3B-103/3C-104: ENHANCING THE IRON CONTENT OF PORK TO PROMOTE HUMAN HEALTH BENEFITS

Report prepared for the Co-operative Research Centre for High Integrity Australian Pork

By

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Executive Summary

The iron content of pork meat is low in relation to lamb and beef even though it can vary considerably according to muscle type, with the 'redder' (more aerobic) muscles generally possessing higher iron levels. Australian pork currently does not meet the Australian Food Standards Code requirements of being a food that is "a good source" of iron, for to achieve such a rating the food must contain no less than 25% of the recommended daily intake (RDI) for that mineral. Furthermore, it appears that the iron content of Australian pork may be lower than that reported by other major pig-producing countries such as Canada and the USA. Evidence to date indicates that various nutritional strategies (e.g., adding more iron to the diet) to elevate the iron content in pork been largely unsuccessful suggesting that muscle iron storage is refractory to dietary iron content, the amount of which can vary in the diet according to the age and feeding phase of the pig. In this context, muscle-specific manipulation of iron levels by examination of the effects housing systems or dietary formulation may be possible assuming any increased iron absorption directly relates to a deposition in the muscle in redder/more aerobic fibre types.

Three experiments were conducted to test the general propositions that (i) pigs raised in deep-litter/eco-shelter systems will have more iron and myoglobin than their counterparts that are raised indoors ("conventional"); (ii) feeding diets lower in iron (to induce mild iron depletion) followed by feeding diets higher in iron (to induce iron repletion) will increase circulating serum iron levels and will increase levels of iron in a redder muscle type; and (iii) there will be differential expression of candidate genes implicated in muscle iron metabolism in relation to feeding different iron concentrations throughout the grower-finisher stages of growth.

In the first experiment, samples of muscle (*longissimus dorsi,* LD; and *rectus abdominus,* RA) were analyzed for iron and myoglobin from female pigs maintained in two housing treatments, C (conventional) or DL (deep litter), that were slaughtered at either 7, 10, 13, 16, 20, 24, 26, 30 or 35 weeks of age. Data obtained from this analysis revealed no effects of housing environment on iron, zinc or myoglobin contents in the muscles although the zinc and myoglobin contents were significantly affected by muscle type and slaughter age, such that an interaction between muscle type and slaughter age was present for zinc and myoglobin but for iron, the two muscles were represented by similar trends. The RA was higher in Fe, Zn and myoglobin concentration ($P<0.05$) than the LD. The concentration of Fe was highest at 7 weeks of age, while both Zn and myoglobin increased with age in the RA but not in the LD.

Experiment 2 built upon these results and examined the effects of feeding one of two grower-stage diets to female pigs for 8 weeks that differed in iron content ('High' iron' or 'Low' iron; 239 versus 50 ppm dietary iron) followed by a cross-over design for 7 weeks of further feeding with half of the 'High' iron pigs fed a high iron (248 ppm iron) finisher diet (High-High) while the other half were fed a low iron (71 ppm) diet (High-Low). A Basal group of pigs was slaughtered at approximately 10 kg live weight before the start of the grower stage. The same design was applied to the 'Low' Iron grower pigs, to create Low-High and Low-Low treatment groups, respectively. Pigs were killed commercially at the end of the grower and finisher stages, with blood samples obtained through the grower-finisher stages and muscle [*m. longissimus dorsi* (LD) and *m. rectus femorus* (RF)] and organ (liver, heart) samples obtained from the abattoir.

The results showed that increases in iron content were only found in the *m. rectus femorus* (RF) at the end of both the grower and finisher stages of growth in response to feeding the High iron diet and diet Low-High, respectively. The increase in iron content in the RF in

the Low-High treatment supports the dietary depletion/repletion model of action. There were no deleterious effects on production parameters despite diets differing markedly in dietary iron content, although pigs fed diet Low-High had more P2 fat depth at slaughter. The LD muscle from High iron pigs was darker and redder than in pigs fed Low iron, and pigs fed High-High had redder meat at the end of the finisher stage in the LD. The liver but not the heart stored iron in pigs fed diets High and diets Low-High and High-High.

In the final experiment, some candidate genes (myoglobin, ferritin, DMT1/SLC11A2, hemojuvelin, ferroportin and deoxyhaemoglobin) involved in iron metabolism and physiological regulation were assessed using RT-PCR methodology in both the LD and RF muscles at both the end of the grower stage and at the conclusion of the finisher stage. The predominant findings confirmed the blood and muscle results obtained in the previous experiment, and underscored the physiological regulation of iron metabolism in muscle; for example, ferroportin gene expression levels (ferroportin is a transmembrane protein that transports iron from the inside of a cell to the outside of it) supported the higher iron concentration in the *rectus femorus* muscle and indicated, physiologically, an increased excretion of iron from this muscle.

Collectively, data obtained from this project using this particular nutritional manipulation model indicate that it is not possible to manipulate, at the whole-pig level, the amount of iron contained in all muscles of the pig at slaughter. The redder muscle (*m. rectus femoris*) was more responsive to the depletion-repletion model implemented, however and as evidenced by blood iron and haemoglobin levels, the grower-finisher pig has tremendous homeostatic propensity to buffer three to four-fold differences in dietary iron intake thereby avoiding deficiency or overload of iron, even though it was arguable that the dietary iron levels (and hence intake) were indeed limiting; in this respect, it was difficult to achieve any lower levels of iron in the diets given the nature of the ingredients available for use.

As a result of the outcomes in this study the following recommendations have been made:

1. There would appear to be no benefit in feeding additional dietary iron *per se* to increase the iron content of muscle, except under circumstances where for example, there might be specific market segments/niches for higher iron pork; in this instance, redder muscle types will deposit more iron if it is supplied in excess to requirements in the diet.
2. Dietary iron levels in young pigs should be re-examined, which will take further research, to eliminate the possibility that over supply of dietary iron early in life is not having a detrimental affect on iron absorption (and hence deposition in muscle) later in life.

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1. Introduction

Pigs are born in an anaemic state and will remain anaemic throughout life unless provided with an exogenous iron supply early in life. Once pigs start to consume solid food after weaning, they receive iron from dietary sources. There are two forms of dietary iron: heme and nonheme. Heme iron is found mainly in animal (muscle) but also in blood products, and nonheme iron is found in plant foods such as grains, pulses and leafy materials. Heme iron is absorbed more efficiently across the gastrointestinal epithelium than nonheme iron. Most of the animal's iron is as haemoglobin with the next major sources as heme iron in cytochromes and myoglobin (10% - especially rich in red muscle), and finally non-heme iron in the form of ferritin (11-18%). Ferritin is the major storage form of Fe in the body with approximately 60% in liver and 40% in muscle and cells of the reticulo-endothethial system. Other nonheme complexes are hemosiderin, a storage depot, and one transport form, transferrin (West and Oates, 2008).

The iron status of pigs, like many animals, is regulated predominately by intestinal iron absorption. Iron absorption occurs predominantly in the duodenum and upper jejunum. A portion of the iron that enters the mucosal cells is retained and sequestered within ferritin. Intracellular intestinal iron is lost when epithelial cells are sloughed from the lining of the gastrointestinal tract, appearing in the faeces. The remaining iron traverses the mucosal cells, to be coupled to transferrin for transport through the circulatory system to various depots (bone marrow, muscle and liver). Despite the mechanism of iron uptake, normally only about 10% of the elemental iron entering the duodenum is absorbed (West and Oates, 2008). However, this value increases markedly with iron deficiency. In contrast, iron overload reduces but does not eliminate absorption. In addition, both anaemia and hypoxia increase iron absorption.

There are a number of dietary and physiological factors that are involved in the amount of iron that the animal absorbs. For instance, gastric acidity as well as solubilizing agents such as ascorbate (vitamin C) and citric acid prevents precipitation of the normally insoluble Fe^{3+}, although this is not the case for organic Fe due to the chelation of the Fe to an amino acid or protein (Fe proteinate). Phytic acid and polyphenols found in plant-based diets are major inhibitors of nonheme iron absorption (West and Oates, 2008). The absorption level of iron from the diet can vary with the age, species, nutritional and physiological status, food sources and the composition of the diet (chemical forms, levels of other dietary components including vitamins, protein types, fiber, secondary plant compounds). In this context, Australian research by Ponnampalan et al. (2009) reported in male pigs that feeding chicory inulin at 5% of the finisher diet significantly increased the haem iron content of pork compared with pigs fed without inulin in their ration, although the increase in muscle haem iron did not significantly increase the total iron content of pork, and feeding an organic iron supplement did not change the haem or total iron content of pork.

The literature shows that the muscle from pigs contains approximately half the level of iron of that observed in beef and lamb. Iron content however varies with muscle type and the pork shoulder contains more than twice the level in pork *longissimus dorsi*; the values were 13 and 5 mg/kg wet tissue, respectively (Leonhardt et al., 1997). From a survey of the literature, Rooke et al. (2010) reported a range of 3-30 mg/kg of iron in pork muscle with a mean value of 6.3 mg/kg. A wide range of iron content was observed in a study carried out among 21 muscle tissues in pig carcasses by Kim et al. (2008). Among the types of pork

muscles examined, *vastus intermedius* and *infraspanatus* muscles, as well as the *rectus femorus*, had the highest iron content while muscle *longssimus dorsi* had the lowest iron content (Kim et al., 2008). This trend of higher iron levels seemed to align well with increased redness of the muscle. Muscle-specific manipulation of iron levels may therefore be possible assuming any increased iron absorption directly relates to a deposition in the muscle in redder/more aerobic fibre types.

Currently, Australian pork does not meet the Australian Food Standards Code requirements of being a food that is "a good source" of iron; indeed and referring to Australian pork, Greenfield et al. (2009) stated "Pork can no longer be promoted as a source of iron" given the decline in iron content that has occurred over time. The average iron content of Australian pork is 0.63 mg/100g (0.5 to 1.5 mg/100g, Food Standards Australia New Zealand 2011), which is lower than that recorded by other major pig-producing countries such as Canada (0.8-1.4 mg/100g, Canada Pork 2008) and USA (0.8–1.95 mg/100g, USDA National Nutrient Database 2010**)**. To date, nutritional strategies to elevate the iron content in pork have been largely unsuccessful suggesting that muscle iron storage is refractory to dietary iron content, the amount of which can vary in the diet according to the age and feeding phase of the pig.

The overall aims of this project were as follows:

1. Examine whether female pigs raised in deep-litter/eco-shelter systems will have more iron and myoglobin than their counterparts that are raised indoors ("conventional") (because of greater levels of physical activity);
2. Investigate a model of feeding diets lower in iron (to induce mild iron depletion) followed by feeding diets higher in iron (to induce iron repletion) throughout the grower-finisher stages of growth, and assess its impact on muscle iron levels as well as blood and organ indicators of iron status; and
3. Examine the expression of a number of candidate genes implicated in muscle iron metabolism in relation to feeding different iron concentrations throughout the grower-finisher stages of growth.

2. Methodology

Experiment 1

Defining age-, weight- and housing-related changes in muscle myoglobin and iron levels in female pigs

Introduction

Existing frozen muscle samples collected as part of Dr Trezona-Murray's PhD thesis were used to indicate whether there were changes in muscle myoglobin and mineral (predominately iron but also zinc) levels in growing female pigs over time. Furthermore, we were interested in the effect of housing as it is known to increase the aerobic capacity of muscle due to increased expression of type IIA muscle fibres, which would almost certainly translate into more iron. Therefore, it is possible that pigs raised in deep-litter/eco-shelter systems have more iron than their counterparts that are raised indoors ("conventional") because of greater levels of physical activity.

Samples collected from two different muscles were analysed, the *longissimus dorsi* (LD) and the *rectus abdominus* (RA). The LD is considered to be a 'fast white' muscle and has a higher proportion of oxido-glycolytic and glycolytic fibres compared to the RA. The RA is considered to be an intermediate muscle and has a higher proportion of oxidative fibres. Glycolytic fibres have a lower myoglobin content compared to oxidative fibres.

In making use of these existing samples, we wished to identify if there were critical ages/weights at which myoglobin and mineral levels were altered that will help ascertain the sampling regimen in the *in vivo* experiment to be conducted. In addition, we wished to examine the variation in levels of muscle iron and myoglobin between individuals. This information would further allow us to determine the number of sample points and sample sizes required in the subsequent experiment.

Materials and Methods

One hundred and forty four Large White x Landrace female pigs were obtained from a high-health-status commercial piggery (Wandalup Farms Pty Ltd, Mandurah Western Australia) and transported to Medina Research Centre (Department of Agriculture and Food Western Australia) at the time of weaning (approximately 3 weeks of age, average LW 5.5 kg). Upon arrival pigs were weighed and identified with individually numbered ear tags. All pigs were individually weighed each week.

The average live weight of the pigs was 5.7 ± 0.09 kg. On arrival, the piglets were stratified by weaning weight into two housing treatments, C (conventional) or DL (deep litter). Pigs allocated to treatment C were stratified by weight into eight groups (therefore nine experimental pigs plus one spare pig per pen) and a slaughter age of 7, 10, 13, 16, 20, 24, 26, 30 and 35 weeks was randomly allocated to each pig within each group. Pigs in the DL treatment were stratified by weight into eight groups and each slaughter age was randomly allocated to the pigs within each group. All pigs had *ad libitum* access to fresh feed and cool fresh water. The Animal Ethics and Experimentation Committee of the Department of Agriculture and Food Western Australia approved the experimental procedures.

Housing
Pigs were group housed in conventional or deep-litter weaning facilities from three to nine weeks of age (approximately 20 kg LW) and then moved into conventional or deep-litter grower-finisher facilities:

(i) Conventional: On arrival each group of C pigs were allocated to a pen within a conventional, insulated weaner facility (eight pens) until nine weeks of age. The weaner pens were 3.3 x 1.2 m where the solid concrete lying area (1.3 x 1.2 m) was an enclosed kennel heated by an infrared heating lamp and fitted with a four-space feeder (1.15 m width). Fresh water was available *ad libitum*, via two wall mounted nipple drinkers located in the mesh-slatted area of the pen (2.0 x 1.2 m). At nine weeks each pen of pigs was randomly allocated to grower-finisher pens, which consisted of pens (total pen dimensions were 3.6 x 1.8 m) with partially slatted floors where the solid concrete floor was 2.25 x 1.8 m and the plastic slatted area was 1.35 x 1.8 m. The pens were within a naturally ventilated, insulated building. The pens were equipped with a single space feeder (30 cm wide), placed in one corner of the pen over the solid floor, and two nipple drinkers located on the side walls over the slatted area. The back wall of the pen (on the slatted area) was mesh, allowing pigs to have visual and physical contact with pigs in the adjoining pen. The sidewalls and the front wall of the pen were solid panels.

