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Original Research Article

Nano-chromium picolinate and heat stress enhance insulin sensitivity in cross-bred sheep

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ABSTRACT

This study evaluated the effects of heat stress (HS) and dietary nano chromium picolinate (nCrPic) on metabolic responses of sheep to an intravenous glucose tolerance test (IVGTT), an intravenous insulin tolerance test (ITT) and an intramuscular adrenocorticotropin hormone (ACTH) challenge in sheep. Thirty-six sheep housed in metabolic cages were randomly allocated within 3 dietary groups (0, 400 and 800 μg/kg supplemental nCrPic) to either thermoneutral (22 °C) or cyclic HS (22 to 40 °C) conditions for 3 wk. Basal plasma glucose tended to be increased during HS ($P = 0.052$) and decreased by dietary nCrPic ($P = 0.013$) while plasma non-esterified fatty acid concentrations were decreased ($P = 0.010$) by HS. Dietary nCrPic reduced the plasma glucose area under the curve ($P = 0.012$) while there were no significant effects of HS on plasma glucose area under the curve in response to the IVGTT. The plasma insulin response over the first 60 min after the IVGTT was decreased by HS ($P = 0.013$) and dietary nCrPic ($P = 0.022$) with the effects being additive. In response to the ITT plasma glucose reached a nadir sooner ($P = 0.005$) in sheep exposed to HS, although there was no effect on the depth of the nadir. Dietary nCrPic decreased ($P = 0.007$) the plasma glucose nadir after ITT. Over the duration of the ITT plasma insulin concentrations were lower in sheep exposed to HS ($P = 0.013$) whereas there was no significant effect of supplemental nCrPic. There was no effect of either HS or nCrPic on cortisol response to ACTH. Dietary nCrPic supplementation decreased ($P = 0.013$) mitogen-activated protein kinase-8 (*JNK*) and increased ($P = 0.050$) carnitine palmitoyltransferase 1B (*CPT1B*) mRNA expression in skeletal muscle. Results of this experiment demonstrated that animals under HS and supplemented with nCrPic had greater insulin sensitivity.

1. Introduction

There will be an increased demand for high-quality animal protein in the future as the worlds' population grows and people in developing countries become more affluent. The detrimental effects of environmental heat stress (HS) on livestock production are likely to increase if the climate continues to warm as predicted (Richardson et al., 2011). Heat stress affects several aspects of livestock productivity, including milk production, growth, reproduction, and carcass traits (Cottrell et al., 2015; Dunshea et al., 2013; Nardone et al., 2010). Therefore, animal agriculture in tropical and subtropical areas will need to expand to keep the same pace with the increasing global appetite for high-quality animal protein, and means will need to be developed to mitigate HS.

Acclimation is a physiological mechanism developed by the animal in response to environmental stress (Nardone et al., 2010). The acclimation of an animal to adapt to the high ambient temperature results in the reduction in feed intake and changes in hormonal signals that affect target tissues to reduce heat load

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(Collier and Zimbelman, 2007). Lower plasma glucose concentrations can sometimes occur in heat-stressed animals, but this reduction is not entirely attributable to the decrease in feed intake at high ambient temperatures (Itoh et al., 1998). These physiological alterations may be responsible for modifying energy metabolism and liver function when animals are under HS (Nardone et al., 2010). For example, Baumgard et al. (2007) and Wheelock et al. (2010) found that glucose disposal was greater in heat-stressed cows, and heat-stressed cows had a greater insulin response to a glucose tolerance test suggesting increased insulin sensitivity. This increase in insulin sensitivity indicates improved or preferential glucose utilisation to reduce metabolic heat production. Also, heat exposure may improve glucose tolerance in rats fed a high-fat diet (Gupte et al., 2009). There is evidence that heat shock proteins (HSPs) may reduce inflammation via mitogen-activated protein kinase-8 (*JNK*) and the nuclear factor-κB (*NFκB*) (Park et al., 2001; Meldrum et al., 2003; Gupte et al., 2009, 2011). *JNK* and *NFκB* are stress kinases implicated in insulin resistance (Gupte et al., 2009). Furthermore, *HSP72* may facilitate fatty acid oxidation as L6 muscle cells under heat treatment activate *HSP72*, resulting in increased mitochondrial oxygen consumption and fatty acid oxidation (Gupte et al., 2009).

Chromium (Cr) is an essential mineral that plays a regulatory role in insulin action and energy metabolism (Anderson, 1997, 2008). Apart from its metabolic function, insulin is a crucial factor controlling lipid peroxidation; thus, Cr may also exert an antioxidant effect (Preuss et al., 1997). Moreover, Cr supplementation benefits livestock, especially when animals are under nutritional or HS because mobilized Cr from body reserves is excreted irreversibly into the urine in stressed animals (Chang and Mowat, 1992). Therefore, animals exposed to stressors such as heat may require more Cr to maintain normal carbohydrate and lipid metabolism (Johnson and Vanjonack, 1975; Lindemann, 1999, 2007; Liu et al., 2017). Cr is usually poorly absorbed by animals when provided in inorganic or even some organic forms (Li and Lien, 2010). Nano-sized Cr, both in organic or inorganic forms, is more digestible than normal-sized particles (Lien et al., 2009; Li and Lien, 2010) and so may be a potential approach to meet the increased demand for Cr during heat or other stress.

The hypothesis addressed in this experiment is that dietary nano chromium picolinate (nCrPic) can improve insulin sensitivity and increase fatty acid oxidation to reduce heat production in sheep under HS. To test this hypothesis, an intravenous glucose tolerance test (IVGTT), an intravenous insulin tolerance test (ITT) and an intramuscular adrenocorticotropin hormone (ACTH) challenge were used to examine the metabolic responses of sheep under HS. Moreover, specific gene expression in white blood cells (WBC), skeletal muscle and adipose tissue were also determined to elucidate the underlying mechanisms of HS and nCrPic on insulin sensitivity and fatty acid oxidation. Thus, this experiment investigates the effect of dietary nCrPic on metabolic responses in sheep under HS.

2. Materials and methods

2.1. Animal ethics statement

All procedures undertaken in this experiment were conducted in accordance with the guidelines set out in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by the University of Melbourne, Science, Optometry & Vision Science and Land & Environment animal ethics committee.

2.2. Animal and experimental treatments

Thirty-six 9-month-old Merino × Poll crossbred ewes and wethers (initial live weight = 42.5 ± 1.04 kg; mean ± standard error) were housed in individual metabolism crates in 1 of 2 climate control rooms which were maintained at either thermoneutral (TN) ($n = 18$) or HS ($n = 18$) conditions. The sheep were acclimatized in climate control rooms for 1 wk, followed by a 3-wk experimental thermal treatment period.

Throughout the experiment sheep were offered barley straw 100 g/sheep per day in conjunction with either a basal control sheep grower pelleted diet (wheat- and barley-based diet containing 11.3 MJ ME/kg and 15.5% CP or a basal diet supplemented with either 400 or 800 μg/kg Cr as nCrPic) (Table 1) provided ad libitum.

The nCrPic particles were prepared according to our previous study (Hung et al., 2015). Nanonized Cr powder were prepared at Hsin-fang Nanotech Co. Ltd. (Tainan) by grinding in a dry cryo-nanonization grinding system through appropriately sized sieve end plate in order to collect nanonized CrPic. No solvent was used and the temperature was controlled under 40 °C during the grinding process. The particles size distribution was determined using the method reported by Gonzales-Eguia et al. (2009) with some modifications. Briefly, the ground CrPic powders (average particle size 310 ± 129 μm) were vibrated in an alcohol solution, depositing the ultrafine fragments on a copper grid covered by a thin carbon film (CF200–Cu, Electron Microscopy Science) in the solution. The bright and dark field images of samples were obtained

Table 1
Ingredients and nutrient composition of experimental diets (%, dry matter).

