Long-term high-grain diet alters ruminal pH, fermentation, and epithelial transcriptomes, leading to restored mitochondrial oxidative phosphorylation in Japanese Black cattle

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To increase intramuscular fat accumulation, Japanese Black beef cattle are commonly fed a high-grain diet from 10 to 30 months of age. Castrated and fistulated cattle (n = 9) were fed a high-concentrate diets during the early, middle, and late stages consecutively (10–14, 15–22, 23–30 months of age, respectively). Ruminal pH was measured continuously, and rumen epithelium and fluid samples were collected on each stage. The 24-h mean ruminal pH during the late stage was significantly lower than that during the early stage. Total volatile fatty acid (VFA) and lactic acid levels during the late stage were significantly lower and higher, respectively, than those during the early and middle stages. In silico analysis of differentially expressed genes showed that “Oxidative Phosphorylation” was the pathway inhibited most between the middle and early stages in tandem with an inhibited upstream regulator (PPARGC1A, also called PGC-1α) but the most activated pathway between the late and middle stages. These results suggest that mitochondrial dysfunction and thereby impaired cell viability due to acidic irritation under the higher VFA concentration restored stable mitochondrial oxidative phosphorylation and cell viability by higher lactic acid levels used as cellular oxidative fuel under a different underlying mechanism in subacute ruminal acidosis.

A high-grain diet promotes the growth, productivity, and quality of meat or milk production in beef and dairy cattle. On a high-grain diet, organic acids such as volatile fatty acids (VFAs) and lactic acid accumulate in the rumen¹,². Ruminal pH is critical in the maintenance of normal, stable fermentation, microbial populations, and absorptive function²–⁴, and is determined by the balance between acid production by microbes and acid removal by absorption, neutralization, and clearance¹,⁵, in a host–microbiome interaction⁷. The occurrence of subacute ruminal acidosis (SARA) defined by a rumen pH <5.6 is due to the non-physiological accumulation of VFAs, while ruminal acidosis defined by a rumen pH <5.0 is associated with accumulated lactic acid in the rumen².

Several short- (days) or mid-term (weeks) studies have shown that SARA or rumen acidosis challenges affected the rumen epithelial structure, gene expression, and transcriptomes⁸–¹⁰. For example, in one study, 3 weeks of a high-grain diet (65% grain) compromised the structural integrity and led to the appearance of undifferentiated cells near the stratum corneum of the rumen papillae in non-lactating dairy cattle⁸. In another, the rumen papillae in dairy cattle fed a total mixed ration had increased epithelial desquamation and sloughing scores during early lactation, as well as upregulation of genes encoding desmosome assembly (desplegin1 and cornedodesmosin), epidermal growth factor (EGF) signaling (epiregulin), transforming growth factor β (TGFβ)
The rumen lipopolysaccharide (LPS) activities were significantly different during the early and middle stages. However, the proportions of individual VFAs and peripheral blood inflammation were significantly different during the early and middle stages, and was significantly lower during the middle stage than during the early stage. The lactic acid concentration was significantly lower during the middle stage than during the early stage (Fig. S1).

Results

**Daily dietary intake, ruminal pH, total VFA, LPS, and LBP.** In this study, we re-analyzed our previously published data regarding rumen fermentation parameters to evaluate the relationships between ruminal pH and candidate gene expression in the RE.

No adverse health condition throughout the study period and effects of ruminal cannulation after surgery were observed for any of the cattle. The daily intake and rumen fermentations were described previously. Briefly, the organic matter basis concentrate intake was significantly higher during the Middle stage than during the early stage (7.6 vs. 6.0 and 6.1 kg/day, respectively), and the forage diet intake was significantly lower during the late stage than during the early and middle stages (2.1 vs. 1.1 and 1.0 kg/day, respectively). Nutrient adequacy rates of dry matter (DM) and total digestible nutrients (TDN) were calculated based on the total consumption amounts of concentrate diet and forage.

No change in pH was observed during calibration. The 24 h mean ruminal pH was significantly lower during the late stage than during the early and middle stages. The 1 h mean pH was significantly lower during the middle stage than during the early stage (Fig. S1).

The total VFA concentration was significantly lower during the late stage than during the early stage (Fig. 2). The lactic acid concentration was significantly higher during the late stage than during the early stage and middle stages, and was significantly lower during the middle stage than during the early stage. The rumen lipopolysaccharide (LPS) activities were significantly higher during the middle and late stages than during the early stage. However, the proportions of individual VFAs and peripheral blood LPS-binding protein (LBP) concentration did not differ (P < 0.10) among the fattening stages (Fig. 2).
“Oxidative Phosphorylation” was activated the most (z-score $P = 0.05$) during the early, middle, and late fattening stages. Different superscripts are significantly different ($P < 0.05$). Values are the mean ± SE.

