Author/s:
Liu, F;de Ruyter, EM;Athorn, RZ;Brewster, CJ;Henman, DJ;Morrison, RS;Smits, RJ;Cottrell, JJ;Dunshea, FR

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DR. FAN LIU (Orcid ID : 0000-0003-2644-2416)

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Effects of L-citrulline supplementation on heat stress physiology, lactation performance and subsequent reproductive performance of sows in summer

Fan Liu[1], Emily M. de Ruyter[1], R. Z. Athorn[1a], Chris J. Brewster[1], David J. Henman[1], Rebecca S. Morrison[1], Robert J. Smits[1], Jeremy J. Cottrell[2], Frank R. Dunshea[2]

[1] Research and Innovation, Rivalea Australia Pty Ltd, Corowa, NSW 2646, Australia
[2] Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, VIC 3010, Australia

Correspondence: Dr. Fan Liu, Research and Innovation Unit, Rivalea Australia Pty Ltd, Corowa, NSW 2646, Australia. Email: fliu@rivalea.com.au

[a] Current address: Australian Pork Limited, Barton, ACT 2600, Australia

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Summary

Lactating sows are susceptible to heat stress (HS). Part of the thermoregulatory response to HS is to increase peripheral blood flow, which is mediated in part by the vasodilator, nitric oxide (NO). Therefore, the aim of this experiment was to determine the effect of supplementation of L-citrulline, a NO precursor, on symptoms of HS, lactation performance, and subsequent reproductive performance of sows in summer. A total of 221 summer farrowing mixed parity sows were fed either a control diet or supplemented with 1% L-citrulline upon entry to the farrowing house (6 ± 1.8 d for mean ± standard deviation (SD) before farrowing) until weaning (26 ± 1.5 d). The average daily minimum and maximum temperature in the farrowing house was 21.0 ± 1.88 °C and 29.2 ± 3.82 °C (mean ± SD). Rectal temperature, respiration rate, and plasma and urinary nitrite and nitrate (NOx) of sows were measured on the 19th day post-farrowing. Supplemental L-citrulline in the diet did not affect the number of piglets born alive, feed intake of sows, body weight or backfat thickness of sows at weaning, or litter weight gain. L-citrulline tended to reduce piglet pre-weaning mortality rate from 18.6% to 15.6% (p = 0.058). L-citrulline reduced the respiration rate of sows compared to the control diet at 17:00 h (Time × Diet, $p < 0.001$), however, rectal temperature was not affected. L-citrulline tended to increase urinary NOx concentrations (127 vs 224 μM, p = 0.057) but not plasma NOx concentrations. L-citrulline did not affect farrowing rate or number of piglets born alive in the subsequent parity. In conclusion, L-citrulline supplementation reduced respiration rate of lactating sows and reduced piglet pre-weaning mortality rate in summer. Whether the effects were due to a NO-dependent mechanism requires further validation.

Keywords: Citrulline, Heat Stress, Lactation, Nitric Oxide, Pig,

Introduction

Lactating sows are particularly susceptible to heat stress (HS) due in part to their high metabolic activity during lactation (Williams et al., 2013). The consequences of HS to the lactating sow are reduced feed intake (Black, Mullan, Lorschy, & Giles, 1993; Prunier 1997; Quiniou & Noblet 1999), litter weight gain (Prunier 1997; Renaudeau, Quiniou, & Noblet, 2001), exacerbated body weight loss (Quiniou & Noblet 1999; Pérez Laspiur & Trottier

2001; Renaudeau et al., 2001), and increased pre-weaning piglet mortality (Stansbury, McGlone, & Tribble, 1987). Because pigs lack eccrine sweat glands, they are particularly reliant on sensible heat loss, whereby blood flow is redistributed to the skin and periphery, where excess body heat is dissipated to the environment by radiation and convection. In one study, blood flow to the ears and skin of pigs are more than doubled when housed under 33°C condition (Collin et al., 2001). As vasodilation is mediated in part by the molecule, nitric oxide (NO), which is enzymatically produced by NO synthase, the aim of this experiment was to determine if supplementing the NO precursor L-citrulline can ameliorate symptoms of HS in the lactating sow in summer.