(ii) **Deep-litter:** On arrival the DL pigs were housed as one group in a low-cost deep-litter shelter fitted out for weaner pigs. The shelter was 14.4 m long by 9 m wide. There was a 3.6 x 9 m concrete feeding platform at the east end where four Trevaskis feeders (64 feeding spaces) and 10 Drik-o-mat drinking bowls were located. The bedded area was soil based and thickly bedded with barley straw. Straw bales were placed near the gates at the west end of the shelter to provide additional protection from the weather. At nine weeks of age the DL pigs were moved into one half of a grower finisher shelter that had been divided length ways by a solid timber fence into two pens. The shelter had solid timber walls to 1.2 m high and the roof (tunnel) was covered in an aluminium coated woven polyethylene tarpaulin, painted matt black on the inside. Each end of the shelter was fitted with mesh gates. The pen was 22 m long by 4.5 m wide and was equipped with a bulk feeder with eight feeding spaces. Six Drik-o-mat drinking bowls provided water. The feeders and drinkers were positioned on a raised, concrete feeding platform (3.6 x 4.5 m) at the east end of the shelter. The bedded area was a base of compacted limestone bedded thickly with barely straw. Fresh straw was added as required to maintain a dry lying area for all pigs at about 0.8 kg/pig day.

Slaughter protocol
Feed was removed from 12 hours prior to slaughter. Animals were transported to a commercial export abattoir (PPC Wholesale Food Services, Wooroloo, WA) (90 minute travel time) and slaughtered within two hours of arrival. Hot carcass weight (AUSMEAT Trim 13; head off, flare off, fore trotters off, hind trotters on) and P2 backfat thickness (Hennessey Grading Probe 4) were measured by abattoir staff as per normal commercial practice. Carcasses entered the chiller (2 $^{\circ}$C, airspeed 4 m/second) approximately 35 minutes after slaughter.

Approximately 5g of muscle, devoid of skin and fat, was collected from the hot carcass from the *longissimus dorsi* (LD; at the P2 site) and *rectus abdominus* (RA) and placed into 5 ml plastic vials. The samples were transported in ice and stored at -80 $^{\circ}$C and were then stored at -20^{o}C until analysis.

Mineral analyses
Analysis of iron and zinc in muscles was conducted at Murdoch University. Samples were stored at 4°C until subsequent freeze-drying. Samples were commercially freeze-dried using a Cuddon FD 1015 freeze dryer (Cuddon Freeze Dry, NZ**)** and 0.2 g dry matter per sample was weighed out in 50 ml digestion tubes for subsequent mineral analysis. Samples were prepared according to the USEPA method 200.3 (USEPA, 1991). Iron and zinc concentrations were determined on a Vista AX CCD simultaneous ICP-AES (Varian Australia Pty Ltd).

The myoglobin concentration in the muscles was determined using the method of Trout (1991) after homogenisation of muscle tissue in 0.04 mol/L phosphate buffer (pH 6.5) using a polytron (Kinematica Polytron, probe PT 10–35; Kinematica Gmbh, Luzern, Steinhofhalde, Switzerland). The assay was performed using a Shimadzu UV-1201 spectrophotometer.

Statistical analyses
The effect of slaughter age, muscle type (LD and RA) and housing treatment (conventional or deep litter) on Fe, Zn and myoglobin were determined using a general linear model in SAS for Windows version 9.0 (SAS Inst. Inc., Cay, NC). Slaughter age, muscle type and housing treatment were used as fixed effects in the model. There were no covariates used in the model. Interactions between all of the fixed effect were tested and removed ($P>0.05$) if not significant.

Results
There was no effect of housing (conventional or deep litter) treatment on Fe, Zn or myoglobin content in the muscle ($P>0.05$) (Table 1). The Fe, Zn and myoglobin contents were significantly affected by muscle type and slaughter age ($P<0.05$). Within this an interaction between muscle type and slaughter age was present for Zn and myoglobin only, meaning that for Fe, the two muscles were represented by similar trends. The RA was higher in Fe, Zn and myoglobin concentration ($P<0.05$) than the LD. The concentration of Fe was highest at 7 weeks of age, while both Zn and myoglobin increased with age in the RA but not in the LD (Table 1).

Discussion
The results of this analysis showed that housing type (conventional versus deep litter housing) had no influence on the mineral or myoglobin content of either the *m.longissimus dorsi* (LD) or *m.rectus abdominus* (RA). There is a dearth of information in the literature pertaining to the effects of housing systems on mineral accumulation in muscles, even though it is recognized that climatic and housing conditions can play a significant role in pig performance in an outdoor production system. Gentry et al. (2004) conducted a study where pigs were farrowed in either indoor crates or outdoor huts. At weaning, indoor- and outdoor-born pigs were allotted randomly to treatments arranged in a 2x2 factorial design with two birth (indoor vs. outdoor) and rearing (indoor vs. outdoor) environments. These authors reported differences in muscle fibre make-up and performance but did not measure blood hemoglobin or muscle iron levels, however they commented that it was "possible that pigs reared outdoors could have a higher plasma iron content than pigs reared indoors". In another study, Hoffman et al. (2003) compared the growth performance and the carcass and physical and chemical characteristics of the meat of Landrace x Large White pigs reared under a free-range [pigs were housed as a group in an 1800 m^2 pen, which had a group of conifers that provided shade as well as an A-frame hut (1.5 m x 1.5 m) built on the ground with a straw roof. The A-frame hut was filled with straw that was replaced as required] or a conventional housing system (pigs were housed indoors in a 100

m^2 pen that formed part of a group of pens that were inside a grower shed. This shed was enclosed with walls and had a roof – manually opening or closing windows controlled airflow. The floor of the pen was concrete with a third of the pen being concrete slats above the faeces and urine drainage channel). Despite a three-fold higher iron muscle content (*M. longissimus lumborum,* between the second and third lumbar vertebrae counting from the caudal end) in the free-range pigs (0.50 *versus* 0.16 mg/100 g muscle), the difference was not statistically significant.

As a result of the findings from this analysis, and from a reading of the relevant literature including the effects of simple supplementation of the diet with additional iron, it was concluded that the iron content of muscle – especially the white muscle – is extremely resilient to both environmental and nutritional influences. Consequently, an alternative experimental model needed to be developed to examine whether fortification of muscle by nutritional means could occur.

Table 1. Least squares means for Fe, Zn and myoglobin content (mg/kg of wet tissue) in the *m.longissimus dorsi* (LD) and *m.rectus abdominus* (RA) in relation to animal age at slaughter (weeks).

		Slaughter age (weeks)								
Item	*Muscle*	7	13	16	20	24	26	30	35	SEM
Fe	LD	7.3^{d}	4.2^{a}	4.5^{ab}	4.9^{ab}	4.8^{ab}	5.9^{c}	5.3^{bc}	4.6^{ab}	0.22
	RA	12.0^{a}	9.1^{b}	n/a	n/a	10.3^{c}	n/a	n/a	n/a	0.38
Zn	LD	10.7^{a}	10.8^{a}	11.5^{ab}	12.5^{ab}	15.4^{c}	15.7^{c}	14.4^{bc}	14.0^{bc}	1.21
	RA	30.0^{a}	37.1^{b}	n/a	n/a	46.5^{c}	n/a	n/a	n/a	1.32
Myoglobin	LD	0.07^{a}	0.07^{a}	0.06^{a}	0.08^{a}	0.12^{a}	0.07^{a}	0.10^{a}	0.11^{a}	0.165
	RA	1.85^{a}	2.57^{b}	n/a	n/a	3.13^{c}	n/a	n/a	n/a	0.071

^{a,b,c} Values within rows not having the same superscript are significantly different ($P<0.05$).
n/a: sample not available for analysis.
SEM: standard error of the mean.

Experiment 2

Mild dietary iron depletion improves muscle uptake of iron in red pork muscles during dietary iron repletion

Introduction

The results from Experiment 1 demonstrated that housing system and, to a lesser extent, age at slaughter, had little or no effect on levels of iron in the LD or RA, but that the RA muscle has greater levels of myoglobin and iron at selected time points. As a consequence, and following extensive discussions with relevant personnel in APL, the CRC, CSIRO and human nutrition experts, it was decided to establish an experimental model to test the proposition that the iron content in pig muscle could possibly be increased by imposing a dietary depletion (through exclusion of iron from the premix and using low iron ingredients to reduce muscle iron levels)/repletion regimen, that might then reset/recalibrate the muscle epithelial transport systems to overcompensate and absorb more iron. This is a new paradigm for pigs, and we are only aware of a single study in rodents (Huang et al., 2000) using a dietary iron depletion and repletion protocol, however this assessed the activities of aconitase (an essential enzyme in the tricarboxylic acid cycle) and iron regulatory protein 1 (IRP-1), which interacts with mRNA to control the levels of iron inside cells, and not muscle levels of iron.

This study aimed to investigate the hypothesis that induction of mild iron depletion followed by dietary repletion in pigs will increase circulating serum iron levels. Additionally, we hypothesized that induction of depletion followed by dietary repletion with a diet high in iron will increase levels of iron in the muscle and this will be more apparent in a redder muscle.

Materials and Methods

Both the Animal Ethics Committee of the Department of Agriculture and Food WA (AEC Number: 6-10-42) and the Murdoch University Animal Ethics Committee (NS 2393/11) approved this experiment.

Experimental design and procedures

Sixty-four female pigs of PIC genetics weighing approximately 10 kg were acquired from a commercial farm in Western Australia and transported to the Medina Research Station. Pigs were given an injection of iron dextran (200 mg) within the first few days of life as per standard commercial practice. Pigs were vaccinated against PCV2 on the farm as per commercial practice. Upon arrival, pigs were weighed, ear tagged, housed in pairs (space allowance of 0.4 m^2 per pig) and were fed an initial adjustment diet (Wesfeeds Weaner Pellet) for 14 days. After this period, 8 median-weight pigs underwent euthanasia (using sodium pentabarbitone) to establish an initial basal iron status. A 40 g piece of *m. longissimus dorsi and m. rectus femorus* was removed from each pig and frozen for subsequent analysis.

The remaining 48 pigs (eight pigs were kept as spares) were allocated on the basis of live weight to one of two grower-stage diets differing in iron content (called 'High Iron' or 'Low Iron', respectively; see Appendix 1 for diets), where they were fed on an *ad libitum* basis for eight weeks. For the first two weeks, pigs were kept in pens of three in crates (1.4 x 1.2 m) in a temperature-controlled room. For the remaining six weeks of this eight-week period, pigs were kept in a naturally ventilated grower house containing 64 individual pens (1 x 2 m). For the first three weeks of this six-week period the pigs were maintained in their same groups of three, and then for the final three weeks, pigs were

separated and kept individually. Pigs remained in this building for the rest of the experiment.

At the end of this eight-week period, eight pigs from each diet group (n=16) were selected for slaughter and sent to a commercial abattoir east of Perth (Linley Valley Pork) and slaughtered according to commercial practices. The same muscle samples as collected at the initial baseline point were collected and frozen immediately for later analysis.

The remaining 32 pigs were subject to a cross-over design with half of the 'High Iron' pigs fed a high iron finisher diet (High-High) while the other half were fed a low iron diet (High-Low). The same design was applied to the 'Low Iron' grower pigs, to create Low-High and Low-Low treatment groups, respectively (see Appendix 1). Therefore there were four separate finisher treatments having eight pigs per treatment combination. Pigs were fed their respective diets on an *ad libitum* for a further seven weeks after which time they were slaughtered at the same commercial abattoir, where the same muscle samples were taken.

Fresh water was available *ad libitum* throughout the experiment. Measurement of the pigs' live weight and feed intake were measured fortnightly.

Diets
The four diets are shown in Appendix 1.

Grower stage
Diets in the grower stage were formulated to contain approximately 14.6 MJ DE/kg and 19-20% CP and 1.1 % available lysine. The High Iron diet fed during the grower stage was achieved by including a premix into the diet at a rate of 1.5 kg/tonne of feed (the premix contained an analyzed iron content of 60,680 mg/kg of premix), and maximizing the use of ingredients high in iron, such as bloodmeal and meat and bone meal. This ensured that the amount of iron in the diet exceeded the pigs' requirement for iron (NRC, 1998). The Low Iron diet was achieved by including a premix (1.5 kg/tonne of feed) devoid of iron (the premix contained an analyzed level of 2,889 mg/kg iron), and maximizing the use of ingredients low in iron such as skim milk powder and whey powder. The analyzed iron content (Appendix 1) showed it to be slightly below the pigs' recommended requirement for pigs 10-50 kg live weight (NRC, 1998).

Finisher stage
Diets in the grower stage were formulated to contain approximately 13.4 MJ DE/kg, 17% crude protein and 0.75% available lysine. The High Iron diet fed during the finisher stage (fed to High-High and Low-High pigs) was achieved by including a premix into the diet at a rate of 1.5 kg/tonne of feed (the premix contained an analyzed iron content of 60,680 mg/kg of premix), and maximizing the use of ingredients high in iron such as bloodmeal and meat and bone meal. This ensured that the amount of iron in the diet exceeded the pigs' requirement for iron (NRC, 1998). The Low Iron diet fed during the finisher stage was achieved by including a premix (1.5 kg/tonne of feed) devoid of added iron (the premix contained an analyzed levels of 2,889 mg/kg iron), and maximizing the use of ingredients low in iron such as whey powder and lupins. The analyzed iron content (Appendix 1) showed it to be above the pigs' recommended requirement for pigs 50-120 kg live weight (NRC, 1998).

Blood sampling and analyses
A blood sample was taken the day after arrival for assessment of baseline measurements. During the grower stage, pigs were bled at three-weekly intervals and then two weeks until the end of this stage. During the finisher stage, pigs were bled at intervals of three

weeks, two weeks and two weeks until they were sent to the abattoir for slaughter. Blood samples were collected by jugular venipuncture using an 18-gauge needle with vacutainer following restraint with a snare. Samples were then stored for subsequent analysis.