Item	Content
Ingredients	
Wheat	40.0
Millmix	24.9
Barley	20.0
Canola meal 36%	2.93
Lupin Kernels 33%	4.53
Water	1.09
Molasses	2.00
Salt	1.30
Limestone	2.13
Calcium sulphate	0.33
Ammonium chloride	0.54
Rumen buffer[1]	0.54
Lamb premix [2,3]	0.11
Lasalocid[4]	0.011
Total	100
Calculated nutrient composition[5]	
ME, MJ/kg	12.3
Fat	2.12
Crude protein	16.8
Calcium	1.08
Total phosphorus	0.52
Analyzed nutrient composition[6]	
Cr, μg/kg	780

[1] Acid Buf (Feedworks Australia, Romsey, VIC, Australia).
[2] Alltech Lienert Australia, Roseworthy, SA, Australia. Provided the following trace mineral per kilogram of diet: Se, 0.2 mg; Fe, 60; Mn, 25 mg; Zn, 50 mg; I, 0.2 mg; Cu, 25 mg.
[3] Provided the following vitamins per kilogram of diet: vitamin A, 2.5 mg; vitamin D_3, 1 mg; vitamin E, 30 mg; niacin, 10 mg; Ca-D-pantothenate, 5 mg; riboflavin, 2 mg; vitamin B_{12} (Cyanocobalamin), 5 mg.
[4] Bovatec 20% (Zoetis, Silverwater, NSW, Australia).
[5] Calculated from formulation package (Saltwater Feedmania, Armidale, NSW, Australia).
[6] Chromium content of the high nano chromium picolinate (nCrPic) diet formulated to be 800 μg/kg.

by using a JEM-2100F field emission transmission electron microscope (FE-TEM; JEOL Ltd, Tokyo, Japan) operating at 200 kV. The images were then manipulated by the software "Digisscan Image Acquisition System" to achieve the area data of each recognized particle. These data were then converted into particle diameter data. Total of 40 random selected recognized particles data were used for particle size distribution. The average particle size of nCrPic, was 49.7 ± 12.37 nm (mean ± SD).

The TN room was maintained at an average 22 °C ambient temperature and 70% to 80% relative humidity (RH) for 24 h throughout the experimental period. The sheep in the HS room experienced cyclical daily temperatures beginning at 09:00 when the temperature was increased (15 °C/h) to a maximum of 40 °C, where it remained until 17:00 before being decreased to 22 °C where it was maintained until 09:00 the following day, and the RH was approximately 50% during the day and 70% overnight. Both rooms were maintained under a 12 h light and 12 h dark cycle. Access to feed and water was provided by troughs and buckets attached to the side of the cage. Feed and water were available ad libitum with feed replenished at 09:00 and 17:00 each day. Feed and water disappearance were recorded daily throughout the experimental period. The temperature-humidity index (THI) was calculated and recorded during experimental periods. The THI was calculated by combining temperature and relative humidity with the following equation (LPHSI, 1990): THI = T - (0.31 - 0.31RH) × (T - 14.4), where T is the temperature (°C) and RH is the relative humidity (%). The THI averaged 21.8 ± 0.83 and 36.7 ± 2.13 for the TN and HS conditions indicating an absence of HS and severe HS, respectively (Marai et al., 2007).

2.3. Intravenous glucose and insulin tolerance tests and adrenocorticotropin hormone challenge

Animals were subjected to IVGTT, ITT and an intramuscular ACTH challenge on d 21, 22 and 23 of thermal treatments. Jugular catheters were inserted 2 h before the IVGTT on d 21. Briefly, the sheep was restrained and a small area of the neck (over the jugular vein) was clipped free of wool and cleaned with 70% ethanol. A catheter (14 G × 3.25 inches, BD Angiocath; NSW, Australia) was inserted into the vein and secured. A 22- cm plastic catheter extension with a Luer-lock was connected and the catheter flushed with 8 to 10 mL heparinised saline (50 IU/L) and sealed with a needle-free valve (Safesite, B. Braun). The insertion site and catheter tubing were then covered in a 10- cm cohesive bandage.

For the IVGTT, basal blood glucose, insulin and non-esterified fatty acid (NEFA) concentrations were determined in animals by taking small samples (approximately 5 mL), 3 times during 30 min before administration of glucose (−30, −15, −1 min) via the jugular catheter. At time 0, a glucose bolus was administered intravenously via the catheter at a dose rate of 0.4 g/kg live weight using a 50% glucose (dextrose) solution (#AHB0253; Baxter Healthcare, Toongabbie, NSW, Australia). Blood samples (5 mL) were taken at 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22, 25, 30, 35, 40, 45, 50, 60, 75, 90, 120, 150, 180, 210 and 240 min post glucose administration. The catheter was flushed with sterile saline and heparin (diluted to 10 IU/mL) between samples.

For the ITT, basal blood glucose, insulin and NEFA concentrations were determined in animals by taking small samples (approximately 5 mL), 3 times during 30 min before administration of insulin (−30, −15, −1 min) from the jugular catheter. Insulin (ActRapid Human Insulin 100 IU/mL; Novo Pharmaceuticals Pty Ltd., Baulkham Hill, NSW, Australia) was administered intravenously via the catheter at a rate of 0.125 IU/kg live weight followed by 4 mL saline. Repeated blood samples were taken at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60,65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 150, 180, 210, 240, 270 and 300 min after insulin administration. The catheter was flushed with sterile saline and heparin (diluted to 10 IU/mL) between samples.

For the ACTH challenge, ACTH at a dose of 2 μg Synacthen/kg live weight (Novartis, North Ryde NSW, Australia) was administered. ACTH was drawn into a 1-mL syringe and the remaining volume of the syringe was filled with sterile saline solution so that the total volume injected remained equal for each animal (approximately 1 mL). The ACTH was injected intramuscularly into the rump of the animal with a 20-g needle. Blood samples were collected via the jugular catheter at −30, −1, 30 and 60 min relative to injection. The catheter was flushed with sterile saline and heparin (diluted to 10 IU/mL) between samples. Synacthen contains a synthetic polypeptide, tetracosactrin, with the first 25 amino acids in the sequence the same as endogenous ACTH and therefore displays similar activity and efficacy as purified ACTH (Watson et al., 1998). The dose of ACTH was chosen based on previous a study that identified the dose required to maximize the cortisol response (Knott et al., 2008).

Plasma insulin (Millipore Corporation, USA) and Cortisol (Diagnostica, Finland) concentrations were determined by radioimmuno-assay using commercial kits. Plasma glucose (Infinity, Thermo Fisher Scientific, Waltham, MA) and NEFA (Wako Chemicals, Kawagoe, Japan) concentrations were quantified by enzymatic colorimetric procedures.

2.4. White blood cell and tissue biopsies

On d 24, blood samples were obtained via the jugular catheter to isolate WBC before muscle and subcutaneous fat biopsies. Heat-stressed animals were sampled after being exposed to elevated temperatures for 2 h before blood and tissue sample collection. For the biopsies, sheep were placed under general anesthesia using 0.1 mg/kg liveweight of xylazine intramuscular injection as a sedative and muscle relaxant, given 10 min before anaesthesia, followed by 5 mg/kg liveweight of ketamine intravenous intravenously. The biopsy site was between the 2nd and 4th lumbar vertebrae midway between the spine and the transverse processes. A 5-cm scalpel incision was made and approximately 2 g of subcutaneous fat removed using dissecting scissors. About 1 g of muscle sample was extracted using an 8-mm biopsy punch, and the internal biopsy site closed using an absorbable suture. Tissue samples were immediately weighed and placed into a sterile labeled Eppendorf tube, snap-frozen in liquid nitrogen and then stored at −80 °C until further analysis. The external site was closed with non-absorbable sutures, and the site was covered with topical antibiotic powder. The non-absorbable sutures were removed 2 wk later.

2.5. Tissue gene expression analysis

RNA was extracted from skeletal muscle, subcutaneous fat tissue and WBC samples. Subcutaneous fat samples were analysed for the mRNA expression of adiponectin, leptin, *TNF-α*, serine/threonine protein kinase (*AKT*), glucose transporter 4 (*GLUT4*), *HSP72*, *HSP90* and peroxisome proliferator-activated receptors-γ (*PPARγ*). Skeletal muscle samples were analysed for the mRNA expression of *AKT*, *GLUT4*, *JNK*, *NFκB*, carnitine palmitoyltransferase-1B (*CPT1B*), *HSP72* and *HSP90*. WBC samples were analysed for the mRNA expression of *HSP72* and *HSP90*. Extracted RNA was transcribed to complimentary DNA (cDNA) using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, USA) according to the manufacturer's instructions. A Light Cycler 480 II (Roche Diagnostics) was used for Q-PCR analysis. Each Q-PCR reaction mix consisted of 4 μL of SYBR Green 1 Master (Roche Diagnostics), 1 μL

of forward primer, 1 μL of reverse primer, 1 μL of cDNA, and 3 μL of RNase free water. The following thermal cycling protocol was followed: 1 cycle of pre-denaturation (95 °C for 10 min), followed by 40 cycles of amplification (95 °C for 10 s, 57 to 63 °C for 10 s (dependent on primer), 72 °C for 10 s), 1 cycle of melting (95 °C for 5 s, 65 °C for 1 min, 97 °C for continuous analysis), and cooling (40 °C for 30 s). Changes in gene expression were calculated as $2^{-\triangle Ct}$, where Ct represents the cycle in which fluorescence threshold is reached and $\triangle Ct = Ct_{target\ gene} - Ct_{housekeeping\ gene}$, with β-actin and r18S utilized as housekeeping genes. Primer characteristics are detailed in Supplemental Table S1.