While the effects of L-citrulline supplementation on NO production has not been studied in pigs, studies in other species suggested that L-citrulline is an immediate precursor of L-arginine (Urschel, Shoveller, Uwiera, Pencharz, & Ball, 2006) and it is even more effective than direct L-arginine oral supplementation in increasing blood L-arginine concentration (Schwedhelm et al., 2008; Lassala et al., 2009) and NO production (Wijnands et al., 2012). Intravenous administration of L-arginine (0.375 g/kg body weight) has shown to facilitate skin vasodilation in human (Bode-Böger, Böger, Galland, Tsikas, & Frölich, 1998). In pigs, increasing L-arginine inclusion rate from 1.0% to 1.8% alleviated the body weight loss of lactating sows during HS conditions and reduced rectal temperature of sows under thermoneutral conditions, although there were no improvements in litter weight gain (Pérez Laspiur & Trottier 2001). A recent study showed that a low oral dosage of L-citrulline (approximately 0.13 g/kg body weight) reduced respiration rate of growing pigs in hot conditions (Kvidera et al., 2016), suggesting that L-citrulline may alleviate HS in pigs. The present experiment investigates the effects of a higher L-citrulline dosage (1% of feed, approximately 0.42 g/kg body weight) on the lactation and subsequent reproductive performance of sows farrowed in summer. The hypothesis tested in this experiment was that supplementing with 1% L-citrulline from late gestation until weaning during summer can reduce HS, resulting in improvements in lactational and subsequent reproductive performance of sows.

Materials and Methods

Animals and experimental design

The procedures that involved animals in current study were in accordance with Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition, 2013), and the protocol (ID:16N068) was approved by the Animal Ethics Committee of Rivalea Australia Pty Ltd, Corowa, NSW, Australia.

A total of 221 mixed parity sows (37 primiparous and 183 multiparous sows, Large White × Landrace, PrimeGro™ Genetics, Corowa, NSW) were allocated to two dietary treatments (Control or L-citrulline) with a similar parity distribution (2.4 ± 1.76 for mean ± SD). The wheat-based control lactation diet contained 14.9 MJ/kg digestible energy and 15% crude protein. The L-citrulline diet was similar as the control diet but 1% wheat was replaced with 1% L-citrulline (Table 1). The sows were fed either the control diet (n=111) or L-citrulline diet (n=110) upon entry to the farrowing house (6 ± 1.8 days (mean ± SD) before farrowing) until weaning (26 ± 1.5 days lactation). The dosage of the L-citrulline used in this experiment is based on an experiment by Morita et al., (2014) in the rabbit model, whereby 0.50 g/kg (body weight) via gavage numerically increased the ear blood flow by approximately 7%. Sows were restrict fed 2.5 to 4 kg from entry until day 3 after farrowing and then fed *ad libitum* as previously described (Liu et al., 2017). Water was supplied *ad libitum* via nipple drinkers. The experiment was conducted in the farrowing house located in the Research and Innovation Unit of Rivalea Australia Pty Ltd (Corowa, NSW, Australia) during a summer period (20 January to 5 April 2017) in the southern hemisphere. The ambient temperature and humidity in the farrowing houses were recorded every hour using an automatic temperature logger (DS1923 Hygrochron, OnSolution Pty Ltd, NSW, Australia). The average daily minimum and maximum temperature was 21.0 ± 1.88 °C and 29.2 ± 3.82 °C (mean ± SD) during the experiment.

Evaluation of farrowing and lactation performance

Body weight and backfat thickness of sows was measured at entry to the farrowing house and at weaning. Backfat thickness was measured using an ultrasound machine (CTS-900V, Shantou Institute of Ultrasonic Instruments Co., Ltd. China) at the P2 site (65 mm from the midline over the last rib). The number of piglets born alive, stillborn and mummified were recorded when the sows finished farrowing. New-born piglets were fostered across treatments within 48 h after birth to standardise litter size between dietary treatments, so that the lactation performance could be compared. Feed intake of sows was recorded daily. Litter size and weight were recorded after cross-fostering and on the 21st day post-farrowing.

