
Selenium and Vitamin E together improve intestinal epithelial barrier function and alleviate oxidative stress in heat stressed pigs

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Running title: Antioxidants protect intestinal barrier integrity in heat stressed pigs

Keywords: antioxidant, intestinal epithelial barrier integrity, heat stress

Word count: 5920 (including abstract, main text, and references)

References: 28

Subject area: GI and epithelial physiology

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1113/EP085746](https://doi.org/10.1113/EP085746).

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What is the central question of this study?

Oxidative stress may play a role in compromising intestinal epithelial barrier integrity in pigs subjected to heat stress, but it is unknown if an increase of dietary antioxidants (selenium and Vitamin E) could alleviate gut leakiness in heat stressed pigs.

What is the main finding and its importance?

Levels of dietary selenium (1.0 ppm) and Vitamin E (200 IU kg⁻¹), greater than usually recommended for pigs, reduced intestinal leakiness caused by heat stress. This finding suggests that oxidative stress plays a role in compromising intestinal epithelial barrier integrity in heat stressed pigs and also provides a nutritional strategy for mitigating these effects.

ABSTRACT

Heat stress compromises intestinal epithelial barrier integrity of mammals through mechanisms that may include oxidative stress. Our objective was to test whether dietary supplementation with antioxidants, selenium (Se) and Vitamin E (VE), protects intestinal epithelial barrier integrity in heat stressed pigs. Female growing pigs (n=48) were randomly assigned to four diets containing from 0.2 ppm Se and 17 IU kg⁻¹ VE (control, NRC recommended), to 1.0 ppm Se and 200 IU kg⁻¹ VE for 14 days. Six pigs from each dietary treatment were then exposed to either thermoneutral 20°C or heat stress (35 °C, 09.00-17.00/ 28°C, overnight) conditions for two days. Trans-epithelial electrical resistance (TER) and FITC-dextran (4 kDa; FD4) permeability were measured in isolated jejunum and ileum using Ussing chambers. Rectal temperature, respiration rate, and intestinal *HSP70* mRNA abundance increased (all $P < 0.001$), and respiratory alkalosis occurred, suggesting that pigs were heat stressed. Heat stress also increased FD4 permeability and decreased TER (both $P < 0.01$). These changes were associated with changes indicative of oxidative stress, a decreased glutathione peroxidase (GPX) activity and an increased glutathione disulfide (GSSG): glutathione (GSH) ratio (both $P < 0.05$). With increasing dosage of Se and VE, *GPX-2* mRNA ($P = 0.003$) and GPX activity ($P = 0.049$) linearly

increased, and the GSSG:GSH ratio linearly decreased ($P = 0.037$) and the impacts of heat stress on intestinal barrier function were reduced ($P < 0.05$ for both TER and FD4 permeability). In conclusion, an increase of dietary Se and VE mitigated heat stress impacts on intestinal barrier integrity, associated with a reduction in oxidative stress in pigs.

Abbreviations

FD4, fluorescein isothiocyanate–dextran 4k Da; GPX-2, glutathione peroxidase-2; GSH, glutathione; GSSG, glutathione disulphide; HIF-1 α , hypoxia induced factor-1 α ; HS; heat stress; HSP70, Heat shock protein 70; IL-8, interleukin-8; ROS, reactive oxygen species; RPL32, ribosome protein L32; Se, selenium; TER, trans-epithelial electrical resistance; TJ, tight junction; TN, thermoneutral; TNF- α , tumor necrosis factor- α ; VE, vitamin E;

1 INTRODUCTION

2 Heat stress compromises intestinal epithelial barrier integrity in pigs (Pearce *et al.*, 2012; Pearce *et al.*, 2013a; Pearce *et al.*, 2013b; Cui & Gu, 2015) and the mechanism may involve oxidative stress.
3
4 Blood is redistributed away from splanchnic tissue to peripheral tissue to maximize the radiant heat
5 dissipation when pigs are in a hot environment (Collin *et al.*, 2001), thus hyperthermia, hypoxia, and
6 even inflammation may occur in the gastrointestinal tract (Lambert *et al.*, 2002; Lambert, 2009; Zuhl
7 *et al.*, 2014), which can all trigger oxidative stress. Although dependent on the duration and intensity
8 of heat stress, markers of intestinal oxidative stress have been reported to increase in the intestine
9 of heat stressed rats (Hall *et al.*, 1999; Oliver *et al.*, 2012) and pigs (Pearce *et al.*, 2013b). Oxidative
10 stress can disrupt tight junctions (TJ) (Rao, 2008) and decrease viability of epithelial cells (Vergauwen
11 *et al.*, 2015). Therefore, oxidative stress may play a role in the etiology of the compromised
12 intestinal barrier integrity in the heat stressed pig, and dietary alleviation of oxidative stress may
13 protect against heat stressed induced intestinal barrier dysfunction.

14 Two common dietary antioxidants include selenium and vitamin E. Selenium (Se) forms
15 selenoprotein such as glutathione peroxidase which is the enzyme that catalyzes the reduction of
16 hydrogen peroxide to water. In this process, monomeric reduced glutathione (GSH) is oxidized to

17 form glutathione disulphide (GSSG). Vitamin E (VE) is a lipid soluble antioxidant capable of reducing
18 free radicals particularly lipid hydroperoxides. Selenium and VE synergistically participate in
19 neutralizing free radicals (Rooke *et al.*, 2004). The recent version of “Nutrient Requirements of
20 Swine” recommends 0.2 ppm Se and 11 IU kg⁻¹ VE as adequate levels for growing pigs (body weight
21 25-50 kg) in a normal physiological state (National Research Council, 2012). However, it is unknown
22 whether additional dietary Se and VE above recommended requirements are beneficial in
23 counteracting the heat stress impacts on the intestine. Therefore the objective of this experiment
24 was to evaluate effects of increasing the levels of dietary Se and VE as a nutritional strategy to
25 mitigate the heat stress impacts on intestinal barrier integrity in pigs. We hypothesize that increased
26 dietary Se and VE may protect intestinal barrier integrity from heat stress damage by alleviating
27 oxidative stress.

