryl group is critical for protein functionality (Giles et al., 2003).

Conversion of the cysteine’s thiol group to the thiolate form will also usually increase the cysteine nucleophilicity, resulting in greater oxidation risk (Gilbert 2006).

Examples of cysteine protein oxidation caused by ROS include S-thiolation and irreversible oxidation (Gilbert 2006). S-thiolation was initially thought to be caused by a protein thiol disulfide exchange with a cysteine thiol group:

$$\text{Protein-S (thiol protein)} + \text{R-} \xleftrightarrow{\text{S-R}} \text{S-S-R} + \text{R-S}^-$$
But according to Gilbert (2006) this reaction occurs rather slowly and requires higher RSSR concentrations than are likely to occur in living tissue. As a results, a different set of mechanisms has been suggested (Thomas and Mallis 2001):

\[
\text{Protein-SH} + R^\circ \rightarrow \text{Protein-S}^\circ + \text{RH (thyl radical)}
\]

\[
\text{Protein-SH} + \text{H}_2\text{O}_2 \rightarrow \text{Protein-SOH (sulfenic acid)} + \text{H}_2\text{O}
\]

These products are reversible and can easily be converted back to their reduced forms by antioxidant enzymes. However, in cases of oxidative stress, thiolation can continue to a point where it is irreversible resulting in sulfinic or sulfonic acid formation. These compounds may accumulate and disrupt normal cellular function if left unchecked:

\[
\text{Protein-SOH} + \text{H}_2\text{O}_2 \rightarrow \text{Protein-SO}_2\text{H (sulfinic acid)} + \text{H}_2\text{O}
\]

\[
\text{Protein-SO}_2\text{H} + \text{H}_2\text{O}_2 \rightarrow \text{Protein-SO}_3\text{H (sulfonic acid)} + \text{H}_2\text{O}
\]

Irreversible protein oxidation normally leads to degradation due to loss of function (Berlett and Stadtman 1997). Apart from S-thiolation of cysteine groups to sulfinic or sulfonic acid, another irreversible oxidation of proteins can be seen in carbonylation.

**Protein Carbonylation**

Protein carbonylation is the addition of a carbonyl group, such as aldehydes or ketones, to a protein molecule as a result of oxidation. Carbonyl groups are characterized by a carbon atom attached to an oxygen molecule by a double bond (Barreiro and Hussain 2010). Protein carbonyl content may be increased as a result of many oxidative reactions and as such is used as a primary marker for oxidative stress in living tissues. Amino acid side
chains particularly susceptible to carbonylation include histidine, arginine, and lysine (Estévez 2011). Carbonylation has a negative impact on proteins in living tissues as their presence will negatively affect structure and function, eventually leading to the need for their removal and degradation. The carbonylation reaction is considered irreversible, though inclusion of a reducing agent within a tissue sample has been seen to reduce total carbonyl formation (Wong et al., 2013). Measuring protein carbonyl content relative to a given amount of protein is an accepted method of determining the degree of protein oxidation in a sample.

The DNPH method of carbonyl quantification is a recognized method of determining carbonyl content and protein oxidation through a spectrophotometric process (Reznick and Packer 1994). The method is based on a reaction between carbonyl compounds and 2, 4-dinitrophenylhydrazine (DNPH) which forms a 2, 4-dinitrophenyl which displays a maximum absorbance at 370 nm. Through the assay process protein content and carbonyl content are determined simultaneously to account for protein lost through the assay. At completion total carbonyl content per mg of protein can be determined (Mario Estévez 2011). Oliver et al., (1987) initially developed the DNPH method to measure differences in protein oxidation between biological samples. Since then it has been widely used in determining protein oxidation in tissues. The DNPH method of carbonyl content analysis remains recognized as the primary method to measure protein oxidation in tissue samples.

**Diagonal Gel Electrophoresis**

Oxidation initiated by ROS can lead to a wide variety of protein modifications including disulfide bond formation of the thiol group of cysteine amino acids. Disulfide bond formation may result in changes in protein function, quaternary structure, and solubility (Murray and Van Eyk, 2012). Through the use of diagonal gel electrophoresis, differences in disulfide bond formation may be compared between protein samples and, when coupled with
mass spectrometry analysis, disulfide bound proteins may be identified. This method has been used in a wide variety of organisms and tissue types to answer questions about disulfide formation and protein oxidation.

The process of diagonal gel electrophoresis makes use of differing masses of proteins caused by disulfide formation. Initially, a sample of protein prepared under non-reducing conditions are run on a non-reducing SDS-PAGE gel. At this point the proteins in a given gel are still in their non-reduced disulfide forms, causing them to migrate higher in the gel if they are conjugated to another protein and are larger in size. The lane containing the protein sample is then excised and incubated in a reducing buffer containing DTT or β-mercaptoethanol. Through this incubation period the disulfide bonds in a protein sample are broken, resulting in proteins that had been bound migrating at their normal molecular mass. The excised piece of gel containing the protein sample is then placed on top of another gel and held there using agarose. This second gel is then run under reducing conditions and stained to view protein. Any proteins that do not initially have a disulfide bond will be seen on a diagonal line running from the top left to the bottom right corner of the gel. Any proteins that contained a disulfide bond will appear off the diagonal. In the case of an intermolecular disulfide the proteins will appear below the diagonal due to a reduction in weight, while in the case of intramolecular disulfides the protein will appear above the diagonal, due to increased resistance due to unfolding (Kim et al., 2010) (Fig. 2.3). Proteins of interest can then be picked from the SDS-PAGE gel following staining and sent for mass spectrometry analysis to determine individual protein identity. Through the use of diagonal gel electrophoresis researchers are able to determine specific proteins that undergo disulfide bond formation following a given treatment and better understand the impact of protein
oxidation on tissues.

Figure 2.3 Depiction of diagonal gel electrophoresis results with ovals and circles representing different proteins within a sample. Proteins appearing below the diagonal represent intermolecular disulfide bond bound proteins which progress further down the gels due to a reduction in molecular weight compared to when they were bound to another protein. Proteins appearing above the line represent proteins with intramolecular disulfide bonds. These proteins are retarded in their migration due to increased resistance caused by unfolding in the protein quaternary structure.

**Antioxidant Proteins Implications for the Live Animal**

Antioxidant proteins play significant roles in the function of animal tissues. They affect the health and efficiency of all commercial livestock production species, as well as the final quality attributes of the protein products derived from muscle tissues. These proteins play a wide variety of roles in maintaining homeostasis and ensuring health and wellbeing in living systems (Karplus, 2015). The main role antioxidant proteins play in the live animal is providing protection from reactive oxygen species by converting them to more stable, less
reactive compounds, for instance converting hydrogen peroxide to water. In doing so, these molecules protect tissues from oxidative damage to nucleic acids, proteins, and lipids. Antioxidant proteins also play a role in the body’s response to oxidative stressors, such as those caused by a disease challenge.

Historically the two best recognized antioxidant proteins are catalase, which was discovered in 1900 (Loew 1900), and glutathione peroxidase, discovered in 1957 (Mills, 1957). Both enzymes have been extensively studied and require the use of specific cofactors in order to activate antioxidant function; a specialized heme group in the case of catalase or a seleno-cysteine group in the case of glutathione peroxidase. Both glutathione peroxidase and catalase are necessary to maintain functional tissue and health status. Glutathione peroxidase gene knockout in mice has resulted in mice with normal life spans, however mice were more susceptible to health issues thought to be caused by oxidative stress resulting in diminished quality of life (Ohlemiller et al., 2000). Glutathione peroxidase can prevent damage to erythrocytes by protecting them from heme iron oxidation through its antioxidant capacity (Mills, 1957). Catalase uses a cofactor heme group to reduce hydrogen peroxide to water and can extend the lifespan and improve health of some invertebrates when overexpressed (Orr and Sohal, 1994; Taub et al., 1999). The enzyme has also assists in protecting immune cells in the blood (Sellak et al., 1994).

For decades, catalase and glutathione peroxidase were thought to be the only two major peroxide reducing enzyme families. However, in the early 1990s protein sequence comparisons between different subfamilies led to the discovery of the peroxiredoxins (Chae et al., 1994). Peroxiredoxins as a group are unique because they reduce radical peroxides without a cofactor, instead utilizing free cysteines to carry out reducing activity. For years
after their discovery, peroxiredoxins were considered to play less of a role in antioxidant defense than other major antioxidant proteins due to the perception of them having a lower efficiency at reducing hydrogen peroxide to water. However, peroxiredoxins are now known to carry out the conversion at a similar efficiency rate to catalase and glutathione peroxidase (Peskin et al., 2007). This realization, combined with the finding that the most abundant peroxiredoxin isomer was present at 50-fold greater concentrations than the most abundant glutathione peroxidase and over 500-fold greater than catalase in yeast (Ghaemmaghami et al., 2003) makes a strong case for peroxiredoxins as the dominant antioxidant protein living systems. Indeed, in human cells it is thought that over 99% of the peroxide in the cytosol and over 90% in the mitochondria will react with peroxiredoxins rather than other antioxidants (Cox, Winterbourn, and Hampton, 2010). As a whole, peroxiredoxins play the role of the “workhorse” of hydrogen peroxide antioxidant defense in living tissues while also serving as invaluable signaling molecules.

**Peroxiredoxins**

Peroxiredoxins are a group of antioxidant proteins that regulate hydrogen peroxide and other hydroperoxide levels through conversion to water and other more stable molecules. They also play a significant role in signaling the presence of oxidative stress and initiating the necessary response. Peroxiredoxins have been seen to be ubiquitously expressed in yeast, plant, animal, protozoan, and bacterial cells. Peroxiredoxins are present in high quantities in cells and as a group are the seventh most abundant protein present in *Escherichia coli* (Link, Robison, and Church 1997) and constituting 0.1 to 0.8% of the water-soluble protein in mammalian cells according to Chae et al. (1999). Different isoforms of peroxiredoxin are found in nature, with multiple forms being found in most species. Six of these isoforms have been found in the majority of mammalian tissue, with variation in quantity being seen
between tissue types. Peroxiredoxins-1, 2, and 6 are associated with the cell cytosol, peroxiredoxin-3 is found in the mitochondria, peroxiredoxin 4 is located in the endoplasmic reticulum, and peroxiredoxin 5 is present throughout the mitochondria, peroxisomes, and cytosol (Poynton and Hampton 2014). The presence of all six of these peroxiredoxins has been documented in skeletal muscle (Leyens, Donnay, and Knoops 2003).

**Structure**

All known peroxiredoxins share a recognizable structural core containing seven central β-strands surrounded by five α-helices which form a pocket around an absolutely conserved Cysteine residue (known as the peroxidatic Cysteine) which is responsible for the reduction of hydroperoxides (Karplus and Hall 2007). Structures of over one hundred peroxiredoxins are known. They range from 160 to 220 amino acids in length with larger variations containing additional secondary structures according to Karplus (2015).

Peroxiredoxins can be organized into five subfamilies based on amino acid sequence and structure including Peroxiredoxin 1 (Prdx-1), Prdx-6, Prdx-5, thioredoxin peroxidase (Tpx), and bacterioferritin-comigratory protein (BCP). It is worth noting that mammalian peroxiredoxins are found only in subfamilies 1, 5, and 6, whereas subfamily Tpx includes only bacterial Prdxs and subfamily BCP contains bacteria and plant cells. Peroxiredoxin-2 belongs to the Prdx-1 subfamily (Karplus and Hall 2007).

**Catalytic Cycle**

The catalytic process peroxiredoxins use to convert peroxide compounds to water utilizes a process including one conformational change and three productive steps (along with two additional competing steps) (Fig. 2.4). This process begins with the hydroperoxide substrate (whether that be an alkyl hydroperoxide or a hydrogen peroxide) entering the fully folded substrate binding pocket containing the peroxidatic cysteine group (S_P) at its base and
binding with it. This first step is known as peroxidation. At this point in the process the peroxide substrate is reduced to its alcohol form, a water molecule in the case of H₂O₂, and the peroxidatic cysteine group is oxidized to its sulfinic acid form (SₚOH) (Karplus 2015).

Resolution, the second step in the catalytic cycle, involves a free thiol group (Sᵣ) attacking the oxidized sulfinic acid and releasing a single water molecule (H₂O) resulting in the formation of a disulfide bond being formed between the two thiol groups. This second attacking thiol group, termed the resolving cysteine (Sᵣ), may be attached to the same peroxiredoxin or a separate molecule depending on the subfamily classification of the particular peroxiredoxin being examined. It is worth noting that the resolution step is only able to occur through conversion of the substrate binding pocket from the fully folded, less spatially available form to a more easily accessible, partially unfolded conformation, which allows for the third step in the process to be carried out (Karplus and Hall 2007).

The third and final step in the catalytic process of all peroxiredoxin subtypes is the recycling phase which returns the peroxiredoxin’s Sₚ and the Sᵣ to unbound reduced forms and converts the binding site surrounding the Sₚ back to its fully folded conformation, allowing the process to be reinitiated. This step is somewhat promiscuous in nature in the fact that it can be initiated by a wide variety of thiol compounds including dithiothreitol and thioredoxin (Karplus and Hall 2007). This recycling step is the rate limiting step the catalytic cycle of peroxiredoxin.

Though it is not a part of a peroxiredoxin molecules productive catalytic cycle, it is important to mention that for all types of peroxiredoxin a fourth step, known as overoxidation or hyperoxidation, may compete with the resolution reaction. In this instance, the fully folded sulfinic acid (CₚOH) form interacts with another peroxide molecule to form
a sulfinic acid (S\textsubscript{P}O\textsubscript{2}H) and release a water molecule. In extreme cases, this molecule of S\textsubscript{P}O\textsubscript{2}H may react with yet another hydrogen peroxide to release a water molecule and form a terminally oxidized sulfonic acid group (S\textsubscript{P}O\textsubscript{3}H). Both of these forms are inactive with respect to peroxidase activity. The S\textsubscript{P}O\textsubscript{3}H conversion is irreversible and C\textsubscript{P}O\textsubscript{2}H can be reduced only through an ATP dependent process. Sensitivity to hyperoxidation varies between peroxiredoxin subfamilies with some members of subfamily Prdx 1 being particularly susceptible. They can utilize a fifth step, known as resurrection (Wood, Poole, and Karplus 2003). Through the process of resurrection S\textsubscript{P}O\textsubscript{2}H is converted back to the S\textsubscript{P}OH sulfenic acid group through an ATP dependent process catalyzed by a sulfiredoxin molecule (Moon et al., 2013). Following the completion of the resurrection step, a peroxiredoxin has the ability to return to the catalytic cycle’s resolution step and carry on peroxidase activity.

It is not completely understood why members of subfamily Prdx 1 are more susceptible to hyperoxidation than others. It bears mentioning that hyperoxidation-sensitive peroxiredoxins are only found in eukaryotes and not prokaryotes, suggesting a positive aspect that led to selection over time. Multiple ideas exist on the topic. The following four hypotheses are the most promising. (1) The idea of a “peroxide floodgate” suggests that hyperoxidation sensitive peroxiredoxins allow peroxides to build up in one area of the cell (where peroxiredoxins are hyperoxidized and inactive) while protecting the rest of the cell, where hydrogen peroxides are present in lower quantity, with active enzyme, thus leading to a localized buildup. The hydrogen peroxide in this “area of buildup” can then act as a signal of oxidative stress leading to a response from exterior systems. Through this “peroxide floodgate” system hyperoxidation sensitive peroxiredoxins are able to function as
antioxidants through periods of low level stress, while enabling a signal induced response in more extreme cases (Wood, Poole, and Karplus, 2003). This concept is demonstrated in Fig. 2.5. (2) Hyperoxidized peroxiredoxins may act to conserve redox power under conditions of high oxidative stress through being inactivated and protected from damage (Karplus and Poole 2012). (3) Hyperoxidized peroxiredoxins may have a protein chaperone function by encouraging the formation of high molecular weight assemblies of stacked decamers of peroxiredoxin (Jang et al., 2004). (4) Hyperoxidized peroxiredoxins may act as redox signal for the cell cycle, signaling arrest or cell proliferation (Phalen et al., 2006). It is worth noting that these hypotheses are not exclusive as they may all be true and that other purposes of the hyperoxidized peroxiredoxins may yet be determined in the future.

