DOES HYPOXIA OR ERYTHROPOIETIN PROMOTE IRON STORAGE IN PORK?

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Report prepared for the Co-operative Research Centre for an Internationally Competitive Pork Industry

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Executive Summary

The nutritional claim that pork is a "good" source of iron cannot be currently supported. Furthermore, the average iron content of pork in Australia (0.63mg/100g) is lower than that recorded by other major pig producing countries such as the US and Canada (> 0.8mg/100g).

The reduction of iron in pork over time has likely arisen from the change in muscle characteristics, such as myofibre composition, that has been brought about by selection for more growth-efficient animals slaughtered at a younger age. As a result there has been an increase in the proportion of white anaerobic myofibres (white, fast twitch glycolytic) in the muscle which are generally associated with a lower level of myoglobin therefore lowering the amount of nutritionally available haem iron. To date, nutritional strategies to elevate iron content in pork have not been effective.

Iron in the haem molecule is an essential element for oxygen-binding proteins, such as myoglobin and haemoglobin, in managing oxygen transport. Exposure to a low oxygen state (hypoxia), such as at high-altitude, causes the activation of factors that increase red blood cells (erythrocytes) and haemoglobin concentration to compensate for increased oxygen demand. This leads to increased iron demand by erythroid cells because synthesis of haemoglobin is up-regulated which in turn stimulates the mobilization and acquisition of iron. Skeletal muscle accounts for 40% of the total body mass and 10-15% of the body's iron, mostly in the form of myoglobin which also contributes to the redness of meat. Myoglobin has been reported to be affected by hypoxia. Moreover, expression of erythropoietin (EPO), the primary growth factor for erythrocytes, is stimulated by hypoxia and in its purified form, is used in medicine as an adjunct to treatment for diseases that result in anaemia e.g. end stage renal failure.

The objective of this study was to perturb iron metabolism and affect iron stores in muscle by hypoxia or by administration of EPO. To that end, pigs were exposed to either intermittent (nightly) hypoxia in a purpose-built chamber over 8 weeks at a simulated altitude of 3800 m (~13% O_2) or received a low dose of EPO intravenously every second day for 8 days. Pigs in both studies were at or near market weight at slaughter (70-90 kg).

Whilst there was a demonstrable increase in red blood cell number, haemoglobin concentration and packed cell volume (haematocrit) due to intermittent hypoxia at 28d and 56d of treatment as predicted, there was no increase in iron content in the muscles of pigs. Moreover, the study confirmed the refractory nature of pig muscle to dietary supplementation of iron. Nonetheless, there was evidence that iron supplementation influenced iron stores in the liver and spleen and circulating levels of serum iron suggesting there had been a differential effect on partitioning and mobilisation of iron resources. There was also evidence to suggest that fortification of the diet with iron had altered the dressing percentage of the pig suggesting there had been a reduction in the weight of viscera of these pigs without compromising the weight of saleable meat. In contrast, serial injection of EPO over 8d resulted in a 20% increase in iron content of skeletal muscles concomitant with the predicted increase in red cell number, haemoglobin and haematocrit. However, it was apparent that the response to EPO had been limited by availability of iron as evidenced by the mobilization of iron from both the liver and spleen. This was despite access to dietary iron in excess of 400mg/day.

Serial injection of EPO is not considered a commercial strategy for increasing the level of iron in pork. Nonetheless, there was proof of the concept that it is possible to perturb iron metabolism in pigs and increase the iron content of their muscles. This work lays the foundation for increasing the effect of EPO and through further research raises the

possibility of manipulating the networks underpinning iron metabolism to provide a commercially viable approach to achieve the desired increase in muscle iron level.

Table of Contents

Executive Summary i

1. **Introduction** 1
2. **Methodology** 2
3. **Outcomes** 7
4. **Application of Research** 13
5. **Conclusion** 13
6. **Limitations/Risks** 14
7. **Recommendations** 14
8. **References** 14

1. Introduction

Currently, Australian pork does not meet the Australian Food Standards Code requirements of being a food that is "a good source" of iron. To achieve such a rating the food must contain no less than 25% of the RDI for that mineral (Food Standards Code). The average iron content of Australian pork is 0.63 mg/100g (0.5 to 1.5mg/100g, Food Standards Australia New Zealand 2011); lower than that recorded by other major pig-producing countries such as Canada (0.8-1.4mg/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 not been successful suggesting that muscle iron storage is refractory to dietary iron, the amount of which can vary in the diet according to the age and feeding phase of the pig (piglet vs weaner vs grower).

Several related factors appear responsible for the reduction in iron in commercial pork. It is likely that as a consequence of selecting for increased efficiency and growth in the production environment, pigs are not only slaughtered at an earlier age but also the composition of porcine muscle has shifted towards a higher proportion of anaerobic, fast twitch, white glycolytic myofibres. Both factors have likely resulted in an overall reduction in myoglobin which constitutes most of the nutritionally available iron, as haem-iron, in skeletal muscle. Skeletal muscle accounts for 40% of the total body mass and 10-15% of the body's iron (Ordway *et al*. 2004), mostly in the form of myoglobin which also contributes to the redness of meat.

Stimulation of red blood cell proliferation (erythropoiesis) affects iron stores. Exposure to a low oxygen state (hypoxia), such as at high-altitude, cause the activation of growth factors that increase the number of red blood cells (erythrocytes) and haemoglobin to compensate for the increased oxygen demand (Gore *et al*. 2006). Such effects have been reported in athletes after exposure to short term normobaric hypoxia (Basset *et al*. 2010). As a consequence of erythropoietic stimulation, there is an increase in iron demand by erythroid cells because synthesis of haemoglobin is up-regulated which stimulates both the mobilization and acquisition of iron stores (Robach *et al*. 2007, 2009). Exposure to prolonged hypoxia has been variously reported to affect myoglobin levels or its RNA (Reynafarje 1962, Terrados *et al*. 1990, Hoppeler & Vogt 2001, Robach *et al*. 2007) and serum erythropoietin (EPO) (Gore *et al*. 2006). EPO is a primary growth factor of erythrocytes (Lacombe & Mayeux 1998) causing them to proliferate and thus, in its purified form, EPO is used as an adjunct to treatment for diseases that result in anaemia e.g. end-stage renal disease (Bhandari *et al*. 1998, Beguin 1999). Serial injection of a low dose of EPO increases the expression of muscle iron proteins in human subjects and doubles the iron content in the muscle without affecting myoglobin levels (Robach *et al*. 2009).