All analyses (apart from haemoglobin) were conducted at the Animal Health Laboratories, DAFWA. Haemoglobin content was analyzed using the HemoCue® blood haemoglobin photometer (HemoCue® AB, Kuvettgatan 1 SE-262 71 Ängelholm, Sweden). This has previously been validated for use in pigs (Gannon et al., 2011). Plasma iron analysis was performed on an Olympus AU400 using the plasma iron reagent kit (OSR6186). Plasma glucose was performed on the Olympus AU400 using the glucose reagent kit (OSR6121). Iron concentration in the diets was performed on the AA280FS, after using the AOAC Official Method 999.10 for digestion (microwave digestion using a mix of nitric acid and hydrogen peroxide). Plasma ferritin was analyzed using an ELISA kit (USCN Life Sciences, Catalogue Number E90518Po). Plasma UIBC (unsaturated iron-binding capacity; this is an alternative to TIBC or total iron-binding capacity and hence to transferrin, that measures the blood's capacity to bind iron with transferrin. When iron stores are low, transferrin levels increase, while transferrin is low when there is too much iron. Usually about one third of the transferrin is being used to transport iron and hence blood serum has considerable extra iron-binding capacity, which is the UIBC; http://www.irondisorders.org/tests-to-determine-iron-levels/) was analyzed using UIBC kit (Beckman Coulter OSR61205, Lot Number 2566) on the AU400 auto-analyzer using commercially available calibrator and controls.

Muscle analysis

The *m. longissimus dorsi* (LD) *and m. rectus femorus* (RF) were excised from the carcasses as soon as possible after slaughter (typically 1-2 hours post mortem). A ~ 40 g sample of muscle was collected, and this was then sub-sampled for mineral analysis and RNA extraction (Experiment 3). The sub-sample (~ 2 g) to be used for subsequent RNA extraction was placed into a sterile specimen pot containing Ambion® RNA*later*® in a ratio of 1 g tissue:5 ml Ambion® RNA*later*®. It was then stored for 24 hours at 4 ^{o} C and then placed into a -20^{o} C freezer for longer-term storage.

For mineral analysis, the sub-sample was placed into a sterile digestion tube, placed on dry ice, and then frozen at -20^{o} C in the laboratory. It was subsequently freeze-dried using a Cuddon FD 1015 freeze-dryer (Cuddon Freeze Dry, Blenheim, New Zealand) prior to analysis. A 10 g sub-sample was also taken, stored on dry ice, and later frozen at -20^{o} C for subsequent myoglobin analysis.

For the mineral analysis, samples containing 0.2 g of dry matter were prepared according to the USEPA method (USEPA 1991). Iron and zinc concentrations were determined using a Vista AX CCD simultaneous ICP-AES (Varian Australia Pty Ltd, Mulgrave, Vic.). Mineral concentrations are presented as mg/kg of wet tissue.

The myoglobin concentration in the muscles was estimated using the method of Trout (1991) after homogenisation of muscle tissue in 0.04 mol/L phosphate buffer (pH 6.5) using a polytron (Kinematica Polytron, probe PT 10–35; Kinematica Gmbh, Luzern, Steinhofhalde, Switzerland). The assay was performed using a Shimadzu UV-1201 spectrophotometer.

Meat quality measurements

Twenty-four hours after slaughter, approximately 1 kg of the LD muscle (with fat and skin attached) was removed from the left side of the carcass in a posterior direction from the point of the last rib, and measurements of objective pork quality were carried out. The pH of the LD was determined using a portable pH/temperature meter. Percent drip loss was

calculated using the suspension method where an 80 g sample of meat devoid of skin and significant fat deposits was cut in duplicate from the LD. The sample was placed in netting and suspended in a sealed plastic container. The samples were refrigerated for 24 hours then removed from the container, gently blotted dry and weighed to determine drip loss. Surface lightness (L*), redness (a*) and yellowness (b*) were measured using a Minolta Chromameter CR-400.

Statistical analyses

Data were analysed using a general linear model procedure in SAS with the main fixed effects being dietary iron feeding regimen (treatment) for the initial eight-week grower period, and then the finisher dietary regimen for the following seven weeks. These effects were used to test the muscle mineral and myoglobin contents, blood analysis and meat quality data. Additional fixed effects of muscle type and time (weeks) and their interactions were used in the muscle mineral analysis and blood analysis models, respectively.

Because the method for measuring myoglobin is not specific to myoglobin (also measures cytochromes), the data produced cannot be used to calculate the number of moles of iron bound to the protein in order to calculate free non-heme iron. Because muscles differ in myoglobin content, this may have an effect on the free iron available to the consumer, thus in a separate analysis the iron in the muscle was adjusted for myoglobin content by including myoglobin concentration as a variable. This allowed for the analysis of differences in trends between total iron and for muscle iron content at a constant myoglobin concentration.

Statistical significance was accepted at $P<0.05$. One pig was unaccounted for at the abattoir in the final slaughter date and was not used in the data analysis.

Results

Muscle mineral content

The statistical output for the model used to test the muscle minerals and myoglobin content is shown in Table 2. All minerals and myoglobin content were higher in the RF compared to the LD muscles (P<0.0001; Table 3). The range of iron concentrations across all treatments was 2.92-11.93 mg/kg wet weight for the LD and 3.60-14.42 mg/kg wet weight for the RF, respectively. For the LD, iron levels were highest in the basal animals (P<0.05) while there were no further differences between dietary iron treatments across both treatment stages (Table 3). For the RF, a decline was observed from the initial basal level to the end of the grower stage, with the Low Iron group depleting further than the High Iron treatment (P<0.05). By the end of the finisher period all treatments produced RF muscles that had iron concentrations between 7.1 to 8.5 mg/kg except the Low-High treatment, which had a mean of 9.3 mg/kg.

Table 2. Statistical outputs for muscle mineral contents and myoglobin (Mb) at both the grower and finisher stages for pigs fed diets differing in iron content.

	Treatment			Muscle		
Term	NDF;DDF	F value	P value	NDF;DDF	F value	P value
Fe	6;96	6.07	<.0001	1;96	53.44	<.0001
Cu	6;96	13.14	<.0001	1;96	45.21	<.0001
Zn	6;96	4.09	0.0011	1;96	19.75	<.0001
Mb	6;96	5.86	<.0001	1;96	44.48	<.0001

Table 3. Least-squares means for muscle mineral contents and myoglobin (Mb) at both the grower and finisher stages for pigs fed diets differing in iron content. The 'Basal' measurement refers to baseline before pigs were placed on experimental diets.

Measure	Treatment							S.E.
	Grower stage			Finisher stage				
	Basal	High	Low	High-High	High-Low	Low-Low	Low-High	
	m.longisimus dorsi							
Fe (mg/kg)	7.91^{b}	4.69^{a}	4.31^{a}	5.14^{a}	4.28^{a}	4.01^{a}	4.20^{a}	0.393
Cu (mg/kg)	0.78^{b}	0.47^{a}	0.45^{a}	0.43^{a}	0.47^{ab}	0.36^{a}	0.37^{a}	0.048
Zn (mg/kg)	10.98^{ab}	9.06^{a}	10.84^{ab}	13.23^{b}	12.23^{b}	11.61^{ab}	11.11^{ab}	0.797
Mb (mg/g)	0.90^{a}	0.94^{a}	0.80^{a}	1.71^{c}	1.37^{bc}	1.15^{ab}	1.34^{bc}	0.123
	m.rectus femorus							
Fe (mg/kg)	9.80^{c}	7.01^{b}	5.54^{a}	7.17^{b}	8.48^{bc}	7.10^{b}	9.27^{c}	0.936
Cu (mg/kg)	1.11^{b}	0.69^{a}	0.61^{a}	0.58^{a}	0.59^{a}	0.57^{a}	0.78^{a}	0.075
Zn (mg/kg)	11.18^{a}	11.57^{a}	12.33^{ab}	14.71^{bc}	15.92^{c}	14.69^{bc}	17.54^{c}	1.377
Mb (mg/g)	1.37^{a}	1.95^{ab}	1.39^{a}	2.77^{c}	2.86^{c}	2.14^{bc}	2.40^{bc}	0.353

^{a,b,c} Within a row, statistical differences between treatments are denoted by different superscripts.

Table 4. Least-squares means for total muscle iron adjusted for myoglobin content.

Muscle	Treatment						
	Basal	High	Low	High-High	High-Low	Low-Low	Low-High
m.longisimus dorsi	8.32[b]	5.04[a]	4.86[a]	4.33[a]	3.98[a]	4.04[a]	3.95[a]
± SE	0.37	0.36	0.38	0.42	0.36	0.35	0.38
m.rectus femorus	10.57[c]	7.18[ab]	6.29[a]	6.51[ab]	7.73[ab]	7.08[ab]	8.98[abc]
± SE	0.91	0.88	0.91	0.90	0.91	0.87	0.94

[a,b,c] Within a row, statistical differences between treatments are denoted by different superscripts.

Finisher animals had more myoglobin than grower animals (P<0.05), while grower animals had similar myoglobin concentrations to the basal animals with the High-Iron grower trending towards higher concentrations (Table 3). The High-High group had the highest myoglobin concentrations (High-Low also in the RF). There was however no correlation with dietary treatment and myoglobin concentration.

There was a decrease in copper from the basal level to all other treatments for both muscles (P<0.05; Table 3). Conversely, zinc increased over the finisher period in both muscles to amount to mostly higher zinc levels compared to the grower and basal animals (P<0.05; Table 3).

Although adjusting the iron content for myoglobin noticeably changed the iron levels, the trends previously observed remained mostly the same (Table 4). The greatest change was observed in the basal animals (and is possibly an over-estimation of iron in these animals).

Blood iron and haemoglobin levels

Grower stage
Blood iron concentrations and haemoglobin increased with time in the High-Iron group (P<0.05). In the Low Iron group, an initial decline at week 3 in both iron and haemoglobin was observed, but both increased at week 5 and from week 8 to finish with similar levels to those at week 0. However, these levels were still lower than the High Iron group, by about 10 μm/L iron and 1.5% haemoglobin, than the High Iron group (P<0.05; Figure 1 and 2; Table 5 and 6).

There was no difference in blood glucose between diet types, however there was a variation with time over the grower period (P<0.05).

Finisher stage
Blood iron increased with time for both the Low-High and Low-Low groups during the finisher periods (P<0.05), which was also correlated with an increase in percent haemoglobin (P<0.05; Figures 1 and 2, Tables 7 and 8). There was no apparent increase in blood iron or haemoglobin for the remaining two groups when compared to those levels at the beginning of the finisher period, except a slight increase in haemoglobin in the High-High group. The Low-Low group finished with the highest blood iron concentrations, having just over 6 μm/L more iron than the High-High pigs. There was no difference in blood glucose between diet types, however there was a variation with time over the finisher period (P<0.05).

Table 5. Statistical outputs for growth performance and blood analyses during the grower period.

	Treatment			Week			Treatment*week		
	NDF;DDF	F value	P value	NDF;DDF	F value	P value	NDF;DDF	F value	P
Liveweight	1;138	0.53	0.468	3;138	2509	<.0001	3;138	0.62	0.604
Daily gain	1;138	0.08	0.779	2;138	116.3	<.0001	2;138	1.55	0.216
Daily consumption	1;138	3.03	0.084	2;138	5031	<.0001	2;138	3.03	0.052
Feed conversion	1;138	0.01	0.929	2;138	39.74	<.0001	2;138	0.8	0.451
Iron	1;138	36.94	<.0001	3;138	7.66	<.0001	3;138	18.16	<.0001
Hemoglobin	1;138	46.57	<.0001	3;138	25.91	<.0001	3;138	23.96	<.0001
Glucose	1;138	0.31	0.579	3;138	33.44	<.0001	3;138	1.64	0.183

Table 6. Least-squares means of blood analyses and performance data during the grower stage.

	Week 0		Week 3*		Week 5*		Week 8*		
	High	Low	High	Low	High	Low	High	Low	SEM
Liveweight (kg)	10.4	10.4	22.2	23.5	32.2	33.3	53.3	53.8	0.84
Iron (μm/l)	22.7^{bcd}	22.3^{bc}	28.4^{de}	8.7^{a}	26.3^{cde}	24.7^{cde}	29.0^{e}	18.2^{b}	1.60
Haemoglobin (%)	11.1^{b}	11.1^{b}	12.5^{c}	10.7^{a}	12.8^{cd}	11.1^{b}	12.9^{d}	11.3^{b}	0.17
Glucose (mM)	5.9	5.9	6.9	6.9	6.3	6.4	6.2	5.9	0.12
Daily gain (kg)	-	-	0.56	0.62	0.62	0.65	1.00	0.98	0.027
Consumption (kg)	-	-	0.99	1.05	1.37	1.36	2.25	2.25	0.013
Feed conversion	-	-	1.83	1.73	1.96	2.01	2.29	2.34	0.068

^{a,b,c,d,e} Within a row, statistical differences between treatments are denoted by different superscripts.

Table 7. Statistical outputs for growth performance and blood analyses during the finisher period.

	Treatment			Week			Treatment*week		
	NDF;DDF	F value	P value	NDF;DDF	F value	P value	NDF;DDF	F value	P value
Liveweight	3;168	0.81	0.4914	6;168	2934.35	<.0001	18;168	1.11	0.3474
Daily gain	3;140	1.07	0.3624	5;140	52.83	<.0001	15;140	0.77	0.7081
Consumption	3;140	0.99	0.4007	5;140	770.69	<.0001	15;140	1.4	0.1565
Feed conversion	3;140	0.5	0.6849	5;140	25.07	<.0001	15;140	0.49	0.9444
Iron	3;168	4.38	0.0054	6;168	13.65	<.0001	18;168	5.2	<.0001
Haemoglobin	3;168	5.59	0.0011	6;168	75.84	<.0001	18;168	4.48	<.0001
Glucose	3;167	0.92	0.4309	6;167	73.61	<.0001	18;167	1.32	0.1809

Table 8. Least-squares means of blood analyses and performance data during the finisher stage.

	Week 11				Week 13				Week 15				
	High-High	High-Low	Low-High	Low-Low	High-High	High-Low	Low-High	Low-Low	High-High	High-Low	Low-High	Low-Low	SEM
Live weight (kg)	77.1	73.9	76.2	71.9	89.1	86.9	88.4	83.5	102.2	100.3	101.7	95.2	2.14
Iron (μm/l)[1]	25.6^{c}	28.1^{b}	31.1^{bd}	28.6^{b}	27.7^{b}	26.1^{c}	28.3^{b}	25.8^{c}	29.0^{b}	33.4^{bd}	30.7^{bcd}	35.8^{d}	2.44
Haemoglobin (%)[2]	13.2^{b}	13.5^{b}	12.6^{b}	13.3^{b}	14.3^{c}	14.2^{c}	13.6^{bd}	14.6^{c}	14.0^{cd}	13.6^{bd}	13.2^{b}	13.8^{bd}	0.32
Glucose (mM)	5.6	5.5	5.9	5.6	5.0	5.2	5.1	4.9	5.0	5.1	5.3	4.9	0.18
Daily gain (kg)	1.03	1.02	0.98	0.94	0.85	0.93	0.87	0.83	0.94	0.96	0.95	0.84	0.051
Consumption (kg)	2.83	2.83	2.78	2.72	3.05	3.18	2.85	2.97	3.08	3.34	3.01	3.21	0.088
Feed conversion	2.78	2.81	2.86	2.92	3.59	3.46	3.43	3.63	4.52	3.48	3.33	3.99	0.347

[a,b,c] Within a row, statistical differences between treatments and week for iron and haemoglobin are denoted by different superscripts.

