**High dietary selenium and vitamin E supplementation ameliorates the impacts of heat load on oxidative status and acid-base balance in sheep**

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ABSTRACT: The objective of this study was to determine the efficacy of supranutritional dietary selenium and vitamin E (Vit E) to ameliorate the effect of heat stress (HS) on oxidative status and acid-base balance in sheep. Thirty-two Merino × Poll Dorset ewes were acclimated to indoor individual pen feeding of a pelleted control diet (0.24 g Se and 10 IU of Vit E/kg DM) for 1 wk. Sheep were then moved to metabolism cages in climatic chambers and randomly allocated to a 2 × 2 × 2 factorial design with the respective factors being dietary Se (0.24 and 1.20 mg/kg DM as Sel-Plex; Alltech, Australia), Vit E (10 and 100 IU/kg DM), and temperature for 2 wk. After 1 wk of acclimation in metabolic cages, 1 climatic chamber continued on thermoneutral (TN) conditions (18°C to 21°C and 40% to 50% relative humidity [RH]), and the other one was set to HS conditions (28°C to 40°C and 30% to 40% RH) for 1 wk. The sheep were then returned to individual pens and fed the control diet for 1 wk before being returned to the same diet as in the first period but a reversed thermal treatment for a further 2 wk. Physiological parameters were recorded 3 times daily, and blood samples were collected on d 1 and 7 of thermal treatment. Average respiration rate and rectal temperature of sheep were increased ($P < 0.001$) during HS; however, combined supranutritional supplementation of Se and Vit E reversed the effects of HS. Sheep given the high Se and high Vit E diet had a lower respiration rate (191 vs. 232 breaths/min; $P = 0.012$) and rectal temperature (40.33°C vs. 40.58°C; $P = 0.039$) under peak HS (1700 h) compared with those fed the low Se and low Vit E diet. Plasma reactive oxygen metabolites concentrations were reduced ($P = 0.048$) by 20%, whereas biological antioxidant potential was increased ($P = 0.17$) by 10% in sheep fed the high Se and high Vit E diet compared with those fed the low Se and low Vit E diet. Blood pH was elevated ($P = 0.007$) and bicarbonate was reduced ($P = 0.049$) under HS, and again, these effects were ameliorated by the high Se and high Vit E diet. Both white blood cell glutathione peroxidase gene expression and red blood cell lysate glutathione peroxidase activity were increased in sheep fed the high Se and high Vit E diet. These data suggest that supranutritional dietary Se or Vit E can reduce some of the negative effects of HS. However, the synergism between the 2 antioxidants improves their potential to ameliorate the impacts of HS in sheep.

**Key words:** acid-base balance, heat stress, oxidative stress, selenium, sheep, vitamin E

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INTRODUCTION

High ambient temperature, relative humidity, and solar radiation compromise the ability of livestock to dissipate heat from the body and reduce their productive performance (growth, meat, and milk yield and quality; St-Pierre et al., 2003; Bernabucci et al., 2010; Baumgard and Rhoads, 2012; Renaudeau et al., 2012). Heat stress (HS) not only is a challenge for animal production but also negatively impacts
animal welfare (Silanikove, 2000). Therefore, there has been considerable interest to develop some suitable strategies to reduce the negative effects of HS on livestock (Beede and Collier, 1986; West, 1999; Dunshea et al., 2013; Rhoads et al., 2013).

Heat stress has been implicated to cause imbalance between antioxidant defenses and reactive oxygen species generation, resulting in oxidative stress in ruminants (Bernabucci et al., 2002; Chauhan et al., 2014b). Recently, antioxidants (vitamins E, A, and C and the micronutrients Se and zinc) have been evaluated as nutrients to potentially reduce the negative effects of HS in ruminants (Sivakumar et al., 2010; Calamari et al., 2011; Alhidary et al., 2012b; Chauhan et al., 2013, 2014b; Sejian et al., 2014). For example, Chauhan et al. (2014b) showed that although HS leads to oxidative stress (OS) in sheep, supranutritional doses of dietary Se and vitamin E (Vit E) can reverse these effects. Given the beneficial effects associated with higher antioxidant supplementation, further research is required to elucidate the effect of these individual antioxidants on oxidative status of sheep exposed to heat. Furthermore, the acid-base physiology of ruminants is perturbed as a consequence of HS because of the respiratory alkalosis resulting from the sharp increase in CO₂ loss via panting during HS (West, 2003; Srikandakumar et al., 2003). Therefore, the present study was designed to test the hypothesis that dietary Se or Vit E at supranutritional doses may reduce the negative effects of heat stress on OS and acid-base balance in sheep.

MATERIALS AND METHODS

All procedures undertaken on the animals in this study were approved by The University of Melbourne Animal Ethics Committee, Faculty of Science, School of Land and Environment, and Department of Optometry and Vision Sciences.

Animals and Experimental Design

Thirty-two Merino × Poll Dorset ewes (10 to 12 mo of age; 45 to 47 kg BW) were acclimated to indoor individual pen feeding of a pelleted control diet with basal levels (NRC 2007) of Se and Vit E (0.24 g Se and 10 IU Vit E/kg DM) for 1 wk. Sheep were then moved to metabolic cages (approximately 1.0 × 0.5 m and standing 1.0 m off the ground with fabricated steel mesh floors and metal walls) housed in climate-controlled rooms and housed under thermoneutral (TN) conditions (18°C to 21°C and 40% to 50% relative humidity [RH]) and randomly allocated to a 2 × 2 × 2 factorial design with the respective factors being dietary Se (0.24 and 1.20 mg/kg DM as Sel-Plex (Alltech, Australia), low selenium [LSe] and high selenium [HSe], respectively) and Vit E (10 and 100 IU/kg DM as α-tocopherol acetate, low Vit E [LVE] and high Vit E [HVE], respectively), and temperature (see below) for 2 wk (Fig. 1). In this design sheep were randomly allocated to 4 diets with different levels of Se and Vit E: 1) control (Se = 0.24 mg/kg and Vit E = 10 IU/kg DM), 2) high Se, low Vit E (Se = 1.20 mg/kg and Vit E = 10 IU/kg DM), 3) low Se, high Vit E (Se = 0.24 mg/kg and Vit E = 100 IU/kg DM), and 4) high Se, high Vit E (Se = 1.20 mg/kg and Vit E = 100 IU/kg DM). After 1 wk of supranutritional dietary Se and Vit E feeding in metabolism cages under thermoneutral conditions, 1 of the climate-controlled rooms was maintained at TN conditions, whereas the other was set to HS conditions (temperature 28°C to 40°C and 30% to 40% relative humidity). The temperature was set to rise daily at 0900 h to eventually reach a maximum of 40°C by 1400 h and then maintained at 39°C ± 1°C until 1700 h followed by decline to reach 28°C at 2000 h (Fig. 2). The temperature was set to 28°C overnight from 2000 to 0900 h and varied from 26°C to 29°C. Relative humidity was set at 35% and varied from 30% to 40%. When the temperature was between 38°C and 40°C, the RH dropped to 30% and varied between 36% and 40% overnight (2000 to 0700h). In the TN room, the ambient temperature varied between 21°C ± 1°C. Relative humidity was set at...
45% and varied from 40% to 50%. Ambient temperature and RH were measured at 10-min intervals inside the climatic chambers with the help of temperature and humidity sensors (HVAC Sensors, Schneider Electric, Melbourne, Australia) installed in the middle of room at a height of 1 m on the walls. The sensors were remotely connected to a computer installed with software (designed by DATACOM, Melbourne, Australia, and maintained by Control Tech, Melbourne, Australia) for online data recording and control of the heaters (Ducted Single Phase Split System Air Conditioner, Temperzone, Sydney, Australia) and humidifiers (Defensor Mk5, Aireven, Hornsby NSW, Australia) to achieve the specific temperature and RH.

After 1 wk of thermal treatments, all sheep were allowed a 1 wk washout period during which all sheep were moved back to individual pens and maintained under TN conditions and fed the control diet. The animals were then subjected to a second thermal treatment period in a single reversal design, such that sheep previously housed in the TN room were now housed in the HS room and vice versa. Each sheep received the same dietary treatment during both thermal treatment periods (Fig. 1).

The feed pellets contained 2.75 Mcal ME/kg and 15.0% CP on a DM basis (Rivalea Australia Pty Ltd., Corowa, Australia). The sheep were fed at 80% of estimated energy requirements (NRC 2007) to minimize the individual variation in antioxidant intake within the same treatment group. Access to feed and water was provided by troughs and buckets attached to the side of the crates. Water was available ad libitum.

**Physiological Parameters and Blood Samplings**

Respiration rate was determined by counting the flank movements for 20 s and then converting to breaths per minute. Heart rate was determined by counting the number of heart beats using a stethoscope (3M Littmann Master Classic, Littman, VIC, Australia) over 20 s and then converting to beats per minute. Rectal temperature was recorded using a digital thermometer (MT-018, Vega Technologies Inc., Dongguan City, China). Skin temperature was also recorded by using a digital thermometer after parting wool on the rump, and care was taken to keep the bulb of thermometer in direct contact with the skin. Respiration rate, heart rate, rectal temperature, and skin temperature were recorded 3 times a day at 0900, 1300, and 1700 h during the thermal treatments. The temperature humidity index (THI) was calculated according to the formula described by Marai et al. (2001): $\text{THI} = \text{db}^\circ \text{C} - [(0.31 - 0.31 \text{RH}) \times (\text{db}^\circ \text{C} - 14.4)]$, where db°C is dry-bulb temperature in degrees Celsius and RH is the relative humidity percentage/100. The severity of heat stress was estimated on the basis of the THI values as follows: $\text{THI} < 22.2 = $ absence of heat stress, $22.2 \leq \text{THI} < 23.3 = $ moderate heat stress, $23.3 \leq \text{THI} < 25.6 = $ severe heat stress, and $\geq 25.6 = $ extreme severe heat stress (Marai et al., 2001, 2007). Feed intake (weight of pellets offered minus refusals) and water intake (amount of water offered minus water left in the bucket) were recorded daily at 0800 h.

Blood samples were collected on d 0, 8, and 14 (period 1) and d 30 and 36 (period 2) at 1300 h just after the measurement of the physiological parameters was completed. Blood samples on d 0 and 30 were obtained by venipuncture to provide blood samples for isolation of white blood cells (WBC) for gene expression. To ensure that multiple blood samples could be obtained for blood gas analyses without the possibility of aeration or contamination with atmospheric gases, animals were catheterized 24 h before blood collection on d 14 and 36 by inserting catheters (14 gauge, 3.25 inch BD Angiocath; BD Australia, North Ryde, Australia) into the jugular vein. A 22-cm plastic catheter extension tube with a lever lock (Heidelberg extension tubing, B Braun, Bella Vista, Sydney, Australia) was secured to the catheter and affixed to the skin at 2 different points leading up the neck. The catheter was flushed with 8 to 10 mL
heparinized saline (50 IU/L) and sealed with a Safesite valve (code: 415068, B Braun). Blood samples (10 mL) were withdrawn by connecting a 10-mL syringe to the Safesite (code: 415068, B Braun) and were transferred immediately to a BD Vacutainer (BD Australia) containing lithium heparin as an anticoagulant. These samples were also used for isolation of WBC and to conduct assays related to antioxidant status.

**Laboratory Analyses**

Within 1 h of blood collection, packed cell volume (PCV) was determined by the microhematocrit method, and blood samples were centrifuged at 3,000 × g for 10 min at 4°C. Separated plasma and WBC were stored at −20°C until further analysis.

The concentration of reactive oxygen metabolite (ROM) in plasma and biological antioxidant potential (BAP) was measured on samples obtained on d 14 and 36 (final day of periods 1 and 2) with commercial kits (d-ROMs and BAP test, respectively, Diaclone Int., Grosseto, Italy). Oxidative stress index (OSI) was calculated as the ratio of ROMs/BAP multiplied by 100 (Celi, 2011). Activity of glutathione peroxidase (GSH-Px) in erythrocyte lysate was measured by a kinetic method with commercial kits (RANSEL, Randox Laboratories, London, UK) as reported by Bernabucci et al. (2002). The concentration of advanced oxidation protein product (AOPP) in plasma was estimated using the method of Witko-Sarsat et al. (1998).

**Blood Gas and Acid-Base Analysis**

Whole-blood pH, partial pressure of carbon dioxide (pCO₂), partial pressure of oxygen (pO₂), bicarbonate ions (HCO₃⁻), and total carbon dioxide (TCO₂) were analyzed in blood samples obtained on d 14 and 36 (final day of periods 1 and 2) using the epoc Blood Analysis System (Alere Inc., Waltham, MA) as per the manufacturer’s instructions. Briefly, epoc Host was connected to the epoc reader. A new epoc BGEM (blood gases, electrolyte, and metabolite) Test Card (Alere Inc.) was inserted into the reader and calibration was performed for each sample. Within 1 min of calibration, 1 mL of blood was obtained from each sheep and immediately introduced into the epoc BGEM Test Card.

**Analysis of Gene Expression**

Gene expression analysis was performed as described previously by Chauhan et al. (2014a). Total RNA was isolated from WBC obtained from blood samples obtained on d 8, 14, 30, and 36 (first and final days of periods 1 and 2) using RiboPure-Blood Kit (Ambion Life Technologies Australia Pty Ltd., Mulgrave, Australia) as per the manufacturer’s protocol. Isolated RNA from WBC was treated with DNase using a DNA-free DNase kit (Ambion Life Technologies) to remove any contaminating genomic DNA. The quality and quantity of RNA extracted from the WBCs were determined using the Experion System automated electrophoresis station (Bio-Rad Laboratories Inc., Gladesville, Australia) with the Experion StdSens Analysis Kit (Bio-Rad Laboratories Inc.). Total RNA (8 μL) was transcribed to cDNA using the SuperScriptIII First-Strand Synthesis System for RT-PCR (Invitrogen, Mount Waverley VIC, Australia), following its quality and quantity evaluation.

Primer sets for β-actin (forward: GTCCGTGACATCAAGGAGAAG and reverse: AGGAAGGAGACTGGGAAGAG), glutathione peroxidase 1 (forward: AGTTTGGGATCGAGAAGAC and reverse: CCGAAGGAGGCGAAGAG), and superoxide dismutase 1 (forward: GGTCCACGTCAGCAGTTT and reverse: CAATGCAACACCATTG) were designed using Primer3 software (http://www.simgene.com/Primer3) on the basis of ovine nucleotide sequences (accession numbers NM0010009784.1, XM_004018462.1, and NM_001145185.1) obtained from the National Center for Biotechnology Information (NCBI) nucleotide database and synthesized by GeneWorks (Thebarton, Australia). Real-time quantitative PCR reaction and amplification were performed in 25-μL reactions as per manufacturer’s instructions using SYBR GreenER Supermix (Life Technologies Australia Pty Ltd., Mulgrave, Australia). PCR quantification of each sample was performed in triplicate, and SYBR green fluorescence was quantified in an iQ5 Single Color Real Time PCR Detection System (BioRad Laboratories Inc., Hercules, CA). The thermocycling conditions employed for each assay run and analyses of amplification plots for all genes were similar, as described previously Chauhan et al. (2014a). Fold change in the relative expression of the target gene was quantified according to the 2−ΔΔCT method (Livak and Schmittgen, 2001).

**Statistical Analysis**

All analyses were undertaken using REML in GenStat (GenStat release 15.1, Hemel Hempstead, UK, VSN Int. Ltd.). Fixed model effects were temperature (HS and TN), selenium (LSe and HSe), and Vit E (LVE and HVE). Carry-over effects of thermal treatment and interactions between heat treatment and period were tested, and there were none. Gene expression was analyzed after normalizing the threshold cycle data with reference gene (ACT = threshold cycle of gene of interest – threshold cycle of reference gene for
same sheep). Effect of time (0900, 1300, and 1700 h) was included in the model for the physiological parameters, and effect of day (d 1 and 7) was included in the model for gene expression analysis. All analysis was undertaken using individual sheep as the random effect. Results were reported as means and pooled SE.

RESULTS

Climatic Conditions and Physiological Parameters

The average THI was 19.0 during TN conditions and was 34.1 between 0900 and 1700 h and 25.0 between 1700 and 1900 h during HS conditions.

Respiration rate, rectal temperature, heart rate, and skin temperature responses to Se and Vit E supplementation during TN and HS are presented in Fig. 3. Respiration rate increased in response to HS (78.3 vs. 169 breaths per minute for TN and HS conditions, respectively; \( P < 0.001 \)) and increased over the day (82.2, 139, and 150 breaths per minute at 0900, 1300, and 1700 h respectively; \( P < 0.001 \); Fig. 3a). There was an interaction (\( P < 0.001 \)) between thermal treatment and time such that respiration rate increased to a greater extent over the day in sheep exposed to heat compared with those housed under TN conditions (Fig. 3a). Although there was no main effect (\( P = 0.71 \)) of Se supplementation on respiration rate, Vit E supplementation decreased respiration rate (132 vs. 116 breaths per minute for LVE and HVE, respectively; \( P = 0.035 \)). There were also significant interactions between Se and thermal treatment (\( P = 0.002 \), Vit E

Figure 3. Relationships between (a) respiration rate, (b) rectal temperature, (c) heart rate, and (d) skin temperature and time of day in sheep fed 0.24 mg Se and 10 IU vitamin E/kg DM (open squares), 0.24 mg Se and 100 IU vitamin E/kg DM (solid squares), 1.20 mg Se and 10 IU vitamin E/kg DM (open triangles), or 1.20 mg Se and 100 IU vitamin E/kg DM (solid triangles) under thermoneutral (TN; 18°C–21°C) or heat stress (HS; 28°C–40°C) conditions. Selenium was provided as SelPlex, Alltech, Australia, and Vitamin E was provided as α-tocopherol acetate. The P-values for the effects of temperature, Se, vitamin E, Se × vitamin E, and temperature × Se × vitamin E were <0.001, 0.71, 0.035, 0.38, and 0.012 for respiration rate, <0.001, 0.02, <0.001, 0.34, and 0.039 for rectal temperature, 0.07, <0.001, <0.001, <0.001, and 0.40 for heart rate, and <0.001, <0.001, <0.001, 0.97, and 0.34 for skin temperature, respectively. See text for other interactions. The standard error of the difference can be found on the values for sheep fed the low levels of both Se and Vitamin E.
and thermal treatment \((P < 0.001)\), thermal treatment, Se, and Vit E \((P = 0.012)\), and thermal treatment, Se, Vit E, and time \((P = 0.048)\) such that respiration rate increased to a lesser extent at both 1300 and 1700 h during high temperatures in the sheep receiving HSe, HVE, or both HSe and HVE (Fig. 3a).

Rectal temperature increased in response to heat load \((39.47°C \text{ vs. } 40.10°C)\) for TN and HS conditions, respectively; \(P < 0.001\) and increased over the day \((39.39°C, 39.90°C, \text{ and } 40.05°C)\) at 0900, 1300, and 1700 h, respectively; \(P < 0.001\); Fig. 3b). However, there was an interaction \((P < 0.001)\) between thermal treatment and time such that rectal temperature increased to a greater extent over the day in the sheep exposed to HS compared with those housed under TN conditions (Fig. 3b). Sheep fed high Se had higher RT \((39.75°C \text{ vs. } 39.81°C)\) for LSe and HSe, respectively; \(P = 0.02\), whereas the sheep fed high Vit E had lower RT \((39.85°C \text{ vs. } 39.71°C)\) for LVE and HVE, respectively; \(P = 0.035\). There were interactions between Vit E and thermal treatment \((P = 0.001)\), thermal treatment, Se, and Vit E \((P = 0.039)\), and time, Se, and Vit E \((P = 0.048)\) such that rectal temperature increased to a lesser extent during the daily heat period in the sheep receiving either HVE or high Se and high Vit E (HSeHVE) compared with those given the LVE diets (Fig. 3b).

Effects similar to those observed for rectal temperature were seen for skin temperature (Fig. 3c). Skin temperature increased in response to HS \((38.89°C \text{ vs. } 37.64°C)\) for HS and TN conditions, respectively; \(P < 0.001\) and increased over the day \((37.69°C, 38.46°C, \text{ and } 38.63°C)\) at 0900, 1300, and 1700 h, respectively; \(P < 0.001\). However, there was an interaction \((P < 0.001)\) between thermal treatment and time such that skin temperature increased to a greater extent over the day in the sheep exposed to heat compared with those housed under TN conditions. There were main effects of both Se and Vit E supplementation on skin temperature such that sheep supplemented with HSe had higher \((38.20°C \text{ vs. } 38.33°C)\) and decreased with the combined effect of HSeHVE \((7.46 \text{ vs. } 7.44 \text{ beats per minute for LSe and HSe, respectively};\ P = 0.001)\) such that respiration rate increased to a lesser extent at both 1300 and 1700 h during high temperatures in the sheep receiving HSe, HVE, or both HSe and HVE (Fig. 3a).

Heart rate tended to increase in response to heat stress \((74.3 \text{ vs. } 75.96 \text{ beats per minute for TN and HS conditions, respectively};\ P = 0.074)\) and increased over the day \((69.15, 79.05, \text{ and } 77.18 \text{ beats per minute at } 0900, 1300, \text{ and } 1700 \text{ h, respectively};\ P < 0.001;\ Fig. 3d). However, there was an interaction \((P = 0.001)\) between thermal treatment and time such that heart rate decreased over the day in sheep that had been exposed to HS but not in the sheep housed under TN conditions (Fig. 3d). There were main effects of both Se and Vit E supplementation such that lower heart rates were observed in sheep supplemented with HSe \((77.4 \text{ vs. } 72.9 \text{ beats per minute for LSe and HSe levels, respectively};\ P = 0.001)\) or HVE \((77.4 \text{ vs. } 72.8 \text{ beats per minute for LVE and HVE diets, respectively};\ P = 0.035)\). There were interactions between thermal treatment and Se \((P = 0.045)\) and Vit E \((P = 0.009)\). Sheep supplemented with HSe or HVE had lower heart rates than those on LSe or LVE diets under HS, respectively (Fig. 3d).

Average daily feed intake decreased in response to HS \((868 \text{ vs. } 723 \text{ g/d for TN and HS conditions, respectively};\ P = 0.042;\ Table 1)\). Although there were no main effects of either Se \((P = 0.29)\) or Vit E \((P = 0.35)\) supplementation, there was an interaction \((P = 0.014)\) between thermal treatment, Se, and Vit E. HS decreased feed intake in sheep fed the LSeLVE diet \((871 \text{ vs. } 723 \text{ g/d})\) but not in the sheep that received the HSeHVE diet \((864 \text{ vs. } 848 \text{ g/d};\ Table 1)\). Average daily water intake increased during HS \((3.1 \text{ vs. } 3.8 \text{ L/d for TN and HS conditions, respectively};\ P = 0.038;\ Table 1)\). However, there were no main effects of either Se \((P = 0.47)\) or Vit E \((P = 0.49)\), nor were there any interactions between thermal treatment and the different dietary levels of Se or Vit E on water intake. The ratio of water to feed intake increased during HS \((3.98 \text{ vs. } 4.84 \text{ for TN and HS conditions, respectively};\ P = 0.038)\). Although there was no main effect of either Se \((P = 0.47)\) or Vit E \((P = 0.49)\) on this ratio, there was an indication \((P = 0.09)\) of an interaction between thermal treatment, Se, and Vit E. Heat stress increased the ratio of water to feed intake in sheep supplemented with the LSeLVE diet \((4.12 \text{ vs. } 5.46)\) but not in sheep that received the HSeHVE diet \((4.15 \text{ vs. } 4.43;\ Table 1)\).

**Acid-Base Balance**

Blood pH increased in response to HS \((7.43 \text{ vs. } 7.46 \text{ for TN and HS conditions, respectively};\ P = 0.004)\) and decreased with the combined effect of HSeHVE \((7.46 \text{ vs. } 7.44 \text{ for LSeLVE and HSeHVE diets, respectively};\ P = 0.05;\ Table 2)\). Similarly, \(pO_2\) tended to increase in response to HS \((42.5 \text{ vs. } 45.7 \text{ mm Hg for TN and HS, respectively};\ P = 0.085;\ Table 2)\). There were no main effects of Se or Vit E or any interactive effects on \(pO_2\). Conversely, \(pCO_2\) decreased \((37.3 \text{ vs. } 32.9 \text{ mm Hg for TN and HS, respectively};\ P < 0.001)\) in response to HS (Table 2). Although there were no main effects of Se or Vit E, there was an indication \((P = 0.09)\) of an interaction between thermal treatment, Se,
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and Vit E such that pCO₂ declined to a greater extent in sheep on the LSeLVE diet (37.59 vs. 31.18 mm Hg) compared with sheep that received the HSeHVE diet (37.83 vs. 34.28 mm Hg) during HS. Total CO₂ also decreased in response to HS (25.8 vs. 24.4 mmol/L for TN and HS conditions, respectively; $P = 0.02$) and tended to be increased by HSe supplementation (24.6 vs. 25.6 mmol/L for LSe and HSe supplementation, respectively; $P = 0.10$; Table 2).

However, blood bicarbonates decreased in response to HS (24.7 vs. 23.4 mmol/L for TN and HS, respectively; $P = 0.03$) and tended to be increased by HSe supplementation (24.6 vs. 25.6 mmol/L for LSe and HSe supplementation, respectively; $P = 0.10$; Table 2).

### Oxidative Stress Biomarkers

Plasma ROM increased in response to HS (93.7 vs. 105 CARR U for TN and HS conditions, respectively; $P = 0.012$; 1 CARR U = 0.08 mg H₂O₂/dL) and tended to be decreased by both HSe (103 vs. 95.9 CARR U for LSe and HSe, respectively; $P = 0.14$) and HVE (103 vs. 95.7 CARR U for LVE and HVE, respectively; $P = 0.093$; Table 2). Although there were no interactions between thermal treatment and Se or Vit E, there was an indication ($P = 0.10$) of 3-way interaction between thermal treatment, Se, and Vit E. HS decreased blood bicarbonates to a greater extent in sheep that received the LSeLVE diet (24.6 vs. 23.0 mmol/L) compared with sheep that received the HSeHVE diet (24.8 vs. 24.1 mmol/L; Table 2).

### Table 1. Effects of chronic heat stress and different dietary doses of selenium and vitamin E on feed and water intake in sheep

<table>
<thead>
<tr>
<th>Item</th>
<th>Thermal treatment (Temp)</th>
<th>P-value</th>
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<tr>
<td></td>
<td>Thermoneutral</td>
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</tr>
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<td></td>
<td>ABW, kg</td>
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<td></td>
<td>ADWI, L/d</td>
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<tr>
<td></td>
<td>ADWI/feed intake, L/kg</td>
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<tr>
<td></td>
<td>ADFI, g/d</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Heat stress</td>
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<td></td>
<td>ABW, kg</td>
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<td>ADWI/feed intake, L/kg</td>
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</tr>
<tr>
<td></td>
<td>ADFI, g/d</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*Within a row means without common superscript differ ($P < 0.05$). Feed and water intake was recorded at 0900 h daily during each thermal treatment period, and the values given are average of 7 d. Vit E = vitamin E, ADWI = average daily water intake.*
Table 2. Effects of chronic heat stress and feeding levels of selenium and vitamin E on the acid-base balance and oxidative stress biomarkers in sheep

<table>
<thead>
<tr>
<th>Item</th>
<th>pH</th>
<th>pCO₂ mmHg</th>
<th>pO₂ mmHg</th>
<th>TCO₂ mmol/L</th>
<th>Bicarbonates mmol/L</th>
<th>WBC GPX1 mRNA abundance</th>
<th>ROM, CARR U</th>
<th>AOPP, mol/L</th>
<th>GSH-Px, units/mL RBC lysates</th>
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<tbody>
<tr>
<td>0.24 mg Se/kg DM</td>
<td>7.43 b</td>
<td>42.4 a,b</td>
<td>42.7 a</td>
<td>24.6 a</td>
<td>24.6 a</td>
<td>7.65 a</td>
<td>11.03 b</td>
<td>11.36 b</td>
<td>34.1 b</td>
</tr>
<tr>
<td>1.20 mg Se/kg DM</td>
<td>7.43 b</td>
<td>42.4 a,b</td>
<td>42.7 a</td>
<td>24.6 a</td>
<td>24.6 a</td>
<td>7.65 a</td>
<td>11.03 b</td>
<td>11.36 b</td>
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<td>0.24 mg Se/kg DM</td>
<td>7.43 b</td>
<td>42.4 a,b</td>
<td>42.7 a</td>
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<td>11.03 b</td>
<td>11.36 b</td>
<td>34.1 b</td>
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<td>1.20 mg Se/kg DM</td>
<td>7.43 b</td>
<td>42.4 a,b</td>
<td>42.7 a</td>
<td>24.6 a</td>
<td>24.6 a</td>
<td>7.65 a</td>
<td>11.03 b</td>
<td>11.36 b</td>
<td>34.1 b</td>
</tr>
<tr>
<td>0.24 mg Se/kg DM</td>
<td>7.43 b</td>
<td>42.4 a,b</td>
<td>42.7 a</td>
<td>24.6 a</td>
<td>24.6 a</td>
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<td>11.36 b</td>
<td>34.1 b</td>
</tr>
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- Within a row, means without a common superscript differ (P < 0.05).
- Blood samples were obtained at 1300 h on d 7 of each thermal treatment period. Vit E = vitamin E; pCO₂ = partial pressure of carbon dioxide; pO₂ = partial pressure of oxygen; TCO₂ = total carbon dioxide; ROM = reactive oxygen metabolites (1 CARR U = 0.08 mg H₂O₂/dL); BAP = biological antioxidant potential; OSI = oxidative stress index; AOPP = advanced oxidation protein products; GSH-Px = glutathione peroxidase.

There were no interactions between thermal treatment and Se (P = 0.31) or Vit E (P = 0.64), there was an indication (P = 0.091) of an interaction between thermal treatment, Se, and Vit E. The greater OSI was observed during HS in sheep that received the LSeLVE diet (3.01 arbitrary units) compared with sheep that received the HSeHVE diet (2.69 arbitrary units; Table 2).

The concentration of AOPP in plasma tended to increase during HS (11.3 vs. 12.4 mol/L for TN and HS conditions, respectively; P = 0.058; Table 2). Although there were no main effects of Se (P = 0.32) or Vit E (P = 0.27), there were indications of interactions between thermal treatment and Vit E (P = 0.071) and thermal treatment, Se, and Vit E (P = 0.063) such that HS increased plasma AOPP to a greater extent in sheep that received the HSeLVE (11.0 vs. 13.4 mol/L) or LSeLVE diets (11.01 vs. 14.91 mol/L) compared with sheep that received the HVE diets (111.0 vs. 11.6 mol/L) during HS, whereas AOPP levels decreased in sheep that received the HSeHVE diet (12.36 vs. 9.54 mol/L) during HS (Table 2).

Packed cell GSH-Px activity was increased in response to HS (33.9 vs. 38.6 units/mL for TN and HS conditions, respectively; P = 0.036) and was also increased by Vit E supplementation (34.4 vs. 38.1 units/mL for LVE and HVE diets, respectively; P = 0.029; Table 2). There were indications of interactions between thermal treatment and Se (P = 0.08) and Vit E (P = 0.08) such that packed cell GSH-Px activity was increased in sheep supplemented with HSe (36.4 vs. 40.8 units/mL) but not in sheep supplemented with HVE (38.3 vs. 38.9 units/mL). There was also a 3-way interaction (P = 0.007) such that packed cell GSH-Px activity increased in sheep supplemented with HSeHVE diet during HS (37.1 vs. 40.5 units/mL) but decreased in sheep that received the LSeLVE E diet (34.14 vs. 28.63) during chronic HS.

**Gene Expression**

White blood cell GPX1 mRNA abundance tended to increase on exposure to HS (1.2-fold; P = 0.10), whereas there was a highly significant (P < 0.001) effect of day such that WBC GPX1 mRNA abundance was increased 3.2-fold on d 7 compared with d 1 (Fig. 4a). Although there were no main effects of Se or Vit E, there were interactions between thermal treatment and day (P < 0.001), day and Vit E (P = 0.05), and day and Se (P = 0.10) such that HS increased WBC GPX1 mRNA abundance by 4.2-fold on d 7 compared with d 1, and similarly, both HVE and HSe also increased WBC GPX1 mRNA abundance on d 7 compared with d 1. However, there was an interaction (P = 0.056) between thermal treatment, day, Se, and VitE such that WBC GPX1 mRNA abundance on d 7 of HS was in-
creased to a greater extent (4- to 5-fold) in sheep that received HSe, HVE, or both compared with sheep that received the LSeLVE diet (1.2-fold; Fig. 4a).

Conversely, WBC SOD1 mRNA abundance decreased ($P < 0.001$) on exposure to HS (2.2-fold), and there was a strong ($P < 0.001$) main effect of day such that WBC SOD1 mRNA abundance was decreased (2-fold) on d 7 compared with d 1 (Fig. 4b). Although there was no main effect of Se, there was an effect ($P = 0.001$) of Vit E such that WBC SOD1 mRNA expression was upregulated by HVE supplementation. There were interactions between thermal treatment and Se ($P < 0.061$) and Vit E ($P = 0.001$) and thermal treatment, Se, and Vit E ($P = 0.063$) such that HS decreased WBC SOD1 mRNA abundance to a greater extent (70%) in sheep that received the LSeLVE diet, whereas the decline in expression was only 45%, 50%, and 30% in sheep fed the HSe, HVE, and HSeHVE diets, respectively (Fig. 4b).

**DISCUSSION**

The major important finding from the present study was that supranutritional dietary Se and Vit E can ameliorate some of the effects of HS on physiological parameters such as respiration rate, rectal temperature, OS, and acid-base balance, particularly when supplemented in combination. Efficient ruminant production can be compromised by HS, and some effective strategies (physical modification of the environment, genetic manipulations, and nutritional interventions) are needed to mitigate the effects of HS on animal health, production, and welfare, so these findings provide an excellent opportunity to reduce the impact of HS.

Sheep in the present study were exposed to severe HS (Marai et al., 2007), and both rectal temperature and respiration rate were elevated in response to HS. As a general homeostatic response to HS, increased respiration rate and panting were expected since the primary cooling mechanism in sheep under severe HS is evaporative cooling via the respiratory tract, which is able to dissipate up to 65% of total heat produced (Hales and Brown, 1974). In agreement with our previous findings (Chauhan et al., 2014b), the combined HSeHVE treatment ameliorated the increase in respiration rate as well as rectal temperature during peak HS conditions (1300 and 1700 h, room temperature 38°C to 40°C). Interestingly, feeding HVE alone without supranutritional supplemental Se (i.e., the LSeHVE diet) reduced respiration rate in a way similar to the HSeHVE diet, whereas rectal temperature was reduced to a greater extent than with the HSeHVE diet. The HSeLVE diet also prevented the increase in respiration rate compared with the LSeLVE diet but not to the extent that was achieved with either of the HVE diets. The greatest difference between dietary treatments in both respiration rate and rectal temperature was observed during peak HS (1300 and 1700 h). Similarly, Sejian et al. (2014) found that the greatest effects of antioxidant and mineral supplementation in Malpura ewes subjected to HS was during the peak HS period. Combined Se and Vit E supplementation has previously been reported to reduce negative effects of HS on respiration rates and rectal temperature in sheep (Alhidary et al., 2012b; Chauhan et al., 2014b; Sejian et al., 2014) and goats (Sivakumar et al., 2010). However, the present study is the first to demonstrate that these antioxidants can also individually reduce the negative effects of HS on respiration rate and rectal temperature. This suggests a potential role of Se or Vit E for mitigation of HS because respiration rate
and rectal temperature are considered reliable markers of HS in sheep (Srikandakumar et al., 2003; Sejian et al., 2010; Alhidary et al., 2012a). It is important to note that the LSe and LVE diets were not deficient in Se or Vit E as they met the recommended requirements for these micronutrients.

During HS, mammalian skin is an important pathway for heat exchange between the body surface and environment. As expected, skin temperature increased in response to elevated ambient temperature in the present study, possibly to dissipate excess body heat to balance the excessive heat load. Skin temperature is the result of the adjustment of blood flow toward skin that culminates with regulation of heat exchange between the body core and skin (Habeeb et al., 1992). Overall, the HVE diet reduced skin temperature of sheep irrespective of thermal treatment. It is tempting to hypothesize that HVE redistributed more blood flow away from skin, possibly toward the splanchnic organs and to the gastrointestinal tract in particular, which is usually compromised during environmentally induced hyperthermia, resulting in decreased intestinal integrity and function (Pearce et al., 2013). The reduced skin temperature coincided with reduced heart rate, suggesting that the HVE diet decreased heart rate and blood flow to the skin, thereby decreasing skin temperature. The effects of HS on heart rate are not well documented in sheep. It was expected that heart rate would increase in response to elevated temperature in an effort to increase blood flow toward the extremities to dissipate heat. Like in our previous findings (Chauhan et al., 2014b), heart rate was reduced by antioxidant supplementation irrespective of thermal treatment, with the decline greater during HS. This outcome may be due to redistribution of blood supply toward the gastrointestinal tract (GIT), which indicates an important role of antioxidants in thermoregulation and GIT function, especially during HS. This may also explain beneficial effects of antioxidant (Vit E) feeding during HS other than reducing the OS in sheep. The beneficial effects of Vit E on the cardiac autonomic nervous system have also been reported to reduce heart rate in rats and humans (Behrens et al., 1986; Manzella et al., 2001; Fahim et al., 2013). An increase in the parasympathetic tone of the autonomic nervous system would be consistent with an improvement in GIT function. Beneficial effects of vit E supplementation on GIT functions during heat stress has been demonstrated in pigs during HS (Fan Liu, The University of Melbourne, Australia, personal communication) but needs to be tested in specifically designed studies in ruminants.

In addition to elevated respiration rate, panting, and drooling of saliva, the general homeostatic responses to HS also include reduced feed intake (Silanikove, 1992). As expected, feed intake was decreased by 16% and water intake was increased by 18% during HS, and feeding the HSeHVE diet reversed these effects. Similarly, when HSe or HVE were fed separately but with NRC (2007) requirements for the other micronutrient, feed intake was also maintained during heat stress, suggesting that these antioxidants individually have the potential to prevent the decline in feed intake during HS when fed at high doses. Although there is little information about the changes in water to feed intake ratio in ruminants, a strong relationship (\( R^2 = 0.84 \)) between ambient temperature and water to feed intake ratio has been reported in pigs (Huynh et al., 2005). Given that this ratio was reduced following Se and Vit E supplementation during HS, it is suggested that these antioxidants reduced the strain imposed by HS on the sheep, possibly via improved antioxidant defenses. Decreased feed intake is a common response to HS in sheep. For example, Nardone et al. (1991) reported a 13% decline in concentrate intake of rams when exposed to 35°C in climatic chambers, whereas Alhidary et al. (2012a) reported an even higher (22%) reduction in feed intake in Australian Merino wethers exposed to a maximum temperature of 38°C for 7 d. We have also shown previously that Se and Vit E supplementation maintained feed intake under HS conditions in sheep (Chauhan et al., 2014b). This ameliorative effect of Vit E and Se may be attributed to their ability to decrease OS and inflammation by improving antioxidant defense and downregulation of cytokines (Chauhan et al., 2014a,b), which are implicated in stimulating systemic responses, including decreased feed intake (Hadden, 1987; Bradford, 2012). Further research is required to determine the possible role of Se and Vit E in maintenance of intestinal membrane integrity and redistribution of blood supply toward GIT, which may also improve feed intake and digestibility during HS.

Heat stress has also been reported to affect acid-base balance in ruminants (Schneider et al., 1988; Sanchez et al., 1994; Srikandakumar et al., 2003; Sivakumar et al., 2010). In the present study, HS decreased blood pCO\(_2\) while increasing the blood pO\(_2\). These changes are suggestive of a greater removal of CO\(_2\) and saturation of blood with O\(_2\). Heat stress appeared to induce respiratory alkalosis due to hyperventilation and a subsequent decrease in pCO\(_2\). As expected, HS increased blood pH, whereas blood bicarbonates (HCO\(_3\)) were decreased. As the CO\(_2\) eliminated via the respiratory system derives from carbonic acid (\( H_2CO_3 = H_2O + CO_2 \)), the observed increase in pH could be ascribed to a decrease in carbonic acid concentration due to increased elimination of CO\(_2\). The bicarbonate buffering mechanism, in which the ratio of HCO\(_3\) to pCO\(_2\) is maintained relatively constant at 20:1, is the most important buffering system in blood (Masero and Siegel, 1977). Since HS decreases pCO\(_2\), to maintain acid-base balance and to counter re-
spiratory alkalosis, HCO$_3^-$ is excreted by the kidney, resulting in decreased blood HCO$_3^-$ (Schneider et al., 1988), as observed in the present study. The observed changes in blood pH and pCO$_2$ during HS are in agreement with those reported by Srikandakumar et al. (2003). A similar increase in pH during HS has been also reported in cows (Sanchez et al., 1994). In this experiment, we have further shown that the combination of HSe and HVE improves the acid-base balance of heat-stressed sheep, as reflected in the correction of blood pH and lower decline in blood bicarbonates. The decline in blood pCO$_2$ was also reduced, whereas total CO$_2$ concentration tended to increase after HSeHVE supplementation during HS. These effects of High selenium high vitamin E (HSHV) on blood acid-base balance could be explained to some extent by the ability to reduce respiration rate and rectal temperature and hence prevent excessive loss of CO$_2$ and respiratory alkalosis. Although a major disruption of blood acid-base balance due to dietary factors is uncommon, under severe HS conditions like those reproduced in this study, the combination of HS and decreased feed intake could have profound negative effects on acid-base physiology (Sanchez et al., 1994). In this scenario the maintenance of feed intake during HS in sheep fed a HSeHVE diet might have also contributed to the observed improvement in acid-base balance.

Heat stress has also been implicated in impaired oxidative status of ruminants (Bernabucci et al., 2002; Di Trana et al., 2006; Chauhan et al., 2014b). In the present study and in line with our previous findings (Chauhan et al., 2014b), plasma ROM concentrations were increased in sheep during HS; this increase was particularly high for the LSeLVE group, whereas the HSeHVE group exhibited reduced plasma ROM concentrations during HS. Overall, both Se and Vit E tended to reduce plasma ROM concentrations, but they also reduced the ROM concentrations during HS when supplemented together at higher doses. As expected, the observed decrease in ROM production in sheep consuming the HSeHVE diet during HS resulted in a lower decline in BAP, thereby resulting in a reduced OSI. Both Se and Vit E reduced the decline in BAP in sheep under HS, suggesting that supranutritional doses of Se and Vit E have the potential to prevent the increase in reactive oxygen species production and improve the oxidative status of sheep during HS. Under normal environmental and physiological conditions, the recommended requirements for Vit E and Se are likely to be sufficient to scavenge oxidants produced during normal metabolic activities. However, when cells are exposed to high oxidant levels such as during HS, the antioxidant network might be overwhelmed, leading to oxidative damage (Lykkesfeldt and Svendsen, 2007), and hence may require higher doses of these micronutrients (Chauhan et al., 2014c).

The improvement in oxidative status of sheep supplemented with HSeHVE during HS in this study is further confirmed by the lower plasma AOPP concentrations. Generally, high oxidant levels overwhelm the antioxidant defenses, resulting in oxidative damage of major macromolecules, including plasma proteins, resulting in accumulation of oxidized proteins. However, the improvement in antioxidant status following HSeHVE supplementation may prevent protein damage during HS. Improved antioxidant defense is also confirmed by increased red blood cells GSH-Px activity, which was increased after high doses of Vit E were supplemented to sheep during HS. The HSeHVE supplementation resulted in the greatest RBC GSH-Px activity during HS, whereas it was markedly lower in sheep fed the LSeLVE diet.

In this study, the increase in RBC GSH-Px activity coincided with an increased expression of WBC GPX1 gene in sheep supplemented with HSeHVE, indicating that feeding HSe and HVE diets results in an increase in the expression as well as in the activity of GSH-Px during chronic HS (i.e., on d 7). GSH-Px is an important component of the antioxidant defense system and plays a critical role in neutralizing hydrogen peroxides and lipid peroxides to prevent further damage due to the formation of highly reactive hydroxyl radicals in the cell (Hefnawy and Tortora-Perez, 2010). High dietary levels of Se are required to maximize the expression of GPX1, which normally receives the lowest priority in relation to the expression of other selenoproteins in the body (Behne and Kyriakopoulos, 2001).

Superoxide dismutase and GSH-Px are high molecular weight enzymatic antioxidants that work together in preventing oxidative damage. Superoxide dismutase is responsible for dismutation of superoxide radicals into hydrogen peroxide, whereas GSH-Px is responsible for the removal of hydrogen peroxide (Lykkesfeldt and Svendsen, 2007). In the present study, the decrease in SOD1 expression under chronic HS might have resulted in excessive accumulation of superoxide radicals. However, this may have not been the case in sheep consuming the HSeHVE diet since Vit E has the potential to directly scavenge superoxide radicals. Vitamin E reacts with free radicals incorporating the unpaired electron of the radical into a more stable Vit E derivative (α-tocopheroxyl radical). In the cell it is likely that α-tocopherol is regenerated from the α-tocopheroxyl radical by a network of other antioxidants, termed the Vit E regeneration system (Machlin and Bendich, 1987). Since some of the enzymes (GSH-Px and thioredoxin reductase) involved in this system are Se dependent, there is a close link between Se and Vit E status and antioxidant status. Previously, increased mitochondrial superox-
Selenium/vitamin E and heat stress in sheep

ide generation and oxidative damage to mitochondrial lipids and proteins were reported in skeletal muscle of chickens during acute HS, possibly because of down-regulation of uncoupling proteins (Mujahid et al., 2005, 2007). Recently, Montilla et al. (2014) reported increased OS following acute HS in red semitendinosus muscle but not in white semitendinosus muscle in pigs, again indicating the involvement of mitochondria as the red muscle (oxidative muscle) is known to have greater mitochondrial density. We have previously shown that combined supplementation of high levels of both Vit E and Se in combination can modulate the expression of heat shock proteins and inflammatory genes in skeletal muscle (LM). However, the individual effects of Vit E and Se on the expression of antioxidant enzymes and proinflammatory genes still need to be investigated further in skeletal muscle.

Conclusion

Heat stress can compromise acid-base balance and impair oxidative status in sheep. Whereas Vit E decreased respiration rate and rectal temperature during HS, Se decreased the former but not the latter. When given at high doses individually, Se or Vit E has little effect on the acid-base balance of sheep. However, when combined, together they ameliorate some of the negative effects of HS on acid-base balance. Although supranutritional Se or Vit E tended to individually decrease OS, the combined supplementation resulted in a synergistic action in preventing oxidative damage of cellular macromolecules such as proteins by reducing OSI as well as increasing the expression and activity of antioxidant enzymes such as GSH-Px. Therefore, it is suggested that high dietary Se or Vit E can ameliorate some of the negative effects of HS in sheep, but combined supplementation may be more beneficial under special circumstances such as HS.

LITERATURE CITED