28 **METHODS**

29 **Ethical Approval**

30 All procedures were approved by the Animal Ethics Committee of the Faculty of Veterinary and
31 Agricultural Sciences, the University of Melbourne, Australia (protocol number: 1413093) and the
32 Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition, 2013) was
33 followed. Authors also acknowledge the ethical principle of Experimental Physiology, and the
34 experiment was conducted in compliance with the animal ethic checklist as detailed by Grundy
35 (2015)

36 **Animal origin, feeding and experimental design**

37 The experiments utilized 48 female growing pigs (Large White × Landrace, 20 ± 3 kg, mean ± SD) and
38 followed a 4 × 2 factorial design (n=6 per treatment). Pigs were assigned into one of the four dietary
39 treatments, with selenium yeast (Selplex[®], Alltech, KY, USA) and synthetic α-tocopherol (Rovimix[®]
40 E50, DSM Nutritional Products, MD, USA) added to diets. The control diet was formulated mainly
41 with wheat and canola meal, and it contained 14 MJ/kg digestible energy and 18.3% crude protein.
42 The control diet contained Se 0.2 ppm and 17 IU kg⁻¹ VE as recommended by National Research
43 Council (NRC 2012) as meeting the nominal requirements of this class of pig. The other three diets
44 contained increased concentrations of Se and VE to reach i) 0.3 ppm Se and 50 IU kg⁻¹ VE, ii) 0.5 ppm
45 Se and 100 IU kg⁻¹ Se, or iii) 1.0 ppm Se and 200 IU kg⁻¹ VE, respectively. After a 14-day
46 supplementation, six pigs from each dietary group were allocated to either 1) a climate-controlled

47 room set at 20°C, humidity 35% as the thermoneutral (TN) condition or 2) a heat stress (HS)
48 condition with a room set to 35°C, 9.00-17.00 h, and 28°C, rest of the day, humidity 35% for two
49 days. Pigs continued their respective diets during the two-day thermal exposure. Given heat stress
50 can reduce voluntary feed intake, and to eliminate the confounding effects of dissimilar feed intake
51 between TN and HS conditions on intestinal blood flow (Collin *et al.*, 2001) and barrier function
52 (Pearce *et al.*, 2013b), all pigs were pair-fed at a rate of 75% of *ad libitum* intake (2.5 times of
53 maintenance energy requirement) which is the predicted voluntary feed intake under this heat load
54 (Huynh *et al.*, 2005). Water was supplied via nipple drinkers *ad libitum*.

55 **Physiological monitoring**

56 Physiological responses to heat stress were assessed by quantifying each pig's respiration rate and
57 rectal temperature which were monitored daily at 9.00, 11.00, 13.00, 15.00 and 17.00 h each of the
58 two days thermal exposure. Researchers were blinded about dietary treatments to minimize bias on
59 physiological recordings. As a precaution, the pig was removed from the heat chamber if its rectal
60 temperature exceeded 41°C, and the pig was then rested under the thermoneutral condition until its
61 temperature was below 40°C. During the experiment only one pig was removed for 1 h due to
62 hyperthermia and then returned to the heat chamber without incident.

63 **Blood sampling, euthanasia, and tissue preparation**

64 At the end of the two-day climate challenge, pigs were sedated with intramuscular injection of
65 Ketamine (13 mg·kg⁻¹ body weight) and Xylazine (1.0 mg·kg⁻¹ body weight). After confirming the level
66 of anesthesia, by lack of response to a toe pinch and touching adjacent to the eye, pigs were killed
67 by cardiac injection of pentobarbitone sodium (162.5 mg kg⁻¹ body weight. Lethabarb, Virbac Animal
68 Health, Australia). Euthanasia was confirmed by an absence of breathing, heart rate (detected with a
69 stethoscope) and palpebral reflex. Thereafter, a midline abdominal incision was made to access the
70 GI tract, and a 5 mL blood sample was taken from the portal vein with heparinized vacutainers (BD
71 vacutainer® Australia) from each pig. Fresh blood was immediately loaded into an automatic blood
72 gas analyzer (EPOC®, Alere, US) for detecting blood gas and chemistry parameters: partial pressure of
73 CO₂ (P_{CO₂}), total CO₂ concentration (ctCO₂), partial pressure of O₂ (P_{O₂}), pH, bicarbonate, lactate, and
74 hematocrit percentage. Sections (20 cm) of proximal jejunum and distal ileum were collected and
75 fresh tissues were rinsed and transported in oxygenated PBS (0.15 M NaCl in 0.01 M sodium
76 phosphate buffer, pH 7.2) to Ussing chamber equipment for assessing mucosal integrity. Jejunum

77 and ileum were collected from the same site of each pig and snap frozen in liquid nitrogen then
78 stored at -80°C for biochemical and RNA analysis.

79 **Intestinal barrier integrity measurements**

80 Ussing chamber sliders (exposed area of 0.71 cm²) with fresh mucosa (muscle layer was removed)
81 were inserted into two-part chambers (EasyMount Diffusion Chambers, Physiologic Instruments, San
82 Diego, USA) that contained physiological saline (115 mM NaCl, 25 mM NaHCO₃, 2.4 mM K₂HPO₄, 1.2
83 mM CaCl₂, 1.2 mM MgCl₂, 0.4 mM KH₂PO₄, pH 7.4) at 37°C and gassed with carbogen (5% CO₂, 95% O₂).
84 Each chamber half contained 5 mL of the Krebs bicarbonate buffer, with the serosal bath having an
85 additional 10 mM glucose to provide an energy substrate and the mucosal bath containing an
86 additional 10 mM mannitol to maintain osmotic balance across the mucosa on the tissues. Each
87 chamber had a set of four electrodes (two voltage sensing and two current passing electrodes)
88 installed on opposite sides of the tissue and connected to the amplifier through agar bridges. A
89 Multichannel voltage-current clamp (Physiologic Instruments, model VCC MC6) was used to record
90 short circuit current. Tissues were allowed to equilibrate for 20-30 min in the chambers before
91 measurements were made. Trans-mucosal voltage was stepped to five graded levels under a voltage
92 clamp conditions and the corresponding currents were measured, TER was calculate by Ohm's law
93 and multiplied the exposed area.