2.6. Statistical analysis

For the IVGTT, ITT and ACTH challenge, the plasma metabolite and hormone data were analysed by restricted maximum likelihood (REML) procedure suitable for repeated measures with the main contrasts being sex (male vs female), temperature (TN vs. HS), time (−30 vs −15, −1, … 300 min), dietary Cr (0 vs 400 and 800 mg/kg Cr as nCrPic) and within dietary Cr (400 vs 800 mg/kg Cr as nCrPic) and their interactions with replicate as a blocking factor. Since there were no main or interactive effects of sex the data have been pooled across sexes. Also, there were no main or interactive effects of within Cr dose, so the Cr effects are presented as contrasts between sheep fed 0 mg/kg supplemental Cr and the pooled data from sheep fed 400 and 800 mg/kg Cr in Figs. 1 and 2. The basal plasma metabolite and hormone concentrations and responses to IVGTT, ITT and ACTH challenges in terms of the concentration × time area under the curve (AUC), peak concentrations and nadir concentrations were analysed by ANOVA with the main contrasts being sex, temperature, dietary Cr (0 vs 400 and 800 mg/kg Cr as nCrPic) and within dietary Cr (400 vs 800 mg/kg Cr as nCrPic) and their interactions with replicate as a blocking factor. The same model was used to analyse the gene expression data (as △Ct) and these data were expressed as radar graphs relative to sheep housed under TN conditions and fed 0 mg/kg supplemental Cr for muscle and adipose tissue. Within a tissue the gene expression data were also subject to a multivariate ANOVA (MANOVA) to determine if there were any generalized responses to the treatments within tissues. All data were analysed using the GenStat statistical package (GenStat release 18; VSN International Ltd., Hemel Hempstead, UK).

3. Results

The production and physiological response to HS and supplemental nCrPic have been reported elsewhere (Hung et al., 2021).

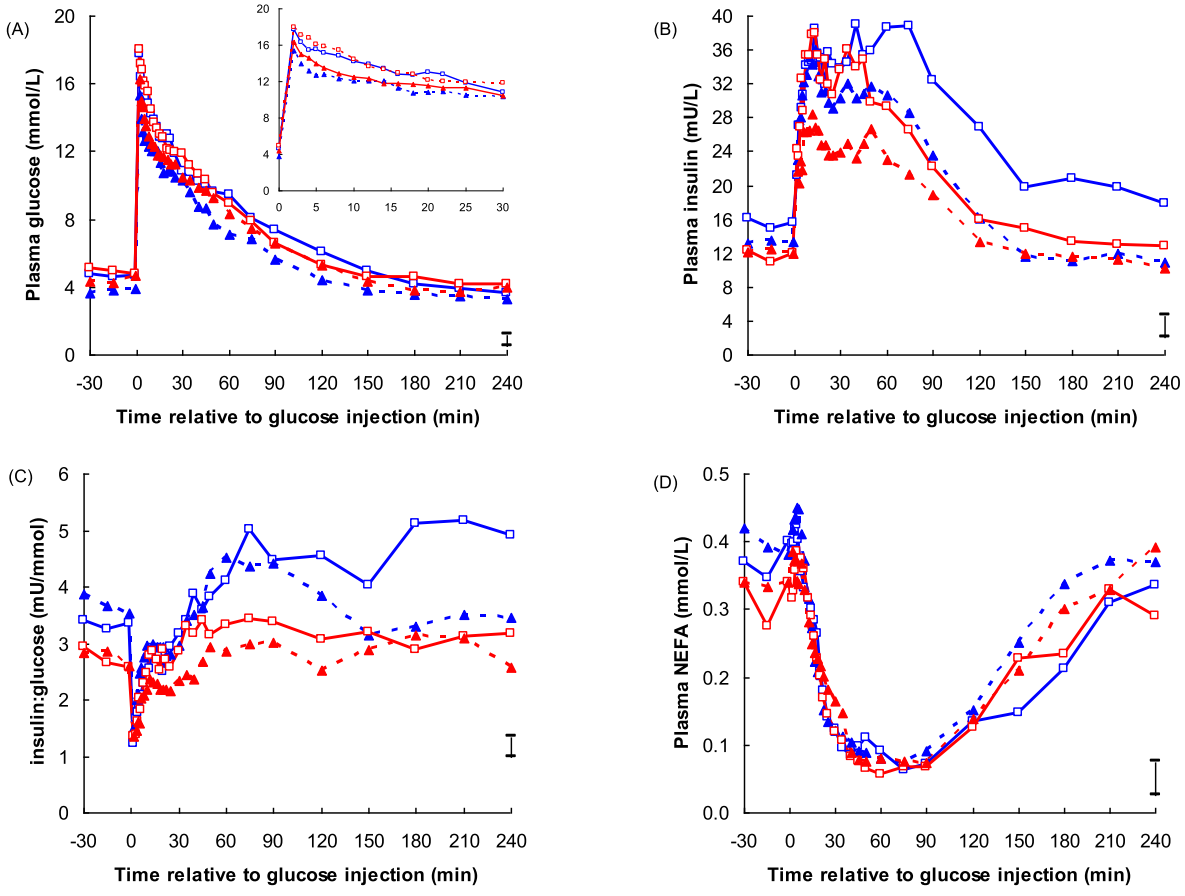

Fig. 1. Relationships between plasma (A) glucose, (B) insulin, (C) ratio of insulin to glucose and (D) non-esterified fatty acids (NEFA) and time relative to glucose (400 mg/kg BW) injection in sheep under thermoneutral (TN) conditions fed either a control (□) or nano chromium picolinate (nCrPic) (▲) supplemented diet and sheep under heat stress (HS) fed either a control (□) or nCrPic (▲) supplemented diet. The standard error of the difference for the interaction between temperature (Temp), dietary nCrPic (Cr) and time (T) is displayed at 240 min. The inserts are for the period between 0 and 30 min. The *P*-values for the effects of temperature Temp, T, Cr, Temp × T, Temp × Cr, T × Cr and Temp × T × Cr were 0.081, < 0.001, < 0.001, 0.39, 0.44, < 0.001 and 0.65 for glucose, 0.004, < 0.001, 0.002, 0.002, 0.50, < 0.001 and < 0.001 for insulin, < 0.001, < 0.001, < 0.28, < 0.001, 0.48, < 0.001 and < 0.001 for the ratio of insulin to glucose and 0.44, < 0.001, 0.61, 0.067, 0.93, 0.34 and 0.85 for NEFA, respectively.

Briefly, HS decreased feed intake (1,195 vs 1,035 g dry matter/d, $P = 0.002$) while sheep under HS had a reduced daily gain (114 vs −55 g/d, $P < 0.001$). Dietary nCrPic increased both ADFI (1,042 vs 1,152 g dry matter/d, $P = 0.040$) and ADG (−24 vs 56 g/d, $P = 0.05$) under both TH and HS conditions. Respiration rate and rectal temperature were increased by HS and decreased by dietary nCrPic with the responses being greatest in the hottest period of the day in the afternoon.

3.1. Basal plasma metabolite and hormone concentrations

Basal plasma glucose tended to be increased during HS (3.89 vs 4.16 mmol/L, $P = 0.052$) and was decreased by dietary nCrPic (4.28 vs 3.90 mmol/L, $P = 0.013$) (Table 2). There were no significant effects of either HS or nCrPic on basal plasma insulin concentration. The ratio of basal plasma insulin to glucose was lower in sheep exposed to HS (3.44 vs 2.74 mU/mmol, $P = 0.042$) but was unaltered by dietary nCrPic (Table 2). Basal plasma NEFA were decreased during HS (0.358 vs 0.288 mmol/L, $P = 0.010$) and were unaltered by dietary nCrPic (Table 2). There were no significant effects of either HS or nCrPic on HOMA. RQUICKI tended to be increased by HS (0.407 vs 0.430 mmol/L, $P = 0.072$) but was unaltered by dietary nCrPic. There were no significant effects of either HS or nCrPic on basal plasma cortisol concentration.

