EFFECT OF NANO-CHROMIUM ON GROWTH PERFORMANCE CARCASS CHARACTERISTICS AND GLUCOSE METABOLISM OF FINISHING PIGS

Report prepared for the

Co-operative Research Centre for an Internationally Competitive Pork Industry

By

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Executive Summary

Chromium (Cr) is an essential mineral element for human and animal. In the past two decades, various forms of Cr^{3+} have been used in pig diets in order to improve growth performance, insulin sensitivity, immune response, carcass trait, pork quality, and to reduce stress responsiveness. However, Cr^{3+} is normally poorly absorbed and utilized even when supplemented in an organic form (Matthews *et al.* 2005) perhaps in part because of the tendency to form large aggregates. The efficiency of uptake of 100 nm size particles by intestinal tissue was 15 to 250 fold higher compared to 1 μm size particles (Desai *et al.* 1996). It is possible that micro- or nano-sized chromium may be a means of improving the poor absorption of chromium and ensuring more consistent responses to dietary supplementation. The aim of this study was to investigate the effect of normal size, micro- and nano- chromium picolinate on growth and carcass characteristics of gilts. In addition, the hypothesis that dietary nano- chromium picolinate would improve body composition by altering glucose metabolism and insulin signaling in muscle and fat was tested by measuring the gene expression of key enzymes and signaling molecules involved in glucose metabolism.

A total of 96 finishing gilts (initial weight 51.9±1.20 kg) were stratified on weight into 4 blocks of 8 pens of 3 pigs and then within each block each pen was randomly allocated to 8 treatment groups in a 2×4 factorial design. The respective factors were dietary fat (25 or 100g/kg) and dietary Cr (0, 400 ppb normal size Cr picolinate (CrP), 400 ppb 1μm Cr picolinate (μCr), or 400ppb 100 nm Cr picolinate (nCr). The μCr and nCr particles were made through grinding CrP through appropriate sized sieve end plates. Feed intake and weight gain were recorded weekly and blood samples were obtained on day 0, 21 and 42. At the end of experiment, pigs were slaughtered at a commercial abattoir and underwent an ultrasound scan to determine eye muscle depth and P2 back fat. Samples of the *Longissimus thoracis* muscle and subcutaneous adipose tissue samples from above the *Longissimus thoracis* were collected 25 minutes post-slaughter and frozen in liquid nitrogen for gene expression using real time polymerase chain reaction. Data were analyzed by ANOVA using GENSTAT Release 11.1. Initial weight was used as a covariate for final weight and carcass weight was used as a covariate for P2 backfat and muscle depth. All gene expression data were analysed as the threshold cycle (cT) relative to that of β-actin (ΔCT) and assessed for main and interactive effects of dietary fat

and nCrPic. A difference in ΔCT of -1.0 is associated with a doubling (200%) and +1.0 a halving (50%) of expression.

Over the first 21 days average daily gain (ADG) was increased by dietary CrPic (944 vs 1011 g/d, respectively, P=0.021) although there was no difference (P=0.17) between the Cr sizes. High dietary fat also increased ADG over this period (963 vs 1026 g/d, P=0.013). However, the responses diminished over time and so there was no effect of CrPic (P=0.35) or dietary fat (P=0.93) on ADG over the full 42 days. However, dietary CrPic increased carcass weight and muscle depth with responses being greatest for nCrPic. Also, dietary CrPic decreased P2 back fat with the greatest response seen in pigs fed nCr and a high fat diet. Furthermore, dietary CrPic tended to decrease plasma insulin (6.9 vs 5.1 μU/mL, P=0.055) without changing plasma glucose (3.55 vs 3.47, P=0.62) indicating an improvement in insulin sensitivity.

Dietary nCrPic increased the expression of the insulin signalling pathway gene Akt (P=0.02) in adipose tissue but had no effect on expression of genes for insulin receptors, phosphoinositide 3-kinase (P=0.58) and glucose transporter-4 (P=0.83) or genes involved in adipocyte differentiation such as PPARγ, C\EBPα, SREBP and FAS, despite a reduction in P2. The UCP3 gene which improves insulin sensitivity was increased by nCrPic (P<0.01) and decreased by fat (P=0.02) supplementation. The adipokines leptin (P=0.04) and TNF-α (P=0.1) were increased with the high fat diet. Also, adiponectin was decreased by high fat (P=0.01) and increased by nCrPic (P=0.06).

Dietary nCrPic increased the expression of skeletal muscle insulin signalling pathway genes PI3K (P<0.01) and Akt (P=0.07) but had no effect on IRS or GLUT4. Expression of SOCS3, which can aggravate insulin resistance, was reduced (P=0.02) by nCrPic. Dietary nCrPic tended to improve UCP3 (P=0.08) and IL-15 (P=0.10) gene expression, both of which facilitate glucose metabolism. These results were generally consistent with improved insulin sensitivity observed *in vivo*.

An associated study has shown that dietary nCrPic tended (P=0.09) to increase average daily gain (ADG) over the entire study with most of the response occurring in the first 2 weeks. As a consequence, dietary nCrPic supplementation tended (P=0.09) to increase final body weight (Collins *et al.* 2011). Dietary nCrPic had no significant effect on HCWT, P2 depth and dressing percentage. These data indicate that dietary nCrPic can have a small effect on growth performance but not on carcass traits in gilts of a very lean genotype during a mild summer. In

other associated research, nCrPic was able to reduce respiration rate and rectal temperature and improve insulin sensitivity in heat-stressed sheep. These data suggest a role for CrPic or nCrPic in meditating heat stress and should be further examines in lactating sows in summer.

In conclusion, dietary CrPic supplementation to finishing pigs can improve carcass traits especially in pigs fed high fat diet and μCr and nCr. The mechanism of action appears to be altering both adipose and skeletal muscle tissue insulin sensitivity. The use of nano particles may present some difficulties with perception and so further studies perhaps should focus on micro particles or organic forms of chromium such as bioplexed material. Regardless of which approach is taken, dose response studies will need to be conducted. While the growth responses to chromium can be variable, the effects of chromium on insulin sensitivity are unequivocal. Associated research has shown that dietary nCrPic improves insulin sensitivity and protects against heat stress in sheep. Further studies need to be conducted with pigs, particularly sows, with either nCrPic or organic chromium, to see if dietary chromium can be used to protect against heat stress. Further work should also look at dietary cinnamon as a mimic for chromium.

1. Introduction

Chromium (Cr) is an essential trace element for humans and animals and has a key role in carbohydrate, fat and protein metabolism through potentiating the action of insulin (Mertz 1993). However, the absorption of Cr is poor with only 0.5-3 % of inorganic Cr being absorbed from the gastro-intestinal tract (Dowling *et al.* 1989; Ducros 1992). Although the organic forms of Cr are considered more bioavailable than the inorganic forms, only 10~25% of organic Cr can be absorbed (Underwood 1977). Recently, a number of researchers demonstrated that nanoparticles of Cr (nano-Cr) were absorbed more efficiently and were more bioavailabile than native CrPic and $CrCl_3$ (Lien *et al.* 2009; Zha *et al.* 2008). There are several factors regulating the intestinal absorption of particles, including their size, nature of polymers, zeta potential and vehicle (Delie 1998). A nano size composite possessing different electrical, magnetic, mechanical and biological properties because of it's reduced dimensions and the high surface area, was investigated in various biomedical applications (Sahoo and Labhasetwar 2003) and exhibited a higher rate of absorption in the gastrointestinal tract (Desai *et al.* 1996; Desai, Labhasetwar *et al.* 1997; Hussain *et al.* 2001). Due to the small size, nanocomposite can enter small capillaries and be taken up by cells (Sahoo and Labhasetwar 2003). For example, the efficiency of uptake of 100 nm size particles by intestinal tissue was 15 to 250 fold higher compared to 1 μm size particles (Desai *et al.* 1996).

There have been a number of studies that have shown improved growth performance, carcass characteristics, pork quality and reproduction in pigs supplemented with dietary Cr (Lindemann *et al.* 1995; Mooney and Cromwell 1995; Page *et al.* 1993; Shelton 2003). Moreover, Cr supplementation to the diet has been shown to increase insulin sensitivity and glucose clearance (Amoikon *et al.* 1995; Kim *et al.* 2004; Matthews *et al.* 2001). However, a number of

studies have reported little or no response to supplemental Cr (Bunting *et al.* 1994; Evock-Clover *et al.* 1993; Matthews *et al.* 2003; Neus Carbóa *et al.* 200; Xi *et al.* 2001). At least some of the variation in response to dietary Cr may be related to low and variable absorption of Cr. (Amoikon *et al.* 1995; Kim *et al.* 2004; Matthews *et al.* 2001). High fat diets are a means of increasing dietary energy for pigs (Brooks *et al.* 2011) although there is a possibility of increased fat deposition and whole body and peripheral tissue insulin resistance (Hansen *et al.* 1998; Storlien *et al.* 1986; Sabin *et al.* 2011). Therefore, the dietary fat content may influence the efficacy of dietary Cr on glucose metabolism. The aim of the present study was to investigate the effect of Cr particle size and dietary fat content on growth performance, carcass characteristics and insulin resistance of finishing pigs.