**Microarray and pathway analyses of the rumen epithelium.** In all, 3,570, 3,856, and 2,477 DEGs (FDR corrected $P < 0.05$) were identified when comparing the middle and early (first period), late and middle (second period), and late and early (total period) stages, respectively. There were 873 DEGs (315 upregulated) during the first period, 1,216 DEGs (656 upregulated) during the second period, and 115 DEGs (34 upregulated) during the total period, and they were mapped to molecular functions (FDR corrected $P < 0.05$, fold change $\geq 2$; Table S3). Twenty-two candidate genes were common to all stages (FDR corrected $P < 0.05$). The principal component analyses (PCA) plots of the fattening stages were similar (2 upregulated, 11 downregulated), second (12 upregulated, 7 downregulated), and total (1 upregulated, 2 downregulated) periods (Table S4). Transporter gene expression of the rumen epithelium (RE) during the first period were reversely downregulated or upregulated during the second and total periods (Table S5).

There were 873 DEGs (315 upregulated) during the first period, 1,216 DEGs (656 upregulated) during the second period, and 115 DEGs (34 upregulated) during the total period, and they were significantly changed during the first (11 downregulated), second (12 upregulated, 7 downregulated), and total (1 upregulated, 2 downregulated) periods (Table S1). Twenty-two candidate genes were common to all stages (FDR corrected $P < 0.05$). The principal component analyses (PCA) plots of the fattening stages were similar within and distinct between stages, particularly in the middle versus late and early stages (Fig. 3).

Canonical pathways of DEGs were identified ($P < 0.05$, z-score $\geq 2$ or $\leq -2$) using IPA software. There was no activated canonical pathway during the first period, whereas “Oxidative Phosphorylation” was the most inhibited (z-score $= -3.32$, $P = 3.72 \times 10^{-10}$) pathway during the same period (Table 2). During the second period, “Oxidative Phosphorylation” was activated the most (z-score $= 2.50$, $P = 6.61 \times 10^{-5}$), and no inhibited pathway was identified (Table 2). Table 3 summarizes the genes integrating oxidative phosphorylation pathway during the first (11 downregulated) and second periods (11 upregulated, 2 downregulated).

Upstream regulator analyses based on the DEGs revealed significant activation or inhibition ($P < 0.05$, z-score $\geq 2$ or $\leq -2$, respectively) within each comparison (Fig. 5). During the first period, 6-[(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437; z-score $= 3.67$, $P = 2.69 \times 10^{-6}$) was the upstream regulator activated the most and nuclear factor, erythroid 2 like 2 (NFE2L2; z-score $= -3.29$, $P = 2.04 \times 10^{-6}$) inhibited the most. During the second period, the most activated and inhibited upstream regulators were genistein (z-score $= 4.61$, $P = 6.93 \times 10^{-5}$) and immunoglobulin G (IgG; z-score $= -3.84$, $P = 2.54 \times 10^{-4}$), respectively, and those during the overall period were 2-(2′-Amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD98059; z-score $= 2.82$, $P = 2.78 \times 10^{-3}$) and MYC proto-oncogene, BHLH transcription factor (MYC; z-score $= -2.39$, $P = 2.97 \times 10^{-5}$). Most of the significantly activated or inhibited upstream regulators during the first period were subsequently inhibited or activated, respectively, during the second period (Fig. 5).

**Validation of DEGs by qPCR.** Fig. S2 shows the fold changes in the expression of nine genes of interest determined by microarray analyses and quantitative real-time polymerase chain reaction (qPCR). For all nine genes, the direction of the estimated fold changes was consistent in the microarray and qPCR analyses.

