Rocchetti, G., Errico, M., Capra, E., Milanesi, M., Fappani, G., Garcia, J., de Paula Nogueira, G., Utsunomiy, Y., Lucini, L., Perez O'Brien, A., Ajmone Marsan, P. (2026) Metabolic indicators of heat tolerance in cattle: blood metabolomics of Angus and Nellore under natural heat stress, *Proceedings 13th World Congress on Genetics Applied to Livestock Production*: 24080 https://doi.org/10.31274/wcgalp.24080
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Metabolic indicators of heat tolerance in cattle: blood metabolomics of Angus and Nellore under natural heat stress

G. Rocchetti[1], M. Errico[1], E. Capra[2], M. Milanesi[1], G. Fappani[1], J.F. Garcia[3], G. de Paula Nogueira[3], Y.T. Utsunomiya[3*], L. Lucini[1], A.M. Perez O'Brien[4], and P. Ajmone Marsan[1]

[1]Università Cattolica del Sacro Cuore, Piacenza, Italy; [2] National Research Council, Lodi, Italy; [3] São Paulo State University, Araçatuba, Brazil; [4] Agropartners Consulting, Araçatuba, Brazil; [*]Corresponding author: yuri.utsunomiya@stgen.com

Introduction

Heat stress (HS) is a major constraint of cattle welfare, impairing performance, reproduction, and immunity and causing significant economic losses (Giannone *et al.*, 2023; Khan *et al.*, 2023; Chen *et al.*, 2024). Understanding the physiological and metabolic mechanisms underlying HS is therefore important to improve heat tolerance in cattle. Nellore (*Bos taurus indicus*) and Angus (*Bos taurus taurus*) breeds exhibit marked differences in their ability to cope with HS (Utsunomiya *et al.*, 2019; Del Corvo *et al.*, 2021). These breeds originate from distinct domestication events, with Nellore cattle evolving in tropical climates and displaying superior heat tolerance compared to the temperate-adapted Angus breed (dos Santos *et al.*, 2023). While genetic and physiological determinants of HS responses in these breeds have been extensively investigated, the metabolic pathways associated with HS tolerance and susceptibility remain insufficiently characterized. In a previous study (Del Corvo *et al.*, 2021), breed-specific DNA methylation patterns were observed in blood samples from Nellore and Angus steers exposed to HS, suggesting differential epigenetic regulation in response to thermal stress. These findings prompted further investigation by untargeted metabolomics, which enables comprehensive profiling of metabolites without prior assumptions, to elucidate biochemical responses to environmental stressors such as HS (Yue *et al.*, 2020; Feng *et al.*, 2022). Blood, as an easily accessible biological matrix, has been widely employed in both targeted and untargeted metabolomic studies (González-Domínguez *et al.*, 2020). This study used untargeted metabolomics to compare blood metabolomes of HS-tolerant Nellore and HS-susceptible Angus cattle, aiming to identify metabolites and pathways linked to HS tolerance. The findings provide a basis for biomarker development, improved management strategies, and breeding programs.

Materials & Methods

Experimental design and data collection. The study involved 25 Nellore (Bos indicus) and 25 Angus (Bos taurus) bullocks (approximately 15 months of age), selected within half-sib families to limit within-breed genetic variability. Animals were sourced from different regions of Brazil and transferred to the UNESP experimental facilities in Araçatuba between 7 and 10 months of age. Management and feeding protocols were based on previously published procedures (Del Corvo *et al.*, 2021). Following a pasture-based rearing period and progressive dietary adaptation, animals were housed under standardized feeding conditions with free access to water. The experimental protocol consisted of three consecutive phases: an adaptation period with shade availability (PRE, 60 days), a heat challenge period induced by shade removal (EXP, 56 days), and a recovery period following shade restoration (POST). Climatic variables were continuously monitored, and the temperature–humidity index (THI) was calculated to assess heat stress conditions (Del Corvo *et al.*, 2021). Blood samples were

collected from five sun-exposed animals per breed during peak heat stress (February 2016) and after recovery (June 2016). Samples were immediately frozen and stored at −80 °C until analysis. Whole blood was used to obtain an integrated representation of circulating and intracellular metabolites. All experimental procedures were approved by the UNESP Ethics Committee (FOA, no. 2014-01445) and complied with ARRIVE guidelines.