2. Materials and methods

2.1 Preparation of nanoparticulate chromium picolinate

Nanoparticulate Cr was processed by a dry polish method in a dry cryo-nanonization grinding system integrated with a size separator (Hsin-Fang Nanotech. Co. Ltd. Tainan, Taiwan). Briefly, the raw chromium picolinate (CrPic) material was ground and then passed through appropriate sized end-plates sieves to collect nano- and micro sized particles of Cr. No solvent was used and the temperature was kept below 40ºC during the milling process.

2.2 Animals and housing

Table 1. Ingredient and nutrient composition of experimental diets

Ingredient	Low Fat	High Fat
Wheat	816.2	555.8
Millmix	73.2	296.9
Soybean meal, 48%	35.7	47.5
Meat meal	32.3	19.7
Water	10	10
Porzyme 9310	0.2	0.2
Ronozyme P Liquid	0.2	0.2
Tallow-mixer	5.0	40.0
Salt	2.0	2.0
Limestone	17.3	19.7
Lysine-HCl	4.5	4.3
M. H. A	0.5	0.7
Threonine	1.4	1.4
Copper proteinate	1.0	1.0
Vitamin and Mineral Premix [a,b]	0.7	0.7
Total	1000.0	1000.0
Calculated nutrient composition		
DE, MJ/kg	13.8	13.8
Fat, %	2.2	5.7
Crude protein, %	16.1	15.9
Calcium, %	1.0	1.0
Total Phosphorus, %	0.5	0.5
Lysine, %	0.9	0.9

[a] Provided the following trace mineral per kilogram of premix: Se, 0.2 mg; Fe, 60; Mn, 25 mg; Zn, 50 mg; I, 0.2 mg; Cu, 10 mg.
[b] Provided the following vitamins per kilogram of premix: Vitamin A, 2.5 mg; Vitamin D3, 1mg; Vitamin E, 30 mg; Niacin, 10 mg; Ca-D-Pantothenate, 5 mg; Riboflavin, 2 mg; Vitamin B12 (Cyanocobalamin), 5 mg.

A total of 96 Large White x Landrace gilts with an initial mean body weight of 51.9±1.20 kg (mean±SEM) were weighed and stratified on live weight into 4 blocks based on quartiles in initial body weight. Within each block pigs were randomly allocated to 8 pens of 3 pigs and were then randomly allocated to a 2×4 factorial design. The respective factors were dietary fat (2.2% or 5.7%) and dietary Cr (0, 400 ppb CrPic, 400 ppb μCrPic, or 400ppb nCrPic). The diets were formulated to meet requirements. The composition of the two diets is reported in Table 1.

Feed intake and live weight were recorded weekly. The procedures used in the experiment were approved by The University of Melbourne, School of Land and Environment Animal Ethics Committee.

2.2 Plasma collection and analysis

Blood samples from pigs fasted for 16h were collected via the anterior vena cava on d 0, d 21 and end of the experiment. After collection of blood, the samples were placed on ice for 1 h, and then centrifuged for 15 min at 1,500 × g. Plasma was collected and frozen (-20℃) until subsequent analysis for glucose, insulin, nonesterified fatty acid (NEFA), and triglyceride (TG) analysis. Plasma insulin levels were determined by RIA procedure (Millipore Corporation, USA). Glucose, NEFA, TG concentration were determined by an enzymatic colorimetric procedure (Glucose, Thermo Fisher Scientific Inc. USA) (NEFA-C and TG kit,Wako Chemical Industries Ltd, Osake, Japan).

2.3 Carcass Quality Evaluation

At the end of the growth trial (average BW= 90.7 kg), all pigs were transport to abattoir for slaughter. Slaughter practices included electrical stunning followed by exsanguination. After slaughter all pigs were underwent an ultrasound scan (Pork Scan Pty. Ltd. Australia) for determined P2 backfat and muscle depth.

2.4 Real time PCR analysis of tissue samples

2.4.1 Tissue homogenization and RNA extraction

Frozen muscle tissue samples were pulverised into a fine power in liquid nitrogen using a pre-cooled mortar and pestle. Approximately 200-250 mg of muscle powder was weighed into

each of two pre-cooled 2 ml screw cap tubes (Axygen Scientific, California, USA) each containing one sterile 5 mm stainless steel bead (Qiagen, Hilden, Germany) and placed into liquid nitrogen until needed. (Two tubes per animal sample were needed to yield a sufficient quantity of high quality RNA for RT-PCR). To each tube, 1 ml of TRIzol™ reagent (Invitrogen, California, USA) was added and placed into a Mini Bead beater (Biospec Products Inc, USA) for two 20 second bursts, each separated by 1 minute. Following that, all samples were incubated at room temperature for 5 minutes to ensure complete nuclear protein dissociation. Following incubation, 200 µl of chloroform (Sigma, Castle Hill, NSW, Australia), was added to each tube and shaken vigorously by hand for 15 seconds. Samples were further incubated at room temperature for 2 to 3 minutes. Following incubation, samples were centrifuged in a Hettich EBA 12 centrifuge (Hettich, Tuttlingen, Germany) at 12,000 x g for 15 minutes at 4°C to fractionate RNA. Following centrifugation, 600 µl of the upper aqueous phase layer containing the RNA was transferred into a new sterile 1.75 ml Eppendorf tube (Axygen Scientific, California, USA). The transferred aqueous solution was centrifuged a second time at 12,000 x g for 15 minutes at 4°C to separate any remaining muscle tissue debris. Following the second centrifugation, 450 µl of the upper aqueous phase layer was transferred to a new sterile 1.75 ml Eppendorf tube. An equal amount of 70 % ethanol (Fronine Pty Ltd, Riverstone, NSW, Australia) was added to the transferred sample and contents mixed by vortexing (IKA, Staufen, Germany). Using the Invitrogen PureLink™ Micro-to-Midi RNA isolation system kit (Invitrogen, California, USA), two tubes of muscle tissue homogenate from identical samples were combined into one spin column. Purelink spin columns containing combined homogenate were centrifuged at 12,000 x g for 15 seconds at room temperature in an Eppendorf MiniSpin plus bench centrifuge (Eppendorf, Hamburg, Germany). The resulting flow through was

discarded and 700 µl of PureLink™ Wash Buffer I was added, and re-centrifuged at 12,000 x g for 15 seconds. The flow through and collection tube were discarded, and the spin column placed into a clean RNA wash tube. To this, 500 µl of Purelink™ Wash Buffer II was added and centrifuged at 12,000 x g for 15 seconds at room temperature. Flow through was discarded and a further 500 µl of wash buffer II was added and re-centrifuged. Spin cartridges were then centrifuged at 12,000 x g for 60 seconds to dry out the membrane with attached RNA. Collection tubes were discarded at this point and spin cartridges were placed inside Purelink RNA recovery tubes supplied with the kit. To the centre of the spin column, 55 µl of Purelink RNase free water was added and incubated for 1 minute at room temperature. Following incubation, the spin cartridges were centrifuged for 2 minutes at 14,500 x g at room temperature. Following centrifugation, spin cartridges were discarded and 3 µl of the eluent containing RNA was transferred into a sterile 1.75 ml Eppendorf tube and stored in a -80°C freezer for subsequent RNA integrity and yield evaluation with the remainder of the RNA sample.

2.4.2 RNA quantification

Yield and integrity of RNA in muscle tissue homogenate was evaluated using the Bio-Rad™ Experion™ automated electrophoresis system (Bio-Rad Laboratories Inc, California, USA) and the Experion StdSens Analysis Kit (Bio-Rad Laboratories Inc, California, USA). All StdSens kit reagents were allowed to equilibrate to room temperature before use with the RNA ladder thawed on ice. The electrodes on the Experion electrophoresis station were cleaned immediately before each run using the recommended procedures, Experion electrode cleaner solution, and DPEC water. The RNA gel from the kit was centrifuged at 1,500 x g for 10 minutes in a spin filter tube provided in the kit prior to use. The Gel stain solution was prepared by combining RNA gel and RNA stain in a 65:1 ratio. A 2 µl sample of the RNA ladder was transferred to a

sterile 1.75 ml Eppendorf tube for each chip run. The tubes containing the 2 µl of RNA ladder and the 3 µl experimental sample tubes were denatured for 2 minutes at 70°C using a dry heat block (Thermoline, Australia). Following heating, samples and ladder were both placed on ice and allowed to cool for 5 minutes. The Experion RNA StdSens chip was primed by addition of 9 µl of filtered gel-stain and subjected to pressure setting B and time setting 1 on the priming station. Following priming, the chip was examined for air bubbles or evidence that would indicate incomplete priming. If adequate priming was not achieved, the chip was discarded and the priming procedure using a new chip initiated again. Following successful priming of the chip, loading of the chip was initiated by addition of a further 9 µl of gel-stain solution, and 9 µl of the filtered gel. To each of the sample wells and the ladder well, 5 µl of loading buffer was added followed by the addition of 1 µl of denatured RNA ladder, and 1 µl of denatured RNA sample into specific wells respectively. The RNA chip was then placed into the Experion vortex station and allowed to mix for 60 seconds. Following vortexing, the chip was placed immediately into the Experion electrophoresis system and analysis was initiated. The electrophoresis system uses a photodiode laser to capture fluorescence detection and display RNA within samples as ribosomal protein bands. The resulting electropherograms were analysed for RNA concentration and RNA integrity. Integrity of RNA was evaluated by the ratio of 28S to 18S ribosomal protein as indicated by the two bands in Figure 1. Samples that exhibited two clean peaks and a ratio close to 1.5 were deemed sufficient quality RNA.