3.2. Intravenous glucose tolerance test

Plasma glucose increased rapidly in response to the IVGTT before declining exponentially and finally returning to baseline by 180 min post-injection (Fig. 1A). Over the duration of the IVGTT plasma glucose concentration tended to be higher in sheep exposed to HS (9.6 vs 10.1 mmol/L, $P = 0.081$) and was lower in sheep fed supplemental nCrPic (10.5 vs 9.2 mmol/L, $P < 0.001$) (Fig. 1A). However, there was an interaction between time and Cr ($P < 0.001$) such that the lower plasma glucose in sheep fed supplemental nCrPic was most pronounced in the first 30 min after the IVGTT (Fig. 1A). The peak in plasma glucose concentration tended to be higher in sheep exposed to HS (16.2 vs 17.0 mmol/L, $P = 0.098$) and was decreased by dietary nCrPic (18.0 vs 15.9 mmol/L, $P < 0.001$) (Table 3). While there was no effect of HS on plasma glucose AUC after the IVGTT, the AUC was decreased by dietary nCrPic over the first 30 min (255 vs 222 mmol·min/L, $P < 0.001$) and 60 min (414 vs 363 mmol·min/L, $P = 0.012$) post-injection (Fig. 1A; Table 3).

Plasma insulin increased rapidly in response to the IVGTT, remaining elevated for at least 1 h before returning to baseline by approximately 180 min post-injection (Fig. 1B). Over the duration of the IVGTT plasma insulin concentrations were lower in sheep exposed to HS (27.7 vs 23.8 mU/L, $P = 0.004$) and was lower in sheep fed supplemental nCrPic (28.1 vs 23.3 mU/L, $P < 0.001$) with

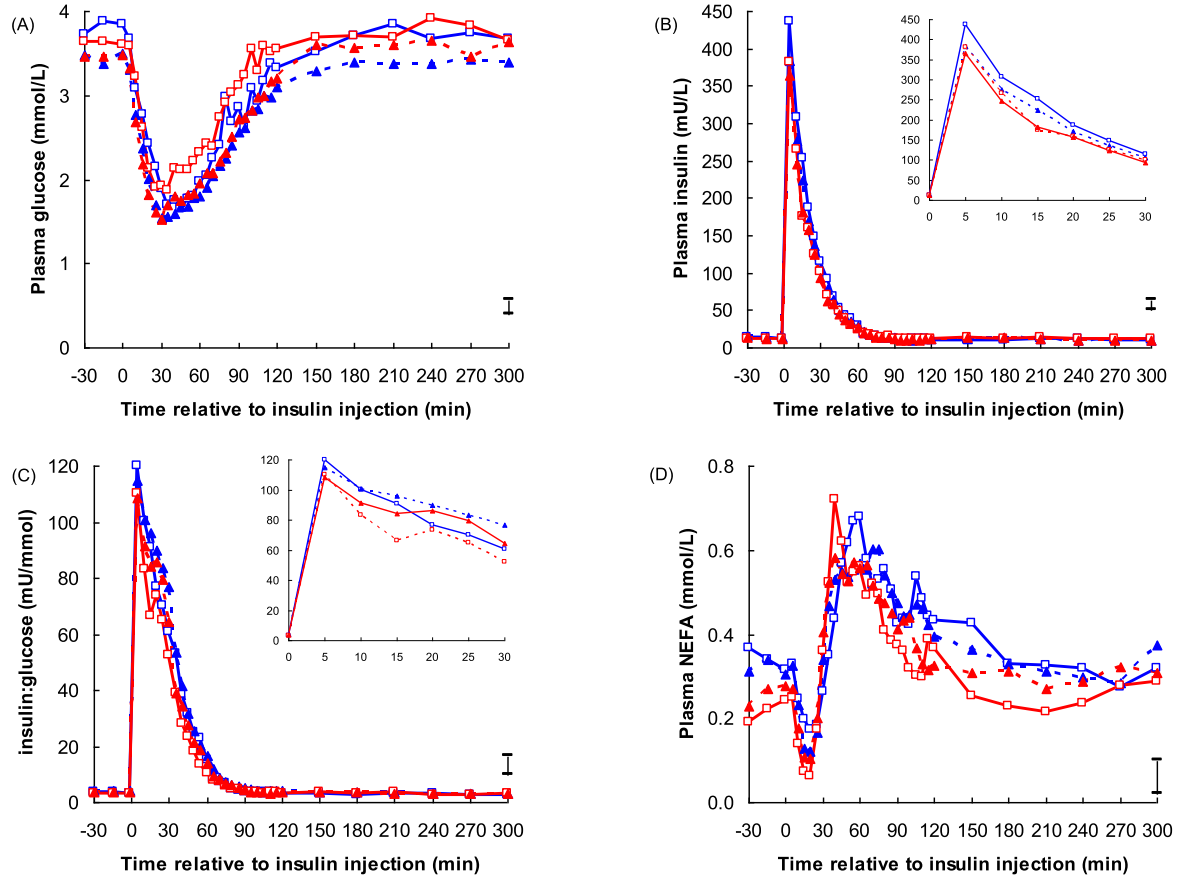

Fig. 2. Relationships between plasma (A) glucose, (B) insulin, (C) ratio of insulin to glucose and (D) non-esterified fatty acids (NEFA) and time relative to insulin (0.125 IU/kg BW) injection in sheep under thermoneutral (TN) conditions fed either a control (□) or nano chromium picolinate (nCrPic) (▲) supplemented diet and sheep under heat stress (HS) fed either a control (□) or nCrPic (▲) supplemented diet. The standard error of the difference for the interaction between temperature (Temp), dietary nCrPic (Cr) and time (T) is displayed at 300 min. The inserts are for the period between 0 and 30 min. The *P*-values for the effects of temperature Temp, T, Cr, Temp × T, Temp × Cr, T × Cr and Temp × T × Cr were 0.11, < 0.001, < 0.001, 0.006, 0.62, 0.39 and 0.26 for glucose, 0.013, < 0.001, 0.41, 0.001, 0.71, 0.84 and 1.00 for insulin, 0.036, < 0.001, 0.20, 0.16, 0.83, 0.54 and 1.00 for the ratio of insulin to glucose and 0.21, < 0.001, 0.80, 0.002, 0.65, 0.99 and 0.80 for NEFA, respectively.

Table 2
Effect of heat stress and dietary nano chromium picolinate on basal plasma glucose, insulin, non-esterified fatty acids (NEFA) and cortisol concentrations and estimated homeostatic model assessment of insulin resistance (HOMA-IR) and revised quantitative insulin sensitivity index (RQUICKI) in sheep.[1]

Item	Thermoneutral			Heat stress			s.e.d.[2]	*P*-value		
	0 Cr, μg/kg	400 Cr, μg/kg	800 Cr, μg/kg	0 Cr, μg/kg	400 Cr, μg/kg	800 Cr, μg/kg		Temperature [3]	Cr[4]	Within Cr[5]
Glucose, mmol/L	4.24	3.61	3.83	4.31	4.12	4.05	0.230	0.052	0.013	0.64
Insulin, mU/L	14.0	15.9	11.7	11.8	12.5	12.2	2.08	0.16	0.89	0.14
Insulin:glucose, mU/mmol	3.10	4.28	2.93	2.63	2.84	2.92	0.533	0.042	0.26	0.13
NEFA, mmol/L	0.356	0.334	0.384	0.269	0.311	0.283	0.0444	0.010	0.58	0.72
HOMA-IR	2.68	2.55	2.05	2.26	2.56	2.22	0.415	0.45	0.44	0.36
RQUICKI	0.401	0.408	0.413	0.431	0.429	0.431	0.0212	0.072	0.73	0.81
Cortisol,[6] nmol/L	20.6	10.7	8.19	7.29	18.2	10.3	7.796	0.78	0.66	0.35

[1] Data are pooled across the glucose and insulin challenge days. There were no interactions between temperature and Cr so main effects are presented except where indicated by superscript and footnote.
[2] Standard error of the difference for effect of temperature × Cr.
[3] Thermoneutral vs heat stress ($n = 18$ vs 18).
[4] Control vs Cr ($n = 12$ vs 24).
[5] 400 vs 800 μg/kg Cr ($n = 12$ vs 12).
[6] Temperature × Cr interaction ($P = 0.067$).

the effects appearing additive (Fig. 1B). Consequently, there were 2- and 3-way interactions (temperature × time, $P = 0.002$; Cr × time, $P < 0.001$; temperature × Cr × time, $P < 0.001$) such that the plasma insulin concentrations peaked lower and returned to baseline sooner in sheep fed supplemental nCrPic and exposed to HS whereas they remained elevated for a prolonged period in sheep consuming the control diet and housed under TN conditions (Fig. 1B). Plasma insulin was most pronounced in sheep fed supplemental nCrPic during the first 30 min after the IVGTT (Fig. 1B). The plasma insulin AUC after IVGTT was decreased by HS over the first 60 min (191 vs 144 mU·min/L, $P = 0.013$) and by dietary nCrPic over the first 30 min (97 vs 70 mU·min/L, $P = 0.012$) and 60 min (198 vs 153 mU·min/L, $P = 0.022$) post-injection and beyond (Fig. 1B; Table 3).