94 The paracellular probe, fluorescently labeled dextran (FITC-dextran; molecular mass = 4kDa, FD4, 1
95 mg mL⁻¹; Sigma-Aldrich, St Louis, MO, USA), was added to the mucosal compartment. Subsequently
96 200 µL of solutions was collected from the both sides of the tissue sheet at 1, 30 and 60 min for
97 quantifying mucosal FD4 permeability. The FD4 fluorecence was measured using a fluorecence
98 reader (FlexStation II; Molecular Devices, Sunnyvale, CA, USA). The apparent permeability
99 co-efficient (PaPP) of FD4 was calculated by the following equation given by Pearce *et al.* (2013a):
100 $PaPP = dQ / (dt \times A \times C_0)$, where dQ/dt = transport rate (mg/min); A = area of the membrane (cm²); C_0
101 = initial concentration in the donor chamber (mg/cm³). Carbachol (100µm; Sigma, Sydney, Australia)
102 was added at the end of the experiment to check tissue viability. Values of TER and FD4 permeability
103 of jejunum and ileum were obtained in duplicates for each pig.

104 **Oxidative stress biomarkers**

105 Frozen jejunum and ileum samples were pulverized in liquid nitrogen and homogenized in Tris-HCL
106 buffer (pH 7.5), then supernatants were collected after centrifuging (1000 g, 15 min, 4°C) for
107 measuring glutathione peroxidase activity (GPX), reduced glutathione (GSH), and oxidized
108 glutathione (GSSG) concentration by commercial kits (Cayman, USA). Antioxidant enzyme activities
109 and glutathione concentrations were normalized in relation to the total protein concentration
110 (Pierce™ BCA kit, Thermo Fisher) of the tissue homogenates.

111 **Gene abundance**

112 Total RNA was isolated from 0.2 g pulverized jejunum and ileum tissue according to the
113 manufactures manual (Purelink™, Life Technologies). The concentration and quality of total RNA
114 were verified using an Experion RNA analysis kit (Bio-Rad Laboratories, Inc.). RNA was stored at -80°C
115 until reverse transcription. The total RNA (0.8 µg) was reverse transcribed using SuperScript™ III
116 First-Strand Synthesis (Invitrogen™, Life Technologies) and the synthesized single-strand cDNA was
117 stored at -20°C until used for Q-PCR. Sequences of primer sets for swine ribosome protein L32
118 (*RPL32*), hypoxia induced factor-1α (*HIF-1α*), interleukin-8 (IL-8), and tumor necrosis factor-α (*TNF-α*)
119 were obtained from Pearce *et al.* (2013b), Heat shock protein 70 (*HSP70*) was referenced from
120 Chauhan *et al.* (2014). Sequences of primers can be found in supporting information **Table S1**. A
121 total volume of 25 µL reaction mix was prepared based on the manufacturer's instruction using
122 SYBR® GreenER™ qPCR Supermix Universal (Invitrogen™, Life Technologies); 100 nM of each forward
123 and reverse primer were applied in each reaction. Each sample was run in triplicate and fluorescence
124 was quantified in iQ5 Real Time PCR Detection System (Bio-Rad Laboratories, Inc.). Each sample
125 plate included a standard curve (five 10-fold dilutions of a pooled cDNA), a non-template negative
126 control, and a blank to determine amplification efficiency of the respective primer pair. The
127 abundances of the mRNA were normalized to *RPL32* according the method described by Livak and
128 Schmittgen (2001).

129 **Statistics**

130 Data were analyzed by ANOVA using Genstat 16th Version (VSNi Ltd, UK). For physiological
131 parameters, treatment factors included "temperature", "diet", "time" and "day". For TER, FD4
132 permeability, blood gas parameters, OS indices and mRNA fold changes, effects of "temperature",
133 "diet" and "intestinal site (jejunum or ileum)" were analyzed. Linear and quadratic response of the
134 Se and VE dosages (defined as 1, 2, 4, 8 for the four antioxidant dosages) of "diet" were in both

135 statistical models. Duncan multiple range test was used for multiple comparison *post-hoc* when the
136 effects of heat stress and antioxidant are both significant. Data were expressed as mean \pm
137 standard error of the mean (SEM). $P \leq 0.05$ was considered significant and $P \leq 0.10$ was considered
138 as a trend. Statistical analysis showed that although the baseline parameters were different between
139 the jejunum and ileum, the interaction of temperature \times diet \times site (jejunum or ileum) was not
140 significant for TER, dextran permeability, *GPX* mRNA, *GPX* activity, or GSSG:GSH ratio, so the data
141 were pooled across the intestinal sites. The separate data for jejunum and ileum are provided in the
142 supplementary data.

143

144 RESULTS

145 Physiological parameters and feed intake

146 As expected, compared to the thermoneutral pigs, heat stress increased the respiration rate from 25
147 to 158 breaths \cdot min⁻¹ ($P < 0.001$). There was an interaction between temperature and time of heat
148 stress ($P < 0.001$, **Fig. 1 (A)**), such that thermoneutral pigs had stable respiration rate, and by
149 comparison, the respiration rate of the heat stressed pigs increased dramatically from 54 to 200
150 breaths \cdot min⁻¹ between 09.00 and 13.00 h and was then maintained around this rate until the end of
151 thermal exposure time. Dietary antioxidants did not affect respiration rate (Diet (linear), $P=0.95$; Diet
152 (quadratic), $P = 0.66$), and there was no interaction with temperature (Temperature \times Diet (linear), P
153 = 0.72; Temperature \times Diet (quadratic), $P = 0.52$).

154 Heat stress increased rectal temperature from 38.3 °C to 39.6 °C ($P < 0.001$). There was an
155 interaction between temperature and time of heat stress ($P < 0.001$, **Fig. 1 (B)**), such that the pigs in
156 thermoneutral conditions exhibited a small change of rectal temperature over time (38.2 °C at 11.00
157 h, 38.3 °C at 13.00 h, 38.5 °C at 17.00 h), whereas the heat stressed pigs markedly increased rectal
158 temperature from 38.5°C to 39.2°C from 9.00 h to 11.00 h and reached a plateau of 40.0 °C at 13.00
159 h which was maintained until 17.00 h, as evidenced by an interaction between time and
160 temperature ($P < 0.001$). There was no dietary effect of on rectal temperature, and neither were
161 interactions of temperature and diet significant (Temperature \times Diet (linear), $P = 0.96$; Temperature
162 \times Diet (quadratic), $P = 0.66$).

163 The design of this experiment was to pair feed the pigs to remove any confounding effect of reduced
164 feed intake due to HS. The feed intake averaged 1.10 kg/d and was not influenced by temperature
165 and dietary antioxidants supplementation (Temperature, $P = 0.57$; Diet (linear), $P = 0.60$; Diet
166 (quadratic) = 0.22; Temperature \times Diet (linear) = 0.96; Temperature \times Diet (quadratic) = 0.70).
167 Therefore pigs under heat stress and thermoneutral conditions received an equal dose of
168 antioxidants, and the impacts of dissimilar feed intake on intestinal barrier function were avoided.