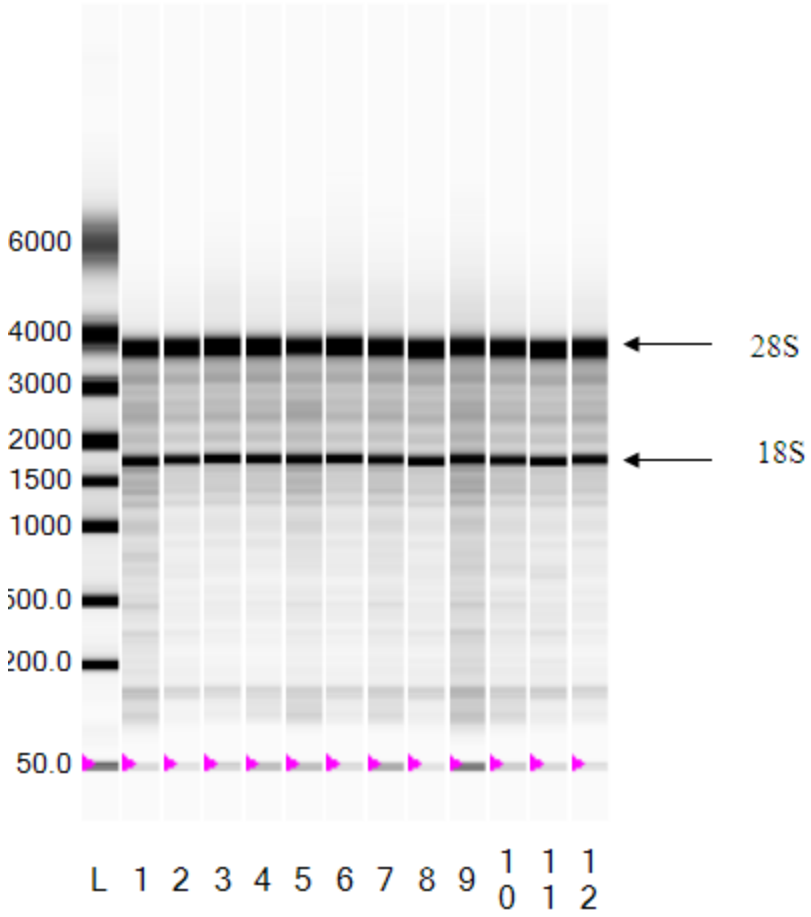

Figure 1. RNA electrograph showing the 28S and 18S ribosomal protein bands, RNA ladder (L) made up of multiple nucleotide base lengths ranging in size from 200 to 6000 bases in length. Lanes 1 thru 12 are ovine adipose tissue sample RNA. The Marker indicating 50 bases in length is a reference lane marker common to all samples as to accurately align samples with ladder to an identical starting point.

2.4.3 cDNA synthesis

Following acceptable RNA being extracted, complimentary DNA (cDNA) was synthesised from transcribed RNA using the Superscript III First Strand Synthesis System for RT-PCR (Invitrogen, California, USA). In brief, this method required 8 µl of RNA from each sample aliquoted in triplicate into a 96 well PCR plate (Simport, Quebec, Canada) and combined with 1 µl of 50 ng/ µl random hexamer and 1 µl of 10 mM dNTP mix. The plate was incubated at 65°C for 5 minutes inside a Corbett Palm Cycler PCR (Corbett Research, Sydney, Australia). A

cDNA synthesis mix was prepared using the following protocol. In brief, 2 µl of 10x RT buffer, 4 µl of 25mM $MgCl_2$, 2 µl of 0.1M Dithiothreitol (DTT), 1 µl of 40 U/µl RNase OUT, and 1 µl of 200 U/µl SuperScript III RT. Following the 5 minute incubation, 10 µl of the cDNA mix was added to each sample well, briefly centrifuged (Eppendorf, Hamburg, Germany) and then placed in the Corbett Palm Cycler for further incubation. This second incubation period consisted of 25°C for 10 minutes, 50°C for 50 minutes and, 85°C for 5 minutes. Following incubation, the plate was removed and cooled on ice for approximately 2 minutes. The final step of cDNA synthesis involved the addition of 1 µl of RNase H to each sample well and incubation at 37°C for 20 minutes. Following cDNA synthesis, 2 µl of each sample was removed from the plate and pooled in a sterile Eppendorf micro tube, and 20 µl portions were aliquoted from this cDNA pool. Following sample pooling, all cDNA plates and pooled aliquots were frozen at -80°C until required for RT-PCR. The 20 µl aliquots were used as positive controls during RT-PCR optimisation and gene expression procedures.

2.4.4 Reverse transcriptase polymerase chain reaction (RT-PCR)

All gene expression evaluation was undertaken using a Bio-Rad MyIQ single colour Real Time PCR Detection System (Bio-Rad Laboratories Inc, California, USA). All PCR reactions were evaluated using 0.2 ml iCycler 96 well PCR plates (Bio-Rad Laboratories Inc, California, USA) and sealed with iCycler IQ optical tape (Bio-Rad Laboratories Inc, California, USA). Each sample PCR reaction consisted of an in-well reaction Supermix containing 12.5 µl of SYBR Green Supermix (Bio-Rad Laboratories Inc, California, USA), 2 µl of cDNA synthesised from sample tissue, 1-3 µl of forward primer (5’ – 3’), and 1-3 µl of reverse primer (3’ – 5’). Primer volume would fluctuate depending on optimised primer concentration, and RNase free

water was added to the Supermix to make up a total volume of 25 µl in each well. Control wells were also implemented on each plate in duplicate, with positive controls comprising 25 µl of the above stated Supermix with cDNA, negative controls comprising 23 µl of Supermix and 2 µl of RNase free water, and plate blanks comprising 25 µl of RNase free water.

2.4.5 Primer design

Because the complete genome of the pig (*Sus scrofa*) is currently only partially sequenced, and the complete genome of the mouse (*Mus musculus*) and human (*Homo sapiens*) have been mapped, nucleotide primers that were used in RT-PCR reactions were designed using these three species messenger RNA (mRNA) sequences. These sequences are published in the internet nucleotide database of the National Centre for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov/sites/entrez. The published genome sequences in NCBI were copied into the Invitrogen OligoPerfect™ Designer software http://www.invitrogen.com/content.cfm?pageid=9716. The OligoPerfect™ Designer software locates primer sequences within the given mRNA sequence that meets individual specifications of size, annealing temperature, Glycine and Cysteine (GC) content, region of analysis, product size, salt concentration and primer concentration. The potential forward and reverse primers were then imported into Premier Biosoft International's Netprimer primer design software http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html to evaluate the structural and biochemical properties of the oligonucleotide sequence. A primer was considered appropriate if its rating out of a possible 100 was above 98, and adverse secondary structures including runs, repeats, palindromes, dimers, and hairpins were minimal. Only the best forward and reverse primer pairs which displayed the least number of adverse characteristics were

selected (Table 1). These custom primers were ordered through GeneWorks Pty Ltd (Hindmarsh, South Australia, Australia). The forward and reverse primers were made up to a stock concentration of 100 µM through the addition of an appropriate amount of 1 x Tris-EDTA (TE) buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0) depending on the concentration (nmol/tube) of the primer ordered. From this 100 µM stock, 5 µM, 3.2 µM and 2500 nM stocks were obtained by diluting an aliquot of the 100 µM stock solution with an appropriate amount of 1 x TE buffer. The 5 µM and 2500 nM stocks were used to optimise the concentration of primer needed to produce the most efficient standard curve for each primer set, while the 3.2 µM stocks were used for sequencing.

2.4.6 RT-PCR optimization

Following primer sequence design, conditions of both temperature, and primer concentration were optimised to obtain the most efficient annealing temperature, and a dilution series for creating an efficient standard curve for accurately evaluating gene expression.

All RT-PCR reactions used the following protocol:

Cycle 1: Step 1, 95.0°C for 03:00 minutes.

Cycle 2: Step 1, 95.0°C for 00:10 minutes,

Step 2, 55.4°C for 00:45 minutes. Cycle 2 was repeated 40 times.

Cycle 3: Step 1, 95.0°C for 01:00 minute.

Cycle 4: Step 1, 95.0°C for 01:00 minute.

Cycle 5: Step1, 55.0°C for 01:00 minute.

Cycle 6: Step 1, 55.0°C for 00:10 minute. Cycle 6 was repeated 80 times.

Table 1. Primer name and nucleotide database accession number, nucleotide sequences of forward and reverse RT PCR primers, and annealing temperature

Primer sequences selected for PCR optimization						
Gene	Abbreviation	Accession number	Primer sequence	Annealing temperature	Total amplicon Size (Base pairs)	GC%
β-actin	β-actin1	DQ845171	Forward 5'AAG GAC CTC TAC GCC AAC AC 3'	56.63	196	55
			Reverse 5'ACA TCT GCT GGA AGG TGG AC 3'	56.87		55
PI3K		NM_213939	Forward 5'AACCTCCAGATCTACTGCGGCAAA 3'	60.1	134	50
			Reverse 5'AGGAAGCGGTGGTCTATCAGCAAT 3'	60.0		50
AKT		NM_001159776	Forward 5'TCGTGTGGCAGGATGTGTATGA 3'	59.0	85	50
			TCGAAATACCTGGTGTCCGTCT 3'	57.5		50
SOCS3		NM_001123196	Forward 5'AGCCTATTACATCTACTCCGGG 3'	55.2	143	50
			Reverse 5'GCAGCTGGGTGACTTTCTCATA 3'	56.7		50
JNK		NM_213880	Forward 5'AAACTTTCCTCCTTCACGGTCC 3'	56.9	238	50
			Reverse 5'GTCATGCTCTGCTTCAGGATCT 3'	56.5		50
PPARγ	PPARγ	AB097926	Forward 5'TCA CGA AGA GCC TTC CAA CT 3'	57.2	238	50
			Reverse 5'TAT GAG ACA TCC CCA CAG CA 3'	56.6		50
FAS	FAS2	AY183428	Forward 5'TCG TGG GCT ACA GCA TGA TA 3'	57.3	208	50
			Reverse 5'GGA GTT AGG CTT CAG CAG GA 3'	57.0		55
GLUT4		NM_001128433	Forward 5'TCTCTGTGGGTGGCATGTTCTCTT 3'	59.9	171	50

			Reverse 5’TGAGGAACCGTCCAAGAATGAGCA 3’	60.0		50
TNF-α		M29079	Forward 5’GCCTACTGCACTTCGAGGTTATCG 3’	59.0	120	54.2
			Reverse 5’CGACGGGCTTATCTGAGGTTTGAG 3’	59.1		54.2
IL-15		NM_214390	Forward 5’GAAGCAACCTGGCAGCACGTAATA 3’	59.4	141	50
			Reverse 5’CAGGAGAAAGCACTTCATCGCTGT 3’	59.1		50
UCP3		NM_214049	Forward 5’CCCACTAGGATGGATGCCTAACAT 3’	58.0	182	50
			Reverse 5’CCTCTAGACTCGGCTGATTTCCAA 3’	57.9		50
C/EBP α	C/EBP α	AF103944	Forward 5’GTGGACAAGAACAGCAACGAGT 3’	57.4	116	50
			Reverse 5’TCCAGCACCTTCTGTTGAGTCT 3’	57.8		50
SREBP-1		AY338729	Forward 5’TCCTTCCACCATGAGCTCCC 3’	58.8	118	60
			Reverse 5’CACCGACGGGTACATCTTCA 3’	56.4		55
Adiponectin		EF601160	Forward 5’ATCACCATGCCTCGAGTCTTTCCT 3’	59.7	172	50
			Reverse 5’AGCATGTGGAGATGGAGCTGACTT 3’	60.0		50
Insulin receptor	IR	XM003123154.2	Forward 5’CAACACTGGTGGTGATGGAG 3’	60.0	150	55
			Reverse 5’CCATCCCATCAGCAATCTCT 3’	60.0		50
Leptin		NM_213840	Forward 5’GCA CGA CAC CAA AGA CAG A 3’	55.0	123	52.6
			Reverse 5’GCT GGA GAT GGA ACA GGA A 3’	54.1		52.6

Based on the Net-primer melting temperature (T_m) data, a range of temperatures were evaluated to determine optimal annealing temperature. An annealing temperature gradient ranging between 51.3°C and 63°C was performed and all genes of interest. The specific temperature that gave the lowest threshold cycle (C_T) value was selected as the optimal annealing temperature (refer to Figure 2). Annealing temperatures for all genes are shown in Table 1.

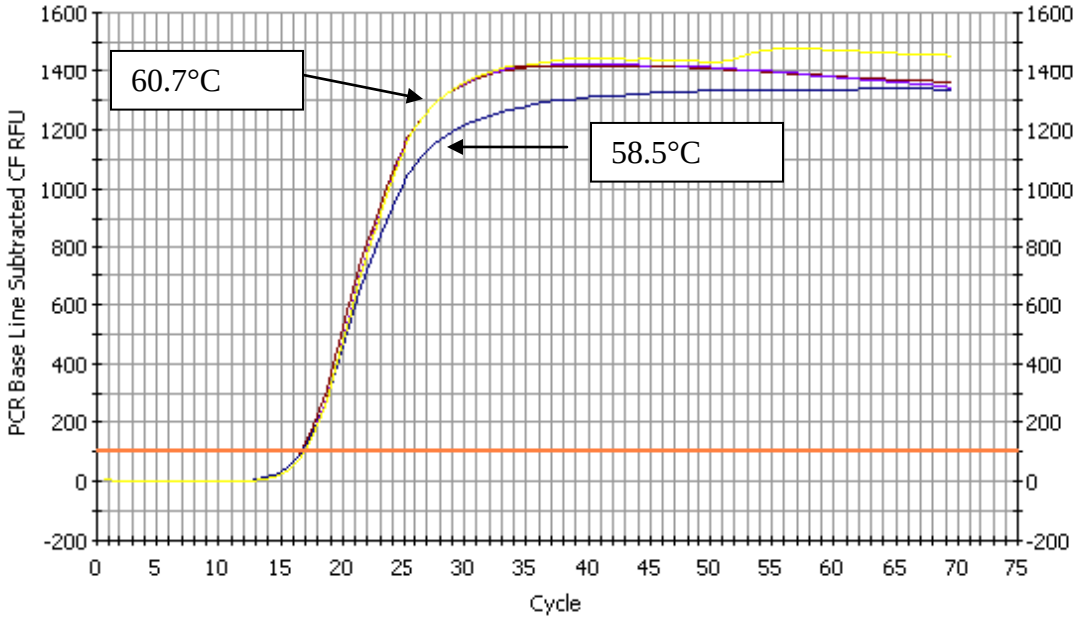

Figure 2. PCR melt curve indicating ultimate annealing temperature for Hormone Sensitive Lipase (HSL) occurred at 60.7 °C.

Following optimal annealing temperature evaluation, optimal primer concentration was elucidated by generating a standard curve using a ten-fold dilution series from 1:1 thru 1:1,000,000 of positive control template cDNA in triplicate. The dilution series produced evenly spaced amplification curves each in close proximity to the ideal 3.32 cycles apart. The data was plotted to determine linearity and efficiency, with a resulting Pearson's correlation coefficient (r) > 0.990, and PCR efficiency > 90% through a minimum of four dilution series points deemed

sufficient to warrant linearity. The C_T number that corresponded with the conditions indicating linearity was the selected C_T used to evaluate expression of genes of interest. Following the determination of C_T number, linearity and efficiency, gene of interest primer sets were deemed efficient and optimised, and the entire PCR plate frozen at -80°C for gene sequencing. The optimised conditions of each gene evaluated are shown in Table 2.

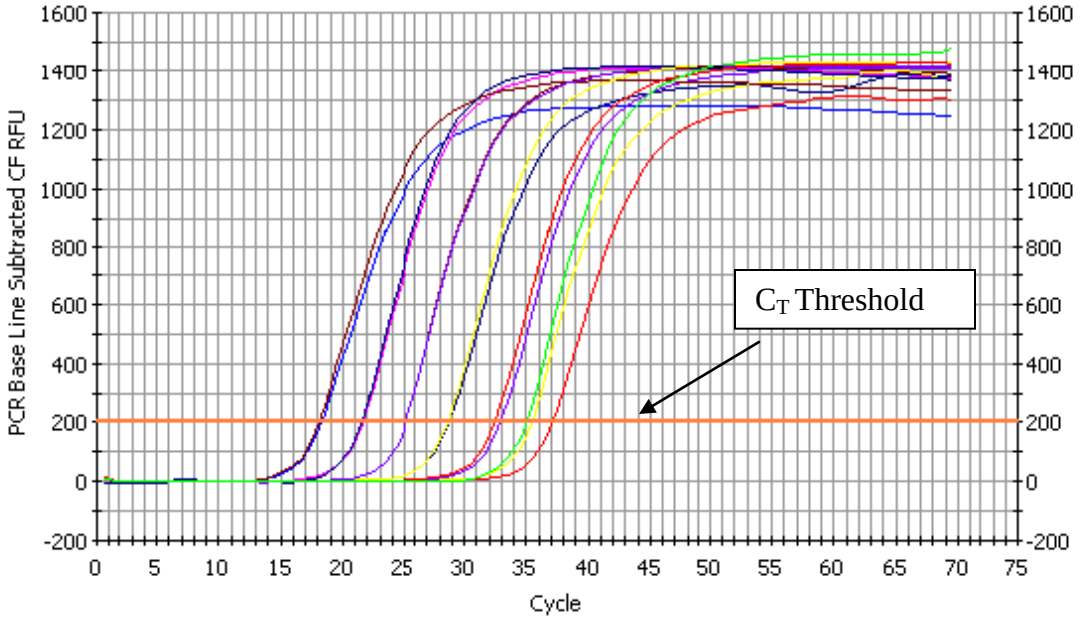

Figure 3. PCR Amplification curve showing a dilution series of 350 nM Hormone Sensitive Lipase (HSL) and the Threshold cycle (C_T) (201.5) which corresponds to the standard curve in Figure 4 located below.