**Discussion**

During the fattening period, Japanese Black beef cattle are raised on a high-grain diet between 10 and 30 months of age to increase intramuscular fat accumulation. In this study, samples were collected throughout the entire fattening period to explore the long-term changes in ruminal pH and fermentation as described previously [44], and their consequences for rumen epithelial transcriptomic dynamics. In cattle, a ruminal pH below the physiological range of 5.8–6.5 can induce inflammatory responses [45], and structural or transcriptomic changes in the RE [5-10]. Consistent with a previous study [44], the 24h mean ruminal pH decreased gradually as the lengths of time at pH <5.6 and <5.8 increased during the middle and late stages of fattening as a result of a long-term high-grain diet.
Table 1. Differentially expressed genes (FDR corrected P < 0.05, fold change ≥ 2) encoding rumen epithelial transporters in the comparisons of the middle and early, late and middle, and late and early stages in Japanese Black beef cattle.

| Gene Symbol | Fold Change vs. Early | P-value | Gene Name |
|-------------|-----------------------|---------|-----------|
| SLC5A12     | 2.24                  | 2.54    | solute carrier family 5 (sodium/glucose cotransporter), member 12 |
| SLC6A8      | −2.01                 | 3.23    | solute carrier family 6 (neurotransmitter transporter, creatine), member 8 |
| SLC7A6OS    | −2.05                 | 3.23    | solute carrier family 7, member 6 opposite strand |
| SLC9A6      | −4.08                 | 4.08    | solute carrier family 9 (sodium/hydrogen exchanger), member 6 |
| SLC10A5     | 2.72                  | 2.05    | solute carrier family 10, member 5 |
| SLC22A5     | −3.68                 | 1.89    | solute carrier family 22 (organic cation/carnitine transporter), member 5 |
| SLC22A8     | −3.50                 | 6.75    | solute carrier family 22 (organic anion transporter), member 8 |
| SLC22A18    | −2.49                 | 2.20    | solute carrier family 22, member 18 |
| SLC23A3     | −2.32                 | 1.46    | solute carrier family 23 (nucleobase transporters), member 3 |
| SLC25A5     | −3.30                 | 2.99    | solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5 |
| SLC25A6     | −5.59                 | 4.80    | solute carrier family 25 (mitochondrial carrier; adenosine triphosphate translocator), member 6 |
| SLC25A11    | −2.48                 | 2.38    | solute carrier family 25 (mitochondrial carrier, oxoglutarate carrier), member 11 |
| SLC25A17    | 2.10                  | 1.19    | solute carrier family 25 (mitochondrial carrier, peroxisomal membrane protein, 34kdA), member 17 |
| SLC26A3     | −6.38                 | 10.80   | solute carrier family 26, member 3 |
| SLC28A3     | −6.71                 | 10.20   | solute carrier family 28 (sodium-coupled nucleoside transporter), member 3 |
| SLC29A4     | 2.45                  | 2.32    | solute carrier family 29 (nucleobase transporters), member 4 |
| SLC35A5     | −2.73                 | 2.52    | solute carrier family 35, member A5 |
| SLC35C1     | −2.09                 | 2.44    | solute carrier family 35, member C1 |
| SLC35F5     | −2.63                 | 3.83    | solute carrier family 35, member F5 |
| SLC39A7     | −2.46                 | 2.03    | solute carrier family 39 (zinc transporter), member 7 |
| SLC39A11    | −2.17                 | −2.92   | solute carrier family 39 (metal ion transporter), member 11 |
| SLC20A1     | −2.08                 | 4.90    | solute carrier organic anion transporter family, member 2A1 |

These observations are sufficient to diagnose SARA during the middle and late stages (ruminal pH < 5.6 or < 5.8 for more than 3 or 5 h per day, respectively). However, lactic acid is 10 times less protonated than VFA in the rumen (pKa 4.9 vs. 3.9; 2), and its concentration peaked during the late stage. Therefore, it is reasonable to postulate that different mechanisms underlie the low ruminal pH in the late (lactic acid accumulation) versus early and middle stages (total VFA accumulation) as an adaptation of rumen bacterial community to and endurance of a long-term, harsh ruminal environment in Japanese Black beef cattle. However, lower ruminal pH during the late stage did not disrupt the gastrointestinal barrier or induce higher LBP levels in peripheral blood, despite inducing significantly higher LPS levels in the rumen.

Transporter genes (SLC family) are considered to be responsible for pH regulation by rumen epithelial absorption. A low ruminal pH or high-grain diet enhances rumen epithelial absorption via enhanced transporter gene expression, such as monocarboxylate transporter (MCT1; 5), sodium hydrogen exchanger isoform 3 (NHE318;), and downregulated in adenoma (DRA19;). In the present study, we observed the opposite, i.e., most of the 22 different SLC family genes identified were downregulated (11/13) during the first period and then upregulated (12/19) during the second period. In detail, the expression of SLC9A6 (NHE6) and SLC26A3 (DRA) were downregulated (fold change = −4.08 and −6.71, respectively) during the first period, while they were upregulated (fold change = 4.08 and 10.80, respectively) during the second period. These results suggest that expression of genes encoding rumen epithelial absorption was suppressed by acidicotic insult during the first period, whereas it was restored during the second, enhancing VFA absorption or transporter gene expression in the rumen.