The ratio of plasma insulin to glucose decreased rapidly after the IVGTT before increasing and eventually reaching a plateau again although the nature of the plateau varied between treatments (Fig. 1C). The ratio of plasma insulin to glucose was lower for exposed to HS (3.23 vs 2.56, $P < 0.001$) while there was no main effect of dietary nCrPic. However, there were two- and three-way interactions ($P < 0.001$) such that the ratio of plasma insulin to glucose retuned to a lower baseline more quickly in sheep exposed to HS (approximate 60 min) compared to those exposed to TN conditions. Indeed, the ratio of insulin to glucose remained elevated for at least 240 min after the IVGTT in sheep fed the control diet and exposed to TN conditions, whereas it had returned to baseline by 150 min in sheep receiving the nCrPic supplemented diet and housed under TN conditions (Fig. 1C).

After a transient spike, plasma NEFA decreased rapidly in response to the IVGTT, reaching a nadir between 60 and 90 min before gradually returning to baseline (Fig. 1D). Over the duration of the IVGTT, there were no main effects of HS or dietary nCrPic on plasma NEFA concentrations. However, there was an indication of a temperature × time interaction ($P = 0.067$), most likely due to the higher basal plasma NEFA concentrations before the IVGTT in sheep housed under TN conditions (Table 2 and Fig. 1D). There was no effect of either HS or dietary nCrPic on the nadir in plasma NEFA concentrations (Table 3). The magnitude of the plasma NEFA AUC up to 30 (−3.5 vs −2.0 mmol·min/L, $P = 0.038$) and 60 min (−12.3 vs −9.2 mmol·min/L, $P = 0.035$) post-injection were lower in sheep exposed to HS, most likely because of the lower basal NEFA concentrations (Table 2 and Fig. 1D). There was no effect of dietary nCrPic on the AUC after the IVGTT.

3.3. Insulin tolerance test

Plasma glucose concentrations decreased rapidly in response to the ITT, reaching a nadir at approximately 30 min post-injection

Table 3
Effect of heat stress and dietary nano chromium picolinate on plasma glucose, insulin and non-esterified fatty acid (NEFA) area under curve (AUC) in response to a glucose infusion in thermoneutral and heat stressed sheep supplemented with dietary nCrPic.[1]

Item	Thermoneutral			Heat stress			s.e.d.[2]	*P*-value		
	0 Cr, μg/kg	400 Cr, μg/kg	800 Cr, μg/kg	0 Cr, μg/kg	400 Cr, μg/kg	800 Cr, μg/kg		Temperature [3]	Cr[4]	Within Cr[5]
Glucose										
Peak, mmol/L	17.8	15.7	15.1	18.1	16.6	16.2	0.73	0.098	<0.001	0.44
AUC $_{0\text{-}30}$, mmol·min/L	258	225	224	253	234	206	14.7	0.62	<0.001	0.20
AUC $_{0\text{-}60}$, mmol·min/L	417	352	368	410	339	392	30.6	0.95	0.012	0.13
Insulin										
AUC $_{0\text{-}30}$, mU·min/L	92.5	83.5	80.6	102	58.7	57.1	18.0	0.22	0.013	0.86
AUC $_{0\text{-}60}$, mU·min/L	217	171	185	178	127	127	31.4	0.012	0.022	0.75
NEFA										
Nadir, mmol/L	0.061	0.058	0.084	0.054	0.069	0.059	0.0216	0.59	0.45	0.61
AUC $_{0\text{-}30}$, mmol·min/L	−2.94	−3.70	−3.77	−1.82	−2.41	−1.82	1.15	0.038	0.44	0.76
AUC $_{0\text{-}60}$, mmol·min/L	−11.2	−12.2	−13.4	−9.02	−9.55	−8.97	2.421	0.035	0.53	0.86

[1] There were no interactions between temperature and Cr so main effects are presented.
[2] Standard error of the difference for effect of temperature × Cr.
[3] Thermoneutral vs heat stress ($n = 18$ vs 18).
[4] Control vs Cr ($n = 12$ vs 24).
[5] 400 vs 800 μg/kg Cr ($n = 12$ vs 12).

Table 4
Effect of heat stress and dietary nano chromium picolinate on plasma glucose, insulin and non-esterified fatty acid (NEFA) area under curve (AUC) in response to an insulin infusion in thermo-neutral and heat stressed sheep supplemented with dietary nCrPic.[1]

Item	Thermoneutral			Heat stress			s.e.d.[2]	*P*-value		
	0 Cr, μg/kg	400 Cr, μg/kg	800 Cr, μg/kg	0 Cr, μg/kg	400 Cr, μg/kg	800 Cr, μg/kg		Temperature [3]	Cr[4]	Within Cr[5]
Glucose										
Nadir, mmol/L	1.88	1.65	1.44	1.92	1.62	1.44	0.144	1.00	0.007	0.20
AUC $_{0\text{-}30}$, mmol·min/L	−30.0	−28.1	−32.5	−27.6	−36.0	−30.8	4.79	0.65	0.30	0.91
AUC $_{0\text{-}60}$, mmol·min/L	−91.7	−77.4	−90.9	−73.5	−86.5	−81.8	8.80	0.25	0.78	0.48
AUC $_{0\text{-}120}$, mmol·min/L[6]	−156	−134	−149	−107	−145	−127	16.2	0.048	0.48	0.91
Insulin										
Peak	444	402	387	381	400	346	43.5	0.17	0.28	0.28
AUC $_{0\text{-}30}$, mU·min/L	6606	5770	5860	5436	5523	5039	598.3	0.041	0.20	0.65
AUC $_{0\text{-}60}$, mU·min/L	8064	7133	7012	6632	6568	6193	685.1	0.025	0.15	0.61
AUC $_{0\text{-}120}$, mmol min/L	8090	7035	7141	6783	6279	6581	684.0	0.029	0.10	0.68
NEFA										
Nadir, mmol/L	0.074	0.102	0.091	0.054	0.082	0.063	0.0206	0.051	0.092	0.30
AUC $_{0\text{-}30}$, mmol·min/L	−3.05	−2.64	−3.44	−1.63	−2.13	−1.40	1.281	0.077	0.93	0.97
AUC $_{0\text{-}60}$, mmol·min/L	2.30	4.57	1.20	8.79	5.15	8.17	3.110	0.014	0.69	0.94
AUC $_{30\text{-}60}$, mmol·min/L	5.35	7.21	4.64	10.4	7.28	9.57	2.611	0.034	0.66	0.94

[1] There were no interactions between temperature and Cr so main effects are presented.
[2] Standard error of the difference for effect of temperature × Cr.
[3] Thermoneutral vs heat stress ($n = 18$ vs 18).
[4] Control vs Cr ($n = 12$ vs 24).
[5] 400 vs 800 μg/kg Cr ($n = 12$ vs 12).
[6] Temperature × Cr interaction ($P = 0.037$).

before gradually returning to baseline (Fig. 2A). Over the duration of the ITT, there were no main effects of HS on plasma glucose concentrations whereas dietary nCrPic decreased plasma glucose concentrations (2.92 vs 2.62 mmol/L, $P < 0.001$) (Table 4 and Fig. 2A). However, there was a temperature × time interaction ($P = 0.005$), such that plasma glucose took less time to reach the nadir and began to return to basal sooner in sheep maintained under HS conditions (Table 4 and Fig. 2A). There was no effect of HS on the nadir in plasma glucose concentrations whereas dietary nCrPic decreased the nadir (1.90 vs 1.54 mmol/L, $P = 0.007$) (Table 4). While there were no significant effects of either HS or dietary nCrPic on the plasma glucose AUC up to 60 min post-injection, the magnitude of the plasma glucose AUC to 120 min was less in those sheep exposed to HS (−146 vs −126 mmol·min/L, $P = 0.048$). This is consistent with the aforementioned temperature × time interaction.