169 **Blood gas parameters**

170 In portal venous blood, heat stress decreased total CO_2 (40.8 vs 37.4 mM, $P = 0.025$) and bicarbonate
171 (39.8 vs 35.5 mM, $P < 0.001$). P_{CO_2} , P_{O_2} , pH, lactate and hematocrit were not affected by heat stress.
172 Selenium and VE supplementation linearly increased bicarbonate (36.7, 37.3, 37.3 and 38.8 mM)
173 going from the low to high doses (Diet (linear), $P = 0.041$; Diet (quadratic), $P = 0.68$). The heat
174 stressed pigs fed on Se 0.3 ppm + VE 50 IU kg^{-1} and Se 1.0 ppm + VE 200 IU kg^{-1} had similar
175 concentrations of blood bicarbonate compared with the pigs housed under thermoneutral
176 conditions. Other blood gas indices in the portal vein blood were not affected by antioxidant
177 supplements (**Table 1**).

178 **Intestinal epithelial barrier integrity**

179 Heat stress reduced TER from 60.4 to 46.7 $\Omega \text{ cm}^2$ ($P = 0.009$). Selenium and VE supplementation
180 linearly increased TER with increasing dosages (45.9, 51.5, 56.1 and 62.4 $\Omega \text{ cm}^2$ for the dosages from
181 low to high; Diet (linear), $P = 0.016$; Diet (quadratic), $P = 0.56$), and the pigs fed on the highest
182 dosage of Se and VE had higher TER than those on the control diet (62.4 vs 45.9 $\Omega \text{ cm}^2$, $P < 0.05$). As
183 **Fig. 2 (A)** shows, Se and VE supplementation exhibited a similar effect in increasing TER under
184 thermoneutral and heat stress conditions, as the interaction between temperature and dietary
185 treatment was not significant (Temperature \times Diet (linear), $P = 0.56$; Temperature \times Diet (quadratic),
186 $P = 0.37$). The TER of the highest dosage of Se and VE group was higher than control diet within heat
187 stress condition (56.7 vs 36.6 $\Omega \text{ cm}^2$, $P < 0.05$). Jejunum had lower TER than ileum (41.0 vs 67.0 Ω
188 cm^2 , $P < 0.001$), as shown in **Fig 2 (B)**.

189 Basal short circuit current was greater in small intestine samples from heat stressed pigs. This
190 tended to be resolved by the higher doses of Se and VE, but the changes did not reach statistical
191 significance (see supplementary **Fig. S1**).

192 Compared with thermoneutral control, intestinal permeability to FD4 was elevated by heat stress
193 from 13.8 to 18.9 $\text{cm min}^{-1} \times 10^{-3}$ ($P = 0.005$). Selenium and Vitamin E supplementation decreased FD4
194 permeability linearly and quadratically with the increasing dosages (23.2, 15.9, 12.9 and 13.4 cm
195 $\text{min}^{-1} \times 10^{-3}$ for from low to high Se and VE dosages; Diet (linear), $P = 0.002$; Diet (quadratic), $P =$
196 0.016). As **Fig. 3 (A)** illustrated, the trend for antioxidants supplementation to decrease FD4
197 permeability was similar in both environmental conditions because the interactions of temperature
198 and diet were not significant (Temperature \times Diet (linear), $P = 0.35$; Temperature \times Diet (quadratic),
199 $P = 0.92$). Within the control diet treatment, heat stressed pigs had higher FD4 permeability than
200 thermoneutral pigs (13 vs 30 $\text{cm min}^{-1} \times 10^{-3}$, $P < 0.05$). The FD4 permeability of the second highest
201 dosage of antioxidants was lower than control diet during thermoneutral condition ($P < 0.05$), and
202 the pigs fed on two highest level of Se and VE had lower permeability than control fed pigs during
203 heat stress (both $P < 0.05$). As shown in **Fig. 3 (B)**, jejunum had a larger FD4 permeability than ileum
204 (19.8 vs 13.0 $\text{cm min}^{-1} \times 10^{-3}$, $P = 0.007$).

205 A statistical data set for showing the barrier integrity in jejunum and ileum separately can be found
206 in supporting information **Table S2**.

207 **GPX mRNA, activity, and GSSG:GSH ratio**

208 Glutathione peroxidase-2 mRNA abundance tended to be reduced by heat stress from 1.58 to 1.22
209 ($P = 0.071$), however, Se plus VE linearly increased *GPX-2* mRNA abundance along with increased
210 dosages (0.97, 1.35, 1.50, 1.78; Diet (linear), $P = 0.007$; Diet (quadratic), $P = 0.39$). As **Fig 4 (A)** shows,
211 the highest dosage of Se and VE had higher *GPX-2* mRNA abundance than control diet ($P < 0.05$)
212 during heat stress condition, whereas *GPX-2* abundance did not differ among treatments during
213 thermoneutral condition. **Fig 4 (B)** shows that *GPX-2* mRNA abundance was similar between ileum
214 and jejunum.

215 Glutathione peroxidase activity was reduced by heat stress from 111 to 91 $\text{unit mg}^{-1} \text{protein}$ ($P =$
216 0.004), conversely, GPX activity was linearly enhanced with increasing dosages of dietary
217 antioxidants (90.3, 100.0, 103.5, and 109.9 $\text{unit mg}^{-1} \text{protein}$; Diet (linear), $P = 0.049$; Diet
218 (quadratic), $P = 0.49$). As **Fig. 5 (A)** demonstrated, the trend of dietary antioxidants in improving GPX
219 activity was not influenced by ambient temperature (Temperature \times Diet (linear), $P = 0.54$;
220 Temperature \times Diet (quadratic), $P = 0.74$). The highest Se and VE group had higher GPX activity than
221 control diet (104.2 vs 78.4 $\text{unit mg}^{-1} \text{protein}$, $P < 0.05$) during heat stress condition, whereas the GPX

222 activity remained similar among dietary treatments during thermoneutral condition. Ileum had
223 higher GPX activity than jejunum (120 vs 82 unit mg⁻¹ protein, P < 0.001), as shown in **Fig 5 (B)**.