[1] Week 8 iron values are not included but are used for comparison. These values were classed as [a] for the Low Iron grower group and [bc] for the High Iron group. See Table 5

[2] Week 8 haemoglobin values are not included but are used for comparison. These values were classed as [a] for the Low Iron grower group and [b] for the High Iron group. See Table 5

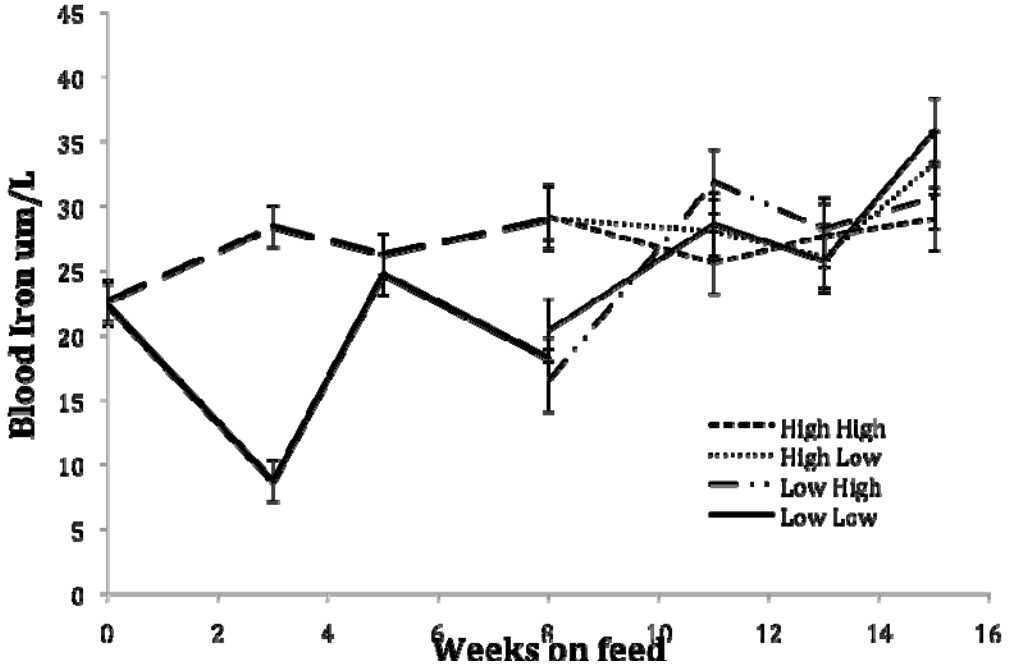

Figure 1. Blood iron concentrations of growing pigs at two feeding stages, grower [weeks 0 to 8: depicted as High (Iron) and Low (Iron)] and finisher (weeks 8 to 15: depicted as High High; High Low; Low High; Low Low). Data are ± SE of the mean.

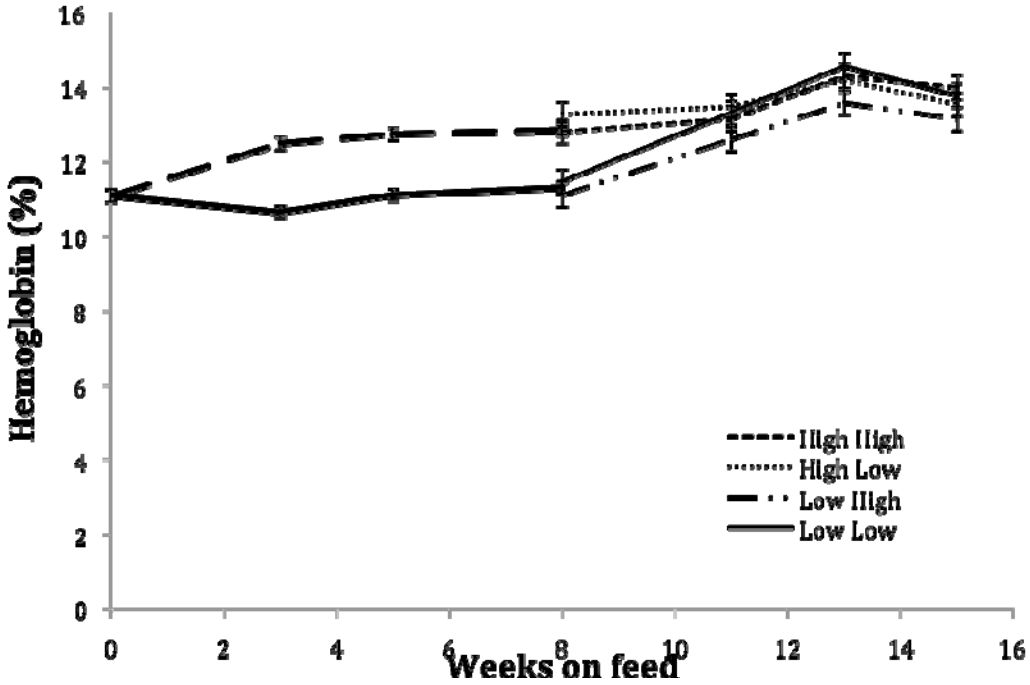

Figure 2. Percentage haemoglobin in the blood of growing pigs at two feeding stages, grower [weeks 0 to 8: depicted as High (Iron) and Low (Iron)] and finisher (weeks 8 to 15: depicted as High High; High Low; Low High; Low Low). Data are ± SE of the mean.

Blood ferritin and UIBC (unsaturated iron binding capacity) levels

There was a main effect of diet (P=0.009), and a tendency (P=0.095) for an interaction between diet and week, for ferritin levels in the finisher stage (Tables 9 and 11; Figure 3). There was a diet x week effect (P<0.001) for the levels of UIBC measured in the plasma in both the grower stage and the finisher stage, with levels increasing over time in the grower stage and decreasing over time in the finisher stage (Tables 9, 10 and 11; Figure 4).

Table 9. Statistical outputs for the effects of diet, week, and diet x week on plasma levels of ferritin and UIBC in the grower and finisher stages of growth.

	Diet			Week			Diet x Week		
	NDF; DDF	F-Value	P-Value	NDF; DDF	F-Value	P-Value	NDF; DDF	F-Value	P-Value
	Grower Stage								
Ferritin	1;138	2.24	0.137	3;138	12.36	<.001	3;138	0.61	0.607
UIBC	1;138	40	<.001	3;138	112.6	<.001	3;138	19.96	<.001
	Finisher Stage								
Ferritin	3;83	4.16	0.009	3;83	6.44	<.001	9;83	1.73	0.095
UIBC	3;84	0.03	0.994	3;84	121.2	<.001	9;84	8.11	<.001

Table 10. Least-squares means for the levels of plasma ferritin (ng/ml) and UIBC (μmol/L) for pigs fed High or Low iron during the grower phase.

	Week				±SEM
	0	3	5	8	
	Ferritin				
High	50.0	73.0	86.6	86.6	9.31
Low	54.8	90.9	93.2	109.9	9.31
	UIBC				
High	51.1	69.9	77.4	80.1	2.61
Low	53.3	105.5	87.8	94.0	2.61

Table 11. Least-squares means for the levels of plasma ferritin ferritin (ng/ml) and UIBC (μmol/L) for pigs fed different diets during the finisher phase.

	Week				±SEM
	8	11	13	15	
	Ferritin				
High High	106.0	95.1	187.0	105.6	19.46
High Low	72.1	91.4	88.5	89.6	19.46
Low High	118.2	132.4	195.6	119.0	19.46
Low Low	115.6	115.7	117.2	72.4	19.46
	UIBC				
High High	79.2	73.3	71.0	63.7	2.92
High Low	82.9	70.1	71.5	62.2	2.92
Low High	96.6	64.4	63.7	62.6	2.92
Low Low	92.6	68.8	66.4	56.3	2.92

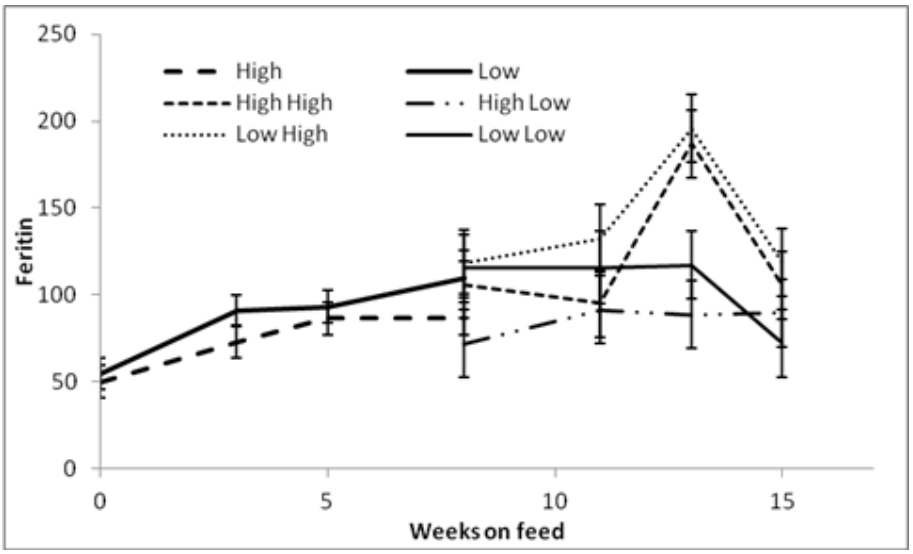

Figure 3. The concentration of ferritin (ng/ml) in the blood of pigs at two feeding stages, grower [weeks 0 to 8: depicted as High (Iron) and Low (Iron)] and finisher (weeks 8 to 15: depicted as High High; High Low; Low High; Low Low). Data are ± SE of the mean.

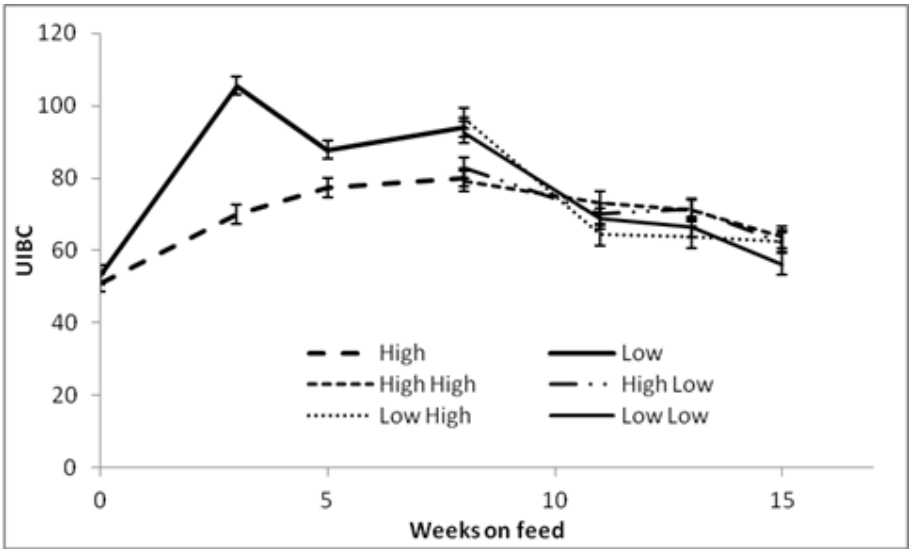

Figure 4. The concentration of UIBC ((μmol/L) in the blood of pigs at two feeding stages, grower [weeks 0 to 8: depicted as High (Iron) and Low (Iron)] and finisher (weeks 8 to 15: depicted as High High; High Low; Low High; Low Low). Data are ± SE of the mean.

Growth performance and carcass characteristics at slaughter

There was no effect of treatment on any growth performance data (Tables 5-8). By the end of the finisher period pigs weighed about 100 kg, were eating over 3kg per day, and gaining just under 1 kg per day indicating very good performance (Table 8).

Differences in P2 fat depth, hot carcass weight and the dressing percentage essentially reflected the difference in the time of slaughter, i.e., either at the end of the grower stage or at the end of the finisher stage (Tables 12 and 13). However and for P2 fat depth at the end of the finisher stage, pigs fed High-High were leaner (P<0.001) than pigs fed Low-High diets (12.2 *vs* 15.4 mm; Table 13).

Table 12. Statistical outputs for P2 fat depth, hot carcass weight (HCW) and dressing percentage of pigs (adjusted for liveweight used as a covariate).

	Diet			Liveweight		
	NDF;DDF	F Value	P Value	NDF;DDF	F Value	P Value
Fat depth	5;41	9.58	<.0001	na		
HCW	5;40	2.21	0.0723	1;40	660.93	<.0001
Dressing %	5;40	2.89	0.0255	1;40	0.13	0.7255

Table 13. Least-squares means for P2 fat depth, hot carcass weight and dressing percentage of pigs killed at either the end of the grower stage (High, Low) or the end of the finisher phase (High-High, High-Low, Low-High, Low-Low). Values in parentheses are ± SE of the mean.

	High	Low	High-High	High-Low	Low-High	Low-Low
Fat depth	8.0^{c} (0.96)	8.9^{c} (0.96)	12.2^{b} (0.96)	14.0^{ab} (0.96)	15.4^{a} (1.03)	14.0^{ab} (0.96)
HCW	54.3^{b} (0.97)	54.8^{b} (0.94)	57.9^{a} (0.70)	58.4^{a} (0.67)	59.0^{a} (0.71)	57.6^{a} (0.59)
Dressing %	63.2^{b} (1.22)	64.1^{b} (1.18)	69.0^{a} (0.88)	69.5^{a} (0.84)	70.1^{a} (0.89)	68.8^{a} (0.74)

$^{a, b}$ Within a row, statistical differences between treatments are denoted by different superscripts.