Untargeted metabolomics analysis. Blood metabolites were extracted in methanolic solution following an ultrasound-assisted protocol adapted from Teruya *et al.* (2021), combined with protein removal using 3-kDa molecular weight cutoff filters. Extracts were analyzed using ultra-high-performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-HRMS) on a Q-Exactive Focus Orbitrap platform equipped with a BEH C18 column, operating in positive ionization mode (Cattaneo *et al.*, 2023). Pooled quality control samples were included and analyzed in data-dependent MS/MS mode. Raw spectral data were processed using MS-DIAL software with LOWESS normalization and compound annotation against the Bovine Metabolome Database, reaching annotation confidence levels 2 and 3 (Foroutan *et al.*, 2020). The complete dataset was deposited in the MetaboLights repository (MTBLS12545). Statistical analyses were conducted in MetaboAnalyst 6.0 (Pang *et al.*, 2024). After median normalization, logarithmic transformation, and Pareto scaling, both unsupervised (HCA, PCA) and supervised (OPLS-DA) multivariate approaches were applied. Model performance was evaluated using R^2 and Q^2 parameters, permutation testing, and Hotelling's T^2. Discriminant metabolites were identified based on variable importance in projection scores, fold-change analysis, and receiver operating characteristic curves. Pathway enrichment analyses were performed using KEGG reference libraries for Bos taurus and Bos indicus (Xia *et al.*, 2013).

Results

Comprehensive untargeted profiling allowed broad coverage of the blood metabolome, with more than 1,400 annotated compounds, predominantly belonging to amino acid, peptide, carbohydrate, and lipid classes. Exploratory hierarchical clustering based on the complete dataset did not produce a clear separation among experimental groups. In contrast, focusing on the most informative features through OPLS-DA revealed a distinct aggregation of Nellore samples collected during the heat challenge phase. Subsequent supervised OPLS-DA modeling indicated that breed represented the primary source of metabolic variation, whereas heat stress accounted for a secondary component, with robust model performance (R^2 = 0.992; Q^2 = 0.883). During the challenge period, OPLS-DA achieved a clear discrimination between Angus and Nellore profiles (Q^2 = 0.917) and identified a limited set of highly informative metabolites encompassing amino acids, lipids, organic acids, peptides, phenolic compounds, and steroid-related molecules. Among these, 4-hydroxyestrone emerged as the strongest discriminator, showing markedly higher levels in Angus. Comparative analyses between challenge and recovery phases highlighted extensive metabolic remodeling in both breeds, with 264 and 277 significantly modulated metabolites detected in Angus and Nellore, respectively. In Angus, major variations involved urobilin, peptide-derived compounds, acylcarnitines, and glycerophospholipids, whereas Nellore exhibited pronounced changes in urobilin, phenolic metabolites, peptides, phosphoenolpyruvate, and S-(hydroxymethyl) glutathione. Approximately one quarter of heat stress–responsive metabolites were common to both breeds, mainly represented by phospholipids, amino acids, and peptides, often displaying opposite accumulation patterns. Notably, differences in lipid-related profiles, particularly in phospholipids and lysophospholipids, underscored a marked breed-specific response to thermal challenge. Consistently, pathway enrichment analyses pointed to

porphyrin, purine, and pyrimidine metabolism as central components of the heat stress response in both genetic backgrounds (Figure 1).