Plasma insulin concentrations increased rapidly in response to the ITT before declining exponentially and finally returning to baseline by 180 min post-injection (Fig. 2B). Over the duration of the ITT plasma insulin concentrations were lower in sheep exposed to HS (58.9 vs 52.1 mU/L, $P = 0.013$) whereas there was no significant effect of supplemental nCrPic (Fig. 2B). However, there was an interaction between time and temperature ($P < 0.001$) such that the lower plasma insulin in sheep exposed to HS was most pronounced in the first 30 to 60 min after the ITT (Fig. 2B). Consequently, although there was no significant effect of HS on peak insulin concentrations, the plasma insulin AUC up to 30 (6,078 vs 5,333 mU min/L, $P = 0.041$), 60 (7,403 vs 6,464 mU min/L, $P = 0.025$) and 120 min (7,422 vs 6,548 mU min/L, $P = 0.029$) were lower in the sheep exposed to HS (Table 4). There was no effect of dietary nCrPic on either peak insulin or plasma insulin AUC up to 60 min post-injection although the plasma insulin AUC until 120 min of the ITT tended to be decreased by dietary nCrPic (7,437 vs 6,759 mU·min/L, $P = 0.10$).

The ratio of plasma insulin to glucose increased rapidly after the ITT before decreasing and eventually reaching baseline again after approximately 90 min (Fig. 1C). The ratio of plasma insulin to glucose was lower for exposed to HS (24.8 vs 21.6 mU/mmol, $P = 0.036$) while there was no main effect of dietary nCrPic and there were not any other interactions.

For plasma NEFA there was the characteristic rapid antilipolytic effect of ITT followed by an equally rapid and sustained rebound in plasma NEFA concentrations (Fig. 2 D) (Ponnampalam et al., 2012). Over the duration of the ITT there were no main effects of either HS or dietary nCrPic on plasma NEFA concentrations. However, there was a temperature × Cr interaction ($P = 0.002$) such that plasma NEFA concentrations commenced lower and returned to a lower baseline earlier in sheep exposed to HS (Fig. 2D). The plasma NEFA nadir occurred 20 to 30 min post-injection and tended to be decreased by HS (0.089 vs 0.066 mmol/L, $P = 0.051$) and increased by nCrPic (0.064 vs 0.085 mmol/L, $P = 0.092$) (Table 4). The magnitude of the plasma NEFA AUC until 30 min tended to be less in sheep under HS (−3.04 vs −1.72 mmol·min/L, $P = 0.077$) while the AUC to 60 min (2.69 vs 7.37 mmol·min/L, $P = 0.014$) and between 30 and 60 min (5.73 vs 9.08 mmol·min/L, $P = 0.034$) were greater in these sheep (Table 4). There were no effects of dietary nCrPic on the plasma NEFA AUC.

3.4. ACTH challenge

Plasma cortisol concentrations were elevated almost 10-fold at 30 min after ACTH injection with a further small increase between 30 and 60 min (10.9 vs 105 and 118 nmol/L at 0, 30 and 60 min, $P < 0.001$; Supplemental Fig. S1) as anticipated (Knott et al., 2008). There were no significant main or interactive effects of HS or dietary nCrPic on the plasma cortisol response to ACTH.

3.5. Gene expression

Dietary nCrPic supplementation significantly decreased *JNK* gene expression in skeletal muscle (94% vs 54% elative expression, $P = 0.013$) (Fig. 3). Also, *CPT1B* gene expression was increased in a dose dependent manner as indicated by significant effect of Cr ($P = 0.050$) and a trend within Cr ($P = 0.094$) treatments (108% vs 124% and 168% relative expression for 0, 400 and 800 μg/kg nCrPic, respectively). While there were no significant main or interactive effects of temperature or Cr on *HSP72*, *HSP90*, *AKT*, *GLUT4* and *NFκB* gene expression in skeletal muscle, the MANOVA quite clearly indicated general changes in the pattern of gene expression as indicated by significant effects of HS ($P = 0.010$) and Cr ($P < 0.001$) (Fig. 3).

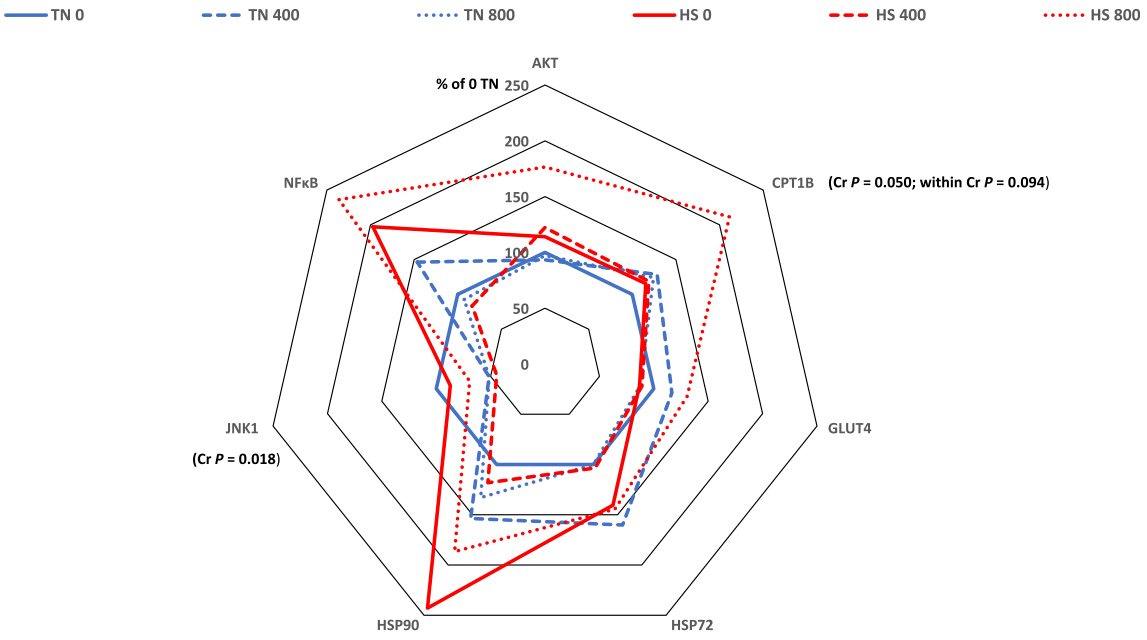

Fig. 3. Protein kinase B (*AKT*), carnitine palmitoyltransferase 1B (*CPT1B*), glucose transporter 4 (*GLUT4*), heat shock protein 72 (*HSP72*), *HSP90*, mitogen-activated protein kinase-8 (*JNK1*) and nuclear factor-κ B (*NFκB*) mRNA expression in skeletal muscle tissue from sheep housed under either thermoneutral (TN) or heat stress (HS) conditions and supplemented with either 0, 400 or 800 μg/kg nano chromium picolinate (nCrPic) (0, 400 or 800). All gene expression data were analysed as the threshold cycle (Ct) relative to that of β-actin and 18s (ΔCt) and assessed for main and interactive effects of temperature (Temp), nCrPic (Cr) and within Cr by restricted maximum likelihood (REML). 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 percentage 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 error term. The *P*-values resulting from a MANOVA of all genes for the effects of Temp, Cr, Temp × Cr, within Cr and Temp × within Cr were 0.010, < 0.001, 0.43, 0.22 and 0.040.

Heat stress reduced the expression of *HSP90* in adipose tissue (74% vs 25% relative expression, $P = 0.013$) while *HSP90* expression was reduced ($P = 0.017$) at the highest but not the lowest dose of nCrPic (64% vs 70% and 16% relative expression) (Fig. 4). Dietary nCrPic supplementation significantly increased *GLUT4* (95% vs 158% relative expression, $P = 0.027$) and tended to reduce *AKT* (95% vs 132% relative expression, $P = 0.081$) gene expression in adipose tissue (Fig. 4). While there were no significant main or interactive effects of temperature or Cr on *HSP72*, *leptin*, *PPARγ* and *TNFα* mRNA expression in adipose tissue, the MANOVA suggested general changes in the pattern of gene expression as indicated by significant effects of HS ($P = 0.003$), and Cr ($P < 0.001$) and within Cr ($P = 0.020$) (Fig. 4).

There were no main or interactive effects of temperature or dietary nCrPic in white blood cells (Supplemental Table S2).