Interestingly, different types of SARA in the earlier and later stages had completely opposite consequences on the candidate gene expression (DEGs) and its analysis results (canonical pathway and upstream regulators). Furthermore, PCA plots also suggested that the middle stage was the stage most affected by long-term high-grain diet. LaMonte et al. suggested that acidosis alters cellular metabolism, such as the pentose phosphate pathway and glutaminolysis, to mitigate increased stress due to reactive oxygen species (ROS). A high-starch diet also leads to increased oxidative stress and changes in oxidative phosphorylation to alleviate the toxic effects of ROS. Moreover, VFAs, particularly butyric acid, are catabolized within rumen epithelial cells via oxidative pathways, which generate ROS in the rumen. However, mitochondrial superoxide dismutase 2 (SOD2; fold change = −2.29, P = 2.16 × 10−4) was significantly downregulated during the first period, and then it was upregulated (fold change = 2.32, P = 5.24 × 10−5) again simultaneously with SOD1 (fold change = 1.52, P = 1.77 × 10−5) during the second period. These results were supported by the fact that the top upregulated candidate gene during the first period, NADH dehydrogenase subunit 6 (ND6), was associated with the significant canonical pathway "Mitochondrial Dysfunction" (P = 2.69 × 10−7; data not shown). Although excess oxidative stress is inferred from the literature, the responses of rumen epithelial cells to oxidative stress might be depressed during the first period in contrast to a short-term (17 days) SARA challenge in dairy cows. These results also imply...
mitochondrial metabolic dysfunction (i.e., handling oxidative stress), suggesting greater rumen epithelial cell vulnerability to cellular damage by acidotic insult. Mitochondrial proficiency and ROS detoxification are critical for cancer cell viability, and proliferating cancer cells must preserve intracellular ATP and NADPH levels through mitochondrial oxidative phosphorylation and the prevention of excessive ROS accumulation. In silico analyses of the DEGs also suggested that the results were in line with mitochondrial dysfunction during the first period. For example, “Oxidative Phosphorylation”, in response to peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α; 29), was the most inhibited canonical pathway with coordinated inhibition of the upstream regulator PPARGC1A (also called PGC-1α) during the first period. In addition, reduced expression of PGC-1α 26,28 and decreased oxidative phosphorylation by coordinated downregulation of PGC-1α-responsive genes 29 are responsible for greater insulin resistance, which might be consistent with the inhibited “Insulin Receptor Signaling” canonical pathway in the present study.

Therefore, long-term high-grain diet induced excessive oxidative stress in the RE due to the higher acidity and VFA metabolism, resulting in mitochondrial oxidative phosphorylation dysfunction via the downregulation of ubiquinone oxidoreductase subunits, which are cell survival factors. Activation of upstream regulators such as CD437 (induction of cellular apoptosis and cell cycle arrest in cancer cells; 31) and inhibition such as NFE2L2 (regulator for normal mitochondrial biogenesis; 32) may indicate regulation of cell proliferation, apoptosis, and mitochondrial biogenesis in the RE. Collectively, the in silico analyses suggest impaired cellular viability and vulnerability to cellular damage due to acidotic irritation during the first period.

During the second period, however, the rumen epithelial transcriptomes restored cellular functions with changes in the underlying mechanism of SARA. The significantly higher lactic acid concentration during the late stage seemed to affect the responses of rumen epithelial cells to acidic irritation differently compared to the early and middle stages. Riemann et al. 33 reported that extracellular acidosis results in a rapid, sustained decrease in intracellular pH (pHi) and tissue hypoxia, and induces increased transcription factor phosphorylation and tumor cells transcriptional activity. The involvement of lactic acid in cancer has attracted attention only recently, and lactic acid directly contributes to tumor growth and progression (i.e., the lactate anion and protons) as an oxidative fuel and support for angiogenesis and metastasis. For example, extracellular lactic acid acidosis causes a lower pH, and higher intracellular lactate concentration, and rescues 4T1 (murine breast cancer) cells from glucose deprivation. In addition, lactic acidosis induces a higher oxidative phosphorylation rate in ATP production in nine different cell lines compared to without lactic acidosis, consistent with the activation of “Oxidative Phosphorylation” during the second period. Although our hypothesis may be preliminary and it is necessary to clarify the positive correlation between the ruminal and intracellular lactic acid concentrations, we postulate that the rumen epithelial transcriptomes were influenced, at least in part, by the significantly increased level of lactic acid in the rumen, resulting in greater cell viability by restoring the mitochondrial oxidative phosphorylation pathway. Our hypothesis may also be supported by the most activated (genistein, tyrosine kinase inhibition that enhances apoptosis and cell cycle arrest in cancer cells) and inhibited (IgG, malignant factor for colorectal cancer cells) upstream regulators during the second period.