**Figure 2.4** The five chemical steps in the peroxiredoxin catalytic cycle for a typical 2-Cys peroxiredoxin including: Peroxidation (1), Resolution (2), Recycling (3), Hyperoxidation (4), and Resurrection (5). Competition between hyperoxidation and resolution steps are determined by hyperoxidation sensitivity of particular peroxiredoxin molecules and hydrogen peroxide concentration. Changes in fold conformation can also be seen. Figure adapted from Karplus (2015).
Figure 2.5 The hyperoxide floodgate hypothesis. The image on the left demonstrates how if peroxide were generated at one site in a cell it would form a radical peroxide gradient (from black to white). The right-hand image shows how the peroxide gradient would look in three different scenarios: with no peroxiredoxins a simple gradient would be generated; with a high amount of a hyperoxidation sensitive peroxiredoxin, peroxide would build up locally where the peroxidase activity was inactivated but not further away where active peroxiredoxin was still present; and with a high amount of a robust peroxidase activity, little peroxide would build up. Thus the “floodgate” caused by sensitive peroxiredoxin does not allow the whole cell to be flooded with peroxides, but creates a barrierless compartmentalization of peroxide buildup, enabling signaling process to occur. Adapted from Karplus (2015).

Fully Folded vs. Locally Unfolded Conformations

Peroxiredoxin molecules utilize a unique conformation change in their catalytic cycle to carry out their peroxidase activity. This conformational change was first described by Wood et al. (2002) and involves the conversion of a fully folded active site state to a locally unfolded state. In the fully folded state the peroxidatic cysteine rests at the bottom of substrate binding pocket, where it is able to react with a peroxide but has limited accessibility to other thiols that it might form a disulfide bond with. After binding with a peroxide molecule, the active site will be converted to the locally unfolded form at which point the active site pocket will structurally cease to exist and the peroxidatic cysteine side chain will
be exposed and readily react with other cysteine groups (whether that be on the peroxiredoxin or on another thiol protein) to form a disulfide bond and allow peroxiredoxins catalytic cycle to continue.

**Classification Based on Reactive Cysteine Number**

The peroxiredoxins may also be sorted solely based the number of cysteines participating in the catalytic cycle: peroxiredoxins with two cysteine participating in antioxidant activity (typical 2-Cys Prdx and atypical 2-Cys Prdx) or those with one cysteine participating in its antioxidant activity (typical 1-Cys Prdx). For typical 2-Cys Prdx, the most common type of mammalian peroxiredoxin, two peroxiredoxin subunits interact to carry out antioxidant activity. The peroxidatic cysteine in its sulfenic acid form from one subunit is attacked by the resolving cysteine of another subunit resulting in the formation of a stable intermolecular disulfide bond and the formation of a dimer. For atypical 2-Cys Prdx, the resolving cysteine is located on the same polypeptide chain and the reaction with the peroxidatic cysteine results in the formation of an intramolecular disulfide bond. In both instances the disulfide can then be reduced by another thiol protein such as thioredoxin back to its active form. For typical 1-Cys Prdx the peroxidatic cysteine is present with the resolving cysteine being absent. In order to perform antioxidant activity glutathione must be available to reduce the peroxide bound cysteine back to an active form (Flohe and Harris 2007).

**Quaternary Structure of Peroxiredoxins**

Typical 2-Cys Peroxiredoxins can assume a variety of oligomeric conformations based on localized redox state and necessary function. All peroxiredoxins found in animal tissues are obligate dimers (Barranco-Medina et al., 2008). As discussed previously, this dimer may form disulfide bonds or exist in the reduced state depending on its stage in the
catalytic cycle. These dimers may form A (alternative)-type or B (β-sheet) -type interfaces based on β-strand interactions (Karplus 2015). B -type dimers (also known as parallel dimers) associate with each other through the edges of their β- strands to form a fourteen stranded β-sheet. These dimers have been seen to be more stable than the A-type form. A-type (or perpendicular) peroxiredoxin dimers associate through loops at the C-terminal ends of β-strands 3 through 7 (Wood et al., 2002).

The first report of peroxiredoxin dimers forming a larger oligomeric structure was in 1969 when transmission electron microscopy analysis of torin (now known to be peroxiredoxin-2, a 2-cys peroxiredoxin) isolated from erythrocytes revealed ring complexes with apparent ten-fold symmetry (Harris 1969). Some ring complexes were also seen to stack into columns of varying lengths forming higher molecular weight conformations. Since these original observations greater study has been carried out to determine the circumstances leading to peroxiredoxin decamer formation. It has been seen to form using B-type type dimers, with dimers coming together to form the decamer structure through interactions at the A-type interface (Flohe and Harris 2007). Biological factors seen to increase decamer formation include high (Kitano et al., 1999) or low (Chauhan and Mande, 2001; Kato et al., 1985) ionic strength, low pH (Kristensen, Rasmussen, and Kristensen, 1999), and high mineral content including magnesium (Kato et al., 1985) and calcium (Allen and Cadman 1979; Plishker et al., 1992). Peroxiredoxin concentration has also been seen to influence decamer formation, with formation threshold level ranging from 1 to 2 µM (Barranco-Medina et al., 2008).

Peroxiredoxin oligomer formation is also strongly influenced by peroxiredoxin redox state. Reduction of peroxiredoxin’s peroxidatic cysteine has been recognized as the primary
factor contributing to the stabilization of the peroxiredoxin decamer with multiple experiments using different methods confirming this theory (Wood et al., 2003), though some reduced peroxiredoxin will generally exist in the dimer form. Increase in the presence of the hyperoxidized form of peroxiredoxin has also been seen to lead to decamer formation and stacked decamer rings that are thought to play a role in cell signaling (Dietz et al., 2006; Schröder et al., 2000). Oxidized (disulfide) peroxiredoxin preferentially exists in the dimer form. This is thought to be caused by changes in peroxiredoxin active site fold conformations related to the catalytic cycle. It is thought that the fully folded active site structure present in all catalytic states excluding the oxidized disulfide buttresses the decamer building A-type interface, while the locally unfolded active site state triggered by oxidation destabilizes the decamer. This suggests that as peroxiredoxin is active in its catalytic cycle it is continuously switching form dimer to decamer conformation (Flohe and Harris, 2007; Wood et al., 2002).

**Gel-based Methods on Measuring Peroxiredoxins**

Because peroxiredoxin structural and oxidation states vary considerably due to oxidative stress status, they can be considered a useful biomarker for oxidative stress in living systems. Gel based methods are commonly used to examine variations in peroxiredoxin profile (Poynton and Hampton 2014). Total peroxiredoxin content in a sample may be analyzed through the use of reducing (+β- mercaptoethanol) SDS-PAGE gels (Feng et al., 2014). Differences in the presence of peroxiredoxin’s quaternary structure and oxidation states can also be compared for different treatments through the use of non-reducing (-β-mercaptoethanol) SDS-PAGE (Cox et al., 2010). Special precautions, such as sample alkylation of biotination, must be taken in some instances to protect the easily oxidized sulphydryl group at the reactive site cysteine due to the antioxidant’s susceptibility to trace peroxide levels in buffers (Poynton and Hampton 2014). Multiple commercial
antibodies are available to measure different peroxiredoxin isoforms through western blot analysis. Antibodies have also been developed for hyperoxidized peroxiredoxin through recognition of the amino acid sequence of the peroxidatic cysteine active site for the sulfinic and sulfonic acid forms (Woo et al., 2003). Through utilizing these methods researchers can gain greater insight into the role of peroxiredoxins on combatting oxidative stress in living tissues.

**Peroxiredoxin-2**

Peroxiredoxin-2 (Prdx-2) is a typical 2-Cys peroxiredoxin that is ubiquitously expressed in mammalian tissues as a cytosol-based enzyme. It can be found at varying concentrations depending on tissue type. Peroxiredoxin-2 plays a role in combatting oxidative stress and in initiating cell signaling through its interaction with hydrogen peroxide (Sobotta et al., 2015). The Prdx-2 enzyme is a member of the peroxiredoxin-1 subfamily and as such is highly susceptible to hyperoxidation in times of high oxidative stress and elevated hydrogen peroxide concentration (Peskin et al., 2007). Peroxiredoxin-2 plays a role in response to various events leading to oxidative stress in human health models, livestock, and in meat quality.

**Peroxiredoxin-2 and Human Health**

Much research has been conducted concerning Prdx-2 and its role in human health. Peroxiredoxin-2 is involved in the body’s response to multiple forms of health-related oxidative stresses, playing a part in blood homeostasis (Low et al., 2007), neural tissue protection (Fang et al., 2007), combatting cancer (Soini et al., 2006), and response to many diseases (Bayer et al., 2013).

One of Prdx-2’s major roles in the body is its maintenance redox homeostasis in the circulatory system and protection of erythrocytes (red blood cells) from ROS.
Peroxiredoxin-2 functions as a H$_2$O$_2$ scavenger in erythrocytes, protecting them from high levels of damage caused by the heme iron (Low et al., 2007). Mice subjected to Prdx-2 gene knockout are fertile and outwardly healthy, however, these mice suffer major issues within the hemolytic system including increased oxidized proteins, increased ROS, decreased erythrocyte numbers, and increased reticulocyte (immature red blood cell) numbers resulting in anemia (Lee 2003). Peroxiredoxin-2 has been considered an oxidative stress biomarker for commercial blood banks, due to its correlation with red blood cell damage caused by oxidative stress and excessive storage time (Rinalducci et al., 2011). Peroxiredoxin-2 also plays a role in protecting the circulatory system and heart through activities such as combatting ROS producing ischemic-reperfusion damage pathways (Leak et al., 2013). It is also to mitigate ROS production in the early stages of pulmonary hypertension, slowing disease progression (Federti et al., 2017).

Peroxiredoxin-2 has been implicated in the protection of neural tissue and has been seen to play a role in neurological disease resistance. The enzyme is recognized as the most highly expressed antioxidant protein in mammalian neurons according to Fang et al. (2007). The nervous system’s reliance on Prdx-2 can be seen in the role it plays in Parkinson’s disease. Normally, Prdx-2 aides in combatting the progression of Parkinson’s disease by neutralizing ROS. However, if the antioxidant protein’s reactive site undergoes a posttranslational modification that negatively impacts its peroxidatic activity, damage to neurons and overall neurodegeneration can be greatly increased. These negatively impactful modifications include phosphorylation (Przedborski 2007) and S-nitrosylation (Fang et al., 2007) of the cysteine active site. Peroxiredoxin-2 also plays a significant role in other neurological diseases. Its expression is upregulated in the lesions of multiple sclerosis.
patients potentially as a response to combat the increased inflammation and tissue damage caused by ROS (Voigt et al., 2017). The brains of aborted fetuses with Down’s syndrome have been found to underexpress Prdx-2, further suggesting that the antioxidant’s activity may play a role in the development of the nervous system (Sánchez-Font et al., 2003). Overexpression of Prdx-2 may also have a benefit in protecting neurons and the nervous system as a whole. Mice with overexpression were seen to have reduced brain injury compared to controls following a cerebral ischemia brain injury, suggesting protective qualities of the peroxiredoxin (Gan et al., 2012).

The role of Prdx-2 in the body’s response to cancer is documented in a wide array of cancer types. Increased circulatory total peroxiredoxin-2 content in patients with renal carcinoma cancers is correlated with improved prognosis and lower grade tumors, suggesting a positive role in combating cancer cell proliferation (Soini et al., 2006). Silencing peroxiredoxin-2 activity through an active site methylation modification is also seen to promote melanoma skin cancer formation (Furuta et al., 2006), further demonstrating the protein’s role in combatting cancer. Peroxiredoxin-2 may also be a viable biomarker for cancer due to its responsive upregulation. For example, total Prdx-2 content in saliva shows promise as an indicator of oral squamos cell carcinomas when used in combination with other protein biomarkers (Heawchaiyaphum et al., 2018). Peroxiredoxin-2 may also protect noncancerous cells from the body’s other defensive systems. Rabilloud et al. (2002) found that Prdx-2 protects Leydig cells from tumor necrosis factor, reducing damage to noncancerous cellular components. However, peroxiredoxin-2 may also play a role in protecting cancer cells. Lu et al. (2014) found that the antioxidant protein protected colorectal cancer cells from ROS and reduced the occurrence of cancer cell apoptosis when
compared to control treatments. Research has also found that overexpression of Prdx-2 may lead to increased resistance to treatments for prostate cancer, possibly due to its signaling function (Shiota et al., 2011).

Though Prdx-2’s participation in response to severe health challenges is clear, it should be remembered that it also functions in healthy individuals. These functions include its signaling capacity with H$_2$O$_2$ and its protection of tissue. Peroxiredoxin-2 is found throughout the tissues of all mammals, where it exerts its antioxidant activity through converting H$_2$O$_2$ to water and preventing oxidative tissue damage (Karplus 2015). With this in mind, Prdx-2 could be a biomarker for the early stages of many disease issues, due to its upregulation in response to health-based oxidative challenge and its role in stress signaling. Diseases where Prdx-2 has showed promise as an early indicator include inflammatory bowel disease (Senhaji et al., 2017), hepatitis-B (Y. Lu et al., 2010), and other inflammation related diseases (Bayer et al., 2013).

Peroxiredoxin-2’s involvement in human health is widely recognized. Its role in livestock health and meat quality is established to a lesser extent.

**Peroxiredoxin-2 and Livestock Oxidative Stress Response**

Peroxiredoxin-2 quantity has been seen to be differentiated in multiple top-down proteomics experiments examining animal response to different types of oxidative stress including heat stress, physiological stress, and immune challenge. Little research has been conducted with the objective of examining differing forms of Prdx-2 despite its role in stress response and hydrogen peroxide reduction. However, Prdx-2’s continuous emergence as a differentially abundant protein in experiments examining different types of stress, from different research groups, suggests the need for further research.

Peroxiredoxin-2 content has been seen to potentially play a role in response to
environmental heat stress in species including pigs (Cruzen et al., 2015), poultry (Akbarian et al., 2016), and dairy cattle (Wang et al., 2017). Cruzen et al. (2015) used 2-dimentional difference in gel electrophoresis (2D-DIGE) to examine proteomic changes in red and white portion of the semitendinosus muscle in pigs following acute heat stress. 2D-DIGE separates proteins based on isoelectric point (first dimension) and weight (second dimension) resulting in differentially abundant protein spots, with some proteins being present in multiple spots. These spots were then picked from gels and identified using mass spectroscopy. Peroxiredoxin-2 was seen to significantly differ (P<0.01) for two distinct protein spots, both of which were decreased in abundance in the heat stressed pigs for both red (-47% and -34%) and white (-56% and -34%) muscle samples. It was thought to be counterintuitive for the antioxidant protein to be reduced in the tissue of animals undergoing oxidative stress, though the authors suggested Prdx-2 may be degraded, depleted, or transported to a more oxidation susceptible tissue. Another potential explanation could be the peroxiredoxin being converted to the hyperoxidized decamer or hyperoxidized stacked decamer, which has been seen to play a signaling role in times of extreme oxidative stress. This high molecular weight conformations may not have been seen in 2D-DIGE, perhaps due to reduced solubility or inability to migrate through the gel but would have decreased the total pool of peroxiredoxin protein that would have been seen in the viewable spots. Similar results were seen by Outhouse et al. (2015) when looking at the impact of immune system stimulation on liver proteomic profile using 2D-DIGE. The liver peroxiredoxin -2 content was reduced by 42% in immune stimulated pigs compared to controls. Regardless, this research shows that Prdx-2 profile is impacted by heat stress and immune system induced oxidative stress.

Peroxiredoxin-2 has been found to be increased in the blood serum of pigs under
physiological stress. Marco-Ramell et al. (2016) examined the effect of pig movement from group housing to individual housing, as a physiological stressor, over the course of a week following movement. Stress impact on the serum protein profile, in addition to various stress biomarkers in the saliva and blood serum were measured. 2D-DIGE and isobaric tag for relative and absolute quantification (iTRAQ). iTRAQ is a method of protein identification and quantification that functions through measuring peptide fractions of a sample, where both used to compare the protein profiles of pigs. Prdx-2 was one of only two proteins that was differentially increased following housing change for both the 2D-DIGE and iTRAQ methods of protein analysis. The Prdx-2 level was increased by 2 to 4-fold depending on the method used. This suggests a potential value of Prdx-2 as an indicator of physiological stress change over time. Peroxiredoxin-2 has also shown potential as a biomarker for disease-related oxidative stress. Genini et al. (2012) showed that Prdx-2 is upregulated in the early stages of Porcine Respiratory and Reproductive Syndrome, suggesting potential as an early disease indicator when analyzed in combination with other proteins.