4. Discussion

The major findings from this study were that sheep fed with the nCrPic diet and exposed to HS had increased insulin sensitivity. Dietary nCrPic decreased basal glucose concentrations, the plasma glucose response to the IVGTT and the plasma glucose nadir and insulin response to ITT all of which are indicative of increased insulin sensitivity. However, there were no effects of dietary nCrPic on any aspects of NEFA metabolism. These observations are consistent with most previous studies. For example, dietary Cr has been reported to decrease the glucose response to IVGTT in sheep (Kitchalong et al., 1995) and to increase glucose clearance rate in calves (Bunting et al., 1994). Amoikon et al. (1995) reported that dietary CrPic supplementation increased glucose clearance rate and decreased the half-life of glucose in pigs during an IVGTT. Hung et al. (2015) also indicated that nCrPic supplementation decreased HOMA and increased QUICKI, thereby improving insulin sensitivity and reducing insulin resistance in pigs. These findings were supported by improvements in some of the genes involved in insulin signalling of skeletal muscle and adipose tissue of pigs supplemented with nCrPic (Hung et al., 2020). On the other hand, Sano et al. (2000) reported that there was no effect of Cr-yeast on insulin sensitivity in sheep, suggesting that some other factors can also affect the function of Cr in glucose metabolism. Borgsa and Mallarda (1998) indicated that the function of Cr is more likely to elicit a greater response when animals are placed under stress.

High ambient temperatures increased basal plasma glucose concentrations and RQUICKI and reduced plasma NEFA concentrations and the ratio of plasma insulin to glucose with these latter 3 measures indicative of improved insulin sensitivity. Also, the plasma insulin response to the IVGTT was decreased in sheep exposed to HS which is again indicative of improved insulin sensitivity. Under high ambient temperature stress, similar improvements in insulin sensitivity have been found in early lactation cows (Mirzaei et al., 2011), periparturient cows (Subiyatno et al., 1996), post weaning calves (Yari et al., 2010), pigs (Liu et al., 2017) and broiler chickens (Sahin et al., 2002) supplemented with Cr. In the present study, the plasma insulin response to glucose infusion was lower in sheep fed with nCrPic and those sheep exposed to HS. These effects were additive suggesting that the effects of nCrPic on insulin sensitivity on more pronounced during HS.

One of the most consistent endocrinal changes during HS in sheep is an increase in plasma prolactin concentrations (Gonzalez-Rivas et al., 2017; Joy et al., 2020). It is over 40 years since Bauman and Currie (1980) proposed possible roles for somatotropin and prolactin as a homeorhetic regulator of homeostatic signals controlling lipid metabolism. It is now well established that somatotropin induces insulin resistance (Boisclair et al., 1994; Dunshea et al., 1995) and stimulates responsiveness to catecholamines (Sechen et al., 1990) while the data for prolactin are more equivocal. Swan (1976) proposed that prolactin inhibited fat mobilization in lactating dairy cows based on an inverse relationship between

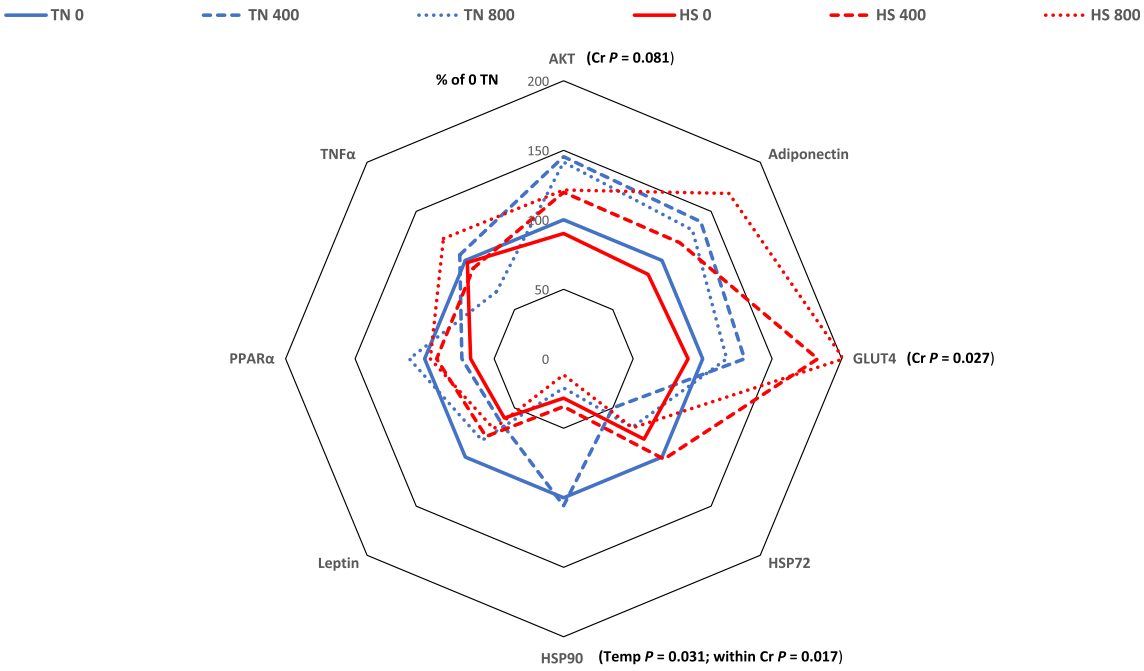

Fig. 4. Protein kinase B (*AKT*), adiponectin, glucose transporter 4 (*GLUT4*), heat shock protein 72 (*HSP72*), *HSP90*, leptin, peroxisome proliferator-activated receptor γ (*PPARγ*) and tumor necrosis factor α (*TNFα*) mRNA expression in subcutaneous adipose tissue from sheep housed under either thermoneutral (TN) or heat Stress (HS) conditions and supplemented with either 0, 400 or 800 μg/kg nano chromium picolinate (nCrPic) (0, 400 or 800). All gene expression data were analysed as the threshold cycle (Ct) relative to that of β-actin and r18s (ΔCt) and assessed for main and interactive effects of temperature (Temp), nCrPic (Cr) and within Cr by restricted maximum likelihood (REML). 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 error term. The *P*-values resulting from a MANOVA of all genes for the effects of Temp, Cr, Temp × Cr, within Cr and Temp × within Cr were 0.003, < 0.001, 0.61, 0.020 and 0.026.

plasma NEFA and prolactin concentrations. Conversely, based on studies in rats Bauman and Currie (1980) proposed that prolactin inhibited lipogenic pathways in adipose tissue and liver. In support of a role for prolactin as a homeorhetic regulator of a homeostatic signal such as insulin, Wagner et al. (2014) found that plasma prolactin concentration was positively related to insulin sensitivity although there were age-related differences in this relationship. It is interesting to speculate that the increase in insulin sensitivity observed during HS may be mediated by increased prolactin concentrations.

The maintenance of plasma glucose homeostasis is a complex process involving several genes. A proper coordinated response in the genes involved in the insulin signaling pathway genes is crucial for maintenance of insulin sensitivity. Normally, insulin binds to the insulin receptor (IR) on the sarcolemma of the cell resulting in an increase in IR tyrosine kinase activity and phosphorylation of the insulin receptor substrates (*IRS*). Tyrosine phosphorylated insulin receptor substrates 1 (*IRS1*) recruits and activates phosphatidylinositol 3 kinase (*PI3K*) and increases serine phosphorylation of downstream *AKT*. Phosphorylated *AKT* facilitates *GLUT4* vesicles translocation and recruitment to the sarcolemma, and consequently mediates cellular glucose uptake (Shulman, 2000; Wei et al., 2008). Phosphorylation of *IRS1* at a serine instead of tyrosine residue is an emerging inhibitory target for impaired insulin signaling (Aguirre et al., 2002). Phosphorylation of the serine residue inhibits insulin-stimulated tyrosine phosphorylation and activation of *IRS1*. In the present study, dietary nCrPic increased the insulin signaling pathway genes *AKT* and *GLUT4* mRNA expression in adipose tissue but not in skeletal muscle. These data are consistent with an increase in insulin sensitivity in adipose tissue. Furthermore, skeletal muscle *JNK* mRNA expression was down-regulated and *CPT1B* mRNA expression was up-regulated by dietary nCrPic supplementation. Chen et al. (2009) indicated that the improvement of insulin signaling by Cr was associated with decreased *IRS1*-Ser^{307} phosphorylation and *JNK* activity. *JNK* is believed to cause insulin resistance by causing the phosphorylation of *IRS1* at the serine residue (Thomas et al., 2003). The results of the glucose challenge were not consistent with gene expression, possibly because mRNA level may not reflect the phosphorylation of *AKT* protein and the translocation of *GLUT4* protein by the reduction of JNK causing phosphorylation of the *IRS1* serine residue. Further work is required to better understand the expression of insulin signaling at the protein level.

