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Modulation of fat mobilization and adipose tissue gene expression in Holstein cows supplemented with omega-3 fatty acids and N-acetyl-tryptophan during the transition period

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Modulation of fat mobilization and adipose tissue gene expression in Holstein cows supplemented with omega-3 fatty acids and N-acetyl-tryptophan during the transition period

Abstract

The transition period is critical for dairy cows, characterized by negative energy balance (NEB), excessive adipose mobilization, and metabolic challenges. This study investigated the effects of dietary omega-3 fatty acids (O3) and N-acetyl-tryptophan (NAT) on blood metabolites and adipose tissue gene expression in Holstein cows. Forty-eight multiparous cows were assigned to four groups (control, O3, NAT, O3+NAT) from -21 to +42 days relative to calving. Body weight (BW) and body condition score (BCS) were recorded, and plasma non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHBA), insulin, and glucose were measured at -21, 0 (calving), +21, and +42 days. Adipose biopsies at +21 and +42 days postpartum were used to perform qPCR analysis of lipogenesis-related genes (Acetyl-CoA carboxylase alpha (ACACA), peroxisome proliferator-activated receptor gamma (PPARγ), lipoprotein lipase (LPL)), fatty acid oxidation (Acyl-CoA oxidase 1 (ACOX1)), lipolysis (hormone-sensitive lipase (LIPE), adipose triglyceride lipase (ATGL)), and adiponectin receptors (AdipoR1, AdipoR2). Statistical analysis used two-way ANOVA with repeated measures. Cows supplemented with O3+NAT maintained higher BW ($p<0.05$) and BCS ($p<0.05$), exhibited lower NEFA and BHBA ($p<0.05$), and had increased insulin ($p<0.05$) and tended to have higher glucose ($p=0.08$) compared with controls. PPARγ, LPL, and adiponectin receptors (AdipoR1, AdipoR2) were upregulated in all supplemented groups compared to controls ($p<0.05$). ACOX1 was downregulated in O3, NAT, and O3+NAT groups compared to control ($p<0.05$). Network analysis revealed strong positive correlations between insulin and AdipoR1/2 ($r>0.7$) and positive correlations between NEFA/BHBA and LIPE/β2AR ($r>0.75$) at d 21. These results demonstrate that O3 and NAT act via complementary mechanisms to attenuate lipolysis, promote lipid storage, and enhance metabolic homeostasis during the transition period.

Key words: Dairy cows, Negative energy balance, Lipolysis, Omega-3 fatty acids, N-acetyl tryptophan, Adipose tissue, Gene expression.

The transition period, spanning approximately three weeks before to three weeks after calving, is considered one of the most critical phases in the productive life of dairy cows[1]. During this time, energy requirements increase rapidly due to fetal growth and the initiation of lactation, while dry matter intake (DMI) typically declines[2]. This imbalance leads to negative energy balance (NEB), triggering adipose tissue mobilization and the release of non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHBA) into circulation[3]. While moderate lipid mobilization is physiologically necessary, excessive NEFA and ketone body accumulation are strongly associated with metabolic disorders such as ketosis, fatty liver, and displaced abomasum[4,5]. Beyond metabolic disorders, NEB

also impairs immune function and delays reproductive performance, emphasizing the need for effective nutritional strategies[6].

One major consequence of NEB is altered adipose tissue metabolism, with reduced insulin action promoting lipolysis through activation of hormone-sensitive lipase (LIPE) and adipose triglyceride lipase (ATGL), exacerbating NEFA release[7,8]. Understanding the regulatory mechanisms that attenuate lipolysis and enhance lipid utilization is therefore crucial. Among nutritional interventions, omega-3 polyunsaturated fatty acids (O3) have received significant attention for their capacity to modulate lipid metabolism, immune function, and inflammation[9,10]. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the two major O3 fatty acids, act as ligands for peroxisome proliferator-activated receptors (PPARs), especially PPARγ, which governs lipogenesis, lipid uptake, and insulin responsiveness[11,12].

Evidence from dairy cow studies supports the beneficial effects of O3 supplementation on energy metabolism. Dietary O3 fatty acids reduced circulating NEFA[13], increased PPARα and PPARδ expression[14], and enhanced adipocyte function, such as improved insulin sensitivity and reduced lipolysis during the transition period[15]. Similarly, Abou-Rjeileh et al.[16] reported that unsaturated fatty acid infusion enhanced insulin sensitivity and reduced lipolysis in periparturient cows. Furthermore, O3 have been shown to improve reproductive outcomes by modulating prostaglandin synthesis and uterine function[9]. These findings highlight the multifaceted role of O3 in supporting transition cow health.

Tryptophan, an essential amino acid, also plays a vital role in metabolic adaptation. Beyond its function as a proteinogenic amino acid, tryptophan is the precursor for serotonin and kynurenine, metabolites that regulate immune function, oxidative balance, and energy homeostasis [17,18]. N-acetyl-tryptophan (NAT), a stabilized derivative of tryptophan, is particularly interesting because of its antioxidant properties and potential to enhance adiponectin signaling [14,19]. Studies in livestock have demonstrated that tryptophan supplementation alleviates stress-induced metabolic disruption and improves antioxidant performance [15,20]. Liu et al.[20] showed that tryptophan supplementation in transition cows improved antioxidant status and energy metabolism. These data suggest that NAT could complement O3 in reinforcing adaptive metabolic capacity during the transition period.

Adipose tissue plays a central role in NEB, with genes such as LIPE, ATGL, and Acyl-CoA oxidase 1 (ACOX1) driving lipolysis, while lipoprotein lipase (LPL) and Acetyl-CoA carboxylase alpha (ACACA) regulate lipogenesis [8,22]. Dysregulated gene expression in ketotic cows highlights the importance of targeted nutritional interventions [23].

Despite these promising observations, the combined effects of O3 and NAT on adipose tissue metabolism and metabolic homeostasis have not been comprehensively investigated. We hypothesized that O3 would primarily act through PPARγ to enhance lipid uptake and lipogenesis, while NAT would attenuate lipolysis through adiponectin receptor (AdipoR1/2) signaling. The objectives were to: (1) evaluate plasma NEFA, BHBA, insulin, and glucose concentrations; (2) assess adipose tissue gene expression of

ACACA, PPARγ, LPL, ACOX1, LIPE, ATGL, AdipoR1, AdipoR2, and β-adrenergic receptors; and (3) examine correlations between metabolites and gene expression to elucidate mechanisms of metabolic regulation.