Physiological symptoms of heat stress

Twenty primiparous and 44 multiparous parity sows from the Control and L-citrulline group were monitored for physiological symptoms of HS including respiration rate and rectal temperature every three hours from 08:00 h until 17:00 h on the 19th day post-farrowing (maximum ambient temperature inside the farrowing house were all above 28°C on the observation days). Respiration rates were visually counted in 30 second periods using a stop watch, and rectal temperature were measured using a digital thermometer (Model DT-K01A, Liberty Health Products, VIC, Australia)

Subsequent reproductive performance

Fourteen sows (including one primiparous sows and 13 multiparous sows) from the control group and 11 sows (including one primiparous and 10 multiparous sows) from the L-citrulline group were removed from the experiment before the subsequent mating due to non-reproductive reasons resulting in 97 sows from the control group and 99 sows from the L-citrulline group being mated after weaning. The weaned sows that entered the subsequent breeding cycle were mated at the first detected standing heat during post-weaning daily boar exposure, and the wean-to-oestrus intervals were recorded for each mated sow. All the experimental sows were fed a same gestation diet in the subsequent breeding cycle. The number of piglets born alive, stillborn and mummified were recorded for each farrowed sow.

Total nitrite and nitrate (NOx) concentration in plasma and urine

Free NO quickly degrades into the stable nitrate and nitrite (NOx), which was quantified as an indirect measure of NO synthesis. Blood samples were taken by jugular venepuncture from 20 primiparous sows and 44 multiparous sows using ethylenediaminetetraacetic acid coated vacutainers (BD vacutainers NJ, USA) at 10:00 h on the 20th day of lactation. Plasma were separated after centrifugation at 3500 rpm for 5 mins at 4°C. Urine samples were collected from six primiparous sows and 14 multiparous sows in 50 mL jars at 07:00 h on the 20th day of lactation. Both plasma and urine samples were stored at -20 °C until analysing for NOx concentrations using a commercial Kit (Cayman Chemicals, Ann Arbor, MI, USA). As per the manufacturer's instructions plasma samples were filtered through a 10 k Da cut off filter to remove interfering proteins and urine samples were diluted 50 times before the assay. The samples were then treated with nitrite reductase to convert all nitrate into nitrite, then the nitrite in the samples were reacted with Griess Reagent (Sulfanilamide and N-(1-Naphtyl ethylenediamine)) to produce an azo chromophore compound which was quantified using a colorimetric plate reader at 540 nm absorbance.

Statistical analysis

The lactation performance data were analysed using UNIVARIATE procedure of General Linear Model for the main effects of dietary treatment (control vs L-citrulline). Data on respiration rate and rectal temperature were analysed using Repeated Measures of General Linear Model with the time of the day as a within-subject factor and dietary treatment as a between-subject factor. Parity of sows (primiparous or multiparous) was used as a block factor in the above statistical models. The pre-weaning mortality rate of piglets and subsequent farrowing rate data were analysed by Pearson *Chi*-square analysis. All the analysis was conducted in SPSS (version 24, IBM, Chicago, USA). Continuous variables were presented as mean ± standard error (SE), and nominal data were report as a percentage of distribution. Means were considered to differ significantly when $p \leq 0.05$, and a trend was identified when $p \leq 0.10$.

Results

Environmental conditions and physiological responses

Daily temperature and relative humidity of farrowing house are presented in Figure 1. The temperature started to increase from around 20 °C at 8:00 h and peaked at 29°C at 16:00 h, then it gradually decreased and returned to 20 °C overnight. The physiological symptoms of HS, rectal temperature and respiration rate, both increased from 8:00 to 17:00 along with the increase of the temperature of farrowing house (Time, $p < 0.001$) (Figure 2 a and b). L-citrulline supplementation did not affect rectal temperature (Figure 2 a). There was a significant interaction between diet and time of the day on respiration rate (Diet × Time, $p < 0.001$), such that the sows supplemented with L-citrulline had a lower respiration rate than the sows fed on the control diet at 17:00 h (68 vs 54 breaths/min for Control vs L-citrulline, $p < 0.05$) (Figure 2 b).

Farrowing performance

L-citrulline supplementation did not affect the number of piglet born alive, still born or mummified (Table 2).

Lactation performance

L-citrulline supplementation did not affect daily feed intake, body weight or backfat of sows over lactation (all $p > 0.10$) (Table 3). L-citrulline supplementation did not affect piglet average daily gain or litter weight gain to the 21st day of lactation ($p > 0.10$). L-citrulline tended to increase post-weaning litter size from 9.5 to 10.1 (p = 0.094) even though cross-fostering was used to standardise the litter size post-farrowing between two dietary groups. L-citrulline tended to decrease piglet pre-weaning mortality rate from 18.6% to 15.6% (χ^2= 5.7, p = 0.058).