Though a fairly limited quantity of information is currently available in regard to Prdx-2’s role in livestock species, the fact that the vast majority of information currently published was the result of top-down proteomic experiments, where the antioxidant protein was not directly chosen for analysis, supports the argument that more research is needed into its actions in livestock stress response. Its appearance in similarly organized experiments with a focus on meat quality furthers this argument.

**Peroxiredoxin-2 and Meat Quality Implications**

Though little literature is currently available on the impact of Prdx-2 profile on meat quality, the protein has emerged in a variety of proteomics experiments as a tool to potentially indicate color stability, tenderness, and even drip loss in meat products.
Tenderness is a major driving factor for consumers of meat products. As such, developing proteomics based methods to determine meat tenderness could have a major impact on the meat production industry. Carlson et al. (2017) defined the proteomic profile of the sarcoplasmic protein portion of tough and tender aged pork loin samples. Peroxiredoxin-2 was seen to significantly differ (P<0.01) in two spots in 2D-DIGE, with both spots being increased in the tougher samples (+37% and +51%). These results were confirmed with western blotting techniques. This suggests Prdx-2 could have promise as a biomarker for tenderness in pork loin (Carlson et al., 2017). This difference in Prdx-2 content between tough and tender pork may be attributed to degradation of the protein in the early postmortem muscle environment. Di Luca et al. (2013) found that a Prdx-2 fragment was released in exudate from pork longissimus muscle from day 1 to day 7 postmortem, supporting this hypothesis.

Peroxiredoxin-2 has been implicated in the maintenance of color stability in fresh meat. Joseph et al. (2012) found through tandem mass spectrometry protein analysis, that Prdx-2 is increased in expression in beef longissimus lumborum muscle relative to the psoas major muscle. More interestingly, Joseph et al. (2012) showed that peroxiredoxin content in this muscle was strongly correlated (0.92 correlation coefficient) to increased ratio of reflectance at 630nm and at 580nm (R630/580). An increased R630/580 indicates lower metmyoglobin and a greater muscle color stability. This makes sense as Prdx-2 may be able to exert its peroxidase activity in muscle tissue on ROS, thus protecting myoglobin, the muscles main color pigment, from oxidation. Similar results related to myoglobin oxidation protection were seen when examining proteins affecting shelf life in longissimus lumborum muscle from Chinese Luxi yellow cattle (Wu et al., 2016). Wu et al. (2016) also found that
Prdx-2 content was strongly correlated to a* value ($r= +0.959$) in the longissimus lumborum muscle indicating increased myoglobin protection. Furthermore, Prdx-2 was strongly positively correlated to pH value ($r=+0.999$) and negatively correlated TBARS value after 15 days of storage. This data further highlights Prdx-2’s positive impact on meat quality through its antioxidant activity.

The meat quality factors of pH decline and water holding capacity are also related to Prdx-2. Wang et al. (2016) found differences in Prdx-2 between high and low drip loss goat loin muscle. Peroxiredoxin-2 and other antioxidant proteins were seen to be increased in high water holding capacity loins compared to those of lower water holding capacity. With respect to Prdx-2’s relationship to pH, Nam et al. (2012) found Prdx-2 spots were increased when using 2D- DIGE methods to compare the sarcoplasmic protein portions of high 24 hour pH pork loins to pork loins with low pH at 24 hours.

**Other Peroxiredoxins in Meat Quality**

Other peroxiredoxin family members besides Prdx-2 have been seen to play a role in meat quality including peroxiredoxin-1 and peroxiredoxin-6. Peroxiredoxin-6 has shown significant potential as a biomarker for tenderness in both live animals, through tissue biopsy, and in meat. Gagaoua et al. (2015) found through comparisons of correlation networks that peroxiredoxin-6 was a robust biomarker across three different breed types and two different muscles. Moreover, they found that peroxiredoxin-6 was strongly correlated with µ-calpain and suggested that peroxiredoxin may protect the proteolytic enzyme from oxidation, thus increasing protein degradation capacity and potentially meat tenderness. More in depth research is needed to determine peroxiredoxin-6 capacity as a tenderness biomarker, but the protein shows promise.
Summary

It is understood that oxidative stress plays a role in factors influencing the efficiency of pork production. Reactive oxygen species production and electron leakage can be increased in animals of lower feed efficiency and when animals are exposed to health challenges. Antioxidant proteins such as Prdx-2 play a major role in mitigating damage caused by ROS by converting $\text{H}_2\text{O}_2$ and other hydroperoxides to more stable molecules. Peroxiredoxin-2 profile is altered in response to a wide array of oxidative health challenges in livestock.

However, to date no study has focused on examining differences in oxidation state and structure of Prdx-2 in any tissue of a livestock species. Greater research is needed in order to determine how the oxidation state of peroxiredoxin-2 is altered in response to oxidative stress challenge and how this influences animal performance.

Therefore, the objective of the study described in this thesis was to determine the impact of a dual respiratory and enteric bacterial health challenge on Prdx-2 protein profile and protein oxidation in the skeletal muscle of pigs selected for differing RFI. It was initially hypothesized that differences would exist in the Prdx-2 profile based on RFI line and infection status (IS) and that high RFI and health challenged pigs would present a higher degree of protein oxidation due to increased oxidative stress.

Literature Cited


CHAPTER 3. NOVEL OBSERVATIONS OF PEROXIREDOXIN-2 PROFILE AND PROTEIN OXIDATION IN SKELETAL MUSCLE FROM PIGS OF DIFFERING RESIDUAL FEED INTAKE AND HEALTH STATUS

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Abstract

This study’s objective was to determine the impact of a dual respiratory and enteric bacterial health challenge on the profile of the antioxidant protein peroxiredoxin-2 (Prdx-2) and protein oxidation in the skeletal muscle of pigs selected for differing residual feed intake (RFI). It was hypothesized that differences would exist in the Prdx-2 profile based on RFI line and infection status and that less efficient high RFI and health challenged pigs would present a higher degree of protein oxidation due to increased oxidative stress.

Barrows (50±7 kg, total n=24) divergently selected for RFI from the 11th generation of the ISU RFI Project were used for this study. To induce a respiratory and enteric health challenge, half of the pigs (n=6 / RFI line) were inoculated with Mycoplasma hyopneumoniae and Lawsonia intracellularis on 0 days post infection (dpi). Uninoculated pigs served as controls (n=6 / RFI line). At dpi 21 (projected peak infection) pigs were necropsied, and longissimus skeletal muscle samples were immediately collected. Protein oxidation, various
forms of Prdx-2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) content were measured on longissimus skeletal muscle sarcoplasmic protein.

Neither RFI selection nor infection status significantly affected protein carbonylation. Based on diagonal gel electrophoresis results, GAPDH was selected for further analysis using western blotting. Under non-reducing conditions MhLi pigs had a greater amount of a slower migrating GAPDH band (P=0.017), potentially disulfide bound oxidized GAPDH. Significant differences were seen based on both RFI line and infection status for Prdx-2 measured on non-reducing gels, suggesting some differences in posttranslational modifications. There was an increase in total Prdx-2 (P=0.035) and Prdx-2 decamer (P=0.0007) in high RFI pigs while hyperoxidized peroxiredoxin relative to Prdx-2 was increased in low RFI pigs (P=0.028).

Pigs divergently selected for RFI appear to differ in antioxidant protein profile and health challenge response. The increased pool of active Prdx-2 in high RFI pigs indicates increased Prdx-2 production to combat chronic increased ROS and tissue damage. This helps to explain the reduced feed efficiency of high RFI pigs. The increase in oxidized GAPDH seen in MhLi pigs, particularly high RFI MhLi pigs, may help to mitigate greater protein oxidation through mediating an increase in reduction of the antioxidant protein glutathione.

**Introduction**

Improving the efficiency of pork production is paramount as global demand for animal protein increases alongside increases in populations and incomes in the developing world. Two factors that have a significant impact on pork production are feed efficiency and response to disease challenges. Both of these factors are influenced by oxidative stress (Bottje et al., 2002; Deblanc et al., 2013; Grubbs et al., 2013; Sies et al., 2017). Oxidative stress is defined as an imbalance between prooxidant and antioxidant forces within the cell in
favor of prooxidant forces (Sies et al., 2017). This leads to damage to cellular components including proteins, lipids, and DNA under oxidative stress conditions. Energy that could otherwise be directed towards growth must then be allocated towards repairing oxidative stress induced tissue damage. More in depth research is needed to improve understanding of the relationship between oxidative stress balance and related factors that impact production, such as feed efficiency and health challenge response.

Animals divergently selected for residual feed intake (RFI) represent a valuable model for comparing animals of differing feed efficiency. As a measure of feed efficiency, RFI represents the difference between observed feed intake and expected feed intake based on average daily gain and backfat measurements with low RFI pigs being more efficient than high RFI counterparts (Boddicker et al., 2011). Grubbs et al. (2013) found that less efficient high RFI pigs exhibited greater electron leakage and reactive oxygen species (ROS) production in the skeletal muscle mitochondria, suggesting a role for oxidative stress in animals of differing efficiency.

Pathogen challenges are known to cause increased oxidative stress in livestock species, most likely due to increased mitochondrial electron leakage and ROS formation (Deblanc et al., 2013; Sies et al., 2017). *Mycoplasma hyopneumoniae* and *Lawsonia intracellularis* are respiratory and enteric pathogens, respectively, which are widespread across commercial swine facilities in the United States.

Antioxidant proteins play an invaluable role in mitigating oxidative stress and protecting living tissues by converting ROS to more stable compounds. The antioxidant protein Peroxiredoxin-2 (Prdx-2) is well recognized for its role in responding to oxidative stress challenges in human health by converting hydrogen peroxide and other hydroperoxides
to more stable molecules such as water. Peroxiredoxin-2 profile is altered in response to a variety of oxidative stress challenges in livestock including heat stress (Cruzen et al., 2015), LPS immune challenge (Outhouse et al., 2015), physiological stress (Marco-Ramell et al., 2016), and health challenge (Genini et al., 2012).

However, to our knowledge, no study has ever focused on comparing the various Prdx-2 oxidation states and oligomeric structures in the skeletal muscle of animals of differing feed efficiency or under health challenge. By better understanding the role of Prdx-2 in feed efficiency and health challenge response, producers may be able to better manage animals towards improved production efficiency without a dramatic increase in inputs. Thus, the objective of this study was to determine the impact dual infection with Mycoplasma hyopneumoniae and Lawsonia intracellularis on Prdx-2 protein profile and overall protein oxidation in the skeletal muscle of pigs divergently selected for RFI. It was hypothesized that differences would exist in the antioxidant protein Prdx-2’s profile based on RFI line and infection status and that high RFI and health challenged pigs would present a higher degree of protein oxidation due to increased oxidative stress.

**Materials and Methods**

**Animals, Treatment, and Experimental Design**

The pigs used in this study were a subset of a larger project (Helm et al., 2018a). All animals in this study were handled in accordance with the Iowa State University Institutional Animal Care and Use committee (IACUC #6-16-8298-S). Six littermate pairs of low RFI and six littermate pairs of high RFI barrows from the eleventh generation of the Iowa State University RFI Project were randomly selected from a larger set of pigs. At 50±7kg in weight, littermate pairs were split and randomly assigned to individual pens across two separate rooms in the same barn, resulting in six high RFI and six low RFI pigs in each room.
The rooms had identical pen size, feeders, flooring, heating and cooling systems, and water supply, but separate manure pits. All pigs were fed an ad libitum commercial corn-soybean diet and had free access to water (Table 3.1). After a three-week acclimation period, pigs in one of the rooms were inoculated with *Mycoplasma hyopneumoniae* and *Lawsonia intracellularis* to elicit a dual respiratory and enteric response. This resulted in a 2 x 2 factorial design with four experimental groups including: low RFI control, low RFI infected, high RFI control, and high RFI infected (n=24 total, n=6 per group) (Helm et al., 2018b).

**Pig Inoculation and Sample Collection**

On 0 days post inoculation (dpi), barrows in the health challenged room were inoculated with *Mycoplasma hyopneumoniae* and *Lawsonia intracellularis*, while those in the control room were inoculated with a sham. Pigs were snare restrained for inoculations. For the respiratory inoculation, *Mycoplasma hyopneumoniae* was dosed in a 10 mL inoculum (strain 232, containing $10^5$ color-changing units/mL) through an intra-tracheal gavage. For the enteric challenge, pigs were intra-gastrically gavaged with 40 mL of *Lawsonia intracellularis* inoculum (2 mL gut homogenate, containing $2 \times 10^7$ *Lawsonia intracellularis* organisms). Inoculums were prepared at the Iowa State University Veterinary Diagnostic Laboratory (Ames, Iowa).

Blood samples (10 mL) were collected via jugular venipuncture from all pigs at dpi 0, 14, and 21. Samples were allowed to clot, centrifuged (2,000 * g* for 10 minutes at 4°C), and serum was collected and stored at -80°C. Individual serum samples were submitted to the Iowa State University Veterinary Diagnostic Laboratory and tested via ELISA methods to quantify *Mycoplasma hyopneumoniae* (IDEXX Laboratories Inc., Westbrook, Maine) and *Lawsonia intracellularis* (SVANOIR® Ileitis ELISA, Boehringer Ingelheim Svanova, Uppsala, Sweden) antibody response. *Mycoplasma hyopneumoniae* antibody response was
Individual feed disappearance and body weights were recorded for each pig on the day of inoculation and on a weekly basis during the acclimation period and following infection. From this data collection, average daily gain, average daily feed intake, and gain:feed were calculated (Table 3.2). On 21 dpi, projected peak infection, pigs were euthanized using a captive bolt stunning device followed by exsanguination. Immediately following euthanasia, longissimus samples were collected and flash frozen in liquid nitrogen. Samples were held at -80°C until protein extraction.

**Sarcoplasmic Protein Extraction and Sample Preparation**

Sarcoplasmic proteins were extracted according to Cruzen et al. (2015). Prior to extraction, frozen longissimus muscle samples were homogenized in the presence of liquid nitrogen using a blender (Waring Blendor, New Hartford, CT, USA). Care was taken to ensure samples did not thaw. 1.5 g of powdered longissimus muscle was homogenized in 5 ml of ice cold (4°C) extraction buffer composed of 50 mM Tris–HCl, 1 mM EDTA, pH 8.5. Samples were homogenized with a Polytron PT 3100 (Lucerne, Switzerland) for approximately 20 seconds. The resulting sample was centrifuged at 40,000×g for 20 min at 4°C (Sorvall Super T21, Newtown, CT, USA). Any insoluble particles were removed from the resulting sarcoplasmic fraction (supernatant protein) by filtering through cheesecloth. The filtered supernatant was assayed for protein concentration according to Lowry et al. (1951) using premixed reagents (BioRad, Hercules, CA, USA). Resulting samples were diluted using ice cold extraction buffer to create to 6.4 mg/ml samples for further use. Four mg/ml reduced and non-reduced (± β-mercaptoethanol) protein samples for sodium dodecyl sulfate
one-dimensional polyacrylamide gel electrophoresis (SDS PAGE) and western blot analysis were produced by adding 0.5 volumes (relative to protein sample) of Wang’s tracking dye (3 mM EDTA, 3% [w:v] SDS, 30% [v:v] glycerol, .001% pyronin-Y [w:v], 30 mM Tris-HCl, pH 8.0) and 0.1 volumes of β-mercaptoethanol in the case of reduced samples or 0.1 volumes extraction buffer in the case of non-reduced samples. Samples were heated for 15 minutes in a dry bath at approximately 50˚ C. Samples were stored at −80 °C until analysis could be carried out.

**One-Dimensional SDS-PAGE**

Reduced and non-reduced sarcoplasmic protein samples were run using one-dimensional SDS-PAGE and western blotting was carried to compare the relative quantities of multiple forms of Prdx-2. Precision Plus Dual Color Protein Standard (BioRad Laboratories, Hercules, CA) was run on each gel to determine apparent molecular weight of proteins for all one-dimensional gels. Gel running conditions and antibody dilutions for western blotting can be seen in Table 3.3.