The aim of the project was to determine if the iron content in pig muscle could be influenced by non-dietary strategies such as exposure to normobaric hypoxia or by the direct administration of erythropoietin (EPO). Both strategies are known to affect iron metabolism in other mammals which suggests the impact and mode-of-action is broadly conserved. However, the efficacy of such treatments to alter iron content in muscles has not been demonstrated in pigs. This study has tested the postulate that prolonged exposure to intermittent hypoxia, or short term serial injection of EPO, will alter the iron content of pig muscle.

2. Methodology

EXPERIMENT 1- HYPOXIA

Experimental design

The effect of intermittent normobaric hypoxia (simulated high altitude) and dietary iron supplementation on the iron content in pork was examined in a 2 x 2 x 2 factorial experimental design consisting of two oxygen levels (normoxia vs. hypoxia) x 2 diets (plus vs. minus dietary iron fortification) x 2 gender (boar vs. gilt).

Twelve pigs (12 week-old, straight bred Large White, 19.0? ± 0.22 kg) of each gender (gilt and boar, n =24)) were randomly assigned to one of 4 treatment groups (n = 6): 1) hypoxia, iron-plus diet; 2) normoxia, iron-plus diet; 3) hypoxia, iron- minus diet; 4) normoxia, iron-minus diet. Each treatment group was balanced equally by gender. Pigs were acclimated to handling and experimental conditions over a 13 d period prior to the experimental start point and were weighed weekly during the course of the 8 week experiment. Pigs were housed in the hypoxia chamber nightly at ~6.00pm and released each morning at ~6.00am into a day-pen identical to that of the normoxia treatment group pens. Blood samples for haematological assay were collected 13 days prior to experimental start point (-13d), at the midpoint (28d) and the end-point (56d) of the eight week experimental period. Tissues were collected post-mortem. At experimental start point the average weight of the pigs was 20.91 ± 0.31 kg.

Diet

Due to a delay in the supply of the experimental diet, pigs were fed a standard grower pellet (Ridley Agriproducts, Dalby QLD) over 13d prior to the experimental start at which time they were fed a specifically formulated weaner diet (Ridley Agriproducts, Dalby, QLD) for the 56d experimental period. Pigs were maintained on the weaner diet over the experimental due to costs associated with re-formulating a phase feeding diet regime. A pig starter premix plus (Fe+) or minus (–Fe-) ferrous sulphate was included in the ration (Table 1). The total iron content of the feed was determined by assay as 207 ± 3.73 mg/kg and 96 ± 1.92 gm/kg of feed for Fe+ and Fe- rations respectively (Appendix1). Access to feed and water was *ad libitum*. Growth performance was measured as the total weight (kg) gained over a 56 d experimental period.

Table1 - Components of diets used in experimental hypoxia

Ridley Code	Raw material component	Iron fortified Inclusion	Iron non-fortified Inclusion
1036	Wheat Cracked (%)	25.00	25.00
1041	Wheat Fine (%)	37.9	37.9
2225	Meat Meal (%)	1.3	1.3
2361	Fishmeal (%)	5.0	5.0
2362	Soybean meal (%)	12.5	12.5
2745	Milk powder (%)	0.8	0.8
2920	Biscuit meal (%)	8.7	8.7
2947	Dextrose monohydrate (%)	5.0	5.0
3181	Limestone Fine (%)	0.8	0.8
3225	Salt (%)	0.2	0.2
3262	DL-methionine (%)	0.03	0.03
3275	L-Lysine HCl (%)	0.32	0.32
3287	L-Threonine (%)	0.07	0.07
3421	Premix (no added iron) (%)	0.0	0.2
3422	Premix (added iron[1]) (%)	0.2	0.0
4255	Copper sulphate (%)	0.04	0.04
4647	Phyzyme XP5000 Pigs (Phantom) (%)	0.01	0.01
4761	Ultracid LAC plus Dry (%)	0.03	0.03
4908	Maxarome RP(%)	0.02	0.02
4971	Ronozyme WX CT(%)	0.03	0.03
	Total iron (mg/kg) (mean ± SD)	207 ± 3.73	96 ± 1.92

[1] Ferrous sulphate- 100g/ton of finished feed or 100g/2kg of Premix

Hypoxia chamber and pens

Two normobaric hypoxia chambers (Fig 1) were constructed by enclosing existing pig pens with high density polyethylene (4 mm) walls (1.45m high) covered by a hinged clear Perspex roof. The roof was supported by a hydraulic ram to allow the chamber to remain open for airing and drying during the day. A door in the front wall allowed access to the chamber and a hinged rear wall panel allowed the waste to be washed from the chamber daily. At floor level, the chamber was sealed with concrete adhesive sealant, while all joins between walls, roof and hinges were sealed with strips of foam rubber to retard the outflow of air. A control (normoxia) pen, a hypoxia chamber and a day pen for the hypoxic pigs were located in each of two adjacent rooms. Each room represented one of the two dietary regimes. Each pen/chamber was identical in living area (2.66m x 2.66m) and equipped with wall-mounted self-feeder bins and drinkers.

Figure 1- Pens constructed for hypoxic and normoxic conditions

Generation of a hypoxic environment

Pigs were acclimated to hypoxia chamber conditions over 5 days prior to experimental start point. No adverse physiological or behavioural effects of hypoxic conditions were observed for the duration of the experiment. Hypoxic conditions within each chamber were generated by the combined output of two Everest Summit Hypoxia Generators (Hypoxico, Altitude Training Systems, USA) with variable settings. Calibrated O_2 and CO_2 meters, equipped with alarms, were used to monitor the level of each gas constantly inside the chamber. HEPA-filtered air from the 2 generators was pumped into the chamber at a combined rate of 15.2 m^3 h^{-1}. Outflow air escaped via the natural leakiness of the chamber allowing ~1.5 chamber atmosphere changes to occur every hour. A stable O_2 and CO_2 atmosphere was achieved in the chambers within 3 h of initiation of hypoxic air conditioning (Fig 1). Pigs were exposed to maximum hypoxia (~13%) for 9 h. Pigs were housed in the chambers each night from 6.00pm and removed to their day pen at 6.00am each morning. The end-point O_2 level in the chamber was recorded prior to release of pigs to their day pen. The O_2% (± sd) of chambers over the 56 d experimental period was 12.9 % (±1.41) or a simulated altitude of ~3800m.

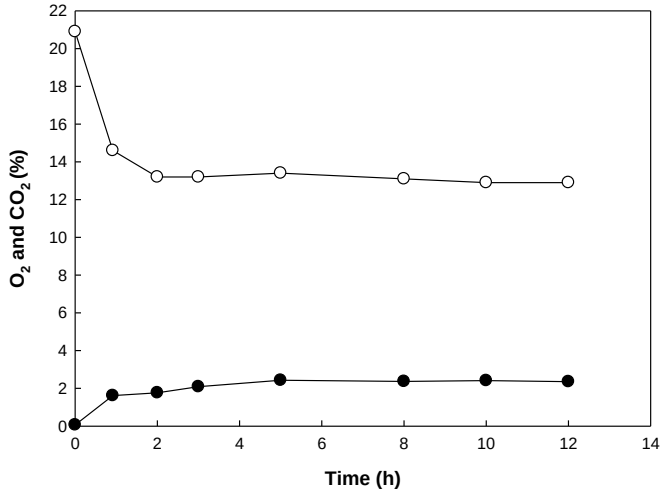

Figure 2 - Percentage O_2 (open circles) and CO_2 (closed circles) in a representative hypoxia chamber over a 12 h period.

Slaughter

Pigs were slaughtered humanely at a commercial abattoir (Swickers Bacon Factory, Kingaroy, Queensland) by rapid emersion into CO_2 atmosphere (81%), followed by exsanguination according to Australian Pork Industry best practice protocols. Samples of tissue were collected from pigs on the chain (liver, spleen and heart) ~20 minutes post slaughter and in the chiller (muscle tissue) ~20 min later. All samples were collected on ice and stored at 4 °C until next day delivery to laboratory where a total iron assay was performed.

EXPERIMENT 2 - EPO

Experimental design

The effect of serial injection of recombinant human erythropoietin (EPO, NeoRecormon, Roche, Melbourne, Australia) on the iron content of pork was examined in an experimental design consisting of two treatment groups: 1) EPO or 2) a saline control, with 6 gilts (Cross bred Landrace x Large White) in each group. Pigs were acclimated to conditions at the Australian Animal Health Laboratory facilities at Werribee, Victoria, for 4 days prior to the initiation of the experiment. Gilts with an average weight of 63.4 ± 2.7 kg were randomly allocated to one of two treatment groups and housed in a pen as a single cohort. Each pig was individually identified by a visible number with respect to treatment group. On days 0, 2, 4 and 6 of the 8-d experimental period, one group received 300 µL EPO (4000U/pig) by ear vein injection whilst a control group received 300 µL of the saline carrier only. Blood was collected for haematology on the days described above prior to administration of the EPO.

Diet

During the experimental period pigs were fed a commercial pig grower pellet (Barastoc) *ad libitum* and had unrestricted access to water. The components of the pellet are described in Table 2. The iron content of the diet was estimated to be 228 ± 16.5mg/kg. Growth performance was measured as the total weight (kg) gained per day over the 8d experimental period.

Table 2 - Components of Barastoc® grower pig diet used in EPO experiment

Component	Inclusion
Protein (minimum)	16 %
Fat (minimum)	2%
Fibre (maximum)	5%
Salt (maximum)	0.25%
Copper	160 mg/kg
Selenium	0.2 mg/kg
Digestible energy estimate	3.5 MJ/kg
Digestible energy	0.64 MJ
Total iron	228 ± 16.5 mg/kg

Slaughter

Pigs were humanely slaughtered using a protocol which consisted of first sedation by injection (IM) of xylazine hydrochloride (Rompun), followed by 20 mL of pentobarbitone IV injection, and then exsanguinated. All tissues for iron assay were collected onto ice immediately upon slaughter and held at 4 °C for delivery the next day to the laboratory where total iron assay was performed.

COMMON METHODS

Haematology

In both experiments, blood (6 mL) was collected by venapuncture into vacutainers for serum iron (μmoles L^{-1}) assay or vacutainers containing anticoagulant (EDTA) for full blood evaluation which consisted of an estimation of the number of erythrocytes (red blood cells, RBC), percentage packed cell volume (haematocrit) and the concentration of haemoglobin (Hgb, g/L). Assays were performed according to established medical laboratory procedures at QML Vetnostics, Brisbane, Queensland (Hypoxia experiment) and at the Gribbles Labs, Melbourne, Victoria (EPO experiment).

Tissue sampling

Post mortem samples (~10g) of liver, spleen, heart and from five skeletal muscles were collected from each pig for tissue iron assay. The skeletal muscles sampled represented the forequarter (m. t*riceps brachii, TB*), the loin (*m. longissimus dorsi,* LD) dissected between the $11/12^{th}$ rib, the hindquarter (m. *semitendinosus* (ST) and m. *semimembranosus* (SM)), and the diaphragm (thick skirt, TSK) as a representative of an oxidative (red) muscle.

Total iron assay

The total iron content in tissues and feed stuffs was determined chemically by Inductively Coupled Plasma Optical emission spectrometry (ICP-OES). Sample preparation was according to established protocols (McDaniel 1991).

Statistics

In Experiment 1 all dependent variables were analysed using a general linear model (Procedure GLM) in SAS (v9.2) in which oxygen level, dietary iron and gender were fixed effects. All first order interactions between fixed effects were included in the model. Effects in the model were deemed significant at $P \leq 0.05$. Where appropriate, covariates were tested and removed if found not to be significant ($P > 0.05$).

In Experiment 2, dependent variables were analysed using a general linear model (Procedure GLM) in SAS (v9.2) in which treatment and day of treatment were fixed effects. In the analysis of the effect of EPO treatment on muscle iron concentration

(mg/kg), muscle was included as a class and analysed using the following model: Muscle Fe = μ + Treat + Covariates (Liveweight (d8) + Serum FE (d8) + muscle + treat*muscle.

Animal welfare and ethics

All animal husbandry, handling, disposal and experimental procedures had prior approval by the University of Queensland Animal Ethics Committees (AEC) (Production and Companion Animal 2 - SAS/425/09/Pork CRC) and the Australian Animal Health Laboratory (AAHL) AEC.

3. Outcomes

HYPOXIA EXPERIMENT

Growth performance

The effects of O_2%, dietary iron and gender on liveweight gain and weight at slaughter (SWT) are shown in Fig 3A and 3B. The effect of O_2% on live weight gain and SWT was not significant ($P > 0.05$). However, iron- fortification (Fe+) significantly ($P < 0.05$) reduced live weight gain by 5.0 kg compared to those pigs fed a reduced iron (Fe-) diet. Gender was a significant ($P < 0.05$) factor contributing to differences in gain and SWT, with boars gaining 5.5 kg more than gilts at the same age. The effects of O_2%, dietary iron and gender on carcass weight are shown in Fig 3C. The effect of O_2% approached significance ($P = 0.055$) suggesting that intermittent hypoxia reduced carcass weight by 3.9 kg compared to pigs that were not exposed. Contrary to the observed effect on liveweight gain, iron fortification did not contribute to variation in carcass weight while the effect of gender approached significance ($P = 0.08$) with boars carcasses heavier than gilts as predicted (Latorre *et al*. 2010). These data suggest that the overall effect of gender on weight was small despite evidence that gilts were 2mm fatter than boars at the P2 site (data not shown). The effects of O_2%, dietary iron and gender on dressing percentage are shown in Fig 3D. Pigs on Fe+ had a significantly ($P < 0.05$) higher dressing percentage than those pigs on Fe-. These data suggest that observed gains in liveweight in Fe- pigs were in the viscera.

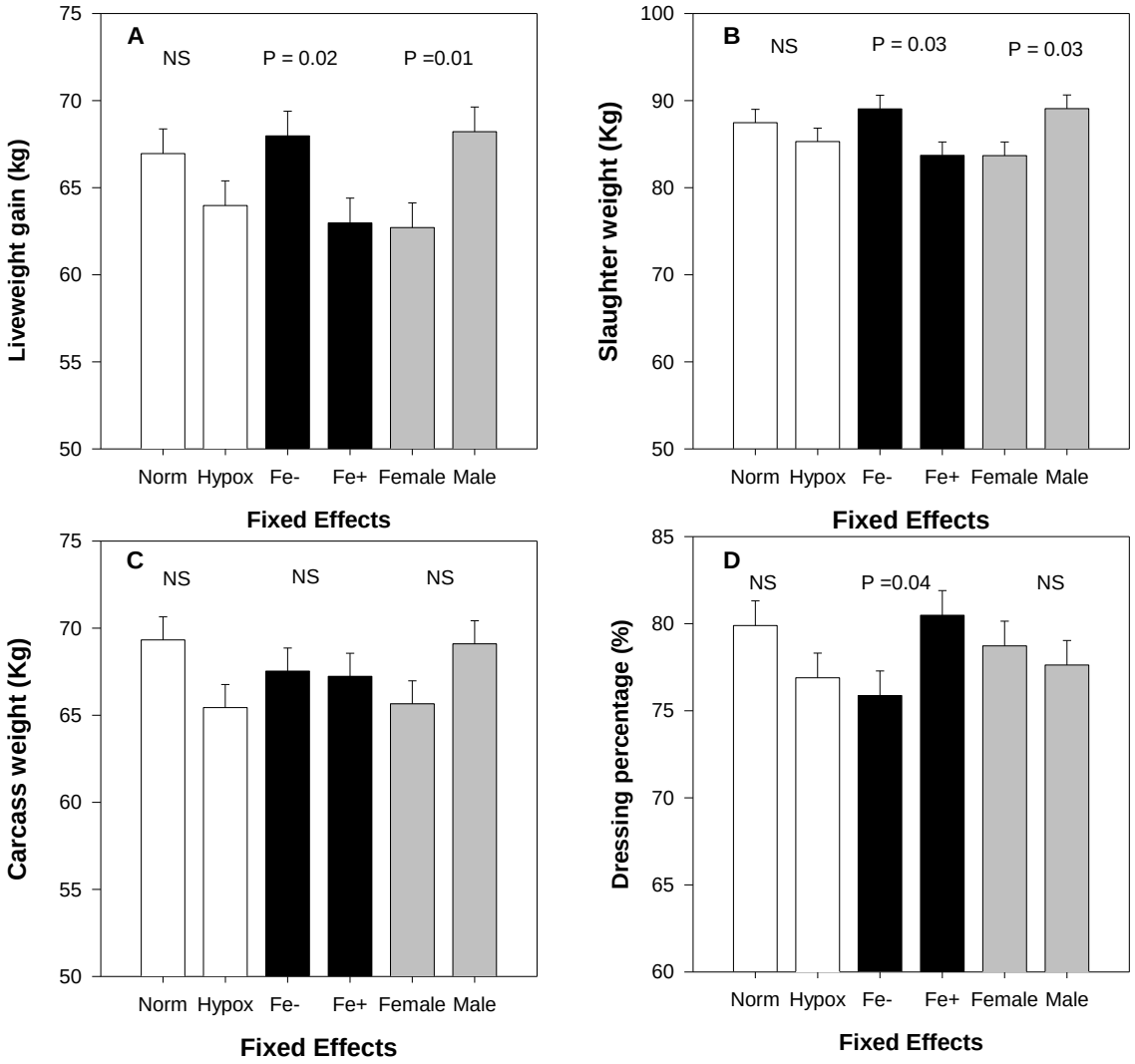

Figure 3 - Effect of oxygen level (Normoxia vs. Hypoxia), dietary iron (Fe+ vs Fe-) and gender (female vs. male) on live weight gain(A), slaughter weight (B), carcass weight (C) and dressing percentage (D).

Haematology

The effects of O_2 %, dietary iron and gender on RBC, haematocrit and haemoglobin concentration are shown in Table 3. Oxygen level contributed significantly ($P < 0.001$) to the variation in RBC at 28 d with pigs exposed to hypoxia having 14% more RBC than those not exposed. The difference in RBC at 56 d remained significant ($P < 0.01$) such that those pigs exposed to hypoxia had 7% more RBC than those not exposed. Dietary iron and gender were not significant factors in the model although there was an age-related increase in RBC independent to the effects of treatment over the experimental period. There was a significant ($P < 0.001$) effect of oxygen level on haemoglobin concentration at 28 d with pigs exposed to hypoxia having a 10% higher concentration compared to normoxic pigs. The difference in haemoglobin concentration between oxygen level treatments at day 56, although lower compared with 28d, approached significance ($P = 0.07$) with hypoxic pigs having a 5% higher concentration than normoxic pigs. There was a significant ($P < 0.001$) difference in haematocrit at 28d with hypoxic pigs having a haematocrit ~13% higher than normoxic pigs. The difference in haematocrit between oxygen level treatments remained significant ($P < 0.05$) at 56d. Dietary iron and gender were not significant factors in the model. The data would support the contention that the effects of a low oxygen state on red blood cell parameters were

attenuated over the last 28d of the experiment and suggest that pigs were adapting to hypoxic conditions.

Table 3 - Effect of oxygen level, dietary iron and gender on red blood cell parameters over the course of the 56 day experiment.

Fixed Effect	Oxygen level		Iron		Gender	
Day of experiment	**Hypox**	**Normox**	**Fe+**	**Fe-**	**Boar**	**Gilt**
			Red blood cells (x 10^{12})			
Baseline at day -13	6.3[a]	6.0[a]	6.3[a]	6.1[a]	6.2[a]	6.1[a]
Midpoint at – day 28	**7.5[a]**	**6.7[b]**	7.1[a]	7.1[a]	7.0[a]	7.1[a]
Endpoint at day 56	**8.2[a]**	**7.6[b]**	7.8[a]	7.9[a]	7.8[a]	7.9[a]
			Haemoglobin (g/L)			
Baseline at day -13	103.1[a]	101.3[a]	100.4[a]	104.1[a]	101.7[a]	102.7[a]
Midpoint at – day 28	**127.6[a]**	**115.2[b]**	121.7[a]	121.[a]1	120.0[a]	122.8[a]
Endpoint at day 56	**136.7[a]**	**129.9[b]**	135.6[a]	130.9[a]	131.8[a]	134.8[a]
			Haematocrit (%)			
Baseline at day -13	35.9[a]	35.0[a]	34.7[a]	36.3[a]	35.9[a]	35.0[a]
Midpoint at – day 28	**40.9[a]**	**36.8[b]**	39.0[a]	38.7[a]	38.3[a]	39.4[a]
Endpoint at day 56	**46.5[a]**	**44.0[b]**	46.0[a]	44.5[a]	44.6[a]	45.9[a]

A different letter in paired comparisons (in bold) beneath columns of fixed effects denotes significant difference at $P < 0.05$.

Serum iron

Oxygen level and gender did not contribute to variation in serum iron over the 56d experimental period (data not shown). There were no interactions between fixed effects. There was evidence to suggest that dietary iron fortification caused an elevation in serum Fe (Fig 4) where, by 56d the difference approached significance ($P = 0.088$).

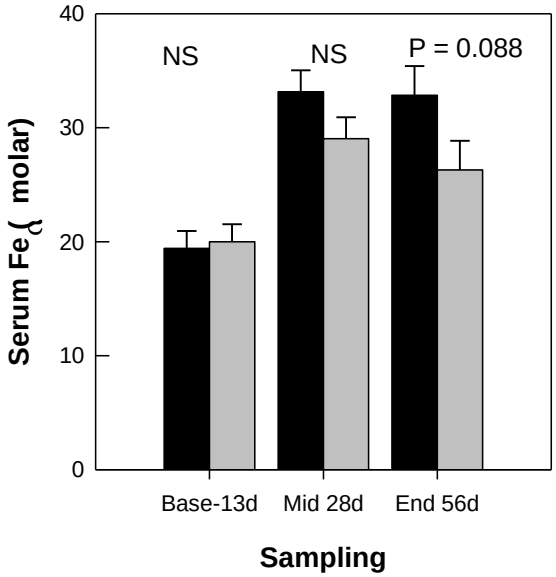

Figure 4 - Effect of dietary iron fortification on the level of iron in the serum Fe-plus, black bars, Fe-minus, grey bars.

Tissue iron

Oxygen level and gender were not significant factors in differences in the iron content of spleen, liver and heart. However, dietary iron fortification was associated

with a significant (P < 0.01) increase in iron in both the liver and spleen (Fig. 5, LS mean ± se).

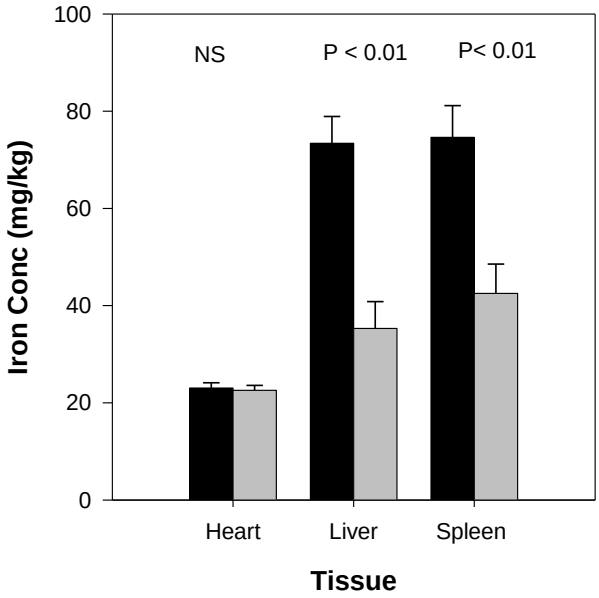

Figure 5 - Effect of dietary iron fortification on the level of iron in the heart, liver and spleen
Fe+, black bars, Fe-, grey bars .

Muscle iron content

The effect of hypoxia on skeletal muscle iron content is shown in Fig 6. Hypoxia did not increase iron levels in any of the muscles sampled. However, those pigs exposed to hypoxia had significantly (P < 0.05) less iron in the loin (LD) than normoxic pigs. The thick skirt (TSK, diaphragm muscle) was included in the design as an example of a red (slow twitch oxidative) muscle and although its iron content was not affected by treatment it was the richest source of iron of all the skeletal muscles sampled.

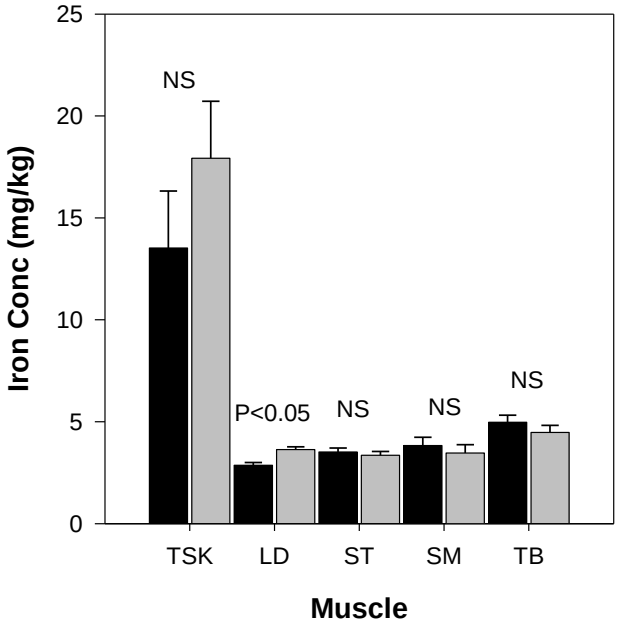

Figure 6 - Effect of hypoxia on the iron content (LS Mean ± se) of five skeletal muscles of the pig
Hypoxia, black bars, Normoxia, grey bars.

EPO Experiment

Growth performance

There was no effect of EPO on health, behaviour or growth of gilts. Over the 8-d period the gilts grew on average (± SD) 1.02 ± 0.08kg/d.

Haematology

The effect of EPO on the red blood cell parameters (red blood cell count, haemoglobin concentration and haematocrit) are shown in Fig 7. All three parameters increased with EPO treatment during the 8 day experiment when compared to those receiving saline alone. Furthermore, linear regression indicated that the rate of change in red cell parameters was ~2 fold higher for EPO-treated pigs compared to those treated with saline only. The similarity of the curves for the 3 parameters anticipated the expected correlation between them.

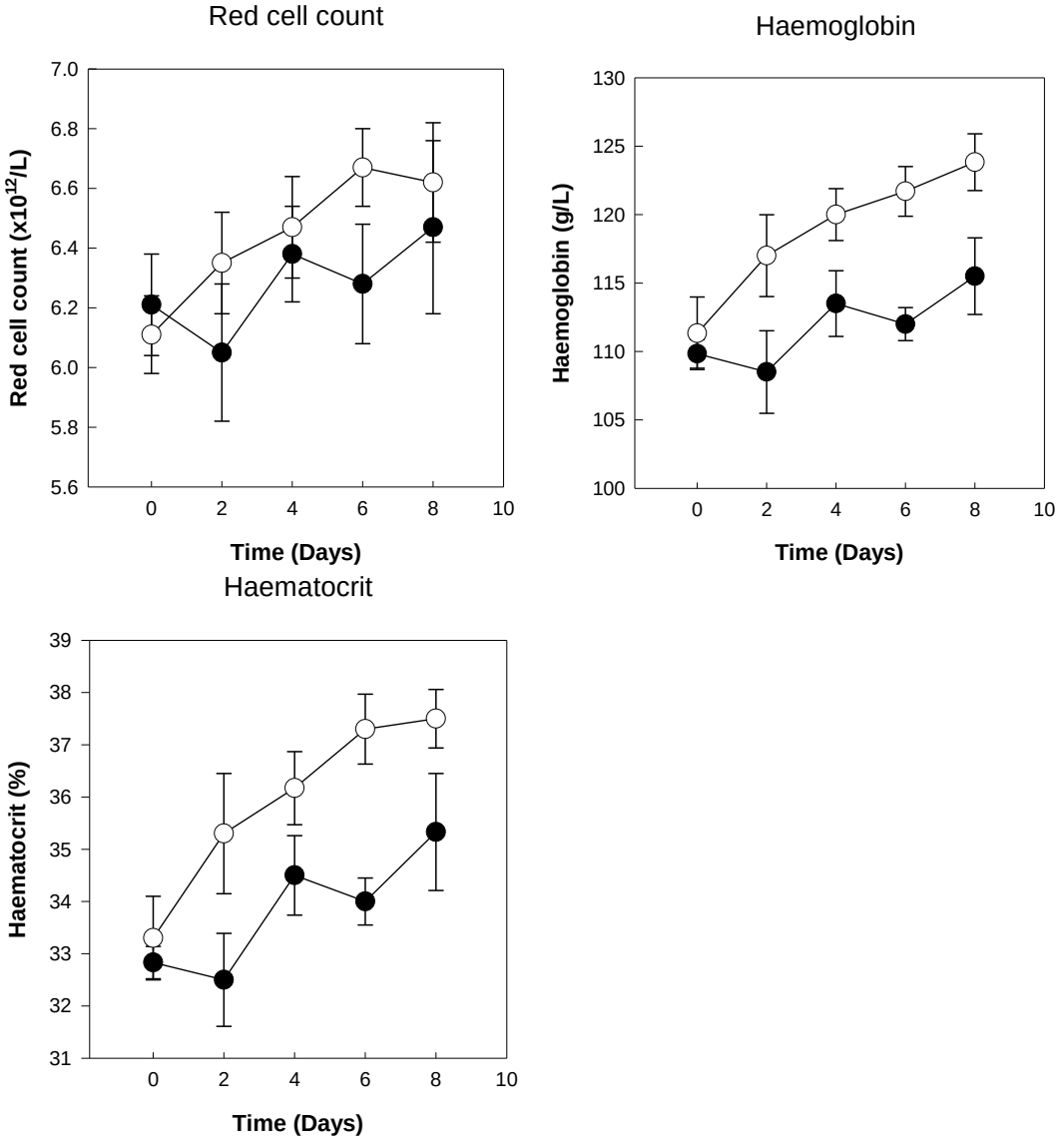

Figure 7 - Effect of serial injection of EPO on red cell count, haemoglobin and haematocrit

LS Means ± se of EPO (open circles) and saline control (closed circles).

Serum iron

Serum iron level (Fig 8) was not affected by EPO treatment although there was significant day to day variation over the 8-d period that was independent of treatment.

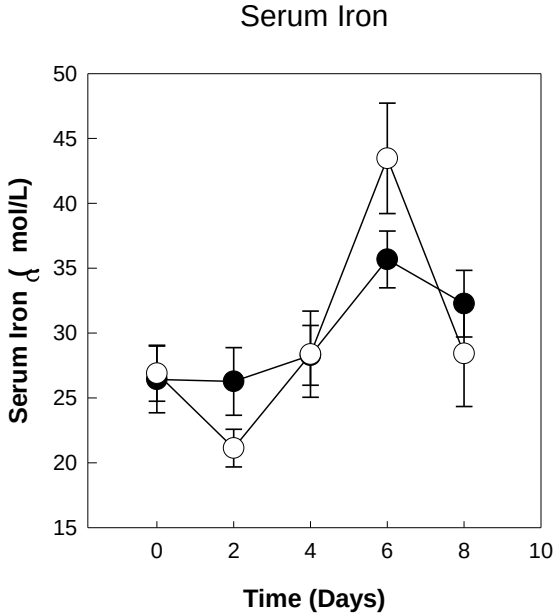

Figure 8 - The effect of serial EPO administration on serum iron concentration (LS means ± se)

EPO, closed circles; Saline control, open cirles

Tissue iron

The effect of EPO on liver and spleen is shown in Fig 9. Treatment with EPO over 8 days caused a significant ($P < 0.05$) reduction (~50%) in iron in the liver and also caused a reduction in iron in the spleen, however, the difference was not significant ($P < 0.10$).

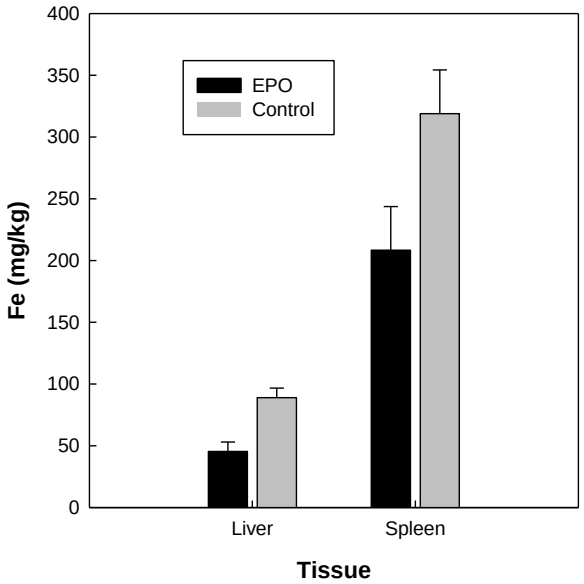

Figure 9 - The effect of EPO administration on the iron content (LS means ± se) of porcine liver and spleen.

Muscle iron

On an individual muscle to muscle basis (Fig 10), treatment with EPO did not significantly increase muscle iron content although of the six muscles including the heart, five muscles had a higher iron content compared to the control treatment. It is reasoned that the small number of animals per treatment group may have factored in the lack of significance. To improve the power of the analysis, it was repeated combining all muscle data into a single class. The revised model included serum iron concentration and liveweight at day 8 as covariates and explained 82% of the variance in muscle iron. Treatment was a significant ($P < 0.05$, $F = 4.72$) factor in the model and indicated that EPO-treated pig muscles had 2.9 ± 2.3 mg/kg (~20%) more iron than those muscles from saline-treated controls. Muscle was also a significant ($P < 0.0001$) factor and accounted for most of the variance in the model ($F = 51.4$). Despite the lack of an effect of EPO on serum iron, the concentration of iron in the serum on day 8 was a significant covariate ($P < 0.05$; $F = 4.04$) and indicated that for every µM increase in serum iron there was a 0.22 mg/kg increase in muscle iron. Neither the first order interactions between muscle and treatment nor the covariate of day 8 liveweight was a significant source of variation.

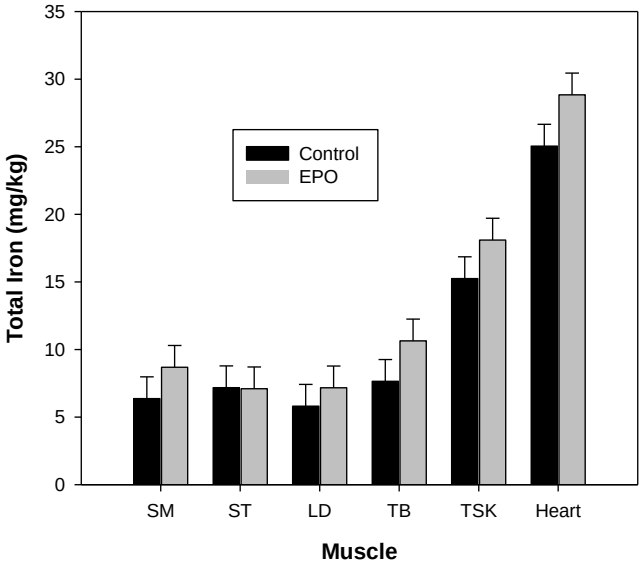

Figure 10 - Effect of EPO treatment on the iron content (LS means ± se) of porcine skeletal and heart muscles

4. Application of Research

The strategies tested in this study were designed to investigate the malleability of the iron metabolic axis given the refractory nature of muscle to dietary iron and were not intended for direct application to Industry.

5. Conclusion

The results indicated that exposure of pigs to intermittent hypoxia over eight weeks prior to slaughter did not affect the iron content of pork despite demonstrating the direct and predicted effect on erythropoiesis; such as increased erythrocyte number, haematocrit and haemoglobin concentration. Whilst dietary iron 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 5 percentage unit increase in dressing percentage without affecting carcass weight. The conclusion is that iron fortification reduces visceral mass through a yet unknown mechanism. This effect could

be titrate-able and worthy of further investigation to determine repeatability and dietary thresholds for iron.

The small increase in iron in muscle together with an erythropoietic response from serial injection of EPO 8 days prior to slaughter would suggest that the strategy had challenged the mobilisation and acquisition of iron by the muscle but not to the fullest extent. EPO therapy in humans can cause a rapid reduction in iron stores (Bhandari *et al* 1995, 1998) and this deficiency can lead to a sub-optimal response to EPO (Fishbane et al 1995). Based on these data, it is postulated that EPO-treated pigs became sub-clinically iron deficient which limited both the EPO response and the extent of iron storage in the muscle.

6. Limitations/Risks

7. Recommendations

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

1) Further research is required to determine if possible gains in efficiency can be gained through tighter control of type and amount of dietary iron.
2) Further research is required to determine if a direct supply of iron dextran can promote an optimal EPO dose-response to achieve a larger increase in iron stores in muscle.

8. References

Australian and New Zealand Food Standards (2011) http://www.foodstandards.gov.au/foodstandards/foodstandardscode.cfm

Basset FA, Joanisse DR, Boivin F, St-Onge J, Billaut F, Dore J, Chouinard R, Falgairette G, Richard D, Boulay MR (2007) Effects of short-term normobaric hypoxia on haematology, muscle phenotypes and physical performance in highly trained athletes. *Experimental Physiology* **91,** 391-402.

Beguin Y (1999) Erythropoietin and platelet production. *Haematologica* **84,** 541-547.

Bhandari S, Brownjohn AM, Turney JH (1995) Response of mean reticulocytes haemoglobin content to intravenous iron therapy in haemodialysis patients. *Nephrology Dialysis Transplantation* **13,** 3276-3277.

Bhandari S, Brownjohn A, Turney J (1998) Effective utilization of erythropoietin with intravenous iron therapy. *Journal of Clinical Pharmacy and Therapeutics* **23,** 73-78.

Canada Pork (2008) Nutrient value of Canadian Pork. Canadian Nutrient File, Health Canada. http://putporkonyourfork.com/media/pdf/nutrient_value_of_canadian_pork.pdf

Fishbane S, Frei G, Maesaka J (1995) Reduction in recombinant human erythropoietin doses by the use of chronic intravenous iron supplementation. *American Journal of Kidney Disease* **26,** 41-46.

Gore CJ, Rodriguez FA, Truijens MJ, Townsend NE, Stray-Gundersen J, Levine B (2006) Increased serum erythropoietin but not red cell production after 4 wk of intermittent hypobaric hypoxia (4000-5500 m*). Journal of Applied Physiology* **101,** 1386-1393.

Hoppeler H and Vogt M (2001) Hypoxia training for sea level performance. Training high-living low. *Advances in Experimental Medicine and Biology* **502,** 61-73

Latorre MA, Lazaro R, Valencia DG, Medel P, Mateos GG (2004) The effects of gender and slaughter weight on the growth performance, carcass traits and meat quality aspects of heavy pigs. *Journal of Animal Science* **82,** 526-533.

Lacombe C, Mayeux P (1998) Biology of erythropoietin. *Haematologica* **83,** 724-732.

McDaniel W (1991) Sample preparation procedure for spectrochemical determination of total recoverable elements in biological tissues. Method 200.3 pp 23-32. Chemistry Research Division, Environmental monitoring systems laboratory, US Environmental Protection Agency, Office of Research and Development, Cincinatti, Ohio,US.

Robach P, Cairo G, Gelfi C, Bernuzzi F, Pilegaard H, Vigano A, Santambrogio P, Cerretelli P, Calbet JAL, Moutereau S, Lundby C (2007) Strong iron demand during hypoxia-induced erythropoiesis is associated with down-regulation of iron-related proteins and myoglobin in human skeletal muscle. *Blood* **109,** 4724-4731.

Robach P, Recalcati S, Girelli D, Gelfi C, Aachmann-Andersen NJ, Thomsen JJ, Norgaard AM, Alberghini A, *et al*. (2009) Alterations of systemic and muscle iron metabolism in human subjects treated with low-dose recombinant erythropoietin. *Blood* **113,** 6707-6715.

Reynefarje B (1962) Myoglobin *conte***nt a**nd enzymatic activity of muscle and altitude adaptation. *Journal of Applied Physiology* 17, 301-305.

Terrados N (1992) Altitude training and muscular metabolism. *International Journal of Sports Medicine* **13,** S206-S209

USDA National Nutrient Database for standard reference, Release 23 (2010) http://www.ars.usda.gov/SP2UserFiles/Place/12354500/Data/SR23/nutrlist/sr23a303