Blood total nitrite and nitrate concentration

L-citrulline supplementation tended to increase (p = 0.057) the urinary NOx concentration from 127 to 224 µM (Figure 3 a) but did not affect the plasma NOx concentration (Figure 3 b).

Subsequent reproductive performance

L-citrulline supplementation did not affect subsequent farrowing rate, wean-to-oestrus interval, number of piglets born alive, number of stillborn piglets, or number of mummified piglets in the subsequent parity (Table 4).

Discussion

The major findings from the present study were that 1% L-citrulline supplementation reduced respiration rate of the lactating sows at the hottest time of the day and decreased pre-weaning mortality rate of piglets during summer. The average maximum daily temperature (29.2 ± 3.82 °C; mean ± SD) recorded during the experiment was above the upper limit of thermal neutral zone (22 °C) of lactating sows (Black et al., 1993; Quiniou & Noblet 1999). The average respiration rate (beyond 50 breaths per min) and rectal temperature from 14:00 to 17:00 h suggesting that the lactating sows experienced a cyclic HS condition in this summer experiment. Similarly, L-citrulline supplementation at a lower dosage (0.13 g/kg body weight) reduced respiration rate of growing pigs housed in heat stress conditions (Kvidera et al., 2016). The effect of L-citrulline in reducing respiration rate in the hot conditions may be due to an enhanced or prolonged skin vasodilation. Heat loss is mainly consisted of sensible heat loss (the heat transferred from skin to the air by radiation and convection) and evaporative heat loss (the heat carried by the moisture from the lung). When the environmental temperature increased to the skin temperature the sensible heat loss of pigs decreases, whereas, the evaporative heat loss increases along with the accelerated respiration rate to maintain or increase total heat loss (Mount 1962; Close & Mount 1978). Theoretically, skin vasodilation can increase the surface area of heat exchange thus allows more sensible heat loss from the skin, therefore a facilitated vasodilation function may spare the evaporative heat loss from the respiratory tract. The increased skin vasodilation may be a reason for the ameliorated respiration rate that was observed in the sows that supplemented with L-citrulline.

Total nitrate and nitrite (NOx) are measured as a marker of endogenous NO synthesis. Our results showed that L-citrulline supplementation tended to increase urinary NOx but not plasma NOx concentrations. The NOx concentration in the urine was 10 times of the concentration in the plasma, suggesting that the accumulated NOx in the urine is more representative of the total NOx production. Furthermore, the blood samples which were collected at a single time point may not be able to fully describe the postprandial NO production. The upregulation of skin vasodilation in response to hot conditions is consisted with a sensory nerve involved initial phase and a NO-dependent maintenance phase (Farrell & Bishop 1995; Charkoudian 2003). The supplementation of L-citrulline was hypothesised to increase precursors for NO production thus facilitate the maintenance phase of skin vasodilation in a hot condition. The increased urinary NOx concentrations indicated that L-citrulline may be a NOx donor for lactating sows under hot conditions. However, it is still unknown whether this magnitude of increased NOx had resulted in an enhanced or prolonged vasodilation function in the lactating sows, because a direct measure of skin vascular conductance or blood flow could not be performed. A study in healthy human showed that an intravenous infusion of L-arginine at 0.375 g/ kg body weight increased urinary nitrite concentration by 97% and reduced total peripheral resistance by 10%, indicating that this level of increased nitric oxide production can effectively stimulate peripheral vasodilation (Bode-Böger et al., 1998). Besides, a topical nitric oxide generating gel was shown to increase skin microcirculatory blood volume by 11 times (Tucker, Pearson, Cooke, & Benjamin, 1999). The reduction of respiration rate observed in the lactating sows supplemented with 1% L-citrulline may be an indirect evidence for an increase sensible heat dissipation that was contributed by an enhanced or prolonged skin vasodilation in the hot condition.