224 The ratio of GSSG: GSH was increased by heat stress from 0.026 to 0.037 (P < 0.001). Selenium and
225 VE supplementation linearly decreased GSSG:GSH ratio with the increasing dosages (0.034, 0.034,
226 0.035 and 0.022; Diet (linear) P = 0.005; Diet (quadratic) P = 0.39). As **Fig. 6 (A)** shows, the dietary
227 antioxidants reduced GSSG:GSH ratio regardless of ambient temperature (Temperature × Diet
228 (linear), P = 0.25; Temperature × Diet (quadratic), P = 0.24). Only within control diet, heat stress pigs
229 had a higher GSSG:GSH ratio than thermoneutral pigs (P < 0.05). As **Fig. 6 (B)** shows, jejunum had
230 higher GSSG:GSH ratio than ileum (P < 0.001).

231 A statistical data set for showing the oxidative stress biomarkers in jejunum and ileum separately
232 can be found in supporting information **Table S2**.

233 ***Stress gene abundance***

234 Heat stress greatly increased *HSP70* mRNA abundance (0.86 vs 3.56, P < 0.001), but antioxidant
235 supplementation did not significantly decrease *HSP70* mRNA abundance (P = 0.13). However, heat
236 stress and antioxidants did not alter *HIF-1α*, *IL-8*, or *TNF-α* mRNA expression (**Table 2**). A statistical
237 data set for showing the expression of stress genes in jejunum and ileum separately can be found in
238 supporting information (**Table S3**).

239

240 **DISCUSSION**

241 In this study we used two measures to assess gut leakiness following heat stress, the *ex vivo*
242 trans-epithelial electrical resistance (TER) and FD4 flux. We related this to the oxidative stress in the
243 tissue, assessed by the ratio of oxidized to reduced glutathione (GSSG:GSH ratio), supplemented by
244 functional measurement of glutathione peroxidase (GPX) activity and measurement of *GPX-2* mRNA
245 abundance. Glutathione peroxidase is important for the removal of free radicals that accumulate
246 during oxidative stress, the major form associated with the intestinal mucosa being GPX-2 (Brigelius
247 Flohe *et al.*, 2012). During oxidative stress, intracellular reduced GSH is depleted and its oxidized
248 form (GSSG) increases (Sentellas *et al.*, 2014). This is reflected in our study, in which the GSSG:GSH
249 ratio in the intestine of heat stressed pigs on the normal recommended diet (Se 0.2 ppm, VE 17 IU

250 kg⁻¹) was almost twice that of pigs kept in thermoneutral conditions on the same diet. This incapacity
251 of the intestine to manage oxidative stress was accompanied by a lower *GPX-2* mRNA abundance
252 and a reduced GPX activity in heat exposed compared to non-exposed pigs. Other studies also point
253 to hyperthermia causing oxidative stress, which has been reported to increase oxidized lipids (Pearce
254 *et al.*, 2013b) and proteins (Oliver *et al.*, 2012) in the small intestine. Under the normal
255 recommended diet (Se 0.2 ppm, VE 17 IU kg⁻¹), the intestinal mucosal barrier was also compromised,
256 the trans-epithelial electrical resistance was 34% less and permeability to FD4 was 57% greater in
257 heat stressed compared to thermoneutral pigs.

258 In heat stressed pigs, there was a dose-dependent linear reduction in the GSSG:GSH ratio as Se was
259 increased to 1.0 ppm and VE was increased to 200 IU kg⁻¹. At this dose, the GSSG:GSH ratio in the
260 mucosa of heat stressed pigs was similar to the ratio in thermoneutral pigs on a normal Se/ VE diet.
261 Thus this dose appears to reverse the oxidative stress. At the same high dose, *GPX-2* mRNA
262 abundance was similar in thermoneutral and heat stressed pigs, and about 1.6 times the level in
263 intestine from thermoneutral pigs on the normal Se/ VE diet. GPX activity was also greater with the
264 highest Se/ VE dose, but to a lesser degree than the gene abundance difference. Thus there is a
265 direct, but non-linear, relation between increased gene abundance, increased enzyme activity, and
266 lowered oxidative stress as measured by the GSSG:GSH ratio.

267 Accompanying the reduced oxidative stress, there was a 61% greater TER, that is, reduced gut
268 leakiness, in the heat stressed pig fed on highest versus the lowest dose of Se plus VE. However,
269 there was only a very small positive difference between the lowest and highest dose in the intestines
270 of pigs kept under thermoneutral conditions, suggesting that Se plus VE does not directly affect the
271 TER when there is no strong indication of oxidative stress. We therefore conclude that it is the
272 reduction of oxidative stress that protects the mucosa. Similarly 1.0 ppm Se and 300 mg kg⁻¹
273 alleviated the increase of epithelial conductance in heat stressed rats (Maseko *et al.*, 2014). On the
274 other hand, the permeability of the mucosa to FD4 was reduced by 53% in heat stressed pigs when
275 Se plus VE was increased from the lowest to the highest dose, and by 44% in pigs kept under
276 thermoneutral conditions. This suggests that Se plus VE may reduce the movement of large
277 molecules across the mucosa even in under thermoneutral conditions. Under thermoneutral
278 conditions, the Se/ VE supplementation tended to increase transepithelial resistance and increase
279 *GPX-2* activity. The effects in thermoneutral conditions are probably a reflection of the intestinal
280 lining always being exposed to micro-organism and potentially injurious substances and thus always

281 being in a state of mild inflammation and immune vigilance (Artis, 2008; Furness *et al*, 2013). Thus,
282 the results suggest that dietary supplementation may improve the barrier function of the intestine
283 even in conditions of mild threat. Pig feed is normally supplemented with both Se and VE because
284 these antioxidants work effectively together. Whether one or other might contribute more under
285 conditions of heat stress is not known.

286 The present results are consistent with other studies that have reported association between
287 oxidative stress, hyperthermia and loss of intestinal barrier function (Lambert, 2009; Pearce *et al.*,
288 2013a; Pearce *et al.*, 2013b). A factor that could link heat stress to reduced intestinal barrier
289 function is inflammation. However, we did not find any increases in *IL-8* or *TNF- α* mRNA abundance,
290 which might suggest that inflammation was not a significant factor. However, we did not look for
291 histopathological changes or a greater range of cytokines, which might have revealed changes.
292 Moreover, gut leakiness might lead to a systemic inflammatory response (Pearce *et al.*, 2013b),
293 which was not investigated. Previous studies have also found that heat stress did not increase
294 inflammatory markers in the intestine of pigs (Pearce *et al.*, 2013b; Gabler & Pearce, 2015). The
295 rectal temperature was increased by 1.3 °C in the heat stressed pigs and *HSP70* mRNA abundance
296 was up-regulated as reported in previous studies (Yu *et al.*, 2010; Cui & Gu, 2015), indicating a
297 generalized stress to the intestine.

