Dietary antioxidants at supranutritional doses improve oxidative status and reduce the negative effects of heat stress in sheep

S. S. Chauhan, P. Celi, B. J. Leury, I. J. Clarke, and F. R. Dunshea

Department of Animal Husbandry, Government of Himachal Pradesh, Shimla 171005, India; Melbourne School of Land and Environment, The University of Melbourne, Parkville, Vic. 3010, Australia; Faculty of Veterinary Science, University of Sydney, Sydney, NSW 2570 Australia; and Department of Physiology, Monash University, Vic. 3800, Australia

ABSTRACT: The present study was undertaken to investigate the impact of heat (thermal) stress and dietary antioxidant supplementation on the oxidative and physiological status of sheep. Twenty-four Merino × Poll Dorset crossbred ewes were housed in 1 of 2 climatic chambers (thermoneutral or heat stress) and offered either a control (10 IU vitamin E/kg DM and 0.24 mg Se/kg DM) or high antioxidant (100 IU vitamin E/kg DM and 1.20 mg Se/kg DM) diet. The sheep were exposed to 2 thermal (temperature) treatments (thermoneutral [TN]: 18–21°C and 26–30% relative humidity; and heat stress [HS]: 28–40°C and 40–50% relative humidity) for 2 wk in a single reversal design. After 1 wk of dietary treatment, animals in 1 chamber were subjected to HS for 1 wk, with the temperature being increased to 40°C between 0900 and 1700 h and then maintained at 28°C overnight. Those sheep in the TN group were maintained at 18 to 21°C. Physiological parameters were recorded 4 times a day (0900, 1300, 1700, and 2100 h) and blood samples were collected on d 1 and 7 of heat treatment. Plasma samples and red blood cell lysates were assayed for oxidative stress biomarkers. The thermal treatments were then reversed and the above measures repeated. All measured physiological parameters were elevated ($P < 0.001$) by thermal treatment. Respiration rate was lower during HS in sheep supplemented with antioxidants as indicated by a diet × temperature × time interaction ($P = 0.010$). There was 13% decline ($P = 0.014$) in feed intake of the unsupplemented animals during HS whereas the same was maintained in sheep supplemented with high doses of antioxidants. Plasma reactive oxygen metabolites concentrations were reduced (114 vs. 85 units/dL; $P < 0.005$) while biological antioxidant potential tended to be increased (3,688 vs. 3,985 μmol/L; $P = 0.070$) in heat stressed sheep supplemented with antioxidants. The oxidative stress index was 30% lower ($P < 0.001$) in supplemented sheep (2.16 ± 0.06 arbitrary units) during HS than in unsupplemented sheep (3.12 ± 0.08 arbitrary units). Plasma advanced oxidation protein products tended ($P = 0.070$) to decrease in antioxidant supplemented heat stressed sheep as compared to their unsupplemented counterparts. It was concluded that heat stress negatively affects the oxidative status of sheep along with the physiological responses and some of these effects can be ameliorated through dietary antioxidants supplementation at supranutritional concentrations.

Key words: antioxidants, heat stress, oxidative stress, sheep

INTRODUCTION

Heat stress is a multibillion dollar problem for the livestock industry as it impairs animal performance during summer months leading to economic losses globally (St-Pierre et al., 2003; Bernabucci et al., 2010). The increasing demand for food coupled with threats of global warming further accentuates the problems of heat stress (Renaudeau et al., 2012). Maintaining animal performance during hot and humid weather requires continued advances in nutritional...
formulations, improved cooling capabilities, and identification of genetic traits for enhanced heat tolerance (Collier et al., 1982; Beede and Collier, 1986; West et al., 2003). Clarity of understanding of the biological mechanisms responsible for impaired animal homeostasis during heat stress may help to generate mitigation strategies (Collier et al., 2006, 2008; Baumgard and Rhoads, 2012) and to develop potential nutritional interventions (West, 1999; Dunshea et al., 2013; Rhoads et al., 2013).

Heat stress has been implicated in promoting oxidative stress either through excessive reactive oxygen species (ROS) production or decreased antioxidant defenses (Trout et al., 1998; Bernabucci et al., 2002; Saker et al., 2004; Di Trana et al., 2006). Excessive ROS production overwhelms the antioxidant defenses leading to oxidative damage of biological molecules including proteins, lipids, and DNA (Machlin and Bendich, 1987; Halliwell and Gutteridge, 1990), disrupting normal metabolism and physiology (Trevisan et al., 2001). Therefore, a robust antioxidant network capable of preventing oxidative damage of biological molecules holds promise for improving the health and performance of animals during heat stress. Supplementation of vitamin E (Vit E) and Se at supranutritional levels have resulted in improvements in animal performance and immune function (Rooke et al., 2004). Recently, an improvement in preventive antioxidant systems was reported in heat stressed lactating dairy cows fed Se yeast (Calamari et al., 2011). Therefore, the present experiment was designed to test the hypothesis that supranutritional doses of dietary antioxidants (Vit E and Se) can ameliorate the negative effects of heat stress in sheep by improving its oxidative status and physiological responses.

**MATERIALS AND METHODS**

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

**Animals and Experimental Design**

Twenty-four Merino × Poll Dorset crossbred ewes (5–6 mo of age; 32–35 kg BW) were used in this study in a single reversal thermal crossover design. After 1 wk (d −7 to d 0) of acclimatization to the indoor facility and pellet feeding (control [CTRL] diet) in the pens, the sheep were allocated randomly to metabolism crates for 29 d, housed inside 1 of 2 climatic chambers that were programmed to either thermoneutral (TN) or heat stress (HS) conditions for the initial period. From d 0 until 29, all the sheep in each room were randomly allocated to 1 of the 2 diets containing antioxidant Vit E and Se at the recommended doses (Vit E as α-tocopherol acetate at 10 IU/kg DM and Se as selenized yeast at 0.24 mg/kg DM) or supranutritional doses (Vit E as α-tocopherol acetate at 100 mg/kg DM and Se as selenized yeast at 1.2 mg/kg DM). From d 0 to 7 and from d 15 to 21, all sheep were exposed to TN conditions with the temperature and relative humidity kept within 18 to 21°C and 40 to 50%, respectively, over the day. Those sheep that were exposed to TN conditions during period 1 continued on this regime while in the HS room, the temperature was increased to 40°C between 0900 and 1700 h and then maintained at 28°C overnight with relative humidity ranging between 26 and 30% from d 7 until 14. After 14 d (Period 1) the temperature regime in each room was reversed for another 14 d (Period 2; i.e., TN for 7 d followed by the opposite thermal treatment to that experienced in Period 1). The feed pellets were formulated by Rivalea Australia Pty Ltd., Corowa, NSW, Australia, and contained 2.65 Mcal of ME/kg, 15.0% CP, and 6.4% Crude Fiber on a DM basis. Ewe lambs were fed at the 80% of the total requirement (as per NRC, 2007; 32–35 kg BW ewe lambs growing at 200 g/d; DM requirement = 1,050 g/d) to minimize the individual variation in antioxidant intake within the same treatment group. Metabolism crates were approximately 1.0 by 0.5 m and stood 1.0 m off the ground with metal mesh floors and metal/gated walls. Access to feed and water was provided by troughs and buckets attached to the side of the cages. Water was available ad libitum.

**Physiological Parameters and Blood Samplings**

Respiration rates, rectal temperature, and skin temperature were recorded 4 times a day at 0900, 1300, 1700, and 2100 h during the thermal treatments. The temperature–humidity index (THI) was calculated according to the formula reported by Marai et al. (2001) as the temperature in Celsius and RH = relative humidity percent-age/100. Feed intake was recorded daily at 0800 h (weight of pellets offered minus refusals). The BW were measured at the beginning of experiment (d 0) and the beginning as well as end of each period and ADG was calculated. Body weights recorded at the end of each period were used for calculation of dose rates for ACTH challenge.

Blood samples were collected on d 0 (before thermal treatments), 7, 13 (period 1), 21, and 27 (period 2) at 0900, 1300, and 1700 h. To minimize the stress due to multiple blood sampling (except d 0; only 1 blood sample collected by vein puncture), animals were catheterized 24 h before blood collection by inserting catheters (14-gauge, 8.255 cm BD Angiocath; BD Australia, North Ryde, NSW, Australia) into the jugular vein. A 22-cm plastic catheter extension tube with a lever lock
Chauhan et al.

(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 valve according to Witko-Sarsat et al. (1996) and was expressed as micromoles per liter of chloramine-T equivalents.

Plasma cortisol concentrations were estimated using a RIA technique as per the method reported by Bocking et al. (1986; analytical sensitivity was 0.30 ng/mL and interassay CV was 6.2%). Estimation of plasma free T3 and free T4 concentration was conducted at Southern Health, Southern Cross Pathology Australia, Monash Medical Center, Clayton. Free T3 estimation was done by using Beckman Coulter, Inc., Access Immunoassay Systems FreeT3 Reagent Kit (reference A13893B; Beckman Coulter Australia Pty Ltd., Lane Cove, NSW 2066, Australia) package insert. Free T4 concentration in plasma was estimated using Beckman Coulter Insert 386902B Access Free T4 (Access/DXI Free T4 Reagent Pack catalog number 33880 and Access/DXI Free T4 Calibrators catalog number 3388; Beckman Coulter Australia Pty Ltd., Lane Cove NSW 2066, Australia).

Statistical Analysis

Data was analyzed using the REML variance component analysis procedure for Genstat (GenStat release 13.1; VSN International Ltd., Hemel Hempstead, UK). Fixed model effects were temperature (HS and TN), diet (CTRL and Se + Vit E), time (effect of hours was tested for physiological parameters), and day (effect of day was tested for physiological parameters, SOD, and GSH-Px activity). The random model effects were sheep and period, keeping in mind that some limitations of crossover design in thermal biology, carryover effects, and interaction between heat treatment and period were tested and there were none. Plasma cortisol concentrations were log-transformed before analyses because of heterogeneity in variances. Multiple comparisons between the means were estimated by conducting the Bonferroni test. Results were reported as means and pooled standard error and means were considered to differ significantly when \( P \leq 0.001 \) or \( P \leq 0.05 \).

RESULTS

Climatic Conditions and Physiological Parameters

Under HS conditions the average value of THI was 34.1 between 0900 and 1700 h and was 25.0 between 1700 and 1900 h. During TN conditions the average THI was 19.0.

Respiration rate, rectal and skin temperature, and heart rate responses (pooled across days) to antioxidant supplementation and thermal treatment are presented in Fig. 1. Respiration rate increased in response to heat...
Antioxidants and heat stress in sheep

stress (89.1 vs. 161 breaths per minute for TN and HS conditions, respectively; \( P < 0.001 \)) and increased over the day before declining again (89.1, 143, 153, and 114 breaths per minute at 0900, 1300, 1700, and 2100 h, respectively; \( P < 0.001 \); Fig. 1a). However, there was an interaction \( (P < 0.001) \) between thermal (temperature) treatment and time such that the rectal temperature increased to a greater extent over the day in the sheep exposed to HS compared to those housed under TN conditions (Fig. 1a). While there was no main effect \( (P = 0.21) \) of antioxidant supplementation on respiration rate, there were interactions between dietary and thermal treatments \( (P = 0.017) \), thermal treatment and time \( (P = 0.001) \), dietary treatment and time \( (P = 0.003) \), and dietary and thermal treatments and time \( (P = 0.012) \) such that respiration rate increased to a lesser extent during the daily heat period in the sheep receiving antioxidants than in CTRL sheep (Fig. 1a).

Rectal temperature increased in response to heat stress (39.45 vs. 40.03°C for TN and HS conditions, respectively; \( P < 0.001 \)) and increased over the day before declining again (39.39, 39.92, 40.01, and 39.64°C at

![Figure 1. Relationships between a) respiration rate, b) rectal temperature, c) skin temperature, and d) heart rate and time of day in sheep fed a control (closed symbols) or a high antioxidant (open symbols) diet under thermoneutral (■ and □) or heat stress (▲ and ∆) conditions. The standard error of the difference for the interaction between thermal and dietary treatments and time is displayed on data for sheep fed the control diet and maintained under thermoneutral conditions. The \( P \)-values for the effects of diet, heat, time, heat × time and diet × time were 0.21, <0.001, <0.001, 0.001, and 0.017 for respiration rate, 0.58, <0.001, <0.001, <0.001, and 0.29 for rectal temperature, 0.97, <0.001, <0.001, <0.001, and 0.23 for skin temperature, and 0.018, 0.001, <0.001, 0.18, and 0.81 for heart rate, respectively. See text for other interactions.](image-url)
Chauhan et al.

Heart rate increased in response to heat stress (88.9 vs. 93.6 beats per minute for TN and HS condition, respectively; \( P = 0.001 \)) and increased over the day before declining again (79.3, 93.9, 96.4, and 90.5 beats per minute at 0900, 1300, 1700, and 2100 h, respectively; \( P < 0.001 \); Fig. 1d). However, there was an interaction \( (P < 0.001) \) between thermal treatment and time such that the rectal temperature increased to a greater extent over the day in the sheep exposed to HS compared to those housed under TN conditions (Fig. 2). There was no main effect \( (P = 0.058) \) of antioxidant supplementation or significant interaction \( (P = 0.29) \) between dietary and thermal treatments on rectal temperature. Similar effects were seen for skin temperature (Fig. 1c). Briefly, skin temperature increased in response to heat stress (38.16 vs. 39.09°C for TN and HS conditions, respectively; \( P < 0.001 \)) and increased over the day before declining again (38.13, 38.91, 38.99, and 38.48°C at 0900, 1300, 1700, and 2100 h, respectively; \( P < 0.001 \)). However, there was an interaction \( (P < 0.001) \) between thermal treatment and time such that the rectal temperature increased to a greater extent over the day in the sheep exposed to heat compared to those housed under TN conditions. There was no main effect \( (P = 0.97) \) of antioxidant supplementation or significant interaction \( (P = 0.23) \) between dietary and thermal treatments on rectal temperature.

Heart rate increased in response to heat stress (88.9 vs. 93.6 beats per minute for TN and HS condition, respectively; \( P = 0.001 \)) and increased over the day before declining again (79.3, 93.9, 96.4, and 90.5 beats per minute at 0900, 1300, 1700, and 2100 h, respectively; \( P < 0.001 \); Fig. 1d). However, there was an interaction \( (P = 0.018) \) between thermal treatment and time such that heart rate remained elevated at 2100 h in those sheep that had been exposed to heat but not in the sheep housed under TN conditions (Fig. 1d). Heart rate was reduced by antioxidant supplementation (93.5 vs. 86.5 beats per minute for CTRL and supplemented sheep, respectively; \( P = 0.018 \)) regardless of thermal treatment indicated by the lack of an interaction \( (P = 0.81) \) between dietary and thermal treatments (Fig. 1d).

Average daily feed intake (as-fed basis) was decreased during heat stress (772 vs. 718 g/d for TN and HS condition, respectively; \( P = 0.006 \); Table 1). Although there was no main effect \( (P = 0.27) \) of diet on feed intake, there was an interaction \( (P = 0.014) \) between thermal and dietary treatments such that HS decreased feed intake in sheep fed the CTRL diet (779 vs. 680 g/d) but not in the sheep supplemented with antioxidants (764 vs. 756 g/d; Table 1). There was no significant \( (P = 0.25) \) effect of diet on ADG, although the sheep supplemented with high levels of antioxidants had greater (132 vs. 67 g) ADG as compared to sheep fed control CTRL diets. There was no effect of temperature \( (P = 0.89) \) on ADG and also no interaction \( (P = 0.88) \) was observed between thermal and dietary treatments (Table 1).

### Biomarkers of Oxidative Stress and Endocrine Parameters

Plasma ROM tended \( (P = 0.097) \) to be increased during HS and was decreased \( (P = 0.046) \) by antioxidant supplementation (Table 1). However, there was a very strong interaction \( (P < 0.001) \) such that antioxidant supplementation reduced ROM during HS (9.12 vs. 6.83 Carr. U) but not under TN conditions (92.8 vs. 101 CARR U). There was no main effect \( (P = 0.21) \) of HS on BAP while BAP was increased by dietary antioxidants (3,776 vs. 3,971 μmol/L; \( P = 0.021 \); Table 1). However, there was an indication \( (P = 0.070) \) such that HS decreased plasma BAP in sheep consuming the CTRL diet (3,863 vs. 3,688 μmol/L) whereas BAP remained high in sheep supplemented with antioxidants and exposed to heat (3,956 vs. 3,985 μmol/L; Table 1), although plasma OSI tended to be increased \( (P = 0.086) \) during HS and increased \( (P = 0.007) \) by antioxidants. However, there was a significant interaction \( (P < 0.001) \) such that HS increased OSI in sheep fed the CTRL diet (2.43 vs. 3.12 arbitrary units) but not in sheep supplemented with antioxidants and exposed to heat (2.58 vs. 2.16 arbitrary units; Table 1). Heat stress increased \( (P = 0.008) \) plasma AOPP, particularly in those sheep fed the CTRL diet as indicated by the interaction \( (P = 0.070 \); Table 1). There was no effect \( (P = 0.82) \) of HS on plasma Se while plasma Se was increased (109 vs. 152 μg/L) by antioxidant supplementation (Table 1). There were no effects of temperature \( (P = 0.82) \) or diet \( (P = 0.32) \) on plasma free T₃ and T₄ (Table 1).
While there were no main effects of thermal treatment (P = 0.74) or dietary antioxidants (P = 0.95) on erythrocyte SOD activity, there tended to be an effect of day (P = 0.10) and a highly significant 3-way interaction (P < 0.001) such that dietary antioxidants decreased SOD except in sheep chronically exposed to HS on d 7 (Table 2). Although there were no main effects of temperature (P = 0.34) or dietary antioxidants (P = 0.18) on packed cell Glutathione peroxidase (GSH-Px) activity, there was a highly significant effect of day (P < 0.001) and a trend for an interaction between temperature treatment and diet (P = 0.068) such that packed cell GSH activity increased with time in all sheep except those consuming the CTRL diet and maintained under TN conditions (Table 2). Plasma GSH activity was not altered (P = 0.65) during HS but was increased (2,448 vs. 2,926 units/L; P = 0.019) by dietary antioxidants (Table 2). Basal plasma cortisol concentrations (cortisol levels in the samples collected before injecting ACTH) tended to be decreased by dietary antioxidant supplementation (5.16 vs. 3.38 nmol/L; P = 0.072) but not altered by HS (4.74 vs. 3.89 nmol/L; P = 0.57; Table 1). Plasma cortisol concentrations were increased (P < 0.001) at 30 and 60 min after administration of ACTH (Fig. 2). While there was no main effect of temperature on plasma cortisol concentrations, the sheep fed the antioxidant supplemented diets had lower (P = 0.028) plasma cortisol concentrations. However, there were significant interactions between diet and temperature (P = 0.039) such that effect of diet was most pronounced under TN where the cortisol response to ACTH was lowered in the sheep supplemented with antioxidants (Fig. 2).

### DISCUSSION

Heat stress reduces the efficiency of animal production leading to multibillion dollar losses to global animal agriculture (Bernabucci et al., 2010). Suitable nutritional strategies and the modifications of the environment may help to reduce the considerable losses associated with reduced productivity, increased mortality, and lower reproductive efficiency due to heat stress (Collier et al., 1982, 2006; West, 1999). Some possible nutritional interventions have been discussed recently (Dunshea et al., 2013; Rhoads et al., 2013) and in this study we have confirmed that supranutritional doses of Vit E and Se can reverse some of the negative effects of heat stress in sheep by improving its oxidative status. Hence, we accepted the hypothesis that supranutritional antioxidant supplementation can reverse some of the negative effects of heat stress on oxidative status and improve some of the physiological responses to heat stress in sheep.

Our thermal treatment protocol resulted in marked hyperthermia in sheep exposed to HS and all the physiological parameters were elevated; however, most intriguing were the interactions between temperature and antioxidants. The general homeostatic responses to thermal stress in mammals include elevated respiration rate, panting, drooling of saliva, reduced heart rates, profuse sweating, and reduced feed intake (Silanikove, 1992). Respiration rates of sheep in this study were increased during HS in an effort to dissipate heat through the respiratory tract. Interestingly, combined supplementation of Vit E and Se reduced this increase in respiration rate, indicating a reduction in the severity of heat stress. The

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Thermoneutral</th>
<th>Heat stress</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed intake, kg/d</td>
<td>CTRL</td>
<td>Se + Vit E</td>
<td></td>
</tr>
<tr>
<td>ADG, g/d</td>
<td>770b</td>
<td>764b</td>
<td>0.014</td>
</tr>
<tr>
<td>ROM, mg hydrogen peroxide/dl</td>
<td>92.8bc</td>
<td>101b</td>
<td>0.001</td>
</tr>
<tr>
<td>BAP, mmol/L</td>
<td>3.46ab</td>
<td>3.96a</td>
<td>0.001</td>
</tr>
<tr>
<td>OSI, arbitrary units</td>
<td>2.43b</td>
<td>2.58a</td>
<td>0.001</td>
</tr>
<tr>
<td>AOPP, nmol/L</td>
<td>18.0b</td>
<td>18.4ab</td>
<td>0.001</td>
</tr>
<tr>
<td>Total Se, μg/L</td>
<td>109a</td>
<td>149b</td>
<td>0.001</td>
</tr>
<tr>
<td>Free T₃, Picomole/L</td>
<td>3.72a</td>
<td>3.85a</td>
<td>0.001</td>
</tr>
<tr>
<td>Cortisol, nmol/L</td>
<td>0.794</td>
<td>0.514</td>
<td>0.001</td>
</tr>
</tbody>
</table>

a-c Within a row means without common superscript differ (P < 0.05).
1ROM = reactive oxygen metabolites; BAP = biological antioxidant potential; OSI = oxidative stress index; AOPP = advanced oxidation protein products.
2CTRL = control; control diet contained recommended levels of Vit E and Se: 10 IU Vit E/kg DM and 0.24 mg Se/kg DM.
3High Vit E and high Se diet (100 IU Vit E/kg DM and 1.20 mg Se/kg DM).
4Temp = temperature (thermoneutral and heat stress).
5Cortisol data were transformed before analyses (y = log₁₀x). Values in parentheses are back-transformed means.
The greatest difference between treatments in respiration rates was observed at 1300 and 1700 h while the ambient temperature in the HS room was 38 to 40°C.

Rectal temperature is another important indicator of thermal balance and may be used to assess the adversity of thermal environment. A rise of 1°C or less in rectal temperature is enough to reduce performance in most livestock species, which makes it a sensitive indicator of physiological response to heat stress (McDowell et al., 1976; West, 1999; Vitali et al., 2009; Bernabucci et al., 2010; Nardone et al., 2010; Berman, 2011) as it is nearly constant under normal conditions (Silanikove, 2000). We also observed an increase in the rectal temperature of sheep in the HS room by 0.6°C as compared to those under TN conditions. The increase in respiration rate and rectal temperature of sheep observed in this study is consistent with the findings in previous studies in Merino sheep (Srikandakumar et al., 2003; Alhidary et al., 2012) and Sardinian ewes (Bernabucci et al., 2009). All of these authors reported the increase in the respiration rate and rectal temperature of sheep subjected to hot conditions. Skin temperature is another physiological parameter that can be used for heat stress assessment as the heat exchange between the body and environment is achieved through the skin. The adjustment of skin blood flow to regulate transfer of heat from body core to the skin results in a shift in skin temperature in response to elevated temperatures (Habeeb et al., 1992). In the present study, there was no effect of dietary antioxidants on rectal or skin temperature during HS despite the improvement in respiration rate, possibly because the sheep supplemented with antioxidants consumed 13% more feed than those consuming the CTRL diet. Previously, dietary antioxidant supplementation was shown to reduce rectal temperature and respiration rate in goats under heat stress where there was a small reduction (approximately –8%) in feed intake in response to heat stress although not as great as in those not supplemented with antioxidants (–15%; Sivakumar et al., 2010). The effects of heat stress on the heart rate has not been reported so far in sheep; however, it was expected to rise in response to the increasing temperature in the HS room in an effort to increase the blood flow towards the extremity to dissipate heat from the body as was indicated by the increased skin temperature. There was no effect of day, although significant effect of time was observed such that increase in the heart rate was recorded at 1300 h as compared to 0900 h irrespective of heat treatment. This increase in heart rate may be associated with increased metabolic activity, as the sheep were fed after the 0900 h (basal measurements) measurements of physiological parameters. Interestingly, the heart rate was reduced by the antioxidant supplementation irrespective of thermal treatment, which may be explained by the beneficial effects of Vit E on the cardiac autonomic nervous system as has been reported in rats and humans (Behrens et al., 1986; Manzella et al., 2001; Fahim et al., 2013).

In general, exposure of sheep to high ambient temperatures results in decline in feed intake along with the increased respiration rate and rectal temperature, compromising the efforts to dissipate heat (Marai et al., 2007). In this study, we found 13% decline in feed intake of CTRL group when exposed to HS. These data corroborate those presented by (Nardone et al., 1991), who also showed a decline of 13% in the concentrate intake of rams when exposed to 35°C in climatic chambers. More recently, Bernabucci et al. (2009) also reported a significant decline in dry matter in Sardinian ewes housed in a climatic chamber for 49 d under elevated THI (THI = 82.0 ± 2.5). However, Alhidary et al. (2012) reported even higher (22%) reduction in feed intake in Australian Merino wethers exposed to a maximum temperature of 38°C for 7 d. Interestingly, in the present study the Se + Vit E group maintained feed intake under HS conditions. This ameliorative effect of Vit E and Se may be attributed to their ability to neutralize ROS, possibly reducing the release of prostaglandins and cytokines, which are implicated in stimulating systemic responses, including decreased feed intake (Hadden, 1987; Bradford, 2012). Vitamin E is the major lipid soluble antioxidant present in all cellular membranes that protects against lipid peroxidation (Miller et
Antioxidants and heat stress in sheep

1.65 kg BW by d 3 and 7 of heat stress, respectively, in pigs, whereas the pair-fed pigs had lost 2.47 kg of BW by d 7. These results may be explained to some extent due to the changes in postabsorptive metabolism and lack of adipose tissue mobilization in heat stressed subjects as Baumgard and Rhoads (2012) had very nicely reviewed and indicated that heat stressed animals use novel homeorhetic mechanism to Yes reprioritize the metabolic and fuel selection strategies independent of nutrient intake.

Heat stress has been reported to affect the oxidative status of dairy cows (Bernabucci et al., 2002) and dairy goats (Di Trana et al., 2006). We also found an increase in plasma ROM levels in sheep exposed to HS; this increase was particularly so for the CTRL group, while the Se + Vit E group exhibited reduced plasma ROM during HS. Therefore, the results of the present study suggest that the current recommended levels of Vit E and Se are insufficient to prevent the increase in ROS production during heat stress. Oxidative status changed significantly when sheep were subjected to HS. In the CTRL group, there was an increase in plasma ROM concentrations, while plasma BAP decreased under HS conditions, possibly as a direct consequence of the increase in ROM. Indeed, changes in the components of antioxidant systems are often not the cause but the consequence of the oxidative stress induced by higher free radical activity (Celi, 2011). The observed decrease in plasma BAP concentrations in the CTRL group during HS might have contributed to the increase in plasma ROM concentrations, as seen in dairy goats (Di Trana et al., 2006). The metabolic pathways and the molecular mechanisms responsible for excessive ROM production or lowered antioxidant defenses under heat stress are still not known in ruminants; however, there are some reports in other species (poultry) indicating involvement of mitochondrial electron transport chain or terminal oxidation. For example, increased mitochondrial superoxide generation and oxidative damage to mitochondrial lipids and proteins have been reported in skeletal muscle of chickens during acute heat stress. It was further indicated that electron transport chain (terminal oxidation) in mitochondria may be responsible for excessive superoxide production due to downregulation of uncoupling proteins (Mujahid et al., 2007a, 2007b, 2007c).

The calculated OSI values clearly indicate that supplementation of Vit E and Se at higher doses reduced the oxidant challenge and improved the antioxidant capacity, thereby improving the oxidative status of heat-stressed sheep. This can be mainly due to the strong reduction of ROM by the antioxidants. Under normal physiological conditions the recommended levels of Vit E and Se are likely sufficient to scavenge the ROS produced during the normal metabolic activity of the body. However, when cells are exposed to high oxidant levels such as occurs during heat stress, the free oxidants can cross the antioxidant barrier and lead to oxidative damage (Lykkesfeldt and Svendsen, 2007). Our data indicate that oxidative damage, as assessed by an individual animal’s OSI (pro-oxidants/antioxidants), is indicative of their capacity to adapt to an environmental challenge. Indeed, when the OSI is low (pro-oxidants < antioxidants) it would be reasonable to expect improved health and well-being conditions for the individual animal that would result in better adaptation to environmental challenges. Our approach is justified by the use of assays that have already been shown to be associated with health status. For example, when oxidative stress was monitored in dairy cows by means of ROM and BAP, the information on oxidative stress level was more accurate when combining ROM and BAP data than when using them separately (Pedernera et al., 2010).

Erythrocyte SOD activity has been used as a biomarker for the oxidative stress in animals during heat stress (Bernabucci et al., 2002; Di Trana et al., 2006; Ferreira et al., 2009). For example, Bernabucci et al. (2002) found that erythrocyte SOD activity was increased in transition dairy cows exposed to moderate heat stress during summer compared with cows during spring. While there were no main effects of dietary or temperature treatments on SOD in the present study, there was a 3-way interaction between diet, temperature, and day such that SOD activity was increased during chronic (d 7) but not acute (d 1) HS in the Se + Vit E group. Glutathione peroxidase activity is the another biomarker of oxidative stress (Erisir et al., 2009; do Reo Leal et al., 2010) and also of Se status (Hafeman et al., 1974; Mahan et al., 1999; Kumar et al., 2009). As expected, there was a concurrent increase in erythrocyte and plasma GSH-Px activity in the Se + Vit E supplemented group, which further indicates the increased antioxidant defense in these animals. The reduced levels of ROM and increased activity of SOD and GSH-Px observed in the Se + Vit E supplemented group under HS conditions underpins the combined action of antioxidants micronutrients and antioxidant enzymes to maintain redox homeostasis. Consistent with the existing data (Hafeman et al., 1974; Kumar et al., 2009; Mahan et al., 1999), erythrocyte and plasma GSH-Px activity was increased in the Se + Vit E supplemented group in the present study; presumably due to the higher availability of Se, which is an integral component of GSH-
Px. Total blood Se concentration as well as erythrocyte GSH-Px activity in animals on control diets is indicative of normal Se status of the sheep (Koller, 1981) and the adaptive response to higher oxidant challenge. However, these concentrations were still insufficient to prevent the oxidative stress induced by heat stress further accentuating the requirement of higher levels of these micronutrients under heat stress.

Higher concentration of AOPP in the CTRL group suggests that these animals suffered oxidative stress and damage to proteins when exposed to heat stress. The observed elevation of AOPP is of particular interest as it is a marker of protein oxidation and is also considered to mediate proinflammatory responses (Celi, 2011). In dairy cows, AOPP concentrations are associated with embryonic losses and are considered an indicator of acute inflammation and oxidative stress (Celi et al., 2011). It has been observed that the concentration of AOPP increases in dairy cows when they are fed maize silage (Celi and Raadsma, 2010; Celi et al., 2012) and in growing dairy calves (Celi and Robinson, 2010) possibly due to lower levels of antioxidant in silage. The observed lower levels of AOPP in the Se + Vit E group under HS could be ascribed to the concomitant decrease in OSI (increase in BAP and decrease in ROM), suggesting that the supranutritional supplementation of Vit E and Se was able to reduce the oxidation of plasma protein and the consequent decrease in AOPP. This further indicates that higher ROM in heat stressed subjects may be the consequence of lower BAP and that in absence of sufficient antioxidant defenses, these uncontrolled ROM lead to damage to macromolecules such as proteins.

It is well recognized that thyroid hormones (T₃ and T₄) are reduced under heat stress in many species including sheep (Bertoni et al., 1991). Thyroid hormones are of the utmost importance in the heat adaptation process, allowing the adjustment of the metabolic rate in favor of body heat balance (Pereira et al., 2008). Thyroid hormone (free T₃ and free T₄) levels were decreased in heat stressed goats compared to those in TN conditions (Sivakumar et al., 2010) in the attempt to reduce metabolic rate and heat production (West, 1999). Selenium plays an important role in thyroid hormone production not only due to the association with the activity of peroxidases in synthesis of thyroid hormones but also due to the activity of selenoenzymes (selenoproteins), iodothyronine deiodinases (5 DI, and 5 DII) that are responsible for activation of T₃ from T₄ in various tissues (thyroid, liver, kidney, brain, and muscle; Hefnawy and Tortora-Perez, 2010; Rayman, 2012). We expected that increased oxidative stress may be responsible for lower Se status (as most of the Se will be diverted for higher expression of GSH-Px required for antioxidant function) under heat stress leading to reduced levels of thyroid hormones. The absence of any changes in free T₃ and free T₄ concentrations observed in this study may be due to that fact that our CTRL diet was not deficient in Se and therefore thyroid function was not compromised during HS; however, the Se + Vit E group were benefited by the supplementation and hence selectively increased GSH-Px activity rather than increasing thyroid hormones production, which would have increased metabolic heat production.

Basal plasma cortisol concentrations (although the estimation of basal cortisol level from 2 samples has limitations) and the response to exogenous ACTH were reduced by dietary antioxidant supplementation under TN conditions but this response was lost under HS. Higher plasma cortisol levels were reported in heat-stressed Malpura ewes and Black Bengal goats and were reduced by antioxidant (Vit E, Se, and vitamin C) supplementation (Sivakumar et al., 2010; Sejian et al., 2014); however, the exact mechanism by which antioxidants alter plasma cortisol concentration is still unknown. (Caroprese et al., 2012) did not find any significant effect of solar radiation on cortisol concentrations in ewes, although an effect of diet and interaction of diet × solar radiation was reported. In the present study, the ACTH challenge was conducted after 7 d of heat stress and, plausibly, cortisol response may have reached a plateau due to the chronic heat phase. Generally, release of cortisol hormone as a consequence of activation of hypothalamic–pituitary–adrenal axis is the main response of the animals to stressful conditions; however, under heat stress plasma cortisol level rises markedly in cattle under acute exposure and decreases under chronic phase (Habeeb et al., 1992; Silanikove, 2000; Ronchi et al., 2001). Decline in cortisol activity under chronic heat stress is considered a thermoregulatory protective action to reduce metabolic heat production (Ronchi et al., 2001) and indicates adaptation of the animal to the stress, whereas the increase in the cortisol concentration over the basal levels under chronic heat stress indicates that the animal became distressed (Silanikove, 2000).

Conclusion

Current study suggests that supranutritional levels of antioxidants (specifically Vit E and Se) are needed to alleviate the negative effects of heat stress on redox homeostasis in sheep. Supplementation of sheep diets with Vit E and Se at supranutritional levels successfully reduced heat-induced oxidative stress. The improved oxidative status resulted in the amelioration of the heat load in sheep as they exhibited lower respiration rates and maintained feed intake. The fact that combined supplementation of Vit E and Se at supranutritional levels improved oxidative status coupled with the maintenance of feed intake has important implications because of the current interest in developing a suitable nutritional strategy for the amelio-
ration of heat stress in sheep and other livestock. Further research is warranted to identify the mechanism responsible and the location of excessive ROS production in ruminants under heat stress and to delineate the potential roles of Vit E and Se individually as strategic nutritional interventions to mitigate heat stress.

LITERATURE CITED