Meat quality data

The LD muscle from the High Iron pigs killed after the grower period was darker and redder than the Low Iron pigs (P<0.05; Tables 14 and 15). There was no difference (P>0.05) in pH drip loss of b* values. The LD muscle from pigs fed the High Iron diet through both stages was redder than other treatments (P<0.05; Table 17). The Low Iron grower pigs were the least red at the end of the finisher period despite which diet they were finished on. There was no further overall effect of the diets on meat quality (Table 17).

Table 14. Statistical output for meat quality data for treatment in the grower stage.

	Treatment		
Measure	NDF;DDF	F value	P value
L*	1;14	6.15	0.0265
a*	1;14	7.77	0.0146
b*	1;14	0	0.963
pH	1;14	0.09	0.7727
Loss	1;14	0.25	0.6219

Table 15. Least-squares means for meat quality data in the grower stage.

	Treatment		
	High	Low	SEM
L*	48.8[a]	51.8[b]	0.867
a*	6.3[a]	4.3[b]	0.502
b*	2.3	2.3	0.243
pH	5.56	5.55	0.027
Loss, %	4.8	4.3	0.710

[a,b] Within a row, statistical differences between treatments are denoted by different superscripts.

Table 16. Statistical output for meat quality data for treatment in the finisher stage.

	Treatment		
	NDF;DDF	F Value	P value
L*	3;31	0.7	0.562
a*	3;30	4.01	0.0175
b*	3;29	1.56	0.2209
pH	3;28	1.86	0.1602
loss	3;27	1.78	0.1749

Table 17. Least-squares means for meat quality data in the finisher stage.

	Treatment				
	High-High	High-Low	Low-High	Low-Low	SEM
L*	49.2	49.5	49.3	51.5	1.279
a*	7.2[a]	6.2[ab]	5.7[b]	5.2[b]	0.433
b*	2.9	2.5	2.0	2.2	0.321
pH	5.54	5.58	5.62	5.53	0.028
Loss, %	4.5	3.9	3.0	3.1	0.501

[a,b] Within a row, statistical differences between treatments are denoted by different superscripts.

Table 18. Statistical output for iron, zinc and copper levels in heart and liver in the finisher stage.

	Treatment		
	NDF;DDF	F Value	P value
		Heart	
Iron	6;48	2.24	0.055
Zinc	6;47	0.11	0.995
Copper	6;48	1.48	0.204
		Liver	
Iron	6;48	29.84	<.0001
Zinc	6;48	4.86	<.001
Copper	6;48	13.43	<.0001

Table 19. Least-squares means for concentrations of iron, copper and zinc (mg/kg) in the heart and liver of pigs fed diets different in iron content.

	Treatment							S.E.
	Basal	High	Low	High-High	High-Low	Low-Low	Low-High	
				Heart				
Iron	46.4[b]	42.7[ab]	38.8[a]	45.0[b]	43.5[ab]	47.1[b]	46.4[b]	1.93
Zinc	18.9	19.4	19.1	19.2	19.3	19.2	19.0	0.58
Copper	3.4	3.7	3.5	3.6	3.8	3.7	3.8	0.11
				Liver				
Iron	84.0[a]	169.4[b]	54.1[a]	294.4[d]	198.5[b]	181.5[b]	247.3[c]	15.38
Zinc	51.0[a]	100.5[b]	82.6[b]	90.2[b]	84.5[b]	94.7[b]	85.3[b]	7.21
Copper	11.7[a]	5.3[b]	5.1[b]	5.6[b]	6.2[b]	6.3[b]	6.1[b]	0.62

[a,b,c,d] Within a row, statistical differences between treatments are denoted by different superscripts.

Mineral levels in heart and muscle

Only the iron concentration showed a tendency to be statistically different (P=0.055) in the heart (Table 15), with pigs fed Low iron during the grower period having less accumulated iron than pigs fed treatments High-High, Low-Low or Low-High, as well as the control animals (Basal) killed at the start of the experiment (38.8 mg/kg; Table 19). There was no difference in other minerals.

In the liver, however, all minerals showed statistically significant differences (Table 18). Iron levels were higher in grower pigs fed the High iron diet than those fed the Low iron diet, and then in the finisher stage, pigs fed diet High-High accumulated the most iron followed by pigs fed Low-High (P<0.001). The zinc and copper contents were highest in control animals (Basal) killed at the start to the experiment (Table 19).

Discussion

The induction of mild iron depletion followed by dietary repletion in pigs increased circulating serum iron levels, thus accepting our initial hypothesis. An increase in blood iron concentration was observed by the end of the finisher stage in those pigs fed the depletion diet (Low iron) during the grower stage. This increase was not observed in those pigs on the High iron diet. Interestingly the increase in circulating iron was also observed in the Low-Low pigs. The iron content in the feed only increased by about 10 mg/kg between the Low Iron (grower) diet and the Low-Low (finisher) diet but was enough to increase the levels of iron by about 17 μm/L. The rapid decline in blood iron from weeks 0 to weeks 3 in the Low iron grower pigs is not currently understood but is possibly due to a physiological acclimation to the decline in dietary iron. This was also reflected in a slight decline in haemoglobin percentages but was not substantial enough to warrant concerns of anaemia. The High iron diet increased the haemoglobin levels to between 13-14% in whole blood, which did not affect the performance or health of the pigs. Because there was no further increase in haemoglobin it is likely that the increased dietary iron sustained storage in other tissues, and in this regard, it was evident that the liver becomes an important storage site for iron (Table 19).

In the grower stage there was an approximately three-fold increase in iron levels in the liver in the High iron as opposed to the Low iron dietary group, and the High-High group had almost 6 times more iron (294 versus 54 mg/kg). Much of this difference could be due to maintaining iron in the muscle, using the blood as transport. It is possible that the liver is an acute indicator of dietary iron levels, given that the High-Low and Low-Low groups are similar whereas there is a difference between the High-High and Low-High groups even though the dietary levels were similar. However some of the iron in this case has gone to replenish the muscles. From an experimental perspective, it may have been of greater benefit to have a shorter repletion period and then possibly a greater contrast would be observed between and within the muscles.

Hansen et al. (2010) examined the circulatory levels of haemoglobin and iron in 36 weaned barrows allocated to a 2x3 factorial design, with the factors being dietary iron levels (97 and 797 mg/kg dietary iron) and three time points for bleeding (20, 41 or 62 days after feeding). Tissue collection occurred at 21, 42 or 63 days after feeding. These authors reported higher haemoglobin levels in blood in pigs fed the high iron diet on all three sampling occasions, and for iron, levels were higher in plasma on days 20 and 41 after feeding, but not on day 62; iron concentrations

decreased over time. In our study, and presumably because of the smaller difference in dietary iron levels compared to those used by Hansen et al. (2010) that was an 8-fold difference, the differences in haemoglobin and iron levels were less apparent and particularly in the finisher stage.

An increase in muscle iron levels was observed after dietary-induced depletion and repletion, but this effect was isolated to the more aerobic RF muscle, thus supporting our second hypothesis. Kim et al. (2008) compared the RF and LD and similarly found significantly higher total iron contents (7.08 *versus* 4.48 mg/kg). Depleting iron available to the muscle followed by repletion allowed the RF muscle to achieve iron levels that can class the meat as a "source" of iron. This result is encouraging considering previous studies have shown no effect on muscle characteristics (O'Sullivan et al., 2002; Shaw et al., 2002; Apple et al., 2007; Ponnampalam et al., 2009). While the depletion/repletion method to increase muscle iron content for products provided to consumers seems to work in the more aerobic RF muscle, it fails to alter the muscle iron content of the LD muscle. This is likely to be due to the physiological requirement of iron between different tissues (Patterson et al., 2008).

In this regard, it is likely that the method of dietary depletion/repletion can influence the muscle iron content in pigs for slaughter in more aerobic muscle types only. Further research is required to understand this effect and why it was only observed in the RF and not the LD. It is suggested that the physiological demand for iron is higher in the RF than the LD and thus the uptake of dietary iron into this muscle was increased while levels in the LD were maintained. Additionally, the expression of certain genes for the regulation or iron metabolism may play a significant role in the difference between tissue types, and this will be examined in Experiment 3. Finally, it may be important to investigate the timing of the repletion period to understand the segregation of iron uptake into the muscle as haem and non-haem iron. Although currently there is no evidence, an acute repletion period may increase the proportion of non-haem iron in the muscle more rapidly considering that myoglobin will increase with age.

In a previous CRC-funded study (Allingham et al., 2011), although feeding a diet higher in iron (207 ± 3.73 mg/kg versus 96 ± 1.92 gm/kg of feed for Fe+ and Fe- rations) did not alter the iron content of the muscles sampled, there was an apparent effect on metabolic efficiency where those pigs offered dietary fortification of iron had a five percentage unit increase in dressing percentage without affecting carcass weight. The conclusion from the authors was that iron fortification reduces visceral mass through a yet unknown mechanism. In our study, we found no effects of diet on fat depth, fat class, dressing percentage or the HSCW (Tables 12 and 13). There was an expected difference in diets for carcass characteristics between pigs killed at the end of the grower and finisher stages.

Ponnampalam et al. (2009) examined the effects of inulin, iron and sex in an interactive manner and did not find any interactions for growth performance, carcass traits, serum iron levels or muscle iron contents. However feeding of chicory inulin at 5% in the finisher ration significantly increased haem iron content of pork compared with pigs fed without inulin in their ration. The increase in muscle haem iron did not significantly increase the total iron content of pork, however. Feeding an organic iron supplement did not change the haem or total

iron content of pork although male pigs fed the chicory inulin diet had higher haem and total iron content than their female counterparts.

Sustained supply of high iron in the diet, especially haem iron, is likely to increase the myoglobin concentration in the muscle. Myoglobin will increase with age (Lawrie, 1950) and this is observed in the current data when differences in myoglobin between the grower and finisher pigs are observed. However this provides evidence that when pigs have a smaller supply of iron at an earlier period then the myoglobin levels are likely to be lower at an older age even when repletion occurs at a rapid rate. Additionally, when the iron content was adjusted for myoglobin concentration, the Low-High RF muscle samples still had significantly higher muscle iron levels than other treatments (Table 4). These data suggest that the depletion/repletion model implemented increased the level of non-heme iron, while a continuous supply of high levels of iron will result in an increased storage of haem iron in the muscle. Furthermore, the increase of iron in the RF muscle did not increase the redness of the product compared to the other treatments. This finding is important for consumer markets that are sensitive to red pork meat.

From a meat quality perspective, the Low-High treatment produced a product with a slightly higher pH with less moisture loss, although no statistical differences were noted. It is not understood why this treatment would cause this effect, however it is a positive attribute of the depletion/repletion method attempted in this experiment. Unfortunately this data was only collected on the LD muscle that did not seem to be sensitive to repletion. In this regard, the experiment would have benefited from having RF meat quality data but unfortunately these samples couldn't be obtained.

Plasma ferritin is a secretory component of intracellular ferritin synthesis and in normal healthy animals, it reflects the amount of ferritin within body cells and hence the size of iron stores (Finch et al., 1986). Ferritin serves to store iron in a non-toxic form, to deposit it in a safe form, and to transport it to areas of requirement. Within cells, iron is stored in a protein complex as ferritin or hemosiderin, and the amount of ferritin synthesized daily is that required to replace catabolized ferritin and hemosiderin and the accommodate any additional iron entering cells (Finch et al., 1986). Determination of plasma ferritin levels therefore provides an indication of iron balance.

In this study, levels of plasma ferritin displayed a significant main effect of diet and a trend for a time x treatment interaction for the finisher stage only (Table 9; Figure 3). Feeding diets High-High and Low-High in the finisher stage elevated the ferritin levels indicating greater physiological storage of iron, possibly in equilibrium with the liver and red muscles. There is a dearth of information in the literature concerning diet and plasma ferritin levels in grower-finisher pigs. Hansen et al. (2010) found that liver iron concentrations were greater, and increased over time, in pigs fed substantially more dietary iron. Similarly, the concentration of iron in the intestine and the heart were greater in pigs fed more dietary iron, with only the heart increasing in iron content over time.

Experiment 3

The expression of selected genes involved in iron metabolism from the muscles of grower-finisher pigs subject to a dietary iron depletion/repletion regimen

Introduction

There are a plethora of studies examining the molecular mechanisms associated with iron regulation in rodents and in man, given its profound importance in human health and nutrition, however there is a scarcity of data in the pig that most likely reflects the fact that the iron requirement of pigs of all stages and phases (except the neonate) is satisfied by the diet.

In pigs, Hansen et al. (2009) showed an up regulation of genes encoding for iron import in the intestines of weaner pigs fed an iron-deficient diet. These same authors (Hansen et al., 2010) then examined the expression of selected genes in 36 weaned barrows allocated to a 2x3 factorial design, with the factors being dietary iron levels (97 and 797 mg/kg dietary iron) and three time points for intestinal tissue collection (21, 42 or 63 days after feeding), which were known or suspected to function in iron metabolism. In the duodenum, they reported a down regulation of genes involved in iron import in pigs fed high dietary iron, however these authors did not measure gene expression in muscle.

The experimental protocol established in Experiment 2 allowed for the collection of muscle samples from pigs at two different slaughter points. The aim of this experiment was to examine the expression of candidate genes implicated in muscle iron metabolism, and to ascertain whether feeding different iron concentrations impacts upon the expression of genes that regulate iron metabolism and therefore, iron deposition in muscles.

Materials and Methods

Samples

Samples of muscle (approximately 2 g) from the *m.longissimus dorsi* (LD) and *m.rectus abdominus* (RA) were collected into Ambion® RNA*later*® tissue collection solution (AM7021) as previously described (see Experiment 2).

Reverse transcription-polymerase chain reaction (RT-PCR)

Tissue preparation and RNA extraction

Sub-samples of each muscle (approximately 50 mg) were taken and placed into Promega isolation denaturing solution reagent (Promega Total RNA Isolation System; Promega, WI, USA). The samples were then homogenized on a bead-beater for 30 seconds. Total RNA was extracted according to the manufacturer's instructions. Contamination genomic DNA was destroyed using RQ1 DNAse 1 (1 U, Promega) in 10 × buffer at 37 °C for 20 min, containing 40 U of RNAsin (Promega) 0.1 M dithiotreitol. RNA was re-extracted with phenol/chloroform/isoamyl alcohol and Phase Lock Gel Tubes (Eppendorf; Brinkman Instruments, NY, USA) and precipitated in 100% ethanol with 2M sodium acetate (pH 4.0) (see Appendix II).