Pre-weaning mortality rate of piglets was slightly reduced by 3% by L-citrulline supplementation, but the litter weight gain or piglet average daily gain during lactation was not affected. Over-lain and unthrifty are the top two reasons for the deaths of pre-weaning piglets in this experiment. The breakdown of the reasons for the deaths of piglets showed that the percentage of piglets destroyed and removed due to unthrifty was numerically decreased by L-citrulline supplementation from 3.7% to 2.8% and from 3.4% to 2.1% respectively, whereas the percentage of overlaid piglets (7.2%) was similar between the two groups. These results suggested that L-citrulline supplementation from late gestation until weaning in sows can slightly increase the viability of piglets; however, the exact mechanism remains to be investigated. Given the null effect on the litter weight gain and piglet average daily gain, the 1% L-citrulline supplementation was unlikely to improve the nutrient composition or the quantity of milk. Likewise, L-arginine supplementation at 25 g/d during gestation and lactation did not affect milk composition or production performance of sows (Krogh, Oksbjerg, Purup, Ramaekers, & Theil, 2016).

The subsequent farrowing outcome was not affected by the L-citrulline supplementation. The subsequent farrowing rate was averaged at 83% which was lower than the normal farrowing rate (above 90%) of the sows mated in the cooler months according to our production records, suggesting a summer infertility scenario. Multiple factors can be responsible for summer infertility, such as the reduced lactational feed intake (Messias de Bragan ca, Mounier, & Prunier, 1998), the impacts of hyperthermia on reproductive physiology (Cabezón, Stewart, Schinckel, & Richert, 2017), and photoperiod (Prunier, Dourmad, & Etienne, 1994; Auvigne, Leneveu, Jehannin, Peltoniemi, & Salle, 2010). L-citrulline did not improve the feed intake or body weight conditions of the lactating sows in summer, therefore it is reasonable that the subsequent reproductive performance was not improved by L-citrulline supplementation.

Future studies should focus on developing improved nutritional strategies to increase NO production. A rodent study showed that a combination of L-citrulline and glutathione supplementation was more effective in increasing the plasma NOx concentration than supplementing with L-citrulline alone (McKinley-Barnard, Andre, Morita, & Willoughby, 2015), therefore the synergism between NO donor and antioxidants could be a strategy to enhance the NO related vasodilation function in lactating sows. Besides, a combination of half dosage of L-citrulline and half dosage L-arginine was more effective in increasing plasma NOx concentration and enhancing vasodilation than separately supplemented with a full dosage of L-citrulline or L-arginine (Morita et al., 2014).

In conclusion, 1% L-citrulline supplementation from late gestation until weaning reduced respiration rate of lactating sows at the hottest time of the day and reduced pre-weaning mortality rate of piglets in summer, while feed intake of lactating sows, litter weight gain, or subsequent reproductive performance was not improved. Whether the beneficial effects are due to the increased NO production remains to be investigated.

Conflict of interest

The authors confirm that they have no conflict of interest**.**

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Tables

TABLE 1 Composition of experimental diets

Ingredient, %	Control diet	L- Citrulline diet
Wheat	53.3	52.2
Barley	20.0	20.0
Canola meal	10.0	10.0
Soybean meal	2.3	2.3
Meat meal	4.4	4.4
Fish oil	0.4	0.4
Tallow	6.1	6.1
Limestone	0.92	0.92
DL-Methionine	0.08	0.08
MgSO4	0.4	0.4
KCL	0.2	0.2
Lysine HCl	0.48	0.48
Threonine	0.2	0.2
L-Citrulline	0	1.0
Premix[†]	1.2	1.2
Calculated nutrients		

DE, MJ/kg	14.9	14.9
CP, %	15.1	16
Fat, %	8.1	8.1
Fibre, %	3.8	3.8
Calcium, %	0.9	0.9
available Phosphorus, %	0.45	0.45
Lysine, %	1.0	1.0
Methionine, %	0.35	0.35

† supplied per kg of diet: copper, 11.7 mg; manganese, 40 mg; zinc, 80 mg; iron, 106 mg; iodine, 2.7 mg; selenium, 0.4 mg; chromium, 530 mg; vitamin A, 15000 IU; vitamin D_3, 3240 IU; vitamin K, 1.0 mg; vitamin B-1, 1.5 mg; vitamin B-2, 5.0 mg; vitamin B-6, 3.0 mg; vitamin B-12, 135 μg; Niacin, 20 mg; pantothenic acid, 15 mg, folic acid, 20 mg; vitamin C, 100 mg; biotin, 200 μg; vitamin E, 95 IU

TABLE 2 Farrowing performance (mean ± standard error) of sows fed control or 1% L-citrulline during late gestation and lactation in summer