Borgsa and Mallarda (1998) indicated that a means by which Cr regulated insulin action may be via altering cortisol secretion since cortisol secretion increases during stress and can act as an insulin antagonist. In this model, cortisol increased plasma glucose concentrations through reduced glucose utilisation by peripheral tissue by inducing insulin resistance. A reduction in plasma cortisol is one of the typical metabolic responses to Cr supplementation in livestock, especially when animals are under stress (Chang and Mowat, 1992; Borgsa and Mallarda, 1998; Hung et al., 2014; Smantha et al., 2008; Zha et al., 2009). Interestingly, in this study, basal cortisol concentration prior to ACTH and cortisol concentration after ACTH infusion were not altered by either dietary nCrPic or heat treatment. Kim et al. (2009) also reported that supplemental Cr had no effects on plasma cortisol concentrations during high ambient temperature compared with pigs in thermoneutral condition. Chauhan et al. (2014a)reported that HS had no effect on plasma cortisol in sheep while dietary antioxidants tended to decrease plasma cortisol concentrations. On the other hand, Chauhan et al. (2016) found that cortisol tended to increase during 2 wk of HS but was unaffected by dietary antioxidants. In the present study, the fasting plasma cortisol concentrations were not affected by heat treatment. Minton and Blecha (1990) reported that cortisol secretion in lambs was increased for 6 h in response to HS

(35 °C) returning to basal within 12 h. This finding suggesting that the effect of HS on cortisol secretion may be acute rather than chronic.

Prior to the IVGTT and ITT, sheep in the HS group had lower plasma NEFA compared with sheep in the TN group. Plasma NEFA are usually lower under hot conditions (Baumgard et al., 2007; Shwartz et al., 2009; Nardone et al., 2010; Wheelock et al., 2010; Rhoads et al., 2011). Torlińska et al. (1987) indicated that HS reduces in vivo lipolytic rates as evidenced by in vitro lipolytic enzyme activity in rats. Furthermore, HS increases lipoprotein lipase in adipose tissue (Christon, 1988), indicating that the heat stressed animal has an increased capacity to liberate fatty acids from circulating triglycerides for storage in fat tissue. Previous studies reported that HS may result in a dissociation of the relationship between energy balance and fat mobilization from adipose tissue (Prunier et al., 1997; Baumgard and Rhoads, 2011). Farm animals typically go into a negative energy balance when they are unable to consume sufficient nutrients to meet requirements for maintenance, growth, milk, wool and egg production. Negative energy balance is associated with a variety of metabolic changes. Circulating NEFA concentrations are positively associated with the severity of negative energy balance, and it is generally agreed that there is a linear relationship between NEFA delivery, tissue NEFA uptake and NEFA oxidation (Armstrong et al., 1961; Dunshea et al., 1990). Plasma NEFA concentrations generally increase during negative energy balance (Dunshea et al., 1990) but there appears to be an uncoupling of this relationship during HS. In the present study there was a decrease in NEFA during HS despite a decrease in feed intake (Hung et al., 2021) consistent with an uncoupling.

McCarty et al. (2009) reported that thermal therapy can improve insulin sensitivity in humans. Moreover, HS can ameliorate biomarkers of insulin sensitivity in diabetic rats (Kokura et al., 2010) and rats fed a high-fat diet (Gupte et al., 2009). Increased insulin sensitivity may be an essential component of the acclimation mechanism in HS. For example, insulin resistant subjects such as diabetic humans are more susceptible to heat-related illness and death (Semenza et al., 1999; Kovats and Hajat, 2008). Increased insulin sensitivity can be associated with a reduction in metabolic heat production, implying that maintenance of insulin sensitivity plays a critical role in the thermoregulatory function, especially when animals are under HS. Therefore, maintaining glucose homeostasis is a possible approach to improve adaption of HS.

Increased insulin sensitivity would ensure that sheep would reduce adipose tissue lipid mobilization and the use NEFA as a preferred energy substrate despite a reduction in feed intake during HS. Rather, glucose would become the preferred energy substrate for peripheral tissues. HS causes a reduction in blood pCO2 and increase in blood pO2 and a resultant decrease in base excess and increase in blood pH in sheep (Gonzalez-Rivas et al., 2017; Joy et al., 2020). These changes in blood gas parameters are most likely a result of increased respiration rate during HS as was the case in the present study (Hung et al., 2021). Use of lipid as a metabolic substrate during HS would result in a low respiratory quotient (Blaxter, 1962, 1967; Fernandez et al., 2012) and would therefore further decrease the blood base excess resulting in respiratory alkalosis. An increase in insulin sensitivity with no change in circulating insulin as observed here would inhibit lipolysis and fat mobilization whilst still ensuring hepatic gluconeogenesis since the effective dose to inhibit plasma NEFA concentrations is within the physiological range and less than that which inhibits gluconeogenesis (Petterson et al., 1993, 1994).

Another possible, but less likely, explanation for the lower plasma NEFA concentrations during HS is increased fatty acid oxidation. *CPT1B* is an important enzyme for mitochondrial fatty acid oxidation, regulating inward translocation of NEFA, and is rate-limiting for β-oxidation of NEFA inside mitochondria (McGarry and Brown, 1997). However, HS had no main effect on *CPT1B* mRNA expression whereas dietary nCrPic increased *CPT1B* mRNA expression in a dose-dependent manner. Also, as noted above, an increase in NEFA oxidation would further exacerbate the impact of HS on respiratory alkalosis.

Animal exposure to high ambient temperatures triggers several signaling pathways, some of which facilitate cell survival and others initiate cell death. The outcome for animals under HS may be either development of a state of tolerance of high ambient temperature and other stresses via survival pathways or cell death via death pathways. Survival pathways activated by HSPs such as *HSP72* and *HSP90* are mediated by heat shock transcription factors (HSFs) (Pirkkala et al., 2001). Gabai and Sherman (2002) indicated that HS can activate either *AKT* (cell survival) or *JNK* (cell death) pathways, and HSPs are the key regulator to control the direction toward either the cell survival or death pathway. A number of in vitro and in vivo studies demonstrated that *HSP72* can inhibit *JNK*, and a lower level of *HSP72* results in *JNK* activation (Chung et al., 2008). In turn, activity of HSPs is regulated by AKT through inhibition of glycogen synthase kinase-3β (*GSK3β*), a negative regulator of *HSF1* (Xavier et al., 2000). The inhibition of *GSK3β* means increased glycogenesis which can help helps maintain a normal glucose homeostasis. These data imply that improved insulin signaling is associated with cell apoptosis when animals are under HS. Chauhan et al. (2014b) found that HS increased both *HSP70* and *JNK* skeletal muscle expression. In the present study, *JNK* mRNA expression was reduced by dietary nCrPic. Moreover, nCrPic was also able to maintain glucose homeostasis as evidenced in the glucose challenge results showed that sheep fed with nCrPic diet had lower glucose AUC. Furthermore, our previous study showed that dietary nCrPic can decrease rectal temperature and increase feed intake in sheep under the same temperature regime as in the present study (Hung et al., 2021). Taken together, these data suggest that dietary nCrPic supplementation ameliorated the negative physiological responses in HS sheep by decreasing *JNK* expression in skeletal muscle and consequently preventing cell apoptosis. Interestingly, dietary nCrPic and HS had no effect on *HSP72* and *HSP90* gene expression in WBC and skeletal muscle although HS and high doses of nCRPic decreased *HSP90* in adipose tissue. The absence of consistent tissue gene expression response to HS or dietary nCrPic may also be due to tissue samples were only collected on one time point during this experiment and therefore, unable to examine temporal gene expression changes response to HS or nCrPic. This is particularly important if the response were transient in nature. Moreover, the metabolic mechanisms involve several feedback systems which may regulate and alter the gene expression.

5. Conclusion

The results of this experiment clearly demonstrated that sheep under HS had greater insulin sensitivity. Moreover, dietary nCrPic supplementation also showed the improvement of insulin sensitivity. The improvement in insulin sensitivity may be due to the reducing of *JNK* mRNA expression in skeletal muscle and hence, prevent cells progressing towards the apoptosis pathway when animals are under HS.

Author contributions

Alex T. Hung: Conceptualization, Investigation, Methodology, Data collection, Data curation, Formal analysis, Writing – Original draft. **Brian J. Leury:** Conceptualization, Writing – Review and Editing, Supervision. **Matthew A. Sabin:** Conceptualization,

Writing – Review and Editing, Supervision. **Fahri Fahri:** Investigation, Data collection. **Kristy DiGiacomo:** Investigation, Data collection, Writing – Review and Editing. **Tu-Fa Lien:** Conceptualization, Resources, Writing – Review and Editing. **Frank R. Dunshea:** Conceptualization, Resources, Formal analysis, Data curation, Writing – Review and Editing, Supervision.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2023.01.003.

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