Reverse transcription

The RNA was reverse transcribed in a 50 μl final volume using Superscript III (RT-SSIII) reverse transcriptase (100 U, Invitrogen, CA, USA) in 5 × RT buffer, with 2.5

ng/μl random primers, 10 mM each deoxynucleoside-triphosphate (dNTPs), 0.1 M dithiotreitol (DTT) and 20 U RNAsin. A heat start was applied for 2 min at 50 °C and then the RT-SSIII was added. Samples were incubated at 45 °C for 50 min and the 55 °C for 30 min. The RT enzyme was heat inactivated (90 °C for 5 min).

Quantitative real-time PCR for genes in muscles

Real-time PCR was performed using a Corbett RotorGene 6 Quantitative Thermal Cycler (Corbett Research, NSW, Australia). The reactions were performed in the presence of conventional forward and reverse primers and SYBR Green (Invitrogen, VIC, Australia). The 11 candidate genes chosen for examination in this experiment are shown in Table 20. These genes were chosen to reflect broad aspects of iron metabolism in muscle, namely iron import (transferring receptor, DMT1/SLC11A2), iron export (ferroportin, ceruloplasmin), iron regulation (hepcidin, hemojuvelin) and iron storage (ferritin) (Polofini et al., 2010). In addition, we examined myoglobin, lactoferrin and deoxyhaemoglobin. The primers used in the experiment were designed using 'primer quest' (www.idtdna.com/scitools/applications/primerquest) and PubMed using the "pick primers" option when the relevant nucleotide sequence was obtained (www.ncbi.nlm.nih.gov/pubmed/). Once primers were designed they were tested using Amplify 3X to determine suitability, and the best primers were chosen (http://engels.genetics.wisc.edu/amplify). Housekeeping genes chosen originally for the experiment were glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin, and hypoxanthine-guanine phosphoribosyltransferase (Table 13), however only GAPDH was eventually selected for normalization against the candidate genes on the basis of its lower variability.

Cycle threshold (Ct) values represented cycle number at which fluorescence emission date exceeded a threshold limit. Expression of the genes was normalized to an endogenous control gene (GAPDH) to give a ΔCt value, which allowed an account for variability in the initial starting amount of cDNA. An aliquot of a previously run sample from a standard curve with a known Ct value was also placed in every run, to compare run-to-run variance. PCR was performed with a Corbett RotorGene 6 Quantitative Thermal Cycler using SYBR green. Cycling conditions consisted of two holds of 50 °C for 2 min, 95 °C for 10 min and then cycling for 40 cycles for 95 °C 15 sec, 60 °C for 1 min and 60 °C for 60 sec. Each sample was run in triplicate.

Real-time PCR data analysis

The analysis of the real-time PCR data was conducted by two methods:

a. Using Rotorgene (ng/μl); the concentration of the gene was determined by running a standard of known concentration in every run, for that particular primer (primers were serially diluted to make a standard curve. The initial 1:1 ratio of cDNA was run on the nano-drop machine to determine its exact concentration in ng/μl. One of the serially diluted samples was included in every run and correlated back to the standard curve, to determine the concentrations of the unknown samples).
b. Using the Pfaffl method; this method determines the efficacy of the primers (relative to housekeeping genes) and then factors this into the determination of the quantity of how much RNA is expressed (amplification efficiency). A full account of the methodology is described by Pfaffl (2001).

Statistical analyses

Data were analysed using a general linear model procedure in SAS with the main fixed effects being dietary iron feeding regimen (treatment) for the initial eight-week grower period, and then the finisher dietary regimen for the following seven weeks, and muscle type together with the interactions. Statistical significance was accepted at $P<0.05$.

Table 20. Primer sequences examined for real-time PCR.

	GenBank Sequence	Size		Primer	Product
Candidate genes					
1. Hepcidin	AF51643	411bp	F	TCCCAGACAAGACAGCTCACA	117bp
Gene=HAMP	NM_214117	411bp	R	GAAGATGCAGATGGGGAAGT	
2. Myoglobin	M14433	1111bp	F	AGCACCTGAAGTCAGAGGATGAGA	166bp
Gene=MB	NM_214236	111bp	R	TTGACAGGGATCTTGTGCTTGGTG	
3. Ferritin	NM_213975	821bp	F	TTTGACCGCGATGATGTGGCTTTG	185bp
FTC=Ferritin, light polypeptide	**(FTH1)**		R	AATTCCATGGCAGTCAGCCCATTC	
FTH1=Ferritin, heavy polypeptide 1					
4. FTMT=Ferritin mitochondrial					
	NM_001244131.1	954bp	F	ATGAGCTCCCAGGTTCGTCAGAAT	126bp
	(FTL)		R	ATCGTCGCGGTTGAAATAGAAGCC	
5. Transferrin	X12386	2258bp	F	ATGCGATGCGTGGAGCATAAACAG	107bp
Gene=TF	NM_001244653.1		R	TCATGGCATCAGCCTCTCCTTTCA	
TFRC=Transferrin receptor	XM_003483241.1				
	XM_003483242.1				
6. Lactoferrin (LF)/	AF222916 (LTF)	159bp	F	TGGTGGTTTGGTGTTTGAAGCAGG	164bp
Lactotransferrin (LTF)	L77887 (LTF)	2342bp	R	TGTGGCAGGACTTTCGACCTTGTA	
	NM_214362 (LTF)	2578bp			
	NM_214362.1				
7. DMT1/SLC11A2 (gene)	AM183784	372bp	F	AGGGATTCCTGAACCTGAAGTGGT	197bp
Divalent metal transporter 1	EU647217	1692bp	R	CCGCAAGCTTGTAAACGTGAGGAT	
	NM_001128440.1				
8. Ferroportin	AX428402	900bp	F	AGCCTGTTGCGCAGCATCAATAAG	128bp
SLC40A1	NM_214009.1		R	AAGGGCTCATCCAGCTTGTCAGAT	
9. Ceruloplasmin	EU714006	1006bp	F	CCGGGAAGTGCTTCAGAGGTGT	198bp
Gene=CP	XM_003132473	4559bp	R	ACGGAAGGTGACTCGGATGGTGT	
10. Hemojuvelin (HJV)	XM_001927637	2181bp	F	TGGACAATCTTCCTGCAGCCTTTG	198bp

Gene=HFE2	XM_003355210	2224bp	R	TGGCCACATCCTCTGCTACTTTGA	
Hemochromatosis type 2 (Juvenile form)	XM_003355211	2206bp			
11. Deoxyhaemoglobin	XM_001144841	106 bp	F	TGTGGACGAAGTTGGTGGTGAGG	106 bp
			R	GACGGCATCGGCATTGGACA	
Housekeeping genes					
1. GAPDH	AF017079	1419bp	F	AGTATGATTCCACCCACGGCAAGT	148bp
	NM_001206359	1341bp	R	TCCACAACATACGTAGCACCAGCA	
Glyceraldehyde 3-phosphate dehydrogenase					
2. ACTB	DQ452569	1102bp	F	GCCCGTCCATCGTCCACCG	127bp
Actin, Beta	XM_003124280	1872bp	R	CAGGAGGCTGGCATGAGGTGTG	
	XM_003357928	1961bp			
3. HPRT1	DQ136030.1	674bp	F	AGCCCCAGCGTCGTGATTAGTG	143bp
	EU395621.1	29529bp	R	TCTCGAGCAAGCCGTTCAGTCC	
	NM_001032376.2	1428bp			
Hypoxanthine-guanine phosphoribosyltransferase					

Results

Primers

Preliminary investigation of the efficacy of expression of the 10 candidate genes showed that only six genes could reliably and accurately be expressed in both muscles at both time points. Therefore, the genes analyzed in this study for further investigation were DMT1/SLC11A2, ferroportin, ferritin, myoglobin, haemojuvelin and deoxyhaemoglobin (Table 20).

Statistical summary of results

a. Concentration of the gene in the muscles

There were significant interactions for dietary treatment with muscle for the concentration of DMT1 only (P=0.002), with there being a strong tendency for an interaction for the concentration of ferroportin (FP; P=0.058) (Table 21). There were main effects of dietary treatment for ferroportin, myoglobin and deoxyhaemoglobin, and there were main effects of muscle type for ferritin, myoglobin, haemojuvelin and deoxyhaemoglobin (Table 21).

b. Pfaffl ratio of the gene in the muscles

There were significant interactions for dietary treatment with muscle for the Pfaffl of DMT1 only (P=0.029; Table 21). There were main effects of dietary treatment for ferroportin and ferritin and a tendency for deoxyhaemoglobin (P=0.082), and there were main effects of muscle type for myoglobin and haemojuvelin with a tendency for DMT1 (P=0.094) (Table 21).

DTM1

The significant interaction for the concentration of this gene was attributable solely to the *rectus femoris* muscle, which were higher than those in the *longissimus dorsi* muscle, with the lowest concentrations occurring in pigs slaughtered at 10 kg liveweight (Basal) and those slaughtered at the end of the grower stage (High, Low) (Table 23).

The significant interaction for the Pfaffl ratio of this gene occurred because values were higher in treatments High-High and Low-High in the *rectus femoris* muscle compared to those in the *longissimus dorsi* muscle (Tables 22 and 23).

Ferroportin (FP)

Values for the concentration of this gene in the treatments Basal, High, Low, High-High, High-Low, Low-High and Low-Low were 730, 1179, 1040, 912, 1182, 3150 and 2761 ng/ml, respectively (P=0.003, Tables 22 and 23). The Pfaffl ratio showed a similar trend (P=0.002; Tables 22 and 23). The tendency for a significant interaction between dietary treatment and muscle type (Table 21) for the expression of ferroportin indicated that at the end of the grower stage, the concentration was higher in High pigs in the *rectus femorus* muscle than in their counterparts in the *longissimus dorsi* muscle (Tables 22 and 23).

Ferritin (FTH1)
Values for the concentration of this gene were higher in the *rectus femoris* muscle compared to those in the *longissimus dorsi* muscle (822 versus 488 ng/ml respectively, P<0.001; Tables 22 and 23). Values for the Pfaffl ratio of this gene in the treatments High, Low, High-High, High-Low, Low-High and Low-Low were 1.0, 1.21, 1.15, 0.74, 0.98 and 0.77, respectively (P=0.039, Tables 22 and 23).

Myoglobin
Values for the concentration of this gene in the treatments Basal, High, Low, High-High, High-Low, Low-High and Low-Low were 107, 357, 262, 441, 512, 471 and 547 ng/ml, respectively (P=0.002, Tables 22 and 23).

Values for the concentration of this gene were higher in the *rectus femoris* muscle compared to those in the *longissimus dorsi* muscle (512 versus 257 ng/ml respectively, P<0.001; Tables 22 and 23).

Haemojuvelin
Values for the concentration of this gene were lower in the *rectus femoris* muscle compared to that in the *longissimus dorsi* muscle (335 versus 510 ng/ml, P<0.001), whereas the Pfaffl ratio was higher in the *rectus femoris* muscle compared to that in the *longissimus dorsi* muscle (0.64 versus 0.62, P=0.013; Tables 22 and 23).

Deoxyhaemoglobin
Values for the concentration of this gene in the treatments Basal, High, Low, High-High, High-Low, Low-High and Low-Low were 2197, 1047, 844, 619, 608, 545 and 580 ng/ml, respectively (P<0.001; Tables 22 and 23).

Values for the concentration of this gene were lower in the *rectus femoris* muscle compared to those in the *longissimus dorsi* muscle (653 versus 1186 ng/ml, P<0.001), whereas there was a tendency for the Pfaffl ratio to be higher in the *rectus femoris* muscle compared to that in the *longissimus dorsi* muscle (0.28 versus 0.26, P=0.013; Tables 22 and 23).

Table 21. Statistical outputs for the effects of dietary treatment fed in both the grower and finisher stage and muscle type, and the interaction, on the expression of selected genes.

	Treatment			*Muscle*			*Treatment x Muscle*		
	NDF;DDF	F value	P value	NDF;DDF	F value	P value	NDF;DDF	F value	P value
DMT1[A]	6;98	2.76	0.016	1;98	0.22	0.638	6;98	3.84	0.002
DMT1[B] *ratio*	5;87	1.27	0.286	1;87	2.87	0.094	5;87	2.64	0.029
FP[A]	6;98	3.52	0.003	1;98	0.69	0.408	6;98	2.12	0.058
FP ratio[B]	5;87	4.25	0.002	1;87	2.13	0.148	5;87	0.58	0.712
FTH1[A]	6;96	1.26	0.284	1;96	26.36	<.001	6;96	0.51	0.798
FTH1 ratio[B]	5;86	2.47	0.039	1;86	2.52	0.116	5;86	0.95	0.451
MB[A]	6;98	3.78	0.002	1;98	19.48	<.001	6;98	0.31	0.933
MB ratio[B]	5;87	0.81	0.543	1;87	9.72	0.002	5;87	0.11	0.989
HJ[A]	6;98	1.46	0.198	1;98	11.93	0.008	6;98	0.97	0.449
HJ ratio[B]	5;87	1.03	0.405	1;87	6.44	0.013	5;87	1.2	0.318
HHB[A]	6;98	9.68	<.001	1;98	16.4	0.001	6;98	1.54	0.172
HHB ratio[B]	5;87	2.03	0.082	1;87	0.29	0.594	5;87	1.77	0.128

[A] DMT1: divalent metal transport 1; FP: ferroportin; FTH1: ferritin; MB: myoglobin; HJ: haemojuvelin; HHB: deoxyhaemoglobin (all expressed as ng/ml).
[B] Expression of the Pfaffl ratio (see text for details).

Table 22. Least-squares means (± standard error of the mean; SEM) for the effects of the dietary treatment fed during the grower and finisher stages on the expression of selected genes in the *longissimus dorsi*.