Total Prdx-2 was quantified using 15 % polyacrylamide separating gels (10 cm x 10 cm; acrylamide: N, N’-bis-methylene acrylamide ratio of 100:1 [w:w], 0.1 % [w:v] SDS, 0.05 % [v:v] TEMED, 0.05 % [w:v] ammonium persulfate [AMPER], and 0.5 M Tris-HCl pH 8.8) with a 5% stacking gel (acrylamide: N,N’-bis-methylene acrylamide = 100:1 [w:w], 0.1 % [w:v] SDS, 0.125% TEMED, 0.075 % [w:v] AMPER, 0.125 M Tris-HCl, pH 6.8) with ten running lanes. Gels were loaded with 60 µg of reduced (+β-mercaptoethanol) protein sample per lane and run at ambient temperature at a constant voltage of 60 volts for 360-volt hours.

Peroxiredoxin-2 posttranslational modifications and hyperoxidized peroxiredoxin content were compared using 12% polyacrylamide separating gels and 5 % stacking gel with 10 lanes. 60 µg of non-reduced (-β-mercaptoethanol) sample were loaded per lane and run at
4°C at a constant voltage of 30 volts for approximately 540-volt hours.

Peroxiredoxin-2 decamer content was determined using 5% continuous gels (acrylamide: N, N'-bis-methylene acrylamide ratio of 100:1 [w: w], 0.1 % [w: v] SDS, 0.05 % [v: v] tetramethylenediamine (TEMED), 0.05 % [w: v] ammonium persulfate [AMPER], and 0.5 M Tris-HCl pH 8.8) with 10 lanes. 80 µg of non-reduced (-β-mercaptoethanol) protein sample was loaded per lane and gels were run at 4°C at a constant voltage of 20 volts for approximately 750-volt hours.

SE 260 Hoefer Mighty Small II electrophoresis units (Hoefer, Inc., 54 Holliston, MA) were used to run all of the previously mentioned gels. The gel running buffer was composed of 25 mM Tris, 192 mM Glycine, 2 mM EDTA, and 0.1% [w: v] SDS.

**Gel Transfer**

Immediately following electrophoresis, all of the previously mentioned gels were transferred to polyvinylidene difluoride (PVDF) membranes with 0.2 µm pore size (ISEQ00010, Immobilon-P SQ, Millipore Sigma, Darmstadt, Germany). The membranes were soaked in methanol prior to the transfer process for approximately 30 seconds to ensure activation. Gels were transferred onto membranes using TE-22 Mighty Small units (Hoefer, Inc., Holliston, MA), running for a constant voltage of 90 volts for 1.5 hours, in transfer buffer cooled 4°C in a constantly circulating water bath. The transfer buffer was composed of 25 mM Tris, 192 mM glycine, 2 mM EDTA, and 15 % [v: v] methanol.

**Western Blotting**

PVDF membranes were blocked in a solution of PBS- Tween (80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, 0.1% [v: v] polyoxyethylene sorbitan monolaurate [Tween-20]) containing 5% non-fat dry milk (NFDM). Membranes were blocked for approximately 1 hour at room temperature.
Primary antibodies diluted in PBS-Tween were added to the blot membranes and incubated for approximately 20 hours at 4°C. Two primary antibodies were used simultaneously so the hyperoxidized peroxiredoxin protein could be analyzed independently and relative to total non-reduced Prdx-2 bands. Test western blots were run to ensure the two antibodies did not interact with each other (Figure 3.1). All antibody dilutions can be viewed in Table 3.3.

Primary antibody dilutions consisted of: total (reduced) Prdx-2- 1:20,000 monoclonal rabbit anti- Prdx-2 (ab109367, Abcam, Cambridge, UK), non-reduced Prdx-2- 1: 15,000 monoclonal rabbit anti- Prdx-2 (ab109367, Abcam, Cambridge, UK), hyperoxidized peroxiredoxin- 1: 2,000 polyclonal rabbit anti- peroxiredoxin SO3 (ab16830, Abcam, Cambridge, UK) and 1: 10,000 monoclonal rabbit anti- Prdx-2 (ab109367, Abcam, Cambridge, UK), Prdx-2 decamer- 1:10,000 monoclonal rabbit anti-Prdx-2 (ab109367, Abcam, Cambridge, UK).

Following incubation in primary antibody, all blots were washed for ten minutes in PBS-Tween three times. Blot membranes were next incubated in secondary antibody diluted in PBS- Tween for one hour at ambient temperature. Secondary dilutions were as follows: reduced Prdx-2 and non-reduced Prdx-2- 1:10,000 goat anti-rabbit-HRP (horseradish peroxidase) (31460, Thermo Scientific, Rockford, IL), hyperoxidized peroxiredoxin and Prdx-2 decamer- 1: 5,000 goat anti-rabbit-HRP (31460, Thermo Scientific, Rockford, IL).

After completion of secondary incubation all blots were washed for three ten minute periods in PBS-Tween. Proteins were detected with a commercial chemiluminescence kit (ECL Prime, GE Healthcare, Piscataway, NJ) with a five minute incubation time. Images were produced and analyzed using a ChemiImager 5500 (Alpha Innotech, San Leandro, CA).
and Alpha Ease FC software (v 3.03 Alpha Innotech). Individual protein bands were quantified for each sample using densitometry and comparisons were made between individual samples by taking the ratio of the measured protein band to an internal reference used in all blots a reference sample of porcine sarcoplasmic protein that was not allowed to age (day 0). For the Prdx-2 analysis on non-reducing gels and hyperoxidized peroxiredoxin western blots, additional within sample comparisons were made. Two distinct bands were seen when Prdx-2 was analyzed on non-reducing gels. The second, faster migrating band, thought to have a posttranslational modification, was compared to the total Prdx-2 seen in each lane/sample to produce a ratio. For hyperoxidized peroxiredoxin western blots, hyperoxidized peroxiredoxin quantity was compared to the total Prdx-2 seen per lane/sample to develop a hyperoxidized band ratio relative to the total Prdx-2. All western blots were performed in at least duplicate to reach a coefficient of variance less than 20%.

**Carbonyl Content Assay**

Differences in protein oxidation were determined through carbonyl content. Carbonyl content for sarcoplasmic proteins of each longissimus muscle sample were determined according to Reznick and Packer (1994). All samples were run in triplicate. Samples from the sarcoplasmic protein fraction were diluted to 6 mg/ml concentration using 1mM EDTA, 50 mM NaHPO₄ pH 7.4 at 4°C. One mL of 6 mg/ml sample was incubated with 4 ml of 10 mM 2, 4-Dinitrophenylhydrazine (DNPH) in 2.5 M HCl. A second 1 ml of 6 mg/ml sample was added to 4 ml of 2.5 M HCl to serve as a control. All samples were vortexed and incubated in the dark at 22°C for 30 minutes, vortexed again, and incubated for an additional 30 minutes. After incubation the reaction was stopped with the addition of 5 ml of 20% (w: v) trichloroacetic acid and placed in an ice bath for 10 minutes before being centrifuged at 3100 xg for 10 minutes at 4°C (Sorvall Super T21, Newtown, CT, USA). Following
centrifugation, the supernatant was discarded, and the pellet was washed in 10% (w/v) trichloroacetic acid before being vortexed and centrifuged at 3100 xg at 4°C. The supernatant was again discarded, and the pellet was washed in a mixture of 1:1 ethyl acetate and ethanol (v:v), vortexed and centrifuged for 10 minutes at 3100 xg at 4°C. This step was repeated once. Following centrifugation, the supernatant was discarded and the pellet was placed 2 ml of 6 M guanidine hydrochloride (H₂NC(NH)NH₂) pH 2.0. Samples were rigorously vortexed and placed in a 37°C hot water bath for 5 minutes. This step was repeated twice. After the pellet was completely dissolved, any remaining insoluble particles were removed by centrifugation at 3700 xg for 10 minutes at 20°C. All DNPH treated samples were read using an Ultraspec 3000 spectrophotometer (Biochem, Ltd., Cambridge, England) at 365nm. Protein contents were determined from the control (no DNPH) samples using a Bradford assay (BioRad Laboratories, Hercules, CA) with sample diluted 1:20 (v:v) in distilled deionized water. The standard curve (0-0.72 mg/ml) was developed using Bovine serum albumin (BSA) dissolved in 300mM guanidine hydrochloride, pH 2.3. Based on results, total carbonyl concentration (nmoles/mg protein) in samples was determined using the following equations:

\[ \text{Extinction coefficient of 10mM DNPH} = 22,000/M = 22,000/10^6 \text{ nmol/ml} \]

\[ \text{Carbonyl concentration} = \text{Absorption}/2.2 \times 10^4/10^6 = \text{Abs (365 nm)} \]

\[ \text{Carbonyl Concentration (nmoles/ml)} = \text{Abs (365 nm)} \times 45.45 \text{ nm/ml} \]

\[ \text{Carbonyl Concentration (nmoles/mg)} = \frac{\text{Carbonyl Concentration (nmoles/ml)}}{\text{Control}} \]

**Diagonal Gel Electrophoresis**

Diagonal gel electrophoresis was used to examine relative differences in disulfide protein content between samples according Kim et al. (2010) with some modifications. For the first dimension, 4mg/ml non-reduced sarcoplasmic protein samples were prepared
without β-mercaptoethanol, as previously described, and loaded onto a 10 cm x 10 cm 12% polyacrylamide gel with a wide lane stacker. Protein was loaded so that a width of 1 cm contained approximately 60 µg of protein (1.14 mg protein loaded in total). Gels were run at 4°C at a constant voltage of 20 volts for approximately 560 volt hours. At completion of running, first dimension gels were incubated for 1 hour at ambient temperature in a reducing SDS buffer (4% w: v SDS, 125 Nm Tris-HCl, 20% v: v glycerol, 0.04% w: v bromophenol blue, pH 6.8) containing 0.2% β-mercaptoethanol. After incubation, a 1 cm wide section was excised from the center of the gel and fixed with agarose to a second 15% polyacrylamide gel. For the second dimension, the gel was run at ambient temperature at a constant voltage of 60 volts for 360 volt hours. 0.1% β-mercaptoethanol was included in the second dimension running buffer. Following electrophoresis, gels were stained overnight in a Colloidal Coomassie Blue Stain. The next day gels were rinsed multiple times in water to remove any residual stain and gels were visually appraised to compare differences in spots appearing below the diagonal line of proteins. These spots appearing below the line were the result of intermolecular disulfide bonds being broken through the reducing process. Gels were run in duplicate. Four spots were selected for identification based on the diagonal gel analysis. Spots were excised and sent to the Proteomics Facility at Iowa State University for liquid chromatography mass spectrometry (LC-MS) identification (Stewart, 1999).

**Glyceraldehyde 3-Phosphate Dehydrogenase Western Blotting**

Based on LC-MS identification results from the diagonal gel electrophoresis, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) non-reducing gel western blot analysis was performed on samples to compare differences in content. One dimensional electrophoresis and western blotting was performed as previously discussed. 60µg of non-reduced protein was load onto a 15% acrylamide gel with ten running lanes. Following
electrophoresis, transfer to PVDF membranes, and blocking, blots were incubated in primary antibody overnight at 4°C. Following primary incubation, blots were subjected to 3 ten-minute washes in PBS-tween, incubated in secondary antibody, washed in PBS-tween for 3 ten-minute periods, and imaged as previously discussed. Primary antibody dilution consisted of a 1:10,000 dilution in PBS-tween of monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase antibody (MAB374, Millipore Sigma, Darmstadt, Germany). Secondary antibody dilution consisted of a 1:10,000 goat anti-mouse-HRP (horseradish peroxidase) (A2554, Sigma Aldrich, Darmstadt, Germany). Western blots were performed in at least duplicate in order to attain a coefficient of variance less than or equal to 20%.

**Statistical Analysis**

A 2*2 factorial design was used to evaluate the fixed effects of line (high RFI and low RFI), infection status (control and infected), and their interaction. The MIXED procedure of SAS (version 9.4; SAS Inst. Inc., Cary, NYC) was used to analyze these data. Day of necropsy and gel repetition (for western blot analysis) were included as random effects. Differences were considered significant when P≤0.05 and tendencies were considered when 0.05<P≤0.10. Partial correlations were generated using PROC CORR. Correlations were considered significant when P≤0.05 and tendencies were considered when 0.05<P≤0.10.

**Results and Discussion**

**Health Challenge Response and Growth performance**

Health challenge was confirmed by pig performance, serum sample collection to assess antibody titer, and lung and small intestine histo-pathology lesion scoring at the time of necropsy. As previously described by Helm et al., (2018a), control pigs were confirmed to be negative for MhLi through the duration of the study. No mortalities occurred and no
antibiotic treatments were necessary during the 21 day period between inoculation and necropsy. All pigs in the infected group were confirmed positive for both pathogens based on antibody presence in the blood sera at dpi 21 (Table 3.4). Less robust signs of infection were seen through daily observations and lung and small intestine lesion scoring at necropsy indicating that this challenge model resulted in subclinical disease. Subclinical disease challenges are important due to their negative impact on production growth traits and profitability (Helm et al., 2018a; Ciprián et al., 2012; Paradis et al., 2012).

Disease challenges have a major impact on the efficiency of pork production across the industry. Health challenges are likely to have a negative impact on growth traits including ADG, ADFI, and G:F (Schweer et al., 2016; Curry et al., 2018). Subclinical health challenges with low mortality such as those used in this study caused by *Lawsonia intracellularis* and *Mycoplasma hyopneumoniae* are still likely to lead to a reduction in growth (Straw et al., 2006). The fact that these subclinical diseases are prevalent throughout commercial swine production systems within the United States (USDA, 2016) further demonstrates their potential for negative impacts on pork production. In the 21 day period following inoculation, health challenged pigs from both RFI lines demonstrated reduced ADG (P<0.0001), ADFI (P<0.0001) and G:F (P=0.014) when compared with their control counterparts (Table 3.2). Challenged pigs showed a 42% reduction in ADG, a 27% reduction in ADFI, and a 24% reduction in G:F. This study’s results support the negative impact of subclinical bacterial infections such as *Lawsonia intracellularis* and *Mycoplasma hyopneumoniae* on growth performance.

Animals of differing feed efficiency may respond differently to health challenge and other oxidative stressors due to differences in reactive oxygen species production and
electron leakage from the mitochondria, as well as antioxidant protein status. Residual feed intake (RFI) is a measure of feed efficiency defined as the difference between the amount of feed consumed relative to an animal’s anticipated consumption based on average daily gain and backfat measurements (Boddicker et al., 2011). Animals divergently selected for RFI represent a valuable model for examining biological or metabolic efficiency due to the statistical fixation of production traits. In previous experiments more efficient, low RFI pigs have demonstrated 10-35% greater feed efficiency through the finishing phase of production relative to less efficient, high RFI counterparts (Boddicker et al., 2011; Harris et al., 2012; Grubbs et al., 2013). In the current study, similar results were seen based on RFI line. High RFI pigs were less efficient than their low RFI counterparts from the beginning of the acclimation period to 21 dpi (42 days total) in terms of both ADFI and G:F. High RFI pigs had 18% higher average daily feed intake (P<0.0001) and a 15% reduction in G:F (P=0.007) compared to low RFI animals (Table 3.2).

No significant interactions were seen between RFI line and infection status for growth traits (Table 3.2). This is in line with previous data as neither low or high RFI pigs have shown an advantage in other studies that examined growth traits in response to inflammatory (Merlot et al., 2016) or pathogenic (Helm et al., 2018a; Dunkelberger et al., 2015) health challenge.

**Protein Oxidation**

Various biological factors are thought to play a role in differences in efficiency between animals divergently selected for residual feed intake. These factors include feeding patterns, body composition, heat increment, activity, digestibility, and the metabolic factors of protein turnover, tissue metabolism, and oxidative stress (Richardson and Herd, 2004). Oxidative stress in living tissue can be defined as an imbalance between prooxidant and
antioxidant forces in favor of prooxidants (Sies et al., 2017). These prooxidants include reactive oxygen species (ROS) that are produced through a wide array of reactions within the cellular environment. Reactive oxygen species are thought to play a role in efficiency variance in animals of differing RFI (Grubbs et al., 2013; Bottje et al., 2002).