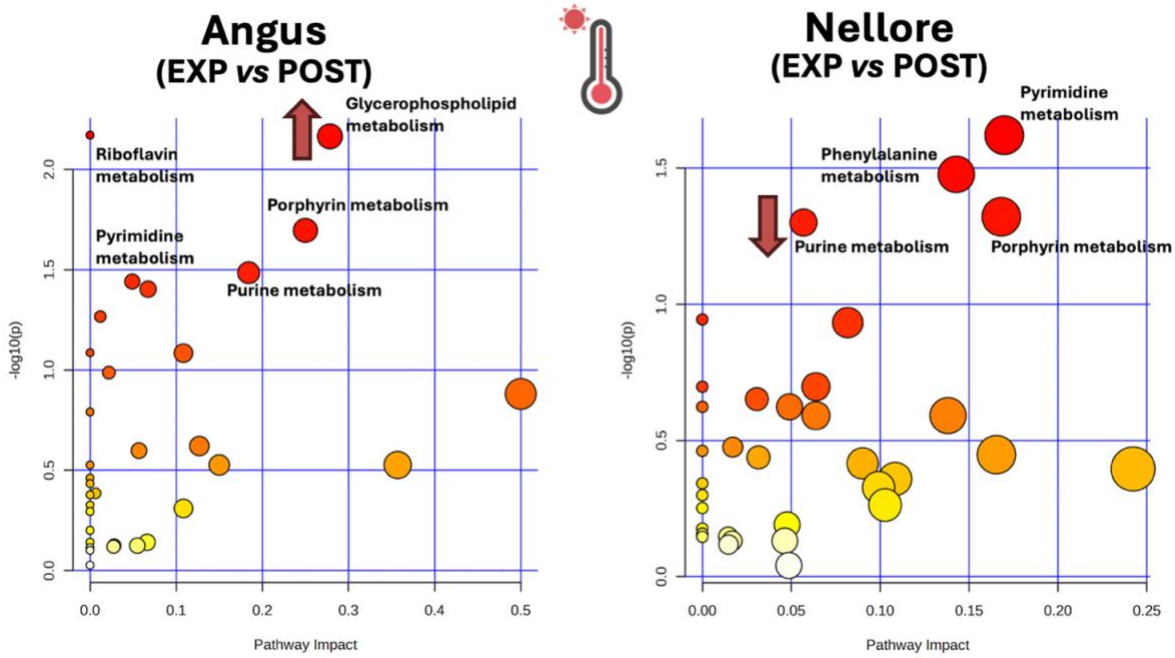

Figure 1. Pathway analysis for both Angus and Nellore cattle considering the pairwise comparisons EXP (heat stress period) *vs* POST (recovery period).

Discussion

In this study, untargeted metabolomics provided an integrated view of the blood metabolic response to heat stress (HS) in Nellore and Angus cattle, revealing both shared and breed-specific adaptive mechanisms. Several metabolites modulated during HS were consistent with previous reports and involved amino acid metabolism, energy metabolism, lipid remodeling, nitrogen and urea cycle pathways, and oxidative stress responses (Tian *et al.*, 2015; Jorge-Smeding *et al.*, 2024). These findings support a breed-dependent metabolic adaptation to HS, in agreement with previous epigenetic evidence obtained from the same animals (Del Corvo *et al.*, 2021). Among breed-discriminant metabolites, 4-hydroxyestrone showed the highest predictive ability, highlighting the sensitivity of steroid metabolism to HS. Additionally, lysine, LysoPC (16:0), and citric acid emerged as highly predictive biomarkers (AUC = 1), previously reported as indicators of HS in cattle. Changes in citric acid likely reflect increased TCA cycle activity under elevated energy demand (Feng *et al.*, 2022), while alterations in lysophospholipids and amino acids are consistent with membrane remodeling and enhanced protein catabolism during HS (Jorge-Smeding *et al.*, 2024). Breed-specific HS responses were particularly evident in lipid and antioxidant metabolism. Nellore cattle showed a significant reduction in lysophospholipids, polyphenols, and lignan-derived metabolites during HS, together with decreased S-(hydroxymethyl)glutathione, suggesting a tightly regulated antioxidant and detoxification response. In contrast, Angus cattle exhibited increased accumulation of phospholipids, lysophospholipids, and long-chain acylcarnitines, indicative of enhanced membrane turnover and lipid mobilization. Elevated acylcarnitines in Angus may reflect incomplete β-oxidation or mitochondrial inefficiency under prolonged HS, whereas their lower levels in Nellore suggest more efficient energy metabolism and greater heat tolerance. Differences in phospholipid dynamics between breeds may represent a key adaptive mechanism. Reduced phospholipid turnover in Nellore could preserve membrane

stability and limit inflammatory signaling, minimizing metabolic costs and oxidative stress (Slimen *et al.,* 2016). Conversely, increased membrane remodeling in Angus may be energetically demanding and exacerbate oxidative damage. These metabolic patterns are consistent with previous findings showing hypomethylation of stress- and lipid-related genes (e.g., ELOVL5, FADS1, PDE5A) in Nellore under HS, supporting enhanced cellular defense and metabolic resilience (Del Corvo *et al.*, 2021). Finally, pathway analyses highlighted porphyrin, purine and pyrimidine metabolism as central components of the HS response. Compared with Angus, Nellore showed a profile consistent with better preserved antioxidant capacity and more efficient stress-regulatory signaling (Giagnoni *et al.*, 2025).

Conclusions

Untargeted metabolomics revealed clear breed-dependent differences in blood metabolic responses to heat stress, with multivariate analyses highlighting both breed and HS effects. Angus cattle showed increased lipid mobilization during HS, accompanied by accumulation of acylcarnitine intermediates, potentially reflecting metabolic strain. In contrast, Nellore exhibited a more balanced energy metabolism and a more efficient reallocation of purine intermediates, consistent with greater heat tolerance. Amino acids, organic acids, phospholipids, and nucleotides emerged as key discriminant metabolites and potential HS biomarkers. Overall, these findings underscore purine metabolism as a relevant pathway in HS adaptation and support the need for future multi-omics and larger-scale studies to refine biomarker identification and mechanistic understanding. *An extended version of this work is currently under review in a peer-reviewed journal.*

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