298 We observed that the jejunum had a higher FD4 permeability and lower TER than the ileum. This
299 difference of barrier integrity may be associated with their different oxidative conditions; for
300 example, we found that the jejunum has lower GPX activity and higher GSSG:GSH ratio compared
301 with ileum. Similarly, Degroote *et al.* (2012) found the mucosa of the proximal small intestine has a
302 2-fold higher GSSG:GSH ratio than the distal small intestine in weaning piglets, although in contrast
303 to the present results they found that the GPX activity of proximal small intestine was higher.

304 In addition to our main objective of this study, we found heat stress triggered respiratory alkalosis
305 which affected acid-base balance in portal vein blood. Respiratory alkalosis was caused by the loss of
306 blood CO₂ concentration due to the increased respiration rate in heat stressed pigs, and the blood
307 bicarbonate decreased corresponding to reduced formation of carbonic acids, so that blood pH
308 could be maintained. The whole process is a typical respiratory alkalosis which was compensated by
309 metabolic acidosis. It is unknown if the compensated respiratory alkalosis had any impacts on
310 intestinal barrier function because of limited literature. Interestingly, 0.3 ppm Se + 50 IU kg⁻¹ VE and
311 1.0 ppm Se + 200 IU kg⁻¹ VE prevented the drop of bicarbonate in response to respiratory alkalosis,

312 the effects possibly due to effect of VE, because our previous study showed VE (200 IU kg⁻¹) but not
313 Se (1.0 ppm) prevented a decrease in bicarbonate in heat stressed pigs (Liu *et al.*, 2014). However,
314 the mechanism remains unknown. Besides, hematocrit was unaffected in current experiment,
315 although the pigs had free access to water. We conclude that there was not a significant dehydration
316 or sufficient excess water intake to have significantly influenced hematocrit.

317 In conclusion, heat stress augmented intestinal oxidative stress and decreased barrier integrity. High
318 levels of dietary Se and VE reduced both oxidative stress and leakiness. It is suggested that elevating
319 Se and VE in pig diets could mitigate against deleterious effects of hot weather on the gut.

320 **Acknowledgement:**

321 The authors express appreciations to Maree Cox, Evan Bittner, Kristy DiGiacomo, Surinder Chauhan,
322 Ashley Gabler, Yaowei Shen, Shannon Holbrook, Paul Eason, Crina Guimaraes De Souza Melo, and
323 Hyun Jung Cho for their technical assistance.

324 **Competing interests:**

325 All authors, no conflicts of interest.

326 **Author contributions:**

327 FL, JJC, LRR, FWK, DW, JBF, PC, BJL, DMB, NKG and FRD developed the conception and designed the
328 experiment. FL, FWK, DW conducted daily feeding, physiological monitoring in live animal phase. JJC,
329 FWK, DW, RVP, JBF, PC participated in blood and tissue samplings. FL, LRR, RVP, LJF quantified
330 intestinal barrier integrity in Ussing chamber. FL, JJC and PC conducted lab analysis. FL, FRD, JJC, JBF
331 participated in the statistical analysis. All the authors participated in drafting or revising the
332 manuscript. The final version of the manuscript was approved by all authors

333 **Funding Support:**

334 Research was primarily funded by the Commonwealth Department of Agriculture and Water
335 Resources Carbon Farming Futures Program (194374-167); work in Professor Furness's laboratory
336 was supported by InVivo Animal Nutrition & Health, through its subsidiary, Pancosma SA; FL received
337 Australian Postgraduate Award, International Postgraduate Research Scholarship, and a study award
338 from Australian Pork Limited; LRR was supported by an NHMRC Peter Doherty Fellowship.

339

340 **Supporting information**

341 Table S1. Primers for Q-PCR

342 Table S2. Barrier function and oxidative stress markers for jejunum and ileum. (statistical data for
343 Fig. 2, Fig. 3, Fig. 4, Fig. 5 and Fig. 6)

344 Table S3. mRNA abundance of stress and inflammatory genes for jejunum and ileum (statistical data
345 for Table 2)

346 Fig. S1. Short circuit currents of the intestines of pigs that were fed with different levels of selenium
347 and Vitamin E and subjected to a 20 °C ambient environment or 35°C.

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418

419 **Tables**

420 Table 1. Blood gas and acid balance in portal vein blood

	20°C				35°C				SEM	T ¹
Selenium (ppm)	0.2	0.3	0.5	1.0	0.2	0.3	0.5	1.0		
Vitamin E (IU kg ⁻¹)	17	50	100	200	17	50	100	200		
P _{CO2} (mmHg)	66.9	61.0	69.7	58.0	68.1	56.2	60.3	61.6	6.55	0.6
ctCO ₂ (mM)	41.2	40.3	42.9	38.6	36.4	37.9	37.0	38.4	1.87	0.01
P _{O2} (mmHg)	27.7	27.8	23.0	29.6	25.4	31.8	28.3	31.2	3.25	0.3
HCO ₃ (mM)	39.2 ^{bc}	38.5 ^{bc}	40.7 ^c	40.9 ^c	34.3 ^a	36.2 ^{ab}	35.1 ^a	36.6 ^{ab}	1.76	<0.0
pH	7.38	7.41	7.39	7.43	7.32	7.43	7.38	7.40	0.040	0.4
Lactate (mM)	4.03	2.30	3.40	3.39	3.30	2.23	3.01	2.79	0.692	0.3
Hematocrit (%)	30.8	32.2	29.8	31.5	29.7	28.8	29.3	29.3	1.90	0.1

421 ^{a b c} Within a row means without common superscript differ (P < 0.05).