Grubbs et al. (2013) studied ROS production from mitochondria isolated from the skeletal muscle of pigs divergently selected for high and low RFI from the eighth generation of selection for the Iowa State University RFI Project. These are the same genetic lines were used for this study. Grubbs et al. (2013) found that less efficient high RFI pigs tended to have greater ROS production in the mitochondria of both red and white semitendinosus skeletal muscle tissue. They also saw positive correlations between RFI and ROS production from mitochondria of longissimus skeletal muscle. Increased ROS production and electron leakage from the mitochondria of less efficient high RFI animals can increase protein oxidation in tissues. Bottje et al. (2006) found that broiler chickens with greater ROS production and electron leakage in skeletal muscle mitochondria were prone to increased protein oxidation measured through protein carbonylation. Health challenges such as those initiated in this study have also been seen to increase oxidative stress, possibly due to increased production and leakage of ROS from the mitochondria as a result of increased energy demand to combat challenges (Deblanc et al., 2013; Sies et al., 2017).

In the current study, protein oxidation was analyzed in the sarcoplasmic protein fraction of each longissimus muscle sample through DNPH-based carbonyl quantification and diagonal gel electrophoresis. Protein carbonyl quantification is a method of measuring irreversible protein oxidation of amino acids (Reznick and Packer, 1994). These damaged amino acids are energetically expensive as they must be removed through cellular processes
to maintain protein function. Diagonal gel electrophoresis evaluates reversible protein oxidation by evaluation of disulfide bonds between proteins (Winger et al., 2007).

Surprisingly, in the current study no significant differences were seen in protein carbonylation in the sarcoplasmic protein extract of longissimus skeletal muscle based on infection status or RFI line (Table 3.5). This may be attributed to a lower level of oxidative stress initiated by this health challenge, possibly due to its subclinical nature. It could also be partially explained by the point that neither of the pathogens used in this project directly target skeletal muscle. Interestingly, a trend was seen for an interaction to occur between RFI line and infection status (P=0.064) with carbonyl content being greater in low RFI MhLi pigs compared to low RFI control pigs and lesser in high RFI MhLi pigs compared to high RFI controls (Table 3.6). This interaction may be explained by subtle differences in how animals of differing feed efficiency respond to oxidative stress.

Four protein spots appearing below the diagonal line of proteins which were selected for identification using liquid chromatography- mass spectrometry (LC-MS). Table 3.7 shows the individual peptides identified in each spot. Two of the spots were identified as creatine kinase and two were identified as glyceraldehyde 3-phosphate dehydrogenase (Figure 3.2). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was chosen for further analysis using western blotting. Glyceraldehyde 3-phosphate dehydrogenase represented the most prominent protein spot that was seen to differ based on RFI line and infection status.

Relative GAPDH content was analyzed using western blotting on one-dimensional SDS-PAGE gels under reducing and non-reducing gel conditions (Figure 3.3). Glyceraldehyde 3-phosphate dehydrogenase is an important catalyst in the glycolysis pathway and has recently become a protein of interest in terms of metabolic and redox
regulation (Hildebrandt et al., 2015; Peralta et al., 2015; Araki et al., 2016). It is a unique protein with respect to its reactivity with hydrogen peroxide (H$_2$O$_2$) generated as a result of oxidative stress. Glyceraldehyde 3-phosphate dehydrogenase is particularly sensitive to oxidation by H$_2$O$_2$ and has been identified as one of its most prominent protein targets (Baty et al., 2005; Hancock et al., 2005). Its cysteine groups react more quickly with H$_2$O$_2$ than free cysteines, glutathione, and the majority of other redox regulated proteins. However, its rate of reaction is still slower than that seen in H$_2$O$_2$-targeting antioxidant proteins, such as the Prdx-2 (Winterbourn and Hampton, 2008). The increased speed of reaction of GAPDH solely with H$_2$O$_2$ is currently thought to be caused by its structural features (Hildebrandt et al., 2015).

Western blot analysis showed total GAPDH content did not differ based on infection status or RFI line when analyzing sarcoplasmic protein samples under reducing conditions, however, significant differences were seen when samples were compared under non-reducing conditions (Figure 3.3). Under non-reducing conditions, a significant increase in a more slowly migrating band of GAPDH was seen in MhLi animals compared to control animals (P=0.017) across RFI line. Moreover, within RFI line, a trend (P=0.083) was seen for a greater difference in GAPDH content between control and MhLi pigs from the high RFI group compared to those in the low RFI group (Table 3.8A). A significant difference existed between high RFI control and high RFI MhLi pigs, with MhLi pigs having a greater amount of the more slowly migrating band, (P=0.0025) but not between low RFI control and low RFI MhLi pigs (Table 3.8B).

This increased slower migrating band of GAPDH on non-reducing gels is most likely the result of an oxidative modification to the protein. Glyceraldehyde 3-phosphate
dehydroghense’s reactive cysteines are susceptible to multiple oxidation modifications including sulfenic, sulfinic, and sulfonic acid formation, s-glutathionylation, s-nitrosylation, and s-sulfhydration (Hildebrandt et al., 2015). This difference seen in GAPDH based on infection status along with the lack of significant difference in protein carbonylation suggests a limited ROS increase in the skeletal muscle in response to MhLi challenge. MhLi challenge may have caused enough of an increase in ROS production to oxidize the more $\text{H}_2\text{O}_2$ oxidation susceptible GAPDH, but not so much as to cause a major change in overall protein carbonylation. This hypothesis has implications in terms of antioxidant response.

It has recently been reported that under oxidative stress conditions in human skin cells, oxidation of GAPDH cysteine active sites leads to a decrease in the glycolysis pathway and increased activation of the pentose phosphate pathway leading to increased reduction of the antioxidant glutathione through the action of glutathione reductase (Kuehne et al., 2015). This increased reductive capacity of glutathione may result in an overall reductive effect within the surrounding tissues, thus helping to mitigate ROS induced damage (Figure 3.4).

This difference in oxidized GAPDH may help to explain the trend for an interaction seen when carbonyl content in sarcoplasmic protein fraction was examined, with greater carbonyls seen in the sarcoplasmic protein from low RFI MhLi pigs compared to low RFI control pigs and less carbonyls in the sarcoplasmic protein of high RFI MhLi pigs compared to high RFI controls. The glutathione response triggered by increased oxidized GAPDH may be great enough in high RFI MhLi pigs to result in lower carbonyl content than that seen in high RFI control pigs.

**Peroxiredoxin-2 Profile**

Peroxiredoxins are a unique family of antioxidant proteins that convert $\text{H}_2\text{O}_2$ and other hydroperoxides to more stable compounds such as water. They also play a major role in
cellular redox signaling. The peroxiredoxins have a greater content in tissues than other major antioxidants such as glutathione peroxidase and catalase (Ghaemmaghami et al., 2003) and have similar reactivity rates (Peskin et al., 2007).

Peroxiredoxin-2 is a typical 2-cys peroxiredoxin which is present in all mammalian tissues (Leyens et al., 2003). It can exist in multiple oxidation states and differing oligomeric structures based on oxidative stress level and function. Peroxiredoxin-2’s role in response to oxidative stress challenge is well documented in human medicine in a wide variety of instances. However, limited research is currently available on the role of Prdx-2 in livestock species. To our knowledge, all of the current literature on the topic is the result of shotgun-based proteomics, with minimal direct investigation into differences in Prdx-2 profile. Thus, we endeavored to better understand the differences in Prdx-2 profile through examining differences based on RFI line and MhLi infection status.

Peroxiredoxin-2 profile has been seen to significantly differ in top down proteomic experiment in response to a variety of oxidative stressors in pigs including environmental heat stress (Cruzen et al., 2015), LPS induced immune challenge (Outhouse et al., 2015), physiological stress (Marco-Ramell et al., 2016), and health challenge (Genini et al., 2012). These changes include an increase in total Prdx-2, hyperoxidized Prdx-2, and potentially an increase in larger stacked decamers of hyperoxidized Prdx-2. These changes in profile in response to a wide variety of stress challenges suggest an important role in maintaining redox balance in livestock species. In the current study, differences were seen in Prdx-2 profile for western blot analysis of the sarcoplasmic protein fraction for RFI line and, to a lesser extent, health status (Table 3.5).

Total Prdx-2 was significantly increased in high RFI pigs (P=0.035) with no
significant difference seen based on infection status or RFI line*infection status interaction
(Table 3.5, Figure 3.5). This increase in total Prdx-2 in the less efficient high RFI pigs could be explained by higher mitochondrial ROS leakage previously seen in high RFI animals (Grubbs et al., 2013) especially when we know that peroxiredoxins can be upregulated in response to oxidative challenge in order to better mitigate oxidative damage (Chang et al., 2007). Similar results were seen by Grubbs et al. (2013) with the antioxidant protein catalase being increased in high RFI pigs. Chronic ROS exposure may lead to greater Prdx-2 production in order to prevent and combat tissue damage. This in turn could lead to an increase in nutrients needed to produce more antioxidant proteins such as Prdx-2. This helps to explain the reduced efficiency in high RFI animals due to an increased diversion of nutrients that could be used for growth towards antioxidant protein production in addition to oxidized tissue repair.

Peroxiredoxin-2 is an obligate dimer. However, the antioxidant protein can form a more complex decamer structure composed of five dimer subunits interacting through their β-strands. This process is dependent upon Prdx-2 oxidation state. Peroxiredoxin-2 exists in three reactive cysteine oxidation states: reduced, oxidized, and hyperoxidized. In the reduced state (S-H) Prdx-2 is readily able to bind H₂O₂ in order to exert its peroxidation reaction. Through this reaction Prdx-2 will convert H₂O₂ into a more stable water molecule (H₂O) resulting in the formation of a sulfenic acid (S-OH) at the reactive cysteine site of Prdx-2. A reduced cysteine on the adjacent Prdx-2 in the same dimer will then form a disulfide bond with the sulfenic acid, releasing a single water molecule and forming an oxidized Prdx-2. Under normal circumstances, a thiol protein in the cell will then break the oxidized Prdx-2 disulfide bond enabling it to return to its reactive reduced state. However, under conditions of
greater oxidative stress and higher H₂O₂ concentrations the Prdx-2’s sulfinic acid may interact with another H₂O₂ molecule prior to disulfide formation resulting in the release of a water molecule and the formation of hyperoxidized Prdx-2 with a sulfinic acid group (SO₂H). The hyperoxidized Prdx-2 is trapped in this nonreactive form until it is returned to the reduced form by an ATP-dependent reaction catalyzed by a sulfiredoxin enzyme (Karplus, 2015).

Peroxiredoxin-2 decamer formation is heavily influenced by oxidation state. Decamer formation is favored by Prdx-2 in the reduced state with increased reduction leading to increased decamer formation (Wood et al., 2003). Increases in the presence of hyperoxidized Prdx-2 is also known to contribute to decamer formation. In some instances hyperoxidized decamers will also come together to form larger stacked structures, which may play a role in cell signaling (Schröder et al., 2000; Dietz et al., 2006). Oxidized (disulfide bound) Prdx-2 exists preferentially in the dimer form. This is thought to be caused by Prdx-2 active site fold conformational changes that occur at the active site surrounding the reactive cysteine through the Prdx-2 catalytic cycle. The partially unfolded conformation triggered by oxidation and disulfide formation is thought to weaken the interaction of the β-strands needed for decamer formation, thus leading to its disassembly. Proxiredoxin-2 will switch from decamer to dimer conformation as it progresses through its catalytic cycle. (Flohe and Harris, 2007). With this in mind increased Prdx-2 decamer formation can suggest a greater amount of either active reduced Prdx-2 or inactive hyperoxidized Prdx-2.

In the current study the Prdx-2 decamer was increased in the skeletal muscle of high RFI pigs (P=0.0007) compared to low RFI counterparts despite the fact similar hyperoxidized peroxiredoxin content was seen between the two groups (Figure 3.6). There
was no significant difference in the detection of the Prdx-2 decamer for infection status or RFI line*infection status interaction. This suggests that a greater amount of Prdx-2 is being kept in the reduced form in the high RFI pigs. This can be explained by the increased total Prdx-2 seen in the high RFI pigs and could suggest that under an extreme oxidative stress challenge the high RFI pigs may exhibit a greater ability to respond to oxidative stress due to increased total Prdx-2 though this was not seen in the current study where a mild health challenge was employed.

Western blot analysis was performed using non-reducing gels in order to make comparisons of Prdx-2 posttranslational modifications in samples (Figure 3.7). Two protein bands were seen using this method. These bands were measured independently by comparison to a reference sample and also by determining a ratio of the second faster migrating band to the total Prdx-2 per sample lane. The non-reducing gel Prdx-2 western blot analysis showed no significant differences were when analyzing the first, slower migrating band. However, a trend was seen for this band to be increased in the high RFI pigs (P=0.055). There was also a trend for an interaction to exist, with high RFI pigs having a greater difference between control and MhLi pigs compared with their low RFI counterparts (P=0.063). There was a significant difference between high RFI control and high RFI MhLi pigs, with MhLi pigs having a greater amount of the first slower migrating band (P=0.0178). High RFI pigs were seen to have a greater quantity of the second, faster migrating band compared to low RFI counterparts (P=0.0006). For Prdx-2 measured on non-reducing gels, the second band was also compared to the total immunoreactive protein per lane as a ratio. This was performed in order to enable comparisons of the two bands irrespective of total Prdx-2 quantity. MhLi pigs had a greater proportion of the second faster migrating band.
compared to control pigs (P=0.014). There was also a significant interaction between RFI line and infection status, with the high RFI pigs having a greater difference in band ratio between control and MhLi pigs relative to that seen in the low RFI pigs (P=0.020). Some common posttranslational modifications that have been documented to have an impact on Prdx-2 activity include phosphorylation (Przedborski, 2007), s-nitrosylation (Fang et al., 2007), and glutathionylation (Salzano et al., 2014). These modifications could lead to differences in migration patterns similar to what was seen in the current study with modifications leading to faster migration. Though limited conclusions can be made due to the fact that we did not determine what posttranslational modifications were present in each of the protein bands analyzed, the fact remains that differences were seen based on both RFI line and infection status. This demonstrates that differences in posttranslational modifications of Prdx-2 exist based on RFI line and infection status. Pigs divergently selected for RFI appear to differ in Prdx-2 stress response as a result of posttranslational modifications on the protein.

Hyperoxidized peroxiredoxin can be an indicator of increased ROS concentration and oxidative stress. Hyperoxidized peroxiredoxin content in longissimus muscle samples was measured as an individual band and relative to the Prdx-2 in each sample (Figure 3.8). This was done by determining a ratio of the hyperoxidized peroxiredoxin to the total immunoreactive protein per sample lane. In this way hyperoxidized peroxiredoxin could be measured as a proportion to the total Prdx-2. As an independent band, no significant differences were seen in hyperoxidized peroxiredoxin, though a trend was present for hyperoxidized peroxiredoxin to be increased in low RFI control compared to low RFI MhLi pigs and decreased in high RFI control pigs compared to high RFI MhLi pigs (P=0.088).
(Table 3.5). The same trend was seen when hyperoxidized peroxiredoxin was compared to the total non-reduced Prdx-2 per lane \( (P=0.056) \). When compared to Prdx-2, hyperoxidized peroxiredoxin was seen to be increased in low RFI pigs relative to their high RFI counterparts \( (P=0.028) \).

Though hyperoxidized peroxiredoxin content did not independently differ between RFI lines, high RFI animals still have a greater available pool of Prdx-2 with hyperoxidation considered, solely due to a greater overall content. Interestingly, a trend was seen for hyperoxidized peroxiredoxin to be increased in low RFI control animals compared to low RFI MhLi counterparts and decreased in high RFI control pigs compared to high RFI MhLi animals. Though there is not a clear explanation for this difference, it may further suggest a difference in oxidative stress response based on selection for feed efficiency.