Finally, activation of upstream regulators such as PD98059 (a mitogen-activated protein kinases kinase inhibitor), sirolimus (immunosuppressant that inhibits cell cycle progression), hexarotene (anti-cancer action by inducing T-cell lymphoma cell apoptosis), and KDM5B (critical regulator of genome stability and DNA repair) and inhibition such as MYC (transcription regulator suppressed by intracellular butyrate) throughout the overall study period implies moderate restoration of cellular functions in response to long-term high-grain diet in Japanese Black cattle during the fattening period. However, the effects of gene expression on SARA occurrence or long-term high-grain diet feeding remain unclear, and further studies are required to provide host-side elucidation of host-microbiome interactions.
### Table 2. Canonical pathways activated (z-score ≥2) and inhibited (z-score ≤−2) generated by Ingenuity Pathway Analysis (IPA) in comparisons of the early and middle stages and late and middle stages. *Values indicate a statistically significant match between up- and down-regulation patterns.

| Canonical Pathways                        | log(p-value) | zScore* | Ratio | Molecules                                                                 |
|------------------------------------------|--------------|---------|-------|---------------------------------------------------------------------------|
| Middle vs. Early stages                  |              |         |       |                                                                           |
| iNOS Signaling                           | 2.10         | −2.00   | 0.133 | FOS, IFNGR1, IFNGR2, C4LM5, JAK1, LBP                                      |
| CCR5 Signaling in Macrophages            | 1.93         | −2.00   | 0.096 | GNA11, FOS, GNG5, CCL5, CD3G, NGG7, C4LM5, GNB1, CACNG1                  |
| IL-22 Signaling                          | 1.87         | −2.00   | 0.167 | IL10RB, AKT1, IL22RA1, JAK1                                              |
| NRF2-mediated Oxidative Stress Response  | 3.39         | −2.11   | 0.094 | SQSTM1, ARCC4, AKT1, UBE2E3, NQO2, HSPB8, MAP2K1, P53B, FOS, AKR7A2, SOD2, CCT7, ACTB, PTPN11, CAT, EPHX1, AKR1A1, VCP, HERPUD1 |
| Insulin Receptor Signaling               | 1.51         | −2.11   | 0.075 | AKT1, PTPN11, PPP1R2A, PPP1CA, PPP1CB, PFRKAR1B, JAK1, SCNN1A, MAP2K1, SYNJ1, PPP1R1 |
| Oxidative Phosphorylation                | 2.43         | −3.32   | 0.101 | SDHB, UQCRCL1, SDHA, NDUFA9, SDHA, NDUFB3, NDUFS6, ATPAF1, ATP5F1C, ATP5F1B, SDHC, NDUFS1 |
| Late vs. Middle stages                   |              |         |       |                                                                           |
| Oxidative Phosphorylation                | 2.18         | 2.50    | 0.119 | COX11, UQCRCL1, SDHA, NDUFA3, NDUFS3, SDHB, UQCRQ, ATP5F1G, NDUFA6, ATPAF1, ATP5F1C, SDHC |
| Role of p14/p19ARF in Tumor Suppression  | 1.46         | 2.45    | 0.133 | SENP3, FGFR1, URTF, PTPN11, MDM2, TTF1                                     |
| Endocannabinoid Cancer Inhibition Pathway | 1.57         | 2.32    | 0.095 | MAP2K5, GNA11, FGFR1, SMPD2, MAP2K1, RH OA, PTPN11, CREB3, SPTLC1, TWIST2, PFRKAR1B, DDIT3, YEGFR, CASP9, PFRKAR2A |

**Conclusions**

In Japanese Black beef cattle, long-term feeding of a high-grain diet (at 10–30 months of age) induced SARA due to the total VFA production during the early and middle stages and due to lactic acid production during the late stage, suggestive of different mechanisms underlying SARA. Total VFA induced a low ruminal pH, which inhibited the oxidative phosphorylation pathway, and was associated with mitochondrial dysfunction and thereby impaired cell viability of the RE during the earlier fattening stage. By contrast, higher lactic acid levels used as cellular oxidative fuel restored the oxidative phosphorylation pathway and cellular viability during the latter fattening stages. Therefore, the specialized fattening stage applied to Japanese Black beef cattle results in unique changes in rumen fermentation characteristics, influencing epithelial transcriptomes through the interaction with altered rumen fermentation as a response to a long-term high-grain diet.