422 ¹Temperature;

423 ²Linear effects of diet;

424 ³Quadratic effects of diet

425

426

427 Table 2. mRNA expression of stress and inflammatory genes

	20°C				35°C				SE M	P-values				
	0.2	0.3	0.5	1.0	0.2	0.3	0.5	1.0		T ¹	D ² (L) ²	D ³ (Q) ³	T× D	T× Q
Selenium (ppm)	0.2	0.3	0.5	1.0	0.2	0.3	0.5	1.0						
Vitamin E (IU kg ⁻¹)	17	50	10 0	20 0	17	50	10 0	20 0						
<i>HSP70</i> fold change	1.20	0.9 0	0.7 7	0.5 5	4.4 0	4.4 0	3.2 5	2.1 8	1.0 2	<0.0 01	0.1 3	0.8 6	0.3 5	0.9 9
<i>HIF-1α</i> fold change	1.04	1.3 7	1.3 7	1.2 8	1.0 2	1.1 4	1.1 4	1.2 1	0.1 3	0.13	0.2 5	0.1 7	0.9 3	0.3 4
<i>IL-8</i> fold change	1.32	1.2 4	1.2 5	1.2 1	1.0 7	1.0 1	1.0 5	0.9 9	0.2 7	0.21	0.8 2	0.9 8	0.9 3	0.6 8
<i>TNF-α</i> fold change	1.56	1.2 9	1.3 9	1.4 5	1.2 4	1.2 4	1.2 0	1.1 2	0.2 4	0.20	0.7 7	0.7 6	0.8 1	0.7 1

428 ¹Temperature; ²Linear effects of diet; ³Quadratic effects of diet;

429

430

431

432 **Figure captions**

433 Figure 1. Respiration rate (A) and rectal temperature (B) of growing pigs that were fed on diets
 434 containing different levels of selenium (Se, ppm) and vitamin E (VE in IU kg⁻¹). Pigs were maintained
 435 under thermoneutral (TN, 20 °C) or a heat stress (HS, 35°C) ambient environment (n=6 per group).
 436 Each point is the average data for the two days of HS or TN conditions. SEM for respiration rate and
 437 rectal temperature were 9.14 breaths min⁻¹ and 0.138°C, respectively. There were no significant

438 differences between dietary treatments at any time for the TN or HS groups. Significance was
439 observed for the effects of time ($P<0.001$), temperature ($P<0.001$) and the interaction ($P<0.05$). For
440 both respiration rate and rectal temperature, all HS time points were different from the
441 corresponding TN time points (brackets marked a). There were no differences in respiration rate
442 with time under TN conditions (bracket marked b). Rectal temperature under TN conditions
443 increased with time such that at 8 hr (*) it was no different from the first time point in HS pigs, but
444 was significantly greater than for TN at 0.1 and 2 h.

445

446

447 Figure 2. (A) Trans-epithelial electrical resistance (TER, $\Omega \text{ cm}^2$) of the intestines of pigs that were fed
448 on different levels of selenium (Se, ppm) and Vitamin E (VE, IU kg^{-1}) when being subjected to a 35°C
449 or 20°C ambient environment (values pooled across jejunum and ileum, and expressed as mean \pm
450 SEM, $n=6$ per group). (B) Trans-epithelial electric resistance of jejunum and ileum (values are
451 expressed as mean \pm SEM, $n=24$ per group). Multiple comparison was conducted by Duncan's
452 multiple range test, “#” indicates that the values differ ($P < 0.05$).

453

454 Figure 3. (A) FITC-dextran (4kDa) permeability of the intestines of pigs that were fed on different
455 level of selenium (Se, ppm) and Vitamin E (VE, IU kg^{-1}) when being subjected to a 35°C or 20°C
456 ambient environment (values pooled across jejunum and ileum, and expressed as mean \pm SEM, $n=6$
457 per group); (B) FITC-dextran permeability of jejunum and ileum (values are expressed as mean \pm
458 SEM, $n=24$ per group). Units of permeability are $\text{cm min}^{-1}\times 10^{-3}$. Multiple comparison was conducted
459 by Duncan's multiple range test, “#” indicates that the values differ ($P < 0.05$).

460

461 Figure 4. (A) Glutathione peroxidase-2 (GPX-2) mRNA fold change in the intestines of pigs that were
462 fed on different levels of selenium (Se, ppm) and Vitamin E (VE, IU kg^{-1}) when being subjected to a
463 35°C or 20°C ambient environment (values pooled across jejunum and ileum, and expressed as
464 mean \pm SEM, $n=6$ per group); (B) GPX-2 mRNA fold change in the jejunum and ileum (values are

465 expressed as mean \pm SEM, n=24 per group). Multiple comparison was conducted by Duncan's
466 multiple range test, “#” indicates that the values differ ($P < 0.05$).

467

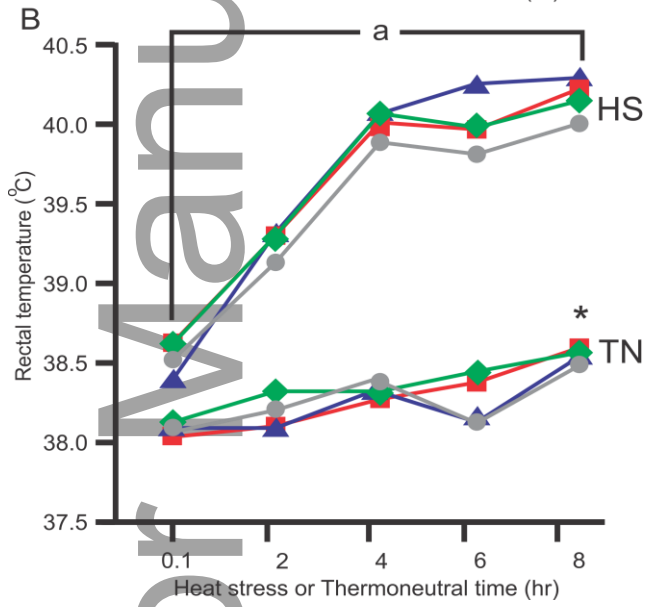
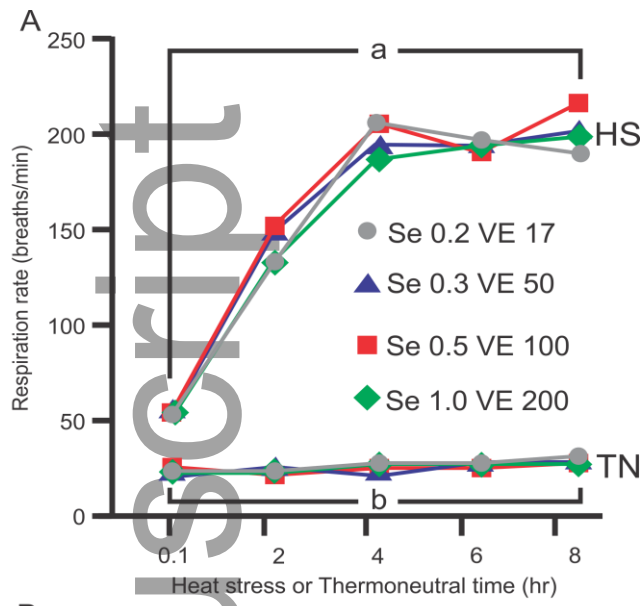
468 Figure 5. (A) Glutathione peroxidase (GPX) activity (units mg^{-1} protein) in the intestines of pigs that
469 were fed on different levels of selenium (Se, ppm) and Vitamin E (VE, IU kg^{-1}) when being subjected
470 to a 35°C or 20 °C ambient environment (values pooled across jejunum and ileum, and expressed as
471 mean \pm SEM, n=6 per group); (B) GPX activity of jejunum and ileum (values are expressed as mean \pm
472 SEM, n=24 per group). Multiple comparison was conducted by Duncan's multiple range test, “#”
473 indicates that the values differ ($P < 0.05$).