Variables	Control (n=111)	Citrulline (n=110)	*p* - values
Born alive piglets per litter	12.0 ± 0.40	11.7 ± 0.37	0.63
Stillborn piglets per litter	0.9 ± 0.26	0.9 ± 0.24	0.88
Mummified piglets per litter	0.2 ± 0.08	0.1 ± 0.07	0.57

TABLE 3 Lactation performance (mean ± standard error) of sows fed control or 1% L-citrulline during late gestation and lactation in summer

Variables	Control (n=111)	Citrulline (n=110)	*p* - values
Sow body weight (110 d), kg	233 ± 4.0	232 ± 3.8	0.87
Sow body weight (wean), kg	214 ± 4.2	214 ± 4.0	0.97
Sow backfat, 110 d, mm	22.2 ± 0.63	23.2 ± 0.59	0.22
Sow backfat, wean, mm	21.8 ± 0.69	22.5 ± 0.64	0.51
Sows backfat change, mm	-0.4 ± 0.59	-0.8 ± 0.55	0.63
Sow daily feed intake, kg	6.4 ± 0.10	6.3 ± 0.09	0.32
Litter size (post-fostering)	11.9 ± 0.18	12.1 ± 0.17	0.51
Litter size (21 d)	9.5 ± 0.25	10.1 ± 0.24	0.094
Piglet pre-weaning mortality, %	18.6	15.6	0.058 (χ^2=5.7)
Litter weight (post-fostering), kg	16.8 ± 0.41	16.9 ± 0.38	0.79
Litter weight (21 d), kg	52.8 ± 1.82	55.2 ± 1.71	0.35
Litter weight gain, kg	36.4 ± 1.61	38.9 ± 1.51	0.25
Piglet average weight (wean), kg	5.5 ± 0.12	5.5 ± 0.11	0.89
Piglet average daily gain, kg/d	0.20 ± 0.005	0.20 ± 0.005	0.92

TABLE 4 Subsequent reproductive performance of the sows fed control or 1% L-citrulline during late gestation and lactation in summer

Variables	Control	L-Citrulline	*p* - values
Number of sows mated [†]	97	99	
Weaning-to-oestrus interval, day	5.7 ± 0.51	5.3 ± 0.45	0.61
Farrowing rate, %	82.5	84.8	0.65 (χ^2=0.20)
Piglet born alive per litter	12.4 ± 0.48	12.1 ± 0.42	0.70
Stillborn piglet per litter	1.1 ± 0.23	1.1 ± 0.20	0.96
Mummified piglet per litter	0.3 ± 0.10	0.3 ± 0.09	1.00

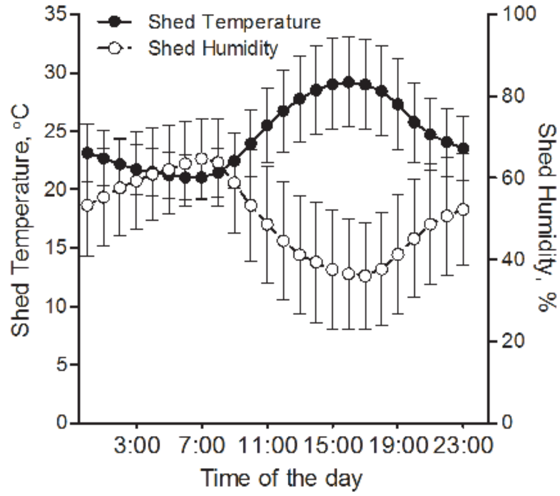
[†] Fourteen sows from the control group and 11 sows from the L-citrulline group were culled before the subsequent mating due to non-reproductive reasons.

Figure legends

FIGURE 1 Temperature and relative humidity of farrowing house (mean ± standard deviation)

FIGURE 2 Rectal temperature (a) and respiration rate (b) (mean ± standard error) of lactating sows fed sows fed control or 1% L-citrulline in summer. Measurements were conducted on the 19th day of lactation, and the maximum shed temperature were above 28°C on the observation days.

FIGURE 3 NOx concentration (mean ± standard error) in urine (a) and plasma (b) of lactating sows fed sows fed control or 1% L-citrulline in summer. Samples were collected on the 20th day of lactation.