Materials and Methods

Animal ethics and welfare. All experimental procedures were approved by the Animal Care and Use Committee of Sari Agricultural Science and Natural Resources University, Sari, Iran. The study was conducted in accordance with the guidelines of the Iranian Ministry of Science, Research and Technology for the ethical use of animals in research. Moreover this study is performed in accordance with ARRIVE guidelines.

Animals and dietary treatments. Forty-eight multiparous Holstein dairy cows (parity 2.8 ± 0.6, range 2–4, average body weight 600 ± 50 kg (SD) at calving) were enrolled in a randomized 2 × 2 factorial design study conducted at a commercial dairy farm [Mahdasht Dairy Farm, Sari, northern Iran; 36°34′ N, 53°05′ E; 16 m above sea level]. The trial began at -20.2 ± 2.1 days prepartum (mean ± SD) and continued through +42 days postpartum. No acclimatization period was required as cows were already adapted to a similar TMR. Cows were housed in freestall barns with ad libitum access to water and a total mixed ration (TMR) formulated to meet or exceed NRC (2001) requirements for lactating cows (Table 1). The TMR, consistent across all groups, included corn silage, alfalfa hay, soybean meal, corn grain, and a vitamin-mineral premix. Cows were randomly assigned to one of four treatment groups (n = 12 per group): (1) control (0 g/cow O3, 0 g/cow NAT), (2) O3 (100 g/cow/day O3, 0 g/cow NAT), (3) NAT (0 g/cow O3, 100 g/cow/day NAT), or (4) combined O3 and NAT (100 g/cow/day O3, 100 g/cow/day NAT). O3 was provided as rumen-protected fish oil (EPA:DHA ratio 2:1, 60% active ingredient; Megalac Omega, Volac International), and NAT was administered as rumen-protected N-acetyl-tryptophan (98% purity, AminoMax, Balchem Corp.). Supplements were top-dressed on the TMR twice daily at 0700 and 1500 h, with complete intake visually confirmed by farm staff. Individual feed intake was measured by subtracting refusals from offered TMR for each cow. Feed intake was similar across groups (ANOVA, $p>0.05$). The cows were milked three times daily at eight-hour intervals. Milk yield from each cow was automatically recorded, and data related to daily examinations, injections, reproductive status, and health conditions were monitored and documented.

All cows were clinically healthy at the beginning of the study, with no evidence of metabolic or infectious diseases. Health status was monitored daily by a veterinarian. Cows that developed clinical disorders during the study were excluded from the experiment and their data were not included in the final statistical analyses. A total of 48 cows completed the study and were included in the results.

Estrus Synchronization. On day 30 postpartum, cows underwent a Presynch protocol consisting of two $PGF_2\alpha$ injections administered 14 days apart. Twelve days after the

second PGF2α injection, the Ovsynch protocol was initiated with a GnRH injection. Sixteen hours following the second GnRH injection, all cows were inseminated.

Body condition scoring and body weight assessment. The body condition score (BCS) and body weight (BW) of all transition cows were assessed at days -20.2, 0, +21, and +42 relative to calving to track changes in energy reserves during early lactation. BCS was conducted by a trained evaluator using a 5-point scale with 0.25-point increments, where a score of 1 indicated a very thin animal and a score of 5 indicated an obese animal, following the method outlined by Edmonson et al.[24]. BW was recorded using a calibrated electronic livestock scale immediately after the morning milking and before feed delivery, thereby minimizing variability caused by rumen fill. These data were used to evaluate the degree of body tissue mobilization and subsequent recovery during the postpartum period.

Blood Sample Collection. Blood samples were collected on days -20.2, 0, +21, and +42 relative to calving at 0600 h, prior to feeding, via coccygeal venipuncture into 10-mL evacuated tubes containing EDTA (for NEFA and BHBA) or heparin (for glucose and insulin). Samples were immediately placed on ice, centrifuged at 3,000 × *g* for 15 min at 4°C, and plasma was aliquoted and stored at -20°C until analysis. Plasma concentrations of glucose, insulin, NEFA, and BHBA were measured using commercial enzymatic kits: Glucose Oxidase Kit (Sigma-Aldrich, Catalog No. GAGO20) for glucose, Bovine Insulin ELISA (Mercodia, Catalog No. 10-1201-01) for insulin, NEFA-HR(2) (Wako Diagnostics, Catalog No. 999-34691) for NEFA, and Beta-Hydroxybutyrate Assay Kit (Cayman Chemical, Catalog No. 700190) for BHBA. Assays were performed on a microplate reader (BioTek Synergy H1) with intra- and inter-assay coefficients of variation <5%. Calibration curves were generated using standards provided in each kit.

Adipose Tissue Biopsies. Subcutaneous adipose tissue samples (~1 g) were collected from ischiorectal fossa (tuber ischium region) using the method described by Zachut et al.[25] at +21 and +42 days postpartum. Hair was shaved from a 25 cm² (5 × 5 cm) area at the sampling site. To further reduce the likelihood of microbial contamination, the skin was thoroughly disinfected using an iodine-based antiseptic solution. A 2.5 cm incision was made in the skin using a scalpel, and a subcutaneous fat sample was aseptically removed with surgical forceps. The collected samples were first rinsed in distilled water, dried, and placed into a microtube. Following collection, the adipose tissue was immediately placed in a microtube and immersed in liquid nitrogen. The frozen sample was then transported to the laboratory under controlled conditions and stored at −80°C for subsequent molecular analyses.

Quantitative Real-Time PCR (qPCR) Analysis of Gene Expression. Total RNA was extracted from 20 mg of adipose using the Parstous Total RNA Extraction Kit (A101231). Genomic DNA was digested using DNase I before reverse transcription**.** RNA Extraction kit includes two reagents, PW and RL, RNase-free water, collection tubes, and centrifuge columns. RNA integrity was verified (RNA Integrity Number > 8.0) using a Bioanalyzer 2100 (Agilent Technologies). Complementary DNA was synthesized from 1 µg RNA using the SuperScript IV First-Strand Synthesis System (Thermo Fisher,

Catalog No. 18091050). Upon completion, the synthesized cDNA was stored at −20 °C until further analysis.

Real-time PCR was conducted using a LightCycler® 96 Instrument (Roche Applied Science), in accordance with MIQE guidelines[26], to measure mRNA levels of β-adrenergic receptors (β1AR, β2AR, β3AR), LIPE, ATGL, LPL, PPARγ, AdipoR1, AdipoR2, ACOX1, and ACACA (Table 2). Each mixture contained cDNA, forward and reverse primers (synthesized by Metabion (Germany), YTA SYBR Green qPCR Mastermix (YT2551), and nuclease-free water, prepared according to the manufacturer's guidelines. The cycling conditions included initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 40 s, and extension at 72 °C for 20 s. A melt curve analysis was performed to verify the specificity of amplification. Relative quantification of mRNA abundance of genes was calculated based on efficiency and the crossing point deviation of an unknown sample vs. a control and expressed in comparison to reference genes (LRP10, GAPDH, and EMERIN, selected for stability based on Dirandeh et al.[8] using the $2^{-\Delta\Delta}$Ct method. qPCR assays were performed in triplicate.

Statistical Analysis. Data were analyzed using SAS 9.4 (SAS Institute Inc., Cary, NC) with a mixed model for a 2 × 2 factorial design with repeated measures. The model included fixed effects of O3 (0 vs. 100 g/cow), NAT (0 vs. 100 g/cow), their interaction, and time (+21 vs. +42 days for gene expression; -20.2, 0, +21, +42 days for metabolites and BW/BCS), with cow as a random effect to account for repeated measures. Normality was assessed using the Shapiro-Wilk test in PROC UNIVARIATE, and non-normal data (NEFA, BHBA) were log-transformed prior to analysis. Treatment means were compared using Tukey's Honestly Significant Difference test. Covariates (parity, initial BW) were tested but not significant and thus excluded. Pearson's correlations and partial correlations were analyzed using PROC CORR, with partial correlations adjusted for NEFA and BHBA concentrations as control variables and network visualization was performed using Cytoscape. Significance was declared at $p<0.05$, and trends were noted at $0.05\leq p<0.10$. Data are presented as least squares means ± standard error of the mean (SEM). Power analysis (PROC POWER) confirmed that n=12 per group provided 80% power to detect a 15% difference in metabolite concentrations and gene expression ($\alpha=0.05$).

Results

Cows supplemented with O3+NAT maintained greater BW and BCS compared with controls ($p<0.05$, Table 3). Plasma NEFA and BHBA concentrations were significantly reduced in O3+NAT cows ($p<0.05$), while insulin concentrations were higher ($p<0.05$), and glucose tended to increase ($p=0.08$, Figure 1).

Gene expression analysis revealed distinct regulatory effects of O3 and NAT. At day +21 postpartum, O3 supplementation significantly upregulated PPARγ and LPL ($p<0.05$), key regulators of lipid uptake and adipogenesis, whereas NAT increased AdipoR1 and AdipoR2 expression ($p<0.05$). O3+NAT suppressed the expression of LIPE ($p<0.05$) but

not ATGL, suggesting selective suppression of lipolysis. ACACA expression was downregulated in the supplemented groups at days 21 and 42 ($p<0.05$) while ACOX1 was downregulated in O3, NAT, and O3+NAT groups compared to control ($p<0.05$) (Table 4). At day +42, the effects of NAT on adiponectin receptor expression persisted, while O3 continued to maintain elevated PPARγ (Table 5). The expression of β2AR was affected by the supplements ($p<0.05$), while β-adrenergic receptors (β1AR, β3AR) showed no significant treatment effects ($p>0.05$).

At day 21 Heatmaps revealed strong positive correlations between glucose/insulin and PPARγ ($r>0.8$), AdipoR1/2 ($r>0.7$), and positive correlations between NEFA/BHBA and β2AR and LIPE ($r>0.75$, Figure 2). Partial correlation analysis, controlling for NEFA and BHBA, showed significant associations ($P<0.05$) identified PPARγ and adiponectin receptors as central regulators (Figure 3). Network mapping highlighted these as major hubs linking systemic metabolites with molecular responses, confirming their integrative role (Figure 4)

Discussion

This study provides novel evidence that supplementation with O3 and NAT during the transition period exerts complementary and synergistic effects on adipose tissue metabolism and metabolic homeostasis in Holstein cows. By integrating plasma metabolites with gene expression patterns, we demonstrated that O3 and NAT both contribute to lipid uptake and lipogenesis via PPARγ and LPL, while downregulating ACOX1, and NAT promotes adiponectin signaling via AdipoR1 and AdipoR2, with combined effects reducing lipolysis. Together, these effects translated into higher body weight and BCS maintenance, reduced NEFA and BHBA, and enhanced insulin concentrations. However, results that do not fit the initial hypothesis, such as upregulation of PPARγ in the NAT group, suggest broader mechanisms where NAT may indirectly influence PPARγ through anti-inflammatory or antioxidant effects, warranting further investigation. Additionally, given measurements of adiponectin receptors, LIPE, ATGL, and data on systemic insulin sensitivity (e.g., elevated insulin and tended higher glucose), we note that systemic and adipose insulin sensitivity, along with related signaling pathways (e.g., AMPK, PI3K-Akt), were not directly assessed but appear improved based on reduced NEFA/BHBA and gene expression patterns. Future studies should validate downstream pathways (e.g., AMPK, PPARα, ACC signaling) to confirm these mechanisms.

Suppression of Lipolysis and Reduction of NEFA: The downregulation of LIPE by O3+NAT, but not ATGL, indicates selective suppression of lipolysis, as LIPE is more responsive to hormonal regulation while ATGL initiates basal lipolysis while ACOX1 downregulation in O3 cows suggests reduced peroxisomal fatty acid oxidation. ACACA downregulation in supplemented groups ACACA upregulation in O3+NAT supports reduced de novo fatty acid synthesis. This is consistent with proteomic analyses by Kra et al. [15], who found that O3 supplementation enriched acute-phase signaling and complement systems, indicating moderated immune response that aligns with our

observed lipid metabolism improvements via reduced inflammation-driven lipolysis. In contrast, Ning et al. [23] reported dysregulated lipid metabolism in ketotic cows, with suppressed lipogenic genes like LPL, which our study counteracted through supplementation. The sustained gene expression effects by day +42 (e.g., PPARγ, AdipoR1/2) suggest a cumulative impact, potentially due to increased O3 incorporation into cell membranes and sustained serotonin production from NAT.

Excessive lipolysis elevates circulating NEFA, predisposing cows to fatty liver and ketosis [1,2]. Our results are consistent with reports that O3 supplementation reduces NEFA and attenuates adipose lipolysis in transition cows [27]. Likewise, tryptophan supplementation in early lactation was shown to enhance insulin signaling and decrease oxidative stress, indirectly restraining lipolytic activation [1,28]. Thus, the combination of O3 and NAT may create a metabolic environment less prone to excessive fat mobilization and associated disorders. A recent study by Liu et al. [29] further supports this, reporting that NAT supplementation in transition cows upregulated genes involved in mitochondrial function, reducing oxidative stress and improving energy metabolism. The synergistic effects in the O3+NAT group may stem from O3's enhancement of serotonin receptor sensitivity, as suggested by human studies [30].

Enhancement of Adiponectin–Insulin Signaling: The marked upregulation of AdipoR1 and AdipoR2 with NAT supplementation highlights the role of tryptophan derivatives in enhancing adiponectin sensitivity. Adiponectin, via its receptors, activates AMPK and PPARα pathways, increasing glucose uptake and fatty acid oxidation while suppressing lipolysis [31]. Liu et al.[20] demonstrated that dietary tryptophan improved antioxidant status in transition cows. Our findings support these results, showing that NAT-fed cows had lower NEFA and higher circulating insulin. However, increased insulin levels do not necessarily indicate enhanced insulin sensitivity, which was not directly measured (e.g., via RQUICKI) in this study. Correlation network analysis revealed a strong positive relationship between insulin and AdipoR1/2 expression, confirming their role in maintaining energy homeostasis postpartum. The observed increase in insulin levels in the O3+NAT groups is consistent with findings by Mezzetti et al. [32], who demonstrated that intravenous infusion of long-chain O3 fatty acids (EPA and DHA) enhanced insulin sensitivity in early lactation, potentially by reducing hepatic lipid accumulation and improving very-low-density lipoprotein (VLDL) secretion. NAT supplementation's impact on glucose and insulin aligns with its role in serotonin synthesis, which may modulate glucose homeostasis through hypothalamic signaling, as suggested by Conejos et al. [33]. Additionally, NAT metabolites such as kynurenine and 5-hydroxytryptophan may enhance antioxidative capacity, contributing to reduced BHBA levels via insulin-dependent glucose uptake, which supports ketone body metabolism [34].

Role of PPARγ and Lipid Storage: O3 supplementation upregulated PPARγ and LPL, both of which are crucial for adipocyte differentiation and lipid storage. PPARγ acts as a master regulator of lipid metabolism, enhancing fatty acid esterification and preventing lipotoxicity by channeling NEFA into triglycerides [35]. Consistent with our study, Kra et al. [36] reported that O3 supplementation improved insulin sensitivity and lipogenic gene expression in periparturient cows. By increasing LPL expression, O3 facilitated the

hydrolysis of circulating triglycerides and uptake of fatty acids into adipose tissue, thereby reducing systemic NEFA concentrations.

Stimulation of Peroxisomal β-Oxidation and Lipogenesis: The downregulation of ACOX1 with O3 suggests reduced peroxisomal β-oxidation, possibly an adaptive mechanism to prioritize lipid storage over oxidation during NEB [37]. Meanwhile, the downregulation of ACACA in O3+NAT cows indicates reduced de novo fatty acid synthesis, which may recycle mobilized NEFA into triglycerides, supporting lipid homeostasis [38]. This dual effect reflects a coordinated metabolic adjustment that stabilizes energy metabolism during NEB.

Integration with Systemic Outcomes: The metabolic improvements at the systemic level—higher insulin, lower NEFA and BHBA, and preserved BCS—are directly linked to the observed transcriptional changes. Previous studies have highlighted the association between improved insulin action, reduced lipolysis, and lower metabolic disease incidence in transition cows [3,6]. Our network analysis further demonstrated that insulin was positively associated with AdipoR1/2 and LPL, and negatively correlated with LIPE and β2AR, reinforcing the mechanistic basis of our findings. The network analysis complements the correlation analysis by visualizing multi-variable interactions, revealing hubs like PPARγ that simple correlations might overlook, thus providing deeper insight into regulatory networks.

Conclusion

Dietary supplementation with O3 and NAT during the transition period exerted synergistic effects on systemic energy metabolism and adipose tissue gene expression in Holstein cows. O3 and NAT both upregulated PPARγ and LPL, as well as adiponectin receptors, while their combination suppressed LIPE but not ATGL and downregulated ACACA, reflecting improved lipid homeostasis.These adaptations contributed to reduced fat mobilization and greater metabolic resilience. This nutritional approach provides a practical strategy to alleviate NEB, reduce metabolic disorders, and potentially enhance reproductive performance in transition dairy cows.

Data availability

The data that support the findings of this study are available, on reasonable request, from the corresponding author.

Author contributions

Essa Dirandeh: Writing – review & editing, Supervision, Project administration, Methodology, Data curation, Conceptualization. **Mansoureh Ghorbanalinia:** Performing project Resources. **Zarbakht Ansari-Pirsaraei:** Formal analysis. **William W. Thatcher** and **Hasan Sadri:** review & editing.

Additional Information

All information presented in text.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1. Ingredients and nutrient composition (g/kg DM unless otherwise noted) of pre and postpartum diets.

Item	**Prepartum**	**Postpartum**
Ingredient (g/kg DM[1])		
Alfalfa hay mid	244	228
Wheat straw	96	--
Corn silage	288	220
Beet pulp, dehydrated	--	41
Corn grain, ground	141	164
Barley grain, rolled	85	145
Soybean meal, 48%	70	145
Soybean whole, roast	--	8
Cottonseed whole	10	--
Wheat	21	--
Sodium bicarbonate	--	9
Salt	--	4
DCP	--	3
Magnesium oxide	--	3
Glucosa	20	13
Choline chloride	8	4
Palm oil[2]	5	3
Mineral and vitamin premix[3]	12	10
Composition (g/kg DM)		
NEL (Mj/kg DM)	6.7	7.3
Fat	32.7	40.8
Crude protein	133.5	171.0
Neutral detergent fiber	368.0	320.0
Acid detergent fiber	231.0	223.0
Non fibrous carbohydrate	365.3	403.9

[1]Gram per kilogram of dry matter.
[2]Energizer-RP10; Iffco, Johor Bahru Johor, Malaysia.
[3]Contained (per kg): 500,000 IU of Vitamin A, 100,000 IU of Vitamin D, 1,000 mg of Vitamin E, 9,000 mg of P, 195,000 mg of Ca, 2,000 mg of Mn, 55,000 mg of Na, 2,000 mg of Zn, 2,000 mg of Fe, 280 mg of Cu, 100 mg of Co, 100 mg of Br, 1 mg of Se, and 3,000 mg of Anti-oxidant.

Table 2. qPCR Primer sequences used for genes associated with fat mobilization. Forward (F) and reverse (R) primer sequences, GenBank accession numbers, and product sizes are shown for each gene.

Gene	Primer sequences 5>3	References	Length (bp)
β1AR	F: CGAGCAGAAGGCACTCAAGAC	AF188187	107
	R: CCAGGTCGCGGTGGAA		
β2AR	F: CCCCAGGCACCGAAAACT	Z86037	69
	R: TCCCTTGTGAATCAATGCTATCA		
β3AR	F: AGGCAACCTGCTGGTAATCG	X85961	72
	R: GTCACGAACACGTTGGTCATG		
LIPE	F: CGAGATCCAGGTGCTATCGT	NM_001080220	106
	R: CGAGGTCAGAGGCATTTCA		
LPL	F: GACAGGATGTGGCCAAGTTT	NM_001075120	61
	R: TTGCCCAGGGGATAGTTAAA		
PPARγ	F: GCGACTTAGCAATATTTATAGCTGTC	NM_181024	350
	R: AGGCTTGCAGCAGATTGTCT		
ATGL	F: TGTGGCCTCATTCCTCCTAC	Palou *et al.,* 2008	271
	R: AGCCCTGTTTGCACATCTCT		
ACOX1	F: CACGCAATAGTTCTGGCTCA	Palou *et al.,* 2008	221
	R: ACCTGGGCGTATTTCATCAG		
ACACA	F: AACATCCCCACGCTAAACAG	NM_174224	61
	R: GAGTCATGCCGTAGTGGTTG		
AdipoR1	F: GCTGAAGTGAGAGGAAGAGTC	NM_001034055	118
	R: GAGGGAATGGAGTTTATTGCC		
AdipoR2	F: GGCAACATCTGGACACATC	NM_001040499.2	200
	R: CTGGAGACCCCTTCTGAG		
LRP10	F: CCAGAGGATGAGGACGATGT	BC149232	139
	R: ATAGGGTTGCTGTCCCTGTG		
GAPDH	F: AATGGAAAGGCCATCACCATC	U85042	204
	R: GTGGTTCACGCCCATCACA		
EMD	F: GCCCTCAGCTTCACTCTCAGA	NM_203361	100
	R: GAGGCGTTCCCGATCCTT		

Genes include *βARs*=β-adrenergic receptors, *LIPE*=hormone-sensitive lipase; *LPL*=lipoprotein lipase; *PPARγ*=peroxisome proliferator-activated receptor gamma; *ATGL*=Adipose triglyceride lipase; *ACOX1*=Acyl-CoA oxidase1; *ACACA*=Acetyl-CoA carboxylase1; AdipoR1=adiponectin receptor1, *AdipoR2*=adiponectin receptor2; *LRP10*= lipoprotein receptor-related protein 10; *GAPDH* =Glyceraldehyde 3-phosphate dehydrogenase, *EMD*= emerin.

Table 3. Body condition score (BCS), BCS changes and body weight (BW) during transition period across treatment (n=12 cows/group)

Variable	Treatments[1]				SEM	P-value (Treatment)
	Control	O3	NAT	O3+NAT		
Overall BCS						
d -20.2	2.89	2.91	2.95	3.02	0.09	0.71
d 0	2.82 [c]	2.88 [b]	2.90 [b]	3.00 [a]	0.01	0.03
d +21	2.25 [c]	2.70 [b]	2.70 [b]	2.89 [a]	0.02	0.01
d +42	2.20 [c]	2.64 [b]	2.62 [b]	2.85 [a]	0.02	0.01
BCS change						
-20.2 to 0	-0.04	-0.07	-0.08	-0.05	0.13	0.57
0 to +42	-0.45 [a]	-0.14 [b]	-0.16 [b]	-0.10 [c]	0.03	0.03
-20.2 to +42	-0.49[a]	-0.25 [b]	-0.28 [b]	-0.14 [c]	0.03	0.01
BW loss -20.2 to +42 (mean,	41.5[a]	20.28[b]	19.05 [b]	13.28[c]	3.05	0.01

Body weight (BW) and body condition score (BCS) at -20.2 (mean start day ± 2.1 SD), 0, +21, and +42 days relative to calving for cows (n=12/group) receiving control, O3 (100 g/cow/day), NAT (100 g/cow/day), or O3+NAT. p-values represent treatment effects (two-way ANOVA with repeated measures). P-value" refers to the overall treatment effect from two-way ANOVA. Different superscript letters (a,b,c) indicate significant differences (P,0.05) between treatments via Tukey's test.

Table 4. Adipose tissues gene expression at day +21 postpartum (LSM±SEM, n=12 cows/group).

Treatment	Relative Gene expression										
	PPARγ	***β3AR***	***β1AR***	***β2AR***	***ADPOR2***	***ADPOR1***	***ACOX1***	***LIPE***	***ATGL***	***LPL***	***ACACA***
Control	1.02 ^{c}	1.00 ^{a}	1.03 ^{a}	1.02 ^{a}	1.03 ^{c}	1.05 ^{c}	1.02 ^{a}	1.12 ^{a}	1.02 ^{a}	1.01 ^{c}	1.04 ^{a}
O3	2.51 ^{b}	1.57 ^{a}	1.13 ^{a}	0.68 ^{b}	2.50 ^{b}	6.68 ^{b}	0.22 ^{b}	0.50 ^{b}	1.49 ^{a}	2.88 ^{b}	0.09 ^{b}
NAT	3.56 ^{b}	1.29 ^{a}	1.16 ^{a}	0.65 ^{b}	3.98 ^{b}	7.57 ^{b}	0.38 ^{b}	0.57 ^{b}	0.93 ^{a}	5.69 ^{a}	0.19 ^{b}
O3+NAT	5.77 ^{a}	1.56 ^{a}	1.36 ^{a}	0.09 ^{c}	7.95 ^{a}	9.22 ^{a}	0.20 ^{b}	0.13 ^{c}	1.22 ^{a}	5.52 ^{a}	0.12 ^{b}
SEM	0.19	0.16	0.18	0.1	0.21	0.13	0.20	0.23	0.31	0.22	0.18
P-Value											
O3	<.01	0.48	0.17	<.01	<.01	<.01	<.01	0.20	0.52	<.01	0.01
NAT	0.01	0.05	0.24	<.01	<.01	0.02	<.01	0.04	0.20	0.03	<.01
O3*NA	0.01	0.45	0.66	0.01	0.01	<.02	<.01	0.01	0.74	0.01	<.01

Relative mRNA abundance at day +21 postpartum in cows (n=12/group) receiving control, O3 (100 g/cow/day omega-3 fatty acids), NAT (100 g/cow/day N-acetyl-tryptophan), or O3+NAT. Data are least squares means (LSM) ± standard error of the mean (SEM), analyzed by two-way ANOVA with repeated measures. Different superscript letters (a,b,c) indicate significant differences ($P<0.05$) between treatments via Tukey's test.

Table 5. Adipose tissues gene expression at day +42 postpartum (LSM±SEM, n=12 cows/group).

Treatment	Relative Gene expression										
	PPARγ	***β3AR***	***β1AR***	***β2AR***	***ADPOR2***	***ADPOR1***	***ACOX1***	***LIPE***	***ATGL***	***LPL***	***ACACA***
Control	1.02 ^{c}	1.04 ^{a}	1.19 ^{a}	1.04 ^{a}	1.07 ^{b}	1.05 ^{c}	1.08 ^{a}	1.04 ^{a}	1.14 ^{a}	1.23 ^{c}	1.04 ^{a}
O3	3.37 ^{b}	0.89 ^{a}	1.11 ^{a}	0.21 ^{b}	3.13 ^{a}	3.57 ^{b}	0.41 ^{b}	0.16 ^{b}	1.42 ^{a}	5.17 ^{b}	0.06 ^{b}
NAT	3.61 ^{b}	0.87 ^{a}	1.37 ^{a}	0.10 ^{b}	3.07 ^{a}	3.95 ^{b}	0.40 ^{b}	0.32 ^{b}	1.41 ^{a}	8.66 ^{a}	0.34 ^{b}
O3+NAT	6.75 ^{a}	1.10 ^{a}	0.91 ^{a}	0.14 ^{b}	4.53 ^{a}	7.13 ^{a}	0.17 ^{b}	0.06 ^{b}	1.02 ^{a}	9.01 ^{a}	0.42 ^{b}
SEM	0.21	0.12	0.20	0.1	0.3	0.10	0.23	0.21	0.4	0.20	0.18
P-Value											
O3	<.01	0.92	0.99	<.01	<.01	<.01	<.04	<.01	0.83	<.01	0.04
NAT	0.01	0.83	0.42	<.01	<.01	0.02	<.02	<.01	0.86	0.20	<.01
O3*NAT	<.01	0.83	0.78	<.01	<.01	<.01	0.04	<.01	0.72	0.01	<.01

Relative mRNA abundance at day +42 postpartum in cows (n=12/group) receiving control, O3 (100 g/cow/day omega-3 fatty acids), NAT (100 g/cow/day N-acetyl-tryptophan), or O3+NAT. Data are least squares means (LSM) ± standard error of the mean (SEM), analyzed by two-way ANOVA with repeated measures. Different superscript letters (a,b,c) indicate significant differences (P<0.05) between treatments via Tukey's test..

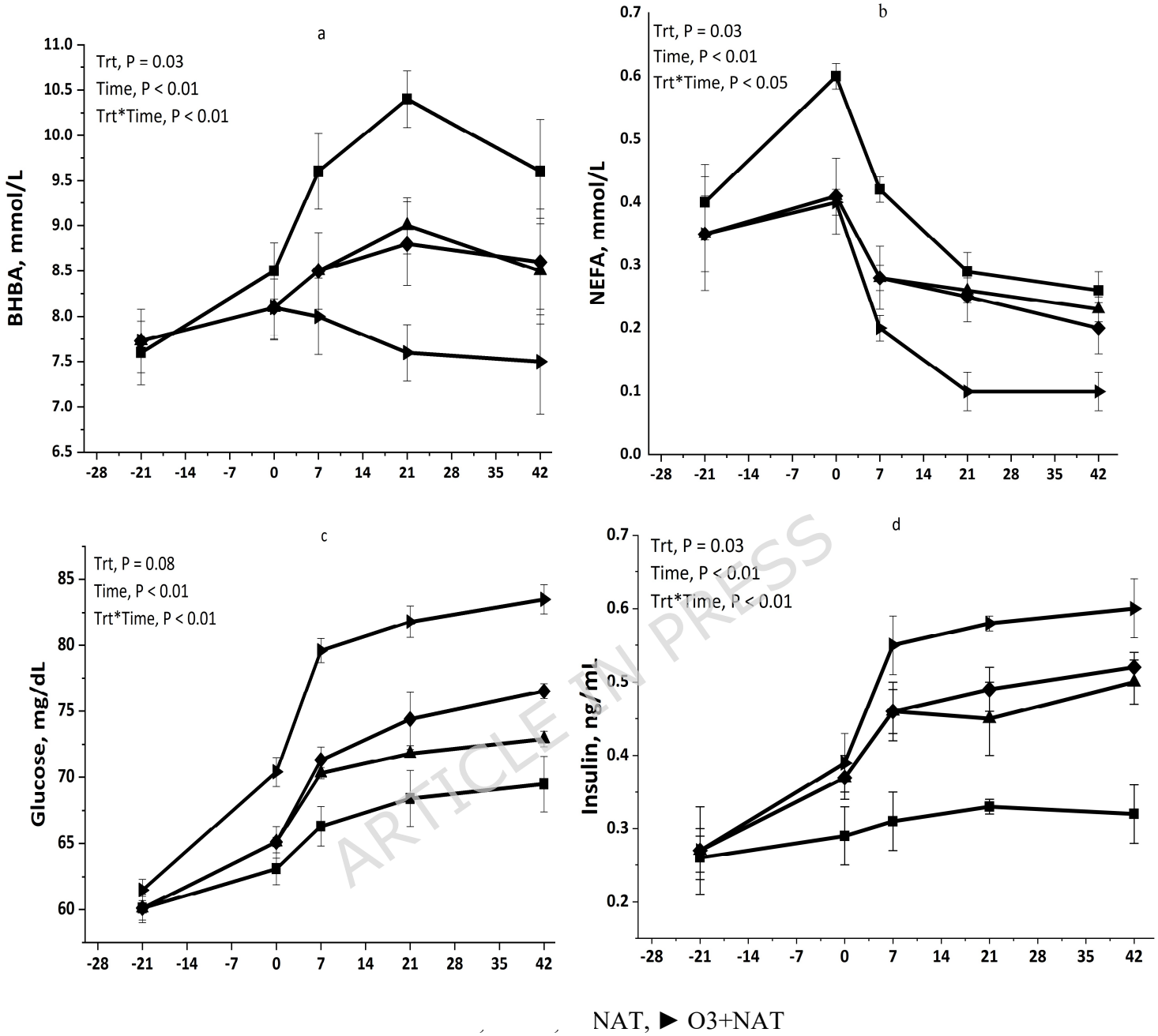

Fig 1. Effects of dietary treatments (Control ■, O3 ▲, NAT ♦, O3+NAT ►) on plasma BHBA (a, log-transformed), NEFA (b, log-transformed), glucose (c), and insulin (d) concentrations at -20.2, 0 (calving), +21, and +42 days relative to calving. Data are least squares means ± SEM (n=12 cows/group), analyzed by two-way ANOVA with repeated measures. "P-value Trt" refers to the overall treatment effect. Post-hoc comparisons via Tukey's test are indicated by different letters where applicable.

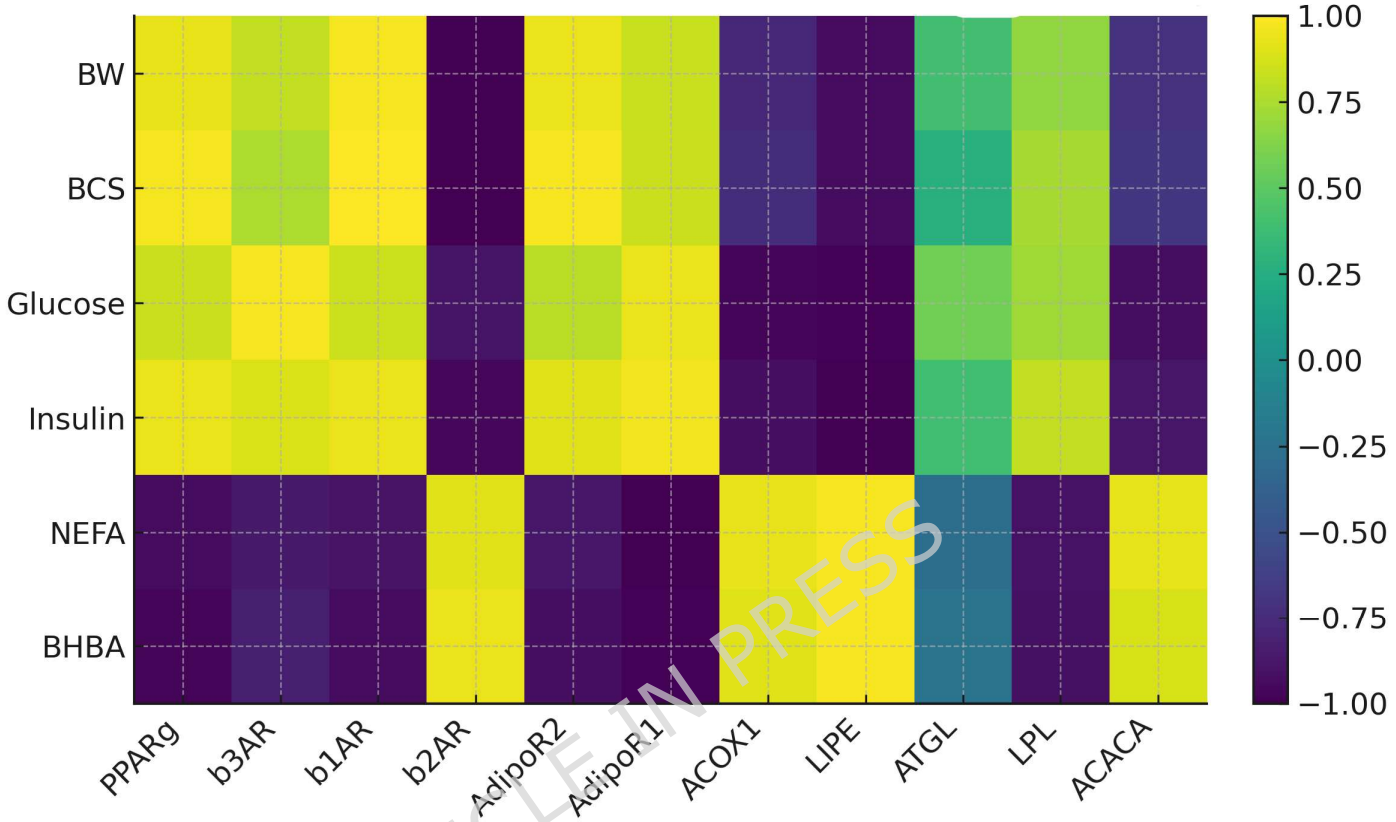

Fig 2. Positive correlations are shown in yellow to violet, negative correlations in blue to yellow (adjusted color legend). Pearson's correlation analysis ($P<0.05$, for all shown correlations). All correlations with $P < 0.05$ are displayed; non significant ones are omitted. Strong associations were observed between insulin and AdipoR1/2, and positive correlations between NEFA/BHBA and LIPE/β2AR.

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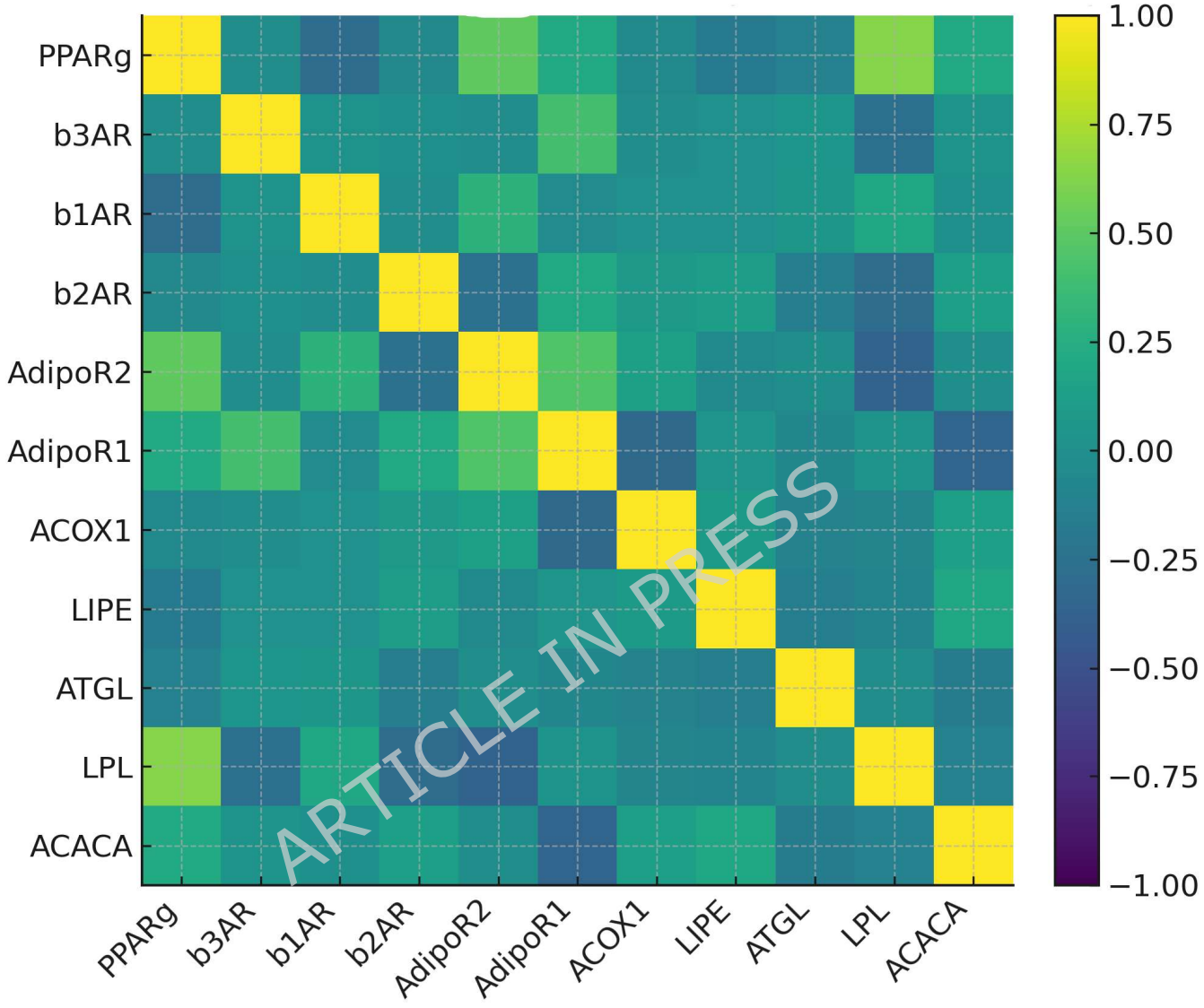

Fig 3. Partial correlation analysis (P<0.05 for all shown, controlling for NEFA and BHBA). All correlations with P< 0.05 are displayed.

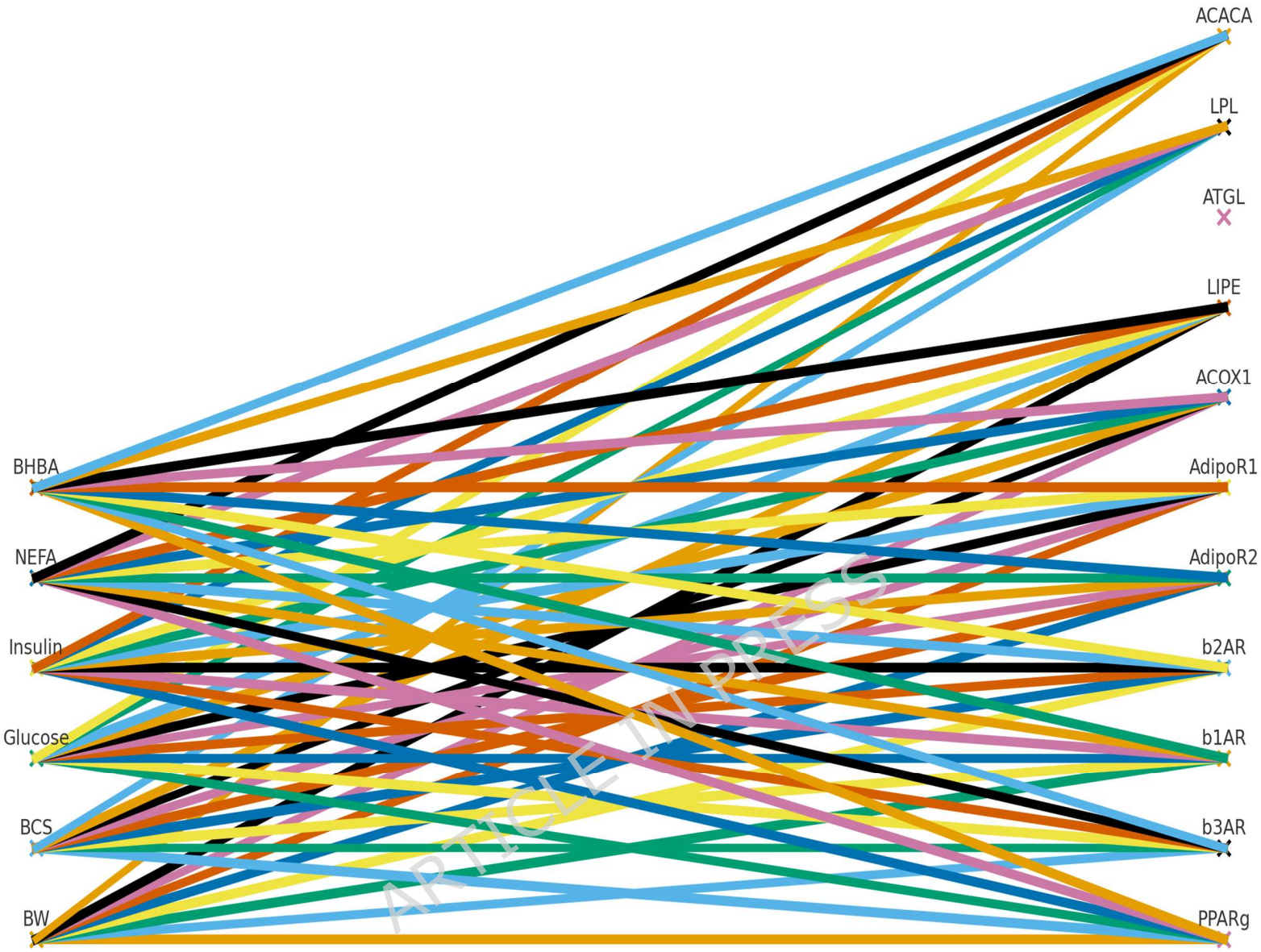

Fig 4. Network map (r≥0.7, P<0.05) of metabolite-gene associations in adipose tissue (blood metabolites and mRNA expression in subcutaneous adipose tissue of dairy cows at +21 and +42 days postpartum). Nodes represent metabolites or genes; edges represent significant associations. The map emphasizes the central roles of PPARγ and adiponectin receptors as hubs, with thicker edges indicating stronger correlations and node size proportional to degree centrality