Though clear differences existed in Prdx-2 profile in longissimus skeletal muscle based on RFI line, infection status did not consistently affect Prdx-2 status. There are many explanations for this unanticipated lack of difference. For one, neither of the bacterial infections used to induce oxidative stress in this study directly target skeletal muscle tissue. Thus, health challenge induced ROS production may have been limited in skeletal muscle. Greater ROS production and a corresponding alteration in Prdx-2 profile may be more likely in tissues directly impacted by health challenge, such as the small intestines and lungs for this experiment. Moreover, the subclinical nature of this challenge may have resulted in limited ROS production. We would expect greater differences in Prdx-2 profile to exist in animals experiencing a major health challenge, to the point of experiencing a reduction in muscle mass and weight.

Greater research is needed to determine the impact of disease challenge on Prdx-2
profile and tissue oxidation across a wider array of tissues and a wider range of health challenge extremes. By better understanding the role of antioxidant proteins such as Prdx-2 in health challenge and feed efficiency we may be better able to manage livestock towards improved production efficiency.

**Correlations**

The 21 dpi antibody titers of both Mh and Li of each MhLi pig (n=11) were used to generate correlations with growth traits, Prdx-2 forms, carbonyl content and calpain features (Helm et al., 2018b) for all infected pigs (Table 3.9). Antibody response for Mh was negatively correlated with study start weight (r=-0.527, P=0.10) and necropsy weight (r=-0.609, P=0.05) and positively correlated with carbonyl content (r=0.539, P=0.09). Li antibody titer was positively correlated with weaning weight (r=0.542, P=0.09) and ADG (r=0.526, P=0.10). Li was also negatively correlated with Prdx-2 non-reducing gel band ratio (r=-0.680, P=0.02).

Greater antibody response is generally associated with a healthier pig with pigs vaccinated against disease exhibiting higher antibody titers (Kim et al., 2011). However, 21dpi Mh antibody titer was negatively correlated to necropsy weight for MhLi pigs. Trends were also seen for Mh antibody titer to be negatively correlated to study start weight and positively correlated to carbonyl content in the longissimus skeletal muscle sarcoplasmic protein extract. This may be explained by greater resource allocation towards immune response in pigs with higher antibody titers.

Correlations were run for sarcoplasmic protein carbonyl content against growth traits, Prdx-2 forms, and calpain features (Helm et al., 2018b) for all pigs (n=24), as well as for subsets based on RFI line (n=12) and infection status (n=12) (Table 3.10). No significant correlations with carbonyl content were seen when all pigs were considered. When separated
based on RFI line and infection status distinct correlations emerged. For control pigs, carbonyl content was negatively correlated to Prdx-2 band ratio measured on non-reducing gels (second band relative to total Prdx-2) \((r=-0.517, P=0.09)\) suggesting a relationship between changes in posttranslational modifications protein oxidation, with pigs presenting a greater proportion of Prdx-2 in the slower migrating band presenting greater carbonyl content. In the case of MhLi pigs, carbonyl content was negatively correlated to necropsy weight \((r=-0.531, P=0.08)\). Carbonyl contents of sarcoplasmic protein from low RFI pigs was negatively correlated with hyperoxidized peroxiredoxin \((r=-0.559, P=0.06)\), total Prdx-2 \((r=-0.520, P=0.08)\), ADG \((r=-0.667, P=0.02)\), and necropsy weight \((r=-0.793, P=0.002)\). The sarcoplasmic protein carbonyl content in high RFI pigs was positively correlated with study start weight \((r=0.636, P=0.03)\) and necropsy weight \((r=0.762, P=0.004)\).

An increase in carbonyl content is generally recognized to be an indicator of oxidative stress and a negative health outcome. Interestingly, an inverse relationship was seen between weight measurements and growth between high and low RFI pigs with low RFI animals demonstrating carbonyl content negatively correlated to necropsy weight and ADG and high RFI animals experiencing positive carbonyl content correlations to study start weight and necropsy weight. This difference may at least be partially explained by differences in Prdx-2 profile with low RFI pigs trending towards having less total Prdx-2 and hyperoxidized peroxiredoxin as carbonyl content increased whereas high RFI pigs lacked these relationships. Based on these results low RFI animals may not be utilizing Prdx-2 to combat oxidative stress at the molecular level as well as high RFI counterparts.

**Conclusions**

Differences were seen in Prdx-2 profile based on RFI line and to a lesser extent infection status, however contrary to our original hypothesis no significant differences were
seen in carbonyl content based on RFI line or infection status. This may be partially explained by the increase in GAPDH oxidation product in MhLi pigs, particularly high RFI MhLi pigs, potentially leading to an increase in reduced glutathione protein and prevention of more widespread protein oxidation (Kuehne et al., 2015).

The fact that neither *Mycoplasma hyopneumoniae* nor *Lawsonia intracellularis* directly target skeletal muscle could also partially explain the lack of difference in carbonyl content. Different results may be seen with a more extreme health challenge with a greater impact on skeletal muscle.

Reduced feed efficiency in high RFI pigs may be partially explained by increased Prdx-2 production, potentially to combat greater mitochondrial ROS production seen in previous work (Grubbs et al., 2013). The resulting increase in nutrient allocation towards antioxidant protein production could in turn lead to a subtle reduction in nutrients available for efficient growth. Moreover, if there is a need for antioxidant protein there may be more oxidative damage that needs to be repaired.

Based on the observed increase in total Prdx-2 and Prdx-2 decamer in high RFI pigs and the increase in hyperoxidized peroxiredoxin relative to Prdx-2 in low RFI pigs, high RFI pigs appear to have a greater pool of total reduced Prdx-2. This suggests that high RFI pigs may be better able to respond to oxidative stress challenges, though this was not seen in the current study which employed a fairly mild health challenge.

Oxidation of GAPDH may be aiding in pig antioxidant response by mediating a shift from glycolysis towards the pentose phosphate pathway. This change could partially explain the reduced efficiency of high RFI pigs due to a subtle reduction in ATP and pyruvate production in addition to leading to an increase in antioxidant response through increased
reduction of the antioxidant protein glutathione, through increased NADPH production and
the action of glutathione peroxidase. The increase in reduced glutathione could then prevent
greater oxidative damage not only through its own antioxidant activity but also by increasing
the efficacy of Prdx-2 and other peroxiredoxins by speeding the rate limiting recycling step
of the peroxiredoxin catalytic process through its reducing activity.

Taken as a whole, the results of this study suggest that animals of differing feed
efficiency may use antioxidant proteins such as Prdx-2 and manage oxidative stress
challenges differently. Further research is needed to evaluate the impact of health challenges
across a wide array of tissue types and health challenge extremities. By better understanding
the role of antioxidant proteins and protein oxidation in health challenge response producers
will be better manage livestock through challenges, thus increasing production efficiency
with limited increases in inputs.

Acknowledgements

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towards to Dr. Ed Steadham and Dr.Wes Schweer for their assistance with this project.

Literature Cited

sensitivities of global cellular cysteine residues under reductive and oxidative stress. J.
Proteome Res. 15:2548–2559. doi:10.1021/acs.jproteome.6b00087.

C. M. Dekkers. 2011. Effects of ad libitum and restricted feed intake on growth performance
and body composition of Yorkshire pigs selected for reduced residual feed intake. J. Anim.


Two primary antibodies were used for the hyperoxidized peroxiredoxin analysis (peroxiredoxin-2 and hyperoxidized peroxiredoxin) so that hyperoxidized peroxiredoxin could be compared to an in gel reference and to the total peroxiredoxin-2 within a sample lane. In this way, hyperoxidized peroxiredoxin relative to peroxiredoxin-2 could be determined. Western blotting was performed to ensure no issue occurred as a result of using two primary antibodies. A was incubated a 1:10,000 dilution of Peroxiredoxin-2 primary antibody (ab109367, Abcam, Cambridge, UK). B was incubated with a 1:2,000 dilution of hyperoxidized peroxiredoxin primary antibody (peroxiredoxin-SO₃, ab16830, Abcam, Cambridge, UK) and a 1:10,000 dilution of Peroxiredoxin-2 primary antibody (ab109367, Abcam, Cambridge, UK). C was incubated with only a 1:10,000 dilution of Peroxiredoxin-2 primary antibody (ab109367, Abcam, Cambridge, UK). Primary incubations were carried out for approximately 20 hours at 4°C. Following primary incubation, the exact same western blot protocols were carried out for blot washing, secondary incubation, and imaging. Samples are labeled based on their feed efficiency and infection status: Low RFI Control (LC), Low RFI Inoculated (LI), High RFI Control (HC), and High RFI Inoculated (HI). Samples are the same in each of the presented images.
Figure 3.2 Representative diagonal gel electrophoresis image. A greater amount of protein spots was seen below the diagonal in the low RFI samples compared to high RFI counterparts. The four marked protein spots were selected for identification using liquid chromatography mass spectrometry (LC-MS). Spots 1 and 2 were both identified as glyceraldehyde 3-phosphate dehydrogenase. Spots 3 and 4 were identified as creatine kinase. Glyceraldehyde 3-phosphate dehydrogenase was selected for further analysis using western blot analysis.
Figure 3.3 Representative western blot of glyceraldehyde 3-phosphate dehydrogenase run on a non-reducing gel (A) and on a reducing gel (B) from sarcoplasmic protein extracts of longissimus muscle collected immediately following necropsy. Glyceraldehyde 3-phosphate dehydrogenase in samples was compared to a sarcoplasmic protein extract from an unaged longissimus sample (REF). Samples are labeled based on their feed efficiency and infection status: Low RFI Control (LC), Low RFI Inoculated (LI), High RFI Control (HC), and High RFI Inoculated (HI).
Figure 3.4 Visual depiction of the shift from glycolysis towards the pentose phosphate pathway mediated through the oxidation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) during periods of increased oxidative stress and hydrogen peroxide ($H_2O_2$) production. This shift towards the pentose phosphate pathway increases production of NADPH, leading to an increase in glutathione reduction through the action of glutathione reductase. This increase in reduced glutathione concentration may in turn lead to an increased reductive capacity, preventing increased protein oxidation. Figure adapted from Kuehne (2015).
**Figure 3.5** Representative western blot of total peroxiredoxin-2 run on a reducing gel. Samples were sarcoplasmic protein extracts from the longissimus muscle collected immediately following necropsy. All samples were compared to a sarcoplasmic protein extract from an unaged longissimus sample (REF). Samples are labeled based on their feed efficiency and infection status: Low RFI Control (LC), Low RFI Inoculated (LI), High RFI Control (HC), and High RFI Inoculated (HI).
Figure 3.6 Representative western blot of the peroxiredoxin-2 decamer run on a non-reducing gel. Samples were sarcoplasmic protein extracts from the longissimus muscle collected immediately following necropsy. All samples were compared to a sarcoplasmic protein fraction from an unaged longissimus sample (REF). Samples are labeled based on their feed efficiency and infection status: Low RFI Control (LC), Low RFI Inoculated (LI), High RFI Control (HC), and High RFI Inoculated (HI).
First, Slower Migrating Band

Second, Faster Migrating Band

Figure 3.7 Representative western blot of the peroxiredoxin-2 run on a non-reducing gel. Samples were sarcoplasmic protein extracts from the longissimus muscle collected immediately following necropsy. The first and second bands were both compared to the corresponding band in a sarcoplasmic protein extract from an unaged longissimus sample (REF). A comparison was also made between the bands within each sample by calculating a ratio of the second band to the total immunoreactive protein per lane. Samples are labeled based on their feed efficiency and infection status: Low RFI Control (LC), Low RFI Inoculated (LI), High RFI Control (HC), and High RFI Inoculated (HI).
Figure 3.8 Representative western blot of hyperoxidized peroxiredoxin run on a non-reducing gel. Samples were sarcoplasmic protein extracts from the longissimus muscle collected immediately following necropsy. Hyperoxidized peroxiredoxin in samples was compared to the corresponding band in a sarcoplasmic protein extract from an unaged longissimus sample (REF). A comparison was also made between hyperoxidized peroxiredoxin and peroxiredoxin-2 within each sample by creating a ratio of the hyperoxidized peroxiredoxin band to the total immunoreactive protein per lane. Samples are labeled based on their feed efficiency and infection status: Low RFI Control (LC), Low RFI Inoculated (LI), High RFI Control (HC), and High RFI Inoculated (HI).
Table 3.1 Diet composition, as fed basis

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>66.67</td>
</tr>
<tr>
<td>Soybean Meal</td>
<td>8.40</td>
</tr>
<tr>
<td>Corn DDGS$^1$</td>
<td>22.50</td>
</tr>
<tr>
<td>Fat</td>
<td>0.51</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.25</td>
</tr>
<tr>
<td>Salt</td>
<td>1.03</td>
</tr>
<tr>
<td>Vitamin-mineral Premix$^2$</td>
<td>0.13</td>
</tr>
<tr>
<td>Ronozyme Phytase$^3$</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Calculated composition

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ME, kcal/kg</td>
<td>3,400</td>
</tr>
<tr>
<td>Crude Protein, %</td>
<td>15.50</td>
</tr>
<tr>
<td>SID Lysine, %$^4$</td>
<td>0.87</td>
</tr>
<tr>
<td>STTD P, %$^5$</td>
<td>0.28</td>
</tr>
</tbody>
</table>

$^1$DDGS = dried distiller’s grains with solubles

$^2$Vitamin-mineral premix supplied (per kg of diet): 66,000,000 IU vitamin A, 12,120,000 IU vitamin D3, 35,200 IU vitamin E, 0.022 g vitamin B, 3.3 g riboflavin, 13.2 g D-pantothenic acid, 16.61 g niacin, 1.496 g ethoxyquin, 0.6 g I as ethylenediamine dihydroiodide, 0.133 g Se as sodium selenite, 1.6 g Cu as copper chloride, 4 g Mn as manganese oxide, 88 g Zn as zinc oxide, and 88 g Fe as ferrous carbonate and ferrous sulfate

$^3$Ronozyme phytase purchased from DSM Animal Nutrition (Heerlen, Netherlands)

$^4$SID= standard ileal digestible

$^5$STTP P= standardized total tract digestibility of phosphorous
Table 3.2 Growth performance parameters of low residual feed intake (LRFI) or high residual feed intake (HRFI) pigs in control and *Mycoplasma hyopneumoniae* and *Lawsonia intracellularis* (MhLi) health challenged groups prior to inoculation (21 day acclimation period) and in the 21 days following inoculation (0 dpi to 21 dpi).

<table>
<thead>
<tr>
<th>Trait</th>
<th>LRFI</th>
<th>HRFI</th>
<th>Control</th>
<th>MhLi</th>
<th>SEM</th>
<th>RFI Line</th>
<th>Infection Status</th>
<th>RFI Line* Infection Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADG&lt;sup&gt;2&lt;/sup&gt; Pre-Inoculation&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.82</td>
<td>0.94</td>
<td>0.86</td>
<td>0.89</td>
<td>0.043</td>
<td>0.065</td>
<td>0.642</td>
<td>0.340</td>
</tr>
<tr>
<td>ADG Post-Inoculation&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.69</td>
<td>0.65</td>
<td>0.85</td>
<td>0.49</td>
<td>0.051</td>
<td>0.590</td>
<td>&lt;0.0001</td>
<td>0.621</td>
</tr>
<tr>
<td>ADG Total&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.72</td>
<td>0.72</td>
<td>0.86</td>
<td>0.59</td>
<td>0.039</td>
<td>0.976</td>
<td>&lt;0.0001</td>
<td>0.477</td>
</tr>
<tr>
<td>ADFI&lt;sup&gt;6&lt;/sup&gt; Pre-Inoculation</td>
<td>2.09</td>
<td>2.61</td>
<td>2.42</td>
<td>2.27</td>
<td>0.067</td>
<td>&lt;0.0001</td>
<td>0.135</td>
<td>0.930</td>
</tr>
<tr>
<td>ADFI Post-Inoculation</td>
<td>2.33</td>
<td>2.80</td>
<td>2.96</td>
<td>2.16</td>
<td>0.092</td>
<td>0.002</td>
<td>&lt;0.0001</td>
<td>0.615</td>
</tr>
<tr>
<td>ADFI Total</td>
<td>2.21</td>
<td>2.70</td>
<td>2.69</td>
<td>2.22</td>
<td>0.061</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.673</td>
</tr>
<tr>
<td>G: F&lt;sup&gt;7&lt;/sup&gt; Pre-Inoculation</td>
<td>0.39</td>
<td>0.36</td>
<td>0.36</td>
<td>0.39</td>
<td>0.019</td>
<td>0.205</td>
<td>0.235</td>
<td>0.382</td>
</tr>
<tr>
<td>G: F Post-Inoculation</td>
<td>0.29</td>
<td>0.22</td>
<td>0.29</td>
<td>0.22</td>
<td>0.018</td>
<td>0.011</td>
<td>0.014</td>
<td>0.510</td>
</tr>
<tr>
<td>G: F Total</td>
<td>0.34</td>
<td>0.29</td>
<td>0.32</td>
<td>0.31</td>
<td>0.012</td>
<td>0.007</td>
<td>0.583</td>
<td>0.354</td>
</tr>
</tbody>
</table>

<sup>1</sup>Significant statistical differences (p≤0.05) are **bolded**. Statistical trends (0.10>p>0.05) were *italicized*

<sup>2</sup>ADG= Average Daily Gain (Kg)

<sup>3</sup>Pre-Inoculation= 21 day acclimation period prior to disease challenge inoculation (-21 dpi – 0dpi)
Post-Inoculation = 21 day period directly following disease challenge inoculation (0 dpi - 21dpi)

Total = 21 day acclimation period and 21 day period directly following disease challenge inoculation (-21 dpi - 21dpi)

ADFI = average daily feed Intake (Kg)

Kilograms of weight gained per kilograms of feed consumed
Table 3.3 Protein fractions, gel loading conditions, acrylamide percentages, and antibody dilutions used to determine peroxiredoxin-2 profile, hyperoxidized peroxiredoxin, and glyceraldehyde 3-phosphate dehydrogenase relative content in samples.

<table>
<thead>
<tr>
<th>Protein Measured</th>
<th>Muscle Fraction</th>
<th>β-mercaptoethanol Presence/Absence</th>
<th>Protein Load</th>
<th>Acrylamide Percentage</th>
<th>Reference</th>
<th>Primary Antibody Dilution</th>
<th>Secondary Antibody Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Peroxiredoxin-2&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Sarcoplasmic</td>
<td>Present</td>
<td>60 µg</td>
<td>15%</td>
<td>0 Day LD (Sarcoplasmic)</td>
<td>1:20,000</td>
<td>1:10,000 GAR&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Non-reducing Gel Peroxiredoxin-2&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Sarcoplasmic</td>
<td>Absent</td>
<td>60 µg</td>
<td>12%</td>
<td>0 Day LD (Sarcoplasmic)</td>
<td>1:15,000</td>
<td>1:10,000 GAR&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peroxiredoxin-2 Decamer&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Sarcoplasmic</td>
<td>Absent</td>
<td>80 µg</td>
<td>5%</td>
<td>0 Day LD (Sarcoplasmic)</td>
<td>1:10,000</td>
<td>1:5,000 GAR&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hyperoxidized Peroxiredoxin&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Sarcoplasmic</td>
<td>Absent</td>
<td>60 µg</td>
<td>12%</td>
<td>0 Day LD (Sarcoplasmic)</td>
<td>1:2,000&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1:5,000 GAR&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glyceraldehyde 3-Phosphate Dehydrogenase&lt;sup&gt;7&lt;/sup&gt;</td>
<td>Sarcoplasmic</td>
<td>Absent</td>
<td>60 µg</td>
<td>15%</td>
<td>0 Day Semitendinosus-Red Portion (Sarcoplasmic)</td>
<td>1:10,000&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1:10,000 GAM&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Acrylamide/Bis-Acrylamide ratio 100:1 for separating and stacking (5%), all analyses used 5% stacking gel excluding the Peroxiredoxin-2 Decamer test which utilized continuous 5% gels.

Primary and secondary antibody incubations were diluted in PBS-Tween solution (80mM Na$_2$HPO$_4$, 20mM NaH$_2$PO$_4$, 100mM NaCl, 0.1% [v: v] polyoxyethylene sorbitan monolaurate [Tween-20]).

GAR= goat-anti rabbit-HRP antibody -31460, Thermo Scientific, Rockford, IL

GAM= goat-anti mouse-HRP antibody- A2554, Sigma Aldrich, Darmstadt, Germany

Peroxiredoxin-2 primary antibody- ab109367, Abcam, Cambridge, UK

Two primary antibodies were used for the hyperoxidized peroxiredoxin analysis (peroxiredoxin-2 and hyperoxidized peroxiredoxin) so that hyperoxidized peroxiredoxin could be compared to an in-gel reference and to the total peroxiredoxin-2 within a sample lane. In this way hyperoxidized peroxiredoxin relative to the total peroxiredoxin-2 pool could be determined.

1:2,000, Hyperoxidized peroxiredoxin(peroxiredoxin-SO$_3$)- ab16830, Abcam, Cambridge, UK

1:10,000 Peroxiredoxin-2 primary antibody- ab109367, Abcam, Cambridge, UK

Glyceraldehyde 3-phosphate dehydrogenase primary antibody- MAB374, Millipore
**Table 3.4** Blood serum antibody titer of barrows (n = 12) inoculated with *Mycoplasma hyopneumoniae* and *Lawsonia intracellularis* (MhLi). Pigs were inoculated on 0 days post inoculation (dpi) and antibody titer was assessed on dpi 0, 14, and 21.

Serum antibody titers to *Mycoplasma hyopneumoniae* (Mh)\(^1\)

<table>
<thead>
<tr>
<th>Dpi</th>
<th>0</th>
<th>14</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (S: P ratio)(^2)</td>
<td>0.072</td>
<td>0.605</td>
<td>0.812</td>
</tr>
<tr>
<td>SE</td>
<td>0.026</td>
<td>0.074</td>
<td>0.063</td>
</tr>
<tr>
<td>Maximum</td>
<td>0.253</td>
<td>1.019</td>
<td>1.130</td>
</tr>
<tr>
<td>Minimum</td>
<td>-0.003</td>
<td>0.125</td>
<td>0.368</td>
</tr>
</tbody>
</table>

Serum antibody titer to *Lawsonia Intracellularis* (Li)\(^3\)

<table>
<thead>
<tr>
<th>Dpi</th>
<th>0</th>
<th>14</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (Percent Inhibition)</td>
<td>9.6</td>
<td>67.3</td>
<td>78.8</td>
</tr>
<tr>
<td>SE</td>
<td>2.4</td>
<td>6.8</td>
<td>3.5</td>
</tr>
<tr>
<td>Maximum</td>
<td>24.5</td>
<td>89.8</td>
<td>90.1</td>
</tr>
<tr>
<td>Minimum</td>
<td>-9.1</td>
<td>21.3</td>
<td>56.5</td>
</tr>
</tbody>
</table>

\(^1\) Serum antibody titers to *Mycoplasma hyopneumoniae* (Mh) in which an S: P ratio between 0.30 and 0.40 is suspect a S: P ratio > 0.40 is positive for Mh

\(^2\) S: P ratio = sample to positive ratio

\(^3\) Serum antibody titers to *Lawsonia intracellularis* (Li) in which percent inhibition between 20% and 30% is suspect and percent inhibition > 30 is positive for Li
Table 3.5 Main effects of residual feed intake (RFI) line, infection status (IS), and interactions of RFI line and Infection Status on peroxiredixin-2 (Prdx-2) features, and carbonyl content for the sarcoplastic protein fraction of the longissimus skeletal muscle for all groups of pigs.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Treatment</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RFI Line</td>
<td>Infection Status</td>
</tr>
<tr>
<td>N</td>
<td>LRFI</td>
<td>HRF</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Total Prdx-2</td>
<td>0.58</td>
<td>0.67</td>
</tr>
<tr>
<td>Prdx-2 Decamer</td>
<td>0.59</td>
<td>0.97</td>
</tr>
<tr>
<td>Prdx-2 Non-reducing Gel 1st Band</td>
<td>0.93</td>
<td>1.02</td>
</tr>
<tr>
<td>Prdx-2 Non-reducing Gel 2nd Band</td>
<td>0.89</td>
<td>1.07</td>
</tr>
<tr>
<td>Prdx-2 Non-reducing Gel Comparison</td>
<td>0.49</td>
<td>0.50</td>
</tr>
<tr>
<td>Hyperoxidized Peroxiredoxin</td>
<td>0.78</td>
<td>0.76</td>
</tr>
<tr>
<td>Hyperoxidized Prdx Comparison</td>
<td>0.48</td>
<td>0.42</td>
</tr>
<tr>
<td>Carbonyl Content (nM/mg protein)</td>
<td>8.39</td>
<td>8.65</td>
</tr>
</tbody>
</table>

1Significant statistical differences (p≤0.05) are **bolded**. Statistical trends (0.10>p>0.05) were *italicized*

2Relative protein content was determined using western blotting by comparing the densitometry of sample protein bands to a reference sample of 0 days aged sarcoplastic extract protein sample. Thus, the observed data are a ratio of sample to reference.

3Comparisons were made through a ratio of the second, faster migrating protein band, and all immunoreactive protein in sample lane

4Comparisons were made between hyperoxidized peroxiredoxin and all immunoreactive protein in sample lane
Table 3.6 Comparisons of RFI line*infection status combinations with main effects of residual feed intake (RFI) line and infection status (IS), and interactions of RFI line and Infection Status on peroxiredoxin-2 (Prdx-2) features, and carbonyl content for the sarcoplastic protein fraction of the longissimus skeletal muscle for RFI line* infection status.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Treatment</th>
<th>P-value (^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LRFI Control</td>
<td>HRFI Control</td>
</tr>
<tr>
<td>N</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Total Prdx-2 (^2)</td>
<td>0.55(^a)</td>
<td>0.65(^{ab})</td>
</tr>
<tr>
<td>Prdx-2 Decamer (^2)</td>
<td>0.55(^a)</td>
<td>0.92(^b)</td>
</tr>
<tr>
<td>Prdx-2 Non-reducing Gel 1(^{st}) Band (^2)</td>
<td>0.91(^a)</td>
<td>1.10(^b)</td>
</tr>
<tr>
<td>Prdx-2 Non-reducing Gel 2(^{nd}) Band (^2)</td>
<td>0.90(^{ab})</td>
<td>1.03(^{bc})</td>
</tr>
<tr>
<td>Prdx-2 Non-reducing Gel Comparison (^3)</td>
<td>0.49(^a)</td>
<td>0.48(^a)</td>
</tr>
<tr>
<td>Hyperoxidized Peroxiredoxin (^2)</td>
<td>0.89(^a)</td>
<td>0.71(^a)</td>
</tr>
<tr>
<td>Hyperoxidized Prdx Comparison (^4)</td>
<td>0.50(^a)</td>
<td>0.39(^b)</td>
</tr>
<tr>
<td>Carbonyl Content (nM/mg protein)</td>
<td>7.34(^a)</td>
<td>9.61(^a)</td>
</tr>
</tbody>
</table>

\(^{a,b,c}\) Means with differing subscripts in the same row indicate a significant difference (P\(\leq\)0.05).

\(^1\)Significant statistical differences (p\(\leq\)0.05) are \textbf{bolded}. Statistical trends (0.10>p>0.05) were \textit{italicized}

\(^2\)Relative protein content was determined using western blotting by comparing the densitometry of sample protein bands to a reference sample of 0 days aged sarcoplastic extract protein sample. Thus, the observed data are a ratio of sample to reference.

\(^3\)Comparisons were made through a ratio of the second, faster migrating protein band, and all immunoreactive protein in sample lane

\(^4\)Comparisons were made between hyperoxidized peroxiredoxin and all immunoreactive protein in sample lane
Table 3.7 Protein spots identified from diagonal gel electrophoresis with individual peptides and Mowse Scores.

<table>
<thead>
<tr>
<th>Spot Number</th>
<th>Protein ID</th>
<th>Species</th>
<th>Accession Number</th>
<th>Coverage(^1)</th>
<th>Peptides</th>
<th>Mowse Score(^2)</th>
</tr>
</thead>
</table>
| Spot 3      | Creatine kinase | Sus scrofa | Q5XLD3            | 52.23%         | FCVGLQK  
GGDDLDPNYVLSSR  
LNFKAEVEYPDSLK  
ALTELIEYKK  
FEEILTR  
SFLVWVNEEDHLR  
LSEALNLTEFEK  
DLFDPIIQDR  
LGSSVEQVQLVVDGVK  
GTGGVDTAAVGSVFDVSNADR  
RGTGVDTAAVGSVFDVSNADR  
ALTELIEYK  
GYTLPHPHCSR  
GIWHNDNK  
TDLNHELNLK  
LMVEMEK  
HKTDLNHELNLK  
GQSIDDMPAQK  
AEEEYPDSLK  
GQSIDDMPAQK  
PFGNTHNK  
IEEIFKK  
HGGYKPTDK | 6928          |
<table>
<thead>
<tr>
<th>Spot Location</th>
<th>Protein ID</th>
<th>Species</th>
<th>Accession Number</th>
<th>Coverage¹</th>
<th>Peptides</th>
<th>Mowse Score²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spot 4</td>
<td>Creatine kinase</td>
<td>Sus scrofa</td>
<td>Q5XLD3</td>
<td>33.59%</td>
<td>GTGGVDTAAVGSFVDVSNADR RGTGGVDTAAVGSFVDVSNADR LGSSEVEQVQLVVDGVK DLFPIIQDR LSVEALNSLTGEFK SFLVWVNEEDHLR GGDDLDPNYVISSR TDLNHENLK GQSIDDMIPAQK AEEEYPDLSK FCVGLQK</td>
<td>4332</td>
</tr>
<tr>
<td>Spot 1</td>
<td>Glyceraldehyde -3-phosphate dehydrogenase</td>
<td>Sus scrofa</td>
<td>P00355</td>
<td>48.05%</td>
<td>TVDGPSAKLWR IVSNASCTTNCLAPIAK LTGMAFR LEKPAKYDDIK VGVNGFGR VGVNGFGR VVDLMVHMASKE VVDLMVHMASK</td>
<td>5678</td>
</tr>
<tr>
<td>Spot Location</td>
<td>Protein ID</td>
<td>Species</td>
<td>Accession Number</td>
<td>Coverage¹</td>
<td>Peptides</td>
<td>Mowse Score²</td>
</tr>
<tr>
<td>---------------</td>
<td>---------------------------------</td>
<td>--------------</td>
<td>------------------</td>
<td>------------</td>
<td>---------------------------------------------------------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Spot 2</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Sus scrofa</td>
<td>P00355</td>
<td>48.65%</td>
<td>TVDGPSGK IVSNASCTTNCLAPLAK GAAQNIIPASTGAAKAVGK AITIFQER LISWYDNEFGYSNR RVIISAPSADAPMFVMGVNHEK AITIFQERDPANIK VIISAPSADAPMFVMGVNHEK VPTPNVSVVDLTCR LTGMAFR AENGKLVINGK QASEGPLK VIELNGK GAAQNIIPASTGAAK VGVNGFGR VVDLMVHMASKE VVDLMVHMASK VGVNGFGR</td>
<td>9302</td>
</tr>
</tbody>
</table>

¹Percentage of the MASCOT protein sequence covered by identified matching peptides from trypsin digest

²MOWSE = Molecular weight search, score used to calculate the similarity in molecular weight of the peptides from trypsin digest and the proteins from the MASCOT database
Table 3.8 Main effects of residual feed intake (RFI) line, infection status (IS), and interactions of RFI line and Infection Status on glyceraldehyde 3-phosphate dehydrogenase non-reducing gel western blot analysis (A). Comparisons of RFI line*infection status combinations with main effects of residual feed intake (RFI) line and infection status (IS), and interactions of RFI line and Infection Status on glyceraldehyde 3-phosphate dehydrogenase non-reducing gel western blot analysis (B).

A

<table>
<thead>
<tr>
<th>Trait</th>
<th>Treatment</th>
<th>P-value(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RFI Line</td>
<td>Infection Status</td>
</tr>
<tr>
<td>N</td>
<td>LRFI</td>
<td>HRFI</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate Dehydrogenase(^2)</td>
<td>.69</td>
<td>0.66</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Trait</th>
<th>Treatment</th>
<th>P-value(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RFI Line</td>
<td>Infection Status</td>
</tr>
<tr>
<td>N</td>
<td>LRFI Control</td>
<td>HRFI Control</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate Dehydrogenase(^2)</td>
<td>0.64(^{ab})</td>
<td>0.35(^{a})</td>
</tr>
</tbody>
</table>

\(^{a,b}\) Means with differing subscripts in the same row indicate a significant difference (P≤0.05).  

\(^1\)Significant statistical differences (p≤0.05) in **bold**. Statistical trends (0.10>p>0.05) were *italicized*  

\(^2\)Relative protein content was determined using western blotting by comparing the densitometry of sample protein band to a reference sample of 0 days aged sarcoplasmic extract protein sample. Thus, the observed data are a ratio of sample to reference.
Table 3.9 Correlations, with $P$-values in parentheses$^1$, of *Lawsonia intracellularis* (Li) or *Mycoplasma hyopneumoniae* (Mh) 21 days post infection antibody responses (peak) and growth traits, peroxiredoxin-2 (Prdx-2) features, or carbonyl content for all infected pigs (n=11).

<table>
<thead>
<tr>
<th>Trait</th>
<th>Li</th>
<th>Mh</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth Weight</td>
<td>0.246</td>
<td>-0.266</td>
</tr>
<tr>
<td></td>
<td>(0.47)</td>
<td>(0.43)</td>
</tr>
<tr>
<td>Weaning Weight</td>
<td>0.542</td>
<td>-0.485</td>
</tr>
<tr>
<td></td>
<td>(0.09)</td>
<td>(0.13)</td>
</tr>
<tr>
<td>Start Weight</td>
<td>-0.181</td>
<td>-0.527</td>
</tr>
<tr>
<td></td>
<td>(0.59)</td>
<td>(0.10)</td>
</tr>
<tr>
<td>Necropsy Weight</td>
<td>-0.060</td>
<td><strong>-0.609</strong></td>
</tr>
<tr>
<td></td>
<td>(0.86)</td>
<td><strong>(0.05)</strong></td>
</tr>
<tr>
<td>Average Daily Gain</td>
<td>0.526</td>
<td>-0.237</td>
</tr>
<tr>
<td></td>
<td>(0.10)</td>
<td>(0.48)</td>
</tr>
<tr>
<td>Daily Feed Intake</td>
<td>0.037</td>
<td>-0.309</td>
</tr>
<tr>
<td></td>
<td>(0.92)</td>
<td>(0.356)</td>
</tr>
<tr>
<td>Total Prdx-2$^2$</td>
<td>-0.435</td>
<td>-0.010</td>
</tr>
<tr>
<td></td>
<td>(0.18)</td>
<td>(0.98)</td>
</tr>
<tr>
<td>Prdx-2 Decamer$^2$</td>
<td>-0.412</td>
<td>0.034</td>
</tr>
<tr>
<td></td>
<td>(0.21)</td>
<td>(0.92)</td>
</tr>
<tr>
<td>Prdx-2 Non-reducing Gel 1$^{st}$ Band$^2$</td>
<td>0.198</td>
<td>0.276</td>
</tr>
<tr>
<td></td>
<td>(0.56)</td>
<td>(0.41)</td>
</tr>
<tr>
<td>Prdx-2 Non-reducing Gel 2$^{nd}$ Band$^2$</td>
<td>-0.457</td>
<td>-0.145</td>
</tr>
<tr>
<td></td>
<td>(0.16)</td>
<td>(0.67)</td>
</tr>
<tr>
<td>Prdx-2 Non-reducing Gel Band Comparison$^3$</td>
<td><strong>-0.680</strong></td>
<td>-0.097</td>
</tr>
<tr>
<td></td>
<td><strong>(0.02)</strong></td>
<td>(0.78)</td>
</tr>
<tr>
<td>Hyperoxidized Peroxiredoxin$^2$</td>
<td>-0.334</td>
<td>-0.298</td>
</tr>
<tr>
<td></td>
<td>(0.32)</td>
<td>(0.373)</td>
</tr>
<tr>
<td>Hyperoxidized Peroxiredoxin</td>
<td>0.007</td>
<td>-0.095</td>
</tr>
<tr>
<td>Comparison to Non-reduced Prdx-2$^4$</td>
<td>(0.98)</td>
<td>(0.78)</td>
</tr>
<tr>
<td>Carbonyl Content</td>
<td>0.299</td>
<td><strong>0.539</strong></td>
</tr>
<tr>
<td>(nM/mg sarcoplasmic protein)</td>
<td>(0.37)</td>
<td><strong>(0.09)</strong></td>
</tr>
<tr>
<td>Calpain I$^5$</td>
<td>0.070</td>
<td>0.168</td>
</tr>
<tr>
<td></td>
<td>(0.84)</td>
<td>(0.62)</td>
</tr>
<tr>
<td></td>
<td>Ratio (Sem)</td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------------</td>
<td></td>
</tr>
<tr>
<td>Calpain II</td>
<td>-0.071 (0.83)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.556 (0.07)</td>
<td></td>
</tr>
<tr>
<td>Calpastatin I</td>
<td>-0.186 (0.58)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.244 (0.47)</td>
<td></td>
</tr>
<tr>
<td>Calpastatin II</td>
<td>-0.047 (0.89)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.478 (0.14)</td>
<td></td>
</tr>
<tr>
<td>Total Calpastatin</td>
<td>-0.108 (0.75)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.370 ((0.26)</td>
<td></td>
</tr>
<tr>
<td>Calpain I:Total Calpastatin</td>
<td>-0.091 (0.79)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.144 (0.67)</td>
<td></td>
</tr>
</tbody>
</table>

1Significant statistical differences (p≤0.05) are **bolded**. Statistical trends (0.10>p>0.05) were *italicized*.

2Relative protein content was determined using western blotting by comparing the densitometry of sample protein bands to a reference sample of 0 days aged sarcoplasmic extract protein sample. Thus, the observed data are a ratio of sample to reference.

3Comparisons were made through a ratio of the second, faster migrating protein band, and all immunoreactive protein in sample lane.

4Comparisons were made between hyperoxidized peroxiredoxin and all immunoreactive protein in sample lane.

5Units of activity/g of protein.

6Ratio of Calpain 1 activity to total calpastatin activity.
Table 3.10 Correlations, with *P*-values in parentheses\(^1\), of carbonyl content and growth traits, or peroxiredoxin-2 (Prdx-2) features for all pigs (n=24), and for subsets of pigs including low RFI, high RFI, control, and MhLi challenged (n=12).

<table>
<thead>
<tr>
<th>Trait</th>
<th>All Pigs</th>
<th>Low</th>
<th>High</th>
<th>Control</th>
<th>MhLi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth Weight</td>
<td>0.039</td>
<td>-0.234</td>
<td>0.409</td>
<td>0.105</td>
<td>-0.043</td>
</tr>
<tr>
<td></td>
<td>(0.86)</td>
<td>(0.46)</td>
<td>(0.19)</td>
<td>(0.75)</td>
<td>(0.89)</td>
</tr>
<tr>
<td>Weaning Weight</td>
<td>-0.048</td>
<td>-0.240</td>
<td>0.182</td>
<td>0.135</td>
<td>-0.328</td>
</tr>
<tr>
<td></td>
<td>(0.82)</td>
<td>(0.45)</td>
<td>(0.57)</td>
<td>(0.68)</td>
<td>(0.30)</td>
</tr>
<tr>
<td>Start Weight</td>
<td>-0.074</td>
<td>-0.474</td>
<td>0.636</td>
<td>0.191</td>
<td>-0.443</td>
</tr>
<tr>
<td></td>
<td>(0.73)</td>
<td>(0.12)</td>
<td>(0.03)</td>
<td>(0.55)</td>
<td>(0.15)</td>
</tr>
<tr>
<td>Necropsy Weight</td>
<td>-0.090</td>
<td>-0.793</td>
<td>0.762</td>
<td>0.175</td>
<td>-0.531</td>
</tr>
<tr>
<td></td>
<td>(0.67)</td>
<td>(0.002)</td>
<td>(0.004)</td>
<td>(0.59)</td>
<td>(0.08)</td>
</tr>
<tr>
<td>Average Daily Gain</td>
<td>-0.10</td>
<td>-0.667</td>
<td>0.441</td>
<td>-0.176</td>
<td>-0.010</td>
</tr>
<tr>
<td></td>
<td>(0.66)</td>
<td>(0.02)</td>
<td>(0.15)</td>
<td>(0.59)</td>
<td>(0.76)</td>
</tr>
<tr>
<td>Daily Feed Intake</td>
<td>0.018</td>
<td>-0.427</td>
<td>0.439</td>
<td>0.462</td>
<td>-0.412</td>
</tr>
<tr>
<td></td>
<td>(0.93)</td>
<td>(0.17)</td>
<td>(0.15)</td>
<td>(0.13)</td>
<td>(0.18)</td>
</tr>
<tr>
<td>Total Prdx-2(^2)</td>
<td>-0.279</td>
<td>-0.520</td>
<td>0.092</td>
<td>-0.055</td>
<td>-0.470</td>
</tr>
<tr>
<td></td>
<td>(0.18)</td>
<td>(0.08)</td>
<td>(0.78)</td>
<td>(0.86)</td>
<td>(0.12)</td>
</tr>
<tr>
<td>Prdx-2 Decamer(^{2})</td>
<td>-0.030</td>
<td>-0.063</td>
<td>-0.046</td>
<td>0.156</td>
<td>-0.244</td>
</tr>
<tr>
<td></td>
<td>(0.89)</td>
<td>(0.85)</td>
<td>(0.89)</td>
<td>(0.63)</td>
<td>(0.44)</td>
</tr>
<tr>
<td>Prdx-2 Non-reducing Gel 1(^{st}) Band(^2)</td>
<td>0.195</td>
<td>0.346</td>
<td>0.035</td>
<td>0.329</td>
<td>0.057</td>
</tr>
<tr>
<td></td>
<td>(0.36)</td>
<td>(0.27)</td>
<td>(0.91)</td>
<td>(0.30)</td>
<td>(0.86)</td>
</tr>
<tr>
<td>Prdx-2 Non-reducing Gel 2(^{nd}) Band(^2)</td>
<td>-0.065</td>
<td>-0.015</td>
<td>-0.216</td>
<td>0.036</td>
<td>-0.184</td>
</tr>
<tr>
<td></td>
<td>(0.76)</td>
<td>(0.96)</td>
<td>(.50)</td>
<td>(0.91)</td>
<td>(0.57)</td>
</tr>
<tr>
<td>Prdx-2 Non-reducing Gel Band</td>
<td>-0.196</td>
<td>-0.478</td>
<td>-0.003</td>
<td>-0.517</td>
<td>-0.005</td>
</tr>
<tr>
<td>Comparison(^3)</td>
<td>(0.36)</td>
<td>(0.12)</td>
<td>(0.99)</td>
<td>(0.09)</td>
<td>(0.99)</td>
</tr>
<tr>
<td>Hyperoxidized Peroxiredoxin(^2)</td>
<td>-0.208</td>
<td>-0.559</td>
<td>0.453</td>
<td>-0.327</td>
<td>-0.008</td>
</tr>
<tr>
<td></td>
<td>(0.33)</td>
<td>(0.06)</td>
<td>(0.14)</td>
<td>(0.30)</td>
<td>(0.98)</td>
</tr>
<tr>
<td>Hyperoxidized Peroxiredoxin</td>
<td>-0.134</td>
<td>-0.264</td>
<td>-0.09</td>
<td>-0.187</td>
<td>-0.066</td>
</tr>
<tr>
<td>Comparison to Non-reduced Prdx-2(^4)</td>
<td>(0.53)</td>
<td>(0.41)</td>
<td>(0.78)</td>
<td>(0.56)</td>
<td>(0.84)</td>
</tr>
<tr>
<td>Calpain I(^5)</td>
<td>0.027</td>
<td>0.148</td>
<td>-0.205</td>
<td>0.471</td>
<td>-0.479</td>
</tr>
<tr>
<td></td>
<td>(0.90)</td>
<td>(0.65)</td>
<td>(0.52)</td>
<td>(0.12)</td>
<td>(0.11)</td>
</tr>
<tr>
<td>Calpain II(^5)</td>
<td>-0.032</td>
<td>-0.414</td>
<td>0.445</td>
<td>0.129</td>
<td>-0.224</td>
</tr>
<tr>
<td></td>
<td>(0.88)</td>
<td>(0.18)</td>
<td>(0.15)</td>
<td>(0.69)</td>
<td>(0.48)</td>
</tr>
</tbody>
</table>
Significant statistical differences (p≤0.05) are **bolded**. Statistical trends (0.10>p>0.05) were *italicized*

Relative protein content was determined using western blotting by comparing the densitometry of sample protein bands to a reference sample of 0 days aged sarcoplasmic extract protein sample. Thus, the observed data are a ratio of sample to reference.

Comparisons were made through a ratio of the second, faster migrating protein band, and all immunoreactive protein in sample lane

Comparisons were made between hyperoxidized peroxiredoxin and all immunoreactive protein in sample lane

Units of activity/g of protein

Ratio of Calpain 1 activity to total calpastatin activity

<table>
<thead>
<tr>
<th></th>
<th>Calpastatin I&lt;sup&gt;5&lt;/sup&gt;</th>
<th>Calpastatin II&lt;sup&gt;5&lt;/sup&gt;</th>
<th>Total Calpastatin&lt;sup&gt;5&lt;/sup&gt;</th>
<th>Calpain I:Total Calpastatin&lt;sup&gt;6&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.012 (0.95)</td>
<td>0.228 (0.48)</td>
<td>-0.161 (0.62)</td>
<td>0.032 (0.92)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.022 (0.92)</td>
<td>0.629 (0.06)</td>
<td>0.174 (0.59)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-0.051 (0.96)</td>
<td>0.353 (0.26)</td>
<td>0.104 (0.75)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.397 (0.20)</td>
<td>0.174 (0.59)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.011 (0.96)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.022 (0.92)</td>
<td>0.629 (0.06)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-0.051 (0.96)</td>
<td>0.353 (0.26)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.397 (0.20)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>Significant statistical differences (p≤0.05) are **bolded**. Statistical trends (0.10>p>0.05) were *italicized*
CHAPTER 4. GENERAL CONCLUSIONS

The objective of this study was to determine the impact of an oxidative stress challenge in the form of a dual respiratory and enteric bacterial health challenge on peroxiredoxin-2 (Prdx-2) protein profile and protein oxidation in the skeletal muscle of pigs selected for differing residual feed intake (RFI). It was originally hypothesized that differences would exist in the antioxidant protein Prdx-2’s profile based on RFI line and infection status and that high RFI and health challenged pigs would present a higher degree of protein oxidation due to increased oxidative stress.

As expected, pigs selected for low RFI and control pigs had improved feed efficiency relative to high RFI and MhLi counterparts. Differences were seen in Prdx-2 profile based on RFI line and infection status, however contrary to our original hypothesis no significant differences were seen in carbonyl content based on RFI line or infection status. This could be partially explained by the increase in GAPDH oxidation product, potentially leading to an increase in glutathione protein and a prevention of more widespread protein oxidation (Kuehne et al., 2015). The fact that neither Mycoplasma hyopneumoniae nor Lawsonia intracellularis directly target skeletal muscle may also partially explain the lack of difference in carbonyl content. Different results could be expected with a more extreme health challenge with a greater impact on skeletal muscle.

Reduced feed efficiency in high RFI pigs may be partially explained by increased Prdx-2 production, potentially to combat greater mitochondrial ROS production seen in previous work (Grubbs et al., 2013). The resulting increase in nutrient allocation towards antioxidant protein production could in turn lead to a subtle reduction in nutrients available for efficient growth.
Based on the observed increase in total Prdx-2 and Prdx-2 decamer in high RFI pigs and the increase in hyperoxidized peroxiredoxin relative to Prdx-2 in low RFI pigs, high RFI pigs appear to have a greater pool of total reduced Prdx-2. This suggests that high RFI pigs may be better able to respond to oxidative stress challenges, though this was not seen in the current study.

Taken as a whole, the results of this study suggest that animals of differing feed efficiency use antioxidant proteins such as Prdx-2 and manage oxidative stress challenges differently. Moreover, longissimus skeletal muscle exhibited limited protein oxidation in response to dual respiratory and enteric bacterial health challenge. Further research is needed to evaluate the impact of health challenges across a wide array of tissue types and health challenge extremities. By better understanding the role of antioxidant proteins and protein oxidation in health challenge response producers will be better manage livestock through challenges, thus increasing production efficiency with limited increases in inputs.

**Literature Cited**