**Methods**

The experimental protocol was approved by the Iwate University Laboratory Animal Care and Use Committee (A201720; Morioka, Japan). All animal experiments adhered to the animal experiment policy of Hyogo Prefectural Technology Center for Agriculture, Forestry and Fisheries (Hyogo Prefecture, Japan).

**Animals and experimental design.** The experimental animals and designs were described previously. Briefly, nine castrated (at age 5–6 months) and subsequently fistulated (at age 12 months) Japanese Black beef bull cattle were housed with free access to food and water throughout the study (10–30 months of age). The fattening period was subdivided into the early, middle, and late stages (10–14, 15–22, and 23–30 months of age, respectively) according to general agreement in Japan. Cattle were fed concentrate and forage (rice straw) diets during all three stages, and the amount of concentrate diet was increased gradually throughout the experimental period. The forage-to-concentrate ratio was 26:74, 13:87, and 14:86 during the early, middle, and late stages, respectively. The concentrate diet was composed of barely, steam-flaked corn, wheat bran, and soybean meal and contains 71.2% total digestible nutrient (TDN) and 15.7% crude protein (CP), 72.2% TDN and 13.9% CP, and 72.8% TDN and 12.0% CP during the early, middle, and late stage, respectively. The mean body weight of the cattle was 335 ± 4.4, 439 ± 7.6, 562 ± 11.6, and 712 ± 18.5 kg on prior to the experiment (10 months of age), and early (14 months of age), middle (21 months of age), and late (29 months of age) fattening stage sampling days, respectively. The forage diet was supplied daily at 0930 and 1530 h in two equal portions, and the concentrate was supplied 1 h after the rice straw to maximize forage diet intake and to prevent excessive consumption of concentrate diet during the early stage. Abnormalities of body condition (body temperature, appetite, hydration, and defecation) were observed daily throughout the study period. The intakes of concentrate and rice straw diets were recorded daily during the final 7 days of the early, middle, and late fattening stages. Table S1 shows the body weight, organic matter intake amount, and chemical composition of the fattening stage diets. Chemical composition of the diets was analyzed according to the official method analysis of the Association of Official Analytical Chemists (AOAC) that registered in the Official Method Feed Analysis of Japan. The adequacy rate of diet was calculated based on the nutrient requirement of Japanese Feeding Standard for Beef cattle.

**Sampling and measurements.** The sample collections and measurements were described previously. Briefly, ruminal pH was measured continuously every 10 min during the final 7 days of the early, middle, and late fattening stages using a radio transmission system (YCW-S; DKK-TOA, Yamagata, Japan), as described
previously. A pH sensor was placed in the ventral sac of the rumen through the rumen fistula and calibrated at standard pH values of 4–7, before and after obtaining data in each fattening stage. Rumen fluid from the ventral sac of the rumen (adjacent to the pH sensor) and blood samples from the jugular vein were collected on day 4 of the pH measurements during the three stages, for analyses of total VFA, individual VFAs, lactic acid concentration, LPS activity, and LBP concentration. The fluid samples were immediately filtered through two layers of cheesecloth and stored at $-80^\circ$C until use. Blood samples were immediately centrifuged ($1,500 \times g, 15$ min, $4^\circ$C) to separate the plasma and then preserved at $-80^\circ$C until analyses.

For the VFA analyses, 1 mL 25% HO$_3$P in 3 N H$_2$SO$_4$ was added to 5 mL rumen fluid. Total VFA and individual VFAs (acetic, propionic, and butyric acids) were separated and quantified by gas chromatography (GC-2014; Shimadzu, Kyoto, Japan) using a packed glass column (Thermon-3000; 3%) with a Shimalite TPA 60–80 mesh support (Shinwa Chemical Industries, Kyoto, Japan). For lactic acid analyses, fluid samples were centrifuged at $2,000 \times g$ for 15 min at $4^\circ$C, and the concentration in the supernatant was determined using a commercial F-kit (D-lactate/L-lactate) (J.K. International, Tokyo, Japan). To measure rumen LPS activity, rumen fluid samples were centrifuged at $11,000 \times g$ for 15 min at $4^\circ$C and assayed using a kinetic Limulus amebocyte lysate assay (Pyrochrome with Glucashield; Seikagaku, Tokyo, Japan) as described previously. The plasma LBP concentration was measured using a commercial kit (HK503; HyCult Biotech, Uden, The Netherlands) as described elsewhere.