474

475 Figure 6. (A) The ratio of oxidized glutathione (GSSG) to reduced glutathione (GSH) in the intestines
476 of pigs that were fed on different level of selenium (Se, ppm) and Vitamin E (VE, IU kg^{-1}) when being
477 subjected to a 35°C or 20 °C ambient environment (values were pooled across jejunum and ileum,
478 and expressed as mean \pm SEM, n=6 per group); (B) GSSG:GSH ratio of jejunum and ileum (values are
479 expressed as mean \pm SEM, n=24 per group). Multiple comparison was conducted by Duncan's
480 multiple range test, “#” indicates that the values differ ($P < 0.05$).

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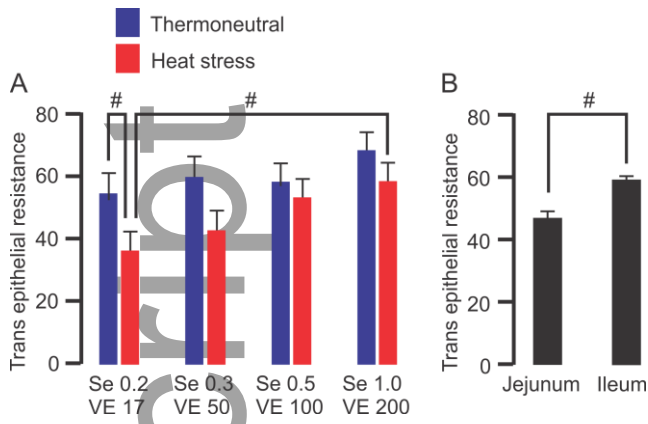
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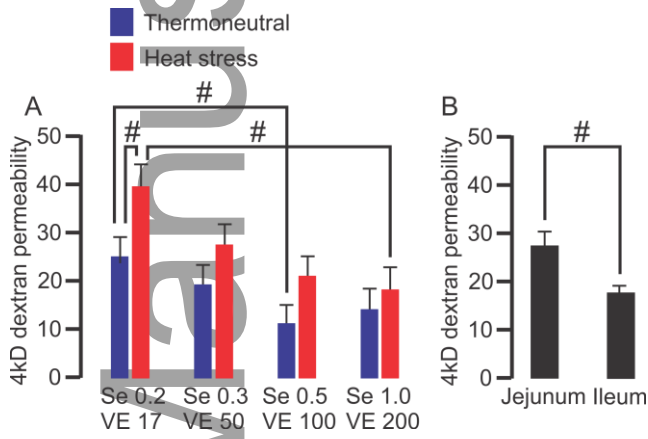
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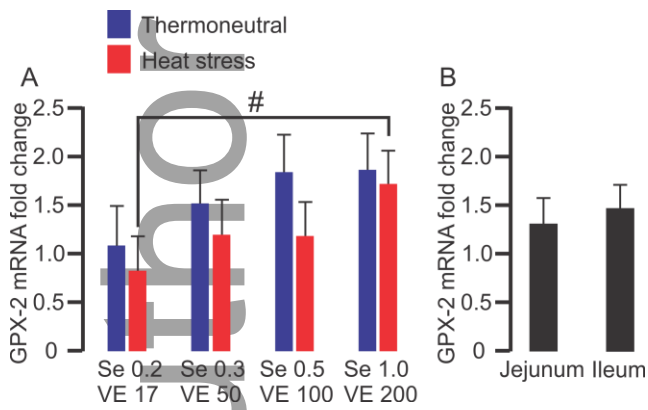
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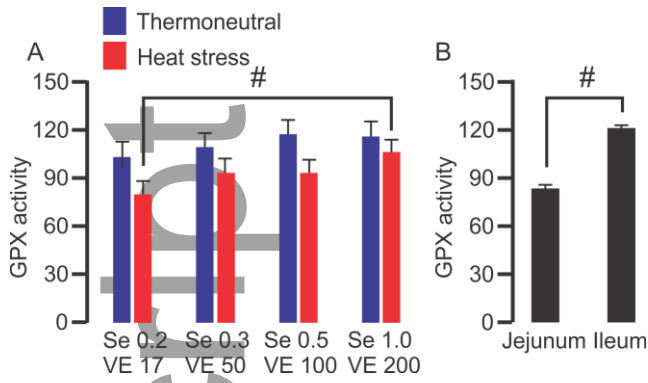
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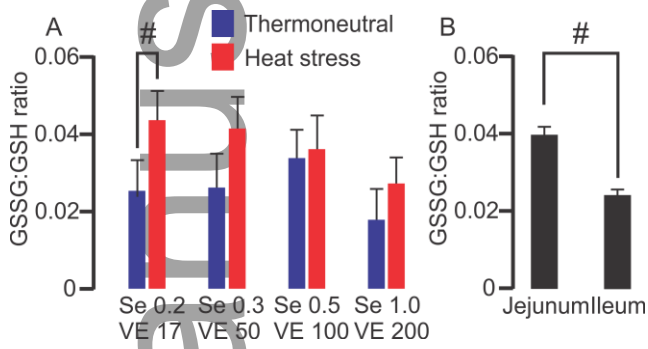
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