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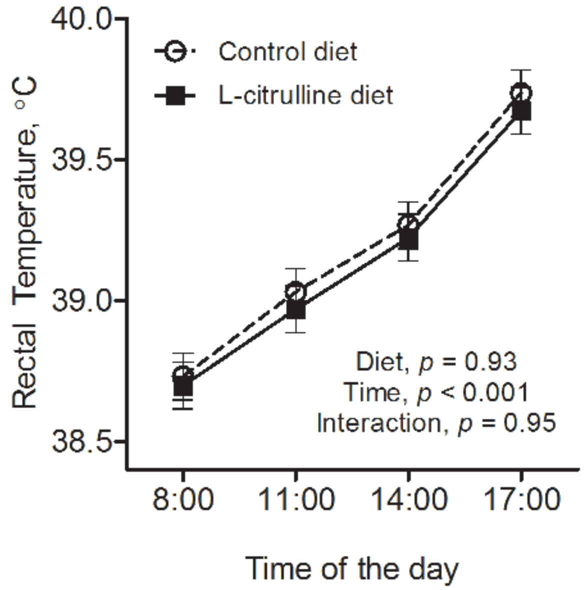

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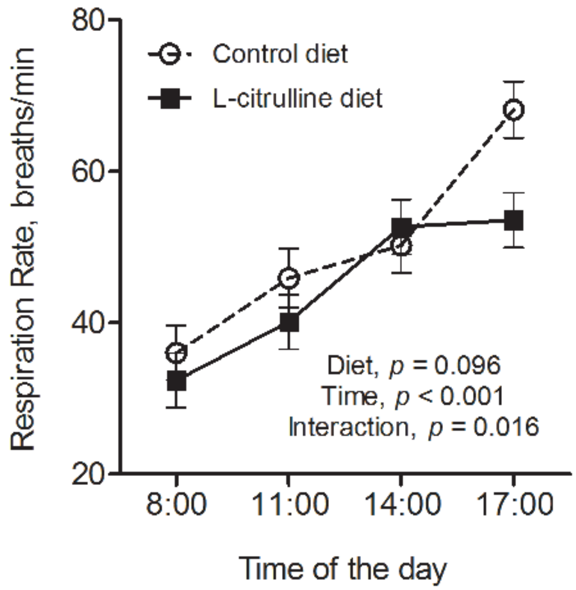

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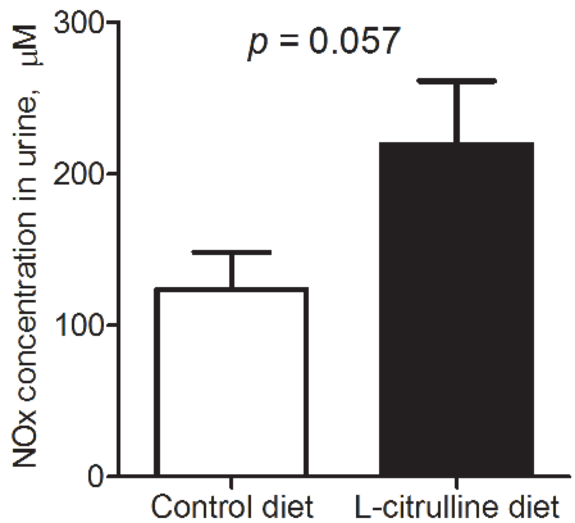

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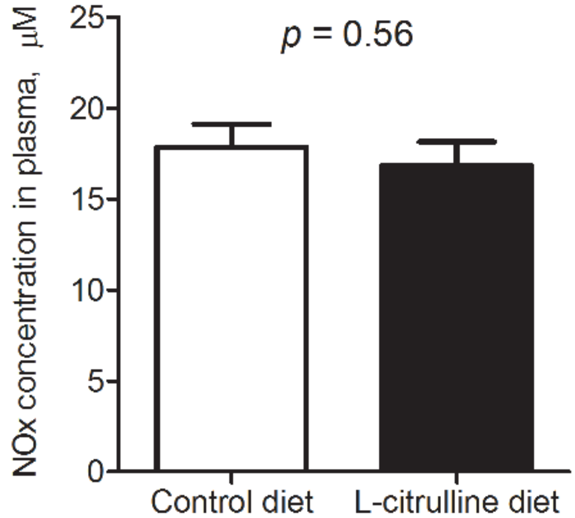

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