**Transcriptome analyses of the rumen epithelium.** The RE was biopsied from the ventral sac of the rumen at a site adjacent to the pH sensor simultaneously with rumen fluid sampling. A sterile, disposable biopsy punch 8 mm in diameter (Kai Industries, Tokyo, Japan) was used to scratch off the RE. Then the RE samples were washed three times in ice-cold PBS and immediately stored at $-80^\circ$C until use. Total RNA was extracted from the RE using TRIzol reagent (Invitrogen) as described previously. The purity of the extracted RNA was increased using an RNeasy RNA Clean-up Kit (QIAGEN, Valencia, CA). Total RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific, Waltham, MA), and its quality...
| Gene Symbol | Fold change | P-value | Gene Name | Location | Type |
|-------------|-------------|---------|-----------|----------|------|
| ATP5F1C     | −3.39       | 2.16    | 3.86 E-05 | ATP synthase F1 subunit gamma | Cytoplasm | Transporter |
| ATP5F1B     | −2.16       | 7.60 E-04 | ATP synthase F1 subunit beta | Cytoplasm | Transporter |
| ATP5PO      | 2.05        | 4.86 E-05 | ATP synthase peripheral stalk subunit OSCP | Cytoplasm | Transporter |
| ATPAF1      | −3.40       | 3.94    | 4.75 E-04 | ATP synthase mitochondrial F1 complex assembly factor 1 | Cytoplasm | Other |
| COX11       | 2.33        | 2.69 E-04 | cytochrome c oxidase copper chaperone COX11 | Cytoplasm | Enzyme |
| NDUFA6      | −4.22       | 2.33 E-08 | NADH:ubiquinone oxidoreductase subunit A6 | Cytoplasm | Enzyme |
| NDUFA9      | −2.06       | 4.26 E-04 | NADH:ubiquinone oxidoreductase subunit A9 | Cytoplasm | Enzyme |
| NDUFA1B     | 2.02        | 3.42 E-04 | NADH:ubiquinone oxidoreductase subunit A1B | Cytoplasm | Enzyme |
| NDUFS1      | −3.20       | 3.07    | 2.89 E-05 | NADH:ubiquinone oxidoreductase core subunit S1 | Cytoplasm | Enzyme |
| NDUFS6      | −2.22       | 2.40 E-03 | NADH:ubiquinone oxidoreductase subunit S6 | Cytoplasm | Enzyme |
| NDUFV3      | −2.06       | 2.18    | 3.07 E-03 | NADH:ubiquinone oxidoreductase subunit V3 | Cytoplasm | Enzyme |
| SDHA        | −7.32       | 7.18    | 2.73 E-06 | succinate dehydrogenase complex flavoprotein subunit A | Cytoplasm | Enzyme |
| SDHB        | −3.18       | 3.57    | 4.64 E-04 | succinate dehydrogenase complex flavoprotein subunit B | Cytoplasm | Enzyme |
| SDHC        | −3.42       | 2.78    | 3.90 E-05 | succinate dehydrogenase complex flavoprotein subunit C | Cytoplasm | Enzyme |
| UQCRCP      | −4.71       | 3.57    | 1.78 E-04 | ubiquinol-cytochrome c reductase core protein 1 | Cytoplasm | Enzyme |
| UQCRQ       | −2.25       | 1.86 E-05 | ubiquinol-cytochrome c reductase complex III subunit VII | Cytoplasm | Enzyme |

Table 3. Genes (raw estimated fold change ≥2, FDR corrected P < 0.05) integrating the oxidative phosphorylation canonical pathway generated by Ingenuity Pathway Analysis (IPA) in comparisons of the early and middle stages and late and middle stages.

was assessed using a 2100 Bioanalyzer and RNA 6000 Nano LabChip Kits (Agilent Technologies, Palo Alto, CA); the mean RNA integrity value was 7.4 ± 0.4 (mean ± SE).