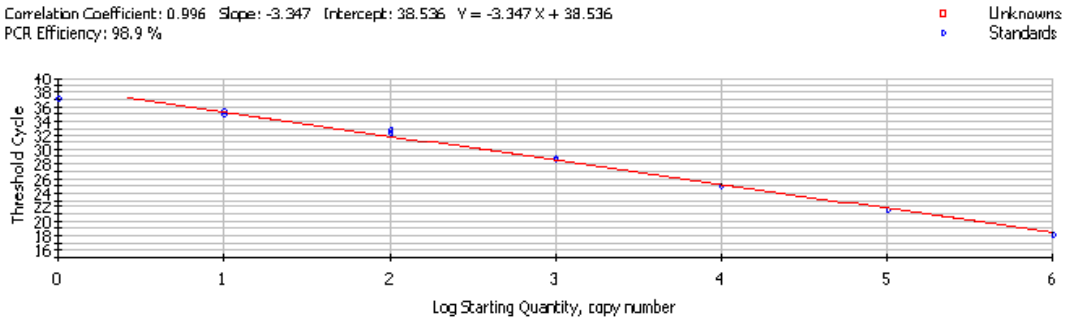

Figure 4. PCR standard curve for 350 nM Hormone Sensitive Lipase (HSL) indicating optimal conditions as indicated by a Pearson's correlation coefficient of 0.996, and PCR efficiency of 98.9%.

2.4.7 PCR product gene sequencing

Gene sequencing of PCR amplicon was necessary to evaluate primer design, and to validate the homology of the sequence with the specific species genome in order to guarantee that the sequence which was amplified during RT-PCR was in fact the sequence specific to the gene of interest. Following RT-PCR optimisation, samples were prepared for gene sequencing using the following procedure. Firstly, an agarose gel (1.5 %) was prepared by dissolving 1.5 grams agarose powder (Scientifix, Australia) in 100 ml of 0.5 x TAE buffer containing Ethidium Bromide ((50 µl per litre), Sigma, Castle Hill, Australia) followed by heating mixture in a microwave oven until boiling. The agarose solution was allowed to cool to approximately 60 °C and poured into an agarose gel mould (Bio-Rad Laboratories Inc, Riverstone, NSW, Australia), and a 20 well comb inserted into the gel and allowed to set. Once the agarose gel had set, the 20 well comb, and gel seals were removed from the mould and the gel placed into an electrophoresis gel tank (BioRad Laboratories Inc, California USA). The gel tank was then filled with 0.5 x TAE buffer until gel was submerged and sample wells had filled. Next, PCR products were prepared by using 15 µl of the 1:1 dilution sample well product and adding 2.5 µl of loading buffer (Yeastern Biotech Corporation, Taiwan) to each sample in a 0.2 ml sterile micro-tube (BioRad Laboratories Inc, California USA). All samples were briefly mixed by vortexing and 5 µl of 100 base pair ladder (Yeastern Biotech Co. ltd) was loaded into lane 1, and 15 µl of each sample mixture was loaded into the remaining wells in duplicate. Following the loading of samples and ladder, an electrical charge was applied to the gel tank at 90 volts for 50 to 60 minutes using a BioRad Powerpac 300 (Bio-Rad, California, USA). Following gel electrophoresis for 60 minutes, the gel was removed from the tank and placed onto an ultraviolet (UV) trans-illuminator (Wealtec Corporation, USA). The Ethidium bromide fluoresced under

ultraviolet light and enabled the DNA fragment from each sample to be excised by using a clean scalpel and forceps to a sterile 2.0 ml micro-tube (Axygen Scientific, California USA). The excised fragment from each sample was weighed and recorded. Using the Mini Elute Gel extraction kit (Qiagen, Germany), DNA fragments were prepared for gene sequencing using the following procedure. To each sample, 3 volumes of buffer QG were added to 1 volume of gel (v/w) which equated to 300 µl of buffer QG to 100 mg of excised gel fragment. Samples were then incubated at 50°C for 10 minutes on a dry heat block (Thermoline, Australia) to dissolve agarose gel. During this incubation step, samples were vortexed briefly every 2 to 3 minutes to assist in dissolving the gel. Following incubation, 1 gel volume of isopropanol (Sigma-Aldrich, St Louis, USA) was added to each sample and mixed by vortex. The samples were then transferred to a mini elute spin column provided with the kit and centrifuged at room temperature for 1 minute at 10,000 x g. The flow through was discarded and spin column placed back into the same collection tube. To each spin column, 500 µl of buffer QG was added and re-centrifuged at room temperature for 1 minute at 10,000 x g. Flow through was discarded and spin column re-inserted into the collection tube. To each spin column, 750 µl of buffer PE was added and allowed to incubate at room temperature for 2-5 minutes and then centrifuged at room temperature for 1 minute at 10,000 x g. Flow through was discarded, the spin column re-inserted into the collection tube and re-centrifuged for a further 1 minute at room temperature at > 10,000 x g. Following centrifugation, the spin column was placed into a sterile 1.5 ml microtube. To elute the DNA fragment, 10 µl of buffer EB was added to the centre of the spin column of each sample and incubated at room temperature for 1 minute. Following incubation, all samples were centrifuged at room temperature for 1 minute at 10,000 x g. Approximately 9 µl of eluent was recovered and 30 µl of 3.2 µM forward primer for each gene being sequenced was also prepared.

Both eluent and primer were kept at -80°C prior to being sent for sequencing the following day. A complete genome sequencing service was carried out by AgGenomics Pty Ltd (Bundoora, VIC, Australia). The complete genome sequencing service produced a chromatograph displaying the corresponding gene sequence and this sequence was then imported into the 'Blast' website of the National Centre for Biotechnology Information (NCBI) and cross matched for nucleotide similarities. The species genome was specified as highly homologous with *Ovis aries* for all genes. All PCR amplicon's sequenced matched genes of interest using this reference database.

2.5 Calculations and statistics

2.5.1 General

Data were analyzed by ANOVA using the REML procedure in GENSTAT Release 11.1 with the respective factors being dietary fat level (2 vs 6%) , dietary chromium level (as CrPic) (0 vs 400 ppb) and within dietary chromium size fractions (normal vs micro vs nano CrPic) and all interactions. Insulin resistance was determine using the homeostatic model assessment (HOMA) and quantitative insulin sensitivity check index (QUICKI) (Katz *et al.* 2000). A decrease in HOMA is associated with reduced insulin resistance and an increase in QUICKI is associated with increased insulin sensitivity.

2.5.2 RT-PCR gene expression evaluation and data analysis

Following confirmation that the designed primers were amplifying the specific gene of interest, gene expression was evaluated on all muscle tissue samples following the procedure outlined earlier. Threshold cycle (C_T) values were calculated as the cycle when fluorescence of the sample exceeded a set threshold level corresponding to the baseline fluorescence determined

during the optimisation process. A sample that contains a higher concentration of the gene of interest will cross the threshold at an earlier cycle than a similar sample with a lower concentration of the gene of interest. All samples had a C_T value for both the gene of interest and the reference/housekeeper gene (β-Actin or ribosomal 18S), with the difference between the two C_T values evaluated as the ΔCt.

When using RT-PCR to evaluate gene expression in samples obtained from multi-factorial experiments, the ΔCt method is required to statistically analyse the data arising from such experiments. The magnitude of the ΔCt of a gene indicates the level of gene expression, with the lower the ΔCt indicating a higher gene expression, and the higher the ΔCt indicating less gene expression. Following ΔCt evaluation of all samples, statistical analysis was carried out by REML using Genstat computational software. All expression data were reported as the threshold cycle (cT) relative to that of ribosomal 18S (ΔCT). A difference in ΔCT of -1.0 is associated with a doubling (200%) and +1.0 a halving (50%) of expression and for ease of presentation data are presented as % relative to expression in tissue from gilts fed the control diet without supplemental fat or nCrPic. This method of presentation prevents the presentation of the SED.

3. Results

3.3.1 Growth performance and carcass quality

Over the first 21 days ADG were increased by dietary CrPic (944 vs. 1011 g/d, P=0.023) although there were no different (P=0.18) between the CrPic fractions (Table 4). High dietary fat also increased ADG over this period (963 vs. 1026 g/d, P=0.023). Dietary CrPic and fat inclusion also tended to improve FCR over this period (2.28 vs. 2.14, P=0.079; 2.24 vs. 2.13, P=0.067, respectively). However, the response diminished over time and so there was no effect of CrPic

(P=0.31) or dietary fat (P=0.96) on ADG and CrPic (P=0.42) and dietary fat (P=0.96) on FCR over the full 42 days.

Final live weight tended (89.2 vs. 91.4 kg, P=0.081) to be increased by dietary chromium but there was no effect of dietary fat (90.6 vs. 91.1 kg, P=0.69) (Table 5). There were no differences in final live weight between the various CrPic fractions. Carcass weight was increased (65.4 vs.68.2 kg, P=0.002) by dietary chromium but there was no effect of dietary fat (67.4 vs. 67.8 kg, P=0.69) (Table 5). However, there tended to be a difference between the CrPic fractions such that carcass weight from pigs fed normal CrPic were not as heavy as those fed the µCrPic or nCrPic (66.7 vs 69 and 69.1 kg, P=0.059). This was particularly so for pigs consuming the low fat diet as indicated by the interaction (P=0.029) between dietary fat and within CrPic fractions (Table 5). Similar effects were observed for dressing percentage (Table 5). Back fat at the P2 site was decreased (8.2 vs.7.4 mm, P=0.009) by dietary chromium but there was no effect of dietary fat (7.7 vs. 7.6 mm, P=0.36) (Table 5). However, there tended to be an interaction (P=0.062) between dietary fat and within CrPic fractions such that P2 was decreased to a greater extent in pigs consuming the high dietary fat diet containing nCrPic (Table 5). Loin muscle depth was increased (49.3 vs 52.1 mm, P=0.024) by dietary chromium but there was no effect of dietary fat (51.6 vs. 51.1 mm, P=0.45) (Table 5). However, there was an interaction (P=0.040) between dietary fat and CrPic such that dietary CrPic increased muscle depth in pigs consuming a high fat diet (47.1 vs 52.4 mm) but not in pigs consuming the low fat diet (51.4 vs 51.9 mm) (Table 5).

Table 4. Effect of dietary fat and chromium picolinate on average daily gain (ADG), average daily feed intake (ADFI) and feed conversion rate (FCR) in finisher gilts.

	Low Fat				High fat					Probability				
	Con	CrPic	μCrPic	nCrPic	Con	CrPic	μCrPic	nCrPic	SED	Fat	Cr	Fat.Cr	within Cr	Fat. within Cr
0-21 days														
ADG, kg/d	0.91	0.99	1.02	0.93	0.98	1.00	1.08	1.06	0.027	0.016	0.023	1.00	0.18	0.20
ADFI, kg/d	2.16	2.15	2.21	2.10	2.14	2.16	2.23	2.21	0.049	0.51	0.62	0.46	0.49	0.64
FCR	2.36	2.20	2.16	2.26	2.19	2.17	2.06	2.10	0.071	0.067	0.079	0.60	0.65	0.76
21-42 days														
ADG, kg/d	0.91	0.80	0.88	0.94	0.80	0.82	0.86	0.85	0.056	0.33	0.92	0.48	0.45	0.73
ADFI, kg/d	2.53	2.41	2.56	2.34	2.38	2.37	2.53	2.54	0.110	0.98	0.99	0.40	0.53	0.61
FCR	2.82	3.15	3.00	2.50	3.01	2.89	2.98	3.00	0.167	0.48	0.98	0.73	0.38	0.19
0-42 days														
ADG, kg/d	0.93	0.90	0.95	0.93	0.88	0.91	0.97	0.96	0.029	0.96	0.32	0.27	0.25	0.98
ADFI, kg/d	2.34	2.28	2.38	2.22	2.26	2.27	2.37	2.38	0.071	0.82	0.84	0.38	0.48	0.55
FCR	2.52	2.56	2.51	2.37	2.54	2.49	2.45	2.49	0.059	0.96	0.42	0.85	0.45	0.36

[a] Fat: low fat vs. high fat; Cr: Control (Con) vs. all CrPic diets; within Cr: CrPic vs. μCrPic vs. nCrPic.

Table 5. Effect of dietary fat and chromium picolinate on carcass characteristics

	Low Fat				High fat					Probability				
	Con	CrPic	μCrPic	nCrPic	Con	CrPic	μCrPic	nCrPic	SED	Fat	Cr	Fat.Cr	within Cr	Fat. within Cr
LWT, kg	89.5	89.6	91.5	91.7	88.9	90.8	93.4	91.1	1.12	0.69	0.081	0.50	0.25	0.68
HCWT, kg	65.5	65.0	70.4	68.5	65.2	68.3	67.6	69.6	0.88	0.65	0.002	0.71	0.059	0.029
Dressing, %	71.9	72.9	76.7	75.3	73.3	76.0	73.2	75.7	1.03	0.69	0.032	0.50	0.69	0.050
P2 fat, mm	8.06	7.62	7.37	7.87	8.38	7.69	7.46	6.67	0.235	0.36	0.009	0.24	0.65	0.062
Muscle, mm	51.4	52.3	50.8	52.5	47.1	52.9	50.3	53.9	1.09	0.45	0.024	0.040	0.13	0.83

[a] Fat: low fat vs. high fat; Cr: Control (Con) vs. all CrPic diets; within Cr: CrPic vs. μCrPic vs. nCrPic.

Table 6. Effect of dietary fat and chromium picolinate on blood glucose (mM), insulin (mM), non-esterified fatty acids (NEFA, μM) and triglycerides (TG, μU/mL) and homeostatic model assessment (HOMA) and quantitative insulin sensitivity check index (QUICKI) in finishing gilts

	Low Fat				High fat					Probability				
	Con	CrPic	μCrPic	nCrPic	Con	CrPic	μCrPic	nCrPic	SED	Fat	Cr	Fat.Cr	within Cr	Fat. within Cr
Glucose	3.88	3.88	3.77	3.42	3.55	3.26	3.74	3.55	0.208	0.11	0.47	0.60	0.33	0.11
Insulin	7.58	4.60	6.06	3.58	7.75	6.23	5.04	5.03	1.232	0.53	0.018	0.80	0.55	0.50
NEFA	201	193	155	173	265	194	273	304	32.2	0.010	0.58	0.76	0.54	0.22
TG	21.8	22.0	23.2	22.5	27.7	23.2	23.5	24.3	1.32	0.056	0.23	0.085	0.85	0.90
HOMA	1.26	0.82	1.01	0.54	1.23	0.93	0.83	0.78	0.148	0.79	0.009	0.77	0.33	0.50
QUICKI	0.51	0.58	0.57	0.65	0.51	0.60	0.52	0.52	0.0278	0.15	0.022	0.38	0.35	0.11

[a] Fat: low fat vs. high fat; Cr: Control (Con) vs. all CrPic diets; within Cr: CrPic vs. μCrPic vs. nCrPic.

3.3.2 Plasma metabolites and hormones

Plasma glucose concentration was not affected by dietary CrPic (3.72 vs 3.60 mM, P=0.47) or fat (3.74 vs 3.53 mM, P=0.11) feeding (Table 5). Plasma insulin was increased by dietary CrPic (7.1 vs 5.1 μU/mL, P=0.018) but was unchanged by dietary fat (5.5 vs 6.0 μU/mL, P=0.53). Plasma NEFA concentrations were not affected by dietary CrPic (233 vs 215 μM, P=0.58) but were higher in pigs consuming a high fat diet (181 vs 259 μM, P=0.010) (Table 5). Plasma triglyceride concentrations were not affected by dietary CrPic (24.8 vs 23.1 mM, P=0.23) but tended to be higher in pigs consuming a high fat diet (22.4 vs 24.7 mM, P=0.056) (Table 5). There was an indication of an interaction (P=0.085) such that dietary CrPic decreased plasma triglycerides in pigs consuming a high fat diet (27.7 vs 23.7) but not in pigs consuming a low fat diet (21.8 vs 22.6 mM). Dietary CrPic decreased insulin resistance as indicted by a decrease in the homeostatic model assessment (HOMA) (1.25 vs 0.82, P=0.009). Similarly, dietary CrPic increased insulin sensitivity as indicated by an increase in the quantitative insulin sensitivity check index (QUICKI) (0.51 vs 0.57, P=0.022). There was no effect of dietary fat on either HOMA or QUICKI.

3.3.3 Adipose gene expression

Dietary nCrPic increased the expression of the insulin signalling pathway gene Akt (P=0.02) but had no effect on expression of genes for insulin receptors, phosphoinositide 3-kinase and glucose transporter-4 or genes involved in adipocyte differentiation such as PPARγ, C\EBPα, SREBP and FAS, despite a reduction in P2 (Table 7). The UCP3 gene which improves insulin sensitivity was increased by nCrPic (P<0.01) and decreased by fat (P=0.02) supplementation. The adipokines leptin (P=0.04) and TNF-α (P=0.1) were increased with the high fat diet. Also, adiponectin was decreased by high fat (P=0.01) and increased by nCrPic

(P=0.06). In conclusion, dietary can improved insulin sensitivity possibly via altering the expression of adiponectin, leptin and TNF-α genes.

Table 7. Effect of dietary fat and nano chromium picolinate (nCrPic) on the expression of adipose tissue genes

	Low Fat		High Fat		P – value		
	Control	nCrPic	Control	nCrPic	Cr	Fat	Cr x Fat
Akt	100	167	106	206	0.03	0.56	0..75
UCP3	100	384	283	479	0.003	0.02	0.10
Adiponectin	100	109	60	90	0.06	0.01	0.21
Leptin	100	320	554	327	0.38	0.04	0.04
TNFα	100	74	167	115	0.23	0.10	0.90
C Jun	100	106	197	153	0.64	0.03	0.48
C\EBP	100	172	366	346	0.49	0.02	0.40
FAS	100	187	57	100	0.85	0.86	1.00
GLUT4	100	246	228	69	0.83	0.76	0.17
ISR	100	94	93	109	0.91	0.93	0.78
PI3K	100	117	110	248	0.59	0.64	0.72
PPARγ	100	79	210	150	0.38	0.06	0.88
SOCS3	100	119	117	143	0.60	0.64	0.96
SREBP	100	113	66	43	0.79	0.22	0.62

3.3.3 Skeletal muscle gene expression

Table 8. Effect of dietary fat and nano chromium picolinate (nCrPic) on the expression of skeletal muscle genes

	Low Fat		High Fat		P – value		
	Control	nCrPic	Control	nCrPic	Cr	Fat	Cr x Fat
IRS	100	94	74	91	0.71	0.38	0.47
PI3K	100	174	135	212	<0.001	0.02	0.56
Akt	100	114	84	129	0.07	0.85	0.33
GLUT4	100	107	82	79	0.97	0.35	0.83
SOCS3	100	90	110	88	0.02	0.54	0.32
UCP3	100	130	80	117	0.08	0.36	0.75
IL5	100	117	86	151	0.10	0.79	0.32
C Jun	100	90	145	85	0.09	0.36	0.24

Dietary nCrPic increased the expression of insulin signalling pathway genes PI3K (P<0.01) and Akt (P=0.07) but had no effect on IRS or GLUT4 (Table 8). Expression of SOCS3,

which can aggravate insulin resistance, was reduced (P=0.02) by nCrPic. Dietary nCrPic tended to improve UCP3 (P=0.08) (Table 8) and IL-15 (P=0.10) gene expression, both of which facilitate glucose metabolism. These results were generally consistent with improved insulin sensitivity observed *in vivo*.

3.4 Discussion

It is generally agreed that small sized particles exhibit high rates of absorption from the gastrointestinal tract (Desai *et al.* 1996; Hussain *et al.* 2001). Due to their small size, nano sized particles can penetrate through small capillaries and be taken up by cells, allowing more efficient delivery to target sites (Sahoo and Labhasetwar 2003). For example, the efficiency of uptake of particles less than 100 nm by the intestinal was 15 to 250 fold higher than larger particles(Desai *et al.* 1996). Also, nano Cr has 2-3 fold higher tissue Cr deposition in pigs (Wang and Xu 2004) and1.66 fold greater Cr digestibility in rats (Lien *et al.* 2009). An *in vitro* study showed that NanoCr exhibited considerably higher absorption efficiency in Caco-2 cell monolayers, and the absorption of NanoCr from the small intestine was mainly via transcellular pathway, whereas larger size Cr particles were mainly absorbed via the paracellular pathway (Zha *et al.* 2008).

The results of previous studies involving Cr supplementation with respect to growth performance have been variable. Some reports have indicated that Cr may improve some aspects of growth performance (Lindemann *et al.* 1995; Mooney and Cromwell 1995; Mooney and Cromwell 1997), other researchers have indicated that Cr does not affect growth performance (Evock-Clover *et al.* 1993; Matthews *et al.* 2005; Matthews *et al.* 2003; Xi *et al.* 2001) or in some cases even decreased growth performance (Boleman *et al.* 1995; O' Quinn, Nelssen *et al.*

1998) of pigs fed with CrPic diet. In the present study, dietary CrPic improved growth performance and carcass quality but there was no difference between sizes of Cr. Thus, both dietary fat or CrPic supplementation improved performance during the early finisher period although the responses diminished with time. This is in agreement with Mooney and Cromwell's study (1995; 1997) that Cr addition improved growth rate and feed efficiency during the grower phase; however, the improvements did not continue during the finisher phase. Research has indicated that Cr deficiency may be exacerbated by dietary addition of 40% lard in rats (Striffler, Law *et al.* 1995). However, Jackson *et al.* (2009) indicated that dietary 7% fat did not affect Cr response in growth performance. In the current study, there were a number of small interactison that suggested that dietary CrPic supplementation was most efficacious in a high fat diet.

The NRC (1997) suggested that addition of Cr to diets for growing-finishing pigs can increase carcass leanness and decrease carcass fatness. Dietary CrPic have been shown to increase carcass leanness and/or decrease carcass fatness in pigs (Lien *et al.* 2001; Lindemann *et al.* 1995; Page *et al.* 1993), although the effects of CrPic on carcass quality were not consistent. Insulin has profound influence on protein synthesis (Wray-Cahen et al. 1998). Moreover, Cr has been shown to exert an anabolic effect *in vitro* in the presence of insulin by increasing amino acid incorporation in heart muscle (Roginski and Mertz, 1969). Lindemann (1999) indicated that supplement with Cr can increase longissimus muscle area (7%) and reduce 10^{th} rib backfat (-13%) in pigs. This is in agreement with our findings that dietary CrPic increased muscle depth (+7.6%) and decreased P2 backfat (-6%).

Cr is an essential nutrient involved in the uptake and metabolism of glucose. It is generally accepted that Cr exerts its effect on glucose metabolism as a component of glucose tolerance factor and thus is an insulinomimic agent. Amoikon *et al.* (1995) reported that CrPic

increased insulin sensitivity as assessed by increased glucose clearance rate and decreased glucose half-life during a glucose tolerance test and insulin challenge test. Also, CrPic is able to increase the rate of insulin internalization and uptake of glucose into skeletal muscle cells, with the reduction in glucose concentration being more pronounced in the non-fasting state (Evans and Bowman 1992). While some authors have reported that dietary supplementation with Cr significantly reduce fasting glucose in pigs (Lien *et al.* 2001; Wang *et al.* 2009; Wang *et al.* 2001), others have reported no effect in fasting glucose concentration (Amoikon *et al.* 1995; Matthews *et al.* 2001). In present study, fasting glucose concentration was not affected by either dietary CrPic or CrPic size. However, fasting insulin and more particularly HOMA (based on both insulin and glucose concentrations) were decreased while QUICKI (again based on both insulin and glucose concentrations) was increased by dietary CrPic indicating a decrease in insulin resistance (or an increase in sensitivity). The improvement of insulin sensitivity may facilitate glucose uptake and/or oxidation by adipocytes and muscle cells (Cupo and Donaldson 1987; Mirsky 1993; Rosebrough and Steele 1981). Insulin is a central regulatory factor in the muscle protein deposition through increasing skeletal muscle protein synthesis (Wray-Cahen, Nguyen *et al.* 1998). Thus, an increase in insulin sensitivity in muscle would result in an increase in skeletal muscle protein synthesis. The quite clear increases in the expression of the insulin signaling genes PI3K and Akt in skeletal muscle in response to dietary CrPic is consistent with an improved insulin sensitivity. Similarly, the increased expression of UCP3 and IL-15 which facilitate glucose metabolism are also consistent with increased glucose uptake by skeletal muscle. Moreover, the improvement of insulin sensitivity also stimulates hydrolysis of circulating lipoprotein triglyceride in to glycerol and NEFA by lipoprotein lipase activity (Dunshea and D' Souza 2003). Dietary energy in excess of nutritional requirements can be

metabolized and stored in adipose tissue as triglycerides. Triglycerides consist of a glycerol backbone and three long-chain fatty acid. These long chain fatty acids may originating from dietary fat intake or are synthesized via de novo fatty acid synthesis. NEFA are released into circulation during the breakdown of adipose tissue (lipolysis) and are an indication of fat mobilization in the pigs (Dunshea and D' Souza 2003). In this study, fasting serum TG and NEFA trended to increase by dietary supplemental fat. However, the fasting serum NEFA and TG were not affected by dietary Cr supplementation in our experiment although there were some interactions with dietary fat level. Page *et al.* (1993) reported that serum TG concentrations were not affected by dietary supplemental CrPic in growing pigs.

4. Associated activity

There were originally plans to conduct a dose response study with dietary µCrPic and nCrPic at the Medina facility in Western Australia. However, delays (>2 years) in obtaining APVMA approval to conduct the study has resulted in the study being cancelled and smaller metabolic studies in pigs are currently being conducted to determine mechanism of action. They will also investigate an alternative approach (cinnamon) which is purported to act through a similar mechanism. In addition, a study has been conducted at Rivalea and has been reported to the Pork CRC (Hung *et al.* 2011). This study is briefly described below.

A total of 60 finisher Large White x Landrace (PrimeGro™ Genetics) gilts (initial weight 67.7±0.46 kg, mean± SE kg) were randomly allocated into 2 treatment groups in three replicates during middle summer (January – February 2011). All pigs were housed in individual pens and had *ad libitum* access to feed and water. Pigs were fed either a control finisher diet or a diet containing 400 ppb Cr as chromium picolinate (CrPic) (nCrPic). Nano particle size Cr was

produced by grinding CrPic through appropriate sized sieve end plates. Feed intake and live weight were recorded weekly. Back fat at the P2 site was measured on d0 and 28 by ultrasound scan. At the end of the experiment, pigs were slaughtered at a commercial abattoir to determine hot standard carcass weight (HCWT), carcass P2 and dressing percentage. Data were analysed by analysis of variance using GENSTAT Release 11.1. Initial weight was used as a covariate for average daily gain (ADG) final weight and HCWT and initial and P2 and HCWT were used as covariate of final P2.

Table 8. Effect of dietary nano- chromium picolinate (nCrPic) on growth performance and carcass characteristics of finisher gilts.

	Control	nCrPic	SED	P- value
ADG (0-14 d)[1] (kg/d)	0.877	0.951	0.0467	0.12
ADG (0-28 d)[1] (kg/d)	0.937	0.985	0.0295	0.09
FCR (0-14 d)	2.72	2.55	0.127	0.20
FCR (0-28 d)	2.61	2.62	0.08	0.96
Final weight[1] (kg)	94.0	95.4	0.83	0.09
HCWT[1] (kg)	70.2	71.1	0.69	0.14
Dressing (%)	74.4	74.6	0.44	0.70
P2[2] (mm)	8.0	8.0	0.19	0.94

[1]initial weight used as covariate; [2]initial P2 and HCWT used as covariate

Dietary nCrPic tended (P=0.09) to increase average daily gain (ADG) over the entire study with most of the response occurring in the first 2 weeks (Table 1). As a consequence, dietary nCrPic supplementation tended (P=0.09) to increase final body weight (Table 1). Dietary nCrPic had no significant effect on HCWT, P2 depth and dressing percentage. These data indicate that dietary nCrPic can have a small effect on growth performance but not on carcass traits in gilts of a very lean genotype during a mild summer.

In addition, we have conducted some work with sheep that shows that dietary nCrPic improves glucose metabolism and insulin sensitivity as well as the ability to protect against heats stress. In brief, thirty six cross-bred ewes and wethers (8-12 months) were used in three replicates of an identical experiment. Within each replicate there were two treatment groups

(thermo neutral and heat). Treatments were administered between 9am-5pm, with heat treated animals being exposed to temperatures up to 41^{o}C. Within each treatment group, three micro-chromium doses were administered (control (0 g/day), low (200 ppb) and medium (400 ppb)). Physiological measures such as respiration rate, heart rate, skin temperature and rectal temperature were taken three times daily at 9am, 1pm and 5pm for the duration of the study. Feed, water and weights of animals were also monitored.

The effect of environmental temperature, time and diet on rectal temperature are shown in Figure 5. As expected there was increase in rectal temperature during heat stress with the temperature being increased ($P<0.001$) at 1300 and 1500 h over that of 0900 h (39.13 vs. 40.30 and 40.48ºC at 0900, 1300 and 1700 h, respectively) in the sheep fed no supplemental chromium. While there was no main effect of dietary chromium on rectal temperature there were significant chromium x time ($P<0.001$) and chromium x time x temperature ($P=0.025$) interactions such that the low dose of chromium decreased rectal temperature during heat stress but not under thermoneutral conditions (39.08 vs 40.12 and 40.25ºC at 0900, 1300 and 1700 h, respectively). The high dose of chromium was intermediate (39.28 vs 40.17 and 40.38ºC at 0900, 1300 and 1700 h, respectively) between the zero and low dose of chromium. Similar responses were observed for respiration rate (-11 breaths/min) indicating that dietary nCrPic protects against heat stress.

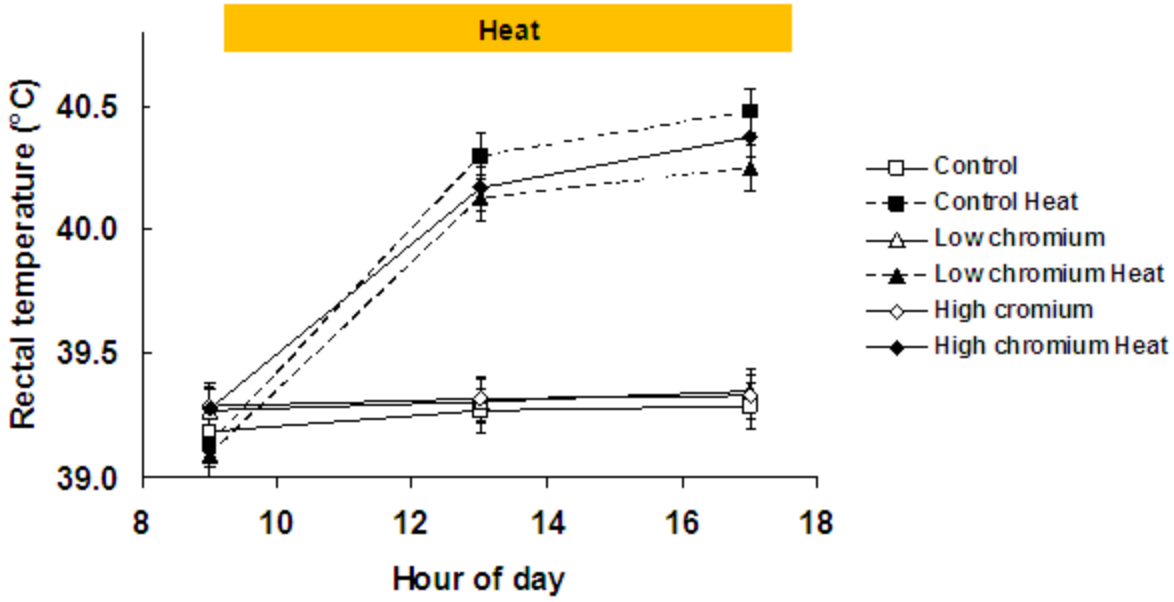

Figure 5. Effect of dietary nCrPic on rectal temperature in sheep under thermoneutrl or heat stress conditions (Hung *et al.* unpublished).

Heat stress appears to induce a state of insulin resistance in ruminants (O'Brien *et al.* 2010). In addition to the effects that insulin resistance has on tissue carbohydrate metabolism, there is now increasing evidence that diabetic individuals suffer from thermal intolerance, exhibiting an inability to control body temperature (Ohtsuka *et al.* 1995). In part this is because skin blood flow and skin thickness are reduced in diabetic individuals (Forst *et al.* 2006), thereby reducing the ability to thermoregulate. In this context, chronic treatment with the insulin sensitiser, rosiglitazone increases skin blood flow and improves the ability to thermoregulate in diabetic individuals (Petrofsky *et al.* 2005). These data that nCrPic may also provide protection against heat stress in sheep. Further studies need to be conducted with pigs, particularly sows, with either nCrPic or organic chromium, to see if dietary chromium can be used to protect against heat stress.

5. Implications and future work

Dietary CrPic improves growth performance of pigs and carcass characteristics with some responses being greater with µCrPic and nCrPic. The mechanism of action appears to be altering both adipose and skeletal muscle tissue insulin sensitivity. The use of nano particles may

present some difficulties with perception and so further studies perhaps should focus on micro particles or organic forms of chromium such as bioplexed material. Regardless, dose response studies will need to be conducted. While the growth responses to chromium can be variable, the effects of chromium on insulin sensitivity are unequivocal. Associated research has shown that dietary nCrPic improves insulin sensitivity and protects against heat stress in sheep. Further studies need to be conducted with pigs, particularly sows, with either nCrPic or organic chromium, to see if dietary chromium can be used to protect against heat stress. Further work should also look at dietary cinnamon as a mimic for chromium.

6. Publications arising

1. Hung, T.Y., Leury, B.J., Sabin, M.A., Lien, T-F. and Dunshea, F.R. 2009.Nano- and micro-size chromium picolinate increase carcases weight and muscle and decreases fat in finisher pigs. In "Manipulating Pig Production XII", ed R.J. van Barneveld. (Australasian Pig Science Association: Werribee), pp 183.
2. Hung, T.Y., Leury, B.J., Lien, T.F. and Dunshea, F.R. (2010). Potential of nano-chromium in improve body composition and performance of farm animals. Proceedings of the 14th Asian-Australasian Association for Animal Production Vol 1 (plenary sessions): 108-112.
3. Dunshea, F.R., Hung, T.Y., Akit, H. and Rikard-Bell, C.V. (2011). Feed additives and feed efficiency in the pork industry. Recent Advances in Animal Nutrition in Australia 18: 105-113.
4. Hung, T.Y., Collins, C.L., Leury, B.J., Sabin, M.A. and Dunshea, F.R. (2011). Effect of dietary nano size chromium picolinate on growth and carcass traits in finisher gilts during summer. In "Manipulating Pig Production XIII", ed R.J. van Barneveld. (Australasian Pig Science Association: Werribee), (in press).
5. Hung, T.Y., Leury, B.J., Sabin, M.A., Lien, T.F. and Dunshea, F.R. (2011). Adipose tissue gene expression in response to dietary nano-chromium and fat supplementation in finisher gilts. In "Manipulating Pig Production XIII", ed R.J. van Barneveld. (Australasian Pig Science Association: Werribee), (in press).
6. Hung, T.Y., Leury, B.J., Sabin, M.A., Lien, T.F. and Dunshea, F.R. (2011). Skeletal muscle gene expression in response to dietary nano-chromium and fat supplementation in finisher gilts. In "Manipulating Pig Production XIII", ed R.J. van Barneveld. (Australasian Pig Science Association: Werribee), (in press).

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