	Basal		*High*		*Low*		*High-High*		*High-Low*		*Low-High*		*Low-Low*	
	Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM
							Longissimus dorsi							
DMT1[A]	1059	201.3	694	180.1	947	189.8	697	189.8	896	189.8	723	201.3	1096	189.8
DMT1[B] *ratio*	na		0.63[a]	0.10	0.81[ab]	0.11	0.83[b]	0.11	0.97[ab]	0.11	0.96[b]	0.11	0.76[ab]	0.11
FP[A]	414[a]	869.4	332[a]	777.6	946[a]	819.6	1041[a]	819.6	1180[a]	819.6	4555[b]	869.4	3616[b]	819.6
FP ratio[B]	na		1.08[a]	2.28	2.49[a]	2.41	3.54[a]	2.41	4.37[a]	2.41	13.12[b]	2.55	7.40[ab]	2.41
FTH1[A]	573[ab]	98.7	594[a]	82.6	685[a]	87.1	381[b]	87.1	390[b]	92.3	319[b]	92.3	474[ab]	87.1
FTH1 ratio[B]	na		1.05	0.15	1.25	0.16	1.10	0.16	1.07	0.17	1.04	0.17	0.84	0.16
MB[A]	48[a]	52.8	206[c]	47.2	187[bc]	49.8	286[b]	49.8	387[ac]	49.8	303[bc]	52.8	385[b]	49.8
MB ratio[B]	na		1.55[a]	0.31	1.68[a]	0.33	2.01[a]	0.33	2.73[b]	0.33	2.58[b]	0.35	2.06[a]	0.33
HJ[A]	745[a]	114.9	586[ac]	102.7	532[ac]	108.3	389[bc]	108.3	395[bc]	108.3	333[bc]	114.9	593ac	108.3
HJ ratio[B]	na		0.71	0.09	0.67	0.09	0.57	0.09	0.54	0.09	0.61	0.10	0.57	0.09
HHB[A]	2771[a]	308.1	1547[b]	275.5	1202[bc]	290.4	748[c]	290.4	786[bc]	290.4	480[c]	308.1	769[bc]	290.4
HHB ratio[B]	na		0.38[a]	0.06	0.37[a]	0.07	0.21[ac]	0.07	0.25[ac]	0.07	0.18[ac]	0.07	0.17[bc]	0.07

[A] DMT1: divalent metal transport 1; FP: ferroportin; FTH1: ferritin; MB: myoglobin; HJ: haemojuvelin; HHB: deoxyhaemoglobin (all expressed as ng/ml).

[B] Expression of the Pfaffl ratio (see text for details).

[a,b,c] Within a row, values not having the same superscript differ significantly (see Table 21).

Table 23. Least-squares means (± standard error of the mean; SEM) for the effects of the dietary treatment fed during the grower and finisher stages on the expression of selected genes in the *rectus femoris*.

	Basal		High		Low		High-High		High-Low		Low-High		Low-Low	
	Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM
							Rectus femorus							
DMT1[A]	58[a]	287.8	505[ac]	243.2	274[a]	243.2	1211[b]	227.5	1486[b]	227.5	1176[b]	243.2	1020[bc]	227.5
DMT1[B] *ratio*	na		1.02[a]	0.15	0.93[ab]	0.15	1.20[a]	0.14	0.59[b]	0.14	1.04[a]	0.15	0.91[ab]	0.14
FP[A]	1047	572.3	2025	483.7	1134	483.7	783	452.4	1184	452.4	1744	483.7	1906	452.4
FP ratio[B]	na		2.31[a]	1.76	1.54[a]	1.76	2.11[a]	1.64	3.73[ab]	1.64	7.74[b]	1.76	3.58[ab]	1.64
FTH1[A]	844	184.1	1001	155.6	803	155.6	730	145.5	804	145.5	813	155.6	760	145.5
FTH1 ratio[B]	na		0.95[ab]	0.16	1.17[a]	0.16	1.20[a]	0.15	0.50[b]	0.15	0.93[ab]	0.16	0.71[b]	0.15
MB[A]	165[a]	189.2	508[ab]	159.9	336[ab]	159.9	595[ab]	149.6	636[ab]	149.6	638[ab]	159.9	708[b]	149.6
MB ratio[B]	na		3.09	0.91	2.55	0.91	3.25	0.85	3.47	0.85	3.63	0.91	3.34	0.85
HJ[A]	404	70.7	338	59.8	252	59.8	298	55.9	374	55.9	330	59.8	349	55.9
HJ ratio[B]	na		0.82[ab]	0.14	0.72[ab]	0.14	0.64[a]	0.13	1.02[b]	0.13	0.82[ab]	0.14	0.63[b]	0.13
HHB[A]	1622[a]	144.9	547[b]	122.4	485[b]	122.4	490[b]	114.5	430[b]	114.5	609[b]	122.4	391[b]	114.5
HHB ratio[B]	na		0.28[ab]	0.09	0.32[ab]	0.09	0.22[a]	0.09	0.20[a]	0.09	0.51[b]	0.09	0.16[a]	0.09

[A] DMT1: divalent metal transport 1; FP: ferroportin; FTH1: ferritin; MB: myoglobin; HJ: haemojuvelin; HHB: deoxyhaemoglobin (all expressed as ng/ml).

[B] Expression of the Pfaffl ratio (see text for details).

[a,b,c] Within a row, values not having the same superscript differ significantly (see Table 21).

Discussion

The overall aim of this experiment was to examine the expression of several candidate genes implicated in iron metabolism in contrasting muscles under conditions of feeding varied dietary iron concentrations, and at either the end of the grower stage or the finisher stage of growth. Data were expressed either as the concentration of the gene in the muscle (determined by running a standard of known concentration in every run, for that particular gene) or using the Pfaffl method, however for the purposes of this report, it is the concentration of the gene that is most physiologically relevant.

The ontogeny of iron metabolism has been described in the suckling rat (Thompson et al., 2007) but appears not to have been fully examined in young pigs, and indeed throughout the entire period from soon after weaning to commercial slaughter weights. Iron homeostasis is heavily dependent on regulation at the point of intestinal absorption (West and Oates, 2008) such that if a young animal has little or no control over dietary iron acquisition, then the animal may be susceptible to tissue iron overload and oxidative stress. The divalent metal transporter 1 (DMT1), also known as natural resistance-associated macrophage protein 2 (NRAMP 2) and divalent cation transporter 1 (DCT1), is a protein that in humans is encoded by the SLC11A2 (solute carrier family 11, member 2) gene. DMT1 represents a large family of metal ion transporter proteins that are highly conserved from bacteria to humans. As its name suggests, DMT1 binds a variety of divalent metals but is best known for its role in transporting ferrous iron (Fe^{2+}); to maintain iron homeostasis, the expression of DMT1 is regulated by body iron stores (West and Oates, 2008).

Previously, Hansen et al. (2009) noted that dietary iron concentration does not appear to regulate DMT1 in the small intestine of weaned pigs to the same extent as in adults of other species. Intestinal transcript levels of DMT1 were significantly greater in pigs receiving a low-iron diet for 32 d (~53 d of age) compared with those receiving either a normal- or high-iron diet, although SLC11A2 protein levels did not significantly differ. This result suggests that regulation of iron acquisition mediated by DMT1 was at least beginning by 53 d of age or there are other factors involved in iron acquisition in the young mammal. Hansen et al. (2010) reported that relative expression of DMT1 in duodenal scrapings was very low in 26-d-old pigs compared with d-21 controls but DMT1 expression in the liver was similar between d-0 pigs and d-21 control pigs. This is interesting because concentrations of iron were similar between the liver and intestine on d 0 and 21, respectively. In the intestine, DMT1 transcript levels increased dramatically from d 0 to 21 in pigs receiving the control diet (97 mg iron/kg DM); however, DMT1 expression remained suppressed throughout the trial in pigs receiving high dietary iron (797 mg iron/kg DM) (Hansen et al., 2010).

This dramatic difference in mRNA suggests that protein expression of DMT1 was likely decreased in high-iron pigs; however, to confirm protein changes, further research is necessary. Expression of the iron exporter ferroportin (also known as SLC40A1 IREG-1) was also relatively low in d-0 pigs, but expression was down regulated by dietary iron on d 21 in a manner similar to DMT1. In general, iron-dependent regulation of DMT1 and ferroportin appeared to develop somewhere between d 0 (pig age 26 d) and d 21 (pig age 47 d), a critical developmental period in which the animal is rapidly growing. Interestingly, pre-starter and starter diets that commercial pigs typically receive during this time are generally over supplemented with iron, which may negatively affect the expression of genes

such as the iron importer (DMT1) and iron exporter (ferroportin) and reduce growth potential.

Additional control of iron absorption comes from the liver-derived hormone HAMP (hepcidin antimicrobial peptide), which is produced in response to high-iron stores, subsequently binding to ferroportin protein on cell surfaces, causing it to be internalized and degraded (Nemeth et al., 2004). Hepatic expression of HAMP was extremely low in d-0 pigs, ~3% of the HAMP expression of control pigs on d 21, despite the 2 groups having very similar liver iron concentrations (~100 mg iron/kg DM). Hansen et al. (2010) suggested that HAMP-mediated iron homeostasis may not yet be fully functional in newly weaned pigs, which in turn suggests that young pigs are not able to appropriately respond to changes in dietary iron. By d 21, hepatic HAMP mRNA expression was dramatically increased in high-iron pigs (6.25- fold that of control expression), suggesting that protein expression of HAMP was likely increased in high-iron pigs as well, but further investigation is needed to confirm this supposition. Nevertheless, the marked accumulation of iron observed in the liver of pigs in the present study (Table 19) adds gravitas to this physiological homeostatic mechanism.

Hansen et al. (2010) concluded that the iron status of young pigs affects expression of SLC11A2, SLC40A1, TFRC, SLC39A14, and ACO1 in a manner similar to the adult animal. Age-dependent expression of certain genes was also observed, including the iron chaperone FXN and the ferritin receptor SCARA5, which were down regulated with pig age. In their study, only gene expression of these molecules was determined and future research could be directed towards examining the effect of animal age and dietary iron concentration on expression of proteins involved in iron metabolism.

These work by Hansen and colleagues represent the only work we could find reporting gene expression in pigs, although not in muscle (they reported data in the intestines). In this regard, the data obtained in our study is unique. Our data for DMT1 in consistent in part with that reported by Hansen et al. (2009, 2010), with their being an age-related decline in DMT1 expression in the *longissimus dorsi* (but not the *rectus femoris*), and levels being greater (but only in the *longissimus dorsi*) for pigs fed the Low diet in the grower stage and the diet Low-Low during the finisher stage. It would appear, therefore, that DMT1 expression in pig muscle depends on both the feeding regimen and the muscle type.

Ferroportin-1 (FP), also known as solute carrier family 40 member 1 (SLC40A1) or iron-regulated transporter 1, is a protein that in humans is encoded by the SLC40A1 gene. Ferroportin is a transmembrane protein that transports iron from the inside of a cell to the outside of it. Ferroportin is found on the surface of cells that store or transport iron, including enterocytes in the duodenum, hepatocytes and macrophages of the reticuloendothelial system. Research suggests that ferroportin is inhibited by hepcidin, which binds to ferroportin and internalizes it within the cell (Nemeth et al., 2004). This results in the retention of iron within cells, and a reduction in iron levels within the plasma. This is especially significant in enterocytes of the gastrointestinal tract, which are shed at the end of their lifespan. The extra iron retained within them is not only prevented from entering the bloodstream but ends up being excreted into the faeces.

Ferroportin levels were greatest at the end of the finisher stage of growth in the treatments Low-High and Low-Low, although the strong tendency for an

interaction suggests that at the end of the grower stage, the concentration was higher in High pigs in the *rectus femorus* muscle than in their counterparts in the *longissimus dorsi* muscle. These data support the higher iron concentration in this muscle (Table 3) and indicate, physiologically, an increased excretion of iron from this muscle.

Ferritin (FHT1) is a ubiquitous intracellular protein that stores iron and releases it in a controlled fashion. The amount of ferritin stored reflects the amount of iron stored. The protein is produced by almost all living organisms, and in humans, it acts as a buffer against iron deficiency and iron overload. In this regard, ferritin serves to store iron in a non-toxic form, to deposit it in a safe form, and to transport it to areas where it is required. The presence of iron itself is a major trigger for the production of ferritin. In this experiment, levels of expression of this gene were higher in the *rectus femoris* relative to those in the *longissimus dorsi* (822 versus 488 ng/ml respectively, $P<0.001$; Tables 22 and 23), supporting the greater accumulation of iron in this muscle (Table 2). Data on circulating ferritin levels support the notion that, at least in the finisher stage, feeding more dietary iron increases causes an increase (Table 11), and this is reflected in a greater expression of this gene at least in the *rectus femoris* muscle.

Myoglobin is an iron- and oxygen-binding protein found in the muscle tissue of vertebrates in general and in almost all mammals. It is related to haemoglobin, which is the iron- and oxygen-binding protein found in blood, specifically in the red blood cells. Myoglobin forms the pigments responsible for making meat red, and in accordance and in the present study, values for the concentration of myoglobin were significantly higher in the *rectus femoris* compared to those in the *longissimus dorsi* (512 versus 257 ng/ml respectively; Tables 22 and 23). The color that meat takes is partly determined by the oxidation states of the iron atom in myoglobin and the oxygen species attached to it. When meat is in its raw state, the iron atom is in the +2 oxidation state, and is bound to a dioxygen molecule (O_2). Myoglobin is found predominately in Type I muscle, Type II A and Type II B.

There are very few studies examining gene expression of myoglobin in the muscle of pigs. Tong et al. (2004) studies the developmental patterns of myoglobin mRNA expression between Erhualian and Large White boars. Semi-quantitative RT-PCR was applied to determine the level of myoglobin mRNA. Different developmental patterns were observed in two breeds of pigs. Myoglobin mRNA expression was low in both breeds at day 3, while divergent trends were followed by different breed of pigs thereafter. No significant changes in myoglobin mRNA expression were observed in Large White boars over the period of investigation, although a higher level was seen at day 120. In Erhualian boars, however, the level of myoglobin mRNA increased significantly ($P<0.01$) from day 3 to day 20 and stayed high consistently afterwards. As a result, Erhualian boars expressed higher levels of myoglobin mRNA in the *longissimus dorsi* muscle compared with Large White boars on 20, 90, 120 and 180 days of age. Similar patterns of myoglobin mRNA expression were found in both sexes of Erhualian pigs, except at day 180, where a large decrease occurred in females ($P<0.01$) resulting in a significant ($P<0.01$) gender difference at day 180 with higher level of myoglobin mRNA expressed in Erhualian boars. Yao et al. (2011) also found breed differences in the myoglobin content of the *longissimus dorsi* muscle that increased from birth, a result supported by our data (Tables 22 and 23; Basal pigs compared to the other treatments).

Haemojuvelin (HJ; also referred to as RGMc/HFE2) is a membrane-bound and soluble protein in mammals that is responsible for the iron overload condition known as juvenile haemochromatosis in man. In humans, the HFE2 gene encodes the haemojuvelin protein. HJV is also called RGMc, a member of a three-gene family (in vertebrates) called the repulsive guidance molecules (RGMs); both RGMa and RGMb found in the nervous system, while RGMc is found in skeletal muscle and the liver. The expression of haemojuvelin was found to be lower in the *rectus femoris* compared to that in the *longissimus dorsi* (335 versus 510 ng/ml, $P<0.001$), suggesting a homeostatic mechanism to dispose of excessive iron from this particular muscle.

Deoxyhaemoglobin is the form of haemoglobin without bound oxygen to the molecule. Data found in the present study showed that the concentration of this gene was highest in the Basal treatment and those pigs fed High iron in the grower stage, but declined thereafter (Tables 22 and 23). Values for the concentration of this gene were also lower in the *rectus femoris* compared to that in the *longissimus dorsi*, suggesting that the higher iron levels in this muscle produce less of this molecule.

3. Outcomes and Conclusions

The major outcomes of this research are as follows:

Experiment 1:

- Housing type (conventional versus deep litter housing) had no influence on the mineral (iron, zinc) or myoglobin content of either the *m.longissimus dorsi* (LD) or *m.rectus abdominus* (RA).
- Age of the pig (7-35 weeks of age at slaughter) had little appreciable effect on iron and myoglobin contents, although in both muscle types, there was an indication that concentrations decreased beyond the first sampling time of 7 weeks of age.
- The RA was higher in iron and myoglobin content than the LD.

Experiment 2:

- The induction of mild iron deficiency in the grower stage followed by iron repletion in the finisher stage increased circulating iron levels with an interaction between diet and time.
- Increases in iron content were only found in the *m. rectus femorus* (RF) at the end of both the grower and finisher stages of growth in response to feeding the High iron diet and diet Low-High, respectively. The increase in iron content in the RF in the Low-High treatment supports the dietary depletion/repletion model of action.
- There were no deleterious effects on production parameters despite diets differing markedly in dietary iron content, although pigs fed diet Low-High had more P2 fat depth at slaughter.
- Pigs on diets Low-High and High-High in the finisher stage showed circulating elevated ferritin levels.
- The LD muscle from High iron pigs was darker and redder than in pigs fed Low iron, and pigs fed High-High had redder meat at the end of the finisher stage in the LD.
- The liver but not the heart stored iron in pigs fed diets High and diets Low-High and High-High.

Experiment 3:

- The results of this experiment ratified many of the physiological findings from Experiment 2.

Finally, a major outcome of the project is an increased understanding of iron regulation and metabolism of pigs, with for example the gene expression data being highly original and meat scientists, physiologists and molecular biologists around the world will meet the publication of these data with interest.

4. **Application of Research**

Given the nature of the experiments following the outcome of Experiment 1, then it was evident there were not going to be any (immediate) commercial opportunities uncovered by the research nor any cost of production benefits to Australian pork producers. The major commercial relevance of the findings from this experiment are as follows:

- Different housing systems do not significantly influence the iron and myoglobin contents of muscle in growing/finishing female pigs.
- The iron content of muscle, particularly the LD, is tightly regulated and relatively unresponsive whereas the red muscle examined (RF) was more responsive to the dietary manipulation regime imposed. Given that an annual genetic gain in iron content in pork of 0.02-0.04 mg/kg per annum is possible, without any unfavorable productivity associations, and with an even higher genetic gain anticipated in the redder muscles (see **5. Limitations/Risks, below**), then there is commercial potential to increase iron levels in selected muscles linked (or not linked, e.g., because of diet cost, practicalities etc.) to an appropriate nutritional strategy.
- Dietary manipulation of iron content was readily evident in the liver, which appears to act as a store for iron not required elsewhere in the body.

5. **Limitations/Risks**

The major limitations/risks to this work can be summarized as follows:

- It was not possible, with the ingredients available and the dietary specifications needed to comply with animal ethics requirements, to formulate a finisher diet that was "deficient" in iron; therefore, the 'Low' iron finisher diet exceeded the animals' requirements (NRC, 1998) and any carry-over effect of the dietary depletion from the grower diet (that was lower than requirements; NRC, 1998) wasn't able to be attenuated in the finisher period of growth. The formulation of an atypical diet (that might induce clinical iron deficiency) would be the only way to examine this proposition.
- The period of feeding (8 weeks for the grower phase and 7 weeks for the finisher stage, respectively) might have been too long to detect more subtle changes in iron absorption/metabolism and hence storage in muscles. Even though blood sampling was done two-weekly to try and account for this (i.e., Figures 1 and 2), the protocol would have missed daily/weekly changes in such events. However to examine such a protocol would mean the use of more pigs and more intense measurements.
- Only two muscles were assessed in this study, representing a 'white' and a 'red' muscle respectively, however a more complete assessment should incorporate measurement of a greater variety of muscles.
- This research was done in only one particular genotype so caution must be exercised with any extrapolation. However and as shown by Dr S. Hermesch and colleagues ("Genetic analyses of haemoglobin levels in pigs and iron content in pork"; AGBU Seminar 15th February, 2012) using many more pigs and two sire lines, a heritability of 0.34 in the iron content in pork exists demonstrating that there is considerable variation. Nevertheless these authors

surmised that an annual genetic gain in iron content in pork of 0.02-0.04 mg/kg per annum is possible, without any unfavorable productivity associations, with an even higher genetic gain anticipated in the redder muscles.

6. **Recommendations**

As a result of the outcomes in this study the following recommendations have been made:

- There is no benefit in feeding additional dietary iron *per se* to increase the iron content of muscle, except under circumstances where for example, there might be specific market segments/niches for higher iron pork; in this instance, redder muscle types will deposit more iron if it is supplied in excess to requirements in the diet.
- Dietary iron levels in young pigs should be re-examined, which could take further research, to eliminate the possibility that over supply of dietary iron early in life is not having a detrimental affect on iron absorption (and hence deposition in muscle) later in life.
- Gene expression studies coupled with appropriate sampling and monitoring is a plausible technique for assessing the physiological changes occurring in the growing-finishing pig.

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10. Appendices

APPENDIX I – Diet composition and analysis (Experiment 2)

	Diets fed in Grower Stage		Diets fed in Finisher Stage	
	High Iron	**Low Iron**	**High Iron**	**Low Iron**
Ingredients, %				
Barley	-	-	79.81	58.80
Wheat	80.59	52.78	-	-
Sweet lupins	-	15	-	25
Soybean meal	-	6.76	5	0.76
Bloodmeal	2	-	2	-
Meat and bone meal	5	2.66	9.88	2.96
Fishmeal	9.94	-	-	-
Skim milk powder	-	10	-	-
Whey powder	-	10	-	10
Canola oil	1.73	2	2.69	1.83
L-Lysine	0.20	0.28	0.05	0.13
DL-methionine	0.08	0.11	0.06	0.08
L-threonine	0.07	0.05	-	-
L-tryptophan	0.02	-	-	-
Salt (NaCl)	0.2	0.2	0.2	0.2
Choline chloride	0.03	-	0.17	0.10
Premix without iron	-	0.15	-	0.15
Premix with iron	0.15	-	0.15	-
Total	100.01	99.99	100.01	100.01
Analysed iron content, *premix* (mg/kg of premix)	60,680	2,889	60,680	2,889
Analysed iron content, *diet* (mg/kg of diet)	239 ± 22.9 (SD)	50 ± 2.9 (SD)	248 ± 15.4 (SD)	71 ± 14.8 (SD)

APPENDIX II: RNA Extraction Protocol

Everything used including solutions should be autoclaved prior to use and all solutions or glassware used pretreated with DEPC 0.1%. Autoclaving will remove these compounds from the solutions. Use gloves at all times.

- ⇒ *Very important to be very sterile (never do anything around post DNA extraction area's*
- ⇒ *Always keep everything on ice!*
- ⇒ *Anything to do with Phenol do in the fume hood and dispose of eppendorfs and tips separately.*

Before you start;

- *Put samples on ice out of the -80 to defrost*
- *Get out phenol:chloroform:isoamyl alcohol (in fridge) so it is at room temperature*
- *Clean lamina flow with DNAaway and leave the UV light on for about half an hour*

PROMEGA PROTOCOL

Cut 50mg of tissue using sterile blade and weigh on foil. Try to keep as sterile as possible and do as quickly as possible.

Place into 600ul of **Promega** Isolation denaturing solution reagent (1.2ml per 100mg tissue)

Homogenize on the beadbeater for 30 seconds. Add ~half a teaspoon of glass beads to each eppendorf. (If it rattles open and retighten handle.)

To Promega Solution add 600ul phenol:chloroform: isoamyl alcohol (P;C;IAA; equivolume to denaturing solution).

Shake vigorously 10 seconds

Incubate samples for 15min on ice

Spin 20 min at 4°C 12,000g (14000 rpm) *(Centrifuge is in the walk in fridge at the end of the corridor)*

Remove aqueous layer and transfer to a new tube *(should be between 400ul and 500ul)*

Add 2M Sodium Acetate *(in fridge)* and Isopropanol *(in hood, the rest is in flammable liquid cupboard in lab 2.030)*

Aqueous phase	*400ul*	*500ul etc..*
2M Sodium Acetate	*60*	*70*
Isopropanol	*400*	*500*

Mix by inverting and incubate at -20 for a minimum of an hour.

Spin at 12,000g (14000 rpm) for 20 min at 4°C

(face tubes all in the same direction so you will know where the pellet is)

Discard the supernatant *(remove excess with pipette)* and wash the pellet with an equal volume (to the total amount of denaturing solution used – *600ul*) of 70% ethanol ice cold *(in freezer)*

Centrifuge at 7500g (12000 rpm) for 5 min at 4°C

Discard supernatant and air dry the pellet and resuspend in 25ul of RNA water *(in fridge)* and then pool the duplicate samples to get a final volume of 50ul.
(air dry for less than 15mins – do not let it get glassy or clear)

Remove 2 ul for a nano drop reading - *SABC*

RNA/DNA nono-drop readings

Nano-Drop

- ⇒ *Take everything with you that you will need (issues, water, pipette (10ul) and tips)*
- ⇒ *Keep all things in the RNA box/container and move them all as little as possible from this box*
- ⇒ *Put tissue over the mouse*
- ⇒ *Take zip drive if want graphs*

On the computer;

- ⇒ *Icon ND-1000*
- ⇒ *Nucleic acid – drop down to RNA*
- ⇒ *Clean points*
- ⇒ *2ul water – PCR water*
- ⇒ *OK – clean again*
- ⇒ *Blank with whatever RNA was dissolved in*
- ⇒ *2ul*
- ⇒ *Hit blank icon*
- ⇒ *Clean*
- ⇒ *Add sample – 2ul*
- ⇒ *Measure*
- ⇒ *Record 260/280 and 260/230*

260/280 (x > 1.8)
If less means contamination
260/230 (x > 2)
If less means contamination

Nano-Drop EXAMPLE
803.8ng/ul
260/280 = 1.64
260/230 = 1.78
DNAse treatment (10-30ug RNA)
803ng in 1ul
x ng in 50ul
x = 803*50
= 40150ng
= 40.15ug in *x* ul

therefore 30ug in *x* ul
= 30*50
40.15
= 37.35ul tRNA

DNA removal

Genomic DNA is then destroyed by incubating total RNA Deoxyribonuclease I.

	Volume Punches	Volume Other	*Final conc (from example above)*
RNA sample	20ul *(x)*	(10-30ug)*	*37.35*
H_2O	57ul *(y)*	ul	*35.65*
10X buffer Promega	10ul	10ul	*10*
0.1M DTT	10ul	10ul	*10*
Rnasin inhibitor (40U/ul)	1ul	2ul	*2*
RQ1 Dnase (1U/ul)	1ul	5ul	*5*
Final volume	100ul	100ul	*100ul*

* Volume calculated from concentration of RNA

Then proceed as follows:
Mix well and incubate for 20min at 37°C
(this will remove the DNA)

At this point store remaining RNA at -80°C

While incubating put the phenol:chloroform: isoamyl alcohol (P;C;IAA) in fume hood, ensuring solution is at room temperature.

To stop reaction and separate phases, add 100ul of phenol:chloroform: isoamyl alcohol (P;C;IAA) and vortex for 30 seconds

Sit on ice for 10 min

Spin phase lock tubes (in RNA cupboard) for 20-30 seconds while waiting

Add (200ul) to 0.5 ml prespun phase lock gel tube (spin for 20-30 sec to pellet PLG), mix gently then spin at no more than 12000g (14000 rpm) for 5 min at room temp.

Collect upper aqueous phase (100 ul) into a new 1.5ml eppendorf, then add 15ul of 2M NaOAc pH 4.0

Add 4 times (~400ul) the aqueous volume of 100% ice cold ethanol *(in freezer)* – mix well

Sit at -80°C overnight or for at least 2 hours

END OF DAY 1

DAY 2

Spin 20min at 12000g (**14000 rpm**) at 4°C to pellet RNA.

Remove supernatant, and rinse pellet with 0.5ml 70% ethanol

Spin for 5 min at 7500g (**12000 rpm**) at 4°C, remove ethanol.

Allow to air dry and re-dissolve in 20ul RNA water

Take 2ul sample for nano-drop reading

Calculate and remove the amount of material required for 5 ug RNA (for both + and – RT reactions) for Reverse Transcription reaction and store the remainder at -80°C

eg 635.7ng/ul*20 = 12714 in 20ul
= 12.714 ug in 20ul
therefore 5.0ug in x ul
x = 5.0*20/12.714
= *7.865 ul*

As you will only have 18ul after nano-dropping you need to make sure that the amount for reverse transcription is less than 9ul as you need to do +RT and –RT.

Reverse Transcription Reaction ~ Standard 50ul reaction for up to 5ug RNA
(all reagents are in a box in the -20 freezer)

	+RT	-RT	Control
RNA (ul)			none
Random primers	2.5	2.5	2.5
Water			25.5
VOLUME	**28**	**28**	**28**

(only need one control)

Heat mix to 65° for 7 minutes then place on ice *(Program RT 1 on the thermocycler)*

Spin briefly to collect contents in the bottom of the tube then leave at room temperature for 10 minutes (primers anneal)

5* RT buffer	10	10	10
10mM dNTP's	5	5	5
0.1M DTT	5	5	5
RNAse inhibitor	1	1	1

Mix contents gently and incubate at 50°C for 2 minutes *(Program RT 2)*

RT-SSIII	1	0	-
H2O	-	1	1
FINAL VOLUME	**50**	**50**	**50**

Take RT-SSIII down to the thermocycler and after 2 min incubation add directly and mix gently by pipetting up and down.

Incubate for 45 minutes at 50°C, then at 55°C for 30 minutes then take down to 4°C *(incase don't remove in time) (Program RT 3)*

Place immediately on ice and into the -20 *(the freezer in the lab).*