Microarray and Ingenuity pathway Analyses approaches. A customized bovine oligonucleotide microarray comprising 15,288 genes (Agilent Technologies) was used for one-color microarray analyses to detect genes expressed in the RE11. Fluorescently labeled (cyanine 3) complementary cRNA probes were hybridized to the samples and washed using the Gene Expression Hybridization Kit and Gene Expression Wash Pack Kit (Agilent Technologies). The arrays were scanned using an Agilent Microarray Scanner (Agilent Technologies). Feature Extraction ver. 9.5 (Agilent Technologies) was used to process microarray images, align spots, and create raw numerical total spot intensity data. The microarray data from each sample were imported into GeneSpring 12.0 (Agilent Technologies) for use in the software’s normalization algorithm and for detection of candidate genes. Normalization was performed by dividing each measurement of each array by the median of all measurements in that array (per-spot normalization). The entire microarray data set has been deposited at the Gene Expression Omnibus (GEO) database. The GEO accession numbers are as follows: Platform, GPL22091; samples, GSM 3901089 to GSM 3901115; series, GSE133152.

Pathway and network analyses of DEGs were performed using Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA). Lists of DEGs identified by GeneSpring 12.0 (Agilent Technologies) that corresponded to raw estimated fold changes ≥2 were uploaded into the software application. The IPA knowledge base was used for the DEG enrichment analyses, and the canonical pathways, top upstream regulators, and statistical calculation were analyzed.

Quantitative real-time PCR. Nine genes (both upregulated and downregulated) representing a range of fold changes in the microarray analyses were selected for validation by qPCR. Total RNA samples were treated with TURBO DNase (Applied Biosystems, Foster City, CA, USA) to remove contaminating DNA. Total RNA (800 ng) was converted into first-strand cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The qPCR was performed using q SYBR Green Supermix (Bio-Rad, Hercules, CA) and the StepOne™ Plus Real-Time PCR system (Applied Biosystems) as described elsewhere10. Primers (Table S2) were designed using Primer Express software 3.0 (Applied Biosystems). Each sample contained 2 μl cDNA, 2 × SYBR green, and 0.6 μM each primer in a final volume of 20 μl. Amplification conditions were as follows: 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C, with collection of fluorescence signal at the end of each cycle. For melting curve data, the temperature was increased from 65 to 95 °C in 0.5 °C increments. The results were recorded as relative changes in gene expression normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein L27 (RPL27), and β-actin (ACTB) by the 2^−△△Ct method10. We examined GAPDH, RPL27, and ACTB to evaluate their use as reference genes for qPCR and there were no significant differences among them. All qPCR experiments were performed in accordance to the guidelines for minimum information for publication of quantitative real-time PCR experiments53,54.

Statistical analyses. The pH data were summarized 1 day before the sample collection to minimize the influence of opening the cannula stopper on the ruminal pH. The normality of the distributions of variables was assessed using the Shapiro–Wilk test. Significant differences in ruminal pH, duration of time where pH was
5.6 and 5.8, VFA components, lactic acid concentration, LPS activity, and peripheral LBP concentration among the early, middle, and late stages were evaluated using paired t-tests for normally distributed variables and the Wilcoxon rank-sum test for non-normal variables (Prism ver. 8.10; GraphPad Software, La Jolla, CA, USA). The microarray data were analyzed using paired Student’s t-tests with the Benjamini–Hochberg FDR multiple testing correction (FDR corrected \( P < 0.05 \)) and summarized using GeneSpring 12.0 (Agilent Technologies). Fold changes were calculated by comparing the middle and early (first period), late and middle (second period), and late and early (third period) stages.

Figure 5. Heatmap of upstream regulators (\( P < 0.05 \)) generated by IPA software (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis) in comparisons of the early, middle, and late stages in Japanese Black beef cattle. Cell colors are based on the activation z-score. Blue represents a negative z-score and red represents a positive z-score. N/A, not applicable.
and late and early (total period) stages. The PCA plot coordinates were calculated using the devtools package with microarray data in R ver. 3.3.2 (http://www.r-project.org; R Foundation for Statistical Computing, Vienna, Austria). Significant differences were determined with a threshold of $P < 0.05$, while trends that suggested possible significance were identified at $0.05 < P < 0.10$.

Data availability
The entire microarray data set has been deposited at the Gene Expression Omnibus (GEO) database. The GEO accession numbers are as follows: Platform, GPL22092; samples, GSM 3901089 to GSM 3901115; series, GSE133152.

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Author contributions

T.O. and S.S. contributed conception and design of the study. T.O. and Y.K. organized the database, performed the statistical analysis and wrote the first draft of the manuscript. T.O., H.M., N.I., E.I., T.M., K.K., Y.K., and S.S. contributed to data collection, manuscript revision, read and approved the submitted version.

Competing interests

The authors declare no competing interests